Inflammation and Coagulation Activity in Unstable Coronary Artery Disease and the Influences of Thrombin Inhibition

BY

JONAS OLDGREN
ABSTRACT


In patients with unstable coronary artery disease, this study evaluated the degree of inflammation and coagulation activity, relations to myocardial cell damage, prognosis, and influences of randomisation to 72 h infusion with three different doses of inogatran, a direct thrombin inhibitor (n=904), or unfractionated heparin (n=305).

Anticoagulant treatment effects were evaluated with aPT time. In inogatran treated patients with aPT times ≥ 44 s (median), the 7-days event rate - death, myocardial infarction or refractory angina – was 11.6%, compared to 6.6% with aPT times < 44 s (p=0.01). Higher aPT times was related to improved outcome during heparin treatment.

Markers of inflammation, i.e. fibrinogen and C-reactive protein (CRP), and coagulation, i.e. prothrombin fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT), soluble fibrin (SF) and D-dimer were analysed in serial samples (n=320). High fibrinogen, F1+2 and D-dimer levels persisted at 30 days. Patients with myocardial damage, detected by elevated troponin, had higher levels of all markers except TAT.

Ischemic events occurred at 30 days in 17% of patients with high (pre-treatment top tertile) and 8.5% of patients with lower fibrinogen levels (p=0.03), while high CRP levels only were related to increased mortality. At 30 days, patients with high compared to low pre-treatment levels of TAT or SF had 40% lower event rate. Patients with early decreased compared to raised F1+2 or TAT levels during treatment had 50% lower 30-days event rate (p<0.05).

Conclusions: The aPT time is an inappropriate indicator of antithrombotic efficacy. The raise in fibrinogen in the acute phase is sustained, and indicates risk of thrombosis and new ischemic events. The pronounced CRP elevation is transient, but associated with increased mortality. Higher coagulation activity may identify patients with a thrombotic condition as the major cause of instability, who are best responders to anticoagulant therapy. However, reactivation of coagulation activity with raised risk of ischemic events is a concern at cessation of treatment.

Key words: Unstable coronary artery disease, inflammation, coagulation, thrombin inhibition.
Man lives with arteriosclerosis, and dies of the complicating thrombosis

Jens Dedichen, 1956
Papers

This thesis is based on the following original papers which will be referred to by their Roman numerals:

I. Activated Partial Thromboplastin Time and Clinical Outcome after Thrombin Inhibition in Unstable Coronary Artery Disease
   Oldgren J, Linder R, Grip L, Siegbahn A, Wallentin L

II. Myocardial Damage, Inflammation and Thrombin Inhibition in Unstable Coronary Artery Disease
    Oldgren J, Wallentin L, Grip L, Linder R, Nørgaard B, Siegbahn A
    In progress

III. The Effect of a Low Molecular Mass Thrombin Inhibitor, Inogatran, and Heparin on Thrombin Generation and Fibrin Turnover in Patients with Unstable Coronary Artery Disease
     Eur Heart J 1999;20(7):506-18

IV. Coagulation Activity and Clinical Outcome in Unstable Coronary Artery Disease
    Oldgren J, Linder R, Grip L, Siegbahn A, Wallentin L

V. Myocardial Damage, Coagulation Activity and the Response to Thrombin Inhibition in Unstable Coronary Artery Disease
   Oldgren J, Siegbahn A, Grip L, Linder R, Thygesen K, Wallentin L
   Submitted

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>aPT</td>
<td>activated partial thromboplastin</td>
</tr>
<tr>
<td>ASA</td>
<td>acetylsalicylic acid</td>
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<tr>
<td>CK-MB</td>
<td>MB isoenzyme of creatine kinase</td>
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<tr>
<td>ECG</td>
<td>electrocardiogram</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunoassay</td>
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<tr>
<td>F1+2</td>
<td>prothrombin fragment 1+2</td>
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<tr>
<td>LMW</td>
<td>low molecular weight</td>
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<tr>
<td>TAT</td>
<td>thrombin-antithrombin complex</td>
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<tr>
<td>TRIM</td>
<td>thrombin inhibition in myocardial ischemia</td>
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<td>UF</td>
<td>unfractionated</td>
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Definition and clinical manifestations of unstable coronary artery disease

Coronary artery disease is the major cause of mortality and morbidity in developed countries. The predominant underlying cause of coronary artery disease is atherosclerosis, a process that starts early in life and progresses slowly and silently for decades (1). The clinical manifestations of coronary artery disease comprises a wide spectrum of conditions, ranging from chronic stable angina to the acute coronary syndromes of unstable angina, myocardial infarction and sudden death.

In chronic stable angina, the ischemia usually results from an increase in myocardial oxygen demand that outweighs the transport capacity of the stenosed coronary artery. In the acute coronary syndrome, the ischemia is caused by plaque disruption with exposure of the lipid-rich and thrombogenic core, leading to the formation of a thrombus (2). The intracoronary thrombus causes aggravated stenosis, transient or persistent total occlusion, and/or distal embolization of the thrombotic material (3, 4). The severity, duration, and extent of the resulting myocardial ischemia or necrosis will determine the clinical presentation.

Unstable angina and non-ST-elevation myocardial infarction (previously called non-q-wave myocardial infarction) are closely related conditions and - because of the similarities in pathogenesis, clinical presentation and initial treatment – they are often merged together into one entity, unstable coronary artery disease. The clinical presentation includes new onset of severe angina (within the latest four weeks), abrupt worsening of previously stable angina or angina at rest (5). Signs of myocardial ischemia or necrosis, such as deviations of the ST-segment or the T-wave in standard 12-lead ECG and/or elevated levels of biochemical markers of myocardial cell damage, are common and supportive of the clinical diagnosis, although not mandatory.

Atherosclerosis and plaque disruption

Atherosclerosis is the result of a complex interaction between blood constituents, disturbed blood flow, and vessel wall abnormalities involving several pathological processes: inflammation, with increased endothelial permeability and monocyte recruitment; growth, with smooth muscle cell proliferation and migration and matrix synthesis; degeneration, with lipid accumulation; necrosis, by cytotoxic effect of oxidised lipids; calcification; and thrombosis (2).
Mature plaques typically consist of two main components: hard, collagen-rich sclerotic tissue and soft, lipid-rich atheromatous “gruel”, devoid of supporting collagen. The risk of plaque disruption depends more on the plaque composition and vulnerability than the degree of stenosis (6). Major determinants of the liability to rupture are the size of the atheromatous core (7), thickness and collagen contents of the fibrous cap covering the core (8) and inflammation in the cap (9). Plaque disruption occurs most frequently where the fibrous cap is thinnest, often the junction between the plaque and the adjacent normal vessel wall, i.e. the shoulder region of the plaque (8). Mechanical and haemodynamic forces may trigger plaque disruption, e.g. increased blood pressure or pulse rate. Cigarette smoking exerts toxic effects on endothelial function and is another potential trigger for acute coronary syndromes (10).

As the disrupted plaque exposes its thrombogenic contents to the flowing blood, thrombotic material immediately layers on top of the plaque (3). Thrombus may also develop over an area of endothelial denudation or erosion, without plaque disruption (10). The expanding thrombus may completely occlude the coronary artery at the site of the plaque disruption, resulting in acute myocardial infarction or sudden death. The evolving thrombus may also be partially or only transiently occlusive resulting in unstable coronary artery disease. Finally, fragments of the thrombus may also embolize and occlude distal arteries and arterioles with subsequent multifocal myocardial cell injury (3, 4).

**Inflammation and markers of inflammation**

Inflammatory processes are involved in the development of atherosclerosis (1). Furthermore, by destabilising the plaque and enhancing thrombus formation, inflammation may also be involved in the initiation of unstable coronary artery disease (11). The potentially dangerous atherosclerotic lesions are difficult to identify by angiography. However, at autopsy active inflammation is evident by the accumulation of macrophages at the sites of the plaque rupture (9). Moreover, elevated levels of interleukins (12), acute-phase proteins (13, 14), activated circulating monocytes (15) and lymphocytes (16) have been reported from clinical studies in unstable coronary artery disease.

The trigger of the active inflammation in unstable coronary artery disease is not known, although infections such as Chlamydia pneunomiae and Helicobacter pylori have been suggested (17). The higher degree of inflammation in patients with unstable coronary artery disease might also, at least in part, be an acute-phase reaction induced by the myocardial cell damage (18-20). However, ischemia in itself does not seem to elicit an acute-phase reaction (21).
Markers of inflammation

Although an increased inflammatory activity has been documented in unstable coronary artery disease, there is limited knowledge about the influence of anticoagulant treatment on the degree of inflammation (14, 21, 22).

In the present study we have measured:

Fibrinogen, an acute-phase protein mainly synthesised by hepatocytes, with a half-life of 5 days. Fibrinogen is regulated by interleukin-6 and is directly involved also in the thrombotic process, both in platelet aggregation by cross-linking the glycoprotein IIb/IIIa-receptors on adjacent platelets (23), and in the coagulation cascade (24), figure 1.

C-reactive protein, a typical acute-phase protein, mainly synthesised in the hepatocytes, with a half-life of 19 hours. The release of C-reactive protein is regulated by interleukin-6 and increased levels are seen in response to infection, inflammation and tissue damage. The biological function is unclear but it has been suggested that C-reactive protein may directly interact with the atherosclerotic process by activating the complement system (25). C-reactive protein has also been shown to induce tissue factor expression on monocytes (26).

Haemostasis and molecular markers of coagulation activity

The endothelial cells outlining the vessels prevent interaction between blood constituents and subendothelial components under normal conditions. However, the exposure of circulating blood to the disrupted or eroded plaque initiates a series of complex reactions including platelets, leukocytes, erythrocytes and haemostatic factors, producing an haemostatic plug.

Platelets

The primary step in haemostasis is the adhesion, activation and aggregation of platelets (23). In response to vascular and tissue trauma, such as plaque disruption, platelets interact with exposed subendothelial structures, such as collagen. Upon activation, platelets change shape, secrete the contents of α-granulae, bind soluble adhesion molecules and become the surface for continuing platelet deposition. The activated platelets express glycoprotein receptors, e.g. Ib-IX-V complex and conformationally changed IIb/IIIa, on their surface and is cross-linked by binding of von Willebrand factor and fibrinogen, thereby aggregating to a platelet plug.

Coagulation

The coagulation system is a series of procoagulant and anticoagulant proteins circulating in plasma as proenzymes or procofactors (24). The activated coagulation factors are rapidly formed by proteolytic cleavage of one or two peptide
bonds, and the activated factor then activates another factor in the coagulation cascade, figure 1.

The lipid-rich core of the disrupted atherosclerotic plaque has high contents of tissue factor, a small glycoprotein expressed on stimulated monocytes, macrophages, endothelial cells and smooth muscle cells (27). In vivo, the exposure of tissue factor to the circulating blood initiates activation of the extrinsic coagulation cascade (24). Factor VII binds tissue factor on the cell surface and the tissue factor/factor VIIa-complex then activates factors IX and X. Factor Xa

Figure 1. Simplified illustration of the extrinsic coagulation cascade, initiated by the tissue/factor VIIa-complex. The molecular markers measured in the present study are underlined. Broken arrows indicate inhibition. TAT = thrombin-antithrombin complex, tPA = tissue-type plasminogen activator.
assembles on the surface of activated platelets as part of the prothrombinase complex, consisting of factors Xa, Va and calcium, and converts prothrombin to thrombin. Thrombin then activates platelets and converts fibrinogen to fibrin. The fibrin is stabilised by factor XIIIa (activated by thrombin) forming a fibrin network, thus stabilising the platelet clot. Factors V, VIII and XI are also activated by thrombin, thereby autoamplifying thrombin generation. Moreover, thrombin inhibits fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor (28).

Several anticoagulant systems operate in order to regulate procoagulant activity (24). Antithrombin forms complexes with coagulation factors Xa, IIa (thrombin), IXa and XIa and these complexes are inactive and removed by the liver. The inhibition by antithrombin is markedly increased in the presence of heparin or heparin sulphate on endothelial cells. Thrombin bound to thrombomodulin activates protein C which, in the presence of protein S, degrades factors Va and VIIIa on platelet surfaces, thereby slowing the blood clotting. Another important anticoagulant is the tissue factor pathway inhibitor, an endothelium-bound protein that inactivates both factors VIIa and Xa by forming quaternary complexes in the presence of phospholipid surfaces and calcium, thereby inhibiting the extrinsic coagulation pathway (29).

**Fibrinolysis**

The fibrinolytic system has a major role in the balance of haemostasis (30). The proenzyme plasminogen is converted to the active enzyme plasmin by tissue-type (or urokinase-type) plasminogen activator. This conversion only occurs efficiently on the fibrin surface. Plasmin is the key enzyme in the fibrinolytic system and degrades fibrin, thus forming fibrin degradation products, one of which is D-dimer. Free plasmin in the blood is very rapidly inactivated by α2-antiplasmin. The tissue-type plasminogen activator is generated in and rapidly released from endothelial cells, and it is inactivated by a specific plasminogen activator inhibitor, thus the balance of these two regulates the fibrinolytic capacity.

**Molecular markers of coagulation activity**

The degree of coagulation activity can reliably be determined by various assays measuring molecular markers in plasma (31). For this purpose, proper blood sampling technique is critical and samples should preferably be acquired with direct venipuncture and a minimum of venous stasis. The blood sample should be rapidly cooled and as quickly as possible centrifuged. Also the timing of blood sampling is important since several markers have a short half-life.

An activation of the coagulation system in the acute phase of unstable angina or acute myocardial infarction has been demonstrated by elevated levels of molecular markers of thrombin
generation, i.e. prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complex (TAT), and of thrombin activity, i.e. fibrinopeptide A and soluble fibrin (32-35). Elevated levels of D-dimer have also been found in patients with unstable angina and myocardial infarction (34, 35). However, there have been conflicting results concerning the effects on these molecular markers of coagulation activity by various thrombin inhibitors (36-41).

In the present study we have measured:

Activated partial thromboplastin (aPT) time is easily available and currently the most widely used method for monitoring of treatment antithrombin agents. It is an in-vitro method that reflects surface-induced activation of the coagulation system and is therefore mainly sensitive to factors of the intrinsic pathway. Despite the long experience of monitoring UF heparin treatment with aPT time, there are rather limited data available on the desirable interval for optimal anticoagulant effect in the treatment of unstable coronary artery disease. Previous recommendations have been similar to the recommendations for treatment of venous thrombosis (42). The differences between arterial and venous thrombosis in thrombus burden and in concomitant treatment with aspirin and other platelet inhibitors such as the glycoprotein Ilia/Ilb-inhibitors might give over-anticoagulation and increased bleeding risk in unstable coronary artery disease (43). In the recent AHA/ACC guidelines for unstable angina/non-ST-elevation myocardial infarction (43, 44) it is recommended to give lower, weight-adjusted, bolus doses and infusions of UF heparin to maintain the aPT times at 1.5 - 2.5 x control, corresponding to approximately 50-70 seconds.

Prothrombin fragment 1+2 (F1+2), a polypeptide released from prothrombin during its conversion to thrombin by the factor Xa/Va-complex, thus reflecting thrombin generation. The normal half-life in plasma is 60-90 minutes. F1+2 is predominantly measured by monoclonal antibody methods (31).

Thrombin-antithrombin complex (TAT) are formed when antithrombin binds and inhibits the active site of thrombin. This binding is markedly increased in the presence of heparin or heparin-like cofactors. TAT is considered as a marker of thrombin generation and activity. The normal half-life in plasma is 5-15 minutes. ELISA:s are commonly used to assay TAT (31).

Soluble fibrin, also called fibrin monomer, is formed when the fibrinopeptides A and B are cleaved from the aminoterminus of fibrinogen by thrombin. Thus, elevated levels reflect increased thrombin activity. A subsequent cross-linking of fibrin is induced by thrombin-activated factor XIIIa. Normal plasma half-life for soluble fibrin is 4-6 hours. There are both ELISA:s and functional assays, the latter measuring the activity of fibrin as a co-factor for tissue-type plasminogen activator-mediated activation of plasminogen (31).
D-dimer, is a fibrin degradation product containing cross-linked gamma-chains. As this is the final product of the coagulation and fibrinolytic cascade, D-dimer mainly reflects fibrin turnover in conditions without pharmacological fibrinolytic treatment. Normal plasma half-life is 15-30 hours. Both semiquantitative latex agglutination methods and more sensitive, quantitative ELISA:s are available (31).

Antithrombin, the natural inhibitor of several serine proteases, including factor Xa and thrombin, is commonly measured with functional assays (45).

Myocardial infarction and biochemical markers of myocardial cell injury
Myocardial ischemia initiates a chain of cellular events which, if not reversed, end with the disruption of the myocardial cell membranes and cell death. The loss of cell membrane integrity leads to diffusion of intracellular markers that can be measured in the systemic circulation as signs of the myocardial cell injury. Until recently, the MB isoenzyme of creatine phosphokinase (CK-MB) has been the most specific marker of myocardial cell injury. The newly developed commercially available methods to analyse troponins, with higher sensitivity and specificity, along with the prolonged time-window have further improved the possibilities to detect small irreversible myocardial cell injuries (46). The diagnosis of myocardial infarction has recently been redefined (47) and the new definition is based on biochemical grounds, e.g. troponins.

Elevated levels of C-reactive protein, as an acute-phase reaction induced by myocardial cell damage, have been reported in unstable coronary artery disease patients with troponin elevation (20). However, there are limited data concerning the inflammatory activity in patients with unstable angina without signs of myocardial cell damage.

Two small studies have reported an association of troponin elevation and signs of coagulation activation, e.g. as indicated by elevated soluble fibrin levels (48, 49). Thus, elevated troponins in patients with unstable coronary artery disease have been suggested as surrogate markers for plaque disruption and thrombus formation (50). This concept is supported by more frequent intracoronary thrombi at angiography in patients with elevated troponin levels (51-53). However, there are limited data on the association of troponins and other molecular markers of coagulation activity.

Prognosis and risk stratification in unstable coronary artery disease
The patients with unstable coronary artery disease are at variable risk for recurrent ischemic events, with death, myocardial infarction or need for revascularisation, at average of about 30% of the patients at 3 months (54). Non-invasive risk stratification in this heterogeneous group of patients might enable the clinician to tailor the therapy based on individual risk evaluations (55).
Clinical presentation
Several easy available demographic and historical features of the patient are indicators of increased risk, such as higher age, prior myocardial infarction, history of congestive heart failure, presence of diabetes mellitus, and known significant coronary artery stenoses. Furthermore, the risk can be estimated from the clinical presentation, with angina at rest, especially during the last 48 hours, haemodynamic instability or congestive heart failure indicating higher risk (44).

Electrocardiogram
Depression of the ST-segment in a standard 12-lead ECG on admission or during hospital stay indicates raised risk for adverse outcome (56, 57). Similarly, ischemic episodes during continuous monitoring with 12-lead electrocardiogram or vectorcardiography also indicate higher risk (58, 59).

Myocardial cell injury (troponins)
Besides their use as diagnostic markers of myocardial infarction, the troponins are powerful predictors of future cardiac events in unstable coronary artery disease (65, 66). However, there are no data on the association of troponins and molecular markers of coagulation activity concerning clinical outcome.

Inflammation
The early levels of fibrinogen and C-reactive protein have in several studies been identified as indicators of increased short- and long-term risk for adverse clinical outcome in unstable coronary artery disease (14, 60-64). There is limited knowledge about the time-course and the influence of anticoagulant treatment of changes in fibrinogen and C-reactive protein in unstable coronary artery disease (14, 21, 22).

Coagulation activity
There are rather limited data concerning the prognostic importance of elevated coagulation markers in patients with unstable coronary artery disease (31). Furthermore, possible relations between the effect of changes in coagulation markers induced by anticoagulant treatment and the clinical outcome in unstable coronary artery disease remain to be elucidated.

Treatment of unstable coronary artery disease
The primary goal of the treatment in unstable coronary artery disease is reduction of symptoms, limitation of myocardial cell damage and prevention of future cardiac events. The initial management of patients with symptoms suggesting unstable coronary artery disease includes admission to a coronary care unit, bed rest with continuous 12-lead ECG or vectorcardiography and repeated blood samples for analyses of biochemical markers of myocardial cell damage for at least 6-8 hours (67). Initial treatment include β-blockers and/or oral long-acting calcium antagonists, nitro-glycerine, angiotensin converting enzyme-inhibitors and statins (67).
Early coronary angiogram and revascularisation have in recent studies been shown to reduce mortality, risk of myocardial infarction and the need for readmission in unstable coronary artery disease patients with ST-depression on admission ECG or elevation of markers of myocardial cell injury, i.e. troponin or CK-MB (68, 69).

Platelet inhibition
Platelet inhibition by ASA is a well-documented standard therapy in unstable coronary artery disease with an average reduction in occurrence of death and myocardial infarction of more than 50% (70-73). ASA ≥ 300 mg should be administered as soon as possible after presentation and should be continued indefinitely with a daily dose of at least 75 mg (74). A thienopyridine, i.e. clopidogrel, should be given to patients unable to use ASA. There are additional benefits by combining clopidogrel and ASA in unstable coronary artery disease (75). In patients for whom an early invasive procedure is planned, a glycoprotein IIb/IIIa-inhibitor might be added to ASA and unfractionated (UF) or low molecular weight (LMW) heparin, while awaiting and during the procedure (69, 76, 77).

Thrombin inhibition by heparins
Heparin is a key component in the antithrombotic management of unstable coronary disease. The results of studies with the combination of ASA and either UF or LMW heparin compared with ASA alone have shown reductions in the short-term rate of death or myocardial infarction of approximately 50% to 60% (72, 73, 78-80). UF heparin is administered as an intravenous bolus followed by an infusion, and should be titrated to an aPT time of 1.5 - 2.5 x control (44). UF heparin has several limitations, including high inter-individual variability of anticoagulant response, a non-linear dose-response, a requirement for endogenous cofactors and non-specific binding to plasma proteins and cells (42). The LMW heparins have, compared with UF heparin, increased bioavailability by subcutaneous administration, less plasma protein binding and 2-4-fold longer half-life (81). A large meta-analysis has shown modest, non-significant 12% reduction in the risk of death or myocardial infarction by LMW heparin compared with UF heparin for an equal duration of therapy (82). Since the LMW heparins are easier to administer and require no monitoring they have become the standard treatment of unstable coronary artery disease.

At discontinuation of UF heparin treatment, the disease process may within hours be reactivated with subsequent new ischemic events (83, 84) and, similarly, clinical reactivation was evident a few days after lowering the dose of LMW heparin (80). Concomitant ASA treatment has been suggested to reduce the reactivation (83).
Direct thrombin inhibitors

Treatment with UF and LMW heparin in unstable coronary artery disease has several potential shortcomings, such as limited effect on clot-bound thrombin, reactivation after cessation of treatment and, despite prolonged therapy, considerable risk for recurrent ischemic events. Therefore, there is an extensive search for new and more effective anticoagulant agents for the treatment of acute coronary syndromes.

Direct thrombin inhibitors work independently of antithrombin or heparin cofactor II and are able to inhibit both fibrin-bound and free thrombin (85). Hirudin, found in the saliva of the medicinal leech, was already in the late 19th century characterised to have antithrombotic properties. Recombinant hirudin and several variants of recombinant hirudin with exchange of individual amino acids have been developed. These direct thrombin inhibitors bind thrombin both at the active site and at exosite 1. Synthetic low-molecular-weight direct thrombin inhibitors, e.g. argatroban, efegatran and inogatran, have also been developed. These only bind the active site of thrombin (86).

Inogatran (AstraZeneca, Mölndal, Sweden) is a synthetic low molecular weight peptidomimetic, based on the tripeptide R-Phe-Pro-Arg, which in turn is based on the structure of fibrinopeptide A close to the thrombin splitting site in the Aα chain of fibrinogen (87). Its biological half-life in plasma is approximately one hour and it is evenly eliminated in urine and bile (88). It is a selective competitive direct thrombin inhibitor, reversibly binding the catalytic site of thrombin, thereby inhibiting both clot-bound thrombin and thrombin in the fluid phase (89, 90). In vitro, inogatran inhibits platelet aggregation and has negligible effects on fibrinolysis (87). Inogatran was more effective than ASA or UF heparin in a porcine model of coronary thrombosis (91), and seemed well tolerated in pilot studies in healthy volunteers and unstable coronary artery disease patients (37). Therefore, the evaluation of the appropriate dose, clinical efficacy and safety of inogatran in a large-scale trial of unstable coronary artery disease was highly warranted.
Aims of the study

The aim of the study was to investigate the degree of coagulation activity and inflammation in unstable coronary artery disease and to evaluate the influences of thrombin inhibition with inogatran, a direct thrombin inhibitor, or UF heparin.

More specific aims were to evaluate:

- the anticoagulant efficacy, i.e. aPT time prolongation, of different doses of inogatran or UF heparin
- the relation of different aPT times during thrombin inhibition and subsequent manifestations of myocardial ischemia
- the degree of inflammation and coagulation activity, reflected by molecular markers of coagulation activity and acute-phase proteins
- the influence of treatment with inogatran or UF heparin on inflammation and coagulation activity
- the reactivation of coagulation activity after cessation of treatment with inogatran or UF heparin
- the relation of myocardial cell injury and the degree of inflammation and coagulation activity
- the degree of inflammation and coagulation activity in relation to short-term manifestations of myocardial ischemia and long-term mortality
TRIM - Thrombin Inhibition in Myocardial Ischemia

Unstable angina or non-Q-myocardial infarction
randomisation within 24 hours from chest pain

72 hours study drug infusion with:

- **Inogatran low dose**
  - 302 patients
  - Substudy inflammation & coagulation: 83 patients*

- **Inogatran medium dose**
  - 303 patients
  - Substudy inflammation & coagulation: 84 patients*

- **Inogatran high dose**
  - 299 patients
  - Substudy inflammation & coagulation: 74 patients*

- **Heparin (unfractionated)**
  - 305 patients
  - Substudy inflammation & coagulation: 79 patients*

* Consecutive patients in 19 of 61 centres

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**Primary composite endpoint**
death, myocardial (re-)infarction, refractory or recurrent angina after 7 days

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**Secondary composite endpoints**
death, myocardial (re-)infarction, refractory or recurrent angina at 72 hours and 30 days
death, myocardial (re-)infarction or refractory angina at 72 hours, 7 and 30 days
death or myocardial (re-)infarction at 72 hours, 7 and 30 days
Material and methods

Patients and design

The Thrombin Inhibition in Myocardial Ischemia (TRIM) study (92), a dose-finding and safety study, enrolled 1209 unstable coronary artery disease patients (paper I) in 61 Scandinavian centres during 1994 and 1995, figure 2. The substudy population (papers II-V) consisted of 320 consecutive patients in 19 of the 61 participating centres of the TRIM study, figure 2. Eligible for inclusion were men and post-menopausal women between 25 and 80 years of age with unstable angina, defined as new onset of ischemic chest pain or rapid deterioration in previously stable angina during the last four weeks, or suspicion of a non-ST-elevation myocardial infarction. This clinical diagnosis had to be supported by either changes in ECG at rest, e.g. ST-depression or T-wave inversion, or previous manifestations of coronary artery disease, e.g. previous myocardial infarction, bypass surgery or positive exercise test.

Patients were, within 24 hours from the qualifying episode of chest pain, randomised to blinded treatment with UF heparin or one of three different fixed doses of inogatran. Low, medium and high dose inogatran patients received intravenous bolus injections of 1.10, 2.75 and 5.50 mg respectively, followed by continuous infusion of 2.0, 5.0 and 10.0 mg/h respectively. Heparin was administered as a 5000 U intravenous bolus injection followed by infusion with 1200 U/hour. All infusions were to be continued for 72 hours and monitored with aPT time, as outlined below. ASA was recommended, while other platelet inhibitors and oral anticoagulants were not allowed. All other medication was to be given at the discretion of the responsible physician.

aPT time and dose adjustments

Blood samples for analyses of aPT time were obtained at baseline and during infusion at 6, 24 and 48 hours. They were immediately analysed with the routine method of the clinical chemistry laboratory in each of the participating centres. The study drug infusion rate was reduced by 10% if the aPT time was 3-4 times the lower reference level at each centre, and by 20% if the aPT time exceeded 4 times the reference level. If the aPT time exceeded 6 times the reference value, the infusion was temporarily stopped for 1-2 hours and then re-started with a 20% reduction. The aPT time was re-measured 4 hours after all adjustments, and if needed further dose reductions were made. Upward adjustments of infusion rates were not allowed.
Inogatran plasma concentrations and thrombin time
Blood samples were after 6 hours of inogatran infusion obtained from 80 low, 82 medium and 71 high dose patients and after 48 hours from 207 low, 199 medium and 208 high dose patients respectively. The plasma concentration of inogatran was determined using a liquid phase chromatography-mass spectrometry method after solid-phase extraction of the compound from plasma (90).

In 67 patients thrombin time was measured after 6 hours infusion to correlate anticoagulant effect of inogatran to plasma concentrations.

Substudy blood samples
Venous blood samples were obtained, preferably by direct veni-puncture, in citrated tubes for the analyses of markers of coagulation and inflammation and in heparin tubes for the analysis of troponin T. The first 2 mL blood were disposed and samples were within 30 minutes centrifuged at 2000G for twenty minutes. Aliquots of 500 µL plasma in Eppendorf tubes were frozen and stored at -70 °C until analysis.

Markers of inflammation
Markers of inflammation were analysed pre-treatment, during study drug infusion after 24 and 72 hours, and after 96 hours, i.e. 24 hours after cessation of treatment, and 30 days after randomisation.

Fibrinogen
Fibrinogen was analysed by rate nephelometry with a Beckman Array protein system (Beckman Instruments Inc). The assay was performed according to the recommendations by the manufacturer except that goat anti-human fibrinogen (Atlantic Antibodies) was used. The assay was calibrated against a human plasma standard (Behring Diagnostics GmbH), reference range 2.0 to 3.6 g/L, total CV ≤ 8%.

C-reactive protein
C-reactive protein was analysed with the Immulite system, a chemiluminescent enzyme-labelled immunometric assay based on ligand-labelled monoclonal antibody and separation by anti-ligand-coated solid phase (Immulite CRP, Diagnostic Products Corporation) (93). The detection limit is 0.1 mg/L and the reference range 0.1 to 4.6 mg/L. Total CV 5.6% at 2 mg/L.

Molecular markers of coagulation activity
Markers of coagulation activity were analysed pre-treatment, during study drug infusion after 6 hours, 24 and 72 hours, and after 76 and 96 hours, i.e. 4 and 24 hours after cessation of treatment, and 30 days after randomisation.

Prothrombin fragment 1+2 (F1+2)
A sandwich ELISA technique (Enzygnost F1+2, Behringwerke AG) was used for the determination of F1+2 (94), with reference intervals for healthy individuals 0.4-1.5
nmol/L and intra- and inter-assay variations (CV) between 5 and 9% (39).

**Thrombin-antithrombin-complex (TAT)**
A sandwich ELISA technique (Enzygnost TAT, Behringwerke AG) was used for the determination of TAT (95) with reference intervals for healthy individuals 1.2 - 5.0 µg/L and intra- and inter-assay variations (CV) between 5 and 9% (39).

**Soluble fibrin**
Soluble fibrin was assessed by a chromogenic method (SF, Chromogenix) where the stimulatory effect of SF on the tissue-type plasminogen activator catalysed conversion of plasminogen to plasmin is exploited, upper reference limit 25 nmol/L, CV 7% (96).

**D-dimer**
A sandwich ELISA technique (TintElize D-dimer, Biopool) was used for the analysis of D-dimer, reference range 10-130 µg/L, CV 10% (97).

**Antithrombin**
Plasma antithrombin was analysed by a functional, chromogenic method (AT 400, Chromogenix) using factor Xa inhibition (45). Reference interval 0.8-1.2 IE/mL, CV <3%.

**Marker of myocardial cell injury**
Troponin T was analysed pre-treatment and after 6 and 12 hours with a sandwich ELISA technique (ELISA Troponin(e) T) on an ES 300 analyser (Boehringer Mannheim GmbH). The detection limit is 0.012 µg/L and the reference range 0.0 to 0.02 µg/L, total CV 12% at 0.22 µg/L. The discriminator value for myocardial cell injury recommended by the manufacturer was 0.1 µg/L (98).

**End-points**
The primary composite end-point in the main TRIM study was the occurrence of death, nonfatal myocardial infarction (or reinfarction), refractory angina or recurrence of angina after 7 days. Secondary end-points were the same composite at 72 hours (the end of infusion) and at 30 days, or the occurrence of death, myocardial (re-)infarction or refractory angina (papers I-V) at 72 hours, 7 days and 30 days, or the occurrence of death or myocardial (re-)infarction at 72 hours, 3 days or 30 days (paper II).

Nonfatal myocardial (re-)infarction was defined as the occurrence of at least two of the following:
1) typical ischemic chest pain,
2) diagnostic ECG with a new Q-wave and/or ST-elevation or
3) typical elevation of cardiac enzymes, i.e. CK-MB (mass) ≥ the upper reference level on one occasion, or catalytic activity of CK, CK-B or CK-MB ≥ twice the upper reference level on one occasion, or catalytic activity of CK, CK-B or CK-MB ≥ the upper reference level on two occasions.

A new infarction (regarded as an event) was differentiated from an ongoing infarction at inclusion by normal cardiac
enzymes during the first six hours or the reappearance of the criteria above after normalisation of initially elevated enzymes. Refractory angina was defined as chest pain lasting ≥ 5 minutes with transient changes in the ECG, despite maximal ongoing medication, leading to an additional coronary intervention. An independent end-point committee evaluated all end-points (99).

Long-term follow-up data were obtained from 286 of the 320 patients at a median of 29 months (min 12 months, max 50 months). This information was obtained from hospital records and local or national registries. If data in these sources were missing the information was collected by telephone interview.

Statistics
Fisher exact test (2-sided) or chi-square tests as appropriate were used to judge significance of differences in proportions. Differences in the levels of markers were judged with non-parametric tests, between-group comparisons with Mann Whitney or Kruskal Wallis tests as appropriate, and within-group differences between different time-points with Wilcoxon signed rank tests. Correlations were evaluated with Spearman rank order coefficients. The influences of pre-treatment levels of different markers of inflammation and coagulation activity on the probability of death during long-term follow-up were evaluated with the log rank test.

The levels of aPT times and markers of inflammation and coagulation activity and their relation to short-term clinical outcome were evaluated for the substudy population, and separately for the inogatran treated patients (all three groups combined) and the UF heparin treated patients.

The change in the levels of the respective coagulation marker after 6 hours of infusion compared to the pre-treatment level was calculated individually and patients were grouped as either early decreasing or increasing in coagulation activity. The latter group included 3, 19, 54 and 6 patients with unchanged levels of F1+2, TAT, soluble fibrin or D-dimer, respectively. The 10-12 patients with a missing blood sample in any of the coagulation markers, either pre-treatment or at 6 hours, were excluded from the analyses of changes in coagulation activity during treatment in relation to clinical outcome.

The maximum change in levels of each coagulation marker from cessation of treatment, at 72 hours, to samples taken at 4 and 24 hours thereafter, was individually calculated to evaluate reactivation in coagulation activity after discontinuation of study drug. Multiple logistic regression analyses were performed evaluating the influence of molecular markers of inflammation, coagulation activity or aPT times, together
with all relevant baseline characteristics – age, gender, weight, smoking habits, congestive heart failure, diabetes mellitus, hypertension, stable angina, previous myocardial infarction and previous angioplasty or bypass surgery – on the clinical end-points of the study.

Possible interactions between inogatran or UF heparin treatment on the markers of coagulation and the risk of ischemic events were evaluated with multiple logistic regression analyses using interaction terms.
The TRIM study
In the main TRIM study (92), there were no significant differences between UF heparin and the combination of the three inogatran groups, or between any of the three inogatran groups concerning the primary end-point, a composite of death, myocardial (re-)infarction, refractory or recurrent angina after 7 days, table 1. Neither were there any differences between the four treatment groups in the same (secondary) composite end-point at 30 days. However, the composite of death and myocardial (re-)infarction and its combinations with refractory and recurrent angina were less common in the UF heparin group compared to the combined three inogatran groups during the 72 hours study drug infusion, table 1. The study drug was given according to protocol in 88 % of the patients, in a similar manner in all four treatment groups.

Table 1. Primary and secondary end-points at 72 hours, 7 and 30 days

<table>
<thead>
<tr>
<th></th>
<th>Inogatran low dose n=302</th>
<th>Inogatran medium dose n=303</th>
<th>Inogatran high dose n=299</th>
<th>Inogatran combined n=904</th>
<th>UF heparin n=305</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary end-point 7 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D+MI+Ref+Rec</strong></td>
<td>138 (45.7 %)</td>
<td>139 (45.9 %)</td>
<td>136 (45.5 %)</td>
<td>413 (45.7 %)</td>
<td>125 (41.0 %)</td>
</tr>
<tr>
<td><strong>Secondary end-points 7 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D+MI</strong></td>
<td>12 (4.0 %)</td>
<td>13 (4.3 %)</td>
<td>20 (6.7 %)</td>
<td>45 (5.0 %)</td>
<td>8 (2.6 %)</td>
</tr>
<tr>
<td><strong>D+MI+Ref</strong></td>
<td>25 (8.3 %)</td>
<td>27 (8.9 %)</td>
<td>31 (10.4 %)</td>
<td>83 (9.2 %)</td>
<td>25 (8.2 %)</td>
</tr>
<tr>
<td><strong>Secondary end-points 72 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D+MI</strong></td>
<td>11 (3.6 %)</td>
<td>6 (2.0 %)</td>
<td>12 (4.0 %)</td>
<td>29 (3.2 %)</td>
<td>2 (0.7 %)*</td>
</tr>
<tr>
<td><strong>D+MI+Ref</strong></td>
<td>15 (5.0 %)</td>
<td>14 (4.6 %)</td>
<td>20 (6.7 %)</td>
<td>49 (5.4 %)</td>
<td>8 (2.6 %)*</td>
</tr>
<tr>
<td><strong>D+MI+Ref+Rec</strong></td>
<td>19 (39.4 %)</td>
<td>114 (37.6 %)</td>
<td>108 (36.1 %)</td>
<td>341 (37.7 %)</td>
<td>90 (29.5 %)**</td>
</tr>
<tr>
<td><strong>Secondary end-points 30 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D+MI</strong></td>
<td>23 (7.6 %)</td>
<td>25 (8.3 %)</td>
<td>27 (9.0 %)</td>
<td>75 (8.3 %)</td>
<td>18 (5.9 %)</td>
</tr>
<tr>
<td><strong>D+MI+Ref</strong></td>
<td>38 (12.6 %)</td>
<td>37 (12.2 %)</td>
<td>39 (13.0 %)</td>
<td>114 (12.6 %)</td>
<td>36 (11.8 %)</td>
</tr>
<tr>
<td><strong>D+MI+Ref+Rec</strong></td>
<td>156 (51.7 %)</td>
<td>158 (52.2 %)</td>
<td>159 (53.2 %)</td>
<td>473 (52.3 %)</td>
<td>146 (47.9 %)</td>
</tr>
</tbody>
</table>

D = death, MI = myocardial (re-)infarction, Ref = refractory angina, Rec = recurrent angina
*p<0.05; **p<0.01; comparison between combined inogatran groups and UF heparin
**Troponin substudy**

In a separate TRIM substudy (100), Lüscher and colleagues related troponins to clinical outcome in 516 patients. Patients with elevated levels of troponin T, i.e. troponin T \( \geq 0.1 \text{ mg/L} \) either pre-treatment or after 6 hours, had an increased mortality at 30 days compared with patients with normal levels, 3.2% versus 0.4%, \( p=0.01 \). Similarly, the composite of death and myocardial (re-)infarction at 30 days was observed in 27 (11%) of the patients with elevated troponin T as compared to 12 (4%) of the patients without troponin elevation, \( p=0.006 \) (100).

**aPT time and the influence of inogatran or UF heparin (paper I)**

During inogatran infusion there was a dose-response relationship concerning median aPT times comparing the low, medium and high dose groups, figure 3. The aPT time levels were fairly stable throughout the infusion period. Dose-reductions were only carried out in four per cent or less in each group. Because of the overlap in aPT times between the three groups and in the absence of significant differences in clinical outcome, all inogatran patients were combined into one group in the further analyses of the

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**Figure 3.** Distribution of activated partial thromboplastin (aPT) times at different time-points before and during infusion in the four randomised treatment groups – low, medium, high dose inogatran and heparin – and for the combined group of all inogatran patients. Box-plots contain median, 1st and 3rd quartiles and in the whiskers 10th and 90th percentiles.
relations between aPT times and clinical outcome. Median aPT times for the combined inogatran group, n=904, were 29 seconds on admission and during infusion 44, 44 and 45 seconds, at 6, 24 and 48 hours respectively.

There was a positive correlation between plasma-concentration of inogatran and aPT times at 6 hours (data not shown) and at 48 hours, figure 4. Thrombin time had, as compared to aPT time, a better and more linear correlation to plasma concentrations of inogatran at 6 hours.

The UF heparin treated patients, n=305, had a clearly different pattern in aPT time response with a peak, median 69 seconds, after 6 hours of infusion and a much larger dispersion in aPT times than among the inogatran treated patients, figure 3. The dispersion diminished and the median aPT times decreased to 54 seconds after 24 hours and 49 seconds after 48 hours, in part because of frequent dose reductions, which were carried out in 41 % of the patients. Still, the median aPT times in patients without any dose reduction also decreased by almost 20 % between 6 and 24 hours.

**Figure 4.** Correlation of plasma concentration of inogatran and activated partial thromboplastin time (aPTT) at 48 hours, n=581, Spearman rank correlation coefficient r=0.7, p < 0.001
Clinical outcome in relation to aPT times (paper I)

Inogatran treatment

Adverse ischemic events, i.e. death, myocardial (re-)infarction or refractory angina, were more frequent in the group of patients with aPT time above the median at 6 hours. This difference in clinical outcome was already seen after the 72 hours of infusion therapy, table 2. A cluster of ischemic events was observed after cessation of treatment and to a similar degree in patients with aPT time levels above or below the median.

Dividing the group of inogatran treated patients into four groups according to quartiles of aPT time at 6 hours indicated direct relationship between higher aPTT and deteriorating clinical outcome, with an approximately doubled event rate in the top quartile as compared to the bottom quartile, p<0.05 (chi² test for trend) at 72 hours, 7 and 30 days respectively, figure 5. Similarly, aPT times above the median after 24 hours inogatran infusion was associated with higher adverse event rate.

Patients with aPT time above the median after 6 hours of inogatran infusion had lower median body weight, higher age and slightly higher pre-treatment aPT time, median 30 vs. 28 seconds, p<0.001. Congestive heart failure was also more common in patients with high aPT time. A multivariate logistic regression analysis - including aPT time on admission and at 6 hours, serum creatinine and all relevant baseline characteristics - demonstrated that age and aPT time above the median at 6 hours remained independent predictors of death, myocardial (re-)infarction or refractory angina up to seven days in patients treated with inogatran.

<table>
<thead>
<tr>
<th>aPT time after 6 hours</th>
<th>&lt; 44 seconds</th>
<th>≥ 44 seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=423</td>
<td>n=464</td>
<td></td>
</tr>
<tr>
<td>Death, MI or refractory angina, 72 hours</td>
<td>15 (3.5%)</td>
<td>33 (7.1%)</td>
</tr>
<tr>
<td>Death, MI or refractory angina, 7 days</td>
<td>28 (6.6%)</td>
<td>54 (11.6%)</td>
</tr>
<tr>
<td>Death, MI or refractory angina, 30 days</td>
<td>44 (10.4%)</td>
<td>68 (14.7%)</td>
</tr>
</tbody>
</table>

MI = myocardial (re-)infarction., Statistics: Chi² test
**UF heparin treatment**

The event rate during UF heparin infusion was low, with no significant difference in adverse events in relation to aPT times above or below median at 6 hours. In contrast to the finding in inogatran treated patients, aPT times above the median at 24 hours were related to favourable outcome. Only 1 (0.6 %) of the 155 patients with aPT time above, and 7 (4.8 %) of the 145 patients with aPT time below the median at 24 hours had an adverse event during the UF heparin infusion. This benefit was lost after cessation of treatment, because of clustering of events within 24 hours, and no difference in clinical outcome was seen at seven days.

**Figure 5.** Composite of death, myocardial (re-)infarction or refractory angina during seven days in inogatran treated patients, in relation to aPT times at 6 hours divided in quartiles; < 38 seconds (n=208), 38-43 seconds (n=215), 44-51 seconds (n=215), > 51 seconds (n=247). Statistics: Chi² test for trend
Baseline characteristics and the degree of inflammation and coagulation activity (papers II-IV)

Baseline characteristics for the substudy patients in the four randomised treatment groups are presented in table 3. There were no significant differences between patients in the four treatment groups in pre-treatment levels of fibrinogen, C-reactive protein, antithrombin, F1+2, TAT, soluble fibrin, D-dimer levels according to the Kruskal-Wallis test. Pre-treatment levels of markers of inflammation, fibrinogen and C-reactive protein, were significantly correlated. There were also significant correlations between pre-treatment levels of all molecular markers of coagulation activity, F1+2, TAT, soluble fibrin and D-dimer, and furthermore weak correlations between pre-treatment levels of markers of inflammation and coagulation.

Patients older than the median age in the substudy, 66 years, had significant higher pre-treatment levels of all the markers of inflammation and coagulation, except antithrombin. Women had significantly higher pre-treatment levels of antithrombin, F1+2 and D-dimer, but significantly lower soluble fibrin levels than men. No significant differences were found in pre-treatment levels of fibrinogen, C-reactive protein or TAT in relation to gender.

Table 3. Baseline characteristics and pre-treatment levels of markers of inflammation and coagulation activity

<table>
<thead>
<tr>
<th></th>
<th>Inogatran low dose n=83</th>
<th>Inogatran medium dose n=84</th>
<th>Inogatran high dose n=74</th>
<th>UF Heparin n=79</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64 (58; 73)</td>
<td>66 (58; 74)</td>
<td>66 (59; 74)</td>
<td>68 (58; 72)</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>78 %</td>
<td>67 %</td>
<td>65 %</td>
<td>78 %</td>
</tr>
<tr>
<td>Current smokers</td>
<td>19 %</td>
<td>19 %</td>
<td>15 %</td>
<td>23 %</td>
</tr>
<tr>
<td>Stable angina &gt;4 weeks</td>
<td>70 %</td>
<td>70 %</td>
<td>68 %</td>
<td>57 %</td>
</tr>
<tr>
<td>Hypertension</td>
<td>42 %</td>
<td>38 %</td>
<td>42 %</td>
<td>33 %</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>12 %</td>
<td>13 %</td>
<td>18 %</td>
<td>13 %</td>
</tr>
<tr>
<td>Previous MI</td>
<td>47 %</td>
<td>49 %</td>
<td>54 %</td>
<td>34 %</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>23 %</td>
<td>15 %</td>
<td>22 %</td>
<td>9 %</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>3.5 (3.0; 4.1)</td>
<td>3.4 (2.7; 4.2)</td>
<td>3.5 (3.1; 4.3)</td>
<td>3.3 (2.8; 3.6)</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>4.0 (2.2; 7.5)</td>
<td>3.9 (2.5; 6.6)</td>
<td>4.5 (2.9; 7.6)</td>
<td>3.5 (2.3; 6.2)</td>
</tr>
<tr>
<td>F1+2, nmol/L</td>
<td>1.30 (0.95; 1.78)</td>
<td>1.30 (0.97; 1.82)</td>
<td>1.32 (1.01; 1.78)</td>
<td>1.41 (1.10; 1.84)</td>
</tr>
<tr>
<td>TAT, mg/L</td>
<td>3.20 (2.40; 5.20)</td>
<td>3.05 (2.40; 5.23)</td>
<td>3.25 (2.38; 5.98)</td>
<td>3.60 (2.60; 7.15)</td>
</tr>
<tr>
<td>Soluble fibrin, nmol/L</td>
<td>11 (9; 14)</td>
<td>11 (9; 14)</td>
<td>12 (9; 15)</td>
<td>11 (9; 15)</td>
</tr>
<tr>
<td>D-dimer, g/L</td>
<td>108 (74; 169)</td>
<td>118 (75; 197)</td>
<td>114 (67; 200)</td>
<td>122 (72; 197)</td>
</tr>
<tr>
<td>Antithrombin, IU/mL</td>
<td>1.01 (0.92; 1.12)</td>
<td>1.07 (0.95; 1.16)</td>
<td>1.05 (0.94; 1.17)</td>
<td>1.01 (0.93; 1.09)</td>
</tr>
</tbody>
</table>

MI = myocardial (re-)infarction. Values are medians (1st and 3rd quartiles) or proportions.
Patients with pre-treatment levels in the upper tertile of fibrinogen, F1+2, TAT, soluble fibrin or D-dimer had in higher proportion concomitant diseases, for instance stable angina pectoris >4 weeks, diabetes mellitus and congestive heart failure. There were no significant differences concerning smoking habits, hypertension, previous myocardial infarction, angioplasty or bypass surgery between patients with high or low pre-treatment levels of markers of inflammation or coagulation activity.

Multivariate logistic regression analyses identified age as independent predictor of high pre-treatment levels, i.e. in the top tertile, of antithrombin, F1+2, soluble fibrin, D-dimer and fibrinogen. Furthermore, female sex was an independent predictor of high F1+2 levels. Congestive heart failure was an independent predictor of high fibrinogen levels. No independent predictors for high pre-treatment levels of TAT or C-reactive protein levels were found in the multivariate logistic regression models.

The influences of thrombin inhibition on inflammation (paper II) and coagulation activity (paper III)

Fibrinogen
There were no significant differences in fibrinogen levels between the four treatment groups, neither during the 72 hours of anticoagulant treatment, nor at 24 hours thereafter. Taking all the groups together, there was a significant increase in fibrinogen during the first 24 hours, which seemed to reach its maximum after 72-96 hours, table 4. After 30 days the fibrinogen levels still remained significantly higher than pre-treatment.

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen, g/L</th>
<th>C-reactive protein, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=314</td>
<td>n=306</td>
</tr>
<tr>
<td>Pre-treatment</td>
<td>3.4 (2.9; 4.0)</td>
<td>4.0 (2.4; 6.9)</td>
</tr>
<tr>
<td>24 hours</td>
<td>3.7 (3.1; 4.5)*</td>
<td>6.6 (3.3; 19.7)*</td>
</tr>
<tr>
<td>72 hours</td>
<td>3.9 (3.3; 5.0)*</td>
<td>6.9 (3.6; 16.3)*</td>
</tr>
<tr>
<td>96 hours</td>
<td>4.0 (3.3; 5.0)*</td>
<td>6.5 (3.2; 13.3)*</td>
</tr>
<tr>
<td>30 days</td>
<td>3.6 (3.0; 4.3)*</td>
<td>3.2 (2.3; 6.3)*</td>
</tr>
</tbody>
</table>

Values are medians (1st and 3rd quartile), *p<0.001 and †p=0.08 for the difference compared to pre-treatment levels, Wilcoxon signed rank test.
C-reactive protein

There were no differences in C-reactive protein levels between the four treatment groups, neither during the 72 hours of anticoagulant treatment, nor at 24 hours thereafter. Overall, the increase in C-reactive protein levels was, compared to fibrinogen, more pronounced with a large upward dispersion during the first 24-96 hours, table 4. In contrast to fibrinogen, the levels of C-reactive were markedly decreased from day 4 to day 30, when the level tended to be lower than pre-treatment.

Prothrombin fragment 1+2

UF heparin was more effective than inogatran in early suppressing the levels of F1+2, and 95% of the UF heparin patients had a decreased F1+2 level at 6 hours as compared to 75%, 79% and 86% of the patients in the low, medium and high dose inogatran groups respectively, p=0.002 (UF heparin vs. inogatran groups combined). After the early decrease in the UF heparin group there was a significant increase in F1+2 to a level slightly above the pre-treatment level at cessation of treatment. In contrast, F1+2 levels during inogatran treatment remained reduced throughout the infusion period, all groups significantly different from the UF heparin group at 72 hours, figure 6.

Figure 6. The median change in relation to baseline for F1+2, TAT, SF and fibrin D-dimers. *p<0.05 **p<0.01 ***p<0.001. Significant differences between the groups, in specific points in time are indicated by dashed lines and levels of significance.

- = LDI, = MDI, = HDI, = Heparin
**Thrombin-antithrombin complex**

The TAT levels decreased during infusion in all groups. There was a greater reduction at 6 hours in the high dose inogatran and the UF heparin groups, than in the medium and low dose inogatran groups, figure 6. A dispersion upwards was seen in different phases of the study in all groups.

**Soluble fibrin**

Soluble fibrin levels were significantly decreased at 6 and 24 hours during infusion only in the UF heparin group. No dose-response relationship could be identified in the inogatran groups, figure 6. There was a marked upwards dispersion from the median value during the infusion period and up to 24 hours thereafter in all inogatran groups, but not in the UF heparin group.

**D-dimer**

The D-dimer levels were unchanged after 6 hours treatment but decreased after 24 h of infusion in all four groups. No dose-response relationship in the inogatran groups could be identified, figure 6.

**Antithrombin**

In the UF heparin group, the level of antithrombin decreased during infusion and reached the lowest level four hours after cessation of infusion. Antithrombin increased significantly within 24 hours after termination of UF heparin infusion, but was still significantly lower than on admission. Antithrombin remained near the admission level in all three inogatran groups without any dose-response relationship, and therefore no further analyses of antithrombin in relation to clinical outcome were performed (paper III).

**Reactivation of coagulation activity after cessation of thrombin inhibition**

There was a significant increase in levels of F1+2 during the first 24 hours after cessation of study drug in all four treatment groups. F1+2 levels at 76 and 96 hours in the UF heparin group were significantly increased compared to the pre-treatment level, while F1+2 levels in the inogatran groups returned to pre-treatment levels, figure 6. The TAT levels increased in a majority of the patients in each treatment group, although to significantly higher levels within 24 hours after cessation only in the low and medium dose inogatran groups. There was an increase to significantly higher D-dimer levels 24 hours after cessation of treatment in all four groups, most pronounced after cessation of UF heparin.

The reactivation increase in levels of F1+2 was significantly greater in patients with pre-treatment levels in the top tertile of F1+2 in the medium and high dose inogatran groups and the UF heparin group. Similarly, in all four groups there was a significantly greater increase in levels of D-dimer after cessation of treatment in patients with high pre-treatment D-dimer levels.
At 30 days there were no significant differences in levels of F1+2, TAT, soluble fibrin and D-dimer between patients in the four treatment groups. The 30-day level of F1+2 in the total substudy cohort was higher than pre-treatment, median 1.54 vs. 1.33 nmol/L, p<0.001, and similarly the 30-day level of D-dimer was higher, 125 mg/L vs. 116 mg/L, p<0.001. There was also a great upward dispersion in D-dimer at 30 days, figure 6. In contrast, TAT was significantly lower at 30 days, median 3.2 mg/L as compared to pre-treatment 3.2 mg/L, p=0.001, and no significant difference was found in levels of soluble fibrin.

Signs of myocardial cell injury and inflammation (paper II) and coagulation activity (paper V)

Pre-treatment elevation of troponin T ≥0.1 µg/L was found in samples from 138 (44 %) of the 317 analysed substudy patients. These troponin-positive patients had significantly higher levels of fibrinogen and C-reactive protein pre-treatment and at 24-96 hours, figure 7a. As well in troponin-positive as troponin-negative patients there was a significant further increase in levels of fibrinogen and C-reactive protein during the first 24-96 hours, figure 7a. This increase in levels of fibrinogen and C-reactive protein in troponin-negative patients was still significant when excluding patients with clinical events, i.e. death, myocardial (re-)infarction or refractory angina during the study.

The F1+2 and D-dimer levels were also significantly higher pre-treatment and at 24-96 hours in troponin-positive patients, figure 7b and c. Pre-treatment levels of soluble fibrin tended to be higher in the troponin-positive patients and soluble fibrin levels were significantly higher at 24-96 hours. The TAT levels were not related to troponin T at any sampling time-point during the study. At 30 days follow-up there was no significant difference in any of the four molecular markers of coagulation activity in relation to pre-treatment troponin T.

Similar results were found using 0.06 µg/L as cut-off limit for pre-treatment troponin T or positive troponin T in serial samples within the first 12 hours.
Figure 7a. Distribution of molecular markers of inflammation in relation to pre-treatment troponin T < 0.1 µg/L (white boxes) or ≥ 0.1 µg/L (shaded boxes). Box-plots contain median, 1st and 3rd quartiles and in the whiskers 10th and 90th percentiles. Statistics: Mann Whitney test for between-group comparisons, Wilcoxon signed rank test (within-group) compared to pre-treatment, *p<0.05, **p<0.01, ***p<0.001
**Figure 7b.** Distribution of molecular markers of coagulation activity in relation to pre-treatment troponin T < 0.1 µg/L (white boxes) or ≥ 0.1 µg/L (shaded boxes). Box-plots, symbols and statistics as in figure 7a.
Figure 7c. Distribution of molecular markers of coagulation activity in relation to pre-treatment troponin T < 0.1 µg/L (white boxes) or ≥ 0.1 µg/L (shaded boxes). Box-plots, symbols and statistics as in figure 7a.
Clinical outcome in relation to the degree of inflammation and coagulation activity (papers II, IV and V)

Pre-treatment inflammation (paper II) and coagulation activity (paper IV)
High fibrinogen levels, i.e. in the pre-treatment top tertile, were related to increased risk of ischemic events at 30 days, table 5. However, the rate of adverse ischemic events tended to be lower during the ongoing anticoagulant treatment in patients with high pre-treatment fibrinogen, but within the first four days after cessation of treatment there was a clinical reactivation with a more than doubled ischemic event rate. Thus, 13 of the 100 patients with pre-treatment levels of fibrinogen in the top tertile (and without ischemic events during treatment) died or experienced a myocardial (re-)infarction or refractory angina from cessation of treatment to 30-days follow-up, as compared to 10 (4.8 %) of the 208 patients with lower pre-treatment fibrinogen levels, p=0.009.

Pre-treatment C-reactive protein levels were not related to the composite of death, myocardial (re-)infarction or refractory angina during or after anticoagulant treatment. However, high pre-treatment C-reactive protein, i.e. in the top tertile, was significantly related to increased mortality at 30 days, table 5.

Table 5. Clinical outcome at 30 days in relation to pre-treatment levels of fibrinogen and C-reactive protein

<table>
<thead>
<tr>
<th>Fibrinogen, g/L</th>
<th>C-reactive protein, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3.12</td>
<td>3.12–3.80</td>
</tr>
<tr>
<td>n=105</td>
<td>n=108</td>
</tr>
<tr>
<td>Death, MI or ref angina</td>
<td>10 (9.5)</td>
</tr>
<tr>
<td>Death or MI</td>
<td>7 (6.7)</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
</tr>
</tbody>
</table>

MI = myocardial (re-)infarction, Ref = refractory. Values shown are number of patients with percentages of the group in parenthesis. P-values are calculated by Chi-square or †Fisher exact test (2-sided) for top versus bottom + middle tertiles.
The pre-treatment levels of F1+2 were not related to short-term clinical outcome. High pre-treatment levels of TAT, i.e. in the top tertile, tended to be associated with better clinical outcome during the anticoagulant treatment, table 6. Similarly, no ischemic event was recorded during anticoagulant treatment in patients with high pre-treatment levels of soluble fibrin. During treatment there was a trend to a lower event rate in patients with high pre-treatment levels of D-dimer, but this difference decreased during follow-up.

These results were more pronounced in the group of inogatran treated patients, in which patients within the top tertile of TAT had a better outcome both at the end of infusion (p=0.02) and at 30 days follow-up (p=0.03). Accordingly, soluble fibrin levels in the top tertile were related to better outcome at the end of inogatran infusion (p=0.02), and with a trend to a lower event rate also at 30 days follow-up (p=0.06).

Table 6. Clinical outcome in relation to pre-treatment levels of coagulation markers

<table>
<thead>
<tr>
<th>prothrombin fragment 1+2, nmol/L</th>
<th>thrombin-antithrombin, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.14 n=103</td>
<td>1.14-1.60 n=105</td>
</tr>
<tr>
<td>Events 72 hours</td>
<td>5 (4.9%)</td>
</tr>
<tr>
<td>Events 7 days</td>
<td>10 (9.7%)</td>
</tr>
<tr>
<td>Events 30 days</td>
<td>11 (11%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>soluble fibrin, nmol/L</th>
<th>D-dimer, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10 n=97</td>
<td>10-13 n=120</td>
</tr>
<tr>
<td>Events 72 hours</td>
<td>5 (5.2%)</td>
</tr>
<tr>
<td>Events 7 days</td>
<td>9 (9.3%)</td>
</tr>
<tr>
<td>Events 30 days</td>
<td>12 (12%)</td>
</tr>
</tbody>
</table>

Events denote a composite of death, myocardial (re-)infarction or refractory angina.
Values shown are number of patients with percentages of the group in parenthesis. P-values are calculated by Chi² or †Fisher exact test (2-sided) for top tertile versus combination of bottom + middle tertiles.
Pre-treatment levels, i.e. in the top tertile, of fibrinogen and D-dimer were predictors of long-term mortality, and there was furthermore a trend to a relation between high pre-treatment levels of C-reactive protein, F1+2 and TAT and increased long-term risk of mortality, figure 8.

Changes in coagulation activity during treatment (paper IV)
High pre-treatment levels were significantly correlated (p<0.001) to decreased levels of all four molecular coagulation markers respectively after 6 and 24 hours of anticoagulant treatment.

In 257 (81 %) of the patients there was, as compared to the pre-treatment levels, a reduction in F1+2 levels after 6 hours anticoagulant treatment. The 51 patients with unchanged or early increased levels had an approximately doubled event rate during the 30-day follow-up, table 7. Similarly, reduction in TAT levels at 6 hours was seen in 189 (61 %) of the patients and the event rate during follow-up was more than two-fold elevated in the group of patients with unchanged or early increased levels compared to the group with decreased TAT levels, table 7.

Decreased levels of soluble fibrin at 6 hours were seen in 145 (47 %) of the patients. The 7-days event rate was doubled for patients with unchanged or increased levels of soluble fibrin at 6 hours compared to the group with decreased levels, table 7. Decreased D-dimer levels were not related to clinical outcome.

| Table 7. Clinical outcome in relation to changes in levels of coagulation markers from pre-treatment to 6 hours |
|-------------------------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Change 0-6 h prothrombin fragment 1+2 | Decreasing n=257 | Increasing n=51 | Decreasing n=189 | Increasing n=121 | Events 72 hours | Events 7 days | Events 30 days |
| | Events | | | | Events | | |
| Events 72 hours | 9 (3.5%) | 3 (5.9%) | 0.4† | 4 (2.1%) | 8 (6.6%) | 0.07† |
| Events 7 days | 17 (6.6%) | 8 (16%) | 0.03 | 10 (5.3%) | 15 (12%) | 0.02 |
| Events 30 days | 25 (9.7%) | 10 (20%) | 0.04 | 15 (7.9%) | 20 (16%) | 0.02 |
| Change 0-6 h soluble fibrin | Decreasing n=145 | Increasing n=164 | Decreasing n=166 | Increasing n=142 | Events 72 hours | Events 7 days | Events 30 days |
| | Events | | | | Events | | |
| Events 72 hours | 2 (1.4%) | 10 (6.1%) | 0.03 | 7 (4.2%) | 5 (3.5%) | 1† |
| Events 7 days | 8 (5.5%) | 17 (10%) | 0.12 | 13 (7.8%) | 12 (8.5%) | 0.8 |
| Events 30 days | 13 (9.0%) | 33 (13%) | 0.2 | 19 (11%) | 16 (11%) | 1 |

Events denote a composite of death, myocardial (re-)infarction or refractory angina.
Values shown are number of patients with percentages of the group in parenthesis.
P-values are calculated by Chi² or †Fisher exact test (2-sided).
**Figure 8.** Probability of death during long-term follow-up, median 29 months; min 12 months, max 50 months; in relation to pre-treatment levels of the respective marker of inflammation or coagulation divided in tertiles. Statistics: Log rank test
**Reactivation after cessation of anticoagulant treatment (paper IV)**

There were signs of reactivation of coagulation activity within 24 hours after cessation of anticoagulant treatment with an increase in F1+2, TAT, SF and D-dimer levels in 85 %, 69 %, 55 % and 83 % of the patients respectively. An adverse clinical event early after cessation of treatment, i.e. at days 4-7, occurred in 9 (5.0 %) of the 181 patients with signs of reactivation in thrombin generation by an increase in TAT levels after discontinuation of study drug infusion. In contrast, none of the 83 patients with unchanged or decreased TAT levels after cessation of treatment died or experienced a myocardial (re-)infarction or refractory angina at days 4-7 (p=0.06). The median increase in TAT levels within 24 hours after cessation of treatment was 4.6 mg/L in patients with an adverse clinical event days 4-7 and 1.0 mg/L in patients without an event after cessation of treatment (p=0.03). Increased F1+2, SF and D-dimer levels after cessation of treatment were, although not significant, related to a 38 %, 64 % and 57 % higher adverse event rate respectively at days 4-7.

**Treatment with ASA**

At inclusion in the study 54 % of the patients were on treatment with ASA, and at cessation of the study drug 95 % of the patients were on ASA. 656 of them with a daily dose equal to or lower than 75 mg (median). In 487 patients the daily ASA doses were higher than the median, predominantly 160 mg, and the highest recorded daily dose was 500 mg. ASA treatment did not protect from clinical reactivation and there was nothing in favour of higher doses (101), figure 9.

**Myocardial cell damage and changes in coagulation activity (paper V)**

Troponin-positive patients tended to have worse short-term clinical outcome (100), without relation to markers of coagulation activity. Among the troponin-negative patients, i.e. with pre-treatment troponin T < 0.1 mg/L, those with unchanged or early increased F1+2 or TAT during treatment had a cluster of ischemic events within 24 hours after cessation of the study drug, figure 10. The 30-day ischemic event rate was 26 % in troponin-negative patients with unchanged or early increased F1+2, and 5.7 % in patients with decreased F1+2, p=0.001. Similarly, 18 % of the troponin-negative patients with unchanged or early increased TAT, and 4.6 % of the

![Figure 9. Composite of death, myocardial infarction or refractory angina in relation to ASA dose, Chi²-test](image-url)
patients with decreased TAT, had an ischemic event within 30 days, \( p=0.005 \). Similar results were found using 0.06 \( \mu \text{g/L} \) as cut-off limit for pre-treatment troponin T or positive troponin T in serial samples within the first 12 hours.

a) Pre-treatment troponin T < 0.1 \( \mu \text{g/L} \)

![Graph showing increased or unchanged levels of prothrombin fragment 1+2 and thrombin-antithrombin complex in patients with decreased TAT](image)

**Figure 10.** Composite of death, myocardial (re-)infarction or refractory angina in patients with a) pre-treatment troponin T < 0.1 \( \mu \text{g/L} \) or b) pre-treatment troponin T \( \geq 0.1 \mu \text{g/L} \), in relation to early decreased versus unchanged or increased levels of molecular markers of thrombin generation. Vertical dotted line represents termination of anticoagulant treatment. Statistics: Chi-square or *Fisher exact test

b) Pre-treatment troponin T \( \geq 0.1 \mu \text{g/L} \)

![Graph showing increased or unchanged levels of prothrombin fragment 1+2 and thrombin-antithrombin complex in patients with decreased TAT](image)
Multivariate logistic regression analyses including molecular markers of coagulation activity (papers IV and V)

Multivariate logistic regression analyses included all relevant baseline characteristics and time from start of qualifying episode of chest pain to randomisation, pre-treatment troponin T (cut-off ≥ 0.1 µg/L) and unchanged/increased levels of either of F1+2 or TAT during the first 6 hours. Unchanged/increased levels of any of these two coagulation markers, together with higher age and congestive heart failure, were found to be independent predictors of death, myocardial (re-)infarction or refractory angina up to 30 days. The use of both these coagulation markers in the model indicated that only unchanged/increased levels of F1+2, age and congestive heart failure were independent predictors of short-term adverse outcome.

Logistic regression models using interaction terms did not indicate a difference between inogatran or UF heparin treatment concerning the prediction of clinical outcome by increasing coagulation activity during treatment.
Discussion

Atherosclerosis, inflammation, plaque disruption and subsequent intracoronary thrombus formation are the predominant causes of unstable coronary artery disease (1, 2). Thus, a key component in the management of these patients is antithrombotic treatment, i.e. platelet inhibition with ASA and antithrombin-dependent thrombin inhibition with heparin (67, 102). Recently, the combination of ASA and heparin with other platelet inhibitors, i.e. glycoprotein IIb/IIIa-receptor inhibitors and ADP receptor inhibitors, have been shown to further reduce the rate of ischemic events (75-77). Despite this regimen and early revascularisation routines, these patients are at considerable risk of new ischemic events within the first months (54, 68, 69). Therefore, and because of the several drawbacks in heparin treatment, there is an extensive search for new and more effective anticoagulant agents, such as direct thrombin inhibitors.

Inogatran (87), a synthetic low molecular weight direct thrombin inhibitor, has an anticoagulant effect independent of antithrombin and heparin cofactor II, and ability to inhibit both fibrin-bound and free thrombin (85, 103). However, none of the doses of inogatran in the TRIM study (92) proved more effective than UF heparin in preventing new ischemic events. In fact, during the 72 hours of study drug infusion the rates of ischemic events were significantly lower in the UF heparin group. Similar discouraging results, demonstrating no or only slight benefits with other direct thrombin inhibitors, such as recombinant hirudin (104, 105) or the synthetic efegatran (106) have been reported in previous clinical trials of these agents in unstable coronary artery disease.

Activated partial thromboplastin time
The aPT time is used to monitor the treatment effects of thrombin inhibitors as well in clinical trials as in routine care of patients with unstable coronary artery disease or myocardial infarction (44). However, there are many differences in dosing regimens as well as in the target aPT times (104, 107-110). This in-vitro method reflects surface-induced activation of the coagulation system and is mainly sensitive to the intrinsic pathway. However, the in-vivo coagulation activation in unstable CAD is mainly triggered by the extrinsic tissue factor/factor VII-pathway (24, 27). The relationship between aPT times and plasma concentrations of heparin or recombinant hirudin, currently the best evaluated direct thrombin inhibitor, is non-linear (111-113). There are also differences in aPT times at different laboratories as there is a considerable variation in available reagents giving widely different results (112, 114). A batch-to-batch variation of a particular aPT
time reagent may also occur (115). In vivo, r-hirudin infusion induces a dose-dependent predictable aPT time prolongation in patients with chronic stable CAD (116). In contrast, large interindividual variations of the aPT time response were seen in healthy volunteers at identical plasma levels of r-hirudin measured by anti-IIa-concentration (113).

The randomised low, medium and high doses of inogatran treatment in the TRIM study (92), aimed at aPT times in the low to medium range. The positive correlation between aPT times and adverse events was unexpected. However, a similar experience has been made in a porcine model of copper-coil-induced coronary artery thrombosis, where inogatran dosages resulting in aPT time prolongation of as little as 1.3 x baseline proved more effective in inhibiting thrombotic occlusion than heparin in dosages resulting in aPT time prolongation of 2.0 – 5.4 x baseline (91).

In contrast, during ongoing UF heparin infusion there was an association between higher aPT times and improved clinical outcome in the present study. This clinical benefit was lost within the first 24 hours after cessation of treatment because of reactivation with clustering of events. It is unknown whether the peak in aPT times at the beginning of the UF heparin infusion is advantageous. However, in a previous trial including almost 30.000 patients receiving intravenous heparin adjunctive to thrombolysis, a direct relationship was found between higher aPT times during the first 12 hours and death or myocardial re-infarction (117).

The results in the present study might be related to the differences in the mechanisms of action between UF heparin and direct thrombin inhibitors. Heparin exerts multiple effects on the coagulation system mainly by amplifying the effects of the natural anticoagulant antithrombin. Antithrombin, besides inactivation of and binding to thrombin, also inhibits several coagulation factors such as XIIa, XIa, IXa, Xa, all of which can alter the aPT time. Direct thrombin inhibitors, on the other hand, have a selective inhibitory effect on thrombin (factor IIa). Therefore, at comparable aPT times the thrombin activity is probably much lower during treatment with a direct thrombin inhibitor than during UF heparin treatment. However, lower aPT times during inogatran treatment were related to better clinical outcome, while better outcome was seen at higher aPT times during UF heparin treatment. It is thus unlikely that the aPT time, in general, is an appropriate indicator of the antithrombotic efficacy of anticoagulant treatment. Other indicators of the influence of different drugs on the thrombotic-antithrombotic balance therefore need exploration.

Thrombin time, another method to assess anticoagulant effect during thrombin inhibition, showed a more linear relationship with plasma concentrations of inogatran in the present study.
Nevertheless, the small number of samples made it impossible to relate the thrombin times to clinical outcome. An alternative sensitive assay to monitor the efficacy of direct thrombin inhibitors is the ecarin clotting time (118). This method is insensitive to UF heparin and has low interindividual variability and strong linear correlation to plasma concentrations of recombinant hirudin in healthy volunteers (119). Further evaluation of this method in clinical trials of direct thrombin inhibitors thereby seems warranted.

The influences of thrombin inhibition on inflammation and coagulation activity

Active inflammation, reflected by elevated levels of interleukins (12) and acute-phase proteins, e.g. fibrinogen and C-reactive protein (13, 14), in unstable coronary artery disease has previously been reported. In the present study, the changes of the levels of fibrinogen and C-reactive protein had different time-courses. The fibrinogen level started to rise at 24 hours, seemed to reach its maximum after 72-96 hours and remained at 30 days higher than before treatment. The initial increase in levels of C-reactive protein occurred somewhat earlier and was more pronounced. However, the C-reactive protein level at 30 days tended to be lower than before treatment. Neither the time-course of levels of fibrinogen nor C-reactive protein levels seemed affected by the treatment with different doses of inogatran or UF heparin. Therefore, the short-lasting elevation of C-reactive protein seems mainly related to a transient increase in inflammatory activity, while the lasting elevation of fibrinogen indicates the co-existence of a chronic low-grade inflammatory condition.

As expected, thrombin inhibitors reduce the degree of coagulation activity, although different molecular markers of coagulation activity have been measured in the clinical studies, with somewhat conflicting results for various thrombin inhibitors. During inogatran infusion there was a persistent reduction in the levels of F1+2, reflecting reduced thrombin generation, and a trend for a dose-response. These results are in accordance with significantly reduced F1+2 levels after 4 hours infusion in a previous small inogatran tolerability study (37). The reduced thrombin generation is probably due to suppression of the feed-back mechanism by which thrombin amplifies its own generation through activation of factors V and VIII (120, 121).

Similarly, in a previous study (41), reduced F1+2 levels were seen at cessation of 3-5 days treatment with recombinant hirudin in 31 unstable coronary artery disease patients. In contrast, in another study (40) there was no significant reduction in F1+2 levels after 6 or 48 hours in 231 patients on treatment with two different doses of recombinant hirudin. As blood sampling time-points and sample size in the latter study were similar to the present, these results suggest differences between inogatran and recombinant hirudin concerning their influence on thrombin generation.
The UF heparin treatment resulted, as compared to inogatran, in a more pronounced early decrease in F1+2 levels in the present study, probably through inhibition of factor Xa. The subsequent increase in F1+2 levels to levels slightly above the pre-treatment levels at cessation of UF heparin infusion might reflect restored thrombin generation, despite the on-going treatment. Similarly, in 164 unstable coronary artery disease patients randomised to UF heparin treatment in a previous study (40), the F1+2 levels were significantly reduced at 6 hours but then increased during continued UF heparin treatment. Likewise, in the FRISC coagulation substudy (39), there was an early reduction in levels of F1+2, followed by significant increase from day 2 to day 5 despite on-going treatment with dalteparin, a LMW heparin, in 170 unstable coronary artery disease patients. In contrast, two studies of 40 and 36 unstable coronary artery disease patients respectively, reported no effect of UF heparin on the levels of F1+2. These discrepant results might be due to the sample size or different time-points in blood sampling, with no assessment between 90 minutes and 24 hours (38, 41). Thus, UF heparin seems associated with an initial reduction in thrombin generation, which however is not maintained during prolonged treatment.

The TAT levels were decreased during infusion in all four treatment groups in the present study, with the greatest reduction seen in the high-dose and UF heparin groups. Inogatran may decrease TAT levels by forming inogatran-thrombin complexes or by inhibiting thrombin generation through the above mentioned positive feed-back mechanism (120, 121). UF heparin accelerates the formation of TAT, but the inhibition of factor Xa and thrombin exerted by UF heparin may also result in a decrease in TAT formation. The observed decrease in TAT levels in the UF heparin group is thus a net of two theoretically counteractive effects. The decreased TAT levels during infusion, observed in both inogatran and UF heparin treated patients, might be interpreted as a reduction in thrombin generation and activity. In the OASIS substudy (40), recombinant hirudin seemed more effective than UF heparin in the reduction of TAT levels. However, a short infusion (4 hours) of argatroban, another low molecular weight direct thrombin inhibitor, did not reduce the TAT levels in 43 unstable coronary artery disease patients (122). Overall, the interactions of heparin with antithrombin and inogatran with thrombin create difficulties in the interpretation of thrombin generation and activity, and comparison between different thrombin inhibitors, on the basis of TAT levels.

In all four treatment groups the D-dimer levels decreased in the present study, but this reduction seemed to be delayed, with unchanged levels at six hours in contrast to the rapid decrease in the molecular markers of thrombin generation. The decrease in D-dimer levels should
probably be interpreted as decrease in fibrin production (123), especially considering the simultaneous reduction in thrombin activity as indicated by reduction of soluble fibrin in the UF heparin group. A similar delayed decrease in levels of D-dimer was noticed in a previous study (40), both in recombinant hirudin and UF heparin treated patients.

Antithrombin decreased consistently only during UF heparin infusion. This gradual decrease is a well-known phenomenon accompanying heparin treatment (38, 124), and may be a therapeutical drawback, especially after cessation of the drug (125).

A reactivation increase in prothrombotic potential after the abrupt cessation of thrombin inhibition has been observed both clinically (83, 84) and biochemically (37, 40, 84, 122, 126), the latter defined as a rapid increase in the levels of molecular markers to levels as high, or even higher than, pre-treatment levels. In the present study there were signs of reactivation of coagulation activity within 24 hours after cessation of study drug treatment, with an increase in levels of F1+2, TAT and D-dimer in a majority of the patients, in accordance with previous studies.

The reactivation increase in F1+2 and D-dimer levels after cessation of treatment was significantly greater in patients with pre-treatment levels in the top tertile of the respective coagulation marker. These results suggest a persistent higher coagulation activity, only temporarily attenuated by anticoagulant treatment. Such a persistent prothrombotic state is furthermore supported by the finding of 30-day levels of F1+2 and D-dimer still higher than pre-treatment. Similarly, sustained high levels of F1+2 at 6 months after an episode of unstable angina or myocardial infarction have been reported (127).

Inflammation and coagulation activity in relation to myocardial cell injury

There are limited data concerning the inflammatory activity in patients with unstable angina in relation to signs of myocardial cell damage. Consistent with previous studies, the troponin-positive patients had significantly higher levels of fibrinogen and C-reactive protein pre-treatment and up to at least 96 hours thereafter in the present study. This enhanced acute-phase reaction is probably induced by the myocardial cell damage (18-20).

In a previous study (21) of unstable angina patients without troponin elevation, there was no increase in the levels of C-reactive protein up to 96 hours after admission, despite ischemic episodes during 24 hours continuous ECG-monitoring in the majority of the patients. In contrast, elevated levels of C-reactive protein despite normal troponin T on admission were reported in another study of unstable angina patients (14). In 8 of these 20 patients the C-reactive protein level was
doubled at 24-72 hours after admission, and all of them had an in-hospital major coronary event (death or myocardial infarction) or underwent urgent revascularisation.

The present study is thus the first to report a significant acute-phase response, with an early increase in fibrinogen and especially C-reactive protein, in a large number of unstable angina patients without any signs of myocardial cell damage. This acute-phase reaction indicates other sources of inflammatory activity besides myocardial cell injury in the acute phase of unstable coronary artery disease (25). However, the finding of a marked reduction in C-reactive protein early after the acute episode, without any influence of the antithrombotic treatment, indicates a spontaneous resolution of the acute inflammatory response. Still, a chronic low grade inflammatory activity might be present as indicated by the remaining high fibrinogen level.

There were higher levels of markers of thrombin generation (F1+2), thrombin activity (soluble fibrin) and fibrin turnover (D-dimer) in patients with elevated troponin T. The higher coagulation activity in troponin-positive patients persisted during the 72 hours treatment with UF heparin or inogatran and up to 24 hours thereafter. The results were similar using different cut-off limits for troponin T or analyses from serial samples of troponin T. Our data confirm earlier findings of enhanced soluble fibrin levels in small cohorts of troponin-positive unstable angina patients (48, 49) and suggest that early troponin elevation in patients with unstable coronary artery disease is associated with raised coagulation activity (50). This concept is also supported by more frequent intracoronary thrombi at angiography in patients with elevated troponin levels (51-53).

**The degree of inflammation and clinical outcome**

Several epidemiological studies have identified elevated levels of markers of inflammation, mainly C-reactive protein, as risk indicators for future cardiovascular events, both in apparently healthy men (128-131) and women (132). Furthermore, both fibrinogen and C-reactive protein have in several studies been found to indicate increased short- and long-term risk for adverse clinical outcome in unstable coronary artery disease (14, 60-64).

The differences in magnitude and time-course of elevations of fibrinogen and C-reactive protein levels observed in the present study might indicate different underlying mechanisms for their associations to new ischemic events in unstable coronary artery disease. High fibrinogen level was related to a trend to a lower rate of clinical events during ongoing anticoagulant treatment, but also a marked clinical reactivation after cessation of treatment with significantly higher rate of ischemic events at 30 days and
increased long-term mortality. Fibrinogen is directly involved in the thrombotic process, both in platelet aggregation cross-linking the glycoprotein IIb/IIIa-receptors on adjacent platelets, and in the coagulation cascade where it is cleaved by thrombin to soluble fibrin which subsequently polymerises to form the fibrin network stabilising the platelet clot (24). The sustained high levels of fibrinogen might thereby be associated to long-term risk of thrombosis and myocardial (re-)infarction (60).

On the other hand, the C-reactive protein level was only related to increased mortality and not to myocardial (re-)infarction in the present as in other trials (60, 63, 133, 134). Similar to these results, elevated levels of interleukin-6 have been related to increased mortality, but not an increase in the combined endpoint of death and myocardial infarction, in patients with unstable coronary artery disease (135). C-reactive protein is mainly regulated by interleukin-6, which is present in the atherosclerotic plaque and secreted by both endothelial cells, smooth muscle cells, macrophages and T-cells (136). High levels of interleukin-6 may thereby reflect greater atherosclerotic burden and/or increased inflammatory activity in the plaques. Thus, these plaques would be more vulnerable and prone to deeper fissuring, causing more severe thrombotic episodes (135). However, the largest increase in C-reactive protein was observed in patients with myocardial cell injury. Also the raised mortality was mainly seen in the top tertile of C-reactive protein. Therefore, much of the relation between the C-reactive protein level and mortality could be explained by an inflammatory reaction in the damaged myocardium.

Recently, polymorphism in exon 2 of the C-reactive protein gene has been described, although no association with C-reactive protein regulation or concentration is known (137). One might speculate that differences in the acute-phase response in unstable coronary artery disease might reflect differences in the individual response to inflammatory stimuli (1, 138). Interestingly, higher C-reactive protein levels have been reported in the offspring of patients with myocardial infarction (139). Thus, a low grade inflammatory activity might be indicated by slight elevation of C-reactive protein and long-lasting fibrinogen elevation and furthermore associated with a propensity to pronounced inflammatory response at plaque ruptures and/or thromboembolic myocardial damage. Furthermore, such a mechanism could explain the relations between transient pronounced elevation of C-reactive protein at acute events and long-term mortality, as well as the relations between slight elevations of fibrinogen and C-reactive protein and the risk of new ischemic events in the chronic stage (128-132).
The degree of coagulation activity and clinical outcome

There are few studies of the influence of anticoagulant treatment on the relation between the molecular markers of coagulation activity and clinical outcome in patients with unstable coronary artery disease. High levels of fibrinopeptide A sampled after the first 24 hours in patients with unstable angina without concomitant anticoagulant treatment have associated with adverse in-hospital outcome (death or myocardial infarction) (140). Lower levels of fibrinopeptide A, sampled after 24 hours treatment, have been connected to improved TIMI flow and greater cross-sectional area on repeated angiography in 163 patients with unstable angina on treatment with unfractionated heparin or recombinant hirudin (141). Another trial of 64 unstable angina or acute myocardial infarction patients, who developed in-hospital recurrent ischemia despite ≥ 72 hours unfractionated heparin infusion, found higher levels of F1+2 and fibrinopeptide A before the event (38).

In the present study we found a relation between higher baseline levels of molecular markers for thrombin generation and activity (TAT and soluble fibrin) and a lower rate of cardiac events, i.e. death, myocardial (re-)infarction or refractory angina, during anticoagulant treatment. These results were more pronounced during and after treatment with inogatran, then at UF heparin treatment. The patients with high pre-treatment levels of molecular markers of thrombin generation and activity and fibrin turnover were at a higher risk, as indicated by higher age and more concomitant diseases such as diabetes mellitus and congestive heart failure. Therefore, the association of high coagulation activity with improved short-term clinical outcome was intriguing. High baseline levels were however correlated to decrease of the respective coagulation marker during treatment. An early decrease in molecular markers of thrombin generation (F1+2 and TAT) and thrombin activity (soluble fibrin) during anticoagulant treatment was also related to significantly improved clinical outcome during 30-days follow-up. Furthermore, among the troponin-negative patients those with unchanged or increased thrombin generation early during treatment had a marked reactivation with a cluster of ischemic events within 24 hours after cessation of anticoagulant treatment, and a higher rate of ischemic events until 30 days. Also the multivariate analyses identified unchanged or early increased F1+2 or TAT, together with age, congestive heart failure and elevated troponin, as independent predictors of death, (re-)myocardial infarction or refractory angina at 30 days.

Thus, a plausible explanation for the relation between high coagulation activity and improved short-term clinical outcome might be that high initial levels of markers of coagulation activity identify patients with a thrombotic condition as the major cause of instability who are the best
responders to anticoagulant therapy. Such an explanation would be in accordance with the more pronounced decrease in coagulation activity during treatment and the decreased risk of ischemic events in patients with initially high coagulation activity. When considering the more pronounced decrease in F1+2, TAT and soluble fibrin during the first six hours of infusion in the UF heparin group, as compared to the inogatran groups, an explanation to the clinical superiority of UF heparin during ongoing treatment might be offered (92).

In contrast, unchanged or early increased levels of molecular markers of coagulation activity could indicate therapeutic failure with continuing thrombus formation and increased risk for new thrombotic events. Alternatively, the unchanged or early increased levels of molecular markers of coagulation activity might identify patients with different pathogenic mechanisms without coagulation activation and, hence, no benefit of anticoagulant treatment. However, the cluster of ischemic events early after cessation of treatment favours the interpretation of therapeutic failure with subsequent clinical consequences of a procoagulant state.

Although reactivation in coagulation activity after discontinuation of UF heparin and direct thrombin inhibitors have previously been described (37, 40, 84, 122, 126), a definite association to adverse ischemic events has not been established. In the present study there were signs of a clinical reactivation with clustering of ischemic events after cessation of the 72 hours anticoagulant treatment. There were also trends to a relation between elevations in thrombin generation and activity and fibrin turnover, as indicated by early increased F1+2, TAT, soluble fibrin and D-dimer levels, and higher cardiac event rate during the first four days after cessation of anticoagulant therapy. Concomitant treatment with ASA did not prevent reactivation, as has previously been suggested (83). Moreover, low ASA doses (≤ 75 mg daily) seemed favourable, similar to previous findings in a study of acute coronary syndromes in the pre-thrombolytic era, where 100 mg ASA daily without loading dose reduced death or myocardial (re-)infarction after two weeks significantly better than 1000 mg daily or placebo (142).

The biochemical and clinical reactivation is of course a major concern for the future development of direct and antithrombin-dependent thrombin inhibitors in the short- and long-term treatment of unstable coronary artery disease. The underlying plaque disruption may take several months to heal, and higher degree of coagulation activity may persist for six months after an episode of acute coronary syndrome (127), thereby suggesting an explanation for the association between higher degree of coagulation activity in the acute phase and increased long-term mortality in unstable coronary artery disease. Several
epidemiological studies have also, similar to the findings in the present study, identified molecular markers of thrombin generation and activity as well as fibrin turnover, such as F1+2, fibrinopeptide A and D-dimer, as risk indicators for future cardiovascular events in apparently healthy men (143-145). Thus, improved long-term antithrombotic treatment in unstable coronary artery disease seems warranted.

**Limitations**

The analyses of F1+2, TAT and D-dimer were performed with ELISA assays, which are well established and utilised in trials in similar patient categories (35, 36, 39, 40, 122). However, the method for analysis of soluble fibrin (96), although different from older methods (146), has the disadvantage of not being entirely specific, since a variety of fibrinogen degradation products can act as co-factors for tissue-type plasminogen activator (147) and thereby influence the analysis. ELISA methods of soluble fibrin analysis may have superior performance (35). The use of Fibrinopeptide A, another sensitive molecular marker of thrombin activity, has been restricted because of its high susceptibility to sampling artefacts (31).

Although the molecular markers of coagulation activity seem promising for identifying groups of patients at high risk for subsequent ischemic events and likely to respond to anticoagulant treatment, the individual risk assessment is nevertheless difficult because of the considerable interindividual dispersion of these markers.

The analyses of the relations of aPT times, levels of troponin and changes in levels of molecular markers of coagulation activity, and clinical outcome are retrospective. Accordingly, they need to be confirmed in future clinical trials.
Summary and implications

In unstable coronary artery disease:

- Inogatran - a synthetic, low molecular weight, selective, direct thrombin inhibitor - did not prove better than UF heparin in the prevention of ischemic events in unstable coronary artery disease.

- Inogatran treatment resulted, compared to UF heparin, in stable and more predictable prolongation of aPT times, although the correlation of inogatran plasma concentrations and aPT times was moderate.

- Higher aPT times during inogatran treatment were, even in the expected therapeutic range, related to increased risk of ischemic events. This might indicate a narrow therapeutic window or a methodological problem when evaluating direct thrombin inhibitors.

- Myocardial cell injury, as detected by elevated troponins, was associated with higher degrees of coagulation activity and inflammation, the latter only in part an acute-phase response due to the tissue damage.

- The elevation of fibrinogen levels in the acute phase was sustained for at least 30 days, which might reflect a low grade inflammatory condition in atherosclerotic lesions and a prothrombotic state, thus explaining the association between the fibrinogen level and the long-term risk for thrombosis and new ischemic events. This risk seems diminished during treatment with thrombin inhibitors, but is recurring early after cessation of treatment.

- The pronounced elevation of C-reactive protein levels in the acute phase was transient, which might indicate a propensity to pronounced inflammatory response at plaque ruptures and/or thromboembolic myocardial damage, both of which might be associated to increased mortality.

- UF heparin seemed more effective than inogatran in early reducing thrombin generation and activity, although this reduction was attenuated during on-going treatment.

- Higher thrombin generation and activity might identify patients with a thrombotic condition as the major cause of instability, who are the best responders to anticoagulant therapy with more pronounced decrease in coagulation activity during treatment and reduced short-term risk of ischemic events.
In contrast, unchanged or early increased levels of molecular markers of thrombin generation and activity during anticoagulant treatment may indicate therapeutic failure, with continuing thrombus formation, and increased risk for new thrombotic events.

Reactivation of coagulation activity was observed within 24 hours of cessation of anticoagulant treatment, which was associated with an increase in ischemic events.

Initially high coagulation activity was related to more pronounced reactivation after cessation of treatment, suggesting a persistent higher coagulation activity only temporarily attenuated by anticoagulant treatment.

The coagulation activity was at 30 days still higher than pre-treatment, furthermore indicating a persistent prothrombotic state.

**Implications of the study:**

- The more pronounced early decrease in thrombin generation and activity by UF heparin treatment, as compared to inogatran, might offer an explanation to the clinical superiority during ongoing UF heparin treatment in the TRIM study.

- The aPT time might be an inappropriate indicator of the antithrombotic efficacy of direct and antithrombin-dependent thrombin inhibitors.

- The mechanisms of the inflammatory response in the acute phase of unstable coronary disease need to be further explored in order to understand its association to future cardiovascular incidents.

- The increased long-term mortality in relation to higher degree of coagulation activity, together with the improved short-term prognosis by treatment with thrombin inhibitors, indicate the need for a refined long-term strategy in patients with unstable coronary artery disease, i.e. continued long-term antithrombotic treatment and/or elimination of the culprit lesion by revascularisation.
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