New Insights in Atherogenesis

COAGULATION

INFLAMMATION

Cardiovascular disease is one of the leading causes of death and complications worldwide. The classic concept of atherosclerosis assigns a pivotal role to inflammation in the onset and progression of this disease. Various inflammatory cell types (e.g., macrophages, neutrophils, and lymphocytes) play crucial roles in the destabilization and subsequent rupture or erosion of an atherosclerotic plaque, ultimately resulting in atherothrombosis. Inflammation is closely linked to coagulation in several pathologic conditions. Intriguingly, extensive bidirectional cross-talk between the two systems has been established in many complex diseases, including atherosclerosis.

This thesis encompasses a series of investigations, which unravel mechanisms through which blood coagulation affects atherosclerosis progression and contributes to the determination of atherosclerotic plaque phenotype. In particular, the dissertation provides important new insights on the role of clotting proteins and coagulation-driven inflammation in atherogenesis, uncovering a potentially significant role for thrombin in controlling the level of inflammation related to atherosclerosis onset and progression.

School for
Cardiovascular
Diseases

Maastricht UMC+

Maastricht University

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THE COAGULATION-INFLAMMATION AXIS IN ATHEROSCLEROSIS

JULIAN I. BORISSOFF

THE COAGULATION INFLAMMATION AXIS

In Atherosclerosis

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The studies presented in this thesis were performed at the departments of Biochemistry / Laboratory for Clinical Thrombosis and Hemostasis (CTH) at Internal Medicine / Cardiology / Pharmacology / Pathology, all within CARIM, School for Cardiovascular Diseases, Maastricht University Medical Center (MUMC+), the Netherlands, which is acknowledged by the Royal Dutch Academy of Arts and Sciences (KNAW).

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DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus Prof. dr. G.P.M.F. Mols,
in accordance with the decision of the Board of Deans,
to be defended in public

on Wednesday, 29th February 2012 at 14:00 hours

by

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*To my lovely wife and daughter, Gergana and Eva,
and my parents, Natalia and Ilcho,
my constant source of love, support and encouragement.*

CONTENTS

Chapter 1	
<i>General Introduction and Outline of the Dissertation</i>	11
Chapter 2	
<i>Is Thrombin a Key Player in the “Coagulation-Atherogenesis” Maze?</i>	21
Chapter 3	
<i>Early Atherosclerosis Exhibits an Enhanced Procoagulant State</i>	49
Chapter 4	
<i>Accelerated In Vivo Thrombin Formation Independently Predicts the Presence and Severity of CT-Angiographic Coronary Atherosclerosis</i>	81
Chapter 5	
<i>Mechanisms of Disease: The Hemostatic System as a Modulator Of Atherosclerosis</i>	101
Chapter 6	
<i>Modifying Atherosclerosis Plaque Stability via the Coagulation-Inflammation Axis</i>	137
Chapter 7	
<i>Main Findings In Perspective / Summary</i>	175
Acknowledgments	183
Curriculum Vitae	187

1

GENERAL INTRODUCTION AND OUTLINE OF THE DISSERTATION

Atherosclerosis is a multifactorial slowly progressive chronic vascular disorder, characterized by the formation of atherosclerotic plaques that cause luminal obstruction of large or medium-sized arteries, thus leading to acute or chronic organ ischemia. Atherosclerosis is the key underlying cause of cardiovascular disease (CVD) and cardiac death, including pathologic conditions such as myocardial infarction, stroke or peripheral artery disease.

Figure 1. (A) Acute myocardial infarction (AMI), **(B)** resulting from an atherosclerotic plaque rupture in a coronary artery (Obtained from NHLBI - Public Domain)

Over the past decades, numerous cardiovascular risk factors have been implicated to play a role in the pathogenesis of atherosclerosis, in-

cluding hyperlipidemia, obesity, diabetes, hypertension, etc. The advancements in understanding some of the pathophysiological mechanisms involved, along with developed distinct pharmacologic and interventional therapies, are paramount in the primary and secondary prevention of atherothrombosis nowadays¹⁻⁶. Nevertheless, CVD remains the primary cause of morbidity and mortality worldwide⁷.

Atherosclerosis – A Chronic Inflammatory Disease

Atherosclerosis is a chronic inflammatory disease^{8,9}. Endothelial dysfunction promotes local inflammation and leukocyte transendothelial migration into the arterial vessel wall by inducing the secretion of various proinflammatory cytokines, chemokines, and endothelial surface

Figure 1.

proteins such as intercellular adhesion molecule (ICAM-1), vascular adhesion molecule (VCAM-1), and monocyte chemotactic protein-1 (MCP-1)^{10,11}. The role of monocytes in atherosclerosis formation and progression is undisputable¹²⁻¹⁴. The transdifferentiation of monocytes into macrophages leads to the up-regulation of various cellular surface receptors, including scavenger receptors. The latter trigger the internalization and cytoplasmic accumulation of modified lipoproteins, which is associated with the formation of so called “foam cells”. Macrophage foam cells secrete numerous mitogenic and chemoattractant products, which facilitate processes such as vascular smooth muscle cell proliferation and migration, fibrous cap formation, eventually leading to the formation of a mature fatty streak. The importance of inflammation in atherosclerosis is further consolidated by the involvement of other leukocyte cell types such as neutrophils¹⁵⁻¹⁸, T-cells, B-cells¹⁹ and dendritic cells²⁰, which all play a role in the initiation and/or destabilization of an atherosclerotic plaque.

Interaction between Blood Coagulation and Inflammation at a Cellular Level – Possible Relevance to Atherosclerosis

Innate immunity and blood coagulation are evolutionary entangled in an intricate network of molecular and cellular interactions, thus forming an

integral part of the host-defense system²¹. There is substantial experimental and clinical data indicating that blood coagulation and inflammation dynamically interact in various medical conditions^{22,23}. Whereas the role of inflammation in atherosclerosis progression and plaque destabilization has been clearly established⁸, the role of blood coagulation in this pathological setting remains unclear to date.

Polymorphonuclear cells, in particular neutrophils, are essential for the primary innate immune response against local and systemic infections or tissue injury²⁴, but are also major cellular mediators supporting inflammation-coagulation interactions²⁵. One of the classic concepts of atherosclerosis asserts that endothelial injury of the vessel wall is integral to lesion formation^{26,27}. Neutrophils actively participate during the onset of atherosclerosis progression^{15,17,18}. Upon inflammation, multiple chemotactic stimuli (cytokines, chemokines, etc.) are released to promote neutrophil activation, extravasation and migration towards the inflammatory foci. A persisting neutrophil hyper-responsiveness may trigger pronounced oxidative stress and proteolysis through an enhanced synthesis of enzymatic proteins such as myeloperoxidase (MPO), neutrophil elastase and cathepsin G, thus contributing to plaque destabilization. These molecular mechanisms can result in the inactivation and degradation of im-

portant anti-coagulant proteins such as antithrombin, thrombomodulin (TM), protein C and tissue factor pathway inhibitor (TFPI), thus inducing a strong inflammation-driven local or systemic pro-coagulant response²². Persisting inflammation may trigger an over-reactive host defense response over time, thus disrupting the immune balance, contributing to tissue injury and thrombosis²⁸. In fact, neutrophils have been indicated to play a role in the pathophysiology of several pathologic conditions including acute coronary syndromes and stroke²⁹⁻³².

Of interest, other inflammatory cell types (e.g. macrophages) and vessel wall constituents (e.g. vascular smooth muscle cells) have been indicated to locally express key clotting and anti-coagulant factors (e.g. tissue factor (TF), factor VII, TFPI, etc.)³⁴⁻³⁷, suggesting an active, cell-based coagulation network in atherosclerotic lesions. Besides their key role in thrombus formation, several hemostatic factors (e.g. thrombin) can also trigger pro-inflammatory actions by activating the so called protease activated receptors (PARs)³⁸. In addition, platelets also represent an intriguing cellular interface between hemostasis and inflammation, secrete clotting factors and are well-known for their potent pro-atherogenic nature³⁹⁻⁴¹. Hence, these data suggest that blood coagulation might also play a role in the modulation of plaque phenotype and

atherosclerosis progression.

Future Perspectives

There is an increasing need for new research and therapeutic strategies, by which to improve the prevention and treatment of CVD in future⁴². The introduction of novel classes of specific anti-coagulants, which are characterized by a superior therapeutic profile, and the development of high-resolution vascular imaging modalities (e.g. CT/MRI) provide a unique opportunity to precisely study the effects of blood coagulation on atherosclerosis progression and plaque phenotype determination in both experimental and clinical situations, *in vivo*.

Focus/Outline of the Thesis

This thesis encompasses the role of blood coagulation in regulating the degree of inflammation related to atherosclerosis progression and modulation of atherosclerosis plaque phenotype.

In blood coagulation, thrombin is the most central clotting enzyme, which has a multifaceted character and regulates a diversity of pro- and anti-coagulant responses. Besides its key hemostatic functions, thrombin is also a potent cell-signaling molecule, which evokes a plethora of molecular and cellular responses in various cell types. In **Chapter 2**, we extensively review the *in vitro* scientific evidence, which demonstrates the

Figure 2. The continuous cycle between inflammation, innate immunity and blood coagulation (Adapted from: ³³). Abbreviations: NETs, neutrophil extracellular traps; MPO, neutrophil myeloperoxidase; TF, tissue factor; TM, thrombomodulin; PC, protein C; TFPI, tissue factor pathway inhibitor; factor (F)V/Va.

multifunctional roles of thrombin signaling within the arterial vessel wall, in particular, we discuss the potential importance of thrombin activity in regulating atherosclerosis plaque formation and progression. To investigate the involvement of procoagulant and anticoagulant clotting factors during atherosclerosis progression, we next assessed the overall distribution, local synthesis and activity of numerous coagulation proteins within human atherosclerotic lesions *ex vivo* (**Chapter 3**). Cardiac computed tomographic angiography (CCTA) is an accurate non-invasive vascular imaging modality, which enables the detection and relatively precise characterization of atherosclerotic plaques. Using CCTA, we further investigated the association between thrombin generation *in vivo* and the presence and severity of coronary atherosclerosis in patients with suspected coronary artery disease (**Chapter 4**). Acute myocardial infarction can very frequently evolve after erosion or rupture of angiographically moderate or even mild non-obstructive lesion⁴³⁻⁴⁷, thus suggesting that phenotyping plaque composition is of great clinical importance. Given the variety of cellular properties, which numerous clotting proteins demonstrate, and the established cross-talk mechanisms between coagulation and inflammation in several disease settings, we anticipated that the hemostatic system might be an important modulator of atherosclerosis plaque

phenotype (**Chapter 5**). To address this matter *in vivo*, we investigated how genetically imposed alterations in blood coagulation potential in transgenic mice determine the fate of atherosclerotic lesions (**Chapter 6**). Finally, in **Chapter 7** we discuss the key findings from the aforementioned studies, as well as the potential clinical implications and future research perspectives.

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2

IS THROMBIN A KEY PLAYER IN THE 'COAGULATION-ATHEROGENESIS' MAZE?

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Editor's Choice

In addition to its established roles in the hemostatic system, thrombin is an intriguing coagulation protease demonstrating an array of effects on endothelial cells, vascular smooth muscle cells (VSMC), monocytes, and platelets, all of which are involved in the pathophysiology of atherosclerosis. There is mounting evidence that thrombin acts as a powerful modulator of many processes like regulation of vascular tone, permeability, migration and proliferation of VSMC, recruitment of monocytes into the atherosclerotic lesions, induction of diverse pro-inflammatory markers, and all of these are related to the progression of cardiovascular disease. Recent studies in transgenic mice models indicate that the deletion of the natural thrombin inhibitor heparin cofactor II promotes an accelerated atherogenic state. Moreover, the reduction of thrombin activity levels in apolipoprotein E-deficient mice, because of the administration of the direct thrombin inhibitor melagatran, attenuates plaque progression and promotes stability in advanced atherosclerotic lesions. The combined evidence points to thrombin as a pivotal contributor to vascular pathophysiology. Considering the clinical development of selective anticoagulants including direct thrombin inhibitors, it is a relevant moment to review the different thrombin-induced mechanisms that contribute to the initiation, formation, progression, and

destabilization of atherosclerotic plaques.

1. Introduction

There is abundant evidence for a close interaction between inflammation and coagulation systems and a bidirectional cooperation between these mechanisms has been proposed^{1,2}. Although the important contribution of blood cells involved in coagulation, particularly platelets and leucocytes, to atherothrombosis is beyond dispute, the properties of several coagulation proteins and their expression in atherosclerotic lesions suggest that they might also contribute to the pathogenesis of cardiovascular disease (CVD). With the current development of highly specific antithrombotic agents including thrombin inhibitors aimed for long-term use in patients with CVD it seemed appropriate to focus on the pleiotropic actions of thrombin, in order to better appreciate possible long-term sequelae related to thrombin inhibition. This is even more important considering a number of physiological functions of thrombin (anticoagulant, vasodilating properties) that are of importance in a healthy vascular system. Taking physiology as a starting point for this review we next focus on the different mechanisms by which thrombin may modulate the formation of the atherosclerotic lesion and the course of atherogenesis.

2. Thrombin's Functional Roles in Physiology

In the coagulation cascade, thrombin is one of the key players. It is a central enzyme generated upon the exposure of tissue factor (TF), which binds and activates circulating factor VII and subsequently enters into the formation of a complex with factor X. The formed prothrombinase complex of factor Xa, factor Va, calcium (Ca^{2+}) cleaves prothrombin into thrombin. Thus the coagulation pathways are amplified by thrombin feedback activation of the cofactors V and factor VIII and the activation of the factor XI zymogen. Hence, generated thrombin leads to the conversion of fibrinogen into fibrin and ultimately to the formation of a fibrin clot. Thrombin activates a subfamily of G protein-coupled receptors named protease-activated receptors (PARs)—1, 3, and 4, affecting processes such as vasomotor regulation. Thrombin depicts a two-faceted role at the level of vascular reactivity, showing diverse vasoactive features, not only with regard to the type of vascular bed but also to the physiological condition of the vessel—whether healthy or diseased one. Several reports indicate that thrombin predominantly causes endothelium-dependent vasorelaxation in different species *in vitro*³⁻⁵. In addition, recent published data show that thrombin induces PAR-1-mediated forearm arterial vasodilation in humans *in vivo*⁶. These en-

dothelium-dependent dilating effects are generally attributed to a PAR-1-mediated production of various vasoprotective factors such as prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor, and mainly nitric oxide (NO)⁷. Similarly to its contrasting functional effects on vasoreactivity, thrombin demonstrates antagonizing actions in haemostasis also, e.g. the procoagulant action of converting fibrinogen into fibrin vs. the anticoagulant action of activating protein C (APC) after binding of thrombin to thrombomodulin (TM)⁸. Moreover, systemically generated thrombin, not captured by receptors is rapidly inactivated by inhibitors such as antithrombin (AT), APC, or heparin-cofactor II (HCII). Thrombin elicits at least 13 different actions (**Figure 1**). Thus, it consolidates its multifaceted character in physiology but it also establishes a strong link between coagulation and inflammation by playing a substantial role in the PAR-dependent initiation of different proinflammatory responses in various cell types including platelets, endothelial cells (EC), macrophages, and vascular smooth muscle cells (VSMC). Thrombin's humoral and cellular actions in normal and pathophysiological conditions are summarized in **Figure 2**.

Figure 1. Antagonizing actions of thrombin in coagulation cascade. Platelets get activated by the collagen that is exposed at sites of vessel dam-

age, leading to the formation of a haemostatic plug. **(1, 2)** Thrombin (FIIa) is generated upon tissue factor (TF) exposure but the reaction is relatively slow. **(3)** Once formed, thrombin activates factor V, factor VIII, and factor XI, which results in a 300 000-fold acceleration, amplification, and thrombin propagation. **(4a)** To prevent a massive conversion of fibrinogen into fibrin and thereby leading to the formation of a stable clot, all natural anticoagulant pathways get activated. Thrombin gets involved into these actions by binding thrombomodulin (TM), which results in the activation of protein C (PC) into activated protein C (APC), which by proteolytic cleavage of activated factors V and VIII reduces the rate of thrombin generation. In addition, antithrombin (ATIII) forms a thrombin-antithrombin (TAT) complex, which irreversibly inhibits thrombin, in association with heparin and heparin cofactor II. **(4b)** In case the procoagulant stimulus overpowers the capacity of the anticoagulant pathways, this would result in more production of fibrin and would lead to the formation of a thrombus. Thrombin-thrombomodulin (T-TM) complex could additionally support the procoagulant actions of thrombin by activating thrombin-activatable fibrinolysis inhibitor (TAFI), thereby inhibiting fibrinolysis. **(5)** Except for the exposed collagen at the site of injury, platelets also get activated by thrombin via PAR-1- and PAR-4-mediated mechanisms but also by cleavage of

glycoprotein V (GPV). Thrombin also prevents destabilization of the platelet plug by inhibiting ADAMTS13 action (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Thrombin facilitates clot stabilization by activating factor XIII (fibrin stabilizing factor) which has the capacity to crosslink fibrin.

3. Thrombin as a Trigger of Endothelial Dysfunction

Endothelial dysfunction, which is characterized with the inability of the endothelium to regulate its key functions (vascular tone, haemostasis, cellular adhesion, electrolyte balance, etc.) is thought to be a prerequisite for the initiation of an atherosclerotic plaque. Endothelial dysfunction results in increased interactions of circulating cells with the endothelium contributing to enhanced permeability. Thrombin signaling in the endothelium, mediated by PARs, might interlace with some of these pathophysiological pathways by triggering a multitude of phenotypic drifts, including changes in vascular tone, EC shape, haemostasis, permeability, downstream gene transcription, and angiogenesis.

3.1. Thrombin, Vascular Tone, and Phenotypic Alterations of the Endothelium

Despite the evolving experimental evidence on the molecular mecha-

Figure 1.

nisms of thrombin's signaling in endothelium, mediated via PAR-1 and -4,9 thrombin's actions in terms of vasomotor physiology are partly elucidated. However, it is known that thrombin induces contrary effects such as endothelium-dependent or direct smooth muscle contraction in healthy animal arteries *in vitro*^{3,10}. Endothelium-independent venoconstriction is observed in humans *in vivo*⁶. Thrombin mediates its vasoconstrictive actions by the secretion of prostaglandin H₂ (PGH₂) or thromboxane A₂ (TxA₂)^{7,10}. NO has a critical impact on the mediation of vascular relaxation and endothelial function. It is synthesized by an endothelial nitric oxide synthase (eNOS) and this enzyme competes with arginase for L-arginine as a substrate. It has been indicated that PARs could regulate eNOS activity by phosphorylating the enzyme at several sites¹¹. Ser1177, Ser615, Ser633, and Tyr81 enhance the production of NO, whereas Thr495 inhibits. Thrombin mediates eNOS-Ser1177 phosphorylation through Gq and a calcium and protein kinase C (PKC)-delta sensitive, but phosphatidylinositol 3-kinase (PI3K)/Akt-independent pathway. The phosphorylation of eNOS-Thr495 and inhibition of NO synthesis is thought to be directed via the activation of the Rho/ROCK pathway^{11,12}. Prolonged incubation with thrombin has been reported to inhibit the synthesis of eNOS in EC^{12,13}. Moreover, multiple *in vitro* studies report that

thrombin increases arginase activity, thereby suppressing NO production¹⁴⁻¹⁶. In addition, the overexpression of arginase by thrombin leads to the depletion of the L-arginine pool, reducing NO production and inducing reactive oxygen species (ROS) synthesis owing to the eNOS uncoupling, which eventually compromises the endothelial function¹⁷. Endothelin-1, a powerful natural vasoconstrictor, also showed an increased expression upon stimulation with thrombin¹⁸. The antagonizing effects of thrombin on vasoactivity seem relevant to the type of vascular bed and the severity of atherosclerotic burden is also dependent on thrombin concentration and continuance of action. In normal arteries, the short-term effect of thrombin is shown to support predominantly the action of vasorelaxants such as NO and PGI₂. On the other hand, increased thrombin generation is usually concentrated at the sites of vascular injury or within formed thrombus *in vivo*¹⁹, but also in patients with advanced CVD or suffering acute coronary syndromes²⁰. In vascular lesions thrombin promotes a pro-inflammatory response, characterized by increased production of diverse chemokines and cytokines, cell adhesion molecules (CAMs), enhanced vascular permeability, VSMC migration and proliferation, wall thickening and vasoconstriction⁷. This might be a result of the combination of a diminished TM and endothelial protein C receptor (EPCR)

Figure 2. Schematic overview of thrombin's humoral and cellular actions in normal and pathophysiological conditions. Relevant protease-activated receptors (PAR) signalling pathways. PLT, platelet; ATIII, antithrombin III; TAT, thrombin-antithrombin complex; APC, activated protein C; HCII, heparin cofactor II; CAMs, cell adhesive molecules; NO, nitric oxide; EDRF, endothelium-derived relaxing factors; PGI₂, prostacyclin; PGH₂, prostaglandin H₂; TxA₂, thromboxane A₂; PDP, platelet-derived products; TGF-β₁, transforming growth factor-β₁; PDGF_{AB}, platelet-derived growth factor_{AB}; ROS, reactive oxygen species; NOR-1, neuron-derived orphan receptor-1.

capacity coupled to an overexpression of PAR-1 and PAR-2 receptors in vascular lesions²¹⁻²³. Various mechanisms have been reported linked to PARs upregulation. First, thrombin-induced activation of PAR-1 in cultured human EC *in vitro* up-regulates PAR-1 gene expression by signaling via Gi1/2 coupled to Src and PI-3K, thus inducing the downstream Ras/MAPK pathway²⁴. Selective augmentation of PAR-2 and -4 gene expression is indicated upon treatment with inflammatory stimuli such as interleukin (IL)-1 α , (IL)-1 β , tumour necrosis factor (TNF)- α , and lipopolysaccharide (LPS)^{25,26}. Finally, high shear stress, also characterized by reduced expression of various atherogenesis-related genes, inhibits PAR-1 expression in human EC *in vitro*²⁷. Thus, the alterations in the vascular tone and the degree of expression of PARs in the vessel wall might have additional impact on the potency of thrombin's cell signalling activity and the progression of atherosclerotic disease.

3.2. Impairing the Barrier Function and Other Thrombin-mediated Effects on the Endothelium

Rabiet et al.²⁸ proposed a mechanism in which thrombin stimulates the intracellular accumulation of Ca²⁺, consecutively activating the PKC pathway, and causing eventual disruption of (VE)-cadherin-catenin complexes at the EC-cell junctions.

Further *in vitro* studies consolidated the participation of PKC in this pathophysiological process²⁹. Moreover, Nobe et al.³⁰ suggested that thrombin-induced endothelial barrier impairment is a biphasic process in which the Rho/Rho kinase pathway is also involved leading to rearrangement of actin stress fibers. A recent study elicits a new mechanism, which gives input to a better comprehension of the thrombin-induced endothelial gap formation and permeability. It was proposed that thrombin activates metalloprotease ADAM10, which mediates VE-cadherin proteolysis by specifically cleaving its ectodomain³¹. Thrombin could also promote the generation of endothelial microparticles (MPs) via ROCK-II activation³². Increase levels of endothelial MPs have been correlated with the morphology and severity of stenosis in patients with CVD³³.

3.3. Thrombin-induced Oxidative Stress

Aside from the induction of pro-inflammatory responses, elevated ROS levels are presumably associated with the promotion of endothelial dysfunction, combined most likely with diminished NO bioavailability. The majority of risk factors of atherosclerosis positively correlate with an enhanced ROS synthesis, which tends to initiate multiple pro-atherogenic effects³⁴. ROS are implicated in cellular signaling mecha-

nisms, such as gene expression, proliferation, migration or apoptosis. Several reports indicate the potentiating effect of thrombin on ROS production in human VSMCs^{35,36} and platelets³⁷. Different enzymatic systems take part in the production of ROS in the vasculature, such as xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and NOS. Nevertheless, NADPH oxidases have been indicated as a major source of superoxide in vascular cells and myocytes³⁸. The importance of NADPH oxidases in thrombin-induced ROS synthesis was studied by the depletion of p22phox subunit, which suppressed ROS formation in VSMCs^{36,39}. Thrombin also triggers the activation of p38 mitogen-activated protein kinases (MAPK) in a NADPH oxidase-dependent manner^{35,40}, which establishes a link between thrombin and the MAPK/ERK pathway, suggesting that it is also indirectly involved in processes like cell differentiation, cell survival, and apoptosis. Djordjevic et al.⁴¹ demonstrated that thrombin induces elevated ROS production in EC *in vitro* by activating p38 MAPK and PI3K/Akt, inducing enhanced proliferation. Intriguingly, thrombin induces its PAR-1 *de novo* re-expression via Src-dependent mechanism, including G proteins, PI3K, p38 MAPK, suggesting that redox pathways are also implicated in the regulation of PAR-1 expression²⁴. The latter was consolidated by two

reports indicating that treating VSMCs with either flavin inhibitor diphenyleneiodonium or antioxidants prevents PAR-1 upregulation upon stimulation by cyclic strain or oxidative agents^{42,43}. Hawkins et al.⁴⁴ indicated a thrombin-induced mechanism, causing the production of mitochondrial-derived superoxide (mROS), which is an outcome of a Ca^{2+} mobilization via inositol (1,4,5)-trisphosphate receptor (InsP_3R), leading to a subsequent mitochondrial uptake of Ca^{2+} , triggering mROS expression and nuclear factor-kappa B (NF- κB) pathway signaling, which strongly promotes the overexpression of intercellular cell adhesion molecule (ICAM)-1 and the adhesion of leucocytes to the vascular endothelium.

4. Thrombin in the Early Stage of Atherosclerotic Plaque Formation

Although several more coagulation serine proteases could function as activators of PARs by cleaving the N-terminal extracellular domain (**Figure 3**) abundant *in vitro* experimental data suggest that thrombin is a critical mediator in the coagulation, inflammation, vessel wall cross-talk. Thrombin enhances ROS production in the arterial vessel wall facilitating lipid peroxidation and apoptotic processes. Thrombin also induces a plethora of pro-inflammatory mediators, causing alterations in gene transcription of IL-6, IL-8, monocyte chemoattract-

ant protein 1 (MCP-1, CCL2), vascular cell adhesion molecule (VCAM)-1, and ICAM-1, etc., facilitating the recruitment of blood circulating monocytes into the arterial vessel wall and encourages early plaque formation. Its signalling mechanisms with a pro-atherogenic impact on the arterial vessel wall are mostly established via PARs⁴⁵.

4.1. Thrombin-induced Pro-inflammatory Responses in Blood and Vascular Wall

Thrombin participates in the selective recruitment of monocytes and T-cells into the vessel wall by inducing the synthesis of MCP-1 in EC and monocytes⁴⁶. MCP-1 is a well-characterized chemokine, which is abundant in human macrophage-rich atherosclerotic plaques⁴⁷. Thrombin has been shown to augment mRNA levels encoding for MCP-1, IL-1 β , IL-6, and TNF- α in human VSMC and less effectively, at high concentrations, in monocytes⁴⁸. It was stated that MCP-1 synthesis in monocytes *in vitro*, co-cultured with EC, is mediated by a thrombin-induced production of fractalkine (FK, CX3CL1), a cytokine, which effectively chemoattracts T-cells, and monocytes and has definite roles in CVD progression⁴⁹. In addition, in human EC *in vitro*, other inflammatory genes such as macrophage inflammatory protein 2-alpha, and neutrophil-activating protein 3, CD69, were reported to be overex-

pressed upon treatment with thrombin⁵⁰. Some of the pro-inflammatory properties of thrombin have been inferred from models of inflammation such as a peritonitis mouse model, in which the administration of the potent thrombin inhibitor hirudin suppressed the antigen- or LPS-stimulated activation of macrophage adhesion. In the same model, the intraperitoneal injection of purified thrombin stimulated the adhesion of macrophages and the accumulation of IL-6 and MCP-1 in a fibrinogen-dependent manner and independently from PAR-1 activation⁵¹. In a mouse heart-to-rat transplant model, a crucial role of PAR-1 activation by thrombin was shown in the initiation of leucocyte cell recruitment *in vivo*⁵². As stated earlier, thrombin is known to potentiate the production of IL-6 both in EC⁵³ and VSMC *in vitro*⁵⁴. IL-6 is an important cytokine with recognized impact on inflammation and is known to exacerbate atherosclerosis⁵⁵. Thrombin upregulates IL-8 expression in the endothelium via p38 MAPK signaling pathway *in vitro*⁵⁶. Similarly, IL-8 triggers monocyte adhesion to the endothelium under flow conditions *in vitro*⁵⁷ and is considered a possible biomarker to predict subclinical atherosclerosis based on data from multiple clinical trials⁵⁸. Finally, thrombin induces the secretion of macrophage migration inhibiting factor in EC and VSMC^{59,60}.

4.2. Thrombin-mediated Leuco-
cyte Adhesion, Rolling, and Migra-
tion on the Activated Endothelium

Once the endothelium has been activated, various molecules get entangled in a molecular network of capture, activation, and rolling. Selectins comprise a family of CAM of trans-membrane glycoproteins. L-, P-, and E-selectins are known to act as main mediator molecules for rolling of monocytes, neutrophils, T cells, and B cells upon binding to the activated endothelium. E- and P- selectins, in particular, play a substantial role in the initial capturing, tethering, and rolling of the leucocyte, relevant to the atherosclerotic development and progression⁶¹. E-selectin, present on EC only, was expressed on the surface upon thrombin stimulation^{62,63}. Much interest was devoted to the

mechanism of this thrombin-mediated expression and it was indicated that thrombin intervenes in the phosphorylation and activation of p38 MAPK, thereby inducing NF-kB-dependent and -independent pathways⁶⁴. Moreover, thrombin has the potential to promptly release P-selectin from the Weibel-Palade bodies in the EC⁶⁵. It was recently demonstrated that there is a differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor (vWF) by PARs and cAMP⁶⁶. Thrombin is not the only potent mediator for the expression of selectins on the endothelium. Many more factors like, e.g. TNF-α and IL-1α intervene in the E and P-selectin synthesis. Elevated expression of adhesion molecules on activated EC is considered a significant feature in the initiation of vascular

Figure 3. Coagulation serine proteases and PARs—activation and cellular expression. PAR, protease-activated receptor; APC, activated protein C; EC, endothelial cells; VSMC, vascular smooth muscle cells.

lesions⁶⁷. These pro-inflammatory responses additionally increase the overall expression of PARs, facilitating the endothelial reaction to thrombin, both with regard to endothelial dysfunction and further atherosclerotic progression⁶⁸.

Thrombin has a powerful potential to activate the endothelium, especially via its PAR-1 and -2 receptors, but also incites the overexpression of important pro-atherogenic immunoglobulin superfamily molecules such as ICAM-1 and VCAM-1^{50,69,70}. Rolling activated leucocytes are exposed to the influence of various chemoattractants, mediated by diverse integrins, and captured to cell adhesion glycoproteins. This eventually leads to the so called 'leukocyte arrest'. Thrombin enhances VCAM- and ICAM-1 synthesis in cultured human EC. NF- κ B- and GATA-dependency was observed with regard to VCAM-1 expression⁷¹. Other *in vitro* studies indicated that PKC- δ and RhoA/ROCK activation independently lead to thrombin-induced NF- κ B-dependent ICAM-1 upregulation^{72,73}. Moreover, the inhibition of both c-Jun N-terminal kinase (JNK) and NF- κ B pathways showed additive inhibitory effect on ICAM-1 expression on the endothelium and highlighted a significant role for JNK signalling⁷⁴. The actual process of transmigration of leucocytes usually occurs on activated endothelial regions thus facilitating the leucocytes to pass through. Thrombin seems to interlace by increasing the release of

Ca²⁺ from the intracellular stores^{75,76}, favoring the ligation of ICAM-1, activating Rho family GTPases^{77,78}, which increases the myosin contractility of EC impairing the inter-endothelial junctions by disrupting VE-cadherin complexes⁷⁹.

4.3. Thrombin and Monocytes/Macrophages in Atherosclerosis

The effects of thrombin on monocytes and monocyte-derived macrophages during atherosclerotic progression remain less elucidated compared with other blood cells such as platelets. Initially, it was indicated that VSMC may be more sensitive to thrombin activation than monocytes and macrophages *in vitro*, the latter needing much higher concentrations of thrombin to achieve increased IL-6, IL-1 β , MCP-1, or TNF- α mRNA expression⁴⁸. Human monocytes, macrophages, and dendritic cells *in vitro* express PARs. PAR-1 was expressed in all cell types, whereas PAR-3 mRNA was less detected in monocytes and macrophages. PAR-1, -2, and -3 levels were upregulated upon thrombin treatment subsequently inducing MCP-1 expression. IL-4 downregulated PAR-1, -2, and -3 expression in dendritic cells derived from monocytes by granulocyte-macrophage colony-stimulating factor (GM-CSF)⁸⁰. Li et al. found PAR-4 protein expression on monocytes, though they failed to detect PAR-4 transcripts. They also

showed that IL-6 was released upon treatment with agonist peptides of PAR-1 and PAR-4, but not of PAR-3, which was associated with PAR-3 incapability of mediating transmembrane signalling⁸¹. Finally, there are multiple pro-inflammatory effects of thrombin on other cell types, which indirectly induce pro-atherogenic reactions in monocytes (as discussed in the text).

5. Thrombin in the Advanced Stage of Atherosclerosis

Intimal thickening, derangement of the arterial vessel wall anatomy in concert with accumulation of lipids, infiltration of cells, and matrix degradation, presented by a necrotic core are the basic histological features of the advanced atherosclerotic lesion⁸². Thrombin is implicated throughout plaque progression and destabilization events (**Figure 4**).

Figure 4. Proposed Mechanism for Thrombin-induced Atherogenesis. All known thrombin-induced pro-atherogenic actions are depicted in a consecutive way, showing its impact throughout the different stages of atherosclerotic development. Square with inverted 'V' indicates activation; encircled plus symbol indicates induction; upward arrow indicates elevated levels; MCP-1, monocyte chemoattractant protein-1; PDGF, platelet-derived growth factor; EDN-1, endothelin-1 gene; ECE-1, endothelin converting enzyme-1 gene; COX-2, cy-

clooxygenase-2; MIF, migration inhibiting factor; ADAM10, A Disintegrin And Metalloproteinase protein-10; ROS, reactive oxygen species; mROS, mitochondrial-derived reactive oxygen species; IL, interleukin; TNF- α , tumour necrosis factor- α ; Mo, monocyte; NO, nitric oxide; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MPs, microparticles; CD40L, CD40 Ligand; MMP, matrix metalloproteinases; PF-4, platelet factor-4; RANTES, Regulated upon Activation, Normal T-Cell Expressed, and Secreted; NAP-2, neutrophil-activating peptide-2; NOR-1, neuron-derived orphan receptor-1; VEGF, vascular endothelial growth factor; PARs, protease-activated receptors; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1.

5.1. Thrombin and Platelet-mediated Effects in Plaque Progression and Destabilization

Besides being a major activator of platelets, thrombin likely induces platelet-mediated atherogenic signals by boosting the synthesis and release of multiple pro-inflammatory mediators by platelets and deploying their interaction with leukocytes to favor chemotaxis, adhesion, and migration into the arterial vessel wall. Platelet activation by thrombin is accomplished exclusively by targeting PAR-1 and -4 receptors, expressed on their surface in humans⁸³. Platelets interfere in athero-

sclerosis in each of its phases—initiation, progression, and late complications^{84–86}. *In vivo*, thrombin-activation of human platelets results in the rapid activation and maximal expression of CD40 ligand (CD40L) on their surface⁸⁷. CD40L is a TNF family protein, expressed on many cell types including platelets, and it binds to CD40 thus forming a trimer, named CD40/CD40L dyad. This established system potentiates downstream of atherogenic signals in the arterial vessel wall constituents, such as EC, VSMC, and monocytes. Downstream signalling of CD40 is mediated by the so-called TNF receptor-associated factors, which are able to recruit kinases and other effectors, which subsequently lead to the activation of NF- κ B pathway, and thus induce the upregulation of various adhesion molecules, matrix metalloproteinases (such as MMPs 1,2,3,9,11,13), cytokines, and growth factors⁸⁸. MCP-1 is induced upon transient interactions of thrombin-stimulated platelets with the endothelium⁸⁹. These pro-inflammatory events, related to MCP-1 production, are observed in VSMC *in vitro* too, probably contributing to VSMC migration and proliferation into the atherosclerotic plaques⁹⁰. Thrombin is also known to induce IL-1 β expression under *in vitro* conditions, both by EC⁹¹ and platelets⁹². An additional number of thrombin-induced platelet mediators, such as platelet factor-4 (PF-4), RANTES (Regulated upon Activation, Normal T-cell Ex-

pressed, and Secreted/CCL5)⁹³, and neutrophil-activating peptide (NAP)-2⁹⁴ are deposited by activated platelets on the endothelium to support leucocyte arrest and to favor the subsequent transmigration events. PF-4 (CXCL4) is a small chemokine also found in atherosclerotic lesions where its concentration correlates with severity of the plaques⁹⁵. PF-4 protects monocytes against apoptosis and induces their differentiation⁹⁶, whereas it serves as a stimulator of oxidative stress in macrophages⁹⁷. Aside from its vessel wall-related oxidative activities, a recent study provides evidence for the role of thrombin in evoking apoptosis in human platelets *in vitro*⁹⁸. It was demonstrated that apoptosis was induced via H₂O₂ production, mediated by mitochondrial cytochrome c release and the activation of caspase-9, leading to caspase-3 activation and ultimately to phosphatidylserine (PS) exposure. On the other hand, it is well known that MPs are mainly released from cells upon activation or apoptosis. Moreover, increased number of circulation procoagulant MPs are positively associated with the initiation and dissemination of pro-inflammatory processes but also with the severity of CVD⁹⁹.

In conclusion, thrombin appears to have an important role in platelet-mediated pro-inflammatory cascades, resulting in a stimulation of ICAM-1, VCAM-1, E-selectin, and MMPs production, all processes that contribute to plaque progression,

subsequent destabilization, and rupture^{88,100–102}.

5.2. Thrombin and VSMC Migration and Proliferation

Besides its functions in the regulation of vascular tone, thrombin mediates migration, proliferation, and hypertrophy of VSMC. VSMC are known to express PAR-1, -2, and -4, thus potentiating the effect of thrombin in the activation of VSMC proliferation and migration¹⁰³. Multiple studies report on situations associated with changes in the expression of PARs in VSMC. We have to take into consideration that the upregulation of these receptors might be as crucial as the direct effect of thrombin alone, because of the fact that they are the main mediators for its further actions. Hence, an upregulation of PAR-1 in human and rat VSMC *in vivo* is demonstrated upon the release of multiple platelet-derived products (PDP) such as transforming growth factor (TGF)- β_1 , platelet-derived growth factor_{AB} (PDGF_{AB}) and to a lesser extent, serotonin¹⁰⁴. Thus a long-term generation of new thrombin receptors at sites of vascular injury might consolidate that thrombin amplifies its pro-atherogenic actions throughout the development of a vascular lesion. Moreover, PAR-1 expression seems responsive to physical stress in both human and rat aortic VSMCs *in vitro* being enhanced when cyclic strain is applied⁴³ and being inhib-

ed upon stimulation with high shear stress¹⁰⁵. This substantiates the idea that VSMC requires physical stimulation (flow or strain) in order to maintain vessel wall homeostasis, and perturbation of this process may be involved in atherosclerosis where an overexpression of PAR-1 and PAR-2 receptors has been demonstrated^{21–23}. Wang et al. studied thrombin-induced VSMC migration in cultured VSMC and demonstrated that the process is p38-MAPK-mediated upon the generation of ROS. Maruyama et al.¹⁰⁶ indicated that thrombin-induced proliferation in cultured human VSMC is regulated by NF-kB. VSMC proliferation appears to be regulated by neuron-derived orphan receptor-1 (NOR-1), a transcription factor overexpressed in human atherosclerotic plaques upon stimulation with thrombin¹⁰⁷.

Finally, the regulation of PDGF in the endothelium also appears to be linked to thrombin. PDGF is related to atherosclerosis for its properties to stimulate VSMC migration and proliferation. PDGF levels rise upon treatment with thrombin of human umbilical vein EC, together with monocyte transmigration and E-selectin expression¹⁰⁸.

5.3. Thrombin and Its Pro-angiogenic Responses

Neoangiogenesis is closely associated with plaque progression. Intraplaque haemorrhage is currently considered a critical factor for

plaque destabilization and is predominantly attributed to the neovascularization of the intima and media by disorganized and immature 'leaky' microvessels¹⁰⁹. Thrombin promotes angiogenesis both *in vitro* and *in vivo*¹¹⁰. It is indicated that it reduces the ability of EC to affix to their anchorage on the basement membrane, thereby promoting early angiogenic events¹¹¹. Furthermore, it has been stated that thrombin increases the mRNA and protein levels of $\alpha_v\beta_3$ -integrin in a concentration-dependent manner in EC¹¹². $\alpha_v\beta_3$ -integrin is a known angiogenic marker in vascular tissue and it directly interacts with thrombin, thereby facilitating EC attachment, migration, and survival. $\alpha_v\beta_3$ -integrin also mediates progelatinase A (MMP-2) activation. Stimulation with thrombin has shown the induction of MMP-2 release in both human EC¹¹³ and rat aorta in a dose-dependent mode *in vitro*¹¹⁴. In addition, thrombin augments the expression of vascular endothelial growth factor (VEGF) and angiopoietin-2 via PAR-1-mediated mechanism^{115,116}. Finally, various studies indicate a relevant role for hypoxia-inducible factor-1 α signaling pathway in the thrombin-induced VEGF gene expression and angiogenesis.

6. Thrombin and Atherosclerosis - In Vivo Animal Studies

Despite the wealth of existing data on thrombin's pro-atherogenic ac-

tions *in vitro*, we should point out that many of these studies have been carried out with cell cultures and purified thrombin, in the absence of receptors and inhibitors, such that the relevance of any of these outcomes may be debated. However, the critical role of thrombin in atherogenesis is supported by recent *in vivo* studies. Indirect evidence shows that heterozygous tissue factor pathway inhibitor (TFPI)-deficient ApoE^{-/-} mice exhibited a significantly greater atherosclerotic burden compared with TFPI wild-type genotype¹¹⁷. TFPI is a potent inhibitor of TF-mediated thrombin generation. Direct evidence for the involvement of thrombin comes from experiments in which the administration of the direct thrombin inhibitor melagatran to ApoE^{-/-} mice reduced lesion progression in brachiocephalic arteries. Total lesion area was significantly decreased in melagatran-treated animals. Thrombin inhibition also contributed to plaque stability (significant increase of immunohistochemical staining against VSMC α -actin), characterized by thicker fibrous caps, increased media thickness, smaller necrotic cores, and a significant decrease of staining against MMP-9¹¹⁸. MMP-9 is considered an important catalyser of plaque rupture. Finally, in a study employing transgenic double knock-out mice, deficient for HCII, a natural thrombin inhibitor, on a ApoE^{-/-} background, HCII deficiency was associated with approximately 64%

Figure 4.

larger total plaque area and increased neointimal formation than in wild-type mice. In support of these findings, the administration of dermatan sulfate, which potentiates the inhibitory function of HCII about 10 000-fold, showed a HCII-dependent antiproliferative effect in wild-type animals¹¹⁹.

7. Clinical Studies

Thrombin's impact on atherosclerotic development is a relatively novel topic to investigate and no specific clinical trials have been conducted yet. However, several reports indirectly demonstrate its importance with regard to CVD progression.

Aihara et al.¹²⁰ found a negative correlation between plasma HCII activity and ultrasound imaged plaque thickness of the carotid arteries in 306 elderly Japanese patients and suggested that HCII inhibits atherogenesis, thereby also showing a possible indirect link between higher thrombin generation and atherosclerosis progression. Moreover, various thrombotic markers measured upon progressive CVD, indicate an indirect link for thrombin and atherosclerosis. The Cardiovascular Health Study (CHS) showed that prothrombin fragments F1-2 (F1-2) and fibrinopeptide A measured in 5201 individuals (399 free of CVD), which are markers for thrombin generation *in vivo*, correlated with various CVD risk factors such as triglycerides, C-reactive protein, low ankle-brachial

pressure index (ABPI), etc¹²¹. F1-2 plasma levels were also independently associated with carotid intima-media thickness in a population of 181 middle-aged adults, free of clinically overt atherosclerosis¹²². Moreover, Nylaende et al. studied the relationship of prothrombotic activity and the severity of peripheral arterial occlusive disease (PAD). Multiple haemostatic markers such as vWF, soluble TM, soluble TF, TAT complex, and D-dimer were determined in a cross-sectional study of 127 patients, diagnosed with PAD. Plasma levels of D-dimer, TAT complex, and fibrinogen significantly correlated with the severity of atherosclerotic burden, evaluated by maximum treadmill walking distance and ABPI¹²³. A recent meta-analysis of 191 studies, investigating seven common haemostatic gene polymorphisms in CVD, indicated that the 1691A variant of the factor V gene and 20210A variant of the prothrombin gene, both of which promote thrombin generation in blood, might be associated with the risk of CAD¹²⁴. Moreover, it was recently shown that long after acute myocardial infarction, patients generate higher, earlier, and faster thrombin in comparison with chronic CAD patients¹²⁵. This strengthens the concept of vulnerable atherosclerotic plaques contributing to the propagation of thrombin generation, thereby leading to aggravation of CVD. Several more indirect cross-relations might be of interest in this

context. Numerous clinical trials postulate that haemostatic factors such as fibrinogen, C-reactive protein, plasminogen activator inhibitor-1 (PAI-1) are risk factors for CVD progression¹²⁶. A recent study associated the progression of symptomatic intracranial large artery atherosclerosis with a pro-inflammatory state and impaired fibrinolysis, characterized with elevated concentrations of the endogenous fibrinolysis inhibitor PAI-1¹²⁷. Despite the fact that thrombin is not a sole mediator of PAI-1 it induces its expression together with TF^{128,129} in EC *in vitro*. TF and PAI-1 are already recognized for their pro-inflammatory features. In addition, many studies demonstrate a relationship between elevated PAI-1 levels and the development of atherosclerosis, not only systemically but also locally¹³⁰. Leukocytosis, and high neutrophil count in particular, may represent another intriguing mechanism for enhancing chronic atherosclerosis via maintaining a hypercoagulable state in CVD patients¹³¹. Neutrophils are a pivotal link between inflammation and coagulation. They produce multiple procoagulant factors and are able to release diverse matrix-destabilizing enzymes (elastase, cathepsin G), which easily activate the coagulation system¹³². They contribute to the liberation of TF-laden MPs into the blood stream upon stimulation with cytokines and consequent platelet adhesion via P-selectin¹³². This seems another potential mechanism

for a continuous thrombin generation *in vivo*, facilitating the amplification of thrombin's pro-atherogenic features.

8. Summary and Perspectives

From histological studies an intense interaction between coagulation, inflammation, and the complex process of atherosclerosis has emerged¹³³. Advanced atherosclerotic lesions show evidence of the presence of active coagulation products including fibrin and fibrin cleavage products. Hence, the presence of an active coagulation cascade within the arterial vessel wall seems likely and our recent immunohistochemical data show that essentially all coagulation proteins are detectable in the atherosclerotic lesion¹³⁴. In the coagulation cascade we and others consider the generation of thrombin as one of the key regulating events. *In vivo*, thrombin is thought to be continuously generated as indicated by measurable quantities of F1-2 and TAT complexes in the plasma of normal individuals. Physiologically, the generation of thrombin is the product of synthesis under influence of TF and inhibition by several inhibitors including AT and HCII. The net amount of thrombin will be determined by the rate of synthesis and inactivation, the localization (free or bound to surfaces), and its associated binding to receptors including PARs and TM. Upon progressive atherosclerosis, there is a diminution in

the level of TM at the endothelium¹, which impairs the anticoagulant action of thrombin and the increased production of thrombin because of TF exposure allows interactions of thrombin with components of the arterial vessel wall, including dysfunctional EC on both initial and advanced lesions and other cell types in ruptured (thrombotic) plaques. The continuous generation of mostly procoagulant thrombin may contribute to a vicious circle in the thrombin-induced atherogenesis process. As discussed, thrombin acts mostly via PARs, inducing multiple vascular proinflammatory reactions. The authors are aware that also other coagulation proteases including factor VIIa, factor Xa, and APC contain PAR-activation properties that may interfere with or add to the actions of thrombin. There has indeed been a public debate on the preference of thrombin vis-à-vis APC in their binding to PAR-1 and this debate has not yet been settled¹³⁵. Atherosclerotic alterations in the vessel wall are known to increase the level of expressed PARs on the surface of most vessel wall constituents²¹⁻²³. Thrombin-mediated proinflammatory events are a powerful trigger for more thrombin formation, which may eventually amplify its contribution to further atherosclerotic progression. Finally, from a clinical perspective the introduction of a number of selective oral anticoagulants that will also be aimed for long-term administration makes it of

actual importance to consider the effects and possible side-effects of thrombin inhibition on the extent and nature of atherosclerosis. Hopefully, thrombin inhibition is, as predicted from animal experiments, associated with a favorable change in atherosclerosis phenotype. However, the typical Janus face of many clotting proteases should warn against overt enthusiasm and calls for prospective clinical studies.

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EARLY ATHEROSCLEROSIS EXHIBITS AN ENHANCED PROCOAGULANT STATE

Borissoff JJ, Heeneman S, Kiliç E, Kassák P, Van Oerle R,
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Background: Thrombin generation (TG) *in vivo* may be important in regulating atherosclerotic progression. In the present study, we examined for the first time the activity and presence of relevant coagulation proteins, in relation to the progression of atherosclerosis.

Methods and Results: Both early (EAL) and stable advanced atherosclerotic lesions (SAAL) were collected pairwise from each individual (n=27) during autopsy. Tissue homogenates were prepared from both total plaques and isolated plaque layers, in which activity of factors (F)II, X, XII, tissue factor (TF) were determined. Microarray analysis was implemented to elucidate local mRNA synthesis of coagulation proteins. Part of each specimen was paraffin embedded and histological sections were immunohistochemically stained for multiple coagulation markers using commercial antibodies. Data are expressed as median (interquartile range - IQR). TF, FII, FX and FXII activities were significantly higher in EAL compared to SAAL. Endogenous thrombin potential (ETP) and thrombin-antithrombin (TAT) complex values consolidated a pro-coagulant profile of EAL (ETP: 1240 nM.min (IQR 1173 - 1311); TAT: 1045 ng/mg (IQR 842.6 - 1376)) vs. SAAL (ETP: 782 nM.min (IQR 0 - 1151); TAT: 718.4 ng/mg (IQR 508.6 - 1151)). TF, FVII and FX co-localized with macrophages and smooth muscle cells. In addition,

multiple pro- and anti-coagulant proteases were immunohistochemically mapped to various locations throughout the atherosclerotic vessel wall in both early and advanced atherosclerotic stages.

Conclusions: This study shows an enhanced pro-coagulant state of early-stage atherosclerotic plaques compared to advanced-stage plaques, which might provide novel insights in a role of coagulation during atherosclerotic plaque progression.

Introduction

Atherosclerosis is widely recognized as a chronic inflammatory disease¹. Rupture of an atherosclerotic plaque is considered the predominant underlying cause of acute atherothrombotic events such as myocardial infarction, ischemic stroke, and vascular death. A close relation between blood coagulation and atherosclerosis²⁻³ is supported by studies revealing the presence of specific coagulation proteins within an atherosclerotic lesion. Tissue factor (TF) and factor(F)VII, of which the complex is the principal initiator of coagulation *in vivo*, are expressed on macrophages (MΦ) and vascular smooth muscle cells (SMC) within the arterial vessel wall and atherosclerotic lesion⁴⁻⁵. Both proteins potentially participate in multiple pro-atherogenic processes such as migration and proliferation of SMC⁶, in-

flammation and angiogenesis⁷. In addition to the single effects of each protein, the local interaction between MΦ/SMC-derived TF and FVII may provide a catalytic complex for subsequent generation of thrombin and fibrin, of which the latter is also detectable in atherosclerotic lesions⁸⁻⁹. The procoagulant condition of the atherosclerotic lesion may be further enhanced by the presence of various pro-inflammatory cytokines (e.g. tumor necrosis factor alpha (TNF-α), interleukin-1¹⁰, etc.), which may down regulate local expression of anticoagulant proteins such as thrombomodulin (TM) and the endothelial protein C receptor on endothelial cells¹¹. Thrombin, a key enzyme in blood coagulation, may also play a critical role in many processes related to the development, progression and atherothrombotic potential of atherosclerotic plaques¹². Direct evidence for the role of thrombin in the atherogenic process comes from experiments showing reduced progression of atherosclerosis in ApoE^{-/-} mice upon pharmacological inhibition of thrombin¹³. Moreover, decreased expression of tissue factor pathway inhibitor (TFPI) on an ApoE^{-/-} background increased the atherosclerotic burden¹⁴. Because of the reported involvement of pro- and anticoagulant coagulation factors during plaque progression, we hypothesized that the overall distribution and activity of coagulation proteins in the arterial vessel wall correlates with the extent and progression

of atherosclerotic lesions. Moreover, we hypothesized that the amount of thrombin that can be generated from atherosclerotic tissue homogenates not only depends on the amount of TF, but also on the presence and activity of other coagulation proteins that either amplify or dampen thrombin generation. Hence, we studied the localization of all coagulation proteins in addition to the TF/FVII complex, on histologically defined early and stable advanced atherosclerotic lesions. In addition to thrombin generation, we determined the procoagulant activity of several coagulation proteins in the same lesions.

Materials and Methods

Patient Characteristics and Tissue Specimens

The tissue specimens were obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage and use of tissue and patient data were performed in agreement with the “Code for Proper Secondary Use of Human Tissue in the Netherlands” (<http://www.fmwv.nl>). Both early and stable advanced atherosclerotic lesions were collected pairwise from each corresponding individual (n=27) during post-mortem dissection of the abdominal aortas within 8 hours of death. Autopsy specimens were obtained from adult men and women with an age range of 45-84 years (mean: 55).

Clinical characteristics of the patients are provided in **Table 1**. The cause of death was diverse (e.g. myocardial infarction, stroke, etc.). Individuals with sepsis or cancer were excluded. All tissue specimens were histologically evaluated on hematoxylin-and-eosin (H&E)-stained sections (4 μ m). Plaque subtypes were determined in compliance with the modified AHA classification, based on morphological description, proposed by Virmani et al¹⁵. Since one of the main goals in this study was to discriminate the overall prothrombotic potential of atherosclerotic lesions between early and advanced stage of development, we classified the plaques as follows: intimal thickenings and xanthomas are uniformly called “Early Atherosclerotic Lesions (EAL)”, whereas all types of stable advanced plaques are called “Stable Advanced Atherosclerotic Lesions (SAAL)”. Complicated lesions, including lesions with intraplaque hemorrhage, a surface defect and/or thrombotic deposit, were not

included in this study.

Preparation of Tissue Homogenates

A section of each of the collected specimens (27 EAL/27 SAAL, obtained in pairs from n=27) was snap frozen upon collection. Snap frozen atherosclerotic tissues were freeze-dried for 3 days, pulverized and subsequently the tissue powders were dissolved in 50 mM n-octyl β -D-glucopyranoside (Sigma-Aldrich) in HN-Buffer (25 mM HEPES, 175 mM NaCl, pH 7.7), vortexed and, centrifuged twice (10 min, 13000 rpm). Total protein content of the tissue homogenates was spectrophotometrically determined using the Biorad DC Protein Assay system according to the manufacturer’s instructions (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). All samples were further diluted into a final concentration of 5 mg/mL.

Table 1. Patient Clinical Characteristics of Autopsy Cases from which Paired EAL and SAAL Were Obtained and Examined

Clinical Characteristics	All (n=27)
Age, y	55.4 $\pm$ 9.2
Male sex	16 (59.3)
Hypertension	14 (51.9)
Smoking	11 (40.7)
Hypercholesterolemia	10 (37.0)
Diabetes mellitus	3 (11.1)
Family history	17 (63)
Previous myocardial infarction	8 (29.6)

Values are n (%) except age.

Effect of Time Delay between Death and Post-Mortem Examination on Coagulation Proteins Activity

See Materials and Methods in the Data Supplement.

EAL & SAAL Layer Preparation and Homogenization

See Materials and Methods in the Data Supplement.

Thrombin Generation, Prothrombin, FX and FXII Activity Assays and Thrombin-Antithrombin Complexes (TAT) Levels / Tissue Factor Activity Assay / TFPI Antigen Assay

The Calibrated Automated Thrombogram (CAT, Thromboscope, the Netherlands) was used to determine the contribution of atherosclerotic tissue homogenates to thrombin generation in human plasma (in triplicates; inter-assay CV<10%). For additional information, see Materials and Methods in the Data Supplement.

Effect of Phospholipid Concentration on Thrombin Generation / Thrombin Generation in Normal Arterial Vessel Wall Homogenates

See Materials and Methods in the Data Supplement.

RNA Isolation and Quantification/**Microarray Hybridization and Data Analysis**

See Materials and Methods in the Data Supplement.

Immunohistochemical (IHC) & Immunofluorescence (IF) Stainings / Immunohistological Evaluation

See Materials and Methods in the Data Supplement.

Statistical Analysis

Data analysis was computed with SPSS, version 17.02 (SPSS Inc, Chicago, IL, USA) and Prism, version 5.00 (GraphPad Software Inc., San Diego, CA, USA). Results are expressed as median (interquartile range (IQR)). An exact-distribution Wilcoxon 2-sample test was used for all intra-individual comparisons. A two-tailed probability value $p < 0.05$ was considered statistically significant. Repeated-measures ANOVA was used to assess differences in coagulation proteins activity over time.

Results**EAL Exhibit Higher Functional Activity of Key Coagulation Proteins Compared to SAAL Ex Vivo**

We determined the dependence of the thrombin generating potential of both EAL and SAAL on their prothrombin, FX and FXII content. From

all 54 specimens (27 pairs - EAL & corresponding SAAL) that we examined, prothrombin activity was detected in 11 samples only. From the latter 11 samples with detected activity, EAL specimens had significantly higher prothrombin activity 0.0% (IQR 0.0 – 7.761), compared to their paired SAAL 0.0% (IQR 0.0 – 0.0) (**Figure 1A**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$). The activity of FX revealed a similar trend with a significant 3-fold up-regulation in the early-staged atherosclerotic lesions 0.276% (IQR 0.164 – 0.536), compared to the stable advanced-staged ones 0.136% (IQR 0.054 – 0.237) (**Figure 1B**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$). Furthermore, FXII also demonstrated significantly higher activity levels in EAL 2.636% (IQR 1.344 – 3.372), compared to SAAL 0.930% (IQR 0.337 – 1.526). (**Figure 1C**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$).

EAL Demonstrate a 3-Fold Increase in TF Activity vs. SAAL

To better appreciate the procoagulant potential of these two sets of atherosclerotic plaque homogenates, we assessed the activity of TF, which is known as a pivotal trigger of coagulation *in vivo*. TF activity was ~ 3-fold higher (0.036 pmol/mg (IQR 0.017 – 0.055)) in EAL compared to SAAL (0.009 pmol/mg (IQR 0.005 – 0.022)) (**Figure 1D**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$). Twenty-six out of 27 early-

staged atherosclerotic lesion homogenates indicated elevated TF activity levels, compared to their corresponding stable advanced-stage lesion specimens. Notably, the 8-hour-window between death and post-mortem collection did not significantly affect the activity of TF, FII, FX and FXII in atherosclerotic lesions, harvested at various time points: 0 hrs (Baseline), 2 hrs, 4 hrs and 8 hrs. No significant differences were found in between the different time points of all tested proteins but also compared to baseline values (**Figure S1**, Data Supplement), strongly suggesting that the post-mortem values reflected actual coagulation activity *in vivo*.

Shift of the TF/TFPI Ratio Suggests an Increased Atherothrombotic Tendency in EAL

TFPI is a potent natural inhibitor of the TF-driven pathway of the coagulation cascade but also plays an important role in regulating inflammation. Furthermore, it has been shown that TFPI modulates thrombus formation in experimental models *in vivo*¹⁶, mostly by attenuating the procoagulant activity and overexpression of TF¹⁷⁻¹⁸. Therefore, we tested the levels of TFPI by utilizing a home-made ELISA. A ~1.6-fold significant increase in TFPI antigen levels was found in EAL compared to SAAL. EAL demonstrated TFPI activity equal to 0.089 nM/mg (IQR 0.072 – 0.140), whereas SAAL showed

Figure 1.

0.056 nM/mg (IQR 0.030 – 0.088) (**Figure 1E**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$). Despite the higher levels of TFPI antigen in the EAL homogenates, SAAL the TF/TFPI balance in the early lesions remained in favor of TF shown by the higher TF/TFPI ratios in EAL homogenates (**Figure 1F**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$).

Figure 1: Activity of coagulation proteases in paired early- and stable advanced-staged atherosclerotic plaque homogenates. **A.** FII activity in FII-deficient plasma, assessed via modified CAT measurement, indicating significantly higher levels in EAL. **B.** Up-regulated FX activity in EAL homogenates, compared to the corresponding SAAL. Factor X activity was significantly elevated (~3-fold increase) in the EAL homogenates than in the advanced ones, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$. **C.** FXII activity comparison between EAL and SAAL, showing significantly higher levels in the early-staged atherosclerotic plaques (~2-fold increase), Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$. **D.** Tissue factor activity is ~3-fold higher in early atherosclerotic lesions homogenates than in the stable advanced ones, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$. **E.** TFPI Activity shows 1.6-fold increase in early atherosclerotic plaques. **F.** TF/TFPI Ratio in EAL (0.294 (IQR 0.109 – 0.770)) and SAAL (0.174 (IQR 0.117 – 0.257)), Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$

Enhanced Thrombin Generation in EAL

In the absence of TF and entirely dependent on the pro-coagulant molecular content in the tissue homogenate, all 27 EAL induced thrombin formation in normal pooled plasma, showing significantly higher values (1240 nM.min (IQR 1173 – 1311)) compared to SAAL (782 nM.min (IQR 0 – 1151)) (**Figure 2B**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$). Twenty-six early atherosclerotic lesions induced higher ETP than their corresponding advanced atheromas. For the SAAL, 10 lesions did not trigger any thrombin generation. Furthermore, EAL showed a significantly increased thrombin generation potential when compared to areas of normal aorta obtained from the same individuals (263.3 nM.min (IQR 117.8 – 350.3); Wilcoxon 2-sample test, 2-tailed exact $p < 0.0001$) (**Figure 2B**). In addition, SAAL also demonstrated significantly higher ETP than their paired normal vessel homogenates (Wilcoxon 2-sample test, 2-tailed exact $p = 0.0053$), thus consolidating the pro-coagulant tendency in early atherosclerosis.

TAT Levels Additionally Point to a Higher Thrombin Generation in EAL Homogenates

Once generated, thrombin is inhibited upon binding to antithrombin, thus forming a stable TAT complex. TAT complexes are considered a

Figure 2. Effect of Phospholipid Concentration on Thrombin Generation. Overall pro-coagulant activity of atherosclerotic plaque homogenates and normal vessels, assessed by the means of thrombin generation and/or TAT complexes.

A. The influence of increasing phospholipid concentrations (1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 μM) on thrombin generation in normal pool plasma triggered by 1pM TF (without addition of plaque homogenates). At concentrations greater than 4 μM phospholipids, thrombin generation is independent from additional phospholipid present in the measured sample (such as present in the added plaque homogenate) (p value was calculated by using Repeated-measures ANOVA test). Furthermore, thrombin generation in EAL and/or SAAL homogenates was established at a final concentration of 4 μM phospholipids upon measurement, irrespective of the plaque type studied.

B. Thrombin generation assessed in paired EAL, SAAL and areas of normal aorta. EAL and SAAL show significantly higher thrombin generation levels compared to normal arterial vessels. These data consolidate the pro-coagulant state in early atherosclerosis, but furthermore indicate that the pro-thrombotic tendency in EAL is not dependent on variations in the cellular density/phospholipid con-

tent as a result of vessel wall structure alterations, which occur upon the atherosclerotic progression.

C. TAT levels measured in paired early and advance stage plaque homogenates

Legend: HAV – Healthy Arterial Vessels

Figure 3. Layer-selective coagulation factors activity analysis, presenting the pro-coagulant state of tunica intima, media and respectively adventitia in EAL vs. SAAL

A. H&E sections, demonstrating histologically controlled layer preparation and confirming the anatomy of the desired vessel wall layer. The activities of coagulation proteins were then tested as per tunica intima, media and adventitia, prepared from the harvested layers of paired EAL and SAAL: ETP (**B**); prothrombin (**C**); FX (**D**); FXII (**E**); TF (**F**) and TFPI (**G**). TF/TFPI ratio as per layers is demonstrated in panel **H**. Statistical significance (Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$) is shown as “*”

Legend: INT – Tunica Intima; MED – Tunica Media, ADV – Tunica Adventitia

marker of *in vivo* intravascular thrombin generation, therefore, the main goal of this experiment was to assess whether there was an excess of FIIa generation in EAL *in situ* compared to their matched SAAL. The concentration of TAT complexes in EAL was significantly higher (1045 ng/mg (IQR 842.6 - 1376)) compared to their paired SAAL homogenates (718.4 ng/mg (IQR 508.6 - 1151)) (**Figure 2C**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$) confirming a more pro-coagulant state in EAL.

Layer-selective Analysis of Coagulation Factors Activities Consolidated a More Pro-coagulant State of EAL vs. SAAL

To give a better insight into pro-coagulant properties of the atherosclerotic lesions, we undertook a more selective, layer-specific analysis in which the potential pro-coagulant effects of the different vessel wall layers were studied. The activity of coagulation factors was analyzed in tissue homogenates prepared from tunica intima, media and adventitia (histologically controlled anatomical separation, **Figure 3A**). All three layers were harvested in 42 specimens (21 pairs of EAL & SAAL from the original tissue collection). ETP values in all layers of EAL were found significantly higher (Intima: 1489 nM.min (IQR 1353 - 1680); Media: 1734 nM.min (IQR 1256 - 1983); Adventitia: 1872 nM.min

(IQR 1655 - 2171)) compared to the corresponding SAAL layers (Intima: 437.9 nM.min (IQR 290.3 - 549.9); Media: 392.1 nM.min (IQR 219.7 - 680.9); Adventitia: 524.1 nM.min (IQR 394.1 - 787.7)) (**Figure 3B**, Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$, all). This strongly pronounced pro-coagulant state of the EAL layers was additionally confirmed by significantly elevated prothrombin, FX and FXII levels (**Figure 3C, D, E**). Intimal layers of both EAL and SAAL showed comparable TF activity, whereas TF was significantly increased in media and adventitia of EAL vs. SAAL (**Figure 3F**). While demonstrating comparable activities in terms of TF, EAL intimal layers contained significantly higher TFPI levels (**Figure 3G**), yielding a significantly lower TF/TFPI ratio in EAL compared to SAAL. EAL and SAAL media layers did not significantly differ in TFPI levels, whereas TFPI in EAL adventitia was significantly higher compared to SAAL one (**Figure 3G**). Tunica adventitia exhibited the most pro-coagulant phenotype of all vessel wall layers in terms of thrombin generation. Its values in both EAL and SAAL were significantly higher compared to the ones measured in tunica intima and media.

Gene-expression of Coagulation Genes in EAL vs. SAAL

To better explore to what extent and which coagulation proteins are ex-

pressed on the genome level within the arterial vessel wall, gene expression profiles of EAL and SAAL were obtained using microarray analysis. In a separate set of patients, early and advanced carotid lesions were collected from the same patient (at autopsy), and fold changes in gene expression were assessed by comparing the advanced lesions to the early lesions. The results indicated that several coagulation factor genes were expressed in both types of atherosclerotic lesions. After correction for multiple testing, 14 coagulation genes showed significant differential transcript levels between EAL and SAAL. **Figure 4** demonstrates the relative mRNA levels described as SAAL/EAL ratio. Of these 14 differentially regulated genes, 6 were up-regulated in EAL (expressed as fold change < -1.0), whereas 8 were up-regulated in SAAL (expressed as fold change $> +1.0$) (**Table S1**). Fold changes ranged from -1.13 to -2.96 for the up-regulated genes in EAL and from 1.08 to 1.29 for the up-regulated genes in SAAL. Additional information is provided in **Table S1** in the Data Supplement.

IHC Staining - Early Atherosclerotic Lesions (EAL)

In EAL, moderate (fibrinogen/fibrin, FIX, TFPI) to strong positivity for von Willebrand factor (vWF), FX, prothrombin/thrombin, protein S (PS), activated protein C (APC) and was observed in the endothelial luminal

cells, indicated by a sharp demarcation of the endothelial lining (**Figure 5, Table 2**). In addition, a positive focal endothelial distribution for TF, FVII, FXII, FXI, kallikrein and TM was shown. MΦ and foam cells stained intensely positive for TF, FVII, FX, prothrombin/thrombin, kallikrein and FXI. Despite that other coagulation proteins such as FXII, FIX, PS, PC and APC were also expressed by MΦ and foam cells, their expression or immunoreactivity was either scarce or focal. Furthermore, EAL were characterized by TF, FVII and FX expression throughout the SMC-rich intima. Medial SMCs associated FVII was located in the cytoplasm and not on the membrane. FXII and FII showed enhanced expression in medial SMC. Tissue factor, FVII, FX, fibrin, kallikrein, TM and TFPI were also associated with medial SMC, whereas FIX demonstrated a more patchy expression. Within the adventitia, the vasa vasorum externa showed positive staining for most of the studied factors while the fibroblasts were positively associated with FX, prothrombin/thrombin, kallikrein, vWF and FXII. An extensive overview of all single and double-staining observations in EAL and SAAL is provided in **Figure 5, Table 2** and **Figure S2** (in the Data Supplement).

IHC Staining - Stable Advanced Atherosclerotic Lesions (SAAL)

In atherosclerotic tissues classified

as SAAL, the endothelial luminal lining was moderately positive for FIX, TM, APC and von Willebrand factor, whereas FXI stained weakly positive (**Figure 5**). Some endothelial segments demonstrated a focal expression of the anticoagulant protein PS. Moreover, all anticoagulant proteins (PS, TM, APC and TFPI) were found associated with MΦ/foam cells. Furthermore, TM, APC and TFPI were also localized in the endothelial cells of the vasa vasorum and in endothelial cells of vessels sprouting into the lesions. Besides TM, intimal and medial SMC contained most of the pro-

coagulant proteins, as well as thrombin and fibrinogen/fibrin (**Figure 5, Figure S2**). A slight focal association between FIX and XI with intimal SMC was observed. In contrast, SMC of the media stained moderately for FXII. Some of the medial SMC stained positive for FXI but also showed double positive staining for both CD68 and anti-smooth muscle actin (ASMA), suggesting either transdifferentiation of SMCs into foam-like cells, or SMC outgrowth from mononuclear cells, the latter was recently reported to be a thrombin-promoted action¹⁹. TF and factors VII, X, XI and

Figure 4. Relative mRNA levels of coagulation genes (microarray analysis), presented as a SAAL/EAL ratio, indicate the differential expression of various coagulation factor genes in EAL vs. SAAL. Statistical significance (Wilcoxon 2-sample test, 2-tailed exact $p < 0.05$) is shown as “*”; Legend: FVIII-A1 - FVIII-associated (Intronic Transcript) 1; FVIII, PC - FVIII, Procoagulant Component; EPCR – Endothelial Protein C Receptor.

Figure 5. Immunohistochemical (IHC) stainings for coagulation proteases in paired EAL and SAAL, 10x magnification. Positive staining is presented in red; Legend: TF – Tissue Factor; FVII - Factor VII; FX – Factor X; FII/FIIa – Prothrombin/Thrombin; FGN – Fibrinogen; FXII – Factor XII; FXI – Factor XI; FIX – Factor IX; TM – Thrombomodulin; PS – Protein S; APC – Activated Protein C; TFPI – Tissue Factor Pathway Inhibitor; TAT – Thrombin-Antithrombin Complex; vWF – von Willebrand factor

XII were either weakly or focally present on MΦ and FXII was also found on some foam cells. FIX showed a pronounced focal distribution on both MΦ and foam cells. Some fibroblasts contained FX, FXII and fibrin. None of the SAAL from the current set of lesions showed a strong or even moderate positive staining for TF, neither in the necrotic core. Necrotic core revealed a focal presence for most of the procoagulant proteins, except for thrombin, fibrin and the anticoagulant APC, which stained weakly positive. A broad histological evaluation is available in **Figure 5, Table 2** and **Figure S2** (in the Data Supplement).

IF Staining – Co-Localizations of TF/FVII/FX with MΦ/VSMC

Double IF staining with CD68 for MΦ, respectively ASMA for SMC, suggests that most of the MΦ and SMC were involved in the synthesis of TF, FVII and FX. The formation of a ternary complex TF/FVII/FX is a potent trigger not only for coagulation (thrombin) but also for many pro-inflammatory cell-signaling pathways that are pivotal in cardiovascular disease. Therefore, we examined the presence of these procoagulant proteins on MΦ and SMC also by the means of immunofluorescence staining on corresponding EAL and SAAL sections which revealed that TF/FVII/FX co-localized with both MΦ/foam cells and SMCs, sug-

gesting a local system of thrombin generation, which may regulate pathophysiological processes such as cell migration and inflammation. Comparing EAL vs. SAAL, co-localizations are scarcer and more diffuse in SAAL, whereas EAL sections show brighter labeling and denser character (**Figure 6**).

Discussion

The present study shows that atherosclerotic plaques exhibit functional activity of many coagulation proteins (prothrombin, FX, FXII and TF) and represents the first study to demonstrate the presence and distribution of all coagulation proteins in both early and advanced human atherosclerotic plaques. We provide new data pointing to local synthesis of several coagulation proteins within the atherosclerotic vessel wall. Furthermore, we indicate a co-localization of key procoagulant proteins with SMC and macrophages, suggesting an active, cell-based coagulation network within the atherosclerotic plaque. Finally, the principal finding of this study is an enhanced pro-coagulant profile of early atherosclerotic plaques compared to stable advanced plaque homogenates, consolidated by an elevated thrombin generation potential and significantly increased TAT levels in the early-staged atherosclerotic tissues. Thus, we provide novel evidence which may help widening the thrombogenic spectrum of “high-

risk" plaques and suggest that local coagulation factors might play an important role not only in the contribution to the onset of atherothrombosis but also to progression of atherosclerotic process.

In contrast to our expectations, these data reject the initial hypothesis that thrombin generation would positively correlate with progression of atherosclerosis. One possible mechanism that might explain the abundant presence and functional activity of coagulation proteins in the early stage of atherosclerosis could be that many of the coagulation proteins

help to propagate the atheromatous plaque by inducing multiple pro-atherogenic actions such as cellular adhesion, migration, angiogenesis and inflammation^{7,12}. In addition to their pro-thrombotic nature, coagulation proteases induce cell proliferation⁶, the latter of a great importance for determining the stability of an atherosclerotic lesion. The abundance of almost all (intrinsic and extrinsic) coagulation proteins suggests that the generation of thrombin is an active process during atherogenesis, supporting a major role of thrombin (and possibly fi-

Figure 6. Immunofluorescence stainings demonstrating cellular co-localizations of TF, FVII and FX with MΦ and VSMC on paired EAL & SAAL, 60x magnification; Legend: TF, FVII and FX – stained with FITC (green color) / MΦ & SMC – stained with Rho (red color)/ Blue color - Nuclei; Co-localizations are demonstrated in yellow/orange color. TF – Tissue Factor; FVII - Factor VII; FX – Factor X; MΦ – Macrophages; SMC – Smooth Muscle Cells; FITC - Fluorescein Isothiocyanate; Rho – Rhodamine

brin) in this condition. Moreover, the enhanced pro-coagulant state of EAL was additionally confirmed by the layer-selective analysis, which also revealed a significantly increased pro-thrombotic phenotype for tunica intima, media and adventitia in early vs. stable advanced atherosclerosis. Previous reports have also documented that adventitial fibroblasts, which surround the arterial walls, contain high amounts of TF, providing a “hemostatic envelope”²⁰. Our study confirmed this latter finding by showing that tunica adventitia was the most pro-coagulant vessel wall layer of all tested. A contributory or distinct effect of other coagulation proteases on atherogenesis, includ-

ing factors XII, XI, IX and X cannot be ruled out. Published data have associated factor XII in blood with cardiovascular disease and while its action has been mainly contributed to promoting arterial thrombosis, the presence in atherosclerosis and in vicinity of macrophages and foam cells suggests cell directed actions of this protein. Indeed, *in vitro* work has demonstrated the localization of contact system proteins on MΦ²¹, suggesting a direct effect on inflammatory pathways. Of interest, although the expression and activity of factor XII diminished upon progression of atherosclerosis in our data, the staining for kallikrein was more abundant in the advanced lesions in

Table 2. Presence of Coagulation Proteins in Various Arterial Vessel Wall-Compartment Throughout the Early and Advanced Stages of Atherosclerotic Development

Types and Structures	TF	FVII	FX	FII/FIla	Fibrin	FXII	FXI	FIX	Kallikrein	Thrombomodulin	Protein S	APC	TFPI	vWF
EAL														
Endothelial cells	F	F	+++	+++	++	F	F	++	F	F	+++	+++	++	+++
Macrophages	+++	+++	+++	+++	+	F	+++	F	++	+	F	F	+	-
Foam cells	+++	+++	+++	+++	+	F	+++	F	++	+	F	F	+	-
Intimal SMC	+++	+++	+++	F	F	F	+	F	+	++	F	F	+	-
Medial SMC	++	+	++	+++	+	+++	+	F	++	++	-	F	++	F
Vasa vasorum	+++	+++	+++	+++	+++	+++	+	+	+++	++	++	+++	++	++
SAAL														
Endothelial cells	F	F	F	F	F	F	+	++	F	++	F	++	F	++
Macrophages	+	F	+	+	+	F	F	+	+	+	F	F	+	-
Foam cells	+	F	+	+	+	F	F	+	+	+	F	F	+	-
Intimal SMC	F	F	+	+	F	F	F	F	+	++	F	F	+	-
Medial SMC	+	+	++	+	F	++	F	F	F	F	-	-	+	-
Necrotic core	F	F	F	+	+	F	F	F	-	-	F	+	F	F
Vasa vasorum	+++	++	++	++	++	+	+	+	++	++	++	++	+	++

The table represents immunohistochemical staining observations in EAL and SAAL, obtained from the same corresponding individuals. FII/FIla indicates prothrombin/thrombin; vWF, von Willebrand factor. Staining grading is as follows: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive; and F, focal (only certain cells stain, not all).

vicinity of MΦ and foam cells. The latter may be compatible with a switch in direction from a procoagulant to a pro-inflammatory action of the factor XII started contact system as established in a recent study²². Switching the action of factor XII into an inflammatory direction may in part explain the diminished thrombin generating capacity in advanced lesions, which are dominated by inflammatory characteristics (including elevated interleukin 6 and TNF-α levels, data not shown). The apparent loss in TM staining within the advanced plaques may also be compatible with increased inflammatory activity as proposed previously¹¹, while apparently the vasa vasorum maintain TM in amounts comparable to the early lesion. The abundant presence of coagulation proteins in the early lesions in particular raises other questions regarding causes and consequences. It is well known that the initial stage of atherosclerotic development (e.g. intimal thickening) is characterized by enhanced SMC migration and proliferation²³. In contrast, an advanced stage of atherosclerotic progression results in decreased cell density, mostly around the fibrous caps and necrotic core/lipid pool²⁴⁻²⁵. However, the decreased pro-coagulant potential in SAAL was found independent of the vessel wall structure alterations which occur upon atherosclerotic progression (**Figure 2A, 2B**), thus suggesting that differences in protein translocation from circulation

towards the vessel wall, as well as local protein expression by different cell types, may also significantly contribute to variations in protein levels. While all coagulation proteins except for tissue factor may end up in the vessel wall by diffusion from the circulating blood, the localization suggest that local synthesis may be involved. Moreover, the microarray analysis clearly shows that multiple coagulation proteins are expressed on the level of mRNA synthesis in the arterial vessel wall (**Figure 4, Table S1**). However, although some of the coagulation proteins were differentially up-regulated in EAL, a similar picture was also observed for other coagulation genes in SAAL, suggesting that the differences in protein expression (as revealed by immunohistochemistry) and activity levels of coagulation proteins were not completely reflected by differences in gene expression levels. Moreover, it is known that RNA expression profiles do not always correlate with protein expression and subsequent biological activity²⁶. Nevertheless, our data point to local synthesis of several coagulation proteins within the atherosclerotic vessel wall, suggesting this may be part of an active regulatory mechanism, subsequently leading to the enhanced procoagulant state in EAL. The pleiotropic effects of proteases like thrombin and activated factor X (FXa), as well as the cell growth promoting effects of fibrin (and its split products), may be also evoked

as part of a response to injury mechanism. This response action of blood coagulation is now well established in inflammatory conditions like sepsis. As a side effect of this process the formation of fibrin may serve to protect the early lesions from rupture and contribute to plaque stability. In addition, a recent study demonstrates that hypercoagulability in transgenic mice promotes plaque stability²⁷. At the same time the activity of coagulation proteases contributes to local inflammation and angiogenesis, so the latter will eventually prevail over processes such as proliferation, thus compromising plaque stability. This pro-inflammatory state of the evolving plaque, including increased apoptosis of SMCs, gradual protein loss and enhanced angiogenesis will herald plaque evolution and greater vulnerability. Hence, EAL may be more stable due to more clotting activity, whereas advanced lesions may be more vulnerable due to instability. In case of a plaque rupture, even relatively small amounts of TF and other proteins may still be highly thrombogenic, precipitating thrombus formation and cardiovascular events. In conclusion, our findings provide substantial new data illustrating the close involvement of coagulation proteins in the entire process of atherogenesis. While in the early lesions essentially all coagulation proteins, including those from the contact/intrinsic system are readily detectable (possibly supporting plaque

stability), upon transformation to advanced lesions the amount and activity of these proteins diminishes. The loss in coagulation activity, possibly due to increased inflammatory pressure, may reduce plaque stability and contribute to the risk of plaque rupture. These results point to various and specific functions of coagulation proteins in regulating progression of atherosclerosis and may provide novel insights in the genesis of atherothrombosis. These data also suggest ways to modulate atherogenesis and possibly reducing atherosclerosis that may eventually be clinically useful. The fact that new specific anticoagulant agents are being clinically tested underlines the necessity of further studies in this area.

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Supplemental Material

Supplemental Materials and Methods EAL & SAAL Layer Preparation and Homogenization

A segment from each of the collected arterial samples was used for an anatomic separation into its three layers – tunica intima, media and adventitia. Once the separated layer strips were obtained, a section of each layer was examined (H&E staining) in order to confirm the desired layer anatomy. The remaining part of the layer specimen was homogenized according to the aforementioned technique and used for further coagulation factors activity analysis. Layer preparation was successful in 21 out of the 27 pairs of EAL and SAAL (42 out of 54 atherosclerotic specimens).

Effect of Time Delay between Death and Post-Mortem Examination on Coagulation Proteins Activity

To address the question about the effect of time on the stability of coagulation proteins activities, we obtained vital atherosclerotic lesions from patients undergoing carotid endarterectomies (Department of Vascular Surgery, Maastricht University Medical Center; n=4). Each specimen was divided into 4 proportionally equal longitudinal segments (consisting of the entire vessel wall/plaque structures) and was kept at 4°C in PBS solution, thus simulating post-mortem conditions. One segment per sample was collected per time point (baseline (0 hours), 2 hours, 4 hours and 8 hours), snap frozen, homogenized and then studied for the activities of TF, FII, FX and FXII as described in the “Materials and Methods” section.

Thrombin Generation, Prothrombin, FX and FXII Activity Assays

The Calibrated Automated Thrombogram (CAT, Thrombinoscope, the Netherlands) was used to determine the contribution of atherosclerotic tissue homogenates to thrombin generation in human plasma (in triplicates; inter-assay CV<10%). We adapted the protocol from the recording of thrombin generation curves in platelet poor plasma as described previously¹: thrombin generation was triggered in 80 μ L of platelet poor pooled human plasma (University Hospital Maastricht, consisted of plasma from 80 healthy volunteers) by adding 15 μ L of tissue

homogenate (5 mg/mL total protein), 16 mM Ca^{2+} and 4 μM phospholipids to the reaction mixture (final concentrations, determined as optimal pre-analytical conditions for CAT method above which a threshold effect is observed²⁻⁴). Endogenous thrombin potential (ETP, the area under the curve) was calculated from the thrombin generation curve using Thrombinoscope software (Thrombinoscope B.V., The Netherlands). FII, FX and FXII activities in atherosclerotic tissue homogenates were assessed via modified thrombin generation assays by adapting the original protocol, as described¹. Prothrombin activity assay was performed in triplicates. The reaction mixture consisted of 80 μl FII-deficient or diluted plasma, in the presence of 10 μl tissue supernatant (final protein concentration of 0.4 mg/mL), 20 μl MP reagent (Thrombinoscope B.V.) containing 4 μM phospholipids, 3 μl corn trypsin inhibitor (CTI, Hematologic Technologies, Inc., final concentration of 40 $\mu\text{g/mL}$), and 2 μl Active site inhibited seven (ASIS, final concentration of 25 nM). CTI was used to specifically inhibit activated FXII (FXIIa), whereas ASIS binds to TF, thereby suspending FVII/VIIa of forming a complex with the latter and blocking the extrinsic pathway-driven coagulation. The reaction was initiated by adding Flu-Ca buffer containing Fluobuffer, 16 mM CaCl_2 , fluorogenic substrate and ecarin (from *Echis carinatus* venom, Sigma-Aldrich). Ecarin is used as a

specific activator of prothrombin. Hence, thrombin generation was completely dependent on the presence and activity of FII in the atherosclerotic lesion homogenate. A reference curve was prepared by serial dilution of FII-deficient plasma in human normal pooled plasma obtained from 80 healthy volunteers within the department of Internal Medicine, Maastricht University Medical Center. Fluorescence was read in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 filter set, and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV) as described previously⁵.

FX and FXII Activity Assay

A method analog to the FII activity described above was implemented for assessing FX activity. FX-deficient plasma (Dade Behring) was utilized for the preparation of the standard curve and 5 μl Russell's viper venom factor X activator (RVV-X, Enzyme Research Laboratories Inc.) was added in the trigger mix as a specific activator of FX. Hence, thrombin generation was determined by the presence and activity of FX in the atherosclerotic lesion homogenate. For the FXII activity assay, FXII-deficient plasma (George King) was used for preparation of the standard curve and analysis of tissue homogenates. CTI was omitted from the reaction to allow for FXII

dependent activation of thrombin generation. Kaolin (Sigma-Aldrich) was used as trigger at a final concentration of 400 µg/mL. The test was carried out in triplicates.

Effect of Phospholipid Concentration on Thrombin Generation / Thrombin Generation in Normal Arterial Vessel Wall Homogenates

Due to the different nature of the atherosclerotic lesions, we assumed that early and stable advanced plaques may also vary in phospholipid content as a result of altered cellular density, the latter normally observed upon atherosclerotic progression. Hence, we estimated that differences in the phospholipid levels in EAL (plaques higher in cellular density) and SAAL homogenates (plaques with more fibrotic and acellular character) may potentially influence thrombin generation, yielding an enhanced pro-thrombotic state in EAL *in vitro*. To address this matter, we first studied the influence of increasing phospholipid concentrations (1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 µM) on thrombin generation in normal pool plasma, triggered by 1pM TF (without addition of plaque homogenates). In addition, areas from normal abdominal aorta were harvested from the same individuals (n=27) from which the original set of atherosclerotic plaques was obtained. All tissue specimens were histologically evaluated and showed no signs of atherosclerosis develop-

ment. Homogenates were prepared and thrombin generation was assessed as described.

Thrombin-Antithrombin Complexes (TAT) Levels, Tissue Factor Activity Assay, TFPI Antigen Assay

TAT complexes were determined in triplicates using a commercial ELISA kit (Cat.#TAT-EIA, Kordia, The Netherlands). This assay was performed in compliance with all manufacturer's directions, however, it was slightly adapted with respect to the use of tissue homogenates. Instead of plasma, the same amount of atherosclerotic homogenate was added per well (100 µl of diluted sample with a final concentration of 5 mg/mL total protein). TF activities in tissue homogenates were determined in triplicates using a home-made activity assay⁶. In brief, dissolved tissue homogenates with a concentration of 5 mg/mL total protein were diluted 160 times in HN-buffer. Samples were incubated for 10 minutes at 37°C in the presence of recombinant FVII (FVII) (Novo Nordisk, Bagsværd, Denmark), 0.2 mM 20/80 PS/PC vesicles, 1 U/mL Bovine FX (Sigma-Aldrich) and 100 mM Ca²⁺. The formation of FXa was then measured kinetically using the chromogenic substrate 2765 (Chromogenix, final concentration of 0.7 mg/mL diluted in 50 mM Tris-HCl, 175 mM NaCl, 30 mM Na₂EDTA, pH 7.4) by measuring the OD at 405 nm each 15 seconds, for

15 minutes at 37°C.

The levels of TFPI antigen in human EAL and SAAL were measured by the means of a home-made high-sensitive total TFPI immunoenzymatic method (ELISA). This assay was performed in triplicates using own monoclonal anti-TFPI K1 fragment antibody for capture and a specific HRP-conjugated monoclonal anti-TFPI K2 fragment antibody for detection (C. F. A. Maurissen, J.R., and T. M. Hackeng, manuscript in preparation).

RNA Isolation and Quantification/ Microarray Hybridization and Data Analysis

In a separate new set, consisting of paired early and advanced carotid lesions (n=4 pairs) collected from each patient upon autopsy, we studied which coagulation proteins show mRNA expression within the arterial vessel wall upon atherosclerotic progression. Total RNA was isolated using the guanidine isothiocyanate/CsCl method⁷, followed by further purification and concentration using RNeasy mini columns (Qiagen, Hilden, Germany). RNA quantity and quality were determined using a nanodrop spectrophotometer (Witec AG, Littau, Switzerland) and a 2100 Bioanalyser (Agilent Technologies, Palo Alto, USA) respectively. Good quality RNA (RIN≥5), from both EAL and SAAL, was successfully collected. Double-stranded cDNA was synthesized from ~2.0 µg of total RNA us-

ing the One-Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA, USA), and used as a template for the preparation of biotin-labeled cRNA using the GeneChip IVT Labeling Kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled cRNA was hybridized in duplicate to the HGU133 2.0 Plus Array (Affymetrix, Santa Clara, CA, USA), washed, stained with phycoerythrin-streptavidin conjugate (Molecular Probes, Eugene, USA), and the signals were amplified by staining with biotin-labeled anti-streptavidin antibody (Vector Laboratories, Burlingame, USA) followed by phycoerythrin-streptavidin. The arrays were laser scanned with the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Data were saved as a raw image file and quantified using GCOS 1.2 (Affymetrix, Santa Clara, CA, USA). Rosetta Resolver Platform Version 4 (specifically developed for Affymetrix GeneChips) was used to correct for multiple testing and analyze differences in single gene expression.

Immunohistochemical (IHC) & Immunofluorescence Stainings, Immunohistological Evaluation

Paraffin sections (4 µm) were deparaffinized and washed 3 times in Tris-buffered saline (5 mmol/L Tris- HCl, pH 7.5, 140 mmol/L NaCl). Before application of the TM and FIX antibody, tissue sections were pretreat-

ed with pepsin (Sigma Chemical Company, St. Louis, MO, #7012) (1 mg/mL in 0.1 M HCl) at room temperature for 30 minutes, in order to increase the visibility. Parallel sections were stained with polyclonal goat anti-human TF (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, # SC-23596), polyclonal goat anti-human K-20 human FVII (1:200, Santa Cruz, # SC-16343), polyclonal goat anti-human FX (1:200, Santa Cruz, # SC-16341), polyclonal goat anti-human FII/FIIa (1:200, Santa Cruz, # SC-16972), polyclonal rabbit anti-human fibrinogen (1:200, Dako Corporation, #A0080), monoclonal mouse anti-human fibrin (1:200, Lifespan Biosciences, LS-C23559), monoclonal mouse anti-human FXII (1:50, in house⁸), monoclonal mouse anti-human FXI (1:50, in house⁸), polyclonal goat anti-human FIX (1:100, Santa Cruz, # SC-16337), monoclonal rabbit anti-human K-15 prekallikrein and kallikrein (1:50, in house⁸), polyclonal rabbit anti-human TM (1:100, Santa Cruz, # SC-9162), polyclonal rabbit anti-human PS antibody (1:200, Dako Corporation, Carpinteria, CA), monoclonal rat anti-human PC (HM 2149, HyCult Biotechnology BV, The Netherlands), specific monoclonal rat anti-human APC PC107 antibody (HM 2151, HyCult Biotechnology BV, The Netherlands), specific monoclonal mouse anti-human APC (generously donated by Charles T. Esmon, OMRF, Oklahoma, US), monoclonal mouse anti-human TFPI C-terminus (1:250, San-

quin, Amsterdam, The Netherlands) and rabbit polyclonal antibodies against human vWF (1:200, Dako Corporation, Carpinteria, CA). For the mouse monoclonal antibodies, biotinylated sheep anti-mouse IgG (1:250, Amersham Life Science, # RPN-1001) was used as the secondary antibody, whereas power vision poly-AP anti-goat (Klinipath, Duiven, the Netherlands # DPVG-110 AP) was used as a secondary antibody for the polyclonal antibodies. After incubation with an alkaline phosphatase-coupled avidin-biotin complex (ABC complex, Dako), antibodies were visualized with an alkaline substrate kit (Vector SK-5100, Vector Laboratories, Inc). Sections were counterstained with hematoxylin (Klinipath, #4085-9002,) and mounted with imsol (Klinipath, # 7961,) and entellan (Merck # 7961,). In negative controls, incubation with primary antibody was omitted. Double staining was performed to colocalize presence of coagulation factors with vascular SMC or MΦ. For this purpose, the single staining procedure was followed by secondary staining just before the hematoxylin counterstaining. Mouse anti-human CD68 (1:100, Dako # M 0814) and mouse anti-human ASMA (1: 500, Dako, # M 0814) were used to identify MΦ and SMC, respectively. Before application of the CD68 antibody tissue sections were pretreated with pepsin (1 mg/mL in 0.1 M HCl) at room temperature for 30 minutes. For visualization, biotinylated sheep

anti-mouse antibody (Amersham, #RPN-1001), strept ABC-alkaline phosphatase (Dako, # K-0391) and Alkaline Phosphatase kit I (blue) (Vector Laboratories, Burlingame, California. #SK-5100,) were used. Localization and co-localization of hemostatic proteases was further assessed by the use of single and double immunofluorescence staining on paired EAL and SAAL sections. The following secondary antibodies were utilized: Rabbit polyclonal anti-goat IgG - H&L (FITC-labeled, Abcam, ab6737, 1:200); goat polyclonal anti-mouse IgG - H&L (FITC-labeled, Abcam, ab6785, 1:200); rabbit polyclonal anti- goat IgG - H&L (Rhodamine-labeled, Abcam, ab6738, 1:200) and goat polyclonal anti-mouse IgG - H&L (Rhodamine-labeled, Abcam, ab6786, 1:200).

A semi-quantitative visual scoring system was used to evaluate the IHC staining. Two investigators (M.Y., S.N.), blinded with respect to the plaque phenotype, independently examined the specimens using light microscopy at 250x magnification. The intensity of the staining was ranked on an arbitrary scale as follows: - = Negative; + = Weak positive; ++ = Moderate positive, +++ = Strong positive; Focal (F) = Only certain cells stain, not all. For this purpose, fifteen random slides were analyzed for each of the stained coagulation proteins as per plaque type. The intra- and inter-observer variability was less than 10%.

Supplemental References

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Supplemental Tables

Table S1. Differential expression of coagulation factor genes in EAL vs. SAAL.
Negative fold change values indicate up-regulation of coagulation factors expres-
sion in EAL, whereas positive values represent up-regulated genes in SAAL.

Sequence Description	Relative mRNA Level - Ratio (SAAL/EAL)	Fold Change	P-value	Intensity - EAL	Intensity - SAAL
Fibrinogen β -chain	0.88219	-1.13355	0.15738	15.07589	14.65217
FIX	1.17648	+1.17648	0.00030	70.02775	75.62477
FV	1.03517	+1.03517	0.49269	16.65244	16.81713
FVII	1.05108	+1.05108	0.32227	14.80140	14.98169
FVIII, Procoagulant Component	0.88213	-1.13362	0.00965	21.98895	20.95785
FVIII-associated (Intronic Transcript) 1	0.76292	-1.31076	9.82422E-9	144.24416	124.44188
FX	0.82084	-1.21826	0.05492	23.81925	22.39022
FXII	1.04966	+1.04966	0.09276	14.50063	14.76679
FXIII, A1 Polypeptide	1.24629	+1.24629	0.01157	208.19531	245.41655
Heparin Cofactor II	0.90993	-1.09899	0.26666	23.46109	22.40778
PAI-1, Member 1	0.64073	-1.56072	6.55047E-8	253.35378	200.89241
PAI-1, Member 2	0.84700	-1.18064	0.01312	429.71500	379.62524
Plasminogen Activator, Urokinase	1.13254	+1.13254	0.00122	17.03220	17.88443
Protein S (α)	1.20045	+1.20045	0.00269	294.18268	327.74210
Protein Z	0.83662	-1.19529	0.01094	15.11580	13.52573
TF	0.97784	-1.02267	0.65523	16.83669	16.48907
TFPI	1.29410	+1.29410	2.77326E-11	171.43971	197.71820
TFPI 2	0.33825	-2.95640	1.38133E-8	726.61249	430.28033
Prothrombin	1.08162	+1.08162	0.04902	37.21303	38.66477
Thrombomodulin	0.93729	-1.06691	0.31647	40.74794	42.61707
von Willebrand Factor	0.94222	-1.06132	0.28796	225.85161	219.74393
α -2 Antiplasmin	1.15311	+1.15311	0.00439	223.69295	245.03899
α -2-Macroglobulin	1.17901	+1.17901	9.19786E-9	420.98053	461.35699
Marker, Positive Control	0.78588	-1.27246	7.67398E-20	265.98102	215.30423
Blank, Background Negative Control	0.90907	-1.10002	0.13188	10.62375	11.85352

Figure S1. Effect of Time Delay between Death and Post-Mortem Examination on (A) TF, (B) FII, (C) FX and (D) FXII activities in homogenized human atherosclerotic plaques (n=4)

Each dotted line, connecting 4 grey dots, represent one single atherosclerotic plaque (n=1), initially divided into 4 proportionally equal segments which were kept at 4°C in PBS and harvested at different time points (0 hrs, 2 hrs, 4 hrs and 8 hrs) after autopsy (p values were calculated by using Repeated-measures ANOVA test). Thus, each dotted line also indicates the effect of time on the activity of the tested coagulation proteins.

Figure S2. Immunohistochemical (IHC) stainings - Localization and co-localization of coagulation proteases in human atherosclerotic plaques at a cellular level, 100x magnification. Positive staining is presented in red. (Images from both EAL and SAAL are shown.)

Legend: TF – Tissue Factor; FVII - Factor VII; FX – Factor X; FII/FIIa – Prothrombin/Thrombin; FXII – Factor XII; FXI – Factor XI; FIX– Factor IX; KLK – Kallikrein; TM – Thrombomodulin; PS – Protein S; APC – Activated Protein C; TFPI – Tissue Factor Pathway Inhibitor; TAT – Thrombin-Antithrombin Complex; vWF – von Willebrand factor; ECs – Endothelial Cells; MΦ – Macrophages; FC – Foam Cells; SMC – Smooth Muscle Cell

	ECs	MΦ/FC	INTIMAL SMCs	MEDIAL SMCs	NECROTIC CORE	VASA VASORUM
TF
FVII
FX
FII/FIIa
FIBRIN
FXII
FXI
FIX
KLK
TM
PS
APC
TFPI
vWF

4

ACCELERATED *IN VIVO* THROMBIN FORMATION INDEPENDENTLY PREDICTS THE PRESENCE AND SEVERITY OF CT-ANGIOGRAPHIC CORONARY ATHEROSCLEROSIS

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Objectives: To investigate the relationship between thrombin generation and the presence and severity of CT-angiographically defined coronary artery disease (CAD).

Background: Besides its pivotal role in thrombus formation, experimental data indicate that thrombin also plays a role in the pathophysiology of atherosclerosis progression and vascular calcification. Nevertheless, the clinical evidence remains limited.

Methods: Using 64-slice coronary computed tomographic angiography (CCTA), we assessed the presence and characteristics of CAD in patients (n=295; median age: 58 years) with stable chest pain. Coronary artery calcification (CAC) was graded as absent (Agatston-score 0), mild (Agatston-score 1-100), moderate (Agatston-score 101-400) and severe (Agatston-score >400). Calibrated automated thrombography was used to assess endogenous thrombin potential in plasma *in vitro*. Thrombin-antithrombin complexes (TATc) levels were measured as a marker for thrombin formation *in vivo*.

Results: TATc plasma levels were substantially higher in patients with CAD vs. patients without CAD (p=0.004). Significant positive correlations were observed between steadily increasing TATc levels and the severity of CAD (r=0.225, p<0.001). In multinomial logistic regression models, after adjusting for established risk factors, TATc levels predicted the degree of CAC:

mild (OR:1.56, p=0.006); moderate (OR:1.56, p=0.007); and severe (OR:1.67, p=0.002). Trends were comparable between the groups when stratified according to the degree of coronary luminal stenosis.

Conclusions: This study provides novel clinical evidence indicating a positive independent association between enhanced *in vivo* thrombin generation and the presence and severity of coronary atherosclerosis, which may suggest that thrombin plays a role in atherogenesis.

Atherosclerosis is a multifactorial chronic inflammatory vascular disorder(1,2). Given the abundant experimental evidence showing extensive interactions between the hemostatic, immune and inflammation systems, we have proposed a role for the clotting proteins in modulating atherosclerosis progression and atherosclerotic plaque phenotype(3). In particular, thrombin, which is the most central coagulation protein, is also recognized as a strong pro-inflammatory mediator. Endowed with a potent cell signaling capacity, thrombin can induce an array of pro-atherogenic and plaque-destabilizing effects such as inflammation, vascular smooth muscle cell migration and proliferation, leukocyte chemotaxis, proteolysis, apoptosis, angiogenesis, etc.(3,4) Recently, we demonstrated that thrombin, as well as other coagulation proteins, are widely expressed and functionally active throughout distinct compartments of

the arterial vessel wall(5), supporting an active cell-based coagulation network within human atherosclerotic plaques. G-protein-coupled protease-activated receptors (PARs), which are selectively cleaved by thrombin, are also abundantly distributed in the vasculature under normal conditions and overexpressed in atherosclerotic lesions(6). Experimental animal studies have clearly indicated that variations in the clotting activity affect the progression and thrombogenicity of atherosclerotic plaques(3). Antithrombotic therapy is a cornerstone in the management and prevention of atherothrombosis in patients(2). Experimental data demonstrate that direct thrombin inhibition substantially attenuates atherosclerosis development in ApoE-null mice(7) and protects against severe plaque progression in prothrombotic mice(8)(abstr). However, the role of blood coagulation proteins in atherogenesis, in particular thrombin, has not been adequately addressed in previously conducted clinical research. Cardiac computed tomographic angiography (CCTA) is a well-established non-invasive imaging modality, which has high diagnostic accuracy for detection and characterization of coronary atherosclerotic plaques(9,10). Using CCTA, we investigated the association between thrombin formation in plasma and the presence and severity of coronary atherosclerosis in patients with suspected coronary artery dis-

ease (CAD).

Methods

Study Population

We studied 295 adult patients who were referred from the cardiology outpatient department for CCTA because of stable chest pain, suspected for CAD. Scans were performed in our university medical center between January 2008 and June 2010 as part of the diagnostic work-up in these patients. Included were patients with a recent history of (a)typical chest pain, who underwent a coronary calcium score scan as well as CCTA. Excluded were patients with acute chest pain suspected for an acute coronary syndrome (ACS), patients with a history of acute myocardial infarction (AMI), percutaneous coronary intervention and/or coronary artery bypass grafting surgery, patients with missing data regarding their cardiac risk profile, patients with an inconclusive CT-scan and patients currently on anti-coagulation therapy (oral vitamin K antagonist/selective anticoagulants or low-molecular weight heparins). *In vitro* hemolysis of blood samples was also an exclusion criterion. We calculated the Framingham risk score (FRS) in all patients to estimate the 10-year risk of suffering a myocardial infarction or cardiovascular death(11). The Institutional Review Board and Ethics Committee at the Maastricht University Medical

Center approved the study, and all patients gave written informed consent.

CCTA Protocol

Scans were performed using a 64-slice multidetector-row CT-scanner (Brilliance 64; Philips Healthcare, Best, The Netherlands) with a 64 x 0.625 mm slice collimation, a gantry rotation time of 420 ms and a tube voltage of 80-120 kV. Tube current varied from 150-210 mAs for the prospectively gated "Step and shoot" protocol and from 600-1000 mAs for the retrospectively gated "Helical" protocol, depending on patients weight and height. Patients received 50 mg Metoprolol tartrate orally, two hours before CCTA. When indicated, an additional dose of 5-20 mg Metoprolol tartrate (AstraZeneca, Zoetermeer, The Netherlands) was administered intravenously to lower the heart rate <65 beats per minute (bpm). 0.8 mg Nitroglycerin spray (Pohl-Boskamp, Hohenlockstedt, Germany) was given sublingually just prior to CCTA. Heart rate and ECG were monitored during CCTA. A non-enhanced scan was performed to determine the amount of coronary artery calcification (CAC), using the Agatston method(12). Subsequently, CCTA was performed using 85-110 mL of contrast agent (Xenetix 350; Guerbet, Roissy CdG Cedex, France), which was injected in the antecubital vein at a rate of 6.0 mL/s, directly followed by 40 mL intravenous sa-

line (6.0 mL/s) using a dual-head power injector. A prospectively gated "Step and shoot" protocol was used in all patients with a stable heart rate <65 bpm. In patients with an irregular heart rate or a stable heart rate >65 bpm, we used a retrospectively gated "Helical" protocol with dose modulation to obtain the best image quality at minimal radiation dose(13,14).

CCTA Analysis

All scans were independently analyzed by two cardiologists with level III expertise in coronary CT-angiography, blinded for patient details, using source images in the Cardiac Comprehensive Analysis software (Philips Healthcare). In case of disagreement, consensus was reached by reviewing findings jointly. CAC was expressed as the Agatston score using calcium scoring software (Philips Healthcare) with a threshold of 130 Hounsfield units (HU). The coronary artery tree was analyzed for the presence and severity of CAD, according to the 16-segment classification of the American Heart Association(15). Coronary plaques were defined as visible structures within or adjacent to the coronary artery lumen, which could be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. Plaques were categorized as calcified (exclusively content with density >130 HU), non-calcified (exclusively content with

density <130 HU) or mixed (characteristics of both calcified and non-calcified plaques). The degree of CAD was classified as absent (no luminal stenosis), mild (<50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis)(16). The degree of CAC was classified as absent (Agatston score 0), mild (Agatston score 1-100), moderate (Agatston score 100-400) or severe (Agatston score >400)(17).

Blood Samples and Laboratory Measurements

Blood samples were taken just before the scan, processed within 2 hours and plasma was stored at -80°C until analysis. Continuous thrombin generation in clotting platelet-poor plasma was monitored *in vitro* by using the Calibrated Automated Thrombography (CAT) method (Thromboscope B.V., Maastricht, The Netherlands)(18). The reaction was triggered by adding 5pM tissue factor (PPP Reagent, Thromboscope B.V., Maastricht, The Netherlands) in the presence of 4 µM phospholipids and 16 mM added CaCl_2 (in duplicate). Endogenous thrombin potential (ETP) was analyzed (corresponds to the area under the curve). ETP values were normalized based on platelet-poor normal pooled plasma (NPP) obtained from healthy volunteers, the latter used as a reference(19). Data are expressed as % of NPP(20). In addition, using a commercially available micro-

enzyme immunoassay kit (Enzygnost® TAT Micro, Siemens Healthcare Diagnostics, Deerfield, IL, USA), we established thrombin-antithrombin complexes (TATc) levels in all patients (in duplicate) as a highly specific marker for thrombin formation *in vivo*.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 19.0.0 (SPSS Inc., Chicago, IL, USA). Categorical variables are presented as numbers (percentages), whereas continuous data are expressed as mean ($\pm$ SD – standard deviation), unless otherwise indicated. TATc plasma levels were normalized by natural logarithm transformation. We used the score plus 1 to also include patients with a TATc plasma level below 1 ng/mL. Demographic differences between patients with or without CAD were tested either using a Student's t-test or Mann-Whitney U test, depending on the distribution characteristics of the data. Pearson's chi-square test (χ^2) was used to compare proportions (binary or categorical), whereas continuous variables were analysed via one-way analysis of variance test (ANOVA), including Bonferroni correction. Correlations are presented as Pearson or Spearman's coefficients according to the observed distribution. Multivariate analyses were conducted using binary/multinomial logistic regression, computed in a

multiple main effects or forward stepwise manner, including variables with $p < 0.05$. Pearson's chi-square test (χ^2) and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to determine which variables demonstrated significant independent associations with atherosclerotic plaque presence, degree of luminal stenosis and CAC. Receiver operating characteristic (ROC) analysis was carried out to evaluate the potential of using TATc and Framingham risk score (separately or in combination) for determining the presence or absence of CAD. Areas under the ROC curve (AUROC) were compared using the Hanley and McNeil's method. We performed the net reclassification index to evaluate the incremental effect of adding TATc to the Framingham risk score for predicting the presence of coronary plaques. A 2-sided p value < 0.05 was considered statistically significant.

Results

Study Population Characteristics

The study population consisted of 295 individuals [182 males (61.7%) and 113 females (38.3%)] with a median age of 57 years (min-max: 30-87). A total of 226 patients underwent a "Step and shoot" scan (mean radiation dose 3.6 mSv), whereas 69 patients underwent a "Helical" scan (mean radiation dose 11.6 mSv). CAD was detected in 205

(69.5%) patients. The prevalence of absent, mild, moderate and severe CAD was 30.5%, 22.7%, 26.4% and 20.3%, respectively. Compared to the non-CAD group, patients with CAD were predominantly male (65.4%) and older, showed increased systolic blood pressure and had lower LDL plasma concentrations. However, the prevalence of statin use in the CAD group was significantly higher compared to the non-CAD group [100 (48.8%) vs. 22 (24.4%)]. Baseline characteristics are presented in **Table 1**.

Increased In Vivo Thrombin Formation Independently Reflects the Presence of Coronary Atherosclerotic Plaques

As depicted in **Figure 1A**, among the population with CAD ($n=205$), the average baseline (lg 10 transformed) TATc levels were significantly higher compared to the group without CAD (mean 0.41; 95% CI 0.38–0.45 vs. mean 0.32; 95% CI 0.29–0.36, $p=0.001$). Multivariate logistic regression analysis showed that higher TATc levels (OR: 1.47, 95% CI 1.10–1.97, $p=0.010$), in addition to other established risk factors such as male gender (OR: 3.36; 95% CI 1.75–6.45, $p<0.001$), age (OR: 1.09; 95% CI 1.05–1.12, $p<0.001$) and smoking (OR: 2.17; 95% CI 1.09–4.33, $p<0.001$), were all independently associated with the presence of CAD (data not shown). AUROC for coronary plaque presence, calculated by

Table 1.

Table 1	Baseline Characteristics of Subjects Stratified into Groups per Presence of CT-Angiographic CAD			
	All participants <i>n</i> =295	Patients without CAD* <i>n</i> =90	Patients with CAD† <i>n</i> =205	<i>p</i> Value
Age, years, median (min-max)	57 (30-87)	54 (30-76)	59 (36 - 87)	<0.001
Male gender, <i>n</i> (%)	182 (61.7%)	48 (53.3%)	134 (65.4%)	0.053
BMI, kg/m ²	26.4 (24.2 - 29.4)	26.6 (23.8 - 29.7)	26.4 (24.3 - 29.3)	0.620
Systolic blood pressure, mmHg	143 (130 - 156)	139 (127 - 153)	145 (132 - 157)	0.023
Diastolic blood pressure, mmHg	80 (73 - 87)	80 (71 - 86)	81 (74 - 87)	0.615
Smoking, <i>n</i> (%)	71 (24.1%)	18 (20.0%)	53 (25.9%)	0.304
Diabetes mellitus, <i>n</i> (%)	26 (8.8%)	6 (6.7%)	20 (9.8%)	0.505
Positive family history, <i>n</i> (%)	126 (42.7%)	36 (40.0%)	90 (43.9%)	0.609
Lipid-lowering therapy, <i>n</i> (%)	122 (41.4%)	22 (24.4%)	100 (48.8%)	<0.005
Anticoagulation therapy (%)	0 (0%)	0 (0%)	0 (0%)	-
Framingham Risk Score	18.9 (12.3 - 30.0)	14.1 (9.8 - 21.4)	21.6 (13.4 - 33.1)	<0.001
Total cholesterol, mg/dL	213 (174 - 244)	217 (192 - 240)	205 (170 - 247)	0.077
LDL, mg/dL	128 (101 - 166)	135 (116 - 163)	124 (97 - 166)	0.043
HDL, mg/dL	46 (35 - 58)	46 (35 - 57)	44 (37 - 58)	0.980
Triglycerides, mg/dL	134 (90 - 208)	146 (91 - 231)	130 (89 - 202)	0.180
Coronary atherosclerotic lesions	2 (0 - 5)	0 (0 - 0)	4 (2 - 7)	<0.001
CAC (Agatston Score)	27 (0 - 214)	0 (0 - 0)	117 (23 - 355)	<0.001

Categorical variables are presented as numbers (percentages). Continuous data are expressed as median (IQR - interquartile range), unless otherwise indicated.

* Patients with no angiographically detected coronary atherosclerotic plaques

† Patients with ≥1 angiographically detected coronary atherosclerotic plaque(s)

CAD = Coronary Artery Disease; BMI = Body Mass Index; LDL = Low-density Lipoprotein; HDL = High-density Lipoprotein; CAC = Coronary Artery Calcification

Statistical significance at the *p*<0.05 level

using the Framingham risk score, was 0.663 (95% CI 0.61–0.72; $p<0.001$). Addition of TATc as a marker to the FRS improved the predictive value, resulting in a significant increase of the AUROC to 0.676 (95% CI 0.62–0.73; $p=0.048$ – difference between areas). The net reclassification index to assess the incremental value of TATc over the Framingham risk score in predicting the presence of coronary plaques was 3.1%, which was not significant ($p=0.68$). CAT measurement, which was carried out to assess the potential to generate thrombin *in vitro*, resulted in ETP values, which are almost the same in the non-CAD group and the CAD group (**Figure 1B**).

TATc as Determinant of CAC and Luminal Stenosis

In the entire study population, TATc levels showed a significant positive association with the degree of CAC (Agatston score, $r=0.209$, $p<0.001$), as presented in **Figure 2A**. In contrast, no significant relationship was noted between TATc concentrations and the number of non-calcified plaques ($r=0.052$, $p=0.376$). Multivariate logistic regression analyses, using multiple main effects and forward stepwise techniques, identified higher TATc formation as an independent risk factor for developing CAC (**Table 2**). Compared to a reference group, consisting of all patients without any coronary calcifications,

the odds ratios associated with CAC burden were as follows: mild CAC (OR: 1.60, 95% CI 1.18–2.16, $p<0.005$); moderate CAC (OR: 1.58, 95% CI 1.16–2.15, $p<0.005$); and severe CAC (OR: 1.71, 95% CI 1.26–2.33, $p<0.005$). As shown in **Figure 2B**, we also found a significant difference in the distribution of the TATc quartiles (Q1–Q4) between the different CAC groups ($p=0.002$). While in the no CAC group, 35.9% of the patients had TATc values in the lowest quartile (Q1) and only 14.6% of them had values in the highest quartile (Q4), the distribution of the TATc quartiles in the severe CAC group was 9.3% and 41.9% in Q1 and Q4, respectively.

There was a strong positive association between Agatston score and severity of CAD ($r=0.712$, $p<0.001$). Nevertheless, we also tested the relationship between TATc formation and the degree of luminal stenosis by performing multivariate logistic regression analyses with degree of luminal stenosis as a dependent variable. Similarly, TATc concentrations accurately identified worsening atherosclerosis. The odds ratios associated with mild, moderate and severe CAD are depicted in **Table 3**.

Furthermore, we found a U-shaped relationship between the potential to generate thrombin *in vitro* and the extent of CAD (**Figure 3**). Within the group of patients with detected CAD, ETP did not correlate to the extent of CAC ($r=-0.036$, $p=0.604$), whereas it was significantly associated with the

Figure 1. In Vivo/In Vitro Thrombin Formation and the Presence of CAD.

A. This panel shows that patients with at least one coronary atherosclerotic plaque exhibit significantly higher in vivo thrombin generation compared to patients without coronary plaques ($p=0.001$); Bars represent average lg 10 transformed TATc levels, whereas data distribution is presented in mean (95% CI) in the table below.

B. This panel shows that patients with CAD show comparable thrombin generation potential compared to patients without any coronary plaques detected ($p<0.004$); Bars represent average ETP levels, whereas data distribution is presented in mean (95% CI) in the table below.

Figure 2. TATc as an Independent Predictor of CAC

A. This panel demonstrates the association between gradually increasing TATc concentrations and severity of CAC; Bars represent lg 10 transformed TATc levels, whereas data distribution is presented in mean (95% CI) in the table below, stratified per CAC group. * Denotes statistical significance at the $p<0.05$ level when compared to severe CAC group; CAC = Coronary Artery Calcification; TATc = Thrombin-antithrombin complex

B. Distribution of TATc quartiles between CAC score groups. Q= Quartile

degree of CAD ($r=0.271$, $p<0.001$). In a multivariate logistic regression analysis, when compared to mild CAD, the odds ratios associated with moderate and severe CAD were as follows: moderate (OR: 1.02, 95% CI 1.00–1.04, $p=0.056$) and severe (OR: 1.04, 95% CI 1.02–1.06, $p<0.001$).

Discussion

Major Findings

The present study examines the relationship between thrombin formation and CAD. In a cohort of 295 patients with suspected CAD, we established baseline TATc concentrations in plasma and used CCTA

imaging to assess the presence and severity of coronary atherosclerotic plaques. We found several novel findings of potential clinical relevance. The primary observation of this study is that higher TATc levels, the latter considered a sensitive marker of thrombin formation *in vivo*, are independently related to the presence and severity of CAD. Net reclassification index analysis showed that incorporation of TATc as an additional test did not significantly improves the cardiovascular risk stratification capacity of the Framingham risk score. On the other hand, the amount of CAC and degree of luminal stenosis were consistently higher with increasing thrombin

Figure 3. U-Shaped Relationship between ETP and CAD. Bars represent average ETP values (% of NPP), whereas data distribution is presented in mean (95% CI) in the table below, stratified per degree of luminal stenosis. * Denotes statistical significance at the $p<0.05$ level when compared to severe CAD group; CAD = Coronary Artery Disease; ETP = Endogenous Thrombin Potential; NPP = Normal Pool Plasma

Table 2.

Table 2 Multivariate Models of Factors Associated with Odds of Coronary Artery Calcification (CAC)						
Model 1 – “Multinomial Logistic Regression: Main Effects Model”						
Variable	Mild CAC AS: 1-100		Moderate CAC AS: 100-400		Severe CAC AS: >400	
	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Reference - No CAC/AS: 0	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Age	1.05 (1.01 – 1.09)	0.021	1.12 (1.07 – 1.17)	<0.005	1.15 (1.09 – 1.22)	<0.005
Gender (Male = 0)	2.61 (1.22 – 5.57)	0.014	3.46 (1.50 – 8.01)	0.004	6.09 (2.17 – 17.07)	<0.005
Smoking (Yes = 1)	0.71 (0.32 – 1.53)	0.377	0.51 (0.21 – 1.22)	0.132	0.34 (0.12 – 0.95)	0.039
Diabetes mellitus (Yes = 1)	1.04 (0.30 – 3.61)	0.951	0.71 (0.19 – 2.61)	0.603	0.50 (0.12 – 2.17)	0.355
Positive family history (Yes = 1)	0.60 (0.31 – 1.16)	0.127	0.42 (0.20 – 0.88)	0.021	0.41 (0.16 – 1.01)	0.053
BMI	0.99 (0.91 – 1.08)	0.788	1.02 (0.93 – 1.12)	0.687	0.94 (0.83 – 1.06)	0.329
Total cholesterol	0.88 (0.66 – 1.15)	0.346	0.96 (0.71 – 1.29)	0.769	0.84 (0.59 – 1.21)	0.354
Systolic blood pressure	1.01 (0.99 – 1.04)	0.219	1.02 (0.99 – 1.04)	0.130	1.03 (1.00 – 1.06)	0.051
Diastolic blood pressure	1.00 (0.96 – 1.03)	0.911	1.00 (0.96 – 1.04)	0.973	0.99 (0.95 – 1.04)	0.752
TATc	1.56 (1.14 – 2.15)	0.006	1.56 (1.13 – 2.15)	0.007	1.67 (1.21 – 2.31)	0.002
Model 2 – “Multinomial Logistic Regression: Forward Stepwise Model”						
Variable	Mild CAC AS: 1-100		Moderate CAC AS: 100-400		Severe CAC AS: >400	
	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Reference - No CAC/AS: 0	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Age	1.05 (1.01 – 1.09)	0.010	1.12 (1.07 – 1.17)	<0.005	1.16 (1.10 – 1.21)	<0.005
Gender (Male = 0)	2.45 (1.25 – 4.81)	0.009	3.14 (1.49 – 6.61)	<0.005	4.86 (1.98 – 11.95)	<0.005
Positive family history (Yes = 1)	0.59 (0.31 – 1.11)	0.104	0.40 (0.20 – 0.82)	0.012	0.40 (0.17 – 0.93)	0.034
TATc	1.60 (1.18 – 2.16)	<0.005	1.58 (1.16 – 2.15)	<0.005	1.71 (1.26 – 2.33)	<0.005

CAC = Coronary Artery Calcification; AS = Agatston Score; BMI = Body Mass Index; OR = Odds Ratio; CI = Confidence Interval; TATc = Thrombin-antithrombin complex. Statistical significance at the $p < 0.05$ level

Table 3.

Multinomial Logistic Regression Models for CAD Severity as the Dependent Variable						
Model 1 – “Multinomial Logistic Regression: Main Effects Model”						
Variable	Mild CAD Stenosis: <50%		Moderate CAD Stenosis: 50% - 70%		Severe CAD Stenosis: > 70%	
	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Reference – No CAD (0%)	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Age	1.08 (1.03 – 1.12)	<0.005	1.09 (1.04 – 1.13)	<0.005	1.10 (1.05 – 1.15)	<0.005
Gender (Male = 0)	2.50 (1.12 – 5.55)	0.025	3.02 (1.37 – 6.69)	0.006	5.70 (2.33 – 13.94)	<0.005
Smoking (Yes = 1)	0.62 (0.26 – 1.47)	0.276	0.54 (0.23 – 1.28)	0.161	0.28 (0.12 – 0.67)	0.004
Diabetes mellitus (Yes = 1)	0.69 (0.20 – 2.36)	0.557	0.82 (0.23 – 2.89)	0.756	0.98 (0.23 – 4.28)	0.980
Positive family history (Yes = 1)	0.60 (0.30 – 1.22)	0.160	0.62 (0.31 – 1.25)	0.177	0.63 (0.30 – 1.34)	0.226
BMI	0.98 (0.90 – 1.08)	0.704	1.04 (0.95 – 1.14)	0.356	1.00 (0.90 – 1.10)	0.944
Total cholesterol	0.77 (0.57 – 1.04)	0.093	0.82 (0.61 – 1.11)	0.198	0.97 (0.72 – 1.32)	0.861
Systolic blood pressure	1.01 (0.98 – 1.03)	0.524	1.02 (0.99 – 1.04)	0.207	1.02 (0.99 – 1.04)	0.232
Diastolic blood pressure	1.00 (0.96 – 1.04)	0.939	1.00 (0.97 – 1.04)	0.870	1.01 (0.97 – 1.05)	0.745
TATc	1.37 (0.99 – 1.88)	0.056	1.57 (1.16 – 2.14)	0.004	1.47 (1.07 – 2.02)	0.017
Model 2 – “Multinomial Logistic Regression: Forward Stepwise Model”						
Variable	Mild CAD Stenosis: <50%		Moderate CAD Stenosis: 50% - 70%		Severe CAD Stenosis: > 70%	
	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Reference – No CAD (0%)	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Age	1.08 (1.03 – 1.12)	<0.005	1.09 (1.05 – 1.13)	<0.005	1.09 (1.05 – 1.14)	<0.005
Gender (Male = 0)	2.46 (1.20 – 5.08)	0.014	3.12 (1.52 – 6.41)	0.002	5.35 (2.38 – 12.03)	<0.005
Smoking (No = 0)	0.73 (0.32 – 1.65)	0.443	0.65 (0.29 – 1.46)	0.296	0.31 (0.14 – 0.69)	0.004
TATc	1.39 (1.02 – 1.89)	0.036	1.59 (1.18 – 2.14)	0.002	1.50 (1.11 – 2.04)	0.009

CAD = Coronary Artery Disease; BMI = Body Mass Index; OR = Odds Ratio; CI = Confidence Interval; TATc = Thrombin-antithrombin complex. Statistical significance at the $p < 0.05$ level

generation, indicating that TATc measurement is useful to detect even mild-grade coronary artery calcification or stenosis. In daily practice, TATc concentrations may therefore contribute to predict which patients are more likely to have CAD. However, as a single biomarker it seems not to have enough power to be a substitute for other diagnostic imaging tools like CCTA, magnetic resonance imaging, ultrasound, nuclear imaging tests or invasive coronary angiography. The detrimental role of thrombin in atherothrombosis is not a matter of dispute(21). Numerous previously published reports have documented increased rates of thrombin synthesis and blood coagulation activation upon the onset of major adverse cardiovascular events(22-25). However, besides being linked to blood thrombogenicity and determining the magnitude of thrombus formation upon atherosclerotic plaque rupture, thrombin activity per se may be also relevant to the pathophysiology of atherosclerosis progression(3,4). Several research groups have attempted to study the relationship between hypercoagulability and atherosclerosis progression by assessing ankle brachial pressure index (ABPI) in patients with peripheral artery disease(26-28) or evaluating other markers of subclinical atherosclerosis such as carotid intima-media thickness (cIMT)(29,30). Nevertheless, these studies do not provide sufficient in-

sight into this problem due to the very limited potential of ABPI and cIMT techniques to evaluate CAD. To our knowledge, this is the first study to precisely examine the relationship between thrombin generation and the angiographic presence, severity and calcification of coronary artery plaques by using CCTA in a population with suspected, but not previously established CAD.

Enhanced In Vivo Thrombin Generation During Atherogenesis: Potential Clinical Implications

Given the capacity of thrombin to modulate pro-atherogenic actions related to plaque destabilization, it becomes important to define what the clinical implications of these findings may be. Previously, we have demonstrated that early atherosclerotic lesions exert an enhanced pro-coagulant state in comparison to stable advanced ones(5). This phenomenon was partially explained by the increased activity of many key coagulation proteins (incl. thrombin), but also by the ability of different vascular cell types to synthesize coagulation factors at a local level. There is abundant histopathological and experimental evidence to demonstrate that thrombosis occurs long before an atherosclerotic plaque ruptures, named as subclinical or "buried" thrombosis(8,31,32). The latter is also considered a potential trigger of plaque vulnerability(33-35). Furthermore, blood coagulation is an important

component of the host-defence system to fight tissue injury and infection(36). Despite that the exact mechanisms of the enhanced TATc formation in blood during atherosclerosis progression remain unclear to date, one may speculate that inflammation and coagulation operate in a perpetual mode to repair worsening atherosclerosis vascular damage. While oral vitamin K-antagonists and antiplatelet therapy remain the cornerstone in primary and secondary prevention therapy against atherothrombosis and reduce cardiovascular mortality by ~30%, numerous clinical trials have failed to account a clear atheroprotective effect(3). In contrast, a few experimental studies have indicated that administration of direct thrombin inhibitors in atherosclerotic mice substantially inhibits plaque volume and results in plaque stability(7,8). Given the improved safety profile that these novel therapeutic agents show(37,38), it becomes important to further investigate the effects of selective thrombin inhibition on plaque volume and phenotype determination in patients.

Although the CAT method is meant to determine the potential to generate thrombin in plasma *in vitro*, the U-shaped association between ETP and CAD, which we demonstrate, may also be of physiological relevance. We have previously reported that early atherosclerotic lesions show an increased thrombin generation potential in comparison to sta-

ble advanced lesions(5). Since the absence of angiographic CAD does not exclude early-stage morphological changes of the arterial vessel wall (e.g. mild pathological intimal thickening), one may assume that the increased thrombin-forming capacity in the non-CAD group may be due to first signs of atherosclerotic alterations. Thrombin is a central enzyme in the coagulation-inflammation axis and represents a potential therapeutic target via which atherosclerosis might be modulated. Moreover, thrombin is well-known for its dual-faceted character in both hemostasis and cell signaling(4). At very low concentrations, thrombin can mediate numerous atheroprotective effects such as endothelial barrier protection, reduction in apoptosis and trans-endothelial migration of leukocytes, and promote atheroprotective interleukin-10 synthesis(39-41). Some of those actions are dependent on the occupancy of endothelial protein C receptor by its natural ligand protein C/activated protein C. Hence, the net effect of specific long-term thrombin inhibition remains hard to predict.

Other Findings

The role of CAC in inducing plaque vulnerability remains controversial. Clinical evidence shows that CAC is associated with coronary vasomotor dysfunction and reduced myocardial perfusion, even in the absence of luminal stenosis(42). Novel concepts

of plaque vulnerability propose that atherosclerotic plaque hypoxia may induce angiogenesis, intraplaque hemorrhage and increased risk for rupture(43,44). Besides serving as a precise indicator of the presence and severity of coronary plaque burden(45), the Agatston score is considered a better predictor of cardiovascular outcomes than the Framingham risk score(46). In the current study, we present new evidence indicating higher thrombin generation as an independent determinant of CAC. Despite that the clinical significance of these findings remains to be further investigated, a recent *ex vivo* human study reports that aortic valve calcification can be induced through increased thrombin generation(47).

Limitations

Our study has several limitations. First, we performed a single-center study in which all patients were of Western European descent. Second, the patient number was relatively small, which limits the options for analyzing the relevance of TATc for predicting follow-up events. Moreover, because the relatively healthy population, we did not found a high rate of (hard) cardiovascular events, especially not in short term, which is in line with other CT-studies. Third, while we screened for angiographic CAD, these findings may reflect other existing atherosclerosis settings (carotid or peripheral artery lesions),

which we did not assess in this study. Therefore, the association between thrombin formation and atherosclerosis in other vascular beds remain open. Fourth, the purpose of this study was to investigate the association of thrombin formation and CAD and was not meant to unravel this complex causal relationship.

Conclusion

Thrombin formation is a useful tool in determining the presence and severity of coronary artery disease, but more importantly, may be also involved in the pathophysiology of vascular calcification and atherosclerosis progression.

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5

**MECHANISMS OF DISEASE:
THE HEMOSTATIC SYSTEM AS A
MODULATOR OF ATHEROSCLEROSIS**

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Cardiovascular disease is one of the leading causes of death and complications worldwide. The classic concept of atherosclerosis assigns a pivotal role to inflammation in the onset and progression of this disease.^{1,2} Various inflammatory cell types (e.g., macrophages, neutrophils, and lymphocytes) play crucial roles in the destabilization and subsequent rupture or erosion of an atherosclerotic plaque, ultimately resulting in atherothrombosis.³ Inflammation is closely linked to coagulation in several pathologic conditions.⁴ Intriguingly, extensive bidirectional cross-talk between the two systems has been established in many complex diseases,^{5,6} including atherosclerosis. Although there is no clinical evidence of a role for the hemostatic system in the progression of atherosclerosis, ample experimental data indicate that platelets and the coagulation system are important determinants of both atherogenesis and atherothrombosis. In numerous clinical trials, the administration of antiplatelet or anticoagulant therapy has not been associated with attenuation or regression of plaque growth. Nevertheless, the hemostatic system is well known for its capacity to exert a multitude of actions on the vasculature, which may influence the molecular and cellular composition of the arterial wall and presumably of the atherosclerotic plaque. This review covers recent advances in this field and discusses mechanisms of hemostasis as potential modula-

tors of plaque phenotype.

Cross-Talk Mechanisms Linking the Hemostatic System with Atherosclerosis

Hemostasis

Hemostasis is accomplished through a network of processes that include the platelet system, coagulation, and anticoagulant and fibrinolytic pathways, which all support the dynamic equilibrium that provides proper blood flow.^{7,8} Such processes evolved to maintain the blood in a fluid state under physiologic conditions and to arrest bleeding after vascular injury⁹⁻¹⁵ (**Fig. 1A and 1B**). Disruption of this well-regulated balance leads to pathologic conditions, such as thrombosis and bleeding.

Figure 1. Platelets and Coagulation Factors in the Regulation of Thrombus Formation.

Panel A shows platelet adhesion and aggregation, in which atherothrombosis begins with an endothelial injury or rupture of an atherosclerotic plaque. This process triggers transient neurohumoral vasoconstrictor mechanisms, which are reinforced by the release of endothelium-derived factors, such as endothelin. The platelet membrane receptors glycoprotein Ib/IX/V and glycoprotein VI elicit platelet tethering to the exposed thrombogenic subendothelial proteins, von Willebrand factor, and collagen. In addition, glycoprotein VI

generates intracellular signals to mediate platelet adhesion and aggregation through the activation of integrin receptors, such as glycoprotein Ia/IIa and glycoprotein IIb/IIIa, with the latter also serving as a receptor for fibrinogen. These molecular events ultimately contribute to the formation of the primary hemostatic plug.¹⁰ Panel B shows the tissue factor

(extrinsic) pathway, in which tissue factor, the major trigger of coagulation, is exposed at the site of plaque erosion or rupture. Tissue factor forms a catalytic complex with factor VIIa that leads to the subsequent activation of factors IX and X. In a so-called prothrombinase complex, activated factor X together with activated factor V promotes a downstream en-

Figure 1.

zymatic cleavage of prothrombin, which yields small amounts of thrombin.¹¹ Thrombin is a pleiotropic, central coagulation enzyme¹² that not only converts fibrinogen into fibrin but also has a substantial role in the activation of platelets and activates factor XIII to induce fibrin polymerization, a fundamental process for the formation of a stable clot, or thrombus. Furthermore, by supporting positive-feedback activation of the upstream factors V, VIII, and XI, thrombin plays a crucial part in the amplification and propagation phases of coagulation. The activated platelet surface is also a critical catalyst for the coagulation cascade. Platelets actively participate in the clotting process by introducing extra amounts of tissue factor, factor V, fibrinogen, and factor XIII into the system, derived from various local sources (fibrinogen and factors V and XIII stored in α granules),¹³ and facilitating the direct activation of factor XI by thrombin and the subsequent activation of factor IX on the platelet surface. Factor IXa forms the so-called tenase complex together with factor VIIIa, thereby igniting a burst of additional thrombin generation, which is essential in forming sufficient fibrin and sealing the defect. Panel C shows the contact activation (intrinsic) pathway, which is not considered to be essential for protection against bleeding in vivo, even though its components may be involved in the pathogenesis of arterial thrombosis.¹⁴ The exposure of plasma prekallikrein,

high-molecular-weight kininogen, and factors XI and XII to anionic surfaces¹⁵ results in the conversion of prekallikrein to kallikrein, which activates factor XII into factor XIIa but also cleaves high-molecular-weight kininogen, leading to the release of the inflammatory mediator and vasodilator bradykinin. Factor XIIa activates factor XI and favors the conversion of more prekallikrein to kallikrein, thereby reciprocally amplifying the cascade. This sequence of proteolytic reactions leads to the activation of factor IX, which ultimately cleaves factor X into its active form and culminates in the convergence of both coagulation pathways. Gray circles indicate the inactive form of a coagulation protein, and green circles indicate the active form.

Molecular and Cellular Responses in the Vasculature

The targeting of genes that encode distinct hemostatic factors and their effect on arterial thrombosis in vivo has been extensively studied (*see Table 1 in the Supplementary Appendix*). Abundant experimental data suggest a role for various constituents of the platelet membrane and coagulation system in the regulation of atherosclerosis progression. Beyond their traditional hemostatic functions, platelets are considered important in proinflammatory conditions, such as atherosclerosis.¹⁶ In addition, numerous coagulation proteins have been implicated in

processes such as the disruption of the endothelial barrier, oxidative stress, leukocyte recruitment, inflammation, migration and proliferation of vascular smooth-muscle cells (VSMCs), immune responses, apoptosis of platelets and other cell types, and angiogenesis.^{17,18} Some of these actions, mostly mediated by the complex of tissue factor and factor VIIa (TF-FVIIa), factor Xa, and thrombin, involve the activation of G-protein-coupled protease-activated receptors (PARs) 1, 2, 3, and 4. PARs are widely distributed on vascular cells under normal conditions and are overexpressed during atherogenesis.¹⁹

Platelets, The Cellular Interface between Hemostasis and Atherosclerosis

Pioneering studies have documented a prominent role of platelets in experimental studies of atherogenesis.^{20,21} Platelets exert a plethora of proatherogenic activities and create an interface between hemostasis, innate immunity, and inflammation in atherosclerosis.¹⁶ A systemic inflammatory environment, independent of vessel wall injury, induces a phenotypic switch to a proatherogenic endothelium. This results in enhanced expression of cell-adhesion molecules, such as P-selectin and E-selectin. The primary adhesion of platelets on a compromised vascular endothelial surface is accomplished through the binding of

platelet glycoprotein Iba receptors to von Willebrand factor, whereas firm adhesion is mediated through β_3 integrins. Once adherent, platelets also secrete atherogenic mediators, such as cytokines, chemokines, growth factors, adhesion molecules, and coagulation factors. The upregulation of P-selectin expression on the surfaces of both platelets and endothelial cells potentiates the interactions with P-selectin glycoprotein ligand 1, which is expressed on leukocyte membranes. The binding between platelets and circulating leukocytes (monocytes and neutrophils), dendritic cells, and progenitor cells produces coaggregates that support further leukocyte activation, adhesion, and transmigration, processes considered to be critical for plaque formation and progression²²⁻²⁹ (**Fig. 2**).

Figure 2. Platelets in Atherogenesis.

Intact endothelium normally expresses CD39 (ecto-ATPase) and CD73 (ecto-5'-nucleotidase), which act in tandem to induce the breakdown of the prothrombotic adenosine 5'-triphosphate (ATP) and adenosine diphosphate (ADP) into the largely antiinflammatory adenosine, thus preventing platelet activation and aggregation. Healthy endothelium also secretes vasodilators, such as prostacyclin and nitric oxide, which have potent antiadhesive and antiaggregating effects. At the time of activation, platelets undergo a sub-

Figure 2.

stantial change in shape and promptly release a variety of autocrine and paracrine mediators such as ADP, epinephrine, and thromboxane A_2 . Studies investigating how platelets orchestrate these widely differing atherogenic actions have provided an increased understanding of the mechanisms involved. Much attention has focused on cytokine-like and chemokine systems such as the CD40–CD40L dyad, CCL5 (RANTES), and platelet factor 4.^{23,24} Platelet factor 4 supports monocyte differentiation into macrophages and down-regulates the atheroprotective receptor CD163, which accounts for the clearance of hemoglobin–haptoglobin complexes. Transgenic mice lacking platelet factor 4 have diminished progression of atherosclerosis. Furthermore, CD40 and its ligand, CD40L, which belongs to the superfamily of tumor necrosis factor receptor and ligand, is widely expressed in the vessel wall (e.g., in endothelial cells, vascular smooth-muscle cells, and fibroblasts) and several immune constituents (monocytes or macrophages, neutrophils, mast cells, T and B cells, and dendritic cells).²⁵ The complex array of proinflammatory, immune-modulating effects and prothrombotic features²⁶ assert an integral role for CD40–CD40L in atherogenesis. Overall, these findings support the hypothesis that platelets are important proinflammatory players that elicit multifaceted cellular interactions and are directly involved in the early development of atherosclerotic lesions. Platelets are

primary mediators in both adaptive and innate immunity.²⁷ Hence, the targeting of platelet chemokines appears to be therapeutically unsuitable in the context of atherosclerosis because of the severe impairment of multiple systemic immune responses, which may also result in carcinogenesis.^{28,29} ADAM15 denotes ADAM metalloproteinase domain-containing protein 15, CCL2/3 chemokine (C-C motif) ligand 2/3, ICAM-1 intercellular cell-adhesion molecule 1, TNF- α tumor necrosis factor α , and VCAM-1 vascular-cell adhesion molecule 1.

Coagulation System during Atherosclerotic Plaque Progression

We have found a local synthesis of several functionally active coagulation proteins, which suggests an active cell-based coagulation network, within human atherosclerotic lesions. The role of these coagulation proteins in atherogenesis is indicated by increased thrombin-generating activity in early atherosclerotic lesions, as compared with that in stable, advanced lesions.³⁰ These findings are supported by experimental data³¹ and a clinical study showing that increased plaque echogenicity (more fibrous structure), rather than plaque echolucency (lipid-rich, higher content of inflammatory cells and thinner fibrous caps), is associated with thrombin generation in plasma from patients with carotid-artery stenosis.³² The abundance of coagulation factors

within early atherosclerotic vessels and local generation of thrombin or fibrin may be attributable to primary protective mechanisms against vascular injury. However, the persistent inflammatory environment within the arterial wall, supported in part by coagulation-mediated actions, may maintain local thrombin generation, which will eventually turn into a vicious cycle, contributing to the formation of intraplaque thrombi^{33,34} and thus ultimately leading to plaque instability.

Tissue Factor (Extrinsic) Pathway

Tissue factor is a transmembrane class II cytokine receptor, which is considered the primary physiologic trigger of the coagulation cascade.⁸ Tissue factor is also physiologically essential for vascular development. In mice, tissue factor deficiency is associated with a high rate of embryonic death and impaired vascular integrity. Tissue factor is differentially distributed among the various cell types of the vessel wall. Under physiologic conditions in normal blood vessels, the inner endothelial lining does not express tissue factor, whereas the surrounding layers, consisting of VSMCs, adventitial fibroblasts, and pericytes, show abundant synthesis of tissue factor. This specific vascular localization of tissue factor is generally attributed to its role in the prevention of bleeding after injury, also referred to as a hemostatic envelope.³⁵

Within the atherosclerotic lesion, tissue factor is predominantly localized on macrophages, VSMCs, and foam-cell-derived debris within the necrotic core.^{30,36-38} Tissue factor activity is significantly higher in lesions obtained from patients with unstable angina or myocardial infarction than in those from patients with a stable form of cardiovascular disease,³⁹⁻⁴¹ suggesting a role of this coagulation protein in plaque thrombogenicity. Factor VII is also extrahepatically expressed within both normal and atherosclerotic vessels and colocalizes with tissue factor on macrophages and VSMCs.³⁰ Apart from its coagulation properties, the TF-FVIIa complex is multifunctional, with a capacity to promote cell signaling, gene transcription, and subsequent protein synthesis. PAR-2 activation is essential in the mediation of TF-FVIIa-induced signaling. The latter may engage several proatherogenic processes, such as monocyte and fibroblast chemotaxis, inflammation, VSMC migration and proliferation (vascular remodeling), angiogenesis (contributing to plaque destabilization), induction of oxidative stress in macrophages, and apoptosis⁴² (**Fig. 3**). Surprisingly, reduced vascular expression of tissue factor does not affect atherosclerosis progression in transgenic mice.⁴³ There are few clinical data regarding the role of TF-FVIIa on atherosclerosis progression. Levels of plasma tissue factor antigen, modulated by known polymorphisms of the tissue factor gene, are positively associated with

both an increased risk of death from cardiovascular causes⁴⁴ and an increased carotid intima-media thickness,⁴⁵ which is considered a marker of subclinical atherosclerosis. A similar relation between factor VII and increased intima-media thickness has been documented both in healthy young adults and in patients with peripheral arterial disease.^{46,47}

Common Coagulation Pathway

Pleiotropic Factor Xa

Once activated, factor Xa initiates intracellular signaling in various cell types of the cardiovascular system, preferentially mediated by PAR-2 or, when in ternary complex with TF-FVIIa, through both PAR-1 and PAR-2.¹⁷ PAR-1, PAR-2, or both are present in abundance on endothelial cells, leukocytes, VSMCs, fibroblasts, and dendritic cells. Factor Xa-dependent, PAR-mediated signaling contributes to the production of pro-inflammatory cytokines, including interleukin-6, interleukin-8, and chemokine (C-C motif) ligand 2 (CCL2), and to the expression of cell-adhesion molecules, including E-selectin, intracellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1), along with tissue factor up-regulation, VSMC proliferation, and the release of growth factors (vascular endothelial growth factor, platelet-derived growth factor, and transforming growth factor β).¹⁷ All these

may contribute to the progression of atherosclerotic plaque, involving inflammation, leukocyte transmigration, restenosis, and angiogenesis (**Fig. 3**). Of note, vascular remodeling and neointimal formation were reduced on targeted delivery of non-specific factor Xa inhibitors (heparin and low-molecularweight heparins) coupled to an antifibrin antibody.⁴⁸

Thrombin

Thrombin is a unique serine protease that is pivotal to coagulation and that may also display various actions toward other systems (e.g., immune, nervous, gastrointestinal, and musculoskeletal systems). Governed by the interaction and proteolytic activation of its direct cellular targets (PAR-1, 3, and 4),^{49,50} thrombin is entwined with the regulation of vascular physiology and pathophysiology⁵¹ (**Fig. 3**). Thrombin is an example of a multifaceted molecule with broad physiologic properties. By binding to thrombomodulin, thrombin favors the transformation of protein C into activated protein C, a potent anticoagulant and anti-inflammatory molecule. Moreover, thrombin can diminish the release of interleukin-12 and promote the up-regulation of interleukin-10 in monocytes, thus inducing immunosuppressive and anti-inflammatory actions. Thrombin may also play a role in normal vasomotor regulation.¹⁸ The endothelial decay of thrombomodulin during atherogenesis may

Figure 3. Nonhemostatic Actions Triggered by the Tissue Factor and Common Activation Pathways in the Phenotypic Modulation of the Arterial Wall. Thrombin, factor Xa, and the tissue factor–factor VIIa complex can activate protease-activated receptors, which are widely expressed on endothelial cells, leukocytes, vascular smooth-muscle cells, fibroblasts, dendritic cells, and platelets, resulting in a plethora of proatherogenic actions. Gray circles indicate the inactive form of a coagulation protein, and green circles indicate the active form. LDL denotes low-density lipoprotein.

allow thrombin to potentiate atherogenic processes, such as endothelial dysfunction and barrier disruption, oxidative stress, apoptosis, inflammation (overexpression of cytokines or chemokines), activation of platelets and leukocytes, leukocyte recruitment, migration and proliferation of VSMCs, and angiogenesis, which suggests an important role in the pathogenesis of cardiovascular disease.¹⁸ Thrombin, factor Xa, factor XIa, factor IXa, and plasmin also show enzymatic activity for cleavage of complement proteins C3 and C5 into their active forms.⁵² Proteins C3 and C5 are known to induce inflammation and chemotaxis of inflammatory cells. Human coronary atherosclerotic lesions overexpress anaphylatoxin receptors C3aR and C5aR, as compared with healthy vessels, primarily localized on macrophages but also on endothelial cells, intimal VSMCs, T cells, and mast cells. Overall, these data establish a new interface between coagulation and inflammation in atherosclerosis. The administration of thrombin-specific inhibitors reduces restenosis in rabbits with atherosclerosis after angioplasty.^{53,54} Another piece of evidence for the *in vivo* relevance of these effects comes from a study showing that the direct thrombin inhibitor melagatran reduces atherosclerosis progression in apolipoprotein E-knockout mice and promotes plaque stability by inhibiting proinflammatory transcription factors and attenuating the synthesis of

matrix metalloproteinases.⁵⁵ Furthermore, mice with combined deficiency of factor VIII and apolipoprotein E had significantly less development of atherosclerotic lesions than control mice, despite having more pronounced hyperlipidemia.⁵⁶ In contrast, hypercoagulability has been linked with atherosclerosis progression in murine studies, showing that homozygosity for factor V Leiden, a known prothrombotic mutation, promotes atherogenesis.⁵⁷ However, a recent study showed an increase in the size of atherosclerotic plaques in procoagulant mice, indicating that a hypercoagulable state contributes to a more stable plaque phenotype.³¹ Overall, these findings suggest that hemostasis exerts various effects on the vasculature and, by the action of distinct regulators, may ultimately contribute to determining the plaque phenotype. The clinical evidence in this regard remains inconsistent. Despite the fact that prothrombotic genetic variants have not been consistently linked to the progression of cardiovascular disease in patients,⁴⁴ clinical data show a positive association between markers of thrombin generation and the atherosclerotic plaque burden.^{58,59} Low levels of factor VIII have not shown atheroprotective effects in patients with hemophilia,⁴⁴ whereas there is clinical evidence that elevated levels of factor VIII promote cardiovascular disease.⁶⁰ In plasma, factor VIII circulates in a complex with von Willebrand factor,

which modulates factor VIII activity in the circulation. Since mice that are deficient in von Willebrand factor have significantly fewer atherosclerotic plaques than control mice, von Willebrand factor may also play a role in atherosclerosis.⁶¹ Like the data regarding factor VIII and other coagulation proteases, clinical data on the association between von Willebrand factor and cardiovascular disease have been inconsistent.^{44,60} More experimental and clinical data are needed to clarify these relationships.

Fibrinogen, Fibrin, and Factor XIII

In clinical studies, there have been strong associations between increased plasma fibrinogen levels and the risk of cardiovascular disease, which suggests hyperfibrinogenemia as an independent predictor of vascular events.⁶² Furthermore, the distribution of fibrinogen and fibrin degradation products in atherosclerotic lesions during progression has been clearly documented.^{63,64} Elevated levels of plasma fibrinogen, a major determinant of the amount of thrombin that is formed,⁶⁵ are closely related to an enhanced rate of coronary-artery calcification and increased intima-media thickness, both measures of premature atherosclerosis.⁶⁶ From a cellular and molecular perspective, fibrinogen may affect the plaque phenotype through several distinct mechanisms: favoring the permeability of endothelial

cells, extracellular accumulation of low-density lipoprotein (LDL) cholesterol, and the formation of foam cells; inducing the migration of monocytes and VSMCs; increasing platelet reactivity or aggregation; and enhancing inflammation⁶⁷ (**Fig. 3**). Studies in animals have shown distinct results on the role of fibrinogen in atherosclerosis, with some studies indicating that fibrinogen deficiency in transgenic mice is associated with accelerated atherogenesis in a thrombin-dependent manner,⁶⁸ and others showing that fibrinogen deficiency is not a prerequisite for the development of advanced atherosclerotic plaque.⁶⁹ Increased plasma levels of d-dimer fragments are also associated with enhanced inflammation and an increased incidence of cardiovascular disease and are considered a biomarker of atherothrombosis.⁷⁰ However, the effect of fibrin degradation products on the vascular-wall phenotype is less clear. Although the results of one study suggested that d-dimers promote a proatherogenic phenotype in human monocytes,⁷¹ other studies have shown that both fragments D and E may prevent the proliferation of VSMCs in vitro.⁷² Finally, blood coagulation factor XIII may also be related to atherogenesis. Factor XIII not only cross-links fibrin chains to fibrin on activation, which contributes to clot stability, but also appears to facilitate the formation of hyperactive dimers of angiotensin II type 1 receptor, thus leading to

chronic sensitization of circulating monocytes and exacerbating atherosclerosis.⁷³

Contact Activation (Intrinsic) Pathway

The contact activation pathway is considered nonessential for hemostasis in vivo (**Fig. 1C and Fig. 4**). However, it may be involved in the pathogenesis of arterial thrombosis.¹⁴ Although experimental data have clearly shown that mice deficient in factor XII are protected against arterial thrombosis and stroke,¹⁴ in several epidemiologic studies, data on the association between factor XII and the risk of cardiovascular disease in humans are inconsistent.⁷⁴⁻⁷⁶ Although additional research is needed in this field, the pharmacologic inhibition of factor XII activation represents a potential therapeutic target,^{77,78} considering that hereditary deficiency of factor XII is not associated with bleeding disorders or other pathologic conditions. At a molecular level, factor XII influences distinct processes mostly through the plasma kallikrein-kinin system.⁷⁹ Factor XII-mediated bradykinin formation not only regulates vasodilatation and vascular permeability but also induces activation of the complement and fibrinolytic systems by activating components C3 and C5 and facilitating the synthesis of tissue-type plasminogen activator from endothelial cells, whereas kallikrein activates urokinase-type

plasminogen activator and plasminogen. Platelet-derived inorganic polyphosphates⁸⁰ and misfolded proteins, which are found abundantly in atherosclerotic arteries,⁸¹ can also activate factor XII, leading to kallikrein formation without triggering coagulation.⁸² Levels of tissue kallikrein and plasma prekallikrein are associated with the severity of cardiovascular disease^{83,84} and have been found to be critical in the process of vascular repair.⁸⁵ Given the proangiogenic and proinflammatory nature of factor XII⁸⁶ and the plasma kallikrein-kinin system, chronic stimulation of these responses may promote a proatherogenic intraarterial environment over time.

Anticoagulant Pathways in Vascular Inflammation

Tissue factor pathway inhibitor (TFPI), which is widely distributed in healthy arterial vessels, tends to be overexpressed in atherosclerotic lesions⁸⁷ (**Fig. 5A**). Although TFPI is expressed on endothelial cells, VSMCs, and macrophages in the fibrous cap and shoulder areas of the plaques, it also colocalizes with tissue factor and attenuates its activity within atherosclerotic lesions.^{30,96,97} This finding suggests a role for TFPI not only in the regulation of tissue factor procoagulant activity but also in the control of tissue factor-induced proatherogenic signaling. The administration of recombinant TFPI has reduced the rates of in-

Figure 4. Contact Activation Pathway and Its Proinflammatory and Proangiogenic Properties. The contact system plays a role in various physiologic processes, such as blood-pressure regulation, coagulation, fibrinolysis, angiogenesis, and inflammation. It consists of factor XII, prekallikrein, and high-molecular-weight kininogen. The activation of the proinflammatory kallikrein–kinin and complement systems is triggered by the proteolytic cleavage of factor XII (autoactivation) in reaction to contact with negatively charged artificial or biologic surfaces. The gray circle indicates the inactive form of a coagulation protein, and green circles indicate the active form. Green circles with plus signs indicate either positive-feedback reactions or induction of a process.

flammation and death in an animal model by decreasing the expression of tumor necrosis factor α (TNF- α), chemokines, and myeloperoxidase.⁸⁸ Moreover, TFPI is a potent inhibitor of matrix metalloproteinases, which are considered key players in plaque destabilization and atherothrombotic complications. Decreased TFPI expression has been associated with up-regulation of the synthesis of matrix metalloproteinases in plaques with a vulnerable phenotype. In addition, TFPI has inhibited endothelial migration and angiogenesis in mice. Several studies in animals have shown that TFPI attenuates neointimal hyperplasia and stenosis but also suppresses the release of proatherogenic platelet-derived growth factor BB, CCL2, and matrix metalloproteinase 2.⁹⁸⁻¹⁰¹ In agreement with these findings, TFPI-deficient mice have significantly more atherosclerotic plaques than control mice,¹⁰² whereas vascular-directed TFPI overexpression appears to regulate lipoprotein clearance and temporarily lowers plasma cholesterol levels, also reducing atherosclerotic plaque development.¹⁰³ Clinical data suggest that plasma TFPI is a marker of endothelial dysfunction; high levels of both free and total TFPI levels are associated with an increased atherosclerotic burden and coronary-artery calcification,^{104,105} whereas low levels of total TFPI are associated with an increased risk of atherothrombosis.^{106,107} In addition to its antico-

agulant properties, the protein C pathway is known for its protective effects on vascular gene-expression profiles involving antiapoptotic and antiinflammatory responses, as well as its stabilizing effect on the endothelial barrier (**Fig. 5B**).¹⁰⁸ Studies of atherosclerosis have shown a substantial downregulation of the local expression of endothelial protein C receptor and thrombomodulin within atherosclerotic vessels, suggesting impaired activation of protein C and hence a reduced antiatherogenic response. Several mechanisms, such as enhanced shedding of thrombomodulin from dysfunctional endothelium, an abundance of LDLcholesterol deposits, and local inflammation within the arterial wall, may account for the attenuation of the anticoagulant activities of protein C within the atherosclerotic plaque. Overexpression of thrombomodulin has been shown to limit neointimal formation in rabbits,¹⁰⁹ whereas a genetic impairment of the protein C-activating cofactor function of thrombomodulin, resulting in diminished formation of activated protein C, is associated with an increased atherosclerotic burden in mice.³¹ The determinants of soluble levels of thrombomodulin in patients with atherosclerosis are poorly understood. The results of various clinical studies that have examined the relationship between thrombomodulin and the extent of atherosclerotic burden have been inconsistent.¹¹⁰⁻¹¹⁴ In monkeys, progressive atheroscle-

Figure 5.

rosis is associated with impaired formation of activated protein C, whereas dietary regression of atherosclerosis was found to enhance the anticoagulant response.¹¹⁵ Mice with a heterozygous deficiency in protein C have enhanced focal arterial inflammation and thrombosis, leading to increased neointima formation and localized thrombosis.¹¹⁶ In agreement with these findings, several clinical studies have confirmed a significant association between circulating low levels of activated protein C and a greater extent or severity of atherosclerosis.¹¹⁷⁻¹¹⁹ Furthermore, protein S, which has been described as linking hemostasis, inflammation, and apoptosis, forms a complex with the complement system regulator C4b-binding protein (C4BP), a major inhibitor of the classical complement pathway, localizing it on the surface of apoptotic cells¹²⁰ and thus promoting phagocytic activity by macrophages.¹²¹ Intriguingly, protein S significantly inhibits the expression of macrophage scavenger receptor A and diminishes the uptake of acetylated LDL cholesterol mediated by this receptor, resulting in a decreased intracellular lipid content in macrophages.¹²² These actions are mostly attributable to the ability of protein S to bind to and induce phosphorylation of the Mer receptor tyrosine kinase. In addition, protein S plays a role in the protection of the integrity of the blood-brain barrier.¹²³ The expression of protein S is reduced

within atherosclerotic plaques obtained from patients with unstable angina, as compared with specimens from patients with stable angina.¹²⁴ Hereditary deficiency of both proteins C and S has been associated with an increased incidence in arterial thromboembolic events¹²⁵ and peripheral-artery disease (**Fig. 5B**).^{89-95,126}

Figure 5. Anticoagulant Pathways and Their Nonhemostatic Features.

The regulation of coagulation operates at three levels: inhibition of thrombin, factor Xa, and factor IXa by antithrombin; inhibition of factor Xa, the tissue factor-factor VIIa (TF-FVIIa) complex, and hence thrombin formation by tissue factor pathway inhibitor; and proteolytic inactivation of factor V and factor VIII by activated protein C. As shown in Panel A, antithrombin is a serine protease that inhibits key coagulation enzymes such as thrombin, factor Xa, and factor IXa. Its action is amplified by as much as 4000 times in the presence of heparin or heparin-like substances, such as heparan sulfate proteoglycan. Antithrombin has apparent antiinflammatory effects,⁸⁸ as seen in an increase in the release of prostacyclin and a decrease in nuclear factor κ B signaling, which is known to have multiple proinflammatory responses. Similar effects have been found after the administration of synthetic direct thrombin inhibitors, which has contributed to plaque stability in vivo.⁵⁵ Antithrombin attenuates leukocyte

recruitment during inflammation, which hints at another potential atheroprotective role. Heparin also stimulates the release from endothelial cells of tissue factor pathway inhibitor, which then binds to factor Xa and the TF–FVIIa complex to form an inactive quaternary complex, thus showing a multitude of antiatherogenic functions. Like antithrombin, heparin cofactor II has the ability to inactivate thrombin, factor Xa, and factor IXa, whereas the plasma form of heparin cofactor II is an inefficient inhibitor in the absence of glycosaminoglycans (e.g., heparan sulfate and dermatan sulfate). Heparin cofactor II is implicated both in vascular remodeling and in atherogenesis. Mice that are deficient in heparin cofactor II have enhanced intimal hyperplasia after vascular injury.⁸⁹ Such mice have increased neointima formation and enhanced atherogenesis, as compared with control mice. However, the findings in clinical studies have been inconsistent, with some indicating that heparin cofactor II is a strong predictive marker against atherosclerosis^{90,91} and one indicating that its presence is not predictive.⁹² Protein Z is a cofactor of another protein, named protein Z–related protease inhibitor, which inhibits factor Xa and factor XIa in the coagulation cascade. Although the roles of protein Z and protein Z–related protease inhibitor in inflammation and the onset of atherosclerosis are poorly understood, a few clinical trials have shown a significant inverse relationship be-

tween levels of these proteins and the clinical severity of atherosclerosis.^{93–95} As shown in Panel B, thrombin also behaves as an anticoagulant molecule physiologically. Binding to the endothelial protein C receptor, protein C is transformed into activated protein C by an activation complex established between thrombin and thrombomodulin. This process is followed by dissociation of activated protein C from the endothelial protein C receptor and the formation of a complex between activated protein C and protein S. The latter allows the inactivation of factor Va and factor VIIIa and thus limits further thrombin generation. Gray circles indicate the inactive form of a coagulation protein, and green circles indicate the active form.

Future Perspectives

Hemostasis is anatomically and functionally entwined with the vasculature. Besides its essential roles in protecting vascular integrity and maintaining normal blood flow, accumulating data suggest an intimate cross-talk between hemostasis and inflammation, underscoring the role of both systems in many complex diseases, including atherothrombosis. Intriguingly, numerous studies in animals have also documented that hemostasis is closely linked to the pathophysiology of atherogenesis. Is this association, mostly based on experimental data, corroborated by clinical data as well? The current concept of a vulnerable plaque sug-

gests that repeated plaque micro-ruptures, followed by subclinical thrombosis, are critical for plaque growth and vulnerability.¹²⁷⁻¹²⁹ In agreement with these findings, histopathological studies showed that two thirds of coronary thrombi obtained from patients who died suddenly from cardiovascular causes were in later stages of maturation, suggesting that thrombi may exist long before a rupture occurs.^{33,34} In addition, the contemporary understanding of atherothrombosis has evolved substantially, establishing new roles for the hemostatic system beyond thrombosis. We have summarized the potential array of actions of hemostasis in relation to the phenotype of the atherosclerotic vascular wall, presumably linked to plaque stability. But is all of this clinically relevant? Antithrombotic therapy with the use of antiplatelet or anticoagulant agents is the key to atherothrombosis prevention in various clinical situations.¹³⁰⁻¹³³ The role of antiplatelet therapy in secondary prevention is no longer questioned, given the strong overall effect of drugs such as aspirin.¹³⁴ A meta-analysis of primary-prevention trials has indicated that the use of aspirin is associated with a reduction of approximately 30% in the risk of myocardial infarction, with more limited effects on the risk of stroke.¹³⁵ In addition to aspirin's antiplatelet actions, the efficacy of this drug may be due in part to its anti-inflammatory actions.¹³⁶⁻¹³⁸ It is difficult to dissect

the contribution of platelets in any of these antiinflammatory effects of aspirin. Also, for more selective antiplatelet drugs, including clopidogrel, prasugrel, and ticagrelor, which target platelet receptors, resulting in impaired platelet activation, antiinflammatory and atherosclerosis-delaying effects have been reported.¹³⁹ However, clinical trials of platelet inhibitors for the prevention of atherosclerosis progression have not shown diminished development of plaque with any consistency.¹⁴⁰ For many years, oral anticoagulants have been used for short- and long-term indications. Studies of heparin and vitamin K antagonists have shown that short-term use of these drugs is not likely to have a major effect on chronic disorders such as atherosclerosis.^{141,142} Despite the fact that long-term administration of vitamin K antagonists did not have any visible effects on angiographic progression in patients who had undergone coronary-artery bypass grafting, an additional followup assessment 3 years after discontinuation of therapy showed a significant 35% reduction in overall mortality in the warfarin group.¹⁴³ Given the powerful effects on risk reduction in thrombotic cardiovascular outcomes, one might speculate that this effect was at least partially mediated by effects of vitamin K antagonists on plaque phenotype rather than plaque size. At the same time, the principal vascular side effect of the long-term admin-

istration of these drugs is accelerated calcification. This effect is mainly due to direct inhibition of other vitamin K-dependent proteins in the vessel wall, including matrix Gla protein. It is not known whether any additional influence of inhibition of thrombin formation may occur.^{144,145} The role of the hemostatic system in atherosclerosis in humans requires further investigation. Only a handful of molecules relevant to hemostasis are targeted by existing medications. As more specific interventions are developed, new therapeutic avenues and research approaches may open up. With the introduction of new oral anticoagulants (e.g., direct inhibitors of factor Xa and thrombin),^{146,147} which are small molecules that can access the vessel wall, it will be possible to document the effects of these drugs on plaque formation and especially on plaque stability. Since both thrombin inhibition⁵⁵ and a prothrombotic state³¹ have been suggested as promoters of plaque stability in atherogenic mice, the net effects in humans, if any, are unpredictable. In conclusion, given the potential of hemostasis to influence molecular and cellular responses in the vasculature, new scientific approaches are required. Notably, the majority of experimental data are entirely based on quantification of plaque burden, rather than on extensive phenotyping of the lesions. This is a major drawback in vascular medicine. Furthermore, most clinical studies predominantly focus on es-

tablishing the thrombotic and mortality outcomes, whereas few investigate plaque progression. During the past decade, ultrasonography has been a major tool in vascular imaging. Unfortunately, this approach is characterized by poor tissue penetration, providing no information on plaque characteristics, and is subject to intraobserver and interobserver variability. With the development of high-resolution magnetic resonance imaging, the assessment of plaque characteristics will improve vessel-wall phenotyping as a means of addressing the role of the hemostatic system in atherosclerosis.

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SUPPLEMENTARY APPENDIX

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Borissoff JI, Spronk HMH, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364:1746-60.

Online Supplementary Appendix

Table 1. Genetically-Altered Mice Studied in Atherosclerosis and Arterial Thrombosis Models; Gene names are presented as follows: Name Aliases (Approved Symbol of the Gene); Denotes either diminished neointima formation/atherogenesis or decreased susceptibility to arterial thrombosis; Denotes either enhanced neointima formation/atherogenesis or increased susceptibility to arterial thrombosis; = Does not play a role; ≠ Controversial Data; - This aspect hasn't been investigated

Table 1.

GENE	FUNCTION	TARGETED MUTATION	ATHEROGENESIS	ARTERIAL THROMBOSIS
<i>GPIba</i> (<i>GP1BA</i>)	Platelet Adhesion Membrane Receptor	<i>IL4Ra</i> <i>GPIba-tg</i>	-	↓ ¹
<i>GPIIb</i> (<i>GP1BB</i>)	Platelet Adhesion Membrane Receptor	<i>GPIIb</i> ^{-/-} <i>GPIIbΔIC</i> ^{-/-}	= 2	↓ ³
<i>GPV</i> (<i>GP5</i>)	Platelet Adhesion Membrane Receptor	<i>GPV</i> ^{-/-}	-	↑ ⁴
<i>GPVI</i> (<i>GP6</i>)	Platelet Adhesion Membrane Receptor	<i>GPVI</i> ^{-/-}	↓ ⁵	↓ ^{6, 7}
<i>FcRγ</i> (<i>FCGR1A/3A</i>)	Co-receptor of <i>GPVI</i>	<i>FcRγ</i> ^{-/-}	↓ ⁸	↓ ⁹
<i>α₂-Integrins</i> (<i>ITGA2</i>)	Platelet Aggregation Membrane Receptors	<i>α₂</i> ^{-/-}	= 10	↓ ^{11, 12}
<i>β₃-Integrins</i>	Platelet Aggregation Membrane Receptors	<i>β₃</i> ^{-/-}	↑ ^{13, 14}	↓ ¹⁵
<i>P2Y₁</i> (<i>P2RY1</i>)	Platelet Aggregation Membrane Receptor	<i>P2Y₁</i> ^{-/-}	↓ ¹⁶	↓ ¹⁷
<i>P2Y₁₂</i> (<i>P2RY12</i>)	Platelet Aggregation Membrane Receptor	<i>P2Y₁₂</i> ^{-/-}	↓ ¹⁸	↓ ¹⁷
<i>P2X₁</i> (<i>P2RX1</i>)	Platelet Aggregation Membrane Receptor	<i>P2X₁</i> ^{-/-}	-	↓ ^{17, 19}
Prostaglandin <i>E2R</i> (<i>PTGER2</i>)	Platelet Aggregation Membrane Receptor	<i>PGES-I</i> ^{-/-} <i>EP3</i> ^{-/-}	↓ ²⁰	↓ ²¹
Prostacyclin <i>PGI2R</i> (<i>PTGIR</i>)	Platelet Aggregation Membrane Receptor	<i>IP</i> ^{-/-}	↑ ²²	↑ ²³
Thromboxane <i>A2R</i> (<i>TBXA2R</i>)	Platelet Aggregation Membrane Receptor	<i>TP</i> ^{-/-}	↓ ²²	- ²⁴
<i>P-selectin</i> (<i>SELP</i>)	Cell Adhesion Molecule	<i>P-selectin</i> ^{-/-}	↓ ^{14, 25-28}	↓ ^{29, 30}
<i>E-selectin</i> (<i>SELE</i>)	Cell Adhesion Molecule	<i>E-selectin</i> ^{-/-}	↓ ²⁸	-
<i>PSGL-1</i> (<i>SELPLG</i>)	<i>P-selectin</i> Glycoprotein Ligand 1	<i>PSGL-1</i> ^{-/-}	↓ ³¹	↓ ²⁹
<i>PECAM-1</i> (<i>PECAM1</i>)	Platelet Endothelial Cell Adhesion	<i>PECAM-1</i> ^{-/-}	≠ ^{32, 33}	↑ ³⁴
<i>ICAM-1</i> (<i>ICAM1</i>)	Intercellular Adhesion Molecule 1	<i>ICAM-1</i> ^{-/-}	↓ ^{28, 35}	-
<i>VCAM-1</i> (<i>VCAM1</i>)	Vascular Cell Adhesion Molecule 1	<i>VCAM D4D</i> ^{-/-}	↓ ^{36, 37}	-
<i>vWF</i> (<i>VWF</i>)	Blood Hemostatic Glycoprotein	<i>vWF</i> ^{-/-}	↓ ³⁸	↓ ^{39, 40}
<i>ADAMTS13</i> (<i>ADAMTS13</i>)	<i>vWF</i> -cleaving Protease	<i>ADAMTS13</i> ^{-/-}	-	↑ ^{41, 42}
<i>Gas6</i> (<i>GAS6</i>)	Ligand Involved in Platelet Aggregation	<i>Gas6</i> ^{-/-}	↓ ⁴³	↓ ^{44, 45}
<i>CD40L</i> (<i>CD40LG</i>)	<i>TNF</i> Receptor-Ligand Superfamily	<i>CD40L</i> ^{-/-}	↓ ^{46, 47}	↓ ⁴⁸
<i>CXCL4</i> (<i>PF4</i>)	<i>CXC</i> Chemokine Family	<i>PF4</i> ^{-/-}	↓ ⁴⁹	↓ ⁵⁰

Table 1 (Continuation).

<i>CCL5</i> (<i>CCL5</i>)	<i>CC Chemokine Family</i>	<i>RANTES</i> ^{-/-}	U ^{51, 52}	-
<i>CD39</i> (<i>ENTPD1</i>)	<i>Ectonucleoside TP Diphosphohydrolase 1</i>	<i>ENTPD1</i> ^{-/-} (<i>CD39</i> ^{-/-})	U ⁵³	U ⁵⁴
<i>eNOS</i> (<i>NOS3</i>)	<i>Endothelial Nitric Oxide Synthase</i>	<i>eNOS</i> ^{-/-}	U ⁵⁵	U ⁵⁶
<i>PAR-1</i> (<i>F2R</i>)	<i>G Protein-coupled PAR</i>	<i>PAR1</i> ^{-/-}	U ⁵⁷	= 57
<i>PAR-3</i> (<i>F2RL2</i>)	<i>G Protein-coupled PAR</i>	<i>PAR3</i> ^{-/-}	-	U ⁵⁸
<i>PAR-4</i> (<i>F2RL3</i>)	<i>G Protein-coupled PAR</i>	<i>PAR4</i> ^{-/-}	= 59	U ⁶⁰
<i>TF</i> (<i>F3</i>)	<i>Protein Acting as Trigger of Coagulation</i>	<i>TF</i> ^{-/-}	Embryonically Lethal Phenotype, Vascular Integrity Impairment	
		Low <i>TF</i>	= 61	U ⁶²
<i>FVII</i> (<i>F7</i>)	<i>Coagulation Factor VII</i>	<i>FVII</i> ^{-/-}	Viable Phenotype, No Vascular Defects, Severe Neonatal Hemorrhage	
		Low <i>FVII</i>	-	-
<i>FX</i> (<i>F10</i>)	<i>Coagulation Factor X</i>	<i>FX</i> ^{-/-}	Partially Viable Phenotype, No Vascular Defects, Severe Neonatal Hemorrhage	
		Low <i>FX</i>	-	-
<i>FII</i> (<i>F2</i>)	<i>Coagulation Factor II</i>	<i>FII</i> ^{-/-}	Partially Viable Phenotype, Vascular Defects, Severe Neonatal Hemorrhage	
		<i>FII</i> ^{+/-}	-	-
<i>FV</i> (<i>F5</i>)	<i>Coagulation Factor V</i>	<i>FV</i> ^{-/-}	Partially Viable Phenotype, Vascular Defects, Severe Neonatal Hemorrhage	
		<i>FV</i> ^{Q/Q} (Leiden)	U ^{63, 64}	U ⁶³
<i>FVIII</i> (<i>F8</i>)	<i>Coagulation Factor VIII</i>	<i>FVIII</i> ^{-/-}	U ⁶⁵	U ⁶⁶
<i>FIX</i> (<i>F9</i>)	<i>Coagulation Factor IX</i>	<i>FIX</i> ^{-/-}	-	U ⁶⁷
<i>FXI</i> (<i>F11</i>)	<i>Coagulation Factor XI</i>	<i>FXI</i> ^{-/-}	-	U ⁶⁷
<i>FXII</i> (<i>F12</i>)	<i>Coagulation Factor XII</i>	<i>FXII</i> ^{-/-}	-	U ⁶⁸
<i>Fibrinogen</i> (<i>FGA/B/G</i>)	<i>Coagulation Factor I</i>	<i>Fbg</i> ^{-/-}	≠ 39, 69	≠ 70, 71
<i>FXIII</i> (<i>F13A1/B</i>)	<i>Coagulation Factor XIII</i>	<i>FXIII</i> ^{-/-}	-	-
<i>TFPI</i> (<i>TFPI</i>)	<i>Anti-coagulant Protein</i>	<i>TFPI</i> ^{-/-} <i>TFPI</i> ^{Tie2}	U ⁷²	U ⁷³
<i>TM</i> (<i>THBD</i>)	<i>Anti-coagulant Protein</i>	<i>TM</i> ^{-/-}	Embryonically Lethal Phenotype	
		<i>TM</i> ^{PRO/PRO}	U ⁶⁴	U ^{74, 75}
<i>PC</i> (<i>PROC</i>)	<i>Anti-coagulant Protein</i>	<i>PC</i> ^{+/-}	U ⁷⁶	U ⁷⁶
<i>EPCR</i> (<i>PROCR</i>)	<i>Endothelial Protein C Receptor</i>	<i>EPCR</i> ^{-/-}	Placental Thrombosis and Early Embryonic Lethality	
<i>PS</i> (<i>PROS1</i>)	<i>Anti-coagulant Protein</i>	<i>PS</i> ^{-/-}	-	-

Table 1 (Continuation).

<i>HCII (SERPIND1)</i>	Anti-coagulant Protein	<i>HCII</i> ^{-/-}	77	78
<i>ATIII (SERPINC1)</i>	Anti-coagulant Protein	<i>AT</i> ^{-/-}	Embryonically Lethal Phenotype	
<i>ZPI (SERPINA10)</i>	Anti-coagulant Protein	<i>ZPI</i> ^{-/-}	-	79
<i>PZ (PROZ)</i>	Cofactor of ZPI	<i>PZ</i> ^{-/-}	-	79
<i>Plasminogen (PLG)</i>	Zymogen of Plasmin	<i>Plg</i> ^{-/-}	80	81
<i>t-PA (PLAT)</i>	Tissue Plasminogen Activator	<i>t-PA</i> ^{-/-}	82	= 82
<i>u-PA (PLAU)</i>	Urokinase-type Plasminogen Activator	<i>u-PA</i> ^{-/-}	82, 83	82
<i>PAI-1 (SERPINE1)</i>	Plasminogen Activator Inhibitor-1	<i>PAI-1</i> ^{-/-}	≠ 84, 85	86-89
<i>TAFI (CPB2)</i>	Thrombin-activable Fibrinolysis Inhibitor	<i>TAFI</i> ^{-/-}	-	≠ 90, 91
<i>α2-Antiplasmin (SERPINF2)</i>	Plasmin Inhibitor	<i>α2-AP</i> ^{-/-}	= 92	= 81
<i>α2-Macroglobulin (A2M)</i>	Coagulation and Fibrinolysis Inhibitor	<i>α2-M</i> ^{-/-}	-	-
<i>Fibronectin (FN1)</i>	Extracellular Matrix Glycoprotein	<i>FN</i> ^{-/-} <i>EIIIA</i> ^{-/-}	93	94
<i>Thrombospondin (THBS1)</i>	Extracellular Matrix Glycoprotein	<i>Tsp-1</i> ^{-/-}	95	96
<i>Vitronectin (VTN)</i>	Extracellular Matrix Glycoprotein	<i>Vn</i> ^{-/-}	97	≠ 87, 88, 98, 99
<i>Adiponectin (ADIPOQ)</i>	Secretory Protein (Adipocytes)	<i>Ad-APN</i> <i>APN</i> ^{-/-}	100	101

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6

MODIFYING ATHEROSCLEROTIC PLAQUE STABILITY VIA THE COAGULATION-INFLAMMATION AXIS

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Summary

Hemostasis and inflammation dynamically interact in various clinical conditions^{1,2}. Whereas inflammation is considered integral to atherogenesis, the role of hemostatic factors in the pathogenesis of atherosclerosis remains elusive³. Here, genetic and pharmacological studies demonstrate that prothrombin and thrombin formation/activity are central not only to the onset and progression of atherosclerosis *in vivo*, but also to plaque stability. Gene-targeted 50% reduction in prothrombin (FII^{-/WT}) was remarkably effective in limiting disease in atherosclerosis-prone ApoE^{-/-} mice. The general diminution in lesion development in FII^{-/WT}:ApoE^{-/-} mice was also associated with significant qualitative benefits, including diminished leukocyte infiltration, altered collagen and vascular smooth muscle cell content. In contrast, genetically-imposed hypercoagulability in TM^{Pro/Pro}:ApoE^{-/-} mice resulted in severe atherosclerotic disease, neutrophil infiltration, plaque vulnerability and spontaneous atherothrombosis. Notably, administration of either the synthetic specific thrombin inhibitor Dabigatran etexilate, or a recombinant form of the natural anticoagulant activated protein C (APC), rescued the pro-inflammatory and pro-atherogenic phenotype of pro-thrombotic TM^{Pro/Pro}:ApoE^{-/-} mice. Overall, these findings reveal that thrombin-

mediated proteolysis is an unexpectedly powerful determinant of atherosclerosis in multiple distinct settings. These studies suggest that selective anticoagulants employed to prevent thrombotic events may also be remarkably effective in clinically impeding the onset and progression of cardiovascular disease.

Main

Blood coagulation and inflammation are evolutionary coupled host-defense mechanisms, which operate via common molecular and cellular pathways, serve as protection against infections or bleeding, promote wound healing and restore the integrity of injured tissues⁴⁻⁶. Atherosclerosis is a progressive chronic inflammatory vascular disorder, which can result in atherosclerotic plaque rupture and subsequent superimposed thrombus formation⁷⁻⁹. Besides the detrimental role of coagulation during the onset of acute atherothrombotic complications, there is evidence that local activation of hemostatic factors within early human atherosclerotic lesions may also be important in atherogenesis¹⁰. Nevertheless, there is limited understanding of the role of blood coagulation in atherosclerosis.

To investigate this matter *in vivo*, we first generated transgenic cross breeds with genetically imposed variations in coagulation potential. Homozygous prothrombin (FII) deficiency in mice results in embryonic

and neonatal lethality due to severe hemorrhagic phenotype and loss of vascular integrity¹¹. Therefore, we employed uniformly viable FII heterozygous ApoE^{-/-} mice (characterized by hypoprothrombinemia and diminished FVII and thrombin generation *but no spontaneous bleeding risk*; *Supplementary Table 1A*) in comparative studies with control, prothrombin-sufficient ApoE^{-/-} mice. TM^{Pro/Pro} mice carry a point mutation in the thrombomodulin (TM) gene, which impairs TM-dependent generation of the natural anticoagulant activated protein C (APC)¹². TM^{Pro/Pro}:ApoE^{-/-} mice demonstrated a profound hypercoagulable state with substantially increased plasma thrombin generation and fibrinogen levels, but also significantly higher factor FVII, FX, and FVIII levels (*Supplementary Table 1A*). We then assessed the extent, as well as the phenotype of the atherosclerotic plaques formed in the aortic arch in experimental cohorts of mice following 35 weeks on a regular chow diet. FII^{+/+}:ApoE^{-/-} mice with a genetic deficit in prothrombin exhibited highly attenuated atherosclerotic lesion formation relative to control ApoE^{-/-} mice (*Figure 1A, 1B*). Macrophage infiltration (MAC-2⁺ cells) and α -smooth muscle actin (SMA; α -SMA⁺ cells) content were unaffected in FII^{+/+}:ApoE^{-/-} mice lesions compared to ApoE^{-/-} control mice (*Figure 1A, 1C, 1D*). However, hypoprothrombinemia was also linked to a significant decrease in neutrophil recruit-

ment (*Figure 1A, 1E*), abundant collagen deposition (*Figure 1A, 1F*), thus showing a more fibrotic appearance, stable plaque phenotype and decreased number of advanced atherosclerotic lesions formed. In sharp contrast, pro-thrombotic TM^{Pro/Pro}:ApoE^{-/-} mice displayed severe atherosclerosis development with remarkably increased total plaque area (*Figure 1A, 1B*). Of note, previous studies have indicated that aggravation of stenosis in TM^{Pro/Pro}:ApoE^{-/-} mice can be in part averted due to positive vascular remodeling during the intermediate phases of progression¹³. Importantly, we demonstrate that plaques of pro-thrombotic mice had profound composition changes, with overt features of plaque vulnerability at later stages of disease development (at 35 weeks on regular chow diet). TM^{Pro/Pro}:ApoE^{-/-} mice showed unstable lesions (*Figure 1A, 1B*), associated with markedly decreased α -SMA and collagen content (*Figure 1A, 1C, 1F*), and significantly higher neutrophil (Ly6G⁺ cells) infiltration (*Figure 1A, 1C, 1F*). These effects were independent of plasma lipid levels (*Supplementary Table 1B*) and could not be attributed to an increased uptake of modified lipoproteins by macrophages (*Supplementary Figure 1A, 1B, 1C, 1D*). Of interest, hypercoagulable TM^{Pro/Pro}:ApoE^{-/-} mice showed significantly increased spontaneous mortality rates, albeit that the exact cause of death could not be pinpointed (*Figure 1G*).

Hence, to further verify the net effects of underlying alterations in clotting potential on plaque phenotype, we also studied the impact of both genetic perturbations on collar-induced carotid artery atherosclerosis¹⁴. High-fat diet fed $FII^{+/-}ApoE^{-/-}$ mice displayed significantly decreased plaque volume, degree of stenosis, intima/media ratio and expansion of the arterial wall, 6 weeks after bilateral perivascular carotid collar placement (*Figure 2A, 2B, 2C, 2F, 2G*). Furthermore, hypo-coagulability ameliorated plaque stability, testified by a significantly increased mean fibrous cap thickness (*Figure 2E*). Conversely, $TM^{Pro/Pro}ApoE^{-/-}$ mice lesions were substantially larger, accompanied by significantly increased luminal stenosis, intima/media ratio and outward remodeling (*Figure 2A, 2B, 2C, 2D, 2F, 2G*). Similar to spontaneous atherosclerosis, collar-induced carotid artery plaques in $FII^{+/-}ApoE^{-/-}$ mice presented a stable pro-fibrotic phenotype, whereas $TM^{Pro/Pro}ApoE^{-/-}$ lesions showed pronounced features of plaque vulnerability, including larger necrotic cores (*Figure 2A, 2D*), thin fibrous caps (*Figure 2A, 2E*) and significant decrease in collagen content (*Supplementary Figure 2A, 2B*). High plaque vulnerability in hypercoagulable mice was also associated with a pro-inflammatory plaque phenotype, signs of intraplaque hemorrhage, plaque dissection, but also spontaneous plaque rupture and atherothrombosis (*Figure 3E,*

Supplementary Figure 3), whereas fibrin deposits were extensively distributed throughout ruptured $TM^{Pro/Pro}ApoE^{-/-}$ lesions (*Supplementary Figure 3*). Intraplaque accumulation of macrophages and neutrophils was significantly diminished in hypocoagulable mice (*Figure 3A, 3B, 3C*), indicating that deficiency in prothrombin results in a less inflammatory plaque profile. In contrast, in $TM^{Pro/Pro}ApoE^{-/-}$ advanced carotid artery lesions were abundantly infiltrated with neutrophils when compared to $ApoE^{-/-}$ control mice, whereas no changes were observed in terms of macrophage content (*Figure 3A, 3B, 3C*). A pronounced Ly6G⁺ cell intraplaque recruitment was also observed during early stages of atherosclerosis in $TM^{Pro/Pro}ApoE^{-/-}$ mice (*Figure 3D*).

To provide further understanding into how alterations in blood coagulation potential affect the thrombogenicity of the arterial vessel wall, we pursued complementary studies of vessel occlusion following ferric chloride injury of healthy arteries. Times to occlusion and cessation of blood flow as a result of thrombus formation were significantly shortened in $TM^{Pro/Pro}ApoE^{-/-}$ mice relative to control $ApoE^{-/-}$ mice, whereas occlusion times in $FII^{+/-}ApoE^{-/-}$ animals was comparable to control $ApoE^{-/-}$ cohorts (*Supplementary Figure 4*). Overall, these data demonstrate that key hemostatic factors are major determinants of atherosclerotic plaque composition and fate, but

also of the thrombogenicity of the arterial vessel wall *per se*.

Neutrophils represent an intriguing cellular interface between blood coagulation and inflammation⁶. Although the importance of neutrophils in atherosclerosis remains to be defined in detail, several studies have highlighted their pro-atherogenic potential and proposed role in atherosclerotic plaque destabilization^{3,15-20}. There appears to be a strong positive correlation between the number of circulating neutrophils in peripheral blood, plasma thrombin generation and the extent of atherosclerotic plaque burden (*Figure 3F, 3G*). Because of the increased neutrophil counts observed in hypercoagulable TM^{Pro/Pro}:ApoE^{-/-} mice after 35 weeks on a regular chow diet (*Supplementary Table 1C*), and the abundant infiltration of neutrophils within vulnerable-appearing atherosclerotic lesions (*Figure 3A, 3C*), we explored the impact of hypercoagulability on neutrophil function and hematopoiesis in the context of atherosclerosis. Intravital microscopy studies revealed that while general leukocyte rolling and arrest was not affected (*Figure 3J*), neutrophils were significantly more adherent to atherosclerotic lesions in the common carotid artery of TM^{Pro/Pro}:ApoE^{-/-} than in ApoE^{-/-} control mice after 6 weeks on a high-fat diet (*Figure 3H, 3K*). These data consolidated our histological findings (*Figure 3A, 3C, 3D*), suggesting that hypercoagulability can promote initiation and

progression of atherosclerotic lesions in a neutrophil-dependent manner. Consistent with this view, hypercoagulability promoted a significant increase in plasma CCL2 and CXCL1 levels (*Supplementary Table 2*), which are critical players in recruitment of monocytes and neutrophils to sites of chronic inflammation²¹⁻²³. TM^{Pro/Pro}:ApoE^{-/-} mice showed significantly higher IL-6 plasma levels after 35 weeks on regular chow diet. Although there were trends toward increased IL-1 β , expression of other key proinflammatory cytokines such as TNF- α , IFN- γ , IL-5 and IL-12 were not statistically different between hypercoagulable and control ApoE^{-/-} mice, suggesting that TM^{Pro/Pro}:ApoE^{-/-} mice exhibited a pro- but not hyper-inflammatory systemic profile (*Supplementary Table 2*). In addition, the higher plasma expression levels of granulocyte-colony stimulating factor (G-CSF) in TM^{Pro/Pro}:ApoE^{-/-} mice raises the possibility that the loss of TM function not only impacts on thrombin activity but also affects granulopoiesis in the bone marrow. However, we did not detect major changes in hematopoiesis between hypercoagulable and control ApoE^{-/-} mice after 10 weeks on regular chow diet (*Supplementary Figure 5*), including any preferential differentiation towards granulocytic-type colonies. Despite a minor but significant increase in the common myeloid progenitor (CMP) cells, lineage-negative (LK, LS, LSK), granulocyte-macrophage progenitor

(GMP) and erythroid / megakaryocyte progenitor (EMP) populations in TM^{Pro/Pro}:ApoE^{-/-} mice remained unaffected (*Supplementary Figure 5*). Nevertheless, the relative percentage of mature granulocytes in bone marrow, as well as of pro-atherogenic Ly6C^{high} monocytes²⁴ as measured by flow cytometry, was significantly increased in the pro-thrombotic mice (*Supplementary Figure 6A, 6B, 6C*). Of interest, another consequence of chronic hypercoagulability was enhanced accumulation of reactive oxygen species in neutrophils and not monocytes, as assessed by DHR fluorescence, the latter considered a measure of neutrophil senescence (*Supplementary Figure 6D, 6E*). The increased number of circulating pro-atherogenic neutrophils (*Figure 3I, Supplementary Table 1C*) in TM^{Pro/Pro}:ApoE^{-/-} mice can be in part explained by enhanced mobilization from the bone marrow as a result of exuberant plasma CCL-2 and CXCL-1 expression, a chemokine recognized for its potent neutrophil chemoattractant activity and capacity to promote vascular inflammation²⁵.

Thrombin is a central coagulation protease and known to promote numerous pro-atherogenic actions *in vitro* such as endothelial permeability, migration and proliferation of VSMC, leukocyte recruitment, cytokine and chemokine production, vascular calcification, angiogenesis and apoptosis²⁶. Distinct pro-thrombotic states have been associated with enhanced atherosclerosis progres-

sion in mice *in vivo*²⁷⁻³². Our data strongly suggests that increased thrombin generation due to diminished APC production in TM^{Pro/Pro}:ApoE^{-/-} mice (*Supplementary Table 1A, Figure 3G*) may be mechanistically-coupled to the pro-atherosclerotic phenotype. To study the role of thrombin in modulating atherogenesis *in vivo*, we administered either the specific oral thrombin inhibitor Dabigatran etexilate or a recombinant form of the natural anticoagulant activated protein C (rAPC) for 6 weeks after carotid collar placement in hypercoagulable TM^{Pro/Pro}:ApoE^{-/-} mice on high-fat diet. Remarkably, both interventions completely rescued plaque formation (*Figure 4A, 4B*), as also evident by the decreased degree of stenosis, intima/media ratio and positive outward remodeling (*Figure 4C, 4F, 4G*). Whereas in the placebo group 5 out of 10 animals had plaques with overt signs of plaque vulnerability (defined as, i.e. plaque dissection, intraplaque hemorrhage or plaque rupture), oral Dabigatran etexilate or rAPC treatments limited the occurrence of plaque destabilization and atherothrombotic phenomena, and resulted in substantially reduced leukocyte recruitment and enhanced plaque stability (*Figure 4A, 4H, 4I; Supplementary Figure 7*). In addition, both interventions led to a pronounced decrease in thrombin generation, suggesting that even ApoE^{-/-} mice exerted a low-grade hypercoagulable state (*Supplementary*

Table 3, Supplementary Table 1). Dabigatran etexilate and rAPC therapies significantly limited systemic inflammation (Supplementary Table 3), as further exemplified by decreased neutrophil and lymphocyte counts and cytokine and chemokine profiles that show a shift to an anti-inflammatory state (Supplementary Table 4).

These studies provide strong evidence suggesting that hemostatic factors, and thrombin in particular, are important modulators of the atherosclerosis plaque phenotype. By simply altering the function of the endothelial cell-associated thrombin receptor, TM, we have effectively established a new mouse model of atherosclerotic plaque vulnerability, which closely resembles the morphology and destabilization features observed in human atherosclerosis. Given the multifactorial nature of atherosclerosis and the well-known capacity of coagulation proteases and their receptors (protease-activated receptors, PARs) and substrates to control inflammatory and reparative processes³, one would anticipate that hemostatic factors might contribute, at least incrementally, to plaque development. However, the present studies directly document that thrombin and other hemostatic system components may be exceptionally powerful determinants of vessel wall disease, and even capable of superseding other pro-atherosclerotic insults. Furthermore, these studies suggest that

these hemostatic factors can influence onset, progression and even the composition and qualitative properties of atherosclerotic plaques. Because of the promising safety profile and significant clinical benefits, which selective anticoagulants offer over traditional anticoagulant therapy^{33,34}, including the reduction of risk of stroke and all-cause mortality after acute coronary syndromes, the potential clinical importance of these findings allows the unique opportunity to study if and how administration of novel classes of anticoagulants modifies atherosclerosis phenotype in patients.

Figures

Figure 1. The effects of variations in coagulation potential on atherogenesis in a spontaneous atherosclerosis model at 35 weeks on a regular chow diet. (A) Top row represents images of the aortic arch and its main branches, stained with hematoxylin and eosin (H&E), used to analyze the extent of atherosclerotic plaque burden. To determine plaque phenotype characteristics, sections were stained against α -smooth muscle actin (vascular smooth muscle cell content – second row), MAC-2⁺ (macrophage infiltration – third row), Ly-6G (neutrophil recruitment – fourth row) and with Sirius red (collagen – bottom row). **(B)** Hypocoagulability in *FII^{-/-}:ApoE^{-/-}* significantly attenuated atherosclerosis plaque development ($90.6 \pm 35.1 \times 10^3 \mu\text{m}^2$ total

plaque burden) when compared to normal ApoE^{-/-} mice ($160.6 \pm 65.9 \times 10^3 \mu\text{m}^2$) ($n=10$ per group, $p=0.0084$). Total plaque area in TM^{Pro/Pro}:ApoE^{-/-} mice was established $389.1 \pm 158.4 \times 10^3$ vs. $187.0 \pm 35.1 \times 10^3 \mu\text{m}^2$ in the corresponding control ApoE^{-/-} group ($n=10$ per group, $p=0.0010$). **(C)** TM^{Pro/Pro}:ApoE^{-/-} mice atherosclerotic plaques demonstrated a significant decrease in intimal vascular smooth muscle cell content ($2.2 \pm 1.3\%$ of plaque area) compared to ApoE^{-/-} mice ($8.7 \pm 2.9\%$ of plaque area) ($n=10$ per group, $p=0.0016$). Recruitment of macrophages within the lesions did not differ between all experimental groups **(D)**. Neutrophil infiltration was significantly diminished in the lesions of hypocoagulable FII^{+/-}:ApoE^{-/-} mice ($n=10$ per group, $p=0.0092$ vs. ApoE^{-/-} mice), and substantially increased in the TM^{Pro/Pro}:ApoE^{-/-} intima ($n=10$ per group, $p=0.0094$ vs. ApoE^{-/-} mice) **(E)**. A similar trend was observed with regard to collagen deposition within the atherosclerotic plaques. In FII^{+/-}:ApoE^{-/-} mice, $29.3 \pm 3.6\%$ of the plaque area stained collagen-positive ($n=10$ per group, $p=0.0002$ vs. ApoE^{-/-} mice). In contrast, Sirius red staining showed only $4.1 \pm 3.0\%$ positivity for collagen in the TM^{Pro/Pro}:ApoE^{-/-} lesions ($n=10$ per group, $p=0.0002$ vs. ApoE^{-/-} mice) **(F)**. To perform a more accurate plaque phenotypic analysis, we aimed at including $n=10$ animals per experimental group. By 35 weeks (established duration of the experiment), we recorded the following fa-

tal events: 6 of 16 TM^{Pro/Pro}:ApoE^{-/-}, 1 of 11 FII^{+/-}:ApoE^{-/-} and 0 of 20 ApoE^{-/-} control mice. Dead mice were excluded from the study analysis. The exact cause of death remained unclear. Kaplan-Meier analysis of the survival data comparing TM^{Pro/Pro}:ApoE^{-/-} vs. ApoE^{-/-} mice, as determined by the Gehan-Breslow-Wilcoxon test, indicated that hypercoagulability is linked to significantly higher spontaneous mortality rates ($p=0.0165$) **(G)**. No significant difference was found between FII^{+/-}:ApoE^{-/-} and ApoE^{-/-} control mice ($p=0.3173$) (data not shown). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent mean $\pm$ SD; Abbreviations: H&E – hematoxylin and eosin; α -SMA – α -smooth muscle actin; SR – Sirius red

Figure 2. Morphometrical analysis of periadventitial cuff-induced atherosclerosis in mice with genetically imposed alterations in blood coagulation potential. (A) Representative hematoxylin and eosin (H&E)-stained sections of carotid arteries of FII^{+/-}:ApoE^{-/-}, TM^{Pro/Pro}:ApoE^{-/-} and control ApoE^{-/-} mice (top row). Necrotic core areas of the atherosclerotic lesions were identified and quantified by using toluidine blue (TB) staining (second and third row). **(B, C)** Whereas hypocoagulable mice were significantly protected against plaque progression ($26.5 \pm 12.6 \times 10^3$ in FII^{+/-}:ApoE^{-/-} vs. $69.2 \pm 18.4 \times 10^3 \mu\text{m}^2$ in ApoE^{-/-} control mice, $n=10$ per group, $p<0.0001$), pro-thrombotic mice developed severe and occlusive

Figure 1.

atherosclerotic burden ($146.4 \pm 52.7 \times 10^3$ in $TM^{Pro/Pro}:ApoE^{-/-}$ vs. $53.9 \pm 27.0 \times 10^3 \mu m^2$ in $ApoE^{-/-}$ control mice, $n=10$ per group, $p=0.0001$). The degree of stenosis in $TM^{Pro/Pro}:ApoE^{-/-}$ reached an average of $88.6 \pm 8.1\%$ (vs. $62.2 \pm 16.1\%$ in $ApoE^{-/-}$ mice, $n=10$ per group, $p=0.0002$), whereas it was substantially lower in $FII^{+/-}:ApoE^{-/-}$ mice ($36.8 \pm 11.9\%$ vs. $64.9 \pm 9.6\%$ in $ApoE^{-/-}$ mice, $n=10$ per group, $p<0.0001$). **(A, D)** Pearson's chi-squared test (χ^2) detected a significant difference in the number of advanced atherosclerotic lesions (presence of fibrous cap atheromata³⁵) formed between $FII^{+/-}:ApoE^{-/-}$ (4 out of 10) and $TM^{Pro/Pro}:ApoE^{-/-}$ mice (10 out of 10) ($n=10$ per group, $p=0.0108$). In fact, the necrotic area within the lesions of the hypercoagulable mice was significantly increased: $56.2 \pm 10.8\%$ of the total plaque area, as compared to $29.0 \pm 17.7\%$ in the control $ApoE^{-/-}$ group ($n=10$ per group, $p=0.0024$). **(E)** Hypocoagulable mice showed more stable advanced lesions, as indicated by the significantly thicker fibrous caps in comparison to $ApoE^{-/-}$ mice ($n=10$ per group, $p=0.0081$). **(F)** Intima/media ratio was significantly increased in $TM^{Pro/Pro}:ApoE^{-/-}$ mice, whereas profoundly decreased in $FII^{+/-}:ApoE^{-/-}$ mice. Of note, the average outer diameter of the common carotid artery is 0.36 mm^{14} , thus suggesting that $TM^{Pro/Pro}:ApoE^{-/-}$ atherosclerotic plaques undergo a dramatic outward remodeling as indicated in panel **(G)**.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent mean $\pm$ SD. Abbreviations: H&E – hematoxylin and eosin; AL – advanced atherosclerotic lesion

Figure 3. Hypercoagulability promotes neutrophil intraplaque recruitment and severe plaque phenotype. **(A)** Representative sections of atherosclerotic lesions formed in the carotid arteries of $TM^{Pro/Pro}:ApoE^{-/-}$ and $FII^{+/-}:ApoE^{-/-}$ mice (incl. control $ApoE^{-/-}$ mice), stained for the presence of macrophages (MAC-2, green color, top row) and neutrophils (Ly-6G, brown color, bottom row). Arrows show examples of positive cells. Macrophage and neutrophil infiltration were expressed as the absolute number of Mac-2⁺ and Ly-6G⁺ cells per plaque. **(A, B)** Hypocoagulability in $FII^{+/-}:ApoE^{-/-}$ mice promoted an anti-inflammatory plaque profile, indicated by a significant decrease in macrophage infiltration compared to control $ApoE^{-/-}$ mice: 26 ± 9 vs. 54 ± 15 cells per plaque ($n=10$ per group, $p=0.0067$). No difference in macrophage content was observed between $TM^{Pro/Pro}:ApoE^{-/-}$ and $ApoE^{-/-}$ mice atherosclerotic plaques: 60 ± 8 vs. 58 ± 11 cells per plaque ($n=10$ per group, $p=0.7479$). **(A, C)** Neutrophil accumulation was significantly increased in $TM^{Pro/Pro}:ApoE^{-/-}$ lesions (151 ± 48 vs. 83 ± 28 cells per plaque in $ApoE^{-/-}$ mice, respectively; $n=10$ per group; $p=0.0260$). Interestingly, the opposite trend was observed in mice with genetically imposed hypoprothrom-

Figure 2.

binemia (51 ± 12 vs. 90 ± 24 cells per plaque in ApoE^{-/-} mice, respectively; $n=10$ per group; $p=0.0127$). **(D)** Representative section of an early atherosclerotic lesion in external carotid artery of a TM^{Pro/Pro}:ApoE^{-/-} mouse, abundantly infiltrated by Ly-6G⁺ cells, suggesting that hypercoagulability triggers lesion formation in a neutrophil-dependent manner. **(E)** The panel represents an atherosclerotic plaque rupture/dissection with superimposed thrombus formation in a TM^{Pro/Pro}:ApoE^{-/-} mouse at 6 weeks after carotid collar placement. Using Perl's Prussian blue stain (blue color), we detected free ferric ions deposited within the sites of plaque dissection, indicating the areas of intraplaque hemorrhage. Whereas the carotid lesions in 5 out of 10 TM^{Pro/Pro}:ApoE^{-/-} mice were associated with either rupture, dissection or intraplaque hemorrhage, none of the control ApoE^{-/-} mice plaques had any signs of severe plaque vulnerability (Pearson's chi-squared test (χ^2), $n=10$ per group, $p=0.0325$). Statistical analysis including all experimental groups indicated that the number of circulating neutrophils in peripheral blood and plasma in vivo thrombin generation (TATc complexes) were both strongly correlated to the extent of atherosclerotic plaque burden **(F, G)**. Using intravital microscopy, we confirmed that the relative percentage of circulating neutrophils in TM^{Pro/Pro}:ApoE^{-/-} mice was found significantly higher than ApoE^{-/-} control mice ($n=6$ per group, $p = 0.0107$). Whereas there

were no differences found in the general leukocyte rolling and arrest between TM^{Pro/Pro}:ApoE^{-/-} and ApoE^{-/-} mice after 6 weeks on a high-fat diet (Rhodamine-labeled leukocytes) ($n=6$ per group, $p= 0.2886$) **(H, J)**, Ly-6G⁺ neutrophils in TM^{Pro/Pro}:ApoE^{-/-} mice were significantly more adherent to atherosclerotic lesions in the common carotid artery than in ApoE^{-/-} control mice ($n=6$ per group, $p=0.0139$). Bar represents 100 μ m.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Error bars represent mean $\pm$ SD

Abbreviations: M Φ – Macrophages; IPH – intraplaque hemorrhage; TAT – thrombin-antithrombin complex

Figure 4. Inhibition of thrombin activity by administration of direct thrombin inhibitor Dabigatran etexilate or recombinant murine APC substantially attenuates leukocyte recruitment and prevents against severe atherosclerosis progression and plaque rupture.

(A) Representative hematoxylin and eosin (H&E)-stained sections of atherosclerotic lesions formed in carotid arteries of TM^{Pro/Pro}:ApoE^{-/-} mice, which were assigned to different intervention arms (oral Dabigatran etexilate - 7.5 mg DE/gram chow; i.p. administered bolus doses of recombinant murine APC - 2.5 mg/kg/per every 5 days; or placebo) for a total of 6 weeks after cuff placement around the common carotid arteries (top row). Toluidine blue (TB) stainings were used to quantify the size of necrotic core areas (second and third

row). Whereas placebo treated $TM^{Pro/Pro}:ApoE^{-/-}$ mice all developed advanced lesions (identified by the presence of necrotic core and fibrous cap formation), Dabigatran etexilate (3 out of 10, Pearson's chi-squared test (χ^2), $n=10$ per group, $p=0.0031$ vs. placebo) and rAPC-treated mice (5 out of 10, Pearson's chi-squared test (χ^2), $n=10$ per group, $p=0.0325$ vs. placebo) had significantly reduced atheromata formed. A total of 5 out of 10 animals in the placebo group showed signs of severe plaque vulnerability, whereas none were observed in the intervention arms. Atherosclerotic plaques were further analyzed for the presence of macrophages (MAC-2, green color, fourth row) and neutrophils (Ly-6G, brown color, bottom row). Arrows show examples of positive cells. Macrophage and neutrophil infiltration were expressed as the absolute number of Mac-2⁺ and Ly-6G⁺ cells per plaque. **(B)** Administration of either Dabigatran etexilate or rAPC rescued the phenotype and pronouncedly reduced atherosclerotic plaque burden (Placebo: $154.3 \pm 35.5 \times 10^3 \mu m^2$; Dabigatran Etexilate: $3.3 \pm 4.4 \times 10^3 \mu m^2$, $p < 0.001$; rAPC: $7.9 \pm 5.5 \times 10^3 \mu m^2$, $p < 0.01$; $n=10$ per group). **(C, F, G)** These findings were further consolidated by a significant decrease in the degree of stenosis (with ~80%), intima/media ratio and outward remodeling within the treatment arms of the study ($n=10$ per group). **(D, E)** Except for a significant reduction of the necrotic core area in the Dabigatran etexilate-

treated mice as compared to placebo group ($n=10$ per group, $p < 0.05$), no other effects were observed with regard to necrotic core formation or fibrous cap thickness. Of note, only mice having advanced lesions were included in these analyses (Dabigatran etexilate: $n=3$; rAPC: $n=5$). **(H, I)** In addition, $TM^{Pro/Pro}: ApoE^{-/-}$ mice treated with direct thrombin inhibitor or natural anticoagulant rAPC developed an anti-inflammatory stable plaque phenotype, associated with substantially reduced levels of macrophage and neutrophil recruitment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent mean $\pm$ SD; Abbreviations: HFD – high-fat diet; AL – advanced atherosclerotic lesion; M Φ - macrophage; rAPC – recombinant murine activated protein C

Supplementary Figure 1. Hypercoagulability in $TM^{Pro/Pro}:ApoE^{-/-}$ mice does not alter lipid uptake in bone marrow-derived macrophages (BMM). **(A)** There were no significant differences found in the lipid uptake in BMM derived from $TM^{Pro/Pro}:ApoE^{-/-}$ and control $ApoE^{-/-}$ mice, as determined by flow cytometry analysis. **(B)** In addition, we also used high performance thin layer chromatography to test the free cholesterol, cholesterol esters and triglycerides accumulation in BMM in response to LDL and oxidized LDL loading and there were no significant differences detected between BMM obtained from $TM^{Pro/Pro}:ApoE^{-/-}$ and control $ApoE^{-/-}$ mice.

Figure 4.

Error bars represent mean $\pm$ SD; Abbreviations: HP-TLC - high performance thin layer chromatography; BMM - Bone marrow-derived macrophages; LDL - low-density lipoprotein; oxLDL - oxidized low-density lipoprotein

Supplementary Figure 2. The role of hypo- and hypercoagulability in plaque fibrosis. Picrosirius red-stained sections assessed by light (**A**, top row) and polarized light (**A**, second row), indicate a significant decrease in the levels of collagen in $TM^{Pro/Pro}:ApoE^{-/-}$ carotid atherosclerotic plaques ($6.7 \pm 4.3\%$ vs. $14.3 \pm 7.8\%$ of total plaque area in $ApoE^{-/-}$ mice, $n=10$ per group, $p=0.0193$) (**B**). Hypocoagulable $FII^{-/-}:ApoE^{-/-}$ mice lesions showed a pro-fibrotic appearance, testified by increased collagen deposition ($24.4 \pm 14.1\%$ vs. $12.0 \pm 6.1\%$ of total plaque area in $ApoE^{-/-}$ mice, $n=10$ per group, $p=0.0435$) and α -smooth muscle actin content ($25.5 \pm 13.6\%$ vs. $6.9 \pm 3.2\%$ of total plaque area in $ApoE^{-/-}$ mice, $n=10$ per group, $p=0.0003$) (**B**, **C**) $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Error bars represent mean $\pm$ SD; Abbreviations: SR - (Picro)sirius red; α -SMA - α -smooth muscle actin

Supplementary Figure 3. Hypercoagulable $TM^{Pro/Pro}:ApoE^{-/-}$ mice - a new mouse model of atherosclerotic plaque vulnerability. Here we present a new hypercoagulable atherosclerosis model, which accurately mimics the composition and events

leading to plaque destabilization, as normally observed in human atherosclerosis. In a series of sections, demonstrating carotid atherosclerotic plaques, obtained from $TM^{Pro/Pro}:ApoE^{-/-}$ mice at 6 weeks after collar placement on high-fat diet regimen, we show multiple signs of plaque vulnerability. (**A**) A non-occlusive but rapidly progressing atherosclerotic lesion, characterized by abundant infiltration of leukocytes. (**B**) $TM^{Pro/Pro}:ApoE^{-/-}$ mice plaques tend to rupture and dissect even during the non-occlusive phase, accompanied by "silent" intraluminal thrombosis. Despite the detrimental pathologic characteristics of those lesions, these data confirm the hypothesis that arterial thrombosis might exist long before a fatal event takes place. This is further consolidated by the presence of so called "buried fibrous caps" in $TM^{Pro/Pro}:ApoE^{-/-}$ mice plaques³⁶, considered a marker of healed plaque ruptures, and also observed in human atherosclerosis. Blue color denotes a massive intraplaque hemorrhage (iron ions deposition) (**C**). Hypercoagulability induces a severe inflammatory and pro-necrotic intraplaque environment, leading to the formation of enormous necrotic core, thin fibrous caps, further plaque destabilization (**D**) and atherothrombosis (occlusive intraluminal thrombosis/abundant fibrin(ogen) deposition) (**E**). Thrombi undergo fibrotic organization involving vascular smooth muscle cells and fibroblasts ingrowth, and are then

partially recanalized by newly formed vessels (arrows, blue color – iron deposition/presence of erythrocytes)(F).

Supplementary Figure 4. 20% FeCl₃-induced arterial injury in hyper- and hypocoagulable atherosclerosis-prone mice. Time to occlusion (TTO) and closing times (CT) were established. TTO is defined as the time after FeCl₃ application required for the blood flow to decline to 90%, whereas CT represents the time from the start of flow reduction to a complete occlusion of the carotid artery. (A, B) Both TTO and CT were significantly shortened in *TM^{Pro/Pro}:ApoE^{-/-}* as compared to *ApoE^{-/-}* control mice (TTO: 4.4 ± 0.9 vs. 14.1 ± 11.1 min., respectively; $n=10$ per group, $p=0.0010$) (CT: 1.2 ± 0.8 vs. 11.3 ± 13.0 min., respectively; $n=10$ per group, $p=0.0010$), suggesting for a pro-thrombotic arterial vessel wall phenotype. In contrast, hypocoagulability in *FII^{-/+}:ApoE^{-/-}* mice had no effect on thrombus formation during FeCl₃-induced arterial injury. Of note, all 10 out of 10 of the *TM^{Pro/Pro}:ApoE^{-/-}* mice formed an occlusive thrombus (animals depicted at 30 min. represent all mice, which did not induce occlusive thrombus formation, indicated by an arrow). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Dotted lines represent mean.

Supplementary Figure 5. The effects of hypercoagulability on hematopoiesis. Using a CFU-C (colony forming unit in culture) assay, we

established that there were no significant differences in the amount of total colonies produced by *TM^{Pro/Pro}:ApoE^{-/-}* as compared to *ApoE^{-/-}* control mice after 8 weeks on a regular chow diet (A). Furthermore, we could not find any changes in the composition, as determined by the CFU subset analysis, indicating that hypercoagulability does not affect hematopoiesis in the bone marrow compartment (B). FACS analysis of the bone marrow consolidated the results of the CFU-C assay (C, D, E). The amount of LSK (Lin⁻/Sca-1⁺/c-Kit⁺) cells showed a tendency towards an increase in the *TM^{Pro/Pro}:ApoE^{-/-}* compared to *ApoE^{-/-}* control mice ($4.2 \pm 0.8\%$ vs. $3.7 \pm 0.7\%$; $n=12$ per group, $p=0.0529$) (F). The amount of CMP (common myeloid progenitor) cells was significantly increased in the *TM^{Pro/Pro}:ApoE^{-/-}* mice compared to the controls ($15.1 \pm 3.3\%$ vs. $12.7 \pm 2.3\%$; $n=12$ per group, $p=0.0402$). (G). In addition, EMP and GMP populations in the bone marrow remained unaffected by the hypercoagulable state in *TM^{Pro/Pro}:ApoE^{-/-}* mice (H, I). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent mean $\pm$ SD; Abbreviations: CFU - colony forming unit; GM - granulocyte-macrophage progenitor; G - granulocyte progenitor; M - macrophage progenitor; LK - Lin⁻c-Kit⁺Sca-1⁺; LSK - Lin⁻c-Kit⁺Sca-1⁺; CMPs - common myeloid progenitors; GMP - granulocyte/macrophage progenitors; EMP - erythroid/megakaryocyte progenitors

Supplementary Figure 6. Hypercoagulability induces oxidative stress in granulocytes within the bone marrow compartment. Granulocytes and monocytes cell fractions in the bone marrow were significantly increased in $TM^{Pro/Pro}:ApoE^{-/-}$ as compared to $ApoE^{-/-}$ control mice after 8 weeks on a regular chow diet (Granulocytes: $26.3 \pm 3.6\%$ vs. $22.9 \pm 3.4\%$; $n=12$ per group, $p=0.0292$) (Monocytes: $12.3 \pm 0.6\%$ vs. $8.8 \pm 0.7\%$; $n=12$ per group, $p<0.0001$) (A, B). The significant increase in monocytes can be explained by the higher relative numbers of $Ly6C^{HIGH}$ monocyte cells in $TM^{Pro/Pro}:ApoE^{-/-}$ mice bone marrow ($Ly6C^{HIGH}$ cells: $9.4 \pm 1.3\%$ vs. $6.3 \pm 0.8\%$; $n=12$ per group, $p=0.0002$) (C). Using DHR123 FACS analysis, we analyzed the amount of oxidative burst activity in granulocytes and monocytes in the bone marrow after PMA stimulation. The monocytes did not show any differences in DHR signal and thus ROS activity, whereas in the granulocytes of the $TM^{Pro/Pro}:ApoE^{-/-}$ mice, a significant increase was observed in the DHR signal when compared to $ApoE^{-/-}$ mice, indicating enhanced oxidative stress upon PMA stimulation in the $TM^{Pro/Pro}:ApoE^{-/-}$ granulocytes present in the bone marrow (D, E). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Error bars represent mean $\pm$ SD; Abbreviations: DHR123 – Dihydrorhodamine 123; ROS – Reactive Oxygen Species; PMA – Phorbol 12-Myristate 13-Acetate

Supplementary Figure 7. The ef-

fects of direct and indirect inhibition of thrombin activity on plaque fibrosis. Picrosirius red-stained sections assessed by light (A, top row) and polarized light (A, second row), indicate no significant changes in collagen content in $TM^{Pro/Pro}:ApoE^{-/-}$ mice after 6-week treatment with either Dabigatran etexilate or mouse rAPC, as compared to placebo (B). Administration of oral Dabigatran etexilate led to a significant increase in α -smooth muscle actin intraplaque content in $TM^{Pro/Pro}:ApoE^{-/-}$ mice vs. placebo treatment ($20.6 \pm 7.4\%$ vs. $6.8 \pm 3.8\%$ of total plaque area, $n=10$ per group, $p<0.05$) (A, C). rAPC therapy did not have an effect ($6.2 \pm 7.1\%$ of total plaque area, $n=10$ per group, $p>0.05$) (A, C). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Error bars represent mean $\pm$ SD; Abbreviations: SR – (Picro)sirius red; α -SMA – α -smooth muscle actin; HFD – high-fat diet; VSMC – vascular smooth muscle cells; rAPC – recombinant mouse activated protein C

Supplementary Table 1. Coagulation profile (A), body weight, lipid profile (B) and complete blood counts (C), assessed after 35 weeks on regular chow diet in $FII^{+/+}:ApoE^{-/-}$, $TM^{Pro/Pro}:ApoE^{-/-}$ and control $ApoE^{-/-}$ mice ($n=10$ per group).

$*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Data are presented as mean $\pm$ SD.

Abbreviations: ETP – Endogenous Thrombin Potential; TAT – Thrombin-Antithrombin Complex; HDL – High-Density Lipoprotein; LDL – Low-

Density Lipoprotein; CBC – Complete Blood Count; RBC – Red Blood Cells; WBC – White Blood Cells; Hgb – Hemoglobin; Hct – Hematocrit

Complete Blood Count; RBC – Red Blood Cells; WBC – White Blood Cells; Hgb – Hemoglobin; Hct – Hematocrit; APC – Activated Protein C

Supplementary Table 2. Cytokine and chemokine profile assessed after 35 weeks on regular chow diet in $FII^{+/-}ApoE^{-/-}$, $TM^{Pro/Pro}ApoE^{-/-}$ and control $ApoE^{-/-}$ mice (n=10 per group).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean $\pm$ SD. Abbreviations: IL – interleukin; TNF- α – tumor necrosis factor-alpha; IFN- γ – Interferon-gamma; G-CSF – Granulocyte colony-stimulating factor; MCP-1 – monocyte chemotactic protein-1; MIP-1 α – Macrophage inflammatory protein-1 α ; MIP-1 β – Macrophage inflammatory protein-1 β ; RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted; KC – keratinocyte chemoattractant

Supplementary Table 3. Coagulation profile (A), lipid profile (B) and complete blood counts (C), assessed at 6 weeks after carotid collar placement in $TM^{Pro/Pro}ApoE^{-/-}$ on high-fat diet and treated with placebo, oral Dabigatran etexilate or mouse recombinant APC (n=10 per group).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Intervention groups compared to placebo group). Data are presented as mean $\pm$ SD. Abbreviations: TAT – Thrombin-Antithrombin Complex; HDL – High-Density Lipoprotein; LDL – Low-Density Lipoprotein; CBC –

Supplementary Table 4. Cytokine and chemokine profile assessed at 6 weeks after carotid collar placement in $TM^{Pro/Pro}ApoE^{-/-}$ on high-fat diet and treated with placebo, oral Dabigatran etexilate or mouse recombinant APC (n=10 per group).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Intervention groups compared to placebo group). Data are presented as mean $\pm$ SD. Abbreviations: IL – interleukin; TNF- α – tumor necrosis factor-alpha; IFN- γ – Interferon-gamma; G-CSF – Granulocyte colony-stimulating factor; MCP-1 – monocyte chemotactic protein-1; MIP-1 α – Macrophage inflammatory protein-1 α ; MIP-1 β – Macrophage inflammatory protein-1 β ; RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted; KC – keratinocyte chemoattractant; APC – Activated Protein C

Supplementary Figure 1.

Supplementary Figure 2.

Supplementary Figure 3.

Supplementary Figure 4.

Supplementary Figure 5.

Supplementary Figure 6.

Supplementary Figure 7.

Supplementary Table 1.

A	COAGULATION PROFILE			
	FII -/WT : ApoE -/-	FII WT/WT : ApoE +/-	TM PRO/PRO : ApoE +/-	TM WT/WT : ApoE -/-
Fibrinogen (g/l)	0.64 ± 0.16	0.66 ± 0.12	1.95 ± 1.13***	0.66 ± 0.22
Prothrombin (%)	61.73 ± 10.27***	123.56 ± 21.02	176.17 ± 31.98**	124.25 ± 31.36
FVII (%)	597.93 ± 111.91**	749.81 ± 65.02	812.23 ± 19.29**	746.44 ± 57.83
FX (%)	373.68 ± 68.60	429.76 ± 93.66	556.78 ± 73.97**	451.45 ± 80.37
FVIII (%)	196.13 ± 52.23	197.25 ± 40.01	291.25 ± 44.81***	214.83 ± 29.09
ETP (nM.min)	352 ± 108***	745 ± 119	1013 ± 257**	734 ± 139
TAT Complexes (ng/ml)	3.01 ± 6.29*	26.62 ± 34.82	420.02 ± 423.33***	20.40 ± 27.11

B	BODY WEIGHTS / LIPID PROFILE			
	FII -/WT : ApoE -/-	FII WT/WT : ApoE +/-	TM PRO/PRO : ApoE +/-	TM WT/WT : ApoE -/-
Body Weight (g.)	29.50 ± 2.92	29.10 ± 1.37	29.30 ± 1.34	29.40 ± 1.43
Total Cholesterol (mmol/l)	4.59 ± 0.88	5.48 ± 1.79	5.52 ± 1.08	5.47 ± 1.78
Triglycerides (mmol/l)	1.53 ± 0.50	1.40 ± 0.48	1.73 ± 0.63	1.53 ± 0.94
HDL (mmol/l)	1.18 ± 0.20*	1.54 ± 0.48	1.56 ± 0.32	1.57 ± 0.53
LDL (mmol/l)	2.63 ± 0.58	3.30 ± 1.24	3.17 ± 0.67	3.20 ± 1.24
HDL/LDL Ratio	0.46 ± 0.07	0.48 ± 0.11	0.50 ± 0.10	0.51 ± 0.13

C	CBC			
	FII -/WT : ApoE -/-	FII WT/WT : ApoE +/-	TM PRO/PRO : ApoE +/-	TM WT/WT : ApoE -/-
RBC (x 10 ¹² /l)	7.0 ± 0.5	7.1 ± 0.4	6.5 ± 1.4*	7.4 ± 0.3
WBC (x 10 ⁹ /l)	3.2 ± 0.8**	4.6 ± 1.1	6.7 ± 0.6***	4.3 ± 0.8
- Lymphocytes (x 10 ⁵ /ml)	28 ± 7*	38 ± 10	41 ± 7.5	35 ± 7.6
- Neutrophils (x 10 ⁵ /ml)	3 ± 1***	6 ± 2	24 ± 5.1***	7 ± 2.0
- Monocytes (x 10 ⁵ /ml)	1 ± 0.5	2 ± 1.3	2 ± 1.2	1 ± 0.7
- Eosinophils (x 10 ⁵ /ml)	0.4 ± 0.5	0.3 ± 0.5	0.4 ± 0.7	0.3 ± 0.4
Platelets (x 10 ⁹ /l)	1058 ± 107	1078 ± 79	844 ± 161***	1228 ± 235
Hgb (mmol/l)	6.6 ± 0.5	6.9 ± 0.5	6.1 ± 1.4	7.1 ± 0.4
Hct (l/l)	0.33 ± 0.02	0.34 ± 0.02	0.31 ± 0.06	0.34 ± 0.01

Supplementary Table 2.

	CYTOKINE/CHEMOKINE PROFILE			
	Flt ^{-/-} WT : ApoE ^{-/-}	Flt ^{WT/WT} : ApoE ^{-/-}	TM PRO/PRO : ApoE ^{-/-}	TM WT/WT : ApoE ^{-/-}
IL-1 α (pg/mL)	6.9 $\pm$ 3.5	9.9 $\pm$ 4.5	11.2 $\pm$ 4.2	8.1 $\pm$ 5.1
IL-1 β (pg/mL)	98.7 $\pm$ 41.0 ^s	138.3 $\pm$ 51.3	167.4 $\pm$ 60.4 ^s	120.9 $\pm$ 55.3
IL-2 (pg/mL)	6.0 $\pm$ 4.0*	3.1 $\pm$ 1.0	4.6 $\pm$ 1.2	3.7 $\pm$ 1.0
IL-3 (pg/mL)	7.8 $\pm$ 4.7	8.9 $\pm$ 4.0	10.9 $\pm$ 4.2	7.1 $\pm$ 5.4
IL-4 (pg/mL)	6.4 $\pm$ 3.7	7.9 $\pm$ 4.0	8.3 $\pm$ 3.0	6.7 $\pm$ 4.8
IL-5 (pg/mL)	11.8 $\pm$ 6.3	13.5 $\pm$ 2.9	13.9 $\pm$ 3.3	16.1 $\pm$ 7.0
IL-6 (pg/mL)	3.9 $\pm$ 1.7*	8.8 $\pm$ 5.3	10.5 $\pm$ 5.0*	6.3 $\pm$ 2.8
IL-9 (pg/mL)	13.9 $\pm$ 9.7	8.8 $\pm$ 9.0	20.2 $\pm$ 7.8	26.2 $\pm$ 13.9
IL-10 (pg/mL)	106.6 $\pm$ 42.5	132.2 $\pm$ 69.0	154.0 $\pm$ 33.1 ^s	107.7 $\pm$ 62.4
IL-12 (p40) (pg/mL)	66.3 $\pm$ 14.9	74.7 $\pm$ 16.2	78.3 $\pm$ 16.0	86.7 $\pm$ 14.9
IL-12 (p70) (pg/mL)	113.2 $\pm$ 46.3	136.0 $\pm$ 18.2	137.4 $\pm$ 11.8	109.8 $\pm$ 54.6
IL-13 (pg/mL)	215.5 $\pm$ 85.7	225.5 $\pm$ 43.7	424.0 $\pm$ 372.0	274.2 $\pm$ 177.0
IL-17 (pg/mL)	8.4 $\pm$ 3.5	11.5 $\pm$ 4.7	12.4 $\pm$ 4.2	12.7 $\pm$ 4.7
TNF- α (pg/mL)	118.4 $\pm$ 44.5 ^s	154.6 $\pm$ 42.2	167.1 $\pm$ 51.0	143.3 $\pm$ 60.2
IFN- γ (pg/mL)	64.1 $\pm$ 42.7	74.6 $\pm$ 20.2	81.0 $\pm$ 35.1	82.8 $\pm$ 43.1
G-CSF (pg/mL)	35.7 $\pm$ 16.5	35.3 $\pm$ 14.8	61.0 $\pm$ 44.0*	31.9 $\pm$ 12.3
GM-CSF (pg/mL)	45.4 $\pm$ 24.5	42.4 $\pm$ 17.9	54.9 $\pm$ 16.9	44.0 $\pm$ 21.2
CCL2 (MCP-1) (pg/mL)	103.5 $\pm$ 42.5	135.5 $\pm$ 45.1	147.4 $\pm$ 53.5*	105.6 $\pm$ 45.3
CCL3 (MIP-1 α) (pg/mL)	9.7 $\pm$ 4.3	13.2 $\pm$ 5.3	13.9 $\pm$ 2.2	11.1 $\pm$ 5.6
CCL4 (MIP-1 β) (pg/mL)	57.6 $\pm$ 24.5	73.2 $\pm$ 23.3	82.5 $\pm$ 29.0	69.0 $\pm$ 30.4
CCL5 (RANTES) (pg/mL)	8.8 $\pm$ 2.2	10.0 $\pm$ 0.9	9.3 $\pm$ 1.2	8.9 $\pm$ 3.1
CCL11 (Eotaxin) (pg/mL)	77.3 $\pm$ 23.9 ^s	98.3 $\pm$ 27.1	124.1 $\pm$ 42.9	111.2 $\pm$ 53.8
CXCL1 (KC) (pg/mL)	14.8 $\pm$ 4.9	14.5 $\pm$ 3.1	17.6 $\pm$ 3.3*	13.3 $\pm$ 5.1

Supplementary Table 3.

A	COAGULATION PROFILE			
	TM PRO/PRO : ApoE +/-			
	PLACEBO (HFD)	DABIGATRAN + HFD	APC + HFD	
	545.7 ± 237.4	10.3 ± 4.5***	21.1 ± 22.0**	
	TAT Complexes (ng/ml)			
B	LIPID PROFILE			
	TM PRO/PRO : ApoE +/-			
	PLACEBO (HFD)	DABIGATRAN + HFD	APC + HFD	
	10.1 ± 0.9	10.4 ± 1.8	10.4 ± 1.0	
	1.5 ± 0.9	1.7 ± 1.0	1.7 ± 0.7	
C	CBC			
	TM PRO/PRO : ApoE +/-			
	PLACEBO (HFD)	DABIGATRAN + HFD	APC + HFD	
	6.5 ± 1.5	6.8 ± 0.5	6.9 ± 0.4	
	8.2 ± 1.1	2.6 ± 0.7***	3.6 ± 1.4***	
	RBC (x 10 ¹² /l)			
	WBC (x 10 ⁹ /l)			
	- Lymphocytes (x 10 ⁵ /ml)	23 ± 7***	30 ± 12***	
	- Neutrophils (x 10 ⁵ /ml)	2 ± 1***	5 ± 2***	
	- Monocytes (x 10 ⁵ /ml)	1.2 ± 0.6	1.0 ± 0.9*	
	- Eosinophils (x 10 ⁵ /ml)	0.2 ± 0.2	0.4 ± 0.5	
	Platelets (x 10 ⁹ /l)	1006 ± 167	935 ± 185	
	Hgb (mmol/l)	6.5 ± 0.5	6.5 ± 0.5	
	Hct (l/l)	0.32 ± 0.02	0.33 ± 0.01	

Supplementary Table 4.

CYTOKINE/CHEMOKINE PROFILE			
TM PRO/PRO : ApoE -/-			
	PLACEBO (HFD)	DABIGATRAN + HFD	APC + HFD
IL-1α (pg/mL)	10.1 ± 6.4	2.6 ± 1.5***	1.8 ± 1.4***
IL-1β (pg/mL)	200.9 ± 44.4	90.9 ± 43.4***	127.1 ± 78.9**
IL-2 (pg/mL)	7.4 ± 3.7	3.4 ± 1.1**	3.5 ± 1.8*
IL-3 (pg/mL)	9.2 ± 5.6	2.2 ± 1.8***	2.2 ± 1.3***
IL-4 (pg/mL)	6.5 ± 4.6	1.5 ± 1.4**	1.3 ± 0.7*
IL-5 (pg/mL)	22.1 ± 3.6	12.3 ± 5.7***	13.6 ± 6.2**
IL-6 (pg/mL)	11.7 ± 6.1	4.2 ± 1.3**	4.0 ± 2.0***
IL-9 (pg/mL)	39.3 ± 29.4	18.9 ± 7.7	13.6 ± 12.5*
IL-10 (pg/mL)	147.1 ± 63.8	55.7 ± 25.7***	46.4 ± 20.7***
IL-12 (p40) (pg/mL)	94.4 ± 40.3	52.1 ± 20.1***	34.6 ± 6.3***
IL-12 (p70) (pg/mL)	127.9 ± 60.1	50.9 ± 31.0***	47.1 ± 28.9***
IL-13 (pg/mL)	488.7 ± 94.3	256.5 ± 65.5***	298.5 ± 158.2***
IL-17 (pg/mL)	14.5 ± 6.2	7.5 ± 2.0***	6.3 ± 2.9***
TNF-α (pg/mL)	212.1 ± 54.2	116.8 ± 27.2**	97.4 ± 36.1***
IFN-γ (pg/mL)	134.7 ± 22.9	75.1 ± 23.9***	77.2 ± 39.9***
G-CSF (pg/mL)	50.8 ± 21.5	20.1 ± 5.1***	19.1 ± 8.1***
GM-CSF (pg/mL)	44.8 ± 8.5	23.8 ± 14.3***	22.8 ± 10.9***
CCL2 (MCP-1) (pg/mL)	184.0 ± 43.1	114.9 ± 37.8**	111.9 ± 50.4**
CCL3 (MIP-1α) (pg/mL)	9.4 ± 4.0	4.0 ± 2.0*	2.6 ± 1.9***
CCL4 (MIP-1β) (pg/mL)	85.2 ± 15.4	44.5 ± 15.1**	39.2 ± 17.0***
CCL5 (RANTES) (pg/mL)	9.9 ± 5.6	4.5 ± 2.5**	3.5 ± 1.3***
CCL11 (Eotaxin) (pg/mL)	255.8 ± 212.5	72.9 ± 52.1*	53.0 ± 40.8**
CXCL1 (KC) (pg/mL)	18.1 ± 7.3	10.6 ± 3.5**	8.1 ± 2.5***

Methods

Animals

TM^{Pro/Pro} mice, carrying a thrombomodulin (TM) gene mutation resulting in diminished TM-dependent generation of activated protein C (APC)¹², and prothrombin (FII) heterozygous mice with genetically imposed hypoprothrombinemia¹¹ were crossed into a pure C57BL/6 background for at least 8 generations and subsequently crossbred to ApoE^{-/-} mice (Charles River, Maastricht, The Netherlands), carrying the same background. Only female mice were used throughout the entire study. All animal experimental protocols were carried out in compliance with the Dutch government guidelines and were approved by the Animal Care and Use Committee of Maastricht University (Maastricht, The Netherlands).

Mouse Models of Atherosclerosis

In a spontaneous atherosclerosis model, female TM^{Pro/Pro}:ApoE^{-/-}, FII^{/WT}:ApoE^{-/-} (age, 8-9 weeks; n=10 per group) and control ApoE^{-/-} mice (age, 8-9 weeks; n=20) received regular chow diet (Hope Farms, Woerden, The Netherlands) for 35 weeks and were then sacrificed for a detailed analysis. In a separate experimental setup, consisting of identical groups, carotid atherosclerotic plaques were induced via placement of perivascular collars around the common ca-

rotid arteries as described before¹⁴. All animals were fed on a high-fat diet (15% cocoa butter, 1% corn oil, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 20% casein, free of cholate, total fat content 16%; Hope Farms, Woerden, The Netherlands) for two weeks before collar placement and for additional six weeks after surgery. Diets and water were provided *ad libitum* throughout all experiments.

Pharmacological Interventions

Female TM^{Pro/Pro}:ApoE^{-/-} mice (n=10 per treatment group; age, 8-9 weeks) fed on a standard high-fat diet (D12451; Research Diets, NJ, USA) for 2 weeks were subsequently subjected to a surgical implantation of non-constrictive perivascular carotid collars and then assigned to different interventions or placebo for a total of 6 weeks. The study design involved an intervention arm with mice receiving standard D12451 high-fat chow supplemented with oral Dabigatran etexilate (DE) (7.5 mg DE/gram chow). In a second intervention arm, TM^{Pro/Pro}:ApoE^{-/-} mice were fed on a standard D12451 high-fat diet and received intraperitoneal (i.p.) administration of recombinant murine APC (mAPC) in bolus doses of 2.5 mg/kg/per every 5 days. Placebo-treated mice received injection of saline and were fed on standard D12451 high-fat chow. Recombinant mAPC was produced in the laboratory of Dr.

Charles T. Esmon (Oklahoma Medical Research Foundation and Howard Hughes Medical Institute, Oklahoma City, Oklahoma, USA). Both DE-supplemented high-fat and placebo chow diets were prepared at Department of CardioMetabolic Disease Research, Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach an der Riss, Germany).

Blood Sampling, Blood Cell Counts, Blood Coagulation and Lipid Profile Analysis

Peripheral blood samples, obtained via vena cava, were collected in 3.2% sodium citrate (weight/volume). Samples were analyzed on automated hematology analyzer (Beckman Coulter, FL, USA) and the following parameters were established: red blood cell count (RBC), white blood cell count (WBC), platelet count, hemoglobin (Hgb) and hematocrit (Hct). Blood samples were centrifuged for 15 minutes at 4500 rpm at room temperature to pellet blood cells. Plasma was transferred to a fresh tube, centrifuged for additional 5 minutes at 14000 rpm in order to discard remaining cells and debris, and then stored at -80 °C. Differential WBC counts were performed manually on May-Grünwald Giemsa stained blood smears (300 cells/smear) and presented as absolute counts. Plasma levels of coagulation factors (fibrinogen, prothrombin, FVII, FX, FVIII) were determined via automated Dade Behring Blood

Coagulation System (BCS, Siemens Healthcare Diagnostics, Deerfield, IL) using human reagents. Murine thrombin-antithrombin complex (TATc) levels were determined with commercially available micro-enzyme immunoassay kit (Enzygnost® TAT Micro, Siemens Healthcare Diagnostics Products, Marburg, Germany). Using a fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC), plasma thrombin generation was recorded via the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, The Netherlands). Thrombin generation curves and endogenous thrombin potential (ETP) were calculated by the means of Thrombinoscope™ software (Thrombinoscope BV, Maastricht, The Netherlands). Plasma samples were analyzed in duplicates in a 96-well plate Fluoroskan Ascent Reader (Thermo Labsystems, Hensinki, Finland; 390/460 filter set) as previously described³⁷. Plasma total cholesterol, triglycerides and HDL cholesterol concentrations were measured in duplicates using automated enzymatic colorimetric assays (CHOD-PAP/GPO-PAP, Roche Diagnostics, Germany). LDL cholesterol concentrations were derived using the Friedewald equation.

Cytokines and Chemokines Profile Analysis

Baseline (TM^{Pro/Pro}:ApoE^{-/-} and FII^{/WT}:ApoE^{-/-} mice) and post-

intervention plasma samples (TM^{Pro/Pro}:ApoE^{-/-} - Placebo/DE/APC) were obtained from all experimental groups (n=10 per group). Samples from non-atherosclerotic C57Bl/6 mice (n=10) were collected as an additional reference group to the control ApoE^{-/-} mice (n=20 per group). Cytokines were measured using Bio-Plex Pro™ mouse cytokine 23-plex immunoassay (#M60-009RDPD, Biorad Laboratories, Hercules, CA, USA) in accordance with manufacturer's manual.

Tissue Harvesting, Preparation and Morphometry

In the model studying spontaneous atherosclerosis development, all mice were exsanguinated and then perfused with freshly prepared sodium nitroprusside for 5 minutes (Sigma-Aldrich St. Louis, MO, USA; dissolved in phosphate-buffered saline (PBS), pH 7.4). After an additional perfusion with 1% paraformaldehyde, the aortic tree (including all major branches) was carefully excised and fixed in 1% paraformaldehyde solution overnight. Samples were embedded longitudinally in paraffin blocks and sectioned at 4μm thickness. Histological evaluation of general plaque parameters was performed on 6 consecutive hematoxylin and eosin (HE)-stained sections, with 100μm intervals, thus representing the average distribution of atherosclerotic plaque burden throughout the entire depth of the

specimen. An identical approach, using 6 successive cross sections (obtained proximally from the collar, 100μm intervals), was undertaken to describe the atherosclerotic plaque parameters in mice subjected to cuff-induced atherosclerosis. Sections were analyzed on research microscopes (Leica DM3000, Leica Microsystems, Wetzlar, Germany/Zeiss Axioskop, Jena, Germany) and captured via a digital camera (Leica DFC 425c/Canon PowerShot G5). Plaque parameters were quantified by two independent investigators using Image-Pro Plus 7.0.1 (Media Cybernetics Inc., Bethesda, MD, USA) and Adobe Photoshop CS5 Extended (Adobe Systems, San Jose, CA, USA) software.

Histology and Immunohistochemistry

Longitudinal aortic tree and cross carotid artery sections were immunolabeled with rabbit polyclonal anti-Mac-2 antibody (Galectin-3; ab53082; Abcam, Cambridge, UK) as a specific marker for macrophages, rat monoclonal anti-Ly6G antibody (551459; BD Biosciences, San Jose, CA, USA) to detect granulocytes (mainly neutrophils), rabbit polyclonal to α-smooth muscle actin (ab5694; Abcam, Cambridge, UK) to detect vascular smooth muscle cells, rabbit polyclonal to fibrin(ogen) (ab3426; Abcam, Cambridge, UK). Biotinylated or FITC-labeled polyclonal anti-rat and anti-rabbit im-

munoglobulins were used as secondary antibodies (E0468; E0431; 1:200; DakoCytomation Denmark A/S, Glostrup, Denmark; ab6717; Abcam, Cambridge, UK; 553896; BD Biosciences, San Jose, CA, USA). For immunohistochemistry, tertiary antibody streptavidin-Alkaline Phosphatase (AP) (1:200; D0396; DakoCytomation Denmark A/S, Glostrup, Denmark) and/or Vector Red Alkaline Phosphatase Substrate, Vectastain Elite ABC and DAB Peroxidase Substrate Kits were additionally used as appropriate (Vector Laboratories; Peterborough, UK). Hematoxylin was used as a counterstaining, whereas in the case of immunofluorescence microscopy, slides were mounted using Vectashield with DAPI. In addition, sections were stained with toluidine blue to perform morphometric analysis of the necrotic core area, whereas Perl's Prussian blue staining was used to detect iron deposition. Sirius red staining determined the relative collagen content in the atherosclerotic lesions.

FeCl₃-induced Carotid Artery Injury Model

TM^{Pro/Pro}:ApoE^{-/-}, FII^{-/WT}:ApoE^{-/-} and control ApoE^{-/-} mice (age, 8-9 weeks; weight, ~25 gr.; on regular chow diet; n=10 per group) were anesthetized by inhalation of 1.5-2.0% isoflurane and placed in a supine position under a dissecting microscope. Exposure of the left common

carotid artery was obtained via a midline cervical incision, and then followed by subsequent blunt dissection. Body temperature was maintained at 37°C using an infrared heating lamp and controlled through a rectal probe. The hemodynamic changes were recorded by using a miniature Doppler flow probe connected to a flow module, equipped with a digital data-acquisition system (IdeeQ, Maastricht, The Netherlands). After extensive rinsing of the surgical site with sterile saline, thrombus formation was induced by the application of a 1x2 mm strip of filter paper, saturated with 20% FeCl₃ solution, to the adventitial surface of the common carotid arteries. Arterial injury with FeCl₃ lasted for a total of 3 minutes before filter paper was removed. The carotid blood flow was recorded throughout the entire experiment: at baseline and continuously for 30 min. following FeCl₃-induced arterial injury.

Lipid Uptake Analysis in Bone Marrow-derived Macrophages (BMM)

Murine BMM were isolated from femurs of both TM^{Pro/Pro}:ApoE^{-/-} and control ApoE^{-/-} mice (n=3 per group), cultured and differentiated into macrophages as described before. BMM from both genotypes were incubated for 24 hours in the presence or absence of 25µg/ml of various LDL types. LDL species were labeled with 1,1'-dioctadecyl-

3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI) in DMSO and incubated overnight. Lipid uptake was established via flow cytometry using FACSCalibur (BD Biosciences Franklin Lakes, NJ, USA)³⁸. In a second experimental setup, in order to determine the free cholesterol, cholesterol esters and triglycerides accumulation in BMM in response to LDL and oxidized LDL (oxLDL) loading, high performance thin layer chromatography (HPTLC) was used as demonstrated before³⁹.

Leukocyte-Endothelium Interactions in Atherosclerotic Carotid Arteries

Female TM^{Pro/Pro}:ApoE^{-/-} and control ApoE^{-/-} mice (age, 8-9 weeks; n=6 per group) were fed on high-fat diet for 6 weeks (Hope Farms, Woerden, The Netherlands) *ad libitum*. Mice were anesthetized by an i.p. injection of ketamine/xylazine and the left carotid artery was exposed via a supraclavicular incision and dissection. Non-specific and neutrophil-specific leukocyte labelling was performed by intravenous injection of Rhodamine 6G (R6G; Molecular Probes; Invitrogen, Carlsbad, CA, USA) and monoclonal rat anti-Ly-6G-FITC (551460; BD Biosciences, San Jose, CA, USA). Using intravital epifluorescence microscopy (Zeiss Axiotech), we studied leukocyte/neutrophil rolling and adhesion in carotid arteries in multiple high-power fields (at least 6, 200x magnification). Leuko-

cytes/neutrophils that remained motionless for more than 30 seconds on the carotid artery were defined as adherent¹⁵.

Characterization of Bone Marrow Cell Populations by CFU-C Assays and Flow Cytometry

Nine-week-old TM^{Pro/Pro}:ApoE^{-/-} and control ApoE^{-/-} mice (n=12 per group) were used for each of the different experiments. All assays were performed as extensively described before⁴⁰. In short, bone marrow cells were isolated from femurs and tibias. Cells were counted twice using a count chamber and the concentration was calculated for each sample. Per well 10,000 bone marrow cells were added to 1 mL methylcellulose medium with recombinant cytokines (MethoCult GF M3434, StemCell Technologies, Grenoble, France). After incubation for 7 days (37°C; 5% CO₂) the total number of colonies was quantified by an independent operator, and GM-CFU, G-CFU, and M-CFU colonies were specified based on morphology. In parallel, bone marrow cells were analyzed for progenitor cell composition by flow cytometry. Lineage positive cells were depleted by MACS LS columns and MACS mouse lineage depletion kit (#130-042-401 and #130-090-858, Miltenyi Biotec, Bergisch Gladbach, Germany). The lineage negative population was stained with antibodies against c-Kit (CD117), Sca-1, CD16/32, and CD34

(17-1171, 45-5981, 25-0161, 11-0341; eBioScience, San Diego, CA, USA) to determine relevant subsets and measured on a FACS Cantoll flow cytometer (BD BioSciences). Oxidative burst capacity of mature leukocytes in the bone marrow was determined by flow cytometry. Full bone marrow was labeled with antibodies against CD11b (25-0118; eBioScience) and Ly6G (560600; BD BioSciences). Next, cells were incubated with dihydrorhodamine (DHR123; 2 μ M; D632; Life Technologies Invitrogen, Bleiswijk, The Netherlands) and subsequently stimulated with phorbol 12-myristate 13-acetate (PMA; 160 nM; P1585; Sigma-Aldrich, St. Louis, MO, USA). Samples were washed and measured within 30 minutes on a FACS Cantoll flow cytometer (BD BioSciences).

Statistical Analysis

All statistics were performed using Prism, version 5.00 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS Statistics 19.0 (SPSS Japan Inc., an IBM company, Tokyo, Japan). Data sets were assessed for normality using Kolmogorov-Smirnov test or Bartlett's test for homogeneity of variance. Data were compared using unpaired 2-tailed *t* test or one-way ANOVA, followed by Newman-Keuls posthoc test for multiple comparisons. In case of non-normal distribution, non-parametric tests such as Mann-Whitney or Kruskal-Wallis

test with Dunn's post hoc analysis were used as appropriate. Data are expressed as mean $\pm$ SD, unless otherwise stated. A 2-tailed *p*<0.05 was considered statistically significant.

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7

SUMMARY/MAIN FINDINGS IN PERSPECTIVE

Vascular injury is considered integral to the onset of atherosclerosis and acute atherothrombosis¹⁻³. There is an enormous body of experimental and clinical evidence demonstrating that the initial response to injury involves the activation of both inflammation and blood coagulation⁴⁻⁶, which both serve as primary host-defense mechanisms to promote wound healing and maintain vascular integrity. Atherosclerosis is a chronic inflammatory disease⁷. Blood coagulation and inflammation reciprocally interact in various clinical conditions^{8,9}. Acute inflammation can induce hypercoagulable states and plays a crucial role in the initiation of arterial thrombosis^{3,10,11}. Histopathological studies document an imbalance in the expression between pro-coagulant and anti-coagulant proteins within human atherosclerotic lesions – a significant increase in tissue factor (TF) and fibrinogen synthesis, accompanied by diminished tissue factor pathway inhibitor (TFPI) and thrombomodulin (TM) expression levels¹²⁻¹⁷. Subclinical thrombosis is known to occur long before an atherosclerotic plaque ruptures¹⁸⁻²⁰, and is even found associated with mild luminal stenosis²¹⁻²⁵. In fact, current concepts of plaque vulnerability assert an important role for thrombosis in triggering atherosclerotic lesion destabilization²⁶⁻²⁸. Nevertheless, the role of clotting in atherosclerosis remains unclear to date. In this thesis, using various *ex vivo* and *in vivo*

methodological approaches, we aimed to elucidate the contribution of blood coagulation to atherosclerosis progression and plaque phenotype determination.

Major Findings

Active Coagulation Network in the Atherosclerotic Vessel Wall – Relevance to Atherogenesis

In **Chapter 3**, we investigated the presence of all coagulation proteins within the arterial vessel wall in relation to atherosclerosis progression. Besides focusing on the expression and overall distribution alone, we also provided novel evidence indicating that both early and advanced atherosclerotic lesions exhibit functional activity of key coagulation proteins such as TF, prothrombin, factor (F)X, and FXII. Furthermore, many of those enzymes were locally synthesized within the atherosclerosis plaques and co-localized with macrophages and vascular smooth muscle cells, suggesting an active, cell-based coagulation network during disease progression. Intriguingly, the primary finding of this study is that early atherosclerotic lesions exhibit a pronounced prothrombotic profile in comparison to stable advanced atheromas. Whereas it seems counterintuitive at first glance, these data suggest that local coagulation proteins may play an important role not only in the onset of atherothrombosis, but also in ath-

erogenesis and plaque phenotype determination. This phenomenon might be in part explained as a “response to injury” mechanism, which prevents atherosclerosis lesions from rupture during the early phases of atherosclerotic development²⁹. However, the persisting inflammation, which is an established feature of atherosclerosis progression⁷, may trigger an overshooting host defense response, thus leading to a continuous coagulation activation, whereas the latter can reciprocally contribute to more local inflammation. The long-term net effects of such perpetual activation of the coagulation-inflammation axis may be detrimental for atherosclerotic plaque stability²⁶⁻²⁸.

Accelerated *In Vivo* Thrombin Formation Independently Predicts the Presence and Severity of Coronary Atherosclerosis

In atherothrombosis, thrombin plays one of the most critical roles in initiating thrombus formation by promoting both the formation of fibrin, which is the ultimate product of the coagulation cascade, but also by acting as a powerful platelet-activating enzyme. Beyond its hemostatic roles, thrombin is also recognized as a potent cell-signaling effector molecule and pro-inflammatory mediator (**Chapter 2**). By selectively cleaving its G-protein-coupled protease-activated receptors (PARs), thrombin can initiate a plethora of pro-

atherogenic and plaque-destabilizing effects such as inflammation, vascular smooth muscle cell migration and proliferation, leukocyte chemotaxis, proteolysis, apoptosis, angiogenesis, etc.^{30,31} PARs are abundantly expressed throughout various compartments in the arterial vessel wall, and overexpressed in atherosclerotic lesions^{32,33}. To help better understand the relationship between blood coagulation potential and atherosclerosis progression *in vivo*, in **Chapter 4** we used a non-invasive vascular imaging modality (cardiac computed tomographic angiography (CCTA)) and studied the association between thrombin formation and the presence and severity of coronary atherosclerosis in patients with suspected coronary artery disease (CAD). The primary finding of this study is that increasing thrombin-antithrombin complex (TATc) levels, known as a sensitive marker for thrombin generation *in vivo*, are independently associated with the presence and severity of CAD, but also coronary calcification. Of note, TATc measurement was efficient in detecting even mild-grade coronary stenosis, whereas the incorporation of TATc as an additional test improved the predictive capacity of the Framingham cardiovascular risk stratification model. Nevertheless, because of the large inter-individual differences in humans, and the multifactorial nature of atherosclerosis, we do not anticipate that a single biomarker can substitute powerful

diagnostic tools such as CCTA. Despite that this study did not unravel the causal complex relationship between thrombin formation, atherosclerosis progression and vascular calcification, it overall suggests that thrombin may be an important player in the pathophysiology of cardiovascular disease in humans.

Thrombin Controls the Level of Inflammation Related to Atherosclerosis

In **Chapter 6** we provide a mechanistic insight into the role of thrombin in atherogenesis. Here we demonstrate the *in vivo* significance of genetic alterations and pharmacologic inhibition of thrombin formation for the onset and progression of atherosclerosis, but also plaque phenotype determination. Genetically imposed hypocoagulability in transgenic mice inhibited atherosclerosis development and promoted atherosclerotic plaque stability, characterized by abundant collagen and vascular smooth muscle cell (VSMC) content, and diminished leukocyte infiltration. Conversely, hypercoagulability induced severe atherosclerosis progression, a marked inflammatory phenotype, plaque vulnerability and spontaneous atherothrombosis. Hypercoagulability-triggered plaque destabilization was mediated in a thrombin-dependent manner through an enhanced activation and oxidative burst response of neutrophils in the bone marrow, followed

by an increased mobilization and infiltration of leukocytes into the atherosclerotic plaques, the latter attributed to pro-inflammatory chemokine and cytokines overexpression. Notably, this severe atherosclerosis phenotype was rescued by the administration of either synthetic specific thrombin inhibitor Dabigatran etexilate or a recombinant form of the natural anticoagulant activated protein C (APC), which both resulted in substantially reduced leukocyte intraplaque recruitment, attenuation of atherosclerotic lesion formation (by ~80%), diminished systemic inflammation and enhanced plaque stability.

Concluding Remarks and Future Perspectives

The main goal of the studies described in this thesis was to unravel part of the mechanisms through which blood coagulation affects atherosclerosis progression and contributes to the determination of atherosclerotic plaque phenotype. This thesis provides important new insights on the role of clotting proteins and coagulation-driven inflammation in atherogenesis. In particular, we here unmask a potentially significant role for thrombin in controlling the level of inflammation related to atherosclerosis onset and progression. Whereas our data clearly implicates the potential of thrombin inhibition in the prevention of atherosclerosis development in mice, fu-

ture research will be needed to establish its effects on the regression and reversibility of atherosclerotic lesions. Of note, numerous studies have shown that very low concentrations of thrombin are associated with beneficial physiological effects such as endothelial barrier protection, apoptosis reduction, and attenuation in leukocyte transmigration³⁴⁻³⁹. Hence, learning the safety and efficacy of long-term thrombin inhibition in atherosclerosis becomes of crucial importance. In future, we also aim to explore the involvement of other key hemostatic proteins with strong cellular signaling capacity (e.g. FXa and TF-FVIIa complex). Overall, extensive studies of the blood coagulation-innate immunity-inflammation axis, and the various molecular pathways involved, may open up new therapeutic avenues in prevention and treatment of atherosclerosis. Antithrombotic therapy is the cornerstone in primary and secondary prevention of atherothrombosis, associated with a reduction in mortality rates by 30%. Despite that numerous clinical trials studying the effects of long-term administration of anticoagulants have failed to demonstrate beneficial side effects on atherosclerosis plaque growth and degree of stenosis, there are no clinical studies to date, which have investigated the effects of anticoagulant therapy on modulating plaque phenotype and stability. The development of new high-resolution vascular imaging modalities, including

molecular magnetic resonance imaging (MRI), and the introduction of new specific coagulation inhibitors will overall enable us to more precisely investigate how alterations in blood coagulation activity can affect atherosclerosis development in humans. The potential clinical impact of our studies involves the unique opportunity to modify atherosclerosis phenotype and progression through the administration of novel classes of anticoagulants³⁰.

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[†] Footnote for future PhD fellows at CTH: If you survive through the Spronk barrier, you may become a scientist!

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Hora est! ☺

Best wishes,

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Summary

Focus of Research:

1. Interaction between coagulation and inflammation systems in atherosclerosis
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3. Vulnerability/Thrombogenicity of atherosclerotic lesions
4. Hypercoagulability and atherosclerosis – clinical studies
5. New markers of arterial thrombosis

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"N.I. Pirogov" University Medical Center
Sofia, Bulgaria
August 1998 - November 2005 (7 years 4 months)
Department of Orthopaedics and Traumatology

Education

- **PhD Fellowship at Maastricht University Medical Center (MUMC+)**
Maastricht, The Netherlands
October 2006 – August 2011
PhD Degree – Prof. Dr. Hugo ten Cate, M.D., Ph.D.
(Promotor); Thesis: "The Coagulation-Inflammation Axis in Atherosclerosis"
- **Medicine at Medical University of Sofia**
Sofia, Bulgaria
1999 -2005
M.D. Degree
 - *15 Active Participations at International Conferences for Medical Students and Young Doctors*
 - *3 Awards for Best Oral Presentation/2 Awards for Best Poster Presentation*
 - *6 Attended Internships and Electives – Bulgaria, The Netherlands and Germany*
 - *5 Publications in Student Journals*

Honors and Awards

- Awarded with "Prof. Assen Zlatarov" Science Achievement Award 2005 at Medical University of Sofia, Sofia, Bulgaria
- Erasmus Fellowship (European Commission)
- Marie Curie Fellowship (European Commission)
- Young Investigator Award at XXII Congress of International Society on Thrombosis and Haemostasis (ISTH), 11-16 July 2009, Boston, USA
- Science Achievement Award in Thrombosis and Hemostasis - NVTH (Dutch Society for Thrombosis and Hemostasis), 31 March - 1 April 2009, Koudekerke, The Netherlands
- Chairman of "Atherosclerosis: Plaque Composition" plenary session at XXII Congress of International Society on Thrombosis and Haemostasis (ISTH), 11-16 July 2009, Boston, USA
- ATVB Award for Young Investigators at AHA Scientific Sessions 2010, 13–17 November 2010, Chicago, USA
- Young Investigator Award for Best Research in Thrombosis and Hemostasis - NVTH (Dutch Society for

Publications and Books

Thrombosis and Hemostasis), 17-18 May 2011,
Koudekerke, The Netherlands

- Young Investigator Award at XXIII Congress of International Society on Thrombosis and Haemostasis (ISTH), 23-28 July 2011, Kyoto, Japan
- Poster Award at XXIII Congress of International Society on Thrombosis and Haemostasis (ISTH), 23-28 July 2011, Kyoto, Japan
- Recipient of Kootstra Talent Fellowship 2011, MUMC+
- Awarded with a Rubicon Grant from the Netherlands Organisation for Scientific Research (NWO) - July 2011
- 4 articles in preparation/submission/revision
- Konings J, Govers-Riemslog JW, Philippou H, Mutch NJ, **Borissoff JL**, Allan P, Mohan S, Tans G, Ten Cate H, Ariens RA. Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin. *Blood*. Oct 6;118(14):3942-51. *Impact Factor: 10.558*
- **Borissoff JL**, ten Cate H. From neutrophil extracellular traps release to thrombosis: an overshooting host-defense mechanism? *J Thromb Haemost*. 2011 Sep;9(9):1791-4. *Impact Factor: 5.439*
- **Borissoff JL**, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med*. 2011 May 5;364(18):1746-60. *Impact Factor: 53.486*
- Kilinc E, van Oerle R, **Borissoff JL**, Oschatz C, Gerlofs-Nijland ME, Janssen NA, Cassee FR, Sandstrom T, Renne T, Ten Cate H, Spronk HM. Factor XII Activation is Essential to Sustain the Procoagulant Effects of Particulate Matter. *J Thromb Haemost*. 2011 Jul;9(7):1359-67. *Impact Factor: 5.439*
- **Borissoff JL**, Heeneman S, Kilinc E, Kassak P, Van Oerle R, Winckers K, Govers-Riemslog JW, Hamulyak K, Hackeng TM, Daemen MJ, Ten Cate H, Spronk HM. Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation*. 2010;122:821-830. *Impact Factor: 14.432*

- **Borissoff JL**, Ten Cate H. Wiskott-Aldrich Syndrome/Chediak-Higashi Syndrome/Thrombomodulin in Lang's Encyclopedia of Molecular Mechanisms of Disease; ISBN: 978-3-540-67136-7; Springer-Verlag GmbH Berlin Heidelberg 2009.
Book
- **Borissoff JL**, Spronk HM, Heeneman S, Ten Cate H. Is Thrombin a Key Player in the "Coagulation-Atherogenesis" Maze? *Cardiovasc Res.* 2009;82:392-403.
Editor's Choice Impact Factor: 6.051

Memberships and Associations

- Member of the American Heart Association (AHA) - Council Affiliations: Council on Arteriosclerosis, Thrombosis, and Vascular Biology
- Member of the International Atherosclerosis Society (IAS)
- Member of the International Society of Thrombosis and Hemostasis (ISTH)
- Member of the Dutch Society of Thrombosis and Hemostasis
- Member of the European Vascular Biology Organisation (EVBO)

Conferences, Workshops and Courses

- **PhD Course in Advanced Microscopy and Vital Imaging**, 18-22 June 2007, Maastricht, The Netherlands
- **XXI Congress of International Society of Thrombosis and Haemostasis** – 6 – 12 July 2007, Geneva, Switzerland; *Poster Presentation*
- **Laboratory Animal Science Course** - 10-28 September 2007, Groningen, The Netherlands, Article 9 License
- **NH Course in "Thrombosis and Haemostasis"** – 8-12 October 2007, Papendal, The Netherlands
Top 5 Posters Award
- **15th International Vascular Biology Meeting** - 1-5 June 2008, Sydney, Australia - *Oral and Poster Presentation*
- **NH Course in "Cardiac Function and Adaptation"** – 27-31 October 2008, Papendal, The Netherlands

- **Annual CARIM Symposium 2008** – 5th November 2008, Maastricht, The Netherlands; *Poster Presentation*
- **Dutch Atherosclerosis Society Symposium**, 13 March 2009, De Reehorst te Ede, The Netherlands; *Poster Presentation*
- **NVTH (Dutch Thrombosis and Hemostasis Society) Symposium** – 31 March - 1 April 2009, Koudekerke/Vlissingen, The Netherlands; *Oral Presentation*
- **Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference 2009** – 29 April– 1 May 2009, Washington D.C., USA; *Poster Presentation*
- **XXII Congress of International Society on Thrombosis and Haemostasis (ISTH)**, 11- 16 July 2009, Boston, USA; *2 Oral Presentations*
- **5th European Meeting on Vascular Biology and Medicine (EMVBM)** - 14-17th September 2009, Marseille, France; *2 Oral Presentations*
- **Joint Meeting GTH & NVTH (Nederlandse Vereniging voor Trombose en Hemostase)** - 24-27th February 2010, Nurnberg, Germany; *Oral and Poster Presentations*
- **Dag Interne Geneeskunde** - 12th November 2010, Maastricht, The Netherlands; *1 Oral Presentation; Nominee for Best Publication 2010*
- **American Heart Association (AHA) Scientific Sessions 2010** - 24-27th November 2010, Chicago, USA *2 Oral Presentations*
Nominee for ATVB Award for Outstanding Research at AHA Scientific Sessions 2010
- **NVTH (Dutch Thrombosis and Hemostasis Society) Symposium** – 17-18 May 2011, Koudekerke, The Netherlands; *Oral Presentation*
- **XXIII Congress of International Society on Thrombosis and Haemostasis (ISTH)**, 23-28 July 2009, Kyoto, Japan; *2 Oral Presentations; 2 Poster Presentations*

Personal Interests

- Web Development, Graphic Design, Digital Photography, Travelling