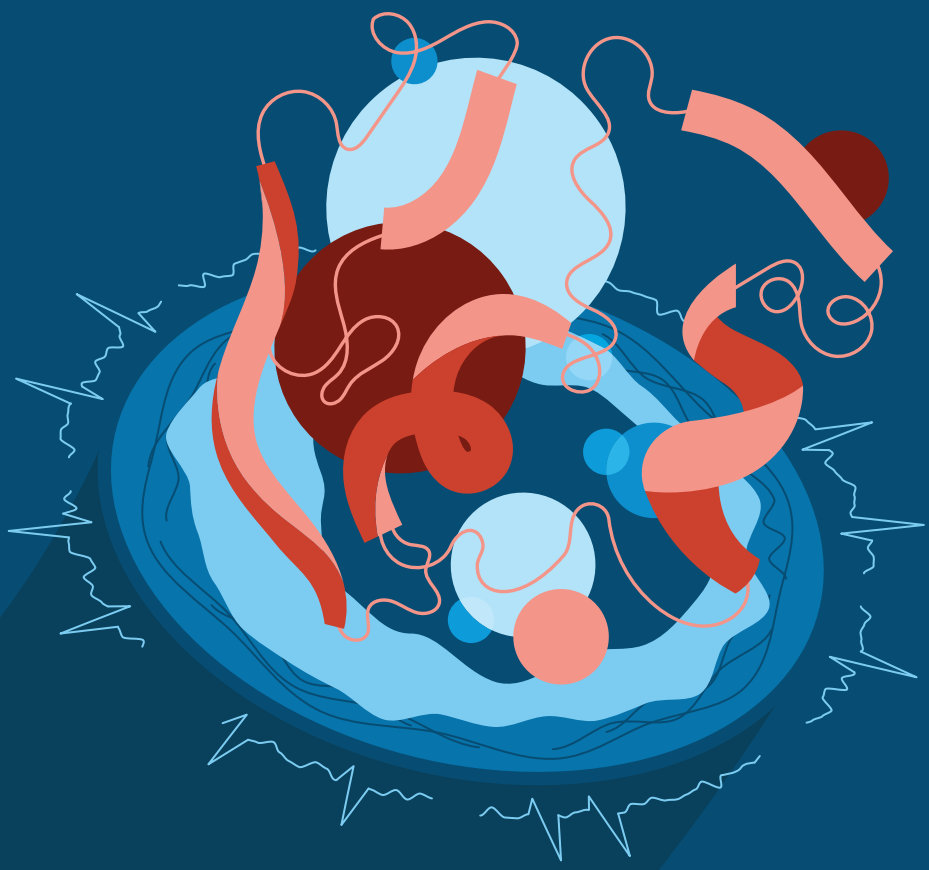
# Coagulation factor Xa as driver of cardiovascular diseases

# COAGULATION FACTOR Xa

## AS DRIVER OF CARDIOVASCULAR DISEASES



Jens Posma

# COAGULATION FACTOR $X_A$

## AS DRIVER OF CARDIOVASCULAR DISEASES

**Jens Posma**

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**COAGULATION FACTOR Xa AS DRIVER OF CARDIOVASCULAR DISEASES**

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# Chapter 1

# Introduction

## HEMOSTASIS

Hemostasis is a tightly regulated process aimed at preventing blood loss after injury. The hemostatic system comprises two major components working in concert: activation of platelets, termed primary hemostasis, and the coagulation cascade, termed secondary hemostasis. Platelets are secreted by megakaryocytes and circulate in blood; once platelets encounter an injured site, they bind to the sub-endothelial collagen, exposed by injury, and become activated. Activated platelets form a hemostatic plug at the damaged site to prevent further blood loss. In parallel to this, local activation of the coagulation cascade helps stabilizing the platelet plug by creating a dense fibrin network on which platelets attach. In a healthy state, hemostasis is a tightly orchestrated process, always balancing between injury-induced activation and inactivation of coagulation proteases by natural inhibitors (1–5). In case of bleeding or thrombosis, the hemostatic system fails to provide sufficient hemostatic protection, or produces excess activity, resulting in thrombosis, respectively (6–8).

It is widely appreciated that proteases of the coagulation system also have pleiotropic functions beyond their effect in hemostasis. Most of the pleiotropic actions of coagulation proteases are mediated via protease activated receptors (PARs), G-protein coupled receptors present on the membrane of multiple cell types (9,10). This thesis focuses on the pleiotropic functions of the coagulation protease factor Xa (FXa) in several cardiovascular diseases.

## ATHEROSCLEROSIS

Cardiovascular diseases are the number one cause of morbidity and mortality worldwide. Despite treatment strategies, numbers are expected to rise in the upcoming decades due to the aging population. Among the cardiovascular diseases, ischemic heart disease is the main cause of death, accounting for over 9 million deaths per annum (11). The underlying cause of most ischemic heart diseases is atherosclerosis: a chronic inflammatory disease of the arterial vessel wall characterized by the formation of vessel-narrowing plaques. Atherosclerotic lesions have different phenotypes, ranging from stable, non-thrombogenic lesions, to unstable, potentially harmful plaques that upon further damage may become highly thrombogenic. Unstable plaques are characterized by a thin fibrotic cap that separates the pro-coagulant content from the blood: a large necrotic core, a high degree of local inflammation, and few stabilizing collagen fibers. When this unstable plaque ruptures in a coronary vessel, an acute myocardial infarction may arise due to the formation of a superimposed thrombus, initiated by the release of the highly pro-coagulant plaque content. The superimposed thrombosis ultimately occludes the coronary vessel. The

less vulnerable plaques are generally less inflammatory and have a higher concentration of stabilizing collagen proteins. While these plaques do not tend to rupture, they can undergo erosion. Ruptured plaques are characterized by a thin fibrous cap, a large lipid core, high presence of macrophages and thrombi are mainly fibrin- and erythrocyte-rich (red thrombi) Eroded plaques often have little to no lipid core, lower presence of inflammatory cells of which mainly neutrophils, vast amount of smooth muscle cells and formed thrombi are generally platelet-rich (white thrombi). Furthermore, erosion is a fairly new concept describing the denaturing of endothelial cells (12,13). Healthy endothelial cells line the lumen of blood vessels and possess anti-coagulant properties; they also prevent platelets from binding to sub-endothelial collagen, thereby preventing their activation (14–16). However, during erosion, endothelial cells are denatured by a mechanism currently not fully known. Healthy endothelial cells are strongly glued to the underlying membrane by ligand/receptor interactions comprising integrins and nonfibrillar type IV collagen and laminins of the basement membrane. However, during erosion parts of the basement membrane or the attached basement membrane-bound integrins (or a combination of both) might get degraded by matrix metallo proteases. Another potential mechanism involves apoptosis of endothelial cells by neutrophil MPO mediated ROS production (13).

Experimental work has shown that coagulation factors are readily present in early and advanced atherosclerotic lesions (17,18). Although the exact contribution of coagulation enzymes in the vessel wall needs to be further clarified, there is strong preclinical data showing that inhibition of FXa or thrombin attenuates atherogenesis (19–23). These protective effects on atherogenesis were associated with more stable atherosclerotic lesions and decreased inflammation. However, these studies did not yet reveal the involved pathways leading to atheroprotection. Moreover, the preclinical models tend to focus on the development of atherosclerosis, whereas in patients with atherothrombotic disease like coronary artery disease, there is mostly a stage of established atherosclerotic lesions. Hence, a question is whether inhibition of different pathways might be involved in early versus late atherosclerosis.

## MYOCARDIAL ISCHEMIA REPERFUSION INJURY

Treatment of an ST-elevated acute myocardial infarction is focused on fast restoration of blood flow to the ischemic zone by combining anti-platelet drugs, anticoagulants and percutaneous coronary intervention in combination with risk factor treatment, such as lipid- and hypertensive lowering drugs (24). Although these treatment strategies have greatly improved the clinical outcome, successful salvage of the ischemic zone also induces reperfusion injury, which, when cell damage is irreversible, contributes to the final infarct size (25–27). Efficient treatment strategies to limit reperfusion injury are currently lacking.

## OUTLINE OF THE THESIS

The research presented in this thesis relates to two major contributors to ischemic heart disease centralizing the coagulation cascade as a key player in the pathologies: the first part focuses on atherosclerosis, the second part on myocardial ischemia/reperfusion injury and the way that coagulation protease inhibition can contribute to salvation of ischemic cardiac tissue.

**Chapters 2 and 3** expand on the pleiotropic effects of coagulation proteases in relation to cardiovascular disease. In **chapter 2**, the focus is on studies that investigate the effect of anticoagulants, such as rivaroxaban and dabigatran, in mouse models of inflammatory diseases. Results are compared and contrasted. Potential challenges in identifying downstream pathways influenced by proteases of the coagulation cascade are described. **Chapter 3** dives further into the signaling functions of protease activated receptors with thrombin as a central player.

In line with the proposed pleiotropic effects of coagulation proteases, in **Chapter 4** the hypothesis is tested whether inhibition of FXa, one of the central enzymes in the coagulation cascade, has a beneficial effect on the development, progression and/or regression of atherosclerosis in mice.

In **chapter 5** the mechanisms behind the beneficial effects of FXa inhibition were explored by isolating aortas from rivaroxaban treated, atherosclerosis-prone mice for RNA sequencing. This chapter highlights novel pathways that potentially explain the atheroprotective effects of FXa inhibition seen in chapter 4.

**Chapter 6** is aimed at identifying novel proteins relevant in risk stratification in patients with peripheral arterial disease, mostly lower limb atherosclerosis.

**Chapter 7** is focused on the crosstalk between coagulation proteases and inflammation in relation to ischemia reperfusion injury of the heart. The potential protective effects of FXa inhibition by rivaroxaban during myocardial ischemia reperfusion were investigated.

**Chapter 8** investigates patients with AF from the RACE V registry. In this chapter patients with self-terminating paroxysmal AF were followed for several years to study whether a hypercoagulable state contributes to AF progression.

**In chapter 9,** the results of this thesis are summarized. **Chapters 10 and 11** bring the results in a broader perspective and discuss the future implications of the findings in this thesis.

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1

# Chapter 2

# Coagulation and Non-Coagulation Effects of Thrombin

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## ABSTRACT

Thrombin is a multifunctional serine protease produced from prothrombin and is a key regulator in hemostatic and non-hemostatic processes. It's the main effector protease in primary hemostasis by activating platelets and plays a key role in secondary hemostasis . Besides its well-known functions in hemostasis, thrombin also plays a role in various non-hemostatic biological and pathophysiological processes, predominantly mediated through activation of protease-activated receptors (PARs). Depending on several factors such as concentration of thrombin, duration of activation, location of PARs, presence of co-receptors, and the formation of PAR heterodimers, activation of the receptor by thrombin can induce different cellular responses. Moreover, thrombin can have opposing effects in the same cell; it can induce both inflammatory and anti-inflammatory signals. Due to the complexity of thrombin's signal transduction pathways, the exact mechanism behind the dichotomy of thrombin is yet still unknown. In this review we will highlight the hemostatic and non-hemostatic functions of thrombin and will specifically focus on the non-hemostatic opposite roles of thrombin under various conditions on cell biology and in relation to cardiovascular disease.

*Keywords*: Protease-activated Receptors, Thrombin, Coagulation, PAR-heterodimers, PAR-homodimers

## INTRODUCTION

Thrombin is a multifunctional serine protease produced from prothrombin and is a key regulator in hemostatic and non-hemostatic processes. It's the main effector protease in primary hemostasis in that it activates platelets with subsequent release of platelet activators ADP, serotonin, and thromboxane A2. In secondary hemostasis, thrombin converts fibrinogen into fibrin monomers finally forming a stable clot that stops bleeding at sites of vascular injury. In addition thrombin attenuates its own generation through formation of a complex with endothelial cell surface receptor thrombomodulin (TM), thereby activating protein C into activated protein C (APC), the main in-activator of factors Va and VIIIa(1).

Besides its well-known functions in hemostasis, thrombin plays an important role in various non-hemostatic biological and pathophysiological processes, predominantly mediated through activation of protease-activated receptors (PARs).

PARs belong to a family of the G protein-coupled receptors(2), which are characterized by a single polypeptide chain with seven transmembrane α-helices that are connected by three intra- and extracellular loops. The N-terminal extracellular domain contains the ligand binding site, whereas the intracellular loops serve as binding sites for G-protein trimer (Gaßg) responsible for signal transduction(3,4). To date, four subgroups of PAR have been identified, PAR1 to -4, which are expressed by a variety of cell types, including platelets, endothelial cells (EC), vascular smooth muscle cells (VSMC), fibroblasts, hepatocytes, T lymphocytes and monocytes. PAR1 and PAR3 are high-affinity receptors for thrombin and can be activated at lower concentrations (<5nM), whereas the low-affinity thrombin receptor PAR4 is only activated at higher concentrations of thrombin, due to the lack of a hirudin-like sequence in the vicinity of the protease cleavage site(5). PAR2 is the only receptor that is not directly activated by thrombin, but is transactivated in cooperation with PAR1 or directly activated by factor Xa or in complex with factor VIIa and tissue factor(6,7).

Activation of PARs occurs through a unique mechanism: proteases cleave the N-terminal extracellular domain thereby generating a new N-terminus which acts as a new N-terminal tethered ligand that activates the cleaved G-protein coupled receptor. The intracellular domains of PARs tightly bind Gα and Gβγ subunits; activation of the receptor stimulates the exchange of GTP for GDP, resulting in phosphorylation of the intracellular Gα subunit, which induces the release of the Gα subunit from its tight binding to the Gßg subunit(8). Depending on several factors such as the duration of activation, concentration of the ligand, and the location and presence of co-receptors of PARs, activation of the receptor stimulates the phosphorylation of either one of the intracellular Gα subunit families: $G\alpha_{12/13,}$ $G\alpha_{i/o}$ or $G\alpha_{q}$. Phosphorylation of Gα subunits regulates gene

expression and intracellular protein synthesis through a complex mechanism of combined secondary signaling pathways involving kinase activities and more than 2000 alternative phosphorylation sites(9).

Signaling through $G\alpha_q$-activation predominantly results in activation of phospholipase C (PLC), which in turn activates mitogen-activated protein kinase (MAPK) and phosphokinase C (PKC). Activation of Gαi results in ERK/MAPK activation, and inhibition of intracellular adenylyl cyclase. The latter, normally activates GTPase Rac1, which is known to promote platelet responses and barrier protective processes(10,11), but also to play a major role in anti-inflammatory responses(5). The Gβγ subunit of $G\alpha_i$-activation can activate phosphoinositide 3-kinase (PI3K)(12), which may contribute to cellular activation by inducing a shape change as well as integrin activation(13). Subunit $G\alpha_{12/13}$ activation predominantly induces activation of MAPK and subsequently the small G proteins such as Rho GTPase for Rho-dependent responses, which are involved in changes of the cytoskeleton(14-16). This in turn might induce cellular permeability and migration of endothelial cells, contributing to apoptotic and pro- and anti-inflammatory processes(5,17,18).

Activated PARs are rapidly internalized and targeted to lysosomal degradation. This mechanism involves phosphorylation of PARs within their C-terminal cytoplasmic domain, which triggers membrane translocation and internalization through clathrin-mediated endocytosis. Recovery of internalized PARs requires either mobilization from the intracellular pool towards the cell membrane or synthesis of new receptors.

Besides the above-described direct PAR activation through proteases, additional modes of activation occur and involve dimer formation between PARs as well as between PARs and other receptors. PAR1 homodimers and PAR1:PAR3 heterodimers are activated at low concentrations of thrombin(19), whereas at high thrombin levels heterodimers of PAR1:PAR4 may be formed with faster activation of PAR4 as a result(20). The formation of PAR heterodimers facilitates additional secondary signal transduction pathways(5,14,21). Furthermore, under certain conditions (e.g. Sepsis), PAR1 can form heterodimers with PAR2 in which the PAR1 tethered ligand transactivates PAR2, thereby initiating distinct signaling pathways(22).

Given the abundant cellular expression of PARs it is hardly surprising that thrombin is involved in many physiological processes such as embryogenic development, angiogenesis, wound healing and inflammation, as well as in pathophysiological processes such as atherosclerosis, sepsis, cancer and neuropathology(23). Depending on the conditions of PAR activation, thrombin can have opposing effects on a cell; for instance, it contributes to both anti- and pro-inflammatory processes, it regulates endothelial integrity as well as permeability, and can induce both vasodilation and vasoconstriction(6,19,23).

Due to the complexity of thrombin's signal transduction pathways, the exact mechanism behind the dichotomy of thrombin is yet still unknown, but seems to be dependent on several factors such as concentration of thrombin, duration of activation, location of PARs, presence of co-receptors, and the formation of heterodimers(9).

In this review we will highlight the hemostatic and non-hemostatic functions of thrombin and will specifically focus on the opposite non-hemostatic dual role (pro-inflammatory vs anti-inflammatory) thrombin has under various conditions.



## 1: HEMOSTATIC FUNCTIONS OF THROMBIN

The main purpose of hemostasis, the formation of a blood clot composed of activated blood platelets intertwined with covalently linked fibrin, is arrest of bleeding from a damaged blood vessel. Platelet adhesion to collagen, activation and subsequent aggregation is considered primary hemostasis, whereas coagulation leading towards conversion of fibrinogen into fibrin is termed secondary hemostasis. Collagen and ADP are well known activators of platelets; in addition, thrombin activates platelets through PAR1 or -4, providing a procoagulant phospholipid membrane surface on which coagulation factors congregate. With multiple functions, thrombin is considered the central enzyme in coagulation. Not only does thrombin convert fibrinogen into fibrin, it also enhances its own generation through activation of the cofactors V and VIII as well as factor XI in the so-called feedback loop. Through activation of factor XIII, thrombin stimulates covalent crosslinking of fibrin molecules. Thrombin attenuates its own generation through activation of the protein C pathway, in which thrombomodulin bound thrombin activates protein C on the endothelial protein C receptor (EPCR). Overall, thirteen functions of thrombin in hemostasis have been described (24).

## 2: NON-HEMOSTATIC FUNCTIONS OF THROMBIN THROUGH ACTIVATION OF PARS

### 2.1: Concentration and time dependent cellular responses to Thrombin

One of the critical factors in the net cellular effect of thrombin mediated PAR signaling concerns the concentration of the agonist (25). For example, studies showed a barrier protective action of thrombin on endothelial cells at low concentrations (20 - 75 pM)(26,27), whereas the cellular response to higher concentrations of thrombin (>100 pM) induced barrier disruptive signals(28). Also, thrombin can both protect astrocytes and induce cell death through activation of PAR1(28). The activation pathways for thrombin-induced apoptosis and cellular protection show strong similarities in which the small GTP-binding

protein, RhoA, plays a major role. Donovan and colleagues demonstrated that inhibition of the PKC inhibitor H7, completely blocked thrombin-induced cellular protection(29). In contrast, activation of the PKC pathway induced apoptosis in the same cell type(18). These two observations suggest that the same or similar second messenger pathways are involved in pro- and anti-apoptotic pathways; these effects may also be partially explained by different cellular responses to high versus low thrombin levels. At relatively high thrombin concentrations (>500 nM), RhoA activity in the cell increases rapidly within minutes resulting in apoptosis, whereas at low levels (1 pM to 100 pM) a small but consistent increase in the available RhoA activity was observed in conjunction with cell protective responses(18,28). Besides the levels of thrombin, exposure time may add to cell response variability. A time course study on thrombin-mediated cell death indicated that cells must be exposed to thrombin for at least 16 hours in order to irreversibly enter the cell death pathway(29). Taken together, short exposure to low concentrations of thrombin induces cellular protective signaling pathways and prolonged exposure to high levels is required for apoptosis (fig. 1). Translated to the clinical setting in case of acute high thrombin generation (e.g., trauma or stroke), there may be a time frame in which the initial protective cellular responses are switched towards inflammation, barrier disruption, and apoptosis.

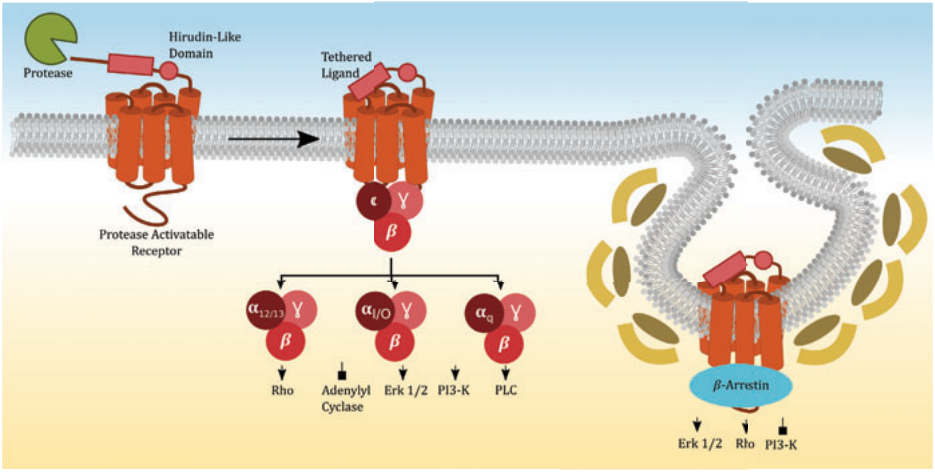


**Figure 1.** Activation of protease activated receptor (PAR). This figure shows the activation of protease activated receptors. Proteases (e.g., Thrombin) cleave the N-terminal extracellular domain of PARs thereby exposing a self-activating ligand. The hirudin-like domain present on PAR1 receptors increases the affinity thrombin has for PAR. After activation, PAR induces a proper signal transduction through various G-proteins, which ultimately leads different effects on the cell. In addition to g-protein dependent signaling, PARs can also signal in a g-protein independent way via ß-arrestin.

**2.2: Homodimers and Heterodimers**

A possible explanation for the previously mentioned concentration dependent cellular response of thrombin could be a mechanism in which PARs form dimers. In addition to inducing cellular responses as a protomer, recent studies revealed that PARs can form homodimers, heterodimers, and oligomers. Homodimers of PAR1:PAR1, PAR2:PAR2, and PAR4:PAR4 as well as heterodimers of PAR1:PAR2, PAR1:PAR3, and PAR1:PAR4 have been described(14). The composition of the dimer partly determines the activated signal transduction cascade, which is distinct from the protomer signaling pathway(22).

PAR3 is known to have a high affinity for thrombin due to its hirudin-like N-terminus domain and to be activated at relatively low concentrations of thrombin(30). Thereby PAR3 is thought to especially regulate cellular processes of PAR1 under physiological conditions(22), whereas PAR4 is only activated upon higher concentrations of thrombin, predominantly found during pathophysiological conditions(21,22,31).

PAR3 was previously thought not to be directly involved in endothelial signal transduction due to the lack of a cytosolic domain(32). However, thrombin mediated Rho- and $Ca^{2+}$-dependent release of ATP in cells that only expresses PAR3, demonstrate that thrombin-induced PAR3 signaling is a functional pathway(33).

PAR-1 forms heterodimers with PAR-3 (PAR1:PAR3) with equal capacity compared to the PAR1 homodimer formation(22). Signaling of PAR1 alone induces equal phosphorylation of both $G\alpha_q$ and $G\alpha_{12/13}$, whereas the PAR1:PAR3 heterodimer favors $G\alpha_{13}$ signaling and the subsequent activation of cytoprotective pathways(30,34). Disruption of PAR3 signaling through anti PAR3 small interfering RNA treatment eliminated thrombin's cytoprotective properties in favor of barrier disruptive signaling(5).

At higher concentrations of thrombin (>10nM), which mainly occur during pathophysiological conditions e.g. early phase of sepsis, endothelial damage and cancer, the lower affinity receptor PAR4 is also activated and can form heterodimers with PAR1(fig. 2)(21,22,31). The PAR1:PAR4 heterodimer provides an additional activation pathway for cells, which is distinct from PAR1 and PAR1:PAR3 signaling(22). Although the specific Gα subunit mapping of the PAR1:PAR4 heterodimer is not known yet, this complex might play a role in inflammation, diabetic vasculopathy, and cancer, by modulating PAR1 signaling (35-37).

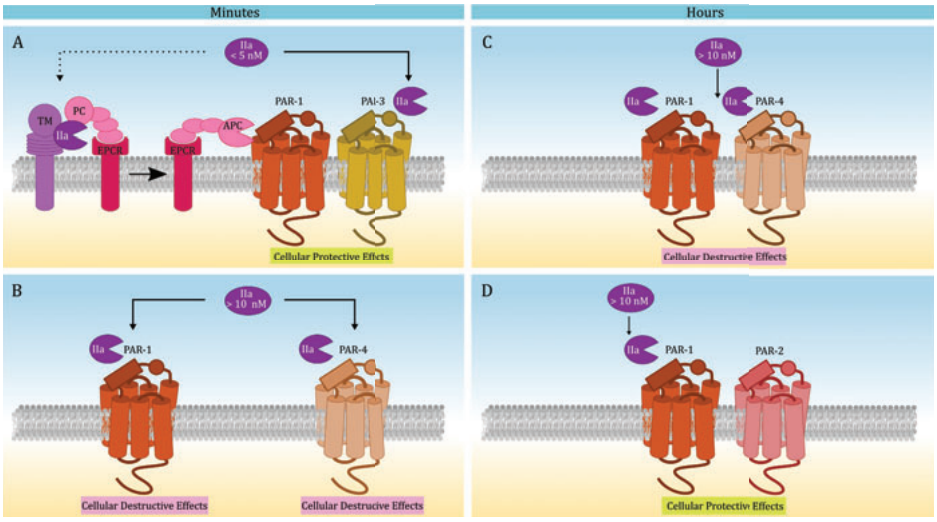


**Figure 2.** Thrombin's (FIIa) non-hemostatic opposite roles on cell biology**.** This figure shows the effects of acute and prolonged exposure to thrombin (respectively minutes to hours), as well as the cellular effects of high and low thrombin concentrations (respectively <5nM and >10nM). **A:** Short term expose to low thrombin levels leads to the activation of PAR3 which in turn can form heterodimers with PAR1. Binding of thrombin to thrombomodulin (TM) activates protein C into activated protein C (APC) presented on the endothelial protein C receptor (EPCR). APC is then able to activate PAR1. Both APC and PAR3 mediated PAR1 activation lead to cellular protective signaling. **B:** High thrombin levels lead to acute cellular destructive effects mediated by PAR1 and PAR4. **C:** High levels of thrombin for a prolonged period leads to the formation of PAR1:PAR4 heterodimers which induce cellular destructive effects. **D:** High concentrations of thrombin for a prolonged period leads to transactivation of PAR2 by PAR1 thereby leading to cellular protective effects.

### 2.3: Transactivation and heterodimerization of PAR2

PAR2 is the only receptor of the four PARs that cannot be directly activated by thrombin. However, PAR2 can be transactivated by PAR1. In this pathway, thrombin generates a N-tethered ligand on PAR1, which is subsequently donated to a nearby localized PAR2(38,39). The PAR1:PAR2 heterodimer especially plays a role in the late stage of sepsis, in hyperplastic responses to arterial injury and cytoprotective processes(22,40,41). For example, during systematic inflammation and enhanced activation of coagulation activation as during sepsis, PAR2 transactivation may be engaged to support thrombin-dependent cellular protective pathways(40). The exact mechanism of PAR1:PAR2 signaling is not fully understood(42); thrombin-activated PAR1 preferentially couples to $G\alpha_q$ and $G\alpha_{12/13}$ and the subsequent RhoA pathway, causing endothelial barrier disruption(43). In contrast, PAR1:PAR2 heterodimer phosphorylates the $G\alpha_i$ subunit, followed by activation of the Rac1 pathway and the subsequent barrier protective processes in endothelial cells(26,40). Furthermore, the transactivation of PAR2 by PAR1 promotes the PKC pathway, leading towards an increased expression of a

complement-inhibitory protein, decay-accelerating factor, which also regulates barrier protective processes in endothelial cells(41).

Additionally, the distinct internalization of the PAR1:PAR2 complex compared to PAR1 or PAR2 alone, contributes to alternative intracellular signaling. Activation of PAR1 results in transient recruitment of β-arrestins, that rapidly dissociates before receptor internalization (44,45), whereas activated PAR2 forms a stable complex with β-arrestins that co-internalizes to endocytic vesicles and functions as a scaffold to promote ERK1/2 activation (46,47). In contrast, PAR1:PAR2 heterodimer activation results in β-arrestin recruitment and co-internalization of the receptor complex to endosomes. Thus, PAR1:PAR2 dimer formation facilitates thrombin-dependent β-arrestin recruitment to endosomes, which does not occur with activation of the PAR1 receptor alone (22). For example, translating this to a clinical setting such as the early stage of sepsis, we assume that thrombin-induced cellular responses are mainly vascular disruptive (e.g., increased endothelial permeability), through activation of subunit $G\alpha_q$ and $G\alpha_{12/13}$ signaling of PAR1, which might be regulated by co-activation of PAR4. In contrast, during the late stage of sepsis, e.g. when cells are exposed to thrombin for a longer period, thrombin activation of PAR1 switches from RhoA to Rac1 and PKC signaling, which requires PAR2 transactivation (40,41). The findings that underlie this scenario suggest that transactivation of PAR2 by PAR1 is dependent on the concentration of thrombin, the duration of activation and the presence of PAR2 in the proximity of an activated PAR1 receptor(fig. 1). The transactivation of PAR2 subsequently affects the intracellular signal transduction of PAR1. The latter hypothesis is supported by Lin and colleagues, demonstrating that the PAR1 protomer directly starts signaling upon activation by thrombin, whereas PAR1:PAR2 heterodimers induce signal transduction after a prolonged period of exposure to thrombin (22).

### 2.4: Endothelial protein C receptor (EPCR) dependent signaling

Besides the modulation of thrombin:PAR1 signaling by PAR2, PAR3, or PAR4, other receptors can affect thrombin dependent cellular processes. Endothelial protein C receptor (EPCR) is an endothelial cell-specific transmembrane glycoprotein activated by coagulation factor VII and activated protein C (APC)(48). Although, thrombin mediated PAR1 activation acts pro-inflammatory, apoptotic, and endothelial barrier disruptive, the opposite effects were observed when EPCR was occupied by APC(fig. 1)(5,49), demonstrating that APC:EPCR switches PAR1 signal transduction towards cytoprotective and regenerative effects(27,50). EPCR interacts with the lipid raft plasma membrane protein caveolin-1, which plays a role in endocytosis. Binding of APC or protein C to EPCR leads to dissociation of EPCR from caveolin-1, thereby enabling activation of PAR1 by APC bound to EPCR. At low thrombin concentration the protease remains bound to thrombomodulin, facilitating the activation of protein C in complex with EPCR; under these conditions, PAR1 activation is mainly mediated through APC:EPCR

inducing cytoprotective $G\alpha_i$ signaling promoting Rac1 activation. On the other hand, increased thrombin levels switch the cytoprotective signaling towards thrombin mediated PAR1 activation with $G\alpha_q$ and $G\alpha_{12/13}$ intracellular signaling and subsequent RhoA activation(5,27,50-52). The switch between thrombin or APC mediated activation of PAR1 may have clinical implications in that intact endothelium supports EPCR:APC mediated cytoprotective effects. However, loss of EPCR and thrombomodulin expression on endothelium facilitates thrombin mediated PAR1 signaling in favor of barrier disruption, apoptosis, and inflammation.

**2.5: G-protein independent PAR signaling**

Although we clearly described the known consequences G-protein dependent activation have on various cellular pathways, new evidence also suggests an important role for G-protein independent PAR signaling. Originally identified as endosomal mediators, ß-arrestins where recently shown to act as scaffold proteins during the activation of G-protein coupled receptors(54,55). To date, two ß-arrestins are known to play a role in PAR activation; ß-arrestin 1 and 2. ß-arrestins are able to work in synergy or in opposing manner with G-proteins by activating or inhibiting the same downstream enzymes (e.g. PI3K and RhoA) (56). As mentioned before in paragraph 2.3, internalization of PAR1 can lead to the recruitment of the scaffold protein ß-arrestin. This induces alternative ERK1/2 signaling compared to regular G-protein activated ERK1/2 where gene expression is modulated. Instead of translocating to the nucleus, the whole ERK1/2 complex scaffolds onto ß-arrestin, thereby promoting prolonged ERK1/2 signaling from the cell membrane resulting in cytoskeleton reorganization(57). Shenoy et al. (2005) showed that G-proteins are responsible for early ERK1/2 activation (0-5 minutes), whereas prolonged activation of ERK1/2 (>5 minutes) was mediated by ß-arrestin (58). G-protein independent signaling was only observed at high agonist concentrations, suggesting a dose dependent switch from G-protein dependent to G-protein independent signaling(59,60). Classical G-protein activation leads to increased activation of PI3K and the subsequent signal transduction. When the PI3K complex scaffolds to ß-arrestins, its activity is inhibited resulting in diminished signaling through G-proteins(61-64). For example, in the Angiotensin II receptor 1, synergistic signaling of ß-arrestin and G-proteins is mandatory for stress fiber formation. The observation that only high agonist concentrations are able to induce ß-arrestin mediated responses, suggests a dose dependent switch from G-protein dependent to G-protein independent signaling(59,60). Although few data exist, ß-arrestin is shown to induce various signaling cascades in addition to the traditional G-protein signaling, which can either counteract or act synergistically with G-protein signaling. However, to clarify the exact contribution of ß-arrestins on PAR signaling more research is needed.

## 3: EFFECTS OF INHIBITION OF THROMBIN ON CARDIOVASCULAR PATHOPHYSIOLOGICAL PROCESSES



As outlined above, thrombin is the key enzyme in fibrin formation during blood clotting and participates in other biological and pathophysiological processes as well. Considered that various *in vivo* thrombin generation profiles (e.g. concentration, time, and location) can induce distinct cellular responses, thrombogenicity and excessive PAR activation may be involved in a range of pathophysiological processes.

Given the role of inflammation and endothelial disruptive cellular responses in the onset and progression of atherosclerosis(53), thrombin might be an important player in this pathology. The finding that early atherosclerotic lesions are more hypercoagulable compared to advanced atheroma's is in support of a more pronounced contribution of thrombin in the development of atherosclerosis(53-55). The role for increased thrombin levels in the onset and development of pathologies associated with inflammation, suggests that (in)direct thrombin inhibition could have beneficial effects on atherosclerotic plaque formation. Indeed, using a spontaneous atherosclerosis model with apolipoprotein E-deficient mice, inhibition of thrombin showed attenuation of atherosclerotic plaque formation and was accompanied by reduced inflammation and PAR1 expression(56-59). Additionally, a hypercoagulable phenotype on an atherogenic background destabilized atherosclerotic plaques, most likely due to increased inflammation, apoptosis, and upregulation of metalloproteinases(57,60). Plaque destabilization is the most important determinant of an acute coronary syndrome, which occurs due to rupturing of atherosclerotic plaque leading to subsequent (occlusive) atherothrombosis(60). Arterial thrombosis in coronary arteries causes myocardial ischemia, often followed by reperfusion injury. Ischemia/reperfusion (I/R) injury initiates cell death mediated inflammatory responses, generation of reactive oxygen species and recruitment of inflammatory cells such as neutrophils and monocytes(61). Inhibition of coagulation by either APC or active-site inhibitor factor VIIa (ASIS) decreased myocardial I/R-injury in mice(62). Furthermore, experiments using PAR1 deficient mice showed reduced infarct sizes after cerebral infarctions(63). In a cardiac I/R-injury model in rats, inhibition of PAR4 attenuated I/R-injury in which the ERK1/2 pathway was involved. More interestingly, specific activation of PAR2 attenuated myocardial I/R-injury in a rat model(64). These data suggest a cytoprotective role for PAR2 signaling, which is in agreement with the earlier described protective role of PAR2 during inflammation at the late stage of sepsis(27,51,52).

Furthermore, coagulation and inflammation are potentially closely linked to atrial fibrillation (AF), through regulating fibrosis, apoptosis, and endothelial dysfunction(65,66). AF is the most common sustained cardiac arrhythmia,

resulting in a variable risk of thromboembolic stroke. AF is caused by conduction disturbance of atrial tissue due to several structural alterations including fibrosis, atrial dilatation, endocardial denudation and cellular hypertrophy(65), which all contribute to the aggravation of the disease(67). Whether these latter processes initiate or maintain AF is still unknown, yet. Nevertheless, it has been described that coagulation plays a key role in fibrosis(68,69), although this has never been investigated in relation to AF. PAR1 and PAR2 are both expressed by cardiomyocytes and are involved in proliferation of embryotic cardiac fibroblasts(70), suggesting a possible role of thrombin in the onset of AF. Furthermore, *in vivo* studies found an upregulation of PAR1 during cardiac hypertrophy and cardiomyopathy, which was reduced by inhibition of coagulation(71). Given the well-known crosstalk between coagulation and inflammation, both may contribute to the onset and/or maintenance of AF(66). Whether inflammation drives the coagulation pathway or the other way around is still unclear. Anti-inflammatory therapy has already been shown to have beneficial effects on the onset of AF, but to our knowledge it has never been investigated for inhibition of coagulation(66).

## 4: CONCLUSION

Thrombin is a multifunction key regulator in hemostatic and non-hemostatic processes. Thrombin mainly regulates cellular responses through activation of PARs but can have opposite effects on the same cell depending on various conditions such as concentration, location, exposure time, and presence of co-factors. Through activation of PARs, thrombin can regulate physiological processes such as embryonic development and wound healing, but also pathophysiological processes like sepsis, cancer, fibrosis, and inflammation. Activation of PARs by thrombin can lead to 2224 different intracellular phosphorylation in the cell, making investigation of the various effects of thrombin on cellular pathways challenging(9).

Altogether, thrombin has primarily a protective role under physiological conditions (<5 nM) upon PAR1 stimulation mainly through phosphorylation of the subunit $G\alpha_i$ and subsequent activation of Rac1. In this case, PAR1 is activated by thrombin:PAR3 or by APC:EPCR(50). Intriguingly, during acute (trauma, sepsis) of chronic (e.g., atherosclerosis) inflammation associated conditions leading to increased thrombin formation, thrombin-dependent cellular responses switch towards barrier disruptive processes. This switch is predominantly mediated through direct activation of PAR1 by thrombin causing phosphorylation of $G\alpha_{12/13}$, and subsequent activation of the RhoA pathway. This pathway is also facilitated by the activation of PAR4 at higher levels of thrombin(5). At the late phase of sepsis, characterized by systemic inflammation and increased coagulation activation, leading to a long-term activation of PAR1 with high concentrations of thrombin, PAR2 is transactivated and switches PAR1 signaling to $G\alpha_i$ and

Rac1 activation, resulting in barrier protective cellular responses. The exact mechanisms behind the different roles of thrombin in different tissues, is not fully understood and needs more work to clarify the complex picture.

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# Chapter 3

# Roles of Coagulation Proteases and Protease-activated Receptors in Mouse Models of Inflammatory Diseases

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## ABSTRACT

Activation of the blood coagulation cascade leads to fibrin deposition and platelet activation that are required for hemostasis. However, aberrant activation of coagulation can lead to thrombosis. Thrombi can cause tissue ischemia, and fibrin degradation products and activated platelets can enhance inflammation. In addition, coagulation proteases activate cells by cleavage of protease-activated receptors (PARs), including PAR1 and PAR2. Direct oral anticoagulants have recently been developed to specifically inhibit the coagulation proteases factor Xa (FXa) and thrombin. Administration of these inhibitors to wild-type mice can be used to determine the roles of FXa and thrombin in different inflammatory diseases. These results can be compared with the phenotypes of mice with deficiencies of either Par1 (F2r) or Par2 (F2rl1). However, inhibition of coagulation proteases will have effects beyond reducing PAR signaling, and a deficiency of PARs will abolish signaling from all the proteases that activate these receptors. We will summarize studies that examine the roles of coagulation proteases, particularly FXa and thrombin, and PARs in different mouse models of inflammatory disease. Targeting FXa and thrombin or PARs may reduce inflammatory diseases in humans.

## INTRODUCTION

The primary function of the blood coagulation cascade is to prevent blood loss after vessel injury. This cascade is initiated by the tissue factor (TF)/factor VIIa (FVIIa) complex. FXa and thrombin are components of the so-called common pathway (1-3). Thrombin is the central protease of the blood coagulation cascade and has numerous roles including cleavage of fibrinogen to fibrin and activation of platelets (Fig. 1). Aberrant activation of the blood coagulation system can contribute to pathology of various diseases. Formation of thrombi within blood vessels leads to ischemia and inflammation. In addition, fibrin degradation products are released during the degradation of fibrin and these can enhance inflammation. For instance, the E1 fragment increases the recruitment of leukocytes to sites of injury by binding to VE-cadherin on endothelial cells and integrin αMβ2 on leukocytes (4). In addition, macrophage activation is enhanced by binding to fibrin(ogen) via αMβ2 (5). Moreover, activated platelets can enhance inflammation by releasing inflammatory mediators and by promoting extravasation of leukocytes (6). Finally, FXa and thrombin activate a variety of cell types, including endothelial cells, vascular smooth muscle cells, macrophages, fibroblasts and cardiac myocytes via protease-activated receptor (PARs) (Fig. 1). There are 4 members of the PAR family (PAR1–4) and they are ubiquitously expressed. These receptors belong to the large family of G-protein coupled receptors but are unique because they are activated by proteolytic cleavage that exposes a tethered ligand. Thrombin activates PAR1, PAR3 and PAR4 whereas FXa primarily activates PAR2 (7-10). Importantly, other proteases also activate PAR1 and PAR2. For example, activated protein C (APC) and matrix metalloproteases activate PAR1 (11-13(, whereas trypsins, tryptase, FVIIa, and matriptase activate PAR2 (14-16). In addition, PAR1 and PAR2 can form heterodimers (17-18) Interestingly, human platelets express PAR1 and PAR4 whereas mouse platelets express a PAR3/PAR4 complex (19-21). This means that any phenotype observed in *Par1*$^{-/-}$ mice cannot be due to an attenuation of platelet activation. Furthermore, there may be differences in PAR signaling in mice and humans.

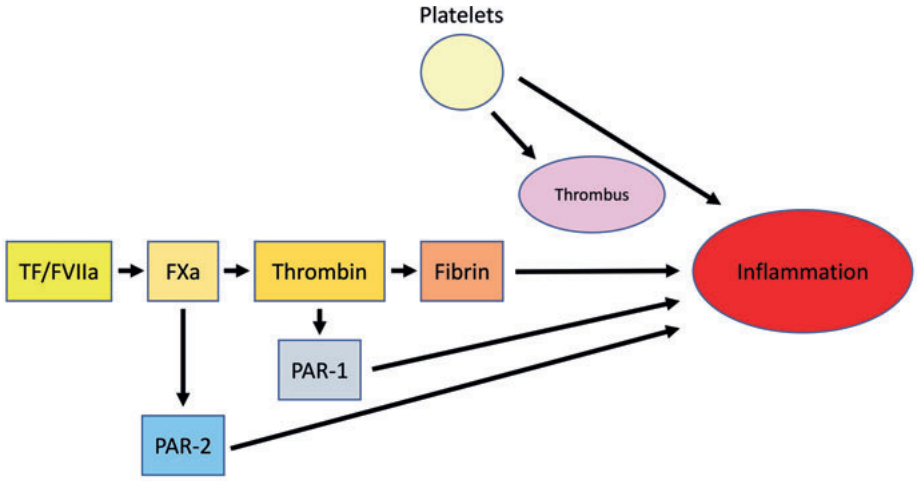


**Figure 1 Roles of coagulation proteases and downstream pathways in inflammatory diseases.** Activation of the coagulation cascade leads to cleavage of fibrinogen into fibrin and platelet activation that can contribute to thrombosis Platelet activation and fibrin degradation products can also enhance inflammation. Additionally, coagulation proteases can activate cells via various PARs that can increase the expression of inflammatory mediators.

Several anticoagulants, such as vitamin K antagonists and various forms of heparins, are used to prevent and treat venous thrombosis and prevent stroke associated with atrial fibrillation (22). Direct oral anticoagulants (DOACs), which are also known as non-vitamin K antagonist oral anticoagulants (NOACs), are a relatively new class of anticoagulants that specifically inhibit FXa and thrombin (23,24). There are several FXa inhibitors (apixaban, betrixaban, edoxaban and rivaroxaban) but only one approved thrombin inhibitor called dabigatran etexilate, which is a pro-dug that is metabolized to dabigatran (24). Various DOACs have been used to study the roles of FXa and thrombin in various mouse models of disease but rivaroxaban and dabigatran are the most popular. As expected, therapeutic doses of these inhibitors reduce thrombosis in mice (25-27). We will not discuss studies that investigated the antithrombotic activities of these drugs. One would expect different effects of rivaroxaban versus dabigatran on PAR signaling. For instance, inhibiting FXa would be expected to primarily inhibit PAR2 signaling whereas inhibiting thrombin would primarily inhibit PAR1 signaling as well as PAR3 and PAR4 signaling (Fig. 1). Additionally, Par2$^{-/-}$ and Par1$^{-/-}$ mice can be used to compare and contrast the effects seen in wild-type mice with pharmacological inhibition of either FXa or thrombin, respectively. However, inhibition of proteases will have effects beyond PAR signaling and a Par deficiency will abolish signaling from multiple proteases. In addition, studies with Par1$^{-/-}$ or Par2$^{-/-}$ mice do not provide any information on the protease(s) that are activating the PARs in a given disease

or the effector cells that are expressing the PARs. Recently, transgenic mouse lines containing floxed Par1 and Par2 genes have been generated (Dr. J. Palumbo, unpublished data; Dr. E. Camerer, unpublished data) and these mice are being used to determine the role of PAR1 and PAR2 in different cell types in multiple diseases. Similarly, transgenic mice have been made that express mutant forms of PAR1 and PAR2 that cannot be activated by particular proteases. For instance, $PAR1^{R41Q}$ cannot be activated by either thrombin or APC whereas $PAR1^{R46Q}$ cannot be activated by APC (28). Another mouse line was generated that expresses $PAR2^{G37I}$ that is resistant to activation by FXa (29). These mice will be useful to determine the role of different proteases in the activation of PARs in various diseases.



In this review, we summarize the effects of inhibiting different coagulation proteases, particularly FXa and thrombin, in wild-type mice and compare these results to the phenotypes of Par1 and Par2 deficient mice in various mouse models of inflammatory disease. We will not discuss studies on cancer because this topic has been previously reviewed (30-33).

## DOSES OF RIVAROXABAN AND DABIGATRAN ETEXILATE USED IN MOUSE STUDIES

The therapeutic dose of rivaroxaban used in humans is between 0.13 and 0.27 mg/kg (based on a patient weight of 75 kg), which gives plasma peak concentrations of 122 – 250 ng/ml (34,35). Dabigatran etexilate has a low bioavailability and therefore it requires a high therapeutic dose in humans between 2 and 4 mg/kg, which gives peak plasma concentrations of 121.6 – 172.9 ng/ml (dose between 2.9–4.0 mg/kg) (36-38). When using DOACs in mice, one has to consider the biological differences between humans and mice, including the faster metabolism of mice, the shorter half-life in mice and the fact that the drugs were developed to inhibit the human coagulation proteases (39). Therefore, higher doses of these drugs are needed for mice.

Rivaroxaban and dabigatran etexilate as well as other DOACs, such as edoxaban, are administrated to mice via either chow, oral gavage, or intravenous injection (Tables 1 and 2 ). Most studies report the dose in terms of milligrams per gram of chow. Only some studies report the plasma levels of the drug and/or present data from plasma-based functional assays that indicate the level of anticoagulation (Tables 1 and 2). Plasma levels of rivaroxaban and dabigatran can be measured using an anti-FXa based assay and chromatography mass spectrometry, respectively (40,41).

**Table 1. Effects of Rivaroxaban in Mouse Models of Inflammatory Disease**

| Animal Model | mg/g chow | [Riva] plasma (ng/ml) | PT (s) Ctr vs Riva | Major findings | Reference |
|---|---|---|---|---|---|
| apoE$^{-/-}$ | 0.006 | 6.5 | | Inflammation ↓<br>Stability → | [42] |
| apoE$^{-/-}$ | 0.031 | 24.2 | | inflammation ↓<br>plaque stability ↑ | [42] |
| apoE$^{-/-}$ WD | 0.031 | 28.5 | | inflammation ↓<br>atherogenesis ↓<br>plaque stability ↑ | [43] |
| apoE$^{-/-}$ WD | 1.2 | 210 | | inflammation ↓<br>atherogenesis ↓<br>plaque stability ↑<br>plaque regression | Unpublished* |
| Ldlr$^{-/-}$ WD | 1.2 | | | atherogenesis ↓ | Unpublished |
| MI I/R-O | 0.54 | 340 | | Inflammation ↓<br>Ejection Fraction ↑ | [44] |
| MI I/R-O | 1.2 | 790 | | Inflammation ↓<br>Ejection Fraction ↑<br>Survival ↑ | [44] |
| MI I/R-C | 1.6 mg/kg Bodyweight IV | | | Infarct size ↓<br>Ejection Fraction ↑ | Unpublished |
| MI LAD | 0.5 | 500 | 10→12 | Ejection Fraction ↑<br>Remodeling ↓ | [40] |
| SCD | 0.013 | ND | No change | | [45] |
| SCD | 0.2 | 75 | 14→22 | | [45] |
| SCD | 0.4 | 190 | 14→28 | Inflammation ↓ | [45] |
| CVB3 | 0.5 | | | Virus ↓ | Unpublished |
| IAV | 0.5 | | | Survival ↑ | Unpublished |

apoE-/- indicates apolipoprotein E deficient; CVB3, coxsackievirus B3; I/R-C, ischemia reperfusion closed chest; I/R-O, ischemia-reperfusion open chest; IAV, influenza A virus; LAD, left anterior descending; Ldlr-/-, low-density lipoprotein receptor deficient; MI, myocardial infarction; ND, not determined; PT, prothrombin time; SCD, sickle cell disease; TAT, thrombin-antithrombin; and WD, Western diet. *Thrombin generation assay lag time: 2.2→3.4 min.

**Table 2. Effects of Dabigatran in Mouse Models of Inflammatory Disease**

| Animal Model | mg/g chow | [Dabi] plasma ng/ml | aPTT (s) Ctr vs DE | Major findings | Reference |
|---|---|---|---|---|---|
| *apoE*$^{-/-}$ WD | 5.6 | 622 | | atherogenesis ↓<br>plaque stability ↑ | 46 |
| *apoE*$^{-/-}$ WD | 7.5 | 100-200 | | atherogenesis ↓<br>plaque stability ↑ | 47 |
| apoE$^{-/-}$ WD | 10 | 372 | [a] | atherogenesis ↓ | 48* |
| *apoE*$^{-/-}$ | 10 | | | atherogenesis ↓<br>plaque stability ↑ | 49 |
| *apoE*$^{-/-}$;<br>*Tm*$^{pro/pro}$;<br>Cuff WD | 7.5 | | [b] | inflammation ↓<br>atherogenesis ↓<br>plaque stability ↑ | 50† |
| SCD | 0.75 | 50 | | | 45 |
| SCD | 5 | 200 | | | 45 |
| SCD | 10 | 190 | 25→75[c] | | 45 ‡ |
| SCD | 15 | 900 | 25→95 | Spontaneous bleeding | Unpublished |
| NAFLD HFD | 10 | | | inflammation ↓<br>hepatic fibrin ↓<br>Steatosis ↓ | 51,52 |
| CVB3 | 10 | | 25→68 | Myocarditis ↑ | 53 |

apoE-/- indicates apolipoprotein E deficient; aPTT, activated partial thromboplastin time; CVB3, coxsackievirus B3; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; SCD, sickle cell disease; TAT, thrombin-antithrombin; TT, thrombin time; and WD, Western diet. *TT: 30→93. †TAT: 546→10 ng/mL. ‡TAT: 14→6 ng/L.

There is a wide range of doses of rivaroxaban that have been used in mice (0.006–1.2 mg/g chow) (Table 1). We found that a rivaroxaban dose of 1.2 mg/chow produced a plasma level of 150–260 ng/mL in apolipoprotein E deficient (apoE$^{-/-}$) mice fed a western diet (40.5% fat and 0.25% cholesterol) (Posma J and Spronk H, submitted). Rivaroxaban induces a linear, concentration-dependent prolongation of the prothrombin time, making it a suitable test to measure the level of anticoagulation with rivaroxaban (34). One study used three different doses of rivaroxaban (0.01, 0.2 and 0.4 mg/g chow) and found a significant increase in prothrombin time with the two higher doses (42). There is a smaller range of dabigatran etexilate doses used in different mouse studies (Table 2). Interestingly, the level of absorption of dabigatran etexilate is affected by the type of diet (43,44). The activated partial thromboplastin time (aPTT) correlates with the level of dabigatran at therapeutic plasma levels and is used to monitor the anticoagulation of this DOAC. However, the aPTT loses sensitivity at supra therapeutic levels of dabigatran (>200ng/ml) (45,46). At plasma levels <60ng/ml, the thrombin time is sensitive to the levels of dabigatran etexilate although no

correlation is observed at higher levels (47). One study evaluated three doses of dabigatran (5, 10 and 15 mg/g chow) in mice and found that the 10 and 15 mg/g doses significantly increased the aPTT (42). Another study found that a dose of 10 mg/g of dabigatran etexilate increased the thrombin time from 30 to 93 seconds (44). An oral dose of 100 mg/kg body weight of dabigatran etexilate did not cause any bleeding complication (48). This study showed that dabigatran is rapidly cleared in mice with a half-life of 1.25 hours. However, sickle cell mice bled spontaneously when dabigatran etexilate was given by chow at a dose of 15 mg/g chow, and wild-type mice bled spontaneously when they received a dose of 500 mg/kg of body weight by oral gavage (Sparkenbaugh E. and Pawlinski R, unpublished data).

## ATHEROSCLEROSIS

High levels of TF expression and procoagulant activity are present in human atherosclerotic plaques (49-51). TF expression has also been observed in a rabbit model of atherosclerosis (52). In addition, levels of TF expression are increased with progression of human lesions (53). ApoE-/- and low-density lipoprotein receptor deficient (Ldlr-/-) mice are the most common models used to study atherosclerosis in mice (54). Humans carry the majority of their lipoproteins in the low density lipoprotein subfraction, while mice transport their lipoproteins via high-density lipoprotein. Feeding apoE-/- and Ldlr-/- a Western diet, which is classically regarded as 42% fat and 0.2% cholesterol, results in very high levels of LDL that resembles distinct genetic disorders in humans (familial dysbetalipoproteinemia and hypercholesterolemia, respectively). However, mice fed a Western diet also have abnormally high chylomicrons and very low-density lipoprotein compared to humans. ApoE-/- mice develop spontaneous atherosclerotic lesions on regular chow but atherosclerosis is markedly accelerated by feeding a WTD (55,56). In contrast, most studies with Ldlr-/- mice use a Western diet because there is limited atherosclerosis on regular chow with young mice. Atherosclerosis is normally measured in the aortic sinus, ascending aorta and innominate artery. Addition of a cuff around the carotid artery creates vascular shear stress and vascular injury that accelerates atherosclerosis. Deletion of the apoE gene interferes with various processes, including macrophage and adipose tissue biology, whereas deletion of the Ldlr gene primarily affects LDL clearance from the liver (57,58). When interpreting mouse data, one also has to consider the differences between mice and humans, including differences in lesion distribution, medial layer size and lipoprotein transport (54,59). Both apoE-/- and Ldlr-/- mice fed a Western diet have a prothrombotic state, as measured by increased levels of plasma thrombin-antithrombin complex (61,61).

## EFFECT OF DEFICIENCIES OF PROCOAGULANT OR ANTICOAGULANT PROTEINS ON ATHEROSCLEROSIS IN MICE

Components of the coagulation system have been implicated in the development of atherosclerosis (60). Increased atherosclerotic lesion development was observed in apoE$^{-/-}$ mice expressing the hypercoagulable Factor V Leiden variant, which is likely due to an increased capacity for thrombin generation (61). Similarly, a 50% reduction in tissue factor pathway inhibitor, the endogenous inhibitor of TF, increased the development of lesions in apoE$^{-/-}$ mice fed regular chow (62). Conversely a 50% reduction of TF did not alter atherosclerotic lesion development in apoE$^{-/-}$ mice fed regular chow (63). Similarly, no difference in atherosclerotic lesion development was observed in Ldlr$^{-/-}$ mice reconstituted with bone marrow expressing low levels of TF on a Western diet (63).

Genetic studies have investigated the role of thrombin in the development of atherosclerosis. Interestingly, apoE$^{-/-}$ mice with 50% levels of prothrombin had reduced lesion burden on regular chow (64). The role for fibrinogen, the major physiological substrate of thrombin, in atherosclerosis is model dependent. In addition, it should be noted that fib$^{-/-}$ mice are difficult to breed. In the apoE$^{-/-}$ model, fibrinogen deficiency did not alter atherosclerotic lesion development in mice on normal chow (65). However, in a transgenic mouse model expressing human apo(a), deletion of fibrinogen significantly reduced lesion development in mice fed a Western diet due to reduced binding of apo(a) to fibrin(ogen) in the vessel wall (66). Finally, fibrinogen deficiency increased plaque development in Ldlr$^{-/-}$ mice with a deficiency in apolipoprotein B mRNA editing enzyme (apobec1) (67). Taken together, these genetic studies suggest that thrombin contributes to atherosclerosis in mouse models.

## EFFECT OF THROMBIN INHIBITION ON ATHEROSCLEROSIS IN MICE

The first study on the effect of the orally available thrombin inhibitor melagatran was performed in 2006 (68). Melagatran decreased progression of advanced lesions in apoE$^{-/-}$ mice on chow (68). In addition, melagatran decreased inflammation and macrophages in the lesion and increased collagen and fibrous cap thickness, which is suggestive of a stable plaque phenotype. Similarly, dabigatran etexilate reduced atherosclerosis in apoE$^{-/-}$ mice on chow (69). Moreover, three studies demonstrated that dabigatran etexilate (5.6 – 10 mg/g chow) reduced atherosclerosis in apoE$^{-/-}$ mice fed a Western diet (43,44,70). These studies also showed that thrombin inhibition reduced inflammation, macrophage accumulation, and necrotic core volume while increasing fibrous cap thickness and improving endothelial function. In the cuff model, dabigatran etexilate reduced atherosclerosis in Western diet fed apoE$^{-/-}$ mice expressing a

mutant version of thrombomodulin with reduced anticoagulant activity (64). In contrast, the thrombin inhibitor bivalirudin did not attenuate atherosclerosis (71). However, bivalirudin has a very short half-life and was administered daily via subcutaneous injection. Taken together, the majority of studies demonstrated that inhibition of thrombin decreased atherosclerosis and inflammatory mediators, such as IL-6, MCP-1, IFN-γ and TNF-α, in apoE$^{-/-}$ mice.

## EFFECT OF FACTOR XA INHIBITION ON ATHEROSCLEROSIS IN MICE

Low dose rivaroxaban attenuated the development of atherosclerosis and inflammation in the aorta of 8-week-old male apoE$^{-/-}$ mice fed a Western diet for 20 weeks (72). Interestingly, low dose rivaroxaban (0.006 and 0.031 mg/g chow) did not induce plaque regression in female apoE$^{-/-}$ mice fed regular chow for 26 weeks and then given rivaroxaban for an additional 26 weeks (72,73). However, both doses of rivaroxaban reduced the expression of inflammatory mediators, such as TNF-α and IL-6 (73). Additionally, the 0.031 mg/g dose of rivaroxaban stabilized the plaque phenotype as reflected by a thicker fibrous cap, smaller necrotic cores, and more collagen (73). We found that high dose rivaroxaban (1.2 mg/g diet) reduced the development of atherosclerosis in 8-week-old female Ldlr$^{-/-}$ mice fed a Western diet for 14 weeks in the cuff model (Posma J and Spronk H, submitted). Additionally, we examined the effect a high dose rivaroxaban (1.2 mg/g chow) on pre-existing plaques by feeding female apoE$^{-/-}$ mice WTD for 14 weeks and then administering rivaroxaban for 6 weeks in the cuff model. Rivaroxaban promoted regression of pre-existing plaques (Posma J and Spronk H, submitted). These differences are likely due to the use of a different dose of rivaroxaban (1.2 mg/g versus 0.03 mg/g chow) with the higher dose more typical for studies with rivaroxaban.

## EFFECT OF PAR1 DEFICIENCY OR PAR1 INHIBITION ON ATHEROSCLEROSIS IN MICE

PAR1 expression is increased in human and mouse atherosclerotic lesions suggesting that it may play a role in atherosclerosis (74). There are several studies that have investigated the role of PAR1 in atherosclerosis in the apoE$^{-/-}$ and Ldlr$^{-/-}$ models (Table 3). However, the data is inconsistent between the two models. In the apoE$^{-/-}$ mouse model with Western diet Par1 deficiency reduced atherosclerosis whereas Par1 deficiency had no effect in the Ldlr$^{-/-}$ model fed Western diet (74-76). Inhibition of PAR1 with the cell penetrating PAR1 pepducin PZ-128 also reduced atherosclerosis, macrophage content of plaques and inflammation in apoE$^{-/-}$ mice fed a WTD (71). However, one must be cautious with the interpretation of this data because PZ-128 also inhibits signaling from a PAR1/

PAR2 heterodimer (77). In vitro studies indicated that the thrombin-PAR1 pathway inhibits cholesterol efflux in macrophages and vascular smooth muscle cells, and contributes to leukocyte migration into lesions (75). These results suggest that PAR1 plays a role in the apoE$^{-/-}$ model but not in the Ldlr$^{-/-}$ model. Future studies should directly compare the phenotypes of apoE$^{-/-}$ mice treated with dabigatran etexilate and apoE$^{-/-}$ lacking Par1.

**Table 3. Effects of *PAR1* Deficiency in Mouse Models of Inflammatory Disease**

| Animal Model | Major findings | References |
|---|---|---|
| *apoE*$^{-/-}$ WD | Atherogenesis ↓ | 80 |
| *Ldlr*$^{-/-}$ WD | Atherogenesis ↔ | 79 |
| I/R-O | Remodeling↓ | 83 |
| SCD | | 45 |
| NAFLD | Inflammation ↓<br>Steatosis ↓ | 84 |
| NAFLD | Inflammation ↓<br>Steatosis ↓ | 51 |
| NAFLD | Inflammation ↓ | 85 |
| CVB3 | Infection ↑<br>Myocarditis ↑ | 53 |
| IAV | Infection ↑ | 53 |
| IAV | Inflammation ↓<br>Survival ↑ | 86 |
| PF | Fibrosis↓,<br>Inflammation↓ | 87 |

WD: Western diet; I/R-O: ischemia reperfusion open chest; SCD: sickle cell disease; NAFLD: non-alcohol fatty liver disease; CVB3: Coxsackievirus B3; IAV: Influenza A virus; PF: Pulmonary Fibrosis

Thrombin also activates PAR4 and is the main thrombin receptor on mouse platelets (19). Platelets have been shown to contribute to atherosclerosis in mice (78). An early study found that Par4 deficiency did not affect atherosclerosis in apoE$^{-/-}$ mice fed a Western diet (79). In contrast, we observed a significant reduction in atherosclerosis in male Ldlr$^{-/-}$ mice lacking Par4 fed a Western diet for 12 weeks (Owens AP 3rd and Mackman N, unpublished data). This suggests that the contribution of the thrombin/PAR4 pathway is relatively mild and can only be detected in the Ldlr$^{-/-}$ model.

## EFFECT OF PAR2 DEFICIENCY ON ATHEROSCLEROSIS IN MICE

PAR2 expression is increased in human atherosclerotic plaques suggesting that it may contribute to plaque progression (74. Several studies have determined the effect of Par2 deficiency on atherosclerosis in mice (Table 4). Par2 deficiency was associated with reduced atherosclerosis, reduced inflammation and increased plaque stability in apoE$^{-/-}$ mice fed a Western diet (80). This phenotype was confirmed and extended by a later study (81). Bone marrow transplantation experiments indicated that Par2 on hematopoietic cells but not non-hematopoietic cells drove atherosclerosis (81). Furthermore, in vitro studies showed that activation of PAR2 on macrophages increased inflammation. We found that a deficiency of Par2 also attenuated atherosclerosis in Ldlr$^{-/-}$ mice fed a Western diet (74). Mice lacking Par2 had decreased expression of the chemokines CCL2 and CXCL1 in the circulation (74). However, in contrast to the studies with the apoE$^{-/-}$ mice, bone marrow transplantation experiments indicated that Par2 on non-hematopoietic cells but not hematopoietic cells drove atherosclerosis in this model. In vitro studies indicated that activation of PAR2 on vascular smooth muscle cells induced Ccl2 and Cxcl1 expression and enhanced monocyte migration (74). Additionally, the PAR2 pepducin inhibitor PZ-235 showed no effects on lesion development in apoE$^{-/-}$ mice fed a Western diet (71). It is surprising that there are cell type differences in the roles of PAR2 in the apoE$^{-/-}$ and Ldlr$^{-/-}$ mice. Additional studies are needed to compare the phenotypes of wild-type mice treated with rivaroxaban and Par2$^{-/-}$ mice in the two models.

**Table 4. Effects of *PAR2* Deficiency in Mouse Models of Inflammatory Disease**

| Animal Model | Major findings | References |
|---|---|---|
| *apoE*$^{-/-}$ WD | inflammation ↓<br>atherogenesis ↓ | 90 |
| *apoE*$^{-/-}$ WD | inflammation ↓<br>atherogenesis ↓ | 43 |
| *LdlR*$^{-/-}$ WD | inflammation ↓<br>atherogenesis ↓ | 79 |
| I/R-O | Infarct Size↓<br>Inflammation ↓ | 91 |
| LAD | Ejection Fraction ↑<br>Remodeling ↓ | 40 |
| SCD | Inflammation ↓ | 45 |
| NAFLD | Glucose tolerance ↑<br>Insulin sensitivy ↑<br>Steatosis ↓<br>Inflammation ↓ | 92,93 |
| Diabetic nephropathy | Fibrosis ↓<br>Inflammation ↓ | 94 |
| CVB3 | Infection ↓<br>Myocarditis ↓ | 95 |
| IAV | Survival ↑ | 96 |
| IAV | Survival ↓ | 97 |

WD; Western diet; I/R-O: ischemia reperfusion open chest; LAD: permanent ligation model; NAFLD: non-alcohol fatty liver disease; CVB3: Coxsackievirus B3; IAV: Influenza A virus



## MYOCARDIAL INFARCTION

Myocardial infarction is a leading cause of morbidity and mortality. The most commonly used models of myocardial infarction in mice are either permanent ligation of the left anterior descending (LAD) artery or temporary ligation of the LAD followed by reperfusion (ischemia-reperfusion model (I/R)) (40,82). I/R can be either an open chest model (I/R-O) similar to LAD, where the opening of the chest precedes I/R on the same day (82), or where the ligature is placed around the LAD without tying and mice allowed to recover for 5–7 days before ligation (I/R-R) (83). In the I/R-O model the surgery contributes to the inflammatory response whereas in the I/R-R model most of the inflammation due to the surgery has resolved at days 5–7 (83). The I/R model provides information on the role of different pathways involved in reperfusion injury.

## EFFECT OF INHIBITION OF COAGULATION ON MYOCARDIAL INFARCTION IN MICE

We found that inhibiting the TF/FVIIa complex in mice with active site-inhibited FVIIa reduced infarct size in an I/R-R model that consisted of 60 minutes of ischemia and 2 hours of reperfusion (84). This was associated with reduced leukocyte infiltration and decreased gene expression of inflammatory mediators, such as IL-6, ICAM-1 and IL-1β (84). Similarly, the thrombin inhibitor hirudin also reduced infarct size in the I/R-O model that consisted of 30 minutes of ischemia and 2 hours of reperfusion (85). We determined the effect of rivaroxaban on infarct size in an I/R-R model by administering two doses of rivaroxaban via intravenous injection (1.6 mg/kg) 15 minutes after ischemia and 5 minutes after reperfusion. Rivaroxaban significantly reduced infarct size after 24 hours (Posma J and Spronk H, unpublished data).

In the LAD permanent ligation model, administration of rivaroxaban (0.5 mg/g chow) immediately after cardiac injury did not alter infarct size but reduced remodeling of the heart and preserved ejection fraction after 3 days and beyond compared to the placebo group (40). Interestingly, no protection was observed when rivaroxaban treatment was started 3 days post ligation. This suggests that FXa inhibition affects the acute inflammatory phase in this model. However, rivaroxaban treatment did not alter inflammatory mediators 2 days after ligation (40). In this study an open chest model was used that promotes inflammation which might have masked the effects of FXa inhibition on inflammation.

These studies indicate that coagulation proteases contribute to inflammation and infarct size after cardiac I/R injury. The protective effects seen with inhibition of coagulation after cardiac injury may be due to a combination of reduced fibrin deposition, activation of PARs and other downstream effects, such as generation of fibrin degradation products and platelet activation.

## EFFECT OF PAR1 DEFICIENCY ON MYOCARDIAL INFARCTION IN MICE

We found that Par1 deficiency did not affect infarct size but reduced remodeling two weeks post infarction in an I/R-O model (Table 3) (85). The lack of effect of PAR1 on infarct size may be because it mediates both pathologic and protective pathways via APC. Indeed, exogenous APC was shown to reduce infarct size after I/R injury and this effect was abolished in $Par1^{-/-}$ mice (86,87). As stated above, Par1 deficiency does not affect platelet activation in mice. However, Par4 deficiency reduced infarct size in an I/R-O model (88). These studies suggest that some of the beneficial effects of thrombin inhibition might be due to a reduction in PAR4 signaling.

## EFFECT OF PAR2 DEFICIENCY ON MYOCARDIAL INFARCTION IN MICE

We found that Par2 deficiency attenuated infarct size in an I/R-O model consisting of 30 minutes of ischemia and 2 hours of reperfusion (Table 4) [89]. In addition, hearts of Par2$^{-/-}$ mice had reduced levels of inflammatory mediators, such as IL-1β and TNF-α, and decreased remodeling compared with hearts from wild-type mice (89) . In the LAD permanent ligation model, Par2$^{-/-}$ mice had reduced remodeling and preserved ejection fraction after 28 days (90). This result is similar to the protective effects of rivaroxaban in the wild-type mice (40). Indeed, rivaroxaban did not provide any protection to Par2$^{-/-}$ mice in the LAD permanent ligation model (40). However, one must be cautious in interpreting these results because FXa and PAR2 may play roles in parallel pathways that contribute to cardiac remodeling after myocardial infarction. For instance, administration of rivaroxaban would reduce levels of the fibrin degradation fragment E1 which has been shown to exacerbate I/R injury (4). (Fig. 1). Additionally, activation of PAR2 by other protease would be abolished in Par2$^{-/-}$ mice.



## DIET-INDUCED OBESITY

Obesity is a global health care crisis with an estimated 34% of adults in the United States classified as obese (91). Obesity leads to chronic activation of the coagulation cascade and is a risk factor for the development of metabolic syndrome (92,93). High levels of TF are expressed in adipose tissue and there is abundant fibrin deposition in adipose tissue (94-96). The role of the coagulation cascade and PARs in diet-induced obesity has been studied using different mouse models that are associated with body weight gain, inflammation, macrophage recruitment to the adipose tissue, and insulin resistance.

Two studies from the Samad group found that Par2$^{-/-}$ mice and mice expressing a mutant form of TF that lacks the cytoplasmic domain had reduced weight gain in a diet-induced obesity model (97,98). It was proposed that hematopoietic cell TF-PAR2 signaling increases adipose inflammation, hepatic inflammation, hepatic macrophage recruitment and steatosis, whereas non-hematopoietic cell TF-FVIIa-PAR2 signaling drives obesity.

We found that mice expressing low levels of TF exhibited significantly less body weight gain when fed a high-fat diet (HFD) (45% kcal fat) for 16 weeks compared with controls (Hisada Y and Mackman N, unpublished data). Notably, adipocyte size was increased in epididymal and subcutaneous fat in wild-type controls but not in low TF mice (Hisada Y and Mackman N, unpublished data). Similarly, low levels of TF expression in hematopoietic cells were also associated with significantly less body weight gain in Ldlr$^{-/-}$ mice fed a Western diet compared with controls (93). Administration of dabigatran etexilate to

C57BL/6J mice reduced body weight gain when fed a HFD (96,99). Dabigatran also suppressed the progression of sequelae in mice with establish obesity (96). In contrast, a deficiency of Par1 did not affect diet-induced body weight gain (92,96). This initiated a search for the effector molecule(s) downstream of thrombin that drove obesity. One obvious candidate was fibrinogen. A previous study found that a sequence in the fibrinogen gamma chain (390–396) binds to macrophages via αMβ2 and that mutation of this sequence abolished binding (100). Strikingly, mice expressing this mutant fibrinogen $Fib\gamma^{390-396A}$ were protected from diet-induced body weight gain in a similar way to thrombin inhibited wild-type mice (96). Similar to the results we observed with low TF mice, the size of adipocytes was not increased in $Fib\gamma^{390-396A}$ mice fed a HFD (96). In addition, $Fib\gamma^{390-396A}$ mice had reduced numbers of macrophages in the adipose tissue (96). These results suggest that thrombin drives diet-induced obesity via fibrin-dependent inflammation in the adipose.

## NON-ALCOHOL FATTY LIVER DISEASE

Non-alcohol fatty liver disease is the livers manifestation of metabolic syndrome and is estimated to affect ~25% of the Western population (101). Mice fed a Western diet develop hepatic inflammation and fatty livers that is mainly due to the accumulation of triglycerides. This is referred to as steatosis. We found that low TF fed a HFD for 16 weeks did not develop steatosis (Hisada Y and Mackman N, unpublished data). Similarly, low levels of TF expression in hematopoietic cells reduced hepatic steatosis in $Ldlr^{-/-}$ mice fed a Western diet (92). Par1 deficiency also reduced hepatic inflammation and steatosis in C57BL/6J mice fed a Western diet for 3 months (92). Finally, $Fib\gamma^{390-396A}$ mice had reduced hepatic inflammation and steatosis when fed a HFD compared with controls (96). These results indicate that TF-thrombin-PAR1 and TF-thrombin-fibrin pathways contribute to hepatic inflammation and steatosis in mice fed a WTD.

## DIABETIC NEPHROPATHY

Diabetic nephropathy is the most common cause of end-stage renal disease in the United States (102). In a mouse model of diabetic nephropathy, FXa inhibition by edoxaban attenuated progression of the disease (103). This was associated with decreased expression of pro-inflammatory genes, such as TNF-α, and pro-fibrotic genes, such as TGF-β. Interestingly, Par2 deficiency gave a similar phenotype and edoxaban did not provide any additional protection to $Par2^{-/-}$ mice (103). The authors speculated that targeting FXa and/or PAR2 may reduce diabetic nephropathy in humans.

## SICKLE CELL DISEASE

A substitution of glutamic acid in normal hemoglobin for valine causes sickle cell disease. The mutant hemoglobin polymerizes and aggregates of hemoglobin tetramers leads to the formation of sickle red blood cells. Patients with sickle cell disease exhibit vaso-occlusion within postcapillary venules and have systemic inflammation and activation of coagulation (104). The two most common mouse models of sickle cell disease (Berkley (BERK) and Townes) have the mouse hemoglobin genes replaced with their human counterparts (105,106). BERK and Townes mice have severe anemia, systemic inflammation, and activation of coagulation (107).



The role of the clotting cascade in sickle cell mice has been analyzed using genetic and pharmacologic approaches. Inhibition of TF reduced both coagulation and the inflammatory mediator IL-6 in both BERK and Townes mice (107). Moreover, reducing TF expression in non-hematopoietic cells to ~1% in BERK mice reduced plasma IL-6, cardiac hypertrophy, and infiltration of neutrophils into the lungs but not activation of coagulation (108). Deletion of TF in endothelial cells also reduced plasma IL-6 but not activation of coagulation (42). Similarly, reducing circulating prothrombin to ~10% of wild-type levels using an antisense oligonucleotide decreased early mortality in BERK mice (109). In addition, a genetic reduction of prothrombin to ~10% of wild-type levels in BERK mice reduced inflammation, endothelial dysfunction and end-organ damage in the kidney, liver, and lung (109).

Interestingly, short-term FXa inhibition with rivaroxaban, but not short-term thrombin inhibition with dabigatran, significantly reduced IL-6 plasma levels in sickle cell mice (42). Similarly, Par2-/- mice but not Par1-/- mice with sickle cell bone marrow had reduced levels of plasma IL-6 (42). These results indicate that TF, FXa, and thrombin contribute to the activation of coagulation in sickle cell mice. In contrast, inflammation and end-organ dysfunction are more complex and appears to be driven by multiple pathways that include endothelial cell TF expression that activates PAR2 and thrombin-dependent pathways. The different phenotypes observed in sickle cell mice may be due to the use of genetic versus pharmacologic approaches and short- versus long-term studies.

## VIRAL INFECTIONS

The blood coagulation cascade is activated in response to viral infection and can lead to disseminated intravascular coagulation (110). Inhibition of the TF/FVIIa complex reduced inflammation and mortality in a primate model of Ebola hemorrhagic fever (111). The roles of PAR1 and PAR2 in mouse models of viral infections are controversial (110).

## COXSACKIEVIRUS B3

We found that inhibition of either TF with an antibody or thrombin with dabigatran etexilate increased viral load and myocarditis after infection of mice with Coxsackievirus B3 (CVB3) (112). Similarly, Par1$^{-/-}$ mice exhibited increased CVB3-induced myocarditis (Table 3). TLR3 is one of the main receptors in the innate immune system that detects single-stranded RNA viruses, such as CVB3. TLR3 can be activated by the double-stranded RNA mimetic poly I:C. Importantly, we found that activation of PAR1 enhanced poly I:C induction of IFN-β expression in murine cardiac fibroblasts, suggesting that PAR1 contributes to the innate immune response to single-stranded RNA viral infection (112). In complete contrast to the results with Par1$^{-/-}$ mice, Par2$^{-/-}$ mice were protected against CVB3-induced myocarditis (113). We also found that administration of rivaroxaban (0.5 mg/g chow) to wild-type mice decreased CVB3-induced myocarditis (Antoniak S and Mackman N, unpublished data). Interestingly, murine cardiac fibroblasts lacking PAR2 had higher levels of IFN-β expression after stimulation with poly I:C compared with wild-type cells, suggesting that PAR2 negatively regulates TLR3-dependent expression of IFN-β (113). Similarly, PAR2 inhibited TLR3-dependent expression of IFN-β in human epithelial cells (114). These studies suggest that TLR3-dependent activation of antiviral pathways is positively and negatively regulated by PAR1 and PAR2, respectively.

## INFLUENZA A VIRUS

Influenza A virus (IAV) is a single-stranded RNA virus. The Riteau group found that Par1 deficiency decreased inflammation and increased survival of mice infected with a mouse adapted H1N1 strain of IAV (Table 3) (115). In contrast, we found that Par1-/- mice exhibited a decrease in the innate immune response and increase in virus genomes after IAV infection (112). We have also observed increased mortality in Par1-/- mice compared with wild-type controls after IAV infection (Antoniak S and Mackman N, unpublished data). These variable results may be due to the use of a different dose of virus. Similarly, the results with Par2-/- are not consistent. The Riteau group observed that Par2-/- mice had increased inflammation and decreased survival after IAV infection (116). In contrast, the Vogel group found that Par2-/- mice exhibited increased survival compared with controls after IAV infection (114). Similarly, administration of rivaroxaban (0.5 mg/g chow) to wild-type mice increased survival after IAV infection compared with controls (Antoniak S and Mackman N, unpublished data). Further studies are needed to determine the roles of coagulation proteases and PARs in IAV infection.

## OTHER VIRUSES

PAR1 inhibition protected mice against respiratory syncytial virus and human metapneumovirus infection (117). Similarly, thrombin inhibition with argatroban reduced the pathogenicity of the infection with no additional effect to PAR1 inhibition. In vitro studies with human A549 cells showed that PAR1 inhibition reduced the replication of respiratory syncytial virus and human metapneumovirus infection (117). Further studies are needed to determine the roles of coagulation proteases and PARs in different viral infections.



## SEPSIS/ENDOTOXEMIA

Sepsis is induced by a systemic infection and activates the coagulation system. Endotoxemia is related to sepsis and is caused by endotoxins, most commonly bacterial lipopolysaccharide (LPS), in the blood. LPS induces TF expression in monocytes (118). Administration of LPS to mice leads to a rapid activation of coagulation. We found that mice expressing low levels of TF had less activation of coagulation and prolonged survival compared with controls in an endotoxemia model (119). In a subsequent publication, we demonstrated endotoxemia-induced activation of coagulation was initiated by both hematopoietic and non-hematopoietic sources of TF (120). Furthermore, inhibition of thrombin with hirudin prolonged survival but did not reduce inflammation. We also found that Par1 and Par2 deficient were not protected against endotoxemia (119). An independent study also showed that a deficiency of Par1, Par2 or Par4 did not affect inflammation or survival in an endotoxemia model that used different doses of LPS and both sexes (121). In contrast to these two studies, one study reported protection of Par1 deficient mice in an endotoxemia model (122). More recently, it was shown that mice expressing PAR1R41Q that cannot be activated by thrombin were not protected in a E.coli-induced pneumonia model (28). These studies indicate a role for TF and thrombin in the activation of coagulation in endotoxemia but the role of PARs in the inflammatory response in this model is controversial.

## IDIOPATHIC PULMONARY FIBROSIS

The coagulation cascade plays a critical role in hemostasis in the lung but also contributes to fibroproliferative lung diseases, such as idiopathic pulmonary fibrosis. The ACE-IPF trial investigated the effect of the anticoagulant warfarin in idiopathic pulmonary fibrosis but was stopped early due to excess risk of mortality (123). Bleomycin induces lung injury and fibrosis in mice and is an established model of human pulmonary fibrosis. We found that bleomycin increased TF expression in the lungs of mice (124). One study showed that

administration of a FXa inhibitor ZK80734 to mice reduced bleomycin-induced lung injury in mice (125). However, it is notable that 3/6 mice that received saline and ZK80734 and 3/9 mice that received bleomycin and ZK80734 were sacrificed due to intraperitoneal hemorrhage after administration of the drug. PAR1 is highly expressed in cells associated with fibrotic foci in idiopathic pulmonary fibrosis suggesting that it may contribute to fibrosis (126). Indeed, Par1$^{-/-}$ mice were protected from bleomycin-induced lung inflammation (127). In vitro studies showed that FXa activated PAR1 on human adult lung fibroblasts (125). PAR1 signaling leads to activation of TGF-β1 which is a key fibrotic mediator in many fibrotic conditions.

## CONCLUSION

Activation of the blood coagulation leads to the generation of multiple coagulation proteases, fibrin deposition, proinflammatory fibrin degradation products, platelet activation and PAR signaling (Fig.1). In atherosclerosis, inhibition of either FXa or thrombin reduces inflammation and lesion development. Similarly, a deficiency of Par2 reduces inflammation and atherosclerosis but PAR2 appears to play different roles in the apoE$^{-/-}$ and Ldlr$^{-/-}$ models. Par1 deficiency reduces atherosclerosis in the apoE$^{-/-}$ model and this may be due to reduced cholesterol influx and monocyte migration into lesions. However, Par1 deficiency had no effect in atherosclerosis in the Ldlr$^{-/-}$ model. In cardiac I/R injury, inhibition of either TF/FVIIa, FXa or thrombin reduces inflammation and infarct size. Similarly, Par2$^{-/-}$ mice but not Par1$^{-/-}$ mice had reduced infarcts compared with controls. In diet induced obesity mouse models, the TF/FVIIa-thrombin-fibrin pathway as well as PAR2-dependent pathways drive inflammation in adipose tissue. In a mouse model of sickle cell disease, the TF/FVIIa-FXa-PAR2 and TF-thrombin-PAR1 pathways drives inflammation and end-organ damage. The roles of PARs in viral infections are controversial. TLR3-dependent antiviral responses appear to be positively and negatively regulated by PAR1 and PAR2, respectively. Further studies are needed with mice that express either mutant forms of the PARs or with cell type-specific deletion of PARs to elucidate how coagulation protease-PAR pathways contribute to different diseases.

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# Chapter 4

# Targeting Coagulation Factor Xa Promotes Regression of Advanced Atherosclerosis in Apolipoprotein-E Deficient Mice

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## ABSTRACT

**Background and aims**

Atherosclerosis is a progressive inflammatory vascular disorder, complicated by plaque rupture and subsequently atherothrombosis. *In vitro* studies indicate that key clotting proteases, such as factor Xa (FXa), can promote atherosclerosis, presumably mediated through protease activated receptors (PARs). Although experimental studies showed reduced onset of atherosclerosis upon FXa inhibition, the effect on pre-existing plaques has never been studied. Therefore, we investigated effects of FXa inhibition by rivaroxaban on both newly formed and pre-existing atherosclerotic plaques in apolipoprotein-e deficient ($ApoE^{-/-}$) mice.

**Methods**

Female $ApoE^{-/-}$ mice (age: 8-9 weeks, n=10/group) received western type diet (WTD) or WTD supplemented with rivaroxaban (1.2mg/g) for 14 weeks. In a second arm, mice received a WTD for 14 weeks, followed by continuation with either WTD or WTD supplemented with rivaroxaban (1.2mg/g) for 6 weeks (total 20 weeks). Atherosclerotic burden in aortic arch was assessed by hematoxylin & eosin immunohistochemistry (IHC); plaque vulnerability was examined by IHC against macrophages, collagen, vascular smooth muscle cells (VSMC) and matrix metalloproteinases (MMPs). In addition, PAR1 and -2 expressions and their main activators thrombin and FXa in the plaque were determined in the plaque.

**Results**

Administration of rivaroxaban at human therapeutic concentrations reduced the onset of atherosclerosis (-46%, $p<0.05$), and promoted a regression of pre-existing plaques in the carotids (-24%, $p<0.001$). In addition, the vulnerability of pre-existing plaques was reduced by FXa inhibition as reflected by reduced macrophages (-39.03%, $p<0.05$), enhanced collagen deposition (+38.47%, $p<0.05$) and diminished necrotic core (-31.39%, $p<0.05$). These findings were accompanied with elevated vascular smooth muscle cells and reduced MMPs. Furthermore, expression of PARs and their activators, thrombin and FXa was diminished after rivaroxaban treatment.

**Conclusions**

Pharmacological inhibition of FXa promotes regression of advanced atherosclerotic plaques and enhances plaque stability. These data suggest that inhibition of FXa may be beneficial in prevention and regression of atherosclerosis, possibly mediated through reduced activation of PARs.

## INTRODUCTION

Atherosclerosis is a multifactorial disease, characterized by progressive chronic inflammation of the arterial wall (1), starting in response to lipid accumulation and subsequent inflammation in the arterial wall and together drive the formation of an atherosclerotic plaque (1). Disruption of atherosclerotic plaque is the underlying cause of luminal thrombosis (atherothrombosis), responsible for most acute coronary syndromes (2). Several studies showed that the plaque phenotype, a thin fibrous cap, large necrotic core, and presence of macrophages, determine atherosclerotic plaque vulnerability for rupturing and thus the risk of atherothrombosis (2-5). The exact processes contributing to plaque instability are poorly understood.



Accumulating evidence shows that key coagulation enzymes, such as FXa and thrombin, can influence a wide range of cellular actions related to cardiovascular function, such as vascular permeability, inflammation, and apoptosis. These non-hemostatic actions are predominantly mediated through activation of protease-activated receptors (PARs) (6-9). PARs belong to the family of G protein-coupled receptors. To date, four PARs have been identified, PAR1 to -4, which are expressed on a variety of cell types involved in atherosclerosis, including endothelial cells (EC), vascular smooth muscle cells (VSMC), fibroblasts, T lymphocytes, and monocytes (9-11). PAR1, -3 and -4 are predominantly activated by thrombin, whereas FXa activates PAR1, -2 and -3 (alone or in complex with tissue factor, factor VIIa) (7,12,13). Experimental animal studies demonstrated that transgenic mice carrying the $TM^{pro/pro}$ and $ApoE^{-/-}$ genes, resulting in respectively a hypercoagulable and pro-atherogenic phenotype, developed more vulnerable atherosclerotic plaques (14). In contrast, administration of direct thrombin inhibitors in atherogenic mice attenuates atherosclerotic plaque formation and promotes plaque stability by reducing inflammation, which is accompanied by a reduced expression of PAR1 (14,15). Similarly inhibition of FXa, utilizing a low dose of rivaroxaban, had a beneficial effect in $ApoE^{-/-}$ mice in terms of plaque stability and inflammation (16). In contrast, Hara et al (2015) reduced the plaque formation, stabilized the plaque, and decreased inflammation with a low dose of rivaroxaban in $ApoE^{-/-}$ mice. Additionally they showed decreased MMP-9 expression (17).

Experimental studies using either a FXa or thrombin inhibitor focused on newly formed atherosclerosis instead of the clinically relevant treatment of already developed atherosclerosis. We therefore studied the effects of FXa inhibition on pre-excising atherosclerotic plaques utilizing optimal direct FXa inhibitor plasma levels.

We hypothesize that pharmacologic inhibition of FXa at human therapeutic levels of rivaroxaban reduces progression of pre-existing atherosclerotic plaques in a mouse model for atherosclerosis.

# MATERIALS AND METHODS

### Animals

Female C57BL-6 ApoE$^{-/-}$ mice (Charles River, Maastricht, The Netherlands) were used throughout all experiments. Animals were housed in a temperature-controlled environment with a 12 h light/ 12 h dark cycle. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Maastricht University (Maastricht, The Netherlands) and all protocols were carried out in compliance with the Dutch government guidelines and the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

### Plaque progression models and pharmacological interventions

Female ApoE$^{-/-}$ mice (age, 8-9 weeks) were fed a western type diet (WTD) *ab libitum* throughout the experiments (15% cocoa butter, 1% corn oil, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 20% casein, free of cholate, total fat content 16%: ABdiets, Woerden, The Netherlands). In a pilot study, therapeutic rivaroxaban levels (150-350 ng/mL) were reached with WTD supplemented with 1.2mg/g rivaroxaban. In the first arm, female ApoE$^{-/-}$ mice (n=10/group) received WTD or WTD supplemented with rivaroxaban (1.2mg/g) for 14 weeks. In our second arm, termed regression model, mice (n=20) received initially a WTD for 14 weeks without rivaroxaban treatment. After 14 weeks, these mice were randomly divided in 2 equal groups (n=10/group): 1 group received WTD for 6 weeks and 1 group received WTD supplemented with rivaroxaban (1.2 mg/g) for 6 weeks to investigate the effects of FXa inhibition on pre-existing atherosclerotic. After experiments, all mice were anaesthetized with inhaled isoflurane (2.3%) and sacrificed using pentobarbital overdose for detailed analysis (Fig .1). In addition to this, blood was collected directly after sacrificing the mice, for further blood analysis.

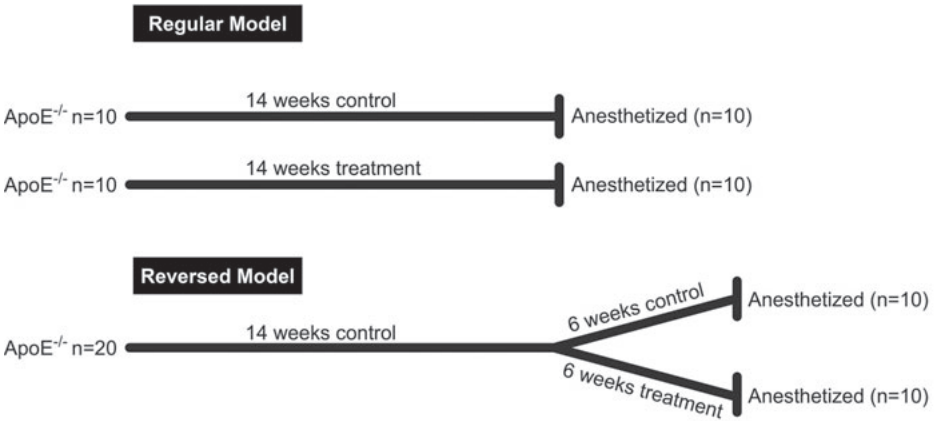


**Figure 1: Animal model of regression**
In the regular model, animals were either put on regular WTD as a control or WTD supplemented with rivaroxaban for 14 weeks. In our reversed model, all animals received WTD during the first 14 weeks. After 14 weeks, the group was divided in 2: 1 group continued with WTD for the remainder of 6 weeks, and one group was switched to WTD supplemented with rivaroxaban.



### Thrombin generation

Thrombin generation in plasma was measured by means of the Calibrated Automated Thrombography (CAT) method (Thrombinoscope BV, Maastricht, The Netherlands), employing a low affinity fluorogenic thrombin substrate (Z-Gly-Gly-Arg-amino-metyl-coumarin) to continuously monitor thrombin activity in clotting plasma. Measurements were conducted in 10 µL of 3.2% (w/v) citrated plasma in a total volume of 120 µL as described previously(16). Coagulation was triggered by adding 4 µM phospholipid vesicles (phosphatidyl serine/phosphatidyl ethanolamine/phosphatidyl choline, 20:20:60) and 1 pM tissue factor, followed by 14.5 mM (final concentrations) $CaCl_2$. To correct for inner-filter effects and substrate consumption, each thrombin generation measurement was calibrated against the fluorescence curve obtained in a sample from the same plasma, added with a fixed amount of thrombin-α2-macroglobulin complex (Thrombin Calibrator, Thrombinoscope BV, Maastricht, The Netherlands). Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and thrombin generation curves were calculated with Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). The curves were automatically analyzed for lag time, thrombin peak height, and endogenous thrombin potential (ETP; area under the thrombin generation curve).

### Determination of lipid levels and rivaroxaban concentration

Plasma concentrations of total cholesterol, triglycerides (TGL), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were determined enzymatically

in 3.2% (w/v) citrated plasma with a Cobas 8000 analyzer (Roche Diagnostics, Almere, The Netherlands). Rivaroxaban concentrations were measured in plasma based on a FXa dependent substrate hydrolysis reaction utilizing a Biophen DiXal kit (Aniara, Hyphen biomed) on an automatic coagulation analyzer (BCS-xp, Siemens Diagnostics Products Corporation, Marburg, Germany).

**Histological and morphometric analysis**

Aortic arches and carotid arteries of mice were acquired at the end of the experiment, fixed in formalin (10%) embedded in paraffin. Paraffinized aortic arches were cut in tissue sections of 5 µm. For immunohistochemical staining, tissue sections were dewaxed, rehydrated, and subsequently stained with hematoxylin and eosin (HE) (Klinipath, Duiven, The Netherlands) for morphometric analysis. Quantification of the atherosclerotic content in the aortic arch was performed by staining longitudinal sections of the aortic arch at 20 µm interval with H&E. Sections in which maximal lesion size was observed were used to measure the total surface of atherosclerotic plaques within the lumen side of the aortic arch.

For antibody-based immunohistochemical staining, endogenous peroxidase activity in aortic arches was inhibited with hydrogen peroxide (0.33% in methanol; Merck Millipore, Billerica, USA), and tissues were incubated with antigen retrieval solution (Sigma-Aldrich, St. Louis, USA) for 30 minutes and blocked with 5% normal serum in TBST pH= 7.4 for 60 minutes. Sections were then incubated overnight with primary antibodies in Tris-Buffered Saline 0.1% Tween (TBST, pH=7.4) and 3% normal serum. Applied primary antibodies: anti-factor X antibody (Novus Biologicals, Littleton, USA), anti-thrombin antibody (Novus Biologicals, Littleton, USA), anti-factor VII antibody (Novus Biologicals, Littleton, USA), anti-PAR1 antibody (Bioss Inc., Woburn, USA), anti-PAR2 antibody (Abcam, Cambridge, UK), anti-tissue factor antibody (Abcam, Cambridge, UK), anti-collagen type 1 antibody (Novus Biologicals, Littleton, USA), anti-alpha smooth muscle actin antibody (Abcam, Cambridge, UK), anti-MAC2 antibody (Abcam, Cambridge, UK). Sections were incubated with biotinylated secondary antibodies for 45 minutes followed by 60 minutes incubation in ABC complex (vectastain elite ABC HRP kit, Vector Laboratories, USA) according to manufacturers' protocol. For visualization ImmPACT NovaRED (Vector Bio-connect) was used. Collagen was visualized by Picrosirius red staining (Sigma-Aldrich, St. Louis, USA), calcification by alizarin red S staining (Sigma-Aldrich, St. Louis, USA) and necrotic core by toluidine blue staining (Sigma-Aldrich, St. Louis, USA), all according to the manufacturers' protocols. The extent of positive staining within the lesions was determined with ImageJ Software (National Institutes of Health, USA) in duplicate by operators blinded for treatment allocation.

### Statistical analysis

Statistical analysis was performed using Prism version 7 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS statistics 23.0 (SPSS Japan Inc., an IBM company, Tokyo, Japan). All data were analyzed using a Mann-Whitney U test. Data are shown as difference compared to 14 weeks or as median (IQR), unless otherwise stated. A 2-tailed $p<0.05$ was considered as statistically significant.

## RESULTS

### Inhibition of FXa reduces thrombin generation and does not affect body weight or plasma lipid profile in ApoE$^{-/-}$ mice

All mice treated with rivaroxaban reached therapeutic plasma levels (median: 210 ng/mL, range: 150-260 ng/mL). Treatment with rivaroxaban reduced *ex vivo* thrombin generation as reflected by increased lag time compared to controls (14 weeks: +56%, *p<0.0001,* and 20 weeks: +60%, *p<0.001*) and reduced ETP (14 weeks: -11%, *p<0.05,* and 20 weeks: -9%, *p<0.05*), whereas no statistically significant differences were observed in peak height (Table 1). During the entire experimental period, no bleeding complications were observed in mice. In addition, administration of rivaroxaban had no significant effects on body weight, total cholesterol, TGL, LDL, and HDL (Table 1).

**Table 1: Effects of rivaroxaban on body weight, metabolic profile, and thrombin generation**

| | 14 weeks WTD | 14 weeks WTD + Riva | *p-value* | 20 weeks WTD | *p-value* | 20 weeks WTD + Riva | *p-value* |
|---|---|---|---|---|---|---|---|
| Body weight (g) | 22 (21 – 24) | 23 (22 – 25) | *0.3726* | 24 (23 – 26) | *0.0446** | 24 (23 – 25) | *0.0138** |
| Rivaroxaban level (ug/L) | | 217 (156 – 349) | | | | 240 (150 – 247) | |
| **Lipid profile** | | | | | | | |
| Total cholesterol (mmol/L) | 18.25 (16.72 – 23.58) | 20.71 (19.50 – 23.40) | *0.4000* | 24.62 (22.10 – 27.67) | *0.1111* | 22.50 (20.20 – 25.13) | *0.3429* |
| TGL (mmol/L) | 0.47 (0.39 – 0.63) | 0.42 (0.41 – 0.47) | *0.5714* | 0.62 (0.48 – 0.74) | *0.4603* | 0.58 (0.50 – 0.62) | *0.4857* |
| LDL (mmol/L) | 15.35 (13.10 – 22.70) | 16.80 (16.50 – 23.20) | *0.3456* | 19.90 (18.00 – 23.10) | *0.4524* | 17.30 (16.65 – 22.23) | *0.3429* |
| HDL (mmol/L) | 5.15 (3.22 – 5.52) | 6.45 (4.73 – 7.45) | *0.4238* | 6.15 (2.67 – 6.72) | *0.2857* | 5.89 (4.95 – 7.89) | *0.4429* |
| **Thrombin generation** | | | | | | | |
| Lag time (min) | 2.08 (1.82 – 2.29) | 3.24 (2.61 – 3.50) | *<0.0001** | 2.33 (2.00 – 2.37) | *0.1322* | 3.33 (3.08 – 3.46) | *0.0002** |
| Peak height (nmol/L) | 71.65 (67.02 – 82.01) | 67.88 (64.21 – 69.59) | *0.0684* | 89.40 (82.54 – 94.51) | *0.0127** | 71.71 (61.70 – 77.72) | *0.7023* |
| ETP (nmol/L.min) | 451 (413 – 476) | 498 (405 – 574) | *0.0322** | 456 (411 – 534) | *0.8272* | 409 (376 – 431) | *0.0432** |

**Note:** All values are median(IQR), *p<0.05 vs 14 weeks WTD, n=10/group.
**Abbreviations:** TGL = Triglyceride, HDL = high-density lipoprotein, LDL = low-density lipoprotein, ETP = endogenous thrombin potential

**Coagulation is a key factor in the onset of atherosclerosis**

Quantitative analysis of atherosclerotic lesions in the aortic arch revealed a significant reduced onset of atherosclerosis in mice receiving rivaroxaban for 14 weeks, as compared to WTD (-46%, *p=0.001*) (Fig. 2). The findings were accompanied by a more stable plaque phenotype, as reflected by increased fibrotic cap thickness (29.50 μm (26.00 – 40.50) vs 41.00 μm (33.25 – 54.75), *p<0.05*), reduced macrophages staining (17.68% (13.25 – 19.46) vs 9.90% (9.23 – 11.24), *p<0.001*) and a smaller necrotic core (34.56% (27.85 – 40.63) vs 22.92% (14.94 – 28.43), *p<0.01*).

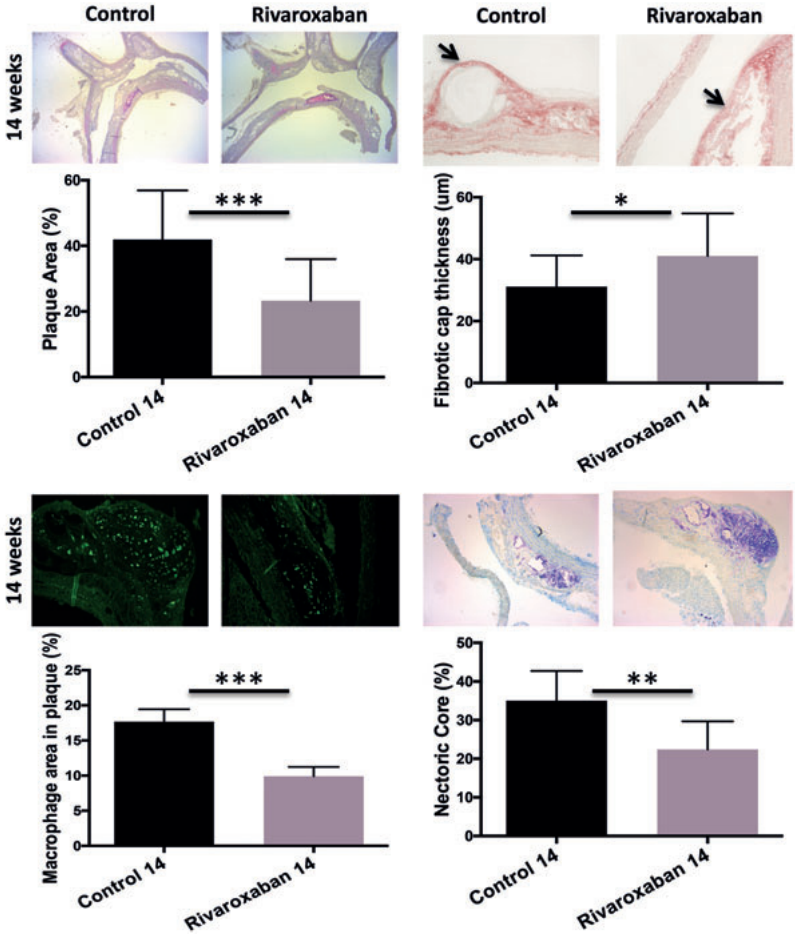


**Figure 2 Plaque development in the aortic arch of ApoE$^{-/-}$ mice.**
The aortic arch of rivaroxaban treated mice showed less plaque formation than control (-46%). This was associated with a more stable phenotype of the plaque as measured by a thicker fibrotic cap (29.50 μm (26.00 – 40.50)) compared to control (41.00 μm (33.25 – 54.75)), reduced macrophages (17.68% (13.25 – 19.46) vs 9.90% (9.23 – 11.24) and decreased necrotic core (34.56% (27.85 – 40.63) vs 22.92% (14.94 – 28.43). * p<0.05, ** p<0.01, *** p<0.001. All data were in median (IQR) n=10 for each group.

**Inhibition of FXa induces regression of advanced atherosclerotic lesions**
In the second arm, after an initial period of 14 weeks WTD, 6 weeks of rivaroxaban treatment at human therapeutic levels significantly reduced pre-existing atherosclerotic plaques (-24%, *p<0.001*), whereas plaque size remained equal in the control group compared to 14 weeks (+10%, *p=0.41*) (Fig. 3).

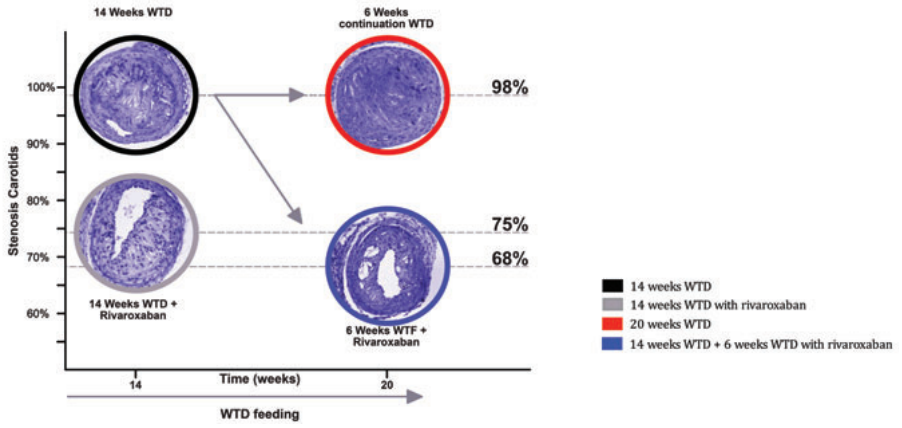


**Figure 3: Plaque regression in the carotids of ApoE$^{-/-}$ mice.**
(A) Carotids stained with hematoxylin & eosin (H&E), used to quantify the extent of atherosclerotic plaque in the luminal side after 14 and 20 weeks. (B) Quantitative analysis of area of atherosclerotic plaque in aortic arch lumen after 14 weeks WTD: 96.48% (87.27-99.33) vs WTD + rivaroxaban: 74.88% (60.83-80.60) and 20 weeks: 98.01% (96.45-99.56) vs 67.66% (56.43-80.97). All data were in median (IQR), n=10 for each group.

**Inhibition of FXa increases atherosclerotic plaque stability in advanced lesions**
The regressed atherosclerotic plaques in the second arm of the study were associated with enhanced plaque stability in rivaroxaban treated mice. This was reflected by elevated total collagen (+35%, *p<0.01*) in plaques of mice treated with rivaroxaban from 14 to 20 weeks, where total collagen tended to decrease in mice receiving WTD for 20 weeks (-18%, *p=0.06*). Furthermore, mean fibrotic cap thickness reduced from 14 to 20 weeks in control mice (-33%, p<0.05), while rivaroxaban preserved the fibrotic cap thickness after 6 weeks of treatment (+19%, *p=0.82*). These findings were supported by increased VSMCs levels (αSMA positive area, +33%, *p<0.05*) after treatment with rivaroxaban, compared to WTD only (-17%, *p=0.11)* (Fig. 4) and reduced collagen degradation proteins MMP9 and -13 (-45%, *p=0.02* and -36%, *p=0.01*).

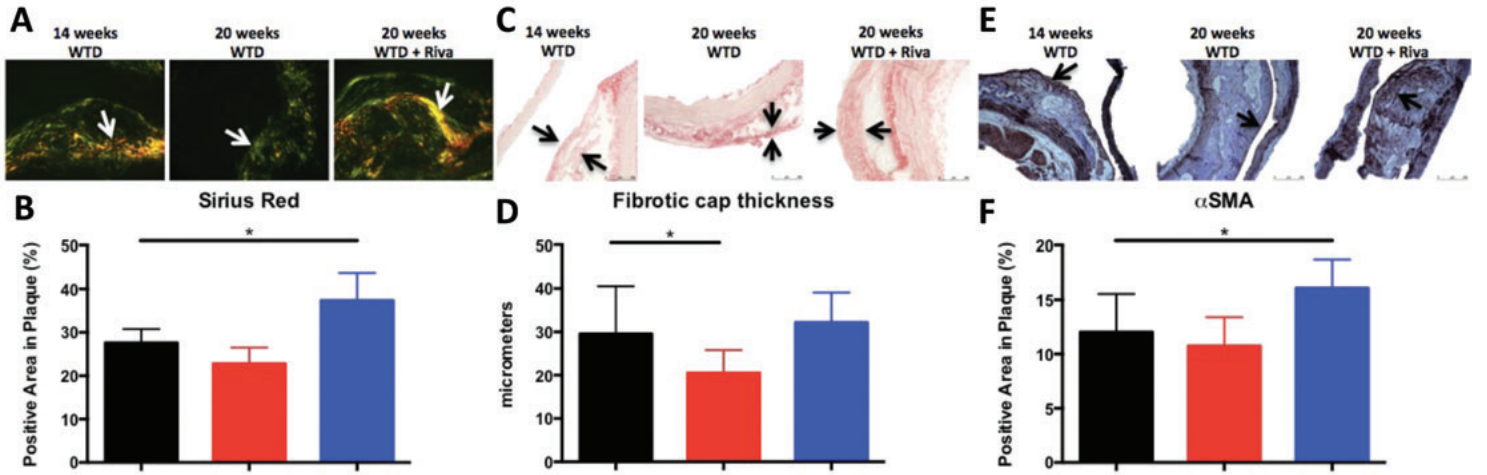


**Figure 4: Enhanced plaque stability in ApoE$^{-/-}$ regression mice treated with rivaroxaban.**
(A)Top row represents images of atherosclerotic plaque in the aortic arch stained with Sirius red, used to quantify total collagen of atherosclerotic plaque under polarized light after 14 and 20 weeks. (B) Quantitative analysis of total collagen in atherosclerotic plaque after 14 weeks: 27.62% (23.79–30.76) and 20 weeks: 22.65% (20.73–25.65) vs 37.03% (31.24–43.21). (C)Atherosclerotic plaque in the aortic arch, stained with Sirius red, used to quantify the mean fibrotic cap thickness of atherosclerotic plaque after 14 and 20 weeks. (D) Quantitative analysis of mean fibrotic cap thickness after 14 weeks: 29.50 μm (26.00–40.50) and 20 weeks: 20.00 μm (15.50–25.75) vs 35.00 μm (28.00–38.00). (E) Top row represents images of atherosclerotic plaque in the aortic arch stained against αSMA, used to quantify the expression of VSMC in atherosclerotic plaques. (F) Quantitative analysis of αSMA expression in atherosclerotic plaques after 14 weeks: 12.02% (10.83-15.55) and 20 weeks: 9.98% (8.98-11.91) vs 16.11% (13.95-19.04). All data were median (IQR), * $p<0.05$ vs 14 weeks, n=10 for each group.

### Targeting FXa reduces inflammation and necrotic core in pre-existing plaques

Treatment with rivaroxaban decreased inflammation in pre-existing atherosclerotic plaques, as revealed by reduced expression of macrophages (mac2 positive area) (-44%, *p=0.009*), while inflammation remained unchanged in mice receiving WTD from 14 to 20 week (-6%, *p>0.9999*). These findings were accompanied by a significantly reduced necrotic core upon rivaroxaban treatment (-25%, *p=0.001*), which increased upon continuation with WTD for 6 weeks (+35%, *p=0.0028)* (Fig. 5).

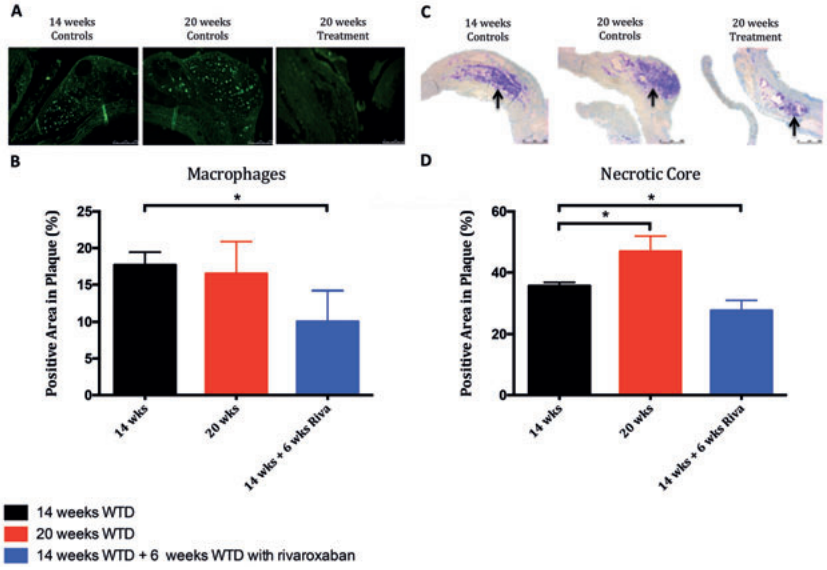


**Figure 5: Reduced inflammation and necrotic core in rivaroxaban treated ApoE$^{-/-}$ regression mice.**
(A) Atherosclerotic plaques in the aortic arch, stained against macrophages by Mac2, used to quantify the infiltration of macrophages in atherosclerotic plaque after 14 and 20 weeks. (B) Quantitative analysis of Mac2 after 14 weeks: 17.68% (13.25-19.46) and 20 weeks: 16.54% (12.39-20.42) vs 9.96% (6.20-13.44). (C) Top row represents images of atherosclerotic plaque in the aortic arch stained with toluidine blue, used to quantify the size of necrotic core within atherosclerotic plaques after 14 and 20 weeks. (D) Quantitative analysis of toluidine blue in atherosclerotic plaque after 14 weeks: 35.58% (33.73-36.75) and 20 weeks: 48.00% (42.00-51.00) vs. 26.84% (25.00-31.00) All data were median (IQR), * p<0.05 vs 14 weeks, n=10 for each group.

### Reduced expression of coagulation factors and PAR 1 and 2 in atherosclerotic lesions

IHC staining revealed a significant reduction of thrombin (-51%, *p<0.01*) and FX (-42%, *p=0.02*) in plaques of mice receiving rivaroxaban from 14 to 20 weeks, whereas levels of thrombin inside the plaque of WTD mice remained unchanged and FX levels significantly increased. In contrast, FVIIa remained unaffected in both groups (data not shown). Additionally, targeting FXa was associated with

a significantly reduced expression of PAR1 (-45%, *p<0.01*) and PAR2 (-48%, *p=0.01*) in atherosclerotic lesions as compared to WTD from 14 to 20 weeks (+5%, *p=0.90* and -5%, *p=0.96*), respectively (Fig. 6).

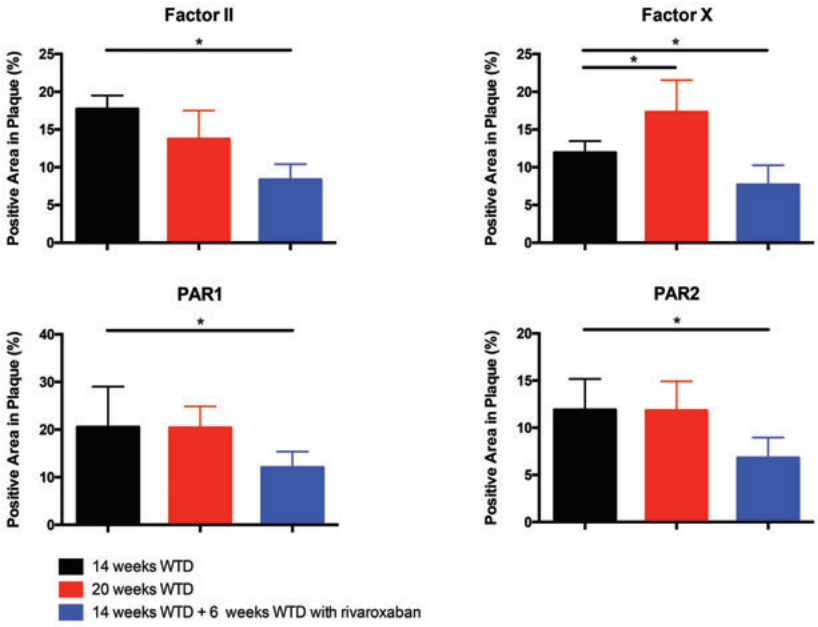


**Figure 6: Diminished coagulation factor II and X and PAR1, PAR2 expression in atherosclerotic plaques of ApoE$^{-/-}$ regression mice treated with rivaroxaban.**
(A) Quantitative analysis of FII after 14 weeks: 17.72% (12.05-19.52) and 20 weeks: 12.94% (10.58-16.55) vs 8.67% (7.11-10.15), (B) FX after 14 weeks: 11.94% (8.11-13.48) and 20 weeks: 18.67% (14.15-20.11) vs 6.98% (5.65-10.35). (C) PAR-1 after 14 weeks: 20.52% (14.53-29.03) and 20 weeks: 21.60% (16.53-23.90) vs 11.34% (9.01-15.89). (D) PAR-2 after 14 weeks: 11.90% (8.52-15.18) and 20 weeks: 11.32% (9.54-13.45) vs 6.20% (5.54-7.45). All data were median (IQR), * p<0.05 vs 14 weeks, n=10 for each group.

## DISCUSSION

In this study we show that treatment with the FXa inhibitor rivaroxaban not only decreases the onset and progression of atherosclerosis but also induces regression of already developed atherosclerotic lesions in ApoE$^{-/-}$ mice. These findings were accompanied by increased plaque stability and decreased macrophages, the latter indicative of reduced inflammation.

Potential mechanisms that led to a reduced progression and regression of atherosclerosis could involve reduced PAR2 activation by FXa. Besides preclinical studies showing the anticoagulant properties of rivaroxaban, inhibition of FXa by rivaroxaban may reduce inflammation in a PAR-2 dependent matter. Zhou et al. used a low dose of rivaroxaban (27.3 ng/mL in plasma) in ApoE$^{-/-}$ mice and observed no change in atherosclerotic volume. However, the expression

of inflammatory cytokines, such as TNF-α, IL-6 and MCP-1, decreased upon rivaroxaban treatment and stabilized the plaque (16). Another study that used a low dose of rivaroxaban (28.5 ng/mL in plasma) was able to attenuate progression of atherosclerosis by roughly 25% , as well as reduced inflammation, as reflected by decreased IL-1β, MCP-1, and TNF-α mRNA and protein levels (17). In our mice study rivaroxaban plasma levels were on average 210 ng/mL, comparable to the human therapeutic levels and 10 times higher compared to the two previous studies. With the higher rivaroxaban plasma levels, the decrease in atherosclerosis progression was more pronounced, moreover inhibition of FXa induced regression of atherosclerosis in the carotid arteries after 6 weeks of anticoagulant treatment. The increased impact of rivaroxaban on both progression and regression of atherosclerosis can potentially be explained by the higher dose of rivaroxaban applied in our model. However, we cannot rule out other pathways involved. Thrombin is also reduced in our model due to FXa inhibition, and is known to play a role in atherogenesis in a PAR1 mediated manner (14,15,18-21). Inhibition of thrombin with melagatran for 22 weeks in 30 weeks old ApoE$^{-/-}$ mice reduced the progression of atherosclerosis, which was accompanied by significant thicker fibrotic cap thickness and smaller necrotic core, but had no effects on macrophages in the plaque (15,22). However, a reduction of macrophages was observed upon inhibition of thrombin with dabigatran in other studies (14,19,21). Although it is difficult to compare the effects of direct FXa inhibition with thrombin inhibition due to different concentrations of anticoagulants used, it is tempting to speculate about the differences on how they affect atherosclerosis. Targeting FXa induces regression of atherosclerosis and enhances plaque stability, whereas effects of thrombin inhibition might be more limited. These potential differences might be explained by the additional inhibition of PAR2 signaling upon targeting FXa along with PAR1, whereas direct inhibition of thrombin only decreases PAR1 activation.

In conclusion, this study suggests the involvement of FXa in atherogenesis and that direct inhibition of FXa stimulates regression of atherosclerotic plaques. The clinical relevance of these findings merits further studies.

**Conflict of Interest**

The author(s) declare the following potential conflicts of interests with respect to the research, authorship, and/or publication of this article: S. Heitmeier is employee of Bayer AG.

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**Author contributions**

JJP, JNP, HS, PL, SH, HC, NM were involved in the study design. JJP, JNP, PL were involved in the execution of the study. JJP wrote the first draft and final manuscript with input from JNP, HC, HS, LS, AJ and NM. JJP performed statistical analysis and produced figures. PL performed animal treatment. JNP, RG, JJP, AJ performed immunohistochemical stainings. JJP and JNP performed immunohistochemical analysis. RO performed coagulation and lipid level assays.



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**Keywords**

Atherosclerosis, Atherothrombosis, Protease activated receptors, Regression, Plaque vulnerability

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Chapter 5

# FXa inhibition by the anticoagulant rivaroxaban modulates vessel wall cellular metabolism in atherosclerotic mice

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In Preparation

## ABSTRACT

**Background**

Atherosclerosis is a chronic inflammatory disease of the vasculature characterized by lumen narrowing lesion formation. We showed that inhibition of Factor Xa (FXa) by rivaroxaban not only reduced atherogenesis, but also promoted regression of plaques in ApoE$^{-/-}$ mice. By analyzing aortic bulk RNAseq data from ApoE$^{-/-}$ mice, we aim to find genes that could explain the beneficial effects of FXa inhibition on atherosclerosis.

**Methods**

Female ApoE$^{-/-}$ mice (age 8-9 weeks) on western type diet (WTD) or WTD+1.2mg/g rivaroxaban for 14 weeks. In a second arm, mice on WTD for 14 weeks, followed by WTD±1.2mg/g rivaroxaban for 6 weeks (total 20 weeks). RNA isolated from aortic arches, were used for sequencing.

**Results**

FXa inhibition modulated gene expression of over 200 genes in the 14-week development group. Gene ontology enrichment analysis revealed that mostly genes related to fatty acid metabolism were affected by FXa inhibition.

**Conclusion**

Direct inhibition of FXa by rivaroxaban attenuated atherosclerosis. Furthermore, inhibition of FXa significantly altered gene expression inside the vessel wall. Modulation of multiple biological pathways related to fatty acid metabolism highlight the multi-faceted role of coagulation FXa.

## INTRODUCTION

Atherosclerosis, as the underlying pathology of most myocardial infarctions and strokes, is the leading cause of mortality worldwide(1). It is characterized by chronic inflammation of the arterial vasculature and plaque deposition which narrows the lumen. In a later stage of atherosclerosis, the plaque may erode or rupture, resulting in atherothrombosis(2,3).

High expression of the hemostatic serine proteases factor VII (FVII), FX, FII, and their cofactors tissue factor (TF) and FV have been found in atherosclerotic lesions in both humans and animals (4–6). Multiple mouse studies have shown that hypercoagulability promotes atherosclerosis (7–10). In contrast, reduced expression of TF does not influence atherosclerosis (11) suggesting downstream effectors of TF, such as FXa and thrombin, mediate the pro-atherogenic effects of a hypercoagulable state. Indeed, we and others have previously shown that inhibition of FXa by the anticoagulant rivaroxaban decreases lesion formation and stabilizes plaques in mice (12–14). Interestingly, two independent studies showed that inhibition of FXa was associated with decreased markers of inflammation in atherosclerosis prone mice (12,14).



Bidirectional cross-talk between the coagulation system and inflammation has been established in both animal models and human studies and is potentially mediated through protease activated receptors (PARs) (15,16). PARs are a family of g protein-coupled receptors which comprise four subtypes: PAR1-4. PAR2 is the main receptor being activated by FXa and is present on multiple cell types involved in atherosclerosis, including vascular smooth muscle cells, fibroblasts, and macrophages. Activation of PAR2 can promote inflammation, among other processes. Two studies with PAR2 deficient mice independently showed an atheroprotective effect with a minor decrease in inflammation (17,18). However, one cannot simply relate the beneficial effects seen in PAR2 deficient mice to a decrease in FXa-mediated PAR2 activation since other proteases can also activate the receptor (15).

It has become evident that the coagulation system does play a pivotal role in the development of atherosclerosis. Although most of its effects are hypothesized to be caused by decreased PAR2-mediated inflammation, no conclusive evidence exists to date. Additionally, the effects of FXa by rivaroxaban on inflammation are sparse (12,14) and do not give strong evidence of an active FXa-PAR2 inflammatory cross-talk during atherosclerosis. To elucidate the mechanisms contributing to the protective effect of FXa inhibition on atherosclerosis, we first explored a wide array of inflammatory markers, both systemically and locally in the vessel wall, in atherosclerosis-prone mice supplemented with rivaroxaban. We could, however, not find strong evidence of decreased inflammation. Therefore, we used RNA-seq as an unbiased approach

to elucidate other potential mechanisms beyond inflammation that may explain the beneficial effects of FXa inhibition by rivaroxaban on atherosclerosis.

## METHODS

### Animals

8-9 weeks old female ApoE-/- mice (C57BL/6K background) originally purchased from Charles River (Maastricht, The Netherlands) were used in this study. All experimental procedures were approved by the Institutional Animal Ethics Committee and Central Commission Animal Testing and were carried out in compliance with the Duch government guidelines and the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Rivaroxaban was supplied by Bayer Pharma AG. To enhance atherogenesis, a carotid cuff-model was used as described previously (19). In the development/progression model, mice received *ad libitum* a western-type diet supplemented with 1.2 mg/g chow rivaroxaban for 14 weeks (fig 1)(13). Sex-age matched ApoE-/- fed a WTD served as controls. In the intervention/regression model, which lasted 20 weeks, mice were fed a WTD diet supplemented with 1.2 mg/g chow rivaroxaban for 6 weeks following a 14-week WTD (fig 1). At this time point (14 weeks), mice had developed advanced atherosclerotic lesions with profound necrotic cores and intraplaque hemorrhage before treatment was initiated. Sex-age matched ApoE-/- mice served as controls. Mice were housed in a 25 0C temperature-controlled room with a 12h light/12h dark cycle.

After the experiments, mice were anesthetized with 2.3% isoflurane and sacrificed with a pentobarbital overdose for further analysis. Blood was collected by I.V. administration of 100µl 3.2% (w/v) sodium citrate into the vena cava prior to blood drawing in the same vein in a syringe (20).

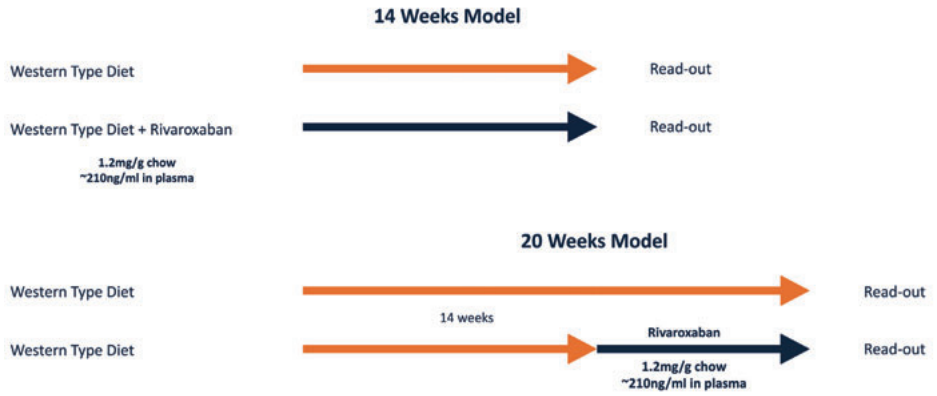


**Figure 1 Mouse model**
In the development model, mice received either a western type diet or a western type diet supplemented with 1.2mg/g chow rivaroxaban for 14 weeks. Mice in the regression model received a western type diet for 14 weeks. After 14 weeks, one group received western type diet supplemented with rivaroxaban for an additional 6 weeks, whereas the other group remained on western type diet without the addition of rivaroxaban.



### Determination of plasma lipids, plasma rivaroxaban levels, and thrombin generation

Plasma concentrations rivaroxaban were measured with BIOPHEN DiXal kit (Aniara, Hyphen biomed) on an automatic coagulation analyzer (BCS-xp, Siemens Diagnostics Products Corporation, Marburg, Germany). Plasma lipid levels were enzymatically determined using the Cobas 80000 analyzer (Roche Diagnostics, The Netherlands) and thrombin generation in plasma was measured by means of the Calibrated Automated Thrombography (CAT) method (Thrombinoscope BV, The Netherlands) (13).

### Preparation of aortic arches and carotid arteries

After sacrifice, aortic arches and carotid arteries were excised and periadventitial fat was carefully removed. After a 0.9% sodium chloride rinse, the arteries were snap-frozen in liquid nitrogen and stored in -80 0C until further use.

### Multiplex analysis of inflammatory markers

Per sample, the left and right carotid arteries of 2 mice were pooled, freeze-dried, and homogenized with a tissue grinder in PBS. For cell lysis, the samples were incubated with equal parts PBS and Cell Lysis Buffer 2 (R&D) for 30 minutes at RT with gentle agitation. The supernatant was collected after two cycles of centrifugation at 10,000g for 10 minutes and subsequently stored at -80 0C. Multiplex analysis of the inflammatory markers in both plasma and carotid artery homogenates was performed using a Millipore MAP Mouse cytokine/chemokine premixed 25-plex Immunology panel and 4 different customized R&D Mouse Magnetic Luminex Assay panels. The manufacturers' protocols were

followed. The multiplex plates were measured on a Luminex Magpix instrument with Magpix xPonent 4.2. Statistical analysis was done in R and heat-maps were created in Prism version 8.4.3 (GraphPad Software Inc, USA). Multiplex data were analyzed using the Mann-Whitney U test. Data are presented as median (IQR). The multiplex analysis was not designed for a large sample size and therefore corrections for multiple testing were not implemented.

**RNA-seq and Analysis**

Total aortic arch RNA was isolated using Trizol The RNA library was prepared using the NEBNext Ultra Direction Library Prep Kit for Illumina according to the manufacture's protocol at the Genetics Department of the University of Münster. Quality control and quantification were done using the Zymo DirectZol kit according to the manufacturer's protocol. The Illumina platform was used to sequence the libraries.

75 Base pair sequence, paired-end, directional reads were aligned to the mm10 reference mouse genome with STAR on the fly (21). After filtering out genes with a lower count than 5 in at least 2 samples, the DESeq2 Bioconductor R package (22) was used to identify significantly differentially expressed genes (p adjusted < 0.1) using the Benjamin-Hochberg procedure. Data were normalized with 5000 empirical controls using the RUVseq Bioconductor R package as described previously (23). The top 200 genes based on *p* adjusted were visualized in an Euclidean hierarchical-clustered heat-map with quantile-ordered color-keys utilizing the Pheatmap Bioconductor R package. The following analyses were only applied to the development model as a result of the low amount of DEG in the regression dataset. Gene ontology enrichment analysis was performed with an explorative *p* adjusted <0.15 using the ClusterProfiler Bioconductor R package (24) and plotted after removing redundant terms with the simplify method using a similarity cutoff > 0.7. The STRING database(25) and Cytoscape version 3.8.0 were used for association networks. Interactions with a high confidence (0.7) were included and disconnected nodes were removed. The interactions were exported to Cytoscape for data visualization. Immune cell repertoire was deconvoluted utilizing seq-ImmuCC as described previously (26).

## RESULTS

We previously reported that FXa inhibition by rivaroxaban attenuates the formation of plaques in ApoE-/- mice fed a WTD for 14 weeks (development model). We additionally showed that 6 weeks of FXa inhibition by rivaroxaban following a 14 week WTD promotes the regression of highly progressed atherosclerotic lesions in the carotid arteries of ApoE -/- mice (regression model) (13). Multiple studies have suggested that the beneficial effects of FXa inhibition are mediated by dampened inflammation. We therefore evaluated a wide

array of inflammatory markers in plasma and carotid artery homogenates of ApoE-/- fed a WTD supplemented with the FXa inhibitor rivaroxaban in both the development and regression model.

**Inflammatory markers in the development model and regression model**

Of the 64 markers measured in the development model, carotid artery CXCL10, MMP12, oncostatin, FABP4, and IL-4 and plasma CXCL10, oncostatin, CCL8, IL-2, IL-4 FAS ligand demonstrated levels outside of the reference range and were therefore excluded for further analysis. Markers with levels outside of the reference range in the regression model included carotid artery CXCL10, oncostatin, FABP4, and IL-4 and plasma CXCL10, oncostatin, IL-2 and IL-4.

A general overview of the cytokines and chemokines in the plasma of rivaroxaban treated mice and their controls is summarized in figures 2a and 3a. In the development model, 14 weeks of rivaroxaban treatment increased plasma levels of BAFF, CXCL16, MMP12, PIGF2 (2a and 2c [$p<0.05$]), while plasma levels of IL7 and PAI-1 were decreased after rivaroxaban treatment (Figures 2b and 2c). In the carotid artery homogenates, FXa inhibition increased protein levels of LIX, MMP8, and MMP10 (Figure 2b). Plasma markers tended to be less affected by rivaroxaban treatment compared to carotid artery proteins.



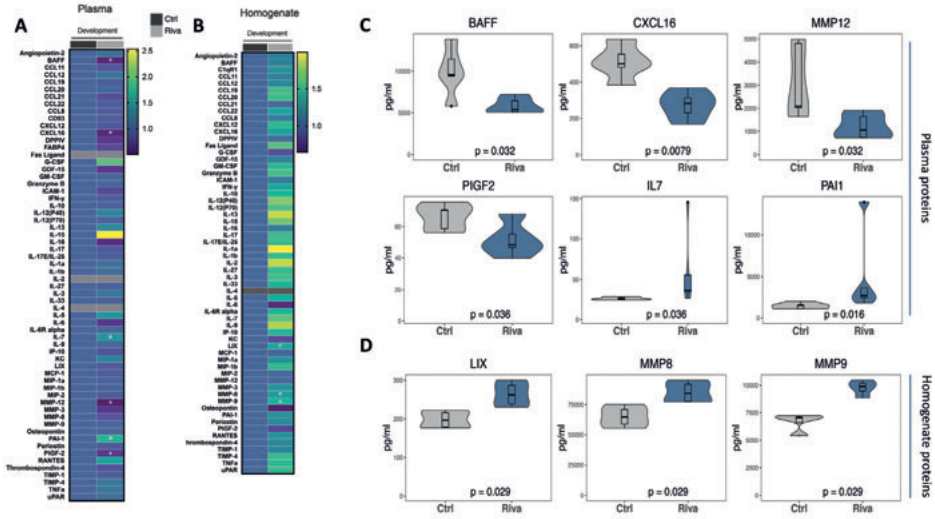


**Figure 2. Inflammatory protein analysis development model**
The heatmap represents a summary of the multiplex analysis of inflammatory proteins measured in **a)** plasma (n=5 per group) and **b)** carotid artery homogenates (n=4 per group). Control mice are depicted in the black columns, whereas the rivaroxaban treated mice are depicted in the gray columns. **c)** Plasma proteins modulated by rivaroxaban treatment. **d)** Carotid artery proteins modulated by rivaroxaban treatment. Values are presented as fold change compared to control. Mann Whitney U test was used to test for statistical differences between the treatment group and control. $P < 0.05$ was considered statistically different.

In the regression model, plasma levels of GM-CSF, IP10, and MIP-1b were increased after 6 weeks of rivaroxaban treatment while no decreased markers could be detected (Figure 3b). In the carotid artery homogenates, BAFF, CCL19, CCL20, FAS ligand, GM-CSF, IL17, IL3, IL6R, IL7, periostin, thrombospondin, and TIMP-4 were decreased after 6 weeks of rivaroxaban treatment (Figure 3b). CCL12 was increased after 6 weeks of rivaroxaban treatment when compared to the control. Similar to the development model, most changes in protein concentration tended to occur in the vessel wall and not in the plasma. However, in the development model, most vessel wall inflammatory proteins tended to be increased, whereas in the regression model, most inflammatory proteins tended to be decreased.

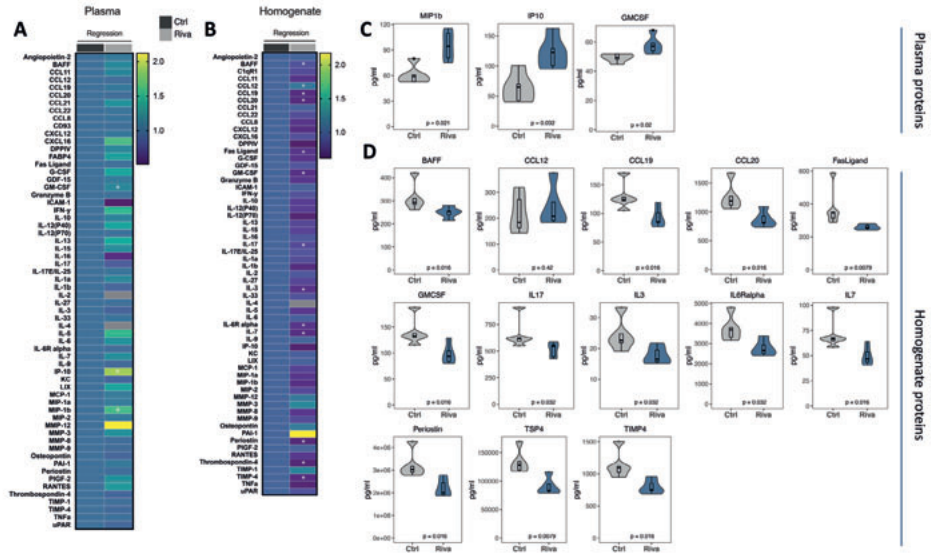


**Figure 3. Inflammatory protein analysis regression model**
The heatmap represents a summary of the multiplex analysis of inflammatory proteins measured in **a)** plasma (n=5 per group) and **b)** carotid artery homogenates (n=4 per group). Control mice are depicted in the black columns, whereas the rivaroxaban treated mice are depicted in the gray columns. **c)** Plasma proteins modulated by rivaroxaban treatment. **d)** Carotid artery proteins modulated by rivaroxaban treatment. Values are presented as fold change compared to control. Mann Whitney U test was used to test for statistical differences between the treatment group and control. $P < 0.05$ was considered statistically different.

### RNAseq development and regression model

Although rivaroxaban modulates the protein expression of some inflammatory markers that were measured, we observed an inconsistent expression profile of these markers in the plasma and homogenates and found an overall unexpected effect on inflammation. This led us to explore the protective effects of rivaroxaban on atherosclerosis with an unbiased bulk RNAseq analysis in aortic tissue. In the development and regression model, respectively, 17825 and

17326 genes were highly expressed in the aortic arch of ApoE-/- mice and were included in differential expression analysis. Two-dimensional representation of the development and regression data in two separate principal component plots showed that rivaroxaban treated mice and control mice tend to cluster in two distinct groups with a more pronounced clustering in the development (fig 4a) model than in the regression model (Figure 5a).

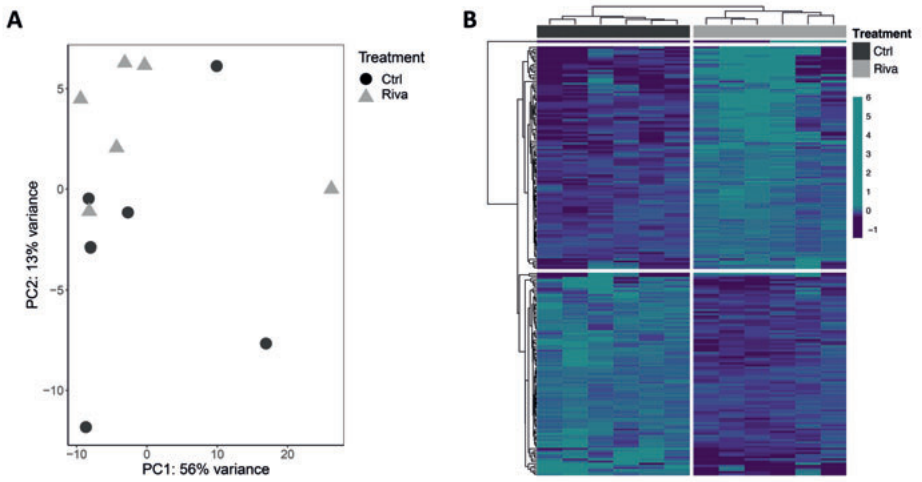


**Figure 4. Development model**
**a)** Principal component analysis of the 14-week model containing 12 entries: 6 control mice and 6 rivaroxaban treated mice. 17825 Genes were included in the analysis. The triangles correspond to control mice and the circles to rivaroxaban treated mice. **b)** The heatmap shows normalized, rlog transformed data of the top 200 DE genes in the development model based on p-adjusted. Euclidean hierarchical clustering was performed in the R Pheatmap package. Quantile-ordered color-keys represent row wise Z-scores. Mice are color-labeled according to their treatment (Rivaroxaban vs Control).

Differential expression analysis identified 94 down-regulated and 107 up-regulated genes in the development model. In the regression model, 34 genes were down-regulated while 18 genes were up-regulated. The top 10 up- and top 10 down-regulated genes are presented in table 1. Heatmap analysis of the 200 most DEG genes based on *p* adjusted value in the development model, distinguished 2 large clusters of genes between the treated and control groups (Figure 4b). In the regression model two similarly large clusters of DEG became evident when visualizing the 200 most DEG, although more variation was present between the mice (Figure 5b).

**Table 1. Top 10 up- and downregulated genes**

| Development Model | | | | Regression Model | | | |
|---|---|---|---|---|---|---|---|
| Gene | BaseMean | log2Fold-Change | *P*-adj | Gene | BaseMean | log2Fold-Change | *P*-adj |
| Gabrq | 114.91 | 0.80 | 0.08 | Ighg2b | 740.08 | 8.09 | 5.97E-05 |
| Chdh | 269.11 | 0.76 | 0.10 | Dbp | 380.33 | 1.73 | 0.07 |
| Rasl10b | 702.17 | 0.72 | 0.00 | Adtrp | 616.47 | 1.51 | 0.01 |
| Srl | 379.12 | 0.70 | 0.10 | Tuba8 | 238.71 | 1.38 | 0.02 |
| Acvr1c | 467.06 | 0.69 | 0.05 | Plin5 | 944.04 | 1.28 | 0.05 |
| Ppl | 472.62 | 0.66 | 0.04 | Klhdc7a | 855.52 | 1.27 | 0.07 |
| Lipe | 3906.70 | 0.60 | 0.05 | Nos1 | 136.65 | 1.1 | 0.08 |
| Prr33 | 245.60 | 0.56 | 0.01 | Aqp7 | 618.05 | 1.08 | 0.08 |
| Plin1 | 3736.27 | 0.56 | 0.08 | Ucp3 | 601.85 | 1.08 | 0.07 |
| Syngap1 | 188.35 | 0.53 | 0.07 | Cntfr | 108.6 | 1.07 | 0.1 |
| Neurl3 | 1126.62 | -0.56 | 0.01 | Dnajb4 | 3511.77 | -0.94 | 0.03 |
| Pianp | 375.91 | -0.58 | 0.08 | Bex1 | 621.01 | -0.95 | 0.07 |
| Lpcat2 | 873.62 | -0.59 | 0.00 | Apold1 | 160.67 | -0.96 | 0.04 |
| Edn1 | 1947.34 | -0.62 | 0.00 | Npas2 | 417.83 | -0.98 | 0.02 |
| Acpp | 189.93 | -0.64 | 0.05 | Hspa1b | 836.53 | -1.03 | 0.06 |
| Cxcr4 | 1636.69 | -0.66 | 0.02 | Hspa1a | 492.16 | -1.1 | 0 |
| Cemip | 1158.12 | -0.67 | 0.06 | Kcnip4 | 141.95 | -1.11 | 0.08 |
| Il12rb2 | 224.22 | -0.70 | 0.04 | Ccdc42 | 140.34 | -1.16 | 0.1 |
| Bcl2a1a | 118.05 | -0.72 | 0.07 | Gm830 | 474.12 | -1.27 | 0.07 |
| Chad | 791.71 | -0.85 | 0.07 | Ptx3 | 216.77 | -1.38 | 0.01 |

Genes with a BaseMean < 100 were filtered out. Top 10 down and upregulated genes are presented in the table and Log2foldchange-ordered. Data is analyzed with the Wald test and corrected with Benjamin-Hochberg.
Abbreviations: *P*-adj = *P* adjusted.

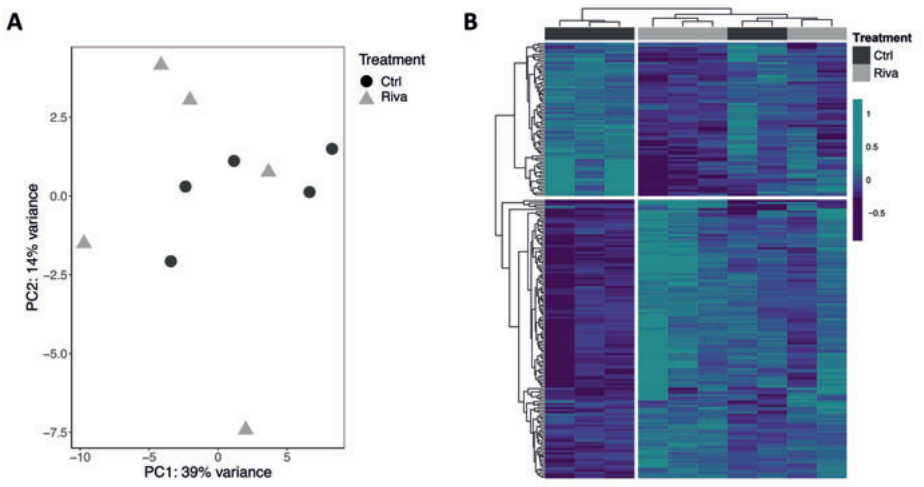


**Figure 5. Regression model**
**a)** Principal component analysis of the regression model containing 10 entries, 5 Control mice and 5 rivaroxaban treated mice. 17326 Genes were included in the analysis. The triangles correspond to control mice and the circles to rivaroxaban treated mice. **b)** The heatmap shows normalized, rlog transformed data of the top 200 DE genes in the regression model based on p-adjusted. Euclidean hierarchical clustering was performed in the R Pheatmap package. Quantile-ordered color-keys represent row wise Z-scores. Mice are color-labeled according to their treatment (Rivaroxaban vs Control).



Gene Ontology enrichment analysis was performed to find enriched biological processes in the development model. Most significantly enriched biological processes induced by FXa inhibition included terms related to lipid energy metabolism, such as fatty acid metabolism, cofactor metabolic process, organic acid metabolic process, energy derivation by oxidation of organic compounds, and oxidative phosphorylation (Figure 6).

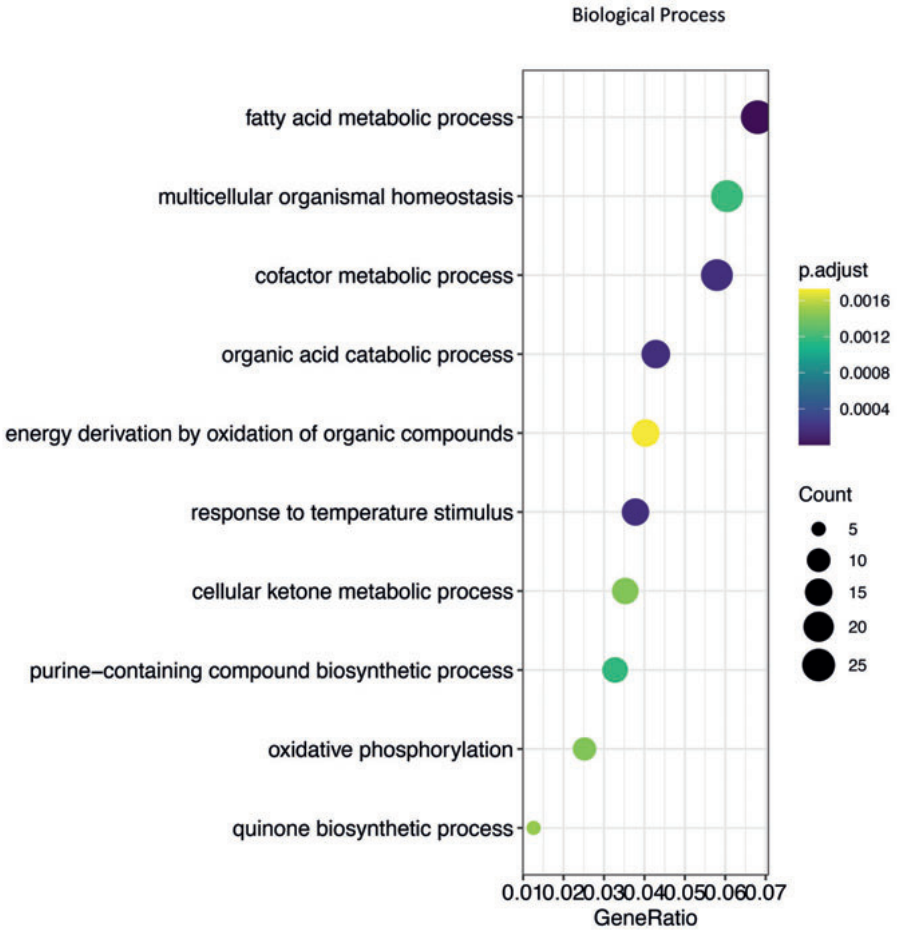


**Figure 6. Gene ontology enrichment analysis of DEG in the development model.** The functional enrichment analysis of DEG genes in the development model was performed with clusterProfiler in R using an explorative threshold of DEG with padj < 0.15. The Simplify method was implemented to remove redundant pathways

Gene association analysis subsequently revealed that the genes related to fatty acid metabolism and mitochondrial energy metabolism were mostly up-regulated in the rivaroxaban treated mice (fig 7). Deconvolution of the immune repertoire did not reveal relative changes in plaque resident immune cells between the rivaroxaban group and the control group (data not shown). Due to the larger variation in gene expression between mice in the regression model and consequently the lower number of DEG, no enrichment analysis was performed with the regression dataset.

Overall, treatment with rivaroxaban in atherosclerosis prone mice up-regulates expression of genes related to lipid metabolism and mitochondrial energy production.

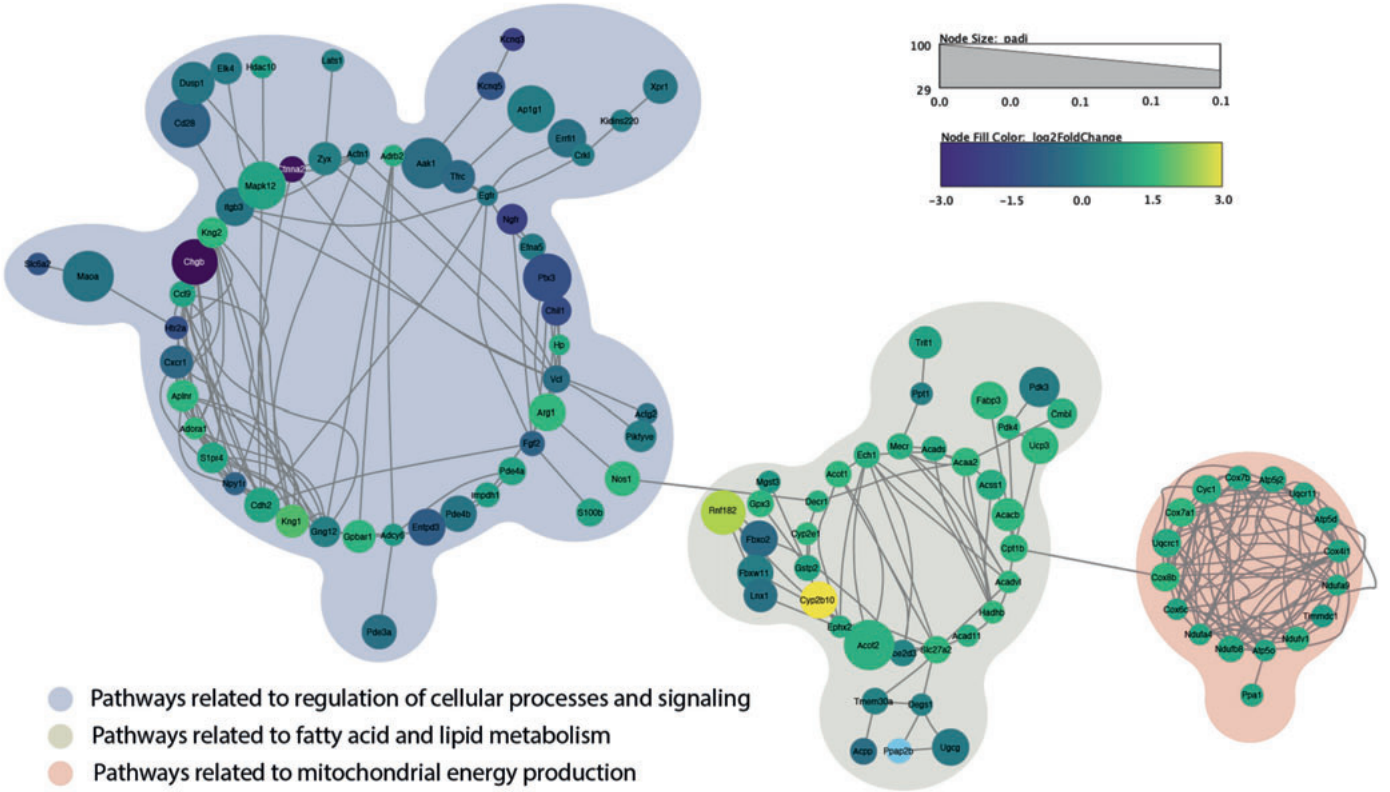


**Figure 7. Gene interaction analysis.**
Gene interaction analysis was performed with String inside the Cytoscape environment. Genes with padj < 0.15 were included in this analysis. **a)** The largest cluster of DEG in our 14-week model belong to pathways related to regulation of cellular processes and signaling. **b)** The second largest cluster is mostly upregulated and belongs to pathways related to fatty avid and lipid metabolism. **c)** All genes in this cluster belong to mitochondrial energy production related pathways and are all enhanced by rivaroxaban treatment.

## DISCUSSION

Accumulating evidence suggests that FXa modulates multiple cellular processes beyond its function in coagulation (15). In line with this, we previously showed that rivaroxaban attenuates the formation of atherosclerotic lesions and promotes regression of already established plaques (13). To further unravel the protective effects of FXa inhibition we measured a broad range of inflammatory markers in plasma and vessel wall. We additionally used RNA sequencing to unravel novel pathways. In both the development and regression model we here confirm previous reports showing that FXa inhibition by rivaroxaban attenuates inflammation systemically and locally (12,14,27). FXa mediated up-regulation of inflammation is considered to be caused by activating the g-protein coupled receptor protease activated receptor 2 (PAR2), a receptor present on multiple cell types involved in atherosclerosis (e.g. endothelial cells, vascular smooth muscle cells and macrophages) (15). Activation of PAR2 promotes up-regulate inflammation, and knockout of PAR2, both systemically (17,18) and on haemapoietic cells (18) results in atheroprotection and decreased inflammation. Additionally, Jones et al showed that PAR2 deficiency in cells other than the haematopoietic compartment (hematopoietic cells were the only cells expressing PAR2) results in the same atheroprotective phenotype (17). Although there is some inconsistency between the cell types involved in the PAR2 phenotype, which is potentially caused by the different mouse strain, modulating PAR2 signaling has profound effects on atherosclerosis. However, none of these studies are able to correlate FXa inhibition with the protective phenotype seen in PAR2 knockout models as multiple proteases activate PAR2, and other downstream effectors of FXa also modulate atherosclerosis. Novel mutant mice that specifically target FXa mediated PAR2 activation such as the G37I PAR2 mutant are needed to shed light on the role of the FXa-PAR2 axis in atherosclerosis.

The anti-inflammatory effects we and others have found after rivaroxaban treatment were not irrefutable and might not fully explain the robust atheroprotective phenotype seen in mice treated with anticoagulants (12,14,27). We therefore used RNA sequencing on aortic arches from ApoE-/- mice treated with rivaroxaban to unravel novel pathways that contribute to the atheroprotective phenotype. Surprisingly, in our RNA seq dataset, differentially expressed inflammatory RNA, present in the aortic arch, were rare in both the development and regression models, suggesting that other pathways are more profoundly involved in atheroprotection. Indeed, gene ontology enrichment analysis, using significantly differentially expressed genes in the development model, revealed that pathways belonging to fatty acid metabolism were mostly influenced by FXa inhibition.

Atherosclerosis is characterized by excessive deposition of LDL particles in the vessel wall where they become oxidized and promote inflammation. Macrophages are key players in clearing these oxLDL particles by engulfing them for transportation back to the circulation (28). Not all macrophages are equally capable of removing oxLDL. Pro-inflammatory subtypes can less efficiently clear LDL particles from the vessel wall, in contrast to anti-inflammatory macrophages (29). Recently it was shown that macrophages exposed to oxLDL are "trained" towards a long-term pro-inflammatory phenotype; this so-called trained immunity is mediated by epigenetic and metabolic reprogramming (30,31). Pro-inflammatory trained macrophages rely on glycolysis as their energy source while anti-inflammatory macrophages use beta oxidation of fatty acids to provide in their energy needs (32).

Recently it was shown that rivaroxaban is able to penetrate tissues and locally interfere with macrophage specific FXa-PAR2 signaling, thereby modulating macrophage polarization(33). Additionally, Chen et al. showed that activation of PAR2 promotes macrophage polarization towards the pro-inflammatory M1-like phenotype (34). It is temping to speculate that the atheroprotective effects of rivaroxaban are caused by inducing a phenotype switch of macrophages, rendering them more capable of clearing oxLDL particles. This phenotypic switch would then be mediated by a change in macrophage metabolism from glycolysis towards lipid metabolism. However, more research is needed to fully elucidate these novel pathways leading to atheroprotection.

Unfortunately, we were not able to find similar effects on gene expression in the development and regression model. One must take into account that different processes might be involved in the development model vs the regression model (early vs late atherosclerosis) as our data suggests. Indeed, targeting inflammation with IL-1β monoclonal antibodies attenuated the development of atherosclerosis in an early phase (35) while IL-1β inhibition negatively affected the composition of atherosclerotic lesions in advanced stage (36). Additionally, the high degree of variation between the mice in our regression model compared to the traditional development model, where mice from the same group readily clustered together, can explain the few significantly differentially expressed genes found in the regression model.

In conclusion, millions of patients worldwide are treated on a daily basis with anti-coagulants and accumulating evidence suggests these drugs influence multiple processes beyond coagulation. For the first time, we now show that FXa inhibition by rivaroxaban modulates cellular metabolism and that this is associated with atheroprotection. To fully understand the effects of rivaroxaban on cellular metabolism and its long-term effects on atherosclerosis, more research should be conducted.

## ACKNOWLEDGMENTS

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Chapter 6

# Targeted protein biomarker discovery to predict cardiovascular outcome in peripheral artery disease

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In Preparation

## ABSTRACT

**Background**
Peripheral artery disease (PAD) is characterized by atherosclerotic plaque formation in peripheral vascular beds. PAD patients are at increased risk of cardiovascular events and mortality despite current treatment strategies. Biomarker research to improve risk stratification thus far focused on pathways known to be associated with atherothrombosis, but other pathways might play more important roles in its pathophysiology.

**Aim**
To explore the association between a broad set of cardiovascular biomarkers with cardiovascular risk.

**Methods**
Between 2018 and 2020, 120 PAD outpatients were enrolled in this observational cohort study. Blood samples and patient data were collected upon inclusion and all patients were followed for one year in which the composite endpoint (myocardial infarction, elective coronary revascularization, ischemic stroke, acute limb ischemia and mortality) was assessed. Citrated platelet-poor plasma was used to analyze 184 biomarkers combined in Olink Cardiovascular panel II and III using a proximity extension assay.

**Results**
Fifteen (12.5%) patients reached the composite endpoint. These patients had more prior strokes (33.3% vs 8.6%, p=0.016) and higher serum creatinine levels (97 (81-154) vs 86 (73-104), p=0.036). Multivariate cox regression analysis with LASSO was performed adjusting for multiple testing and correcting for age, gender, prior myocardial infarction or stroke and creatinine levels. Increased plasma levels of protease-activated receptor 1 (PAR1), galectin-9 (Gal-9), tumor necrosis factor receptor superfamily member 11A (TNFRSF11A) and interleukin 6 (IL-6) were most predictive for cardiovascular events and mortality. Positive regulation of acute inflammatory responses and leukocyte chemotaxis were identified as involved biological processes.

**Conclusion**
This study identified IL-6 and three new biomarkers (PAR1, Gal-9, TNFRSF11A) as potent predictors for cardiovascular events and mortality in PAD.

## INTRODUCTION

Peripheral artery disease (PAD) involves atherosclerotic plaque formation in peripheral vascular beds leading to progressive blood flow restriction in large and medium-sized arteries. PAD patients are at increased risk of atherothrombotic events such as myocardial infarction, ischemic stroke or cardiovascular death, with an incidence of 5% to 14% each year (1). This high incidence of cardiovascular events within PAD populations is in part caused by concomitantly affected vascular beds, apart from the peripheral vascular beds, in more than 60% of all PAD patients (2, 3). We previously characterized a cohort of PAD patients managed according to current guidelines with lipid-lowering drugs, antihypertensive drugs and antiplatelet drugs (Kremers et al. submitted). Despite the good compliance to this combined medication, there was still a 10.6% incidence of thrombotic complications and mortality within one year. This high rate of complications illustrates the needs to better identify patients at highest risk that would benefit from more intensive cardiovascular risk management. Although several biomarkers have been identified in previous studies, as summarized in our recent systematic review (4), it is striking to see that none have been implemented yet in clinical management. One reason is the perception that current biomarkers including high-sensitivity c-reactive protein (hs-CRP), neutrophil-lymphocyte ratio (NLR), fibrinogen, d-dimer, N-terminal pro brain natriuretic peptide (NT-proBNP) and high-sensitivity cardiac troponin T (hs-cTnT) still lack power to tailor individual patient management. In other vascular diseases, like atrial fibrillation, the ABC-score comprising the biomarkers NT-proBNP and hs-cTnT, can be used to estimate stroke risk (5). Pursuing a similar strategy in PAD patients should start with searching candidate biomarkers with the potential to identify patients with an increased cardiovascular risk.

We therefore decided to explore a broad set of cardiovascular biomarkers from different biological processes that were not known to be associated with risk stratification in PAD.



## METHODS

### Study design

Outpatients of the department of Vascular Surgery of the Maastricht University Medical Center (MUMC+) were screened for PAD between 2018 and 2020. Eligibility for study participation was based on the ankle-brachial index, which had to be 0.9 or below. Within the selection of patients with an abnormal ankle-brachial index, we selected patients with Fontaine II (intermittent claudication) or Fontaine III (chronic limb ischemia). Patients with Fontaine IV were not eligible due to increased inflammatory parameters rising from ulcer formation. Active malignancy, chronic inflammatory disease, coagulation disorders or

anticoagulant therapy, pregnancy and an age below eighteen were also reasons or exclusion. All eligible patients willing to participate were included after written informed consent was obtained. Upon inclusion, patient characteristics were collected and blood was drawn from the patient. All patients were followed for one year in which the primary outcome was assessed. The Medical Ethics Committee of the MUMC+ approved the study (NL63235.068.17).

**Blood collection and sample storage**

Venous blood was drawn upon inclusion by antecubital venipuncture with 21-gauge needles and 3.2% (w/v) citrated Vacutainer glass tubes. The blood collection tubes were immediately processed using the standard platelet-poor plasma centrifugation (4000 x g for 5 minutes and 11000 x g for 10 minutes). After centrifugation, the plasma aliquots were frozen and stored at -80°C.

**Data collection and outcome**

Patient characteristics were recorded at baseline including gender and age of each patient as well as a history of myocardial infarction, ischemic stroke and PAD revascularization. Information on the presence of traditional risk factors smoking, renal insufficiency, diabetes mellitus type 2 (DM2) and body mass index (BMI) was also obtained. Kidney function was evaluated by measuring plasma creatinine levels. The outcome of the study comprised a composite endpoint of myocardial infarction, stroke, acute limb ischemia, elective percutaneous coronary intervention or coronary artery bypass grafting and all-cause mortality during one year of follow-up. Outcome verification took place by a combination of telephone calls to the patient and hospital records.

**Biomarker analysis**

Citrated platelet-poor plasma was used to measure protein concentrations using the ProSeek Cardiovascular II and III panels (Olink Biosciences, Uppsala, Sweden). These panels are based on proximity extension assay (PEA) technology allowing simultaneous measurements of 92 protein biomarkers per panel. Pairs of oligonucleotide-labeled antibodies bind pairwise to target proteins present in 1mL of plasma, leading to the formation of a new polymerase chain reaction (PCR) target sequence formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified by standard real-time PCR. Measurements are specified as Normalized Protein Expression (NPX), generated from the PCR quantification cycles. NPX data are then used to establish protein signatures where high NPX values equal high proteins concentrations and low NPX values equal low proteins concentrations.

**Statistical analysis**

Statistical analysis was performed on the whole cohort and a comparison was made between patients who reached the composite endpoint (event group) and patients who did not (no event group). Differences in baseline characteristics for continuous variables were presented as mean with standard deviation or median with interquartile range, as appropriate. Dichotomous and categorical variables were defined as frequencies with percentages and compared using the Fisher's Exact test or chi-square testing, while continuous variables were compared using the parametric two-samples t-test or the non-parametric Mann-Whitney U test. Protein expression levels following non-normal distributions were transformed into normal distributions using logarithmic transformation. First, the relation of the standardized biomarker levels (mean=0 and SD=1) with the cardiovascular outcome was assessed using individual Cox Hazard proportional regression models adjusted for age, gender, prior myocardial infarction, prior stroke and plasma creatinine levels. Then, to identify a subset of best predictive biomarkers for cardiovascular events and mortality during follow-up, LASSO regression analysis was performed with a 10-fold cross validation to increase generalizability of the models (glmnet package (6)). The selected biomarkers were shown as hazard ratios and 95% confidence intervals from previous individual Cox regression models. Due to the explorative nature of this study, a nominal p-value < 0.05 was used to reach statistical significance. All analyses were performed using R (R Core Team (2013) version 3.5.3. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).



## RESULTS

Baseline characteristics for the full cohort are shown in *Table 1*. All 120 patients completed the one-year follow-up, although 4 patients were unable to give blood and could therefore not be included in the biomarker analysis. The cohort comprised 70 (58.3%) male patients with an average age of 67.7 (±9.6) years. Most of the patients had a prior PAD revascularization 84 (70%) while 39 (32.5%) had a prior myocardial infarction and another 14 (11.7%) suffered from a prior stroke. The average BMI was 26.5 (±4.5) kg/m$^2$ and 53 (44.2%) patients were current smokers upon inclusion. DM2 was diagnosed in 31 (25.8%) patients and the median plasma creatinine level was 90 (74-105 mmol/L).

Within the whole cohort 15 patients reached the composite endpoint with recordings of 9 myocardial infarctions, 3 elective coronary interventions, 2 ischemic strokes and 1 death. Age and gender did not differ between patients with and without events, however more prior strokes were observed in patients with events (5 (33.3% vs 9 (8.6%), p=0.016). Kidney function was significantly worse in patients with events (plasma creatinine 97 (81-154) mmol/L vs 86 (73-104)

mmol/L, p=0.036) while other cardiovascular risk factors were similar between the groups.

**Table 1 Baseline characteristics for the whole cohort and distribution between patients with and without cardiovascular events during follow-up.**

| | Whole cohort | Event group | No event group | P-value |
|---|---|---|---|---|
| | Mean±SD / Median (IQR) / n (%) | Mean±SD / Median (IQR) / n (%) | Mean±SD / Median (IQR) / n (%) | |
| Age (years) | 67.7±9.6 | 72.1±7.7 | 67.1±9.7 | 0.056 |
| Male gender | 70 (58.3) | 8 (53.3) | 62 (59) | 0.440 |
| Prior myocardial infarction | 39 (32.5) | 8 (53.3) | 31 (29.5) | 0.064 |
| Prior ischemic stroke | 14 (11.7) | 5 (33.3) | 9 (8.6) | **0.016*** |
| Prior PAD revascularization | 84 (70) | 12 (80) | 72 (68.9) | 0.281 |
| Current smoking | 53 (44.2) | 8 (53.3) | 45 (42.9) | 0.312 |
| Body Mass Index (kg/m²) | 26.5±4.5 | 27.1±5.2 | 26.4±4.5 | 0.557 |
| Diabetes Mellitus type 2 | 31 (25.8) | 5 (33.3) | 26 (24.8) | 0.335 |
| Creatinine (µmol/l) | 90 (74-105) | 97 (81-154) | 86 (73-104) | **0.036*** |

Significance was reached when p < 0.05 (*).

All biomarkers were added to the multivariate cox regression analysis with correction for age, gender, prior myocardial infarction or stroke and creatinine levels, revealing 13 proteins to be positively predictive for cardiovascular events and mortality. Placenta growth factor (PGF) appeared to have the highest hazard ratio (HR [95% Confidence interval (CI)]) (HR 4.03 [1.48-10.95]) followed by heat shock protein 27 (HSP; HR 3.18 [1.37-7.35]), protease-activated receptor 1 (PAR1; HR 3.15 [1.40-7.07]), adrenomedullin (ADM; 3.10 [1.16-8.29]), galectin-9 (Gal-9; HR 3.03 [1.45-6.32]), tumor necrosis factor superfamily member 11A (TNFRSF11A; HR 2.46 [1.20-5.03]), interleukin-6 (IL-6; HR 2.02 [1.35-3.02]), brain natriuretic peptide (BNP; HR 2.02 [1.20-339]), N-terminal pro brain natriuretic peptide (NT-proBNP; HR 2.01 [1.01-4.00]), interleukin-4 receptor subunit alpha (IL4ra; HR 1.99 [1.22-3.26]), Dickkopf-related protein 1 (Dkk1; HR 1.93 [1.01-3.68]), matrix metalloproteinase-12 (MMP12; HR 1.89 [1.06-3.39]) and chitinase-3-like protein 1 (CHI3L1; HR 1.83 [1.03-3.27]). Another 2 proteins were negatively predictive for cardiovascular events and mortality, being p-selectin glycoprotein ligand 1 (PSGL1; HR 0.57 [0.34-0.98]) and plasminogen activator inhibitor 1 (PAI-1; HR 0.45 [0.23-0.88]) *(Figure 1)*.

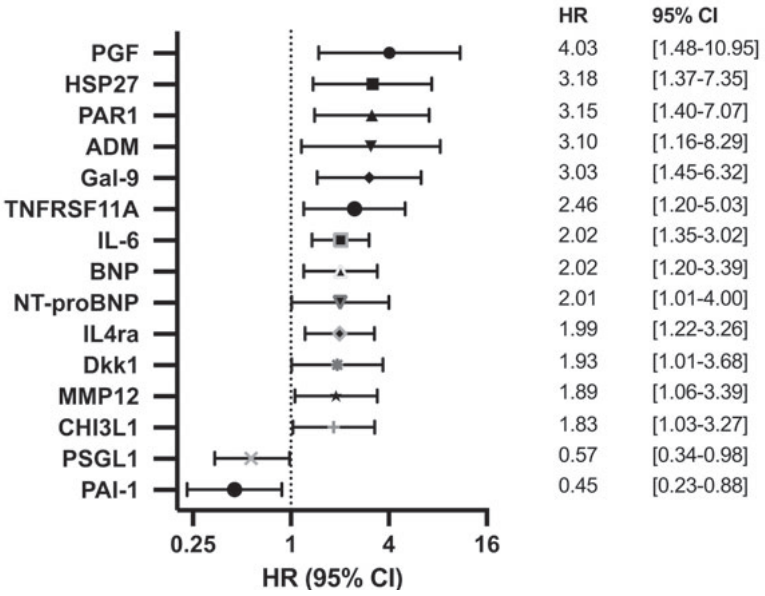


**Figure 1.** Biomarkers with a significant positive or negative predictive value for cardiovascular events and mortality, identified by multivariate cox regression analysis.



To identify the most predictive biomarkers across all proteins, LASSO regression analysis was performed and revealed protease-activated receptor 1 (PAR1), galectin-9 (Gal-9), tumor necrosis factor receptor superfamily member 11A (TNFRSF11A) and interleukin 6 (IL-6) as most predictive biomarkers for cardiovascular events and mortality *(Figure 2)*. PAR1 showed the highest predictive value with a hazard ratio of 3.15 [1.40-7.07] followed by Gal-9 (HR 3.03 [1.45-6.32]), TNFRSF11A (HR 2.46 [1.20-5.03] and IL-6 (2.02 [1.35-3.02]).

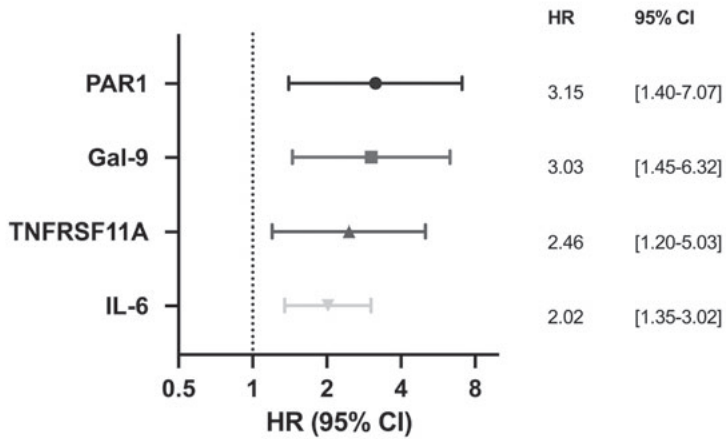


**Figure 2.** Biomarkers with significantly increased plasma levels in patients with events, identified by multivariate cox regression analysis with LASSO.

## DISCUSSION

Patients with PAD are at increased risk of future cardiovascular events and death despite current optimal treatment strategies. Biomarkers may potentially help to identify patients with different risk profiles. The main finding of our study is that four proteins (IL-6, PAR1, TNFRSF11A and Gal-9) are linked to cardiovascular outcomes in PAD patients and therefore are novel candidate biomarkers for risk assessment in PAD.

The pro-inflammatory cytokine IL-6 is locally synthesized at inflammatory sites and IL-6 induces the synthesis and release of several acute phase proteins such as C-reactive protein (CRP) and fibrinogen (7). IL-6 has a variety of functions, making it a key player in the inflammatory response in different stages of atherosclerosis. In early stages, IL-6 coordinates influx of inflammatory cells into atherosclerotic lesions. Complex formation between endothelial cells and IL-6 increases expression of intercellular adhesion molecule 1 (ICAM-1) and thereby enables leukocytes to be recruited and transmigrated into the vessel wall (8). IL-6 has also been shown to increase the surface expression of tissue factor on cultured monocytes, thereby enhancing hemostasis (9). In later stages of atherosclerosis, growth and progression of atherosclerotic lesions are promoted by IL-6 through induction of platelet-derived growth factor (PDGF) which causes growth of vascular smooth muscle cells (10). In the final stages of atherosclerosis, including atherothrombosis, IL-6 induces aggregation and activation of platelets and thereby accelerates thrombus formation. Aggregation of platelets is stimulated through the production of fibrinogen (11) while activation is enhanced through increased expression of P-selectin (12). Combining these functions, IL-6 appears to play an important pro-atherogenic role throughout all stages of atherosclerosis, including thrombus formation and arterial occlusion.

PAR1 is a membrane-bound protein mostly found on endothelial cells, platelets and vascular smooth muscle cells. As PAR1 is a transmembrane receptor, measured plasma concentrations of PAR1 do not represent functional receptor. However, PAR1 is known to be internalized by multivesicular bodies (MVB). Cargo from MVBs can be degraded by lysosomes or secreted as exosomes. This suggests that PAR1 concentrations in the plasma might reflect exosome PAR1 concentrations. Furthermore, PAR1 plasma levels can also reflect cell death (13). Thrombin is the main activator of PAR1 (14), indicating a prominent role for the receptor in the hemostatic system and thus in late stages of atherosclerosis. Here, increased activation of the hemostatic system with upregulation of circulating thrombin might cause an increase in PAR1 receptor expression(15). PAR1 however plays, just like IL-6, an important role throughout various stages of atherosclerosis. In early stages, matrix metalloproteinase-9 (MMP-9)-mediated PAR1 activation induces endothelial dysfunction leading to a loss of vascular integrity (16). PAR1 on endothelial cells can also be activated by activated protein

C (APC) upregulating monocyte chemotactic protein 1 (MCP-1). MCP1 however promotes not only pro-inflammatory effects, but also anti-inflammatory effects, indicating anti-atherogenic effects of PAR1 activation (17, 18). In later stages however, pro-atherothrombotic effects become more visible as activation of PAR1 on platelets leads to platelet activation through thromboxane A2 production and aggregation through P-selectin upregulation (19, 20). Overall, activation of PAR1 induces mostly pro-atherosclerotic effects and thereby increases the cardiovascular risk, making it a potent predictive plasma biomarker.

TNFRSF11A is commonly known as receptor activator of nuclear factor kappa-B (RANK) and is part of the RANK/RANKL/OPG signaling pathway which regulates osteoclast differentiation and activation (21). Activation of NF-kB is usually mediated by RANK ligand, however overexpression of RANK itself is sufficient to activate the pathway (22). Via NF-kB signaling, endothelial cells become activated and express various chemokines (e.g., MCP1) and adhesion molecules (e.g., ICAM-1) responsible for chemotaxis and transmigration of leukocytes into atherosclerotic plaques (23). Progression and evolvement of plaques is further stimulated through NF-kB by accumulation and proliferation of VSMCs (24). In late stages of atherosclerosis, NF-kB plays an important role in regulating activation and aggregation of platelets however underlying mechanisms remain to be elucidated (25).

Gal-9 is an important immune regulator which is abundantly present in several inflammatory diseases such as inflammatory bowel disease (26) and systemic lupus erythematosus (27). Expressed by many different cell types such as endothelial cells, macrophages, and T-lymphocytes (28), Gal-9 is thought to be anti-inflammatory via TIM-3 signaling. Important effects of this signaling include apoptosis of pro-inflammatory Th1 and Th17 cells (28, 29) and stimulation of regulatory T cell activity (30), both to dampen atherosclerotic progression. Serum levels of Gal-9 were found to be decreased in patients with coronary artery disease, specifically those with acute coronary syndrome. However, other studies reported higher serum Gal-9 levels in patients with DMII and chronic kidney disease, two morbidities that were abundantly present in our cohort (31). Gal-9 seems to be anti-inflammatory and thus atherosclerosis-dampening, however certain factors such as kidney function and the presence of DMII may alter levels of Gal-9. Therefore, Gal-9 should be used with caution and the presence of co-morbidities should be considered.



**Limitations**

This study has several limitations. First, all biomarkers were measured in Olink panels using the Proximity Extension Assay (PEA) technology followed by PCR. This semiquantitative technology provides relative concentrations of a biomarker in plasma rather than absolute concentrations. Therefore, these results must be validated in a quantitative assay to measure quantitative concentrations.

Furthermore, our sample size was limited to 120 patients, yielding higher confidence intervals in several biomarker hazard ratio calculations.

## CONCLUSION

Risk stratification models in PAD patients are necessary to predict future cardiovascular events and death. This study identified IL-6, PAR1, TNFRSF11A and Gal-9 as promising biomarkers to aid in risk stratification. These proteins are involved in prominent atherosclerotic biological processes including activation of endothelial cells, positive regulation of acute inflammatory responses, leukocyte chemotaxis and platelet activation. This semi-quantitative biomarker discovery is a first step to improve risk stratification in PAD. Quantitative assays are required to confirm the association with cardiovascular outcome. Potent biomarkers can then be implemented in risk stratification models.

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# Chapter 7

# FXa inhibition reduces myocardial damage in mice subjected to myocardial ischemia reperfusion injury

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In Preparation

## ABSTRACT

Acute myocardial infarction is one of the leading causes of death world wide. Although treatment strategies revolve around fast reperfusion of the ischemic heart to prevent further tissue damage, reperfusion injury per se also promotes cell death. Factor Xa (FXa), one of the central enzymes in coagulation, has pleiotropic roles and is a known modulator of inflammation. In the present study mice were subjected to myocardial ischemia reperfusion injury (1 hour ischemia followed by either 24 hours or 4 weeks reperfusion). The mice were treated with two doses of the FXa inhibitor rivaroxaban or placebo in the acute phase, 15 minutes after the induction of ischemia and 5 minutes after induction of reperfusion. Mice in the rivaroxaban treated arm had a decreased infarct size after 24 hours of reperfusion with potential long lasting benefical effects on heart function. Assessment of inflammatory proteins in the plasma and left ventricle revealed that the cardiac protective effects of FXa inhibition were associated with long-lasting increased angiogenic potential and decreased inflammation. Overall, our data reveals that early FXa inhibition in the acute phase of a myocardial infarction chronically modulates the inflammatory response and protects the heart.

## INTRODUCTION

Acute myocardial infarction (MI) is one of the world's leading causes of death resulting from atherothrombotic occlusion of one or more coronary arteries (1). Emergency management of an acute MI revolves around immediate restoration of blood flow to the ischemic myocardium to prevent further tissue damage and cell necrosis (2). Although reperfusion strategies including thrombolysis, anti-platelet therapy and percutaneous coronary intervention with stenting have greatly improved the clinical outcome after an acute MI, successful salvage of the ischemic zone also induces reperfusion injury, which when cell damage is irreversible, contributes to the final infarct size (3,4). A timely reperfusion and prevention of reperfusion injury is essential to maximize cardio-protection after an acute MI. Efficient therapeutic strategies that prevent or even limit reperfusion injury are, however, currently lacking.

Reperfusion injury is a multifactorial entity that involves excessive production of reactive oxygen species, abnormal calcium handling, mitochondrial damage, endothelial dysfunction, microvascular thrombosis and inflammation (5,6). FXa and thrombin are central players in the coagulation cascade and are involved in reperfusion injury due to their pro-thrombotic properties (7,8). However, animal work supports the concept that proteases of the coagulation system also modulate pleiotropic processes by activating protease activated receptors (PARs). PARs are membrane-bound, G-protein coupled receptors comprising 4 subtypes: PAR1-4 (9). Indeed, thrombin is the main activator of PAR1, while FXa is the main activator of PAR2. The activation of these receptors enhances pro-inflammatory cytokine production and the expression of endothelial cell adhesion molecules, among others (9). Upon reperfusion, leukocytes are attracted to the ischemic zone; endothelial cells will up-regulate their cell adhesion molecules, and cells in the ischemic zone will produce cytokines and chemokines, thereby creating a chemical gradient for additional leukocyte attraction. Both thrombin and FXa have been implicated with endothelial cell activation and leukocyte attraction (10).

Previous studies demonstrated that inhibition of the coagulation cascade attenuated myocardial reperfusion injury and infarction. Administration of activated protein C (APC), the natural anticoagulant of the coagulation system, decreased infarct size in a model of myocardial ischemia reperfusion injury with an early attenuation of leukocyte accumulation to the infarcted area and a decrease in IL-6 concentration (7). We also showed that inhibition of Factor VIIa (FVIIa), in complex with TF the activator of FX, attenuates reperfusion injury in mice. This was associated with reduced expression of inflammatory proteins in the myocardium, such as TLR-4 and CD14 (8). Although both strategies were experimentally successful, translation to the clinic is hampered by the risk for major bleeding upon administration of APC, or inhibitors of TF mediated FVII

activation in clinical studies (sepsis) (11–14). Direct oral anticoagulants targeted at FXa or thrombin are widely clinically used to prevent or treat thrombosis. In experimental models of complex disease, including atherosclerosis, we and others have demonstrated that rivaroxaban inhibited atherogenesis and reversed atherosclerosis (15–17). Since FXa can also be synthesized by extrahepatic cells including monocytes/macrophages, which are prominent in the vessel wall, the inhibitory actions of rivaroxaban likely reflect tissue penetrating properties of this agent (18)

Given the beneficial effects and tissue penetrating characteristics of the FXa inhibitor rivaroxaban, it is tempting to speculate that FXa inhibition by rivaroxaban protects the myocardium during reperfusion of the ischemic zone by modulating the inflammatory response. We therefore evaluated the effects of FXa inhibition by rivaroxaban in mice subjected to myocardial ischemia reperfusion injury.

## METHODS

### Experimental procedure

Male C57/Bl6 mice (Charles River Laboratories, Maastricht, The Netherlands), 8-10 weeks old, were used for all experiments. Myocardial ischemia, reperfusion injury was induced according to a closed chest model, as described previously (19). Mice received 0.03 mg/kg buprenorphine 30 minutes prior to surgery and 2 times daily for 2 days after the pre-surgery. Mice were anesthetized with 3-4% isoflurane and maintained with 1.5-2.5% during the procedure. During the ischemic period, mice were ventilated at a frequency of 210/min and a volume of 200-250μL. Ischemia was induced for 60 minutes followed by 2 different reperfusion times: 4 hours and 4 weeks (Figure 1a). 0.086 mg/kg Rivaroxaban was administered via the jugular vein 15 minutes after the induction of ischemia and 5 minutes after reperfusion. Echocardiography verified the induction of ischemia and reperfusion.

### 4 hours model

After 4 hours reperfusion, one group of mice were re-anesthetized, the chest was opened, the coronary artery was re-occluded and 50-100 μL 2% Evans blue dye (Sigma) was injected directly in the vena cava to differentiate the perfused area from the ischemic area (area at risk) (19). Hearts were rapidly excised, rinsed in 0.9% normal saline and fixated at -20°C in a cooled cutting block for 10 minutes. Hearts were then cut in approximately 6 short-axis slices from the apex towards the atria. Viable tissue was visualized by incubating the slices in 2% freshly prepared 2,3,5 TriphenylTetrazoliumChloride (TTC) (Sigma) for 15 minutes at 37 °C with gentle agitation. To increase tissue contrast, slices were incubated in 10% neutral-buffered formalin for 10 minutes (20). Each section was

photographed and the area at risk (AAR) surface, as visualized by the Evens blue dye, was determined and compared to the area of infarction (AOI) utilizing Image J. Mean values of AOI/AAR were used after correcting for slice size.

In a second group of mice, blood was collected by injecting sodium citrate (3.2%) directly into the vena cava before drawing blood with the same syringe, as described previously (21). Hearts were rapidly excised, split into left and right atria and ventricles, snap frozen in liquid nitrogen and stored at -80°C until further use.

**4 week model**

During the experimental procedure in mice subjected to 4 weeks of reperfusion, echocardiography was utilized to determine heart function by means of left ventricle ejection fraction at baseline and after 4 weeks. Before sacrificing the mice, blood was drawn as described previously (21). Hearts were rapidly excised, and either snap-frozen in liquid nitrogen, split into left and right atria and ventricles and stored at -80°C until further use, or formalin fixed and paraffin embedded for immunohistological analysis.

**Multiplex analysis**

Frozen left ventricles were freeze-dried, and homogenized with a tissue grinder in PBS. For cell lysis, the samples were incubated with equal parts PBS and Cell Lysis Buffer 2 (R&D) for 30 minutes at RT with gentle agitation. The supernatant was collected after two cycles of centrifugation at 10,000g for 10 minutes and subsequently stored at -80 °C. Multiplex analysis of the inflammatory markers in both plasma and carotid artery homogenates was performed using a Millipore MAP Mouse cytokine/chemokine premixed 25-plex Immunology panels and 4 different customized R&D Mouse Magnetic Luminex Assay panels. The manufacturers' protocols were followed. The multiplex plates were measured on a Luminex Magpix instrument with Magpix xPonent 4.2.

**Statistical analysis**

Statistical analysis was performed with Prism version 9.1.2 (GraphPad Software Inc, USA). Data were analyzed using the Kruskal Wallis test and are presented as median with their ranges unless otherwise stated. The multiplex analysis was not designed for a large sample size and therefore corrections for multiple testing were not implemented.

## RESULTS

### Infarcted area and heart function

To assess the role of FXa in myocardial ischemia reperfusion injury, we treated mice with the FXa inhibitor rivaroxaban in the short-term (60 minutes ischemia and 4 hours reperfusion) and long-term (60 minutes ischemia and 4 weeks reperfusion) model. Mice who received two doses of 0.086 mg/kg rivaroxaban reached plasma levels comparable to the human situation (Figure 1A) (22).

In the short-term model, we found that mice treated with rivaroxaban had significantly reduced infarct size after myocardial ischemia reperfusion: after rivaroxaban treatment the AOI/AAR was 39.96% (31.09 - 48.01) vs 62.11% (52.26 - 67.36), $p < 0.001$, in the control group (Figure 1B). These short-term protective effects of rivaroxaban did not lead to significantly better heart function in the long-term, although the left ventricle ejection fraction in the rivaroxaban treated group tended to be better preserved -41% (-53 to -12) compared to -58% (-75 to -41) in the control group (Figure 1C-D).

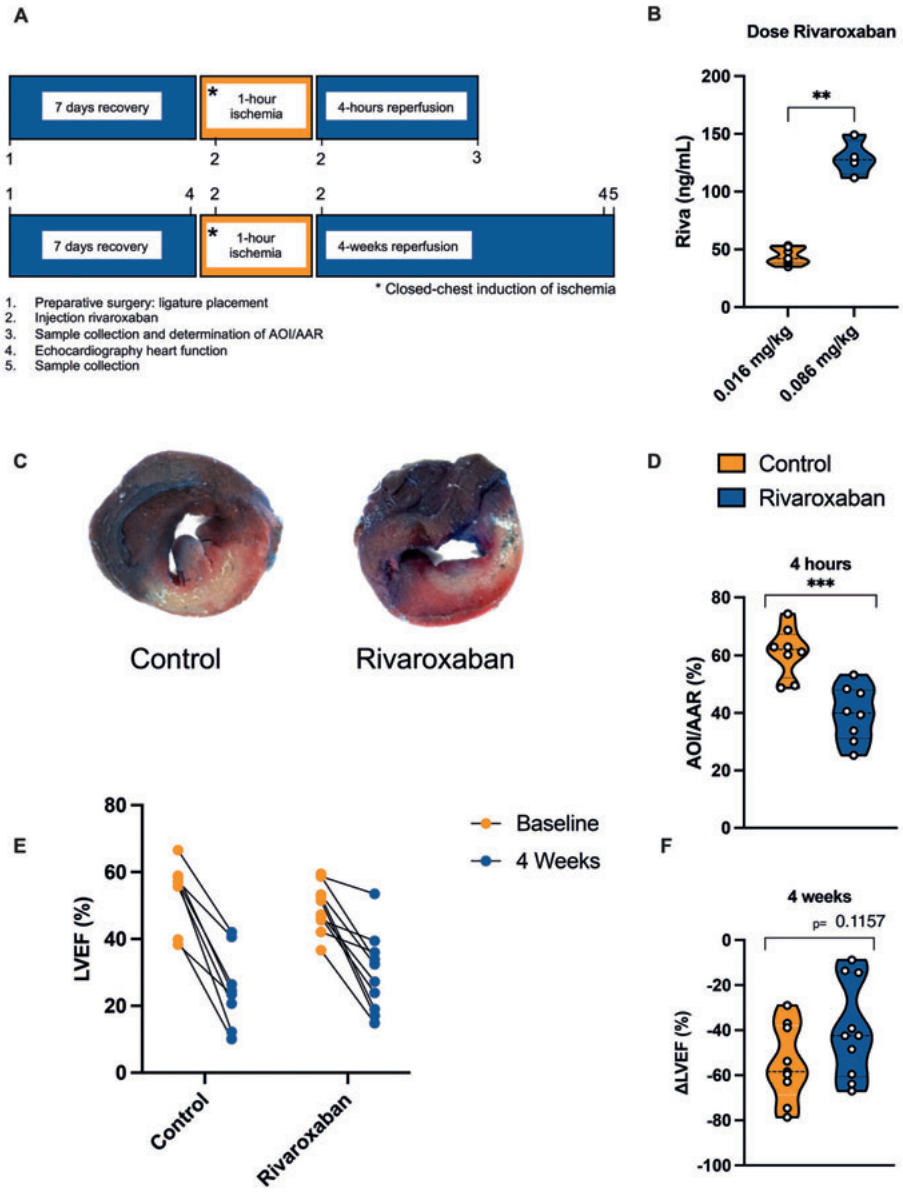


**Figure 1. Rivaroxaban attenuates ischemia reperfusion injury.**
**A.** Protocol myocardial ischemia reperfusion. After a 7-day recovery period following placement of a ligature around the left carotid artery, ischemia was induced for 1 hour and subsequently reperfusion for 4 hours or 4 weeks. **B.** Two doses of 0.086 mg/kg rivaroxaban yielded clinically relevant plasma concentrations. The 0.086 mg/kg dose was therefore used in the following experiments. **C.** Representative images of the AAR (non blue area) and the AOI (pale area in the AAR) following myocardial ischemia reperfusion **D.** AOI/AAR was decreased after 4 hours of reperfusion (n=8 per group). **E-F** Two doses of rivaroxaban in the acute phase tended to preserve the LVEF at 4 weeks after ischemia. ** *p* value < 0.01; *** *p* value < 0.001.

**Factor Xa inhibition decreases inflammation locally**

Inhibition of FXa has been shown to reduce inflammation in animal models (16,17). Since, inflammation contributes to reperfusion injury, we next explored concentrations of an array of inflammatory markers. Out of the 53 markers that were measured in plasma and homogenates, CXCL10 and IL-4 were below the detection limit of the assay in the short- and long-term model (data not shown).

In the short-term model, most profound effects were seen in the left ventricle: ICAM-1, MMP-12 and CCL12 were down-regulated in the rivaroxaban group (Figure 2A), whereas IFN-γ was significantly up-regulated compared to the control group (Figure 2B). Although left ventricle MCP-1, IL-9, IL-1β, CXCL-16, C1qR1 and TIMP-1 were not significantly different between the treatment group and control, they clearly deviate towards decreased levels in the rivaroxaban treated group (Figure 2A). In contrast, CXCL12 tended to be increased as well, although not significant (Figure 2B).

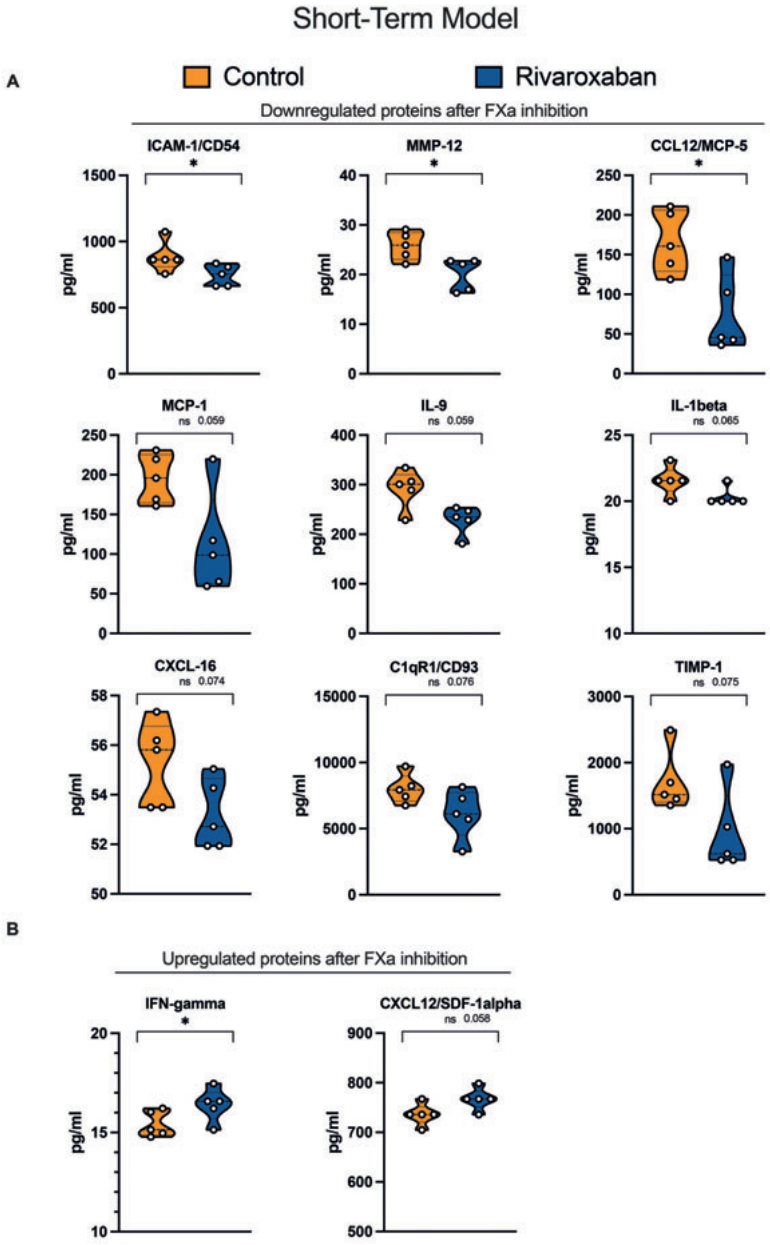


**Figure 2 FXa inhibition modulates inflammatory proteins in the left ventricles in the acute phase of myocardial ischemia reperfusion injury.** Selected violin plots (*p* value <0.1) of inflammatory proteins measured in left ventricle homogenates. The orange color represents the control mice and the blue the treated mice. **A.** Protein concentrations of ICAM-1, MMP-12, CCL12/MCP5 were significantly down-regulated after FXa inhibition. **B.** The protein concentration of IFN-γ was significantly up-regulated in the left ventricle of rivaroxaban treated mice. * *p* value < 0.05.

Most of the inflammatory markers that were significantly different between the groups of the short-term model were not altered in the long-term model (supplemental Tables 1 and 2). However, two doses of rivaroxaban in the acute phase of myocardial infarction reperfusion injury led to long-term up-regulation of plasma concentrations of CXCL12 and LIX (Figure 3). No differences could be found in left ventricle in the long-term model.

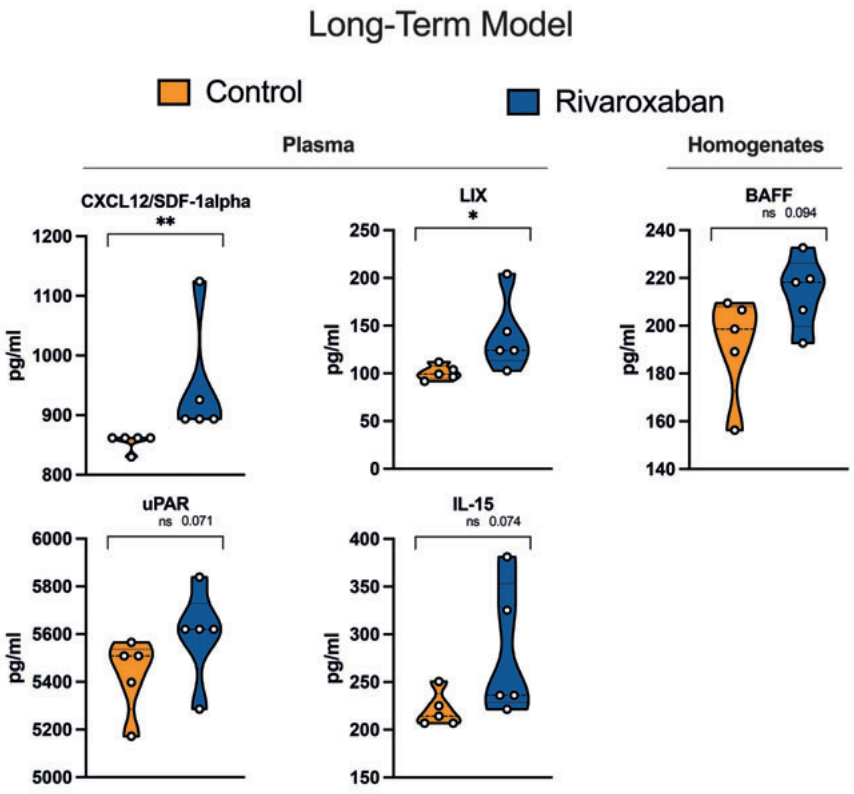


**Figure 3 Acute phase FXa inhibition modulates inflammatory proteins chronically after myocardial ischemia reperfusion injury.**

Selected violin plots ($p$ value <0.01) of inflammatory proteins measured in left ventricle homogenates. The orange color represents the control mice and the blue the treated mice. Plasma protein concentrations of CXCL12/SDF-1α were significantly up-regulated down-regulated after 4 weeks reperfusion following acute-phase FXa inhibition.* $p$ value < 0.05; ** $p$ value <0.01.

## DISCUSSION

Direct oral anticoagulants (DOACs) are widely prescribed to prevent and treat thrombosis. Accumulating animal work demonstrates the pleiotropic effects of inhibition of coagulation by DOACs on biological pathways beyond the scope of hemostasis, such as inflammation (9). During an acute myocardial infarction, tissue damage is caused by oxygen deprivation during ischemia. Timely reperfusion is therefore needed to decrease damage to the ischemic zone, however, reperfusion per se also promotes cell death (23,24). We showed that two early intravenous injections of the FXa inhibitor rivaroxaban protect the mouse heart after myocardial ischemia reperfusion injury with potential long-lasting protective effects. Furthermore, early FXa inhibition modulated several inflammatory biomarkers in both the acute phase and chronic phase of myocardial ischemia reperfusion injury.

MMP12 expression was decreased in the acute phase, whereas CXCL12 and CCL5 were among the proteins that were chronically increased in plasma after FXa inhibition; these proteins are well-known for their angiogenic functions (25,26). CXCL12 is the main ligand for CXCR4 and both proteins are expressed by cardiomyocytes (27). Activation of the CXCL12/CXCR4 axis attracts stem cells to injured sites and modulates cardiogenesis and vasculogenesis among other processes (28). In a rat permanent ligation model, cardiomyocyte specific over-expression of CXCL12 preserved ejection fraction, whereas cardiomyocyte specific knockout of CXCL12 decreased the ejection fraction when compared to their respective controls. However, comparing the ejection fraction of the cardiomyocyte specific CXCL12 over-expressing rat versus the CXCL12 knockout rat suggests that the former has a better ejection fraction than the latter (approximately 39% vs 32%) after 28 days permanent ligation (29). Additionally, it was recently shown that cardiomyocyte survival and function were preserved after cardiomyocyte-induced angiogenesis during myocardial ischemia (30). FXa inhibition might promote activation of the CXCL12/CXCR4 axis in the ischemic zone and thereby stimulate early angiogenesis, contributing to less myocardial damage. The pathways contributing to the increased expression of these proteins and their precise role in the setting of ischemia reperfusion injury have to be established.

Our data further demonstrates that FXa inhibition decreases inflammation, which is in line with previous reports (16,31,32). However, it is currently not known which pathways contribute to these effects since multiple downstream pathways of FXa can modulate inflammation (9). For example, inhibition of FXa also reduces thrombin generation and these coagulation proteins have different signaling functions via protease activated receptors (PARs). PARs are g-protein coupled receptors present on the membrane of multiple cells and can be activated by coagulation proteases and other serine proteases. FXa predominantly activates

PAR2, whereas PAR1 is the main target of thrombin, the downstream protein of FXa (33).

Interestingly, in an experimental diabetic model for wound healing, the inflammasome blocking agent BAY 11-7082 increased protein levels of CXCL12 and VEGF and improved wound healing (34). In contrast, *in vitro* work revealed that the inflammasome inhibitor Oridonin decreased cell migration and angiogenesis in VEGF-treated HUVEC cells (35). Low-grade inflammasome activation might therefore be warranted to promote angiogenesis. Recently it was shown that FXa or thrombin inhibition have different effects on myocardial ischemia reperfusion injury. While both FXa and thrombin inhibition attenuated myocardial ischemia reperfusion injury, FXa but not thrombin reduced the activity of pathways related to sterile inflammation and inflammasome activation (31). The involvement of various PARs in activation of the inflammasome was, however, not further studied. Furthermore, the effects of FXa inhibition on NLRP3 mediated angiogenesis in ischemia reperfusion injury have to be established.

PAR2 deficiency in myocardial ischemia reperfusion injury resembles the protective effects we found with FXa inhibition by rivaroxaban (36). However, other serine proteases activate PAR2 as well, and therefore one cannot specifically link the protective effects of PAR2 deficiency found by Antoniak et al. towards inhibited FXa-PAR2 signaling. Novel PAR mutants such as the the G37I PAR2 mutant mouse, a FXa resistant PAR2 mutant that resembles rivaroxaban mediated inhibition of FXa-PAR2 signaling without affecting coagulation, have to be implemented to fully elucidate the role of FXa/PAR2 axis in ischemia reperfusion injury.

In conclusion, we showed that the FXa-PAR2 axis is involved in myocardial ischemia reperfusion injury in mice and that inhibition of this axis partly protects the heart after ischemia reperfusion injury potentially by enhancing angiogenic properties. It is however, not clear how the FXa inhibition mediates neovascularisation and what clinical implications early FXa inhibition has on the clinical outcome after a myocardial infarction.

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## SUPPLEMENTS

**Supplemental table 1 - Plasma and left ventricle biomarkers**

| | Homogenates 4 hours reperfusion | | | Homogenates 4 weeks reperfusion | | |
|---|---|---|---|---|---|---|
| | 4h Ctrl (N=5) | 4h Riva (N=5) | p value | 4w Ctrl (N=5) | 4w Riva (N=5) | p value |
| Angiopoietin-2 | 1159.00 (1159.00, 1221.00) | 1098.00 (1098.00, 1251.00) | 0.100 | 1874.00 (1159.00, 2159.00) | 1816.00 (1522.00, 2382.00) | 0.834 |
| BAFF/BLyS/TNFSF13B | 162.88 (128.98, 180.42) | 137.86 (83.92, 178.96) | 0.347 | 198.61 (156.28, 209.49) | 218.19 (192.80, 232.64) | ***0.094*** |
| C1qR1/CD93 | 7937.00 (6757.00, 9706.00) | 6117.00 (3286.00, 8146.00) | **0.076** | 14092.00 (8456.00, 18192.00) | 10272.00 (6826.00, 29033.00) | 0.465 |
| CCL11/Eotaxin | 76.55 (75.27, 82.81) | 77.82 (72.69, 85.25) | 1.000 | 173.42 (85.25, 206.82) | 180.32 (151.92, 200.34) | 1.000 |
| CCL12/MCP-5 | 160.65 (118.93, 210.71) | 45.67 (36.16, 146.65) | ***0.028*** | 103.59 (90.54, 124.71) | 97.57 (79.81, 147.89) | 0.602 |
| CCL19/MIP-3 beta | 64.60 (63.81, 66.97) | 66.18 (62.22, 66.97) | 0.395 | 66.18 (50.93, 67.75) | 65.39 (63.81, 66.97) | 1.000 |
| CCL20/MIP-3 alpha | 642.70 (636.23, 655.50) | 655.50 (629.71, 655.50) | 0.735 | 642.70 (616.52, 642.70) | 642.70 (629.71, 655.50) | 0.572 |
| CCL22/MDC | 38.40 (36.95, 39.82) | 36.95 (36.95, 39.82) | 0.658 | 49.06 (37.68, 51.53) | 47.17 (41.21, 56.28) | 0.834 |
| CXCL12/SDF-1 alpha | 735.96 (704.86, 767.18) | 767.18 (735.96, 798.55) | ***0.058*** | 925.86 (767.18, 1024.00) | 893.72 (830.08, 1057.00) | 0.916 |
| CXCL16 | 55.81 (53.49, 57.34) | 52.72 (51.94, 55.04) | ***0.074*** | 105.64 (86.34, 130.38) | 96.05 (73.52, 112.33) | 0.347 |
| DPPIV/CD26 | 9322.00 (7997.00, 13655.00) | 13173.00 (10164.00, 24094.00) | 0.117 | 18853.00 (16068.00, 43826.00) | 24830.00 (16673.00, 79347.00) | 0.249 |
| Fas Ligand/TNFSF6 | 135.64 (135.64, 141.70) | 141.70 (135.64, 147.73) | 0.419 | 177.69 (177.69, 207.32) | 177.69 (153.75, 230.83) | 0.589 |
| G-CSF | 66.56 (36.69, 82.14) | 39.55 (18.19, 76.29) | 0.251 | 15.57 (15.57, 22.68) | 17.31 (14.72, 19.07) | 0.525 |
| GDF-15 | 17.77 (16.69, 18.61) | 17.77 (16.69, 25.20) | 0.748 | 22.74 (18.61, 37.28) | 32.48 (16.93, 56.95) | 0.917 |
| GM-CSF | 73.53 (71.70, 77.10) | 73.53 (67.98, 73.53) | 0.280 | 71.70 (67.98, 71.70) | 71.70 (66.07, 73.53) | 0.737 |
| Granzyme B | 46.40 (44.26, 47.47) | 44.26 (42.11, 48.53) | 0.452 | 77.30 (42.11, 89.13) | 73.29 (60.05, 96.88) | 0.917 |
| ICAM-1/CD54 | 861.44 (754.97, 1072.00) | 754.97 (664.37, 834.91) | ***0.033*** | 1599.00 (914.34, 2787.00) | 1241.00 (781.67, 4094.00) | 0.754 |

**Supplemental table 1 - Plasma and left ventricle biomarkers (continued)**

| | Homogenates 4 hours reperfusion | | | Homogenates 4 weeks reperfusion | | |
|---|---|---|---|---|---|---|
| | 4h Ctrl (N=5) | 4h Riva (N=5) | p value | 4w Ctrl (N=5) | 4w Riva (N=5) | p value |
| IFN-γ | 15.14 (14.78, 16.21) | 16.57 (15.14, 17.46) | ***0.045*** | 26.45 (20.13, 41.37) | 29.23 (22.25, 41.20) | 0.917 |
| IL-10 | 38.22 (37.18, 46.02) | 43.93 (37.70, 53.33) | 0.249 | 150.92 (53.33, 155.29) | 134.03 (124.81, 156.39) | 0.602 |
| IL-12(P40) | 31.69 (29.78, 34.25) | 32.01 (31.69, 34.89) | 0.341 | 157.30 (71.37, 258.89) | 210.52 (113.31, 237.59) | 0.602 |
| IL-12(P70) | 56.33 (54.19, 60.58) | 56.33 (52.04, 58.46) | 0.502 | 58.46 (55.27, 62.68) | 60.58 (54.19, 62.68) | 0.523 |
| IL-13 | 35.47 (35.47, 41.77) | 40.72 (34.41, 44.90) | 0.289 | 128.52 (53.24, 255.73) | 175.25 (116.49, 234.72) | 0.602 |
| IL-15 | 272.07 (268.49, 293.45) | 272.07 (254.13, 286.34) | 0.395 | 286.34 (272.07, 321.75) | 293.45 (279.21, 314.70) | 0.752 |
| IL-17 | 3.56 (3.45, 3.78) | 3.67 (3.45, 3.89) | 0.747 | 4.89 (3.56, 7.06) | 5.87 (4.17, 8.29) | 0.530 |
| IL-17E/IL-25 | 296.91 (281.94, 296.91) | 289.43 (281.94, 296.91) | 0.729 | 517.52 (371.16, 561.03) | 473.85 (400.63, 618.82) | 0.834 |
| IL-1a | 72.20 (64.69, 100.68) | 82.43 (66.21, 95.15) | 0.754 | 405.94 (183.00, 617.81) | 506.28 (339.00, 578.89) | 0.602 |
| IL-1b | 21.56 (20.00, 23.11) | 20.00 (20.00, 21.56) | ***0.065*** | 26.23 (20.00, 27.78) | 26.23 (21.56, 30.88) | 0.595 |
| IL-2 | 3.77 (2.72, 6.04) | 3.20 (2.24, 4.35) | 0.116 | 54.78 (12.09, 78.82) | 64.10 (42.87, 72.63) | 0.754 |
| IL-27 | 107.07 (103.12, 111.93) | 106.08 (99.11, 107.07) | 0.161 | 158.76 (118.62, 174.54) | 154.51 (139.69, 176.17) | 0.675 |
| IL-3 | 12.56 (12.06, 13.06) | 12.56 (12.56, 13.06) | 0.212 | 12.31 (10.55, 12.81) | 12.56 (11.56, 12.56) | 0.577 |
| IL-33 | 417.57 (384.85, 439.25) | 406.70 (373.89, 428.43) | 0.340 | 498.38 (423.00, 609.63) | 599.11 (514.39, 1104.00) | 0.116 |
| IL-5 | 5.78 (5.10, 5.78) | 5.78 (5.10, 5.78) | 1.000 | 5.44 (5.44, 5.78) | 5.44 (5.10, 5.78) | 0.606 |
| IL-6 | 25.02 (22.45, 30.34) | 12.80 (5.31, 37.65) | 0.602 | 9.16 (6.02, 11.33) | 9.51 (6.63, 14.01) | 0.602 |
| IL-6R alpha | 1851.00 (1820.00, 1881.00) | 1836.00 (1820.00, 1912.00) | 0.828 | 1820.00 (1404.00, 1881.00) | 1820.00 (1759.00, 1958.00) | 0.525 |
| IL-7 | 35.34 (35.34, 36.34) | 36.34 (34.33, 39.35) | 0.190 | 39.35 (35.34, 43.89) | 39.35 (35.34, 40.36) | 0.831 |

**Supplemental table 1 - Plasma and left ventricle biomarkers (continued)**

| | Homogenates 4 hours reperfusion | | | Homogenates 4 weeks reperfusion | | |
|---|---|---|---|---|---|---|
| | 4h Ctrl (N=5) | 4h Riva (N=5) | p value | 4w Ctrl (N=5) | 4w Riva (N=5) | p value |
| IL-9 | 300.73 (228.34, 334.04) | 234.74 (181.11, 253.48) | ***0.059*** | 1172.00 (496.16, 1611.00) | 1225.00 (801.91, 1385.00) | 0.917 |
| IP-10 | 15.93 (12.19, 21.49) | 10.14 (2.51, 18.44) | 0.117 | 22.37 (7.66, 28.08) | 23.99 (18.49, 25.05) | 0.754 |
| KC | 413.86 (288.25, 464.60) | 142.12 (17.69, 470.30) | 0.347 | 46.85 (20.75, 88.86) | 55.56 (30.52, 63.37) | 0.754 |
| LIX | 186.82 (164.32, 220.93) | 132.41 (88.90, 261.14) | 0.463 | 129.07 (76.15, 226.43) | 97.38 (76.15, 195.54) | 0.753 |
| MCP-1 | 196.00 (160.71, 231.03) | 98.91 (59.69, 219.64) | ***0.059*** | 65.48 (62.62, 69.68) | 65.48 (65.48, 69.68) | 1.000 |
| MIP-1a | 184.16 (174.29, 188.93) | 184.16 (179.28, 193.60) | 0.915 | 280.58 (188.93, 339.61) | 304.53 (243.48, 345.13) | 0.465 |
| MIP-1b | 102.89 (98.41, 104.77) | 103.52 (98.41, 106.61) | 0.674 | 100.99 (100.35, 104.77) | 103.52 (97.09, 104.77) | 0.670 |
| MIP-2 | 193.45 (175.72, 199.01) | 181.82 (175.72, 204.41) | 0.338 | 187.72 (184.79, 193.45) | 193.45 (181.82, 199.01) | 0.163 |
| MMP-12 | 25.92 (22.11, 29.08) | 22.11 (16.33, 22.75) | ***0.036*** | 30.96 (21.47, 45.92) | 37.22 (20.83, 61.35) | 0.834 |
| MMP-8 | 101029.00 (90025.00, 129680.00) | 66180.00 (47897.00, 134372.00) | 0.530 | 83837.00 (42520.00, 86319.00) | 73813.00 (61030.00, 96158.00) | 0.600 |
| PIGF-2 | 191.53 (139.63, 211.59) | 119.49 (75.66, 288.50) | 0.175 | 97.62 (83.27, 148.01) | 84.12 (77.35, 146.33) | 0.175 |
| RANTES | 13.98 (12.79, 14.13) | 13.24 (12.79, 14.28) | 0.589 | 36.93 (14.72, 51.45) | 44.11 (39.32, 48.89) | 0.602 |
| TIMP-1 | 1517.00 (1355.00, 2491.00) | 622.21 (528.77, 1970.00) | ***0.075*** | 894.22 (576.52, 1153.00) | 739.48 (510.26, 1793.00) | 0.602 |
| TIMP-4 | 574.51 (559.85, 574.51) | 574.51 (559.85, 588.91) | 0.212 | 588.91 (559.85, 657.53) | 574.51 (559.85, 574.51) | 0.443 |
| TNFa | 14.21 (13.14, 14.57) | 13.86 (12.41, 15.27) | 0.395 | 17.97 (14.57, 19.28) | 17.31 (15.96, 19.28) | 1.000 |
| uPAR | 5620.00 (5509.00, 5730.00) | 5509.00 (5509.00, 5730.00) | 0.174 | 12102.00 (9081.00, 13172.00) | 11491.00 (8808.00, 14699.00) | 0.602 |

| | Plasma 4 hours reperfusion | | | Plasma 4 weeks reperfusion | | |
|---|---|---|---|---|---|---|
| | 4h Ctrl (N=5) | 4h Riva (N=5) | p value | 4w Ctrl (N=5) | 4w Riva (N=5) | p value |
| Angiopoietin-2 | 22295.00 (14822.00, 28591.00) | 19465.00 (5490.00, 26097.00) | 0.602 | 29890.00 (21952.00, 31862.00) | 25686.00 (23627.00, 26775.00) | 0.465 |
| BAFF/BLyS/TNFSF13B | 5371.00 (3918.00, 5925.00) | 4564.00 (4165.00, 6291.00) | 0.465 | 6147.00 (4407.00, 7878.00) | 5040.00 (3104.00, 6082.00) | 0.175 |
| C1qR1/CD93 | 59679.00 (51215.00, 68992.00) | 58170.00 (51248.00, 63790.00) | 0.602 | 46931.00 (44676.00, 64923.00) | 49243.00 (45399.00, 57092.00) | 0.754 |
| CCL11/Eotaxin | 518.01 (388.78, 612.96) | 536.21 (441.17, 619.22) | 0.347 | 387.51 (237.27, 592.94) | 431.33 (276.26, 492.52) | 0.917 |
| CCL12/MCP-5 | 86.50 (80.23, 96.80) | 78.08 (54.14, 91.34) | ***0.094*** | 42.64 (31.94, 78.95) | 45.08 (41.39, 62.38) | 0.834 |
| CCL19/MIP-3 beta | 70.11 (68.54, 73.24) | 71.67 (70.11, 73.24) | 0.240 | 70.11 (68.54, 74.01) | 71.67 (68.54, 71.67) | 0.914 |
| CCL20/MIP-3 alpha | 655.50 (616.52, 680.56) | 655.50 (642.70, 680.56) | 0.915 | 668.12 (629.71, 680.56) | 642.70 (629.71, 655.50) | 0.202 |
| CCL22/MDC | 73.51 (66.26, 86.09) | 73.51 (39.82, 79.44) | 0.401 | 67.32 (39.82, 77.97) | 61.93 (41.21, 77.97) | 1.000 |
| CXCL12/SDF-1 alpha | 861.80 (861.80, 990.83) | 909.76 (861.80, 1040.00) | 0.234 | 861.80 (830.08, 861.80) | 893.72 (893.72, 1124.00) | ***0.006*** |
| CXCL16 | 251.69 (234.76, 341.91) | 229.54 (152.23, 289.86) | 0.117 | 224.64 (92.83, 295.66) | 220.05 (214.15, 279.54) | 0.602 |
| DPPIV/CD26 | 123862.00 (85850.00, 156843.00) | 110278.00 (104579.00, 135633.00) | 0.347 | 183656.00 (117109.00, 186449.00) | 169289.00 (139649.00, 184838.00) | 0.602 |
| Fas Ligand/TNFSF6 | 159.76 (153.75, 159.76) | 153.75 (147.73, 159.76) | 0.265 | 153.75 (153.75, 162.76) | 153.75 (147.73, 159.76) | 0.178 |
| G-CSF | 2014.00 (1499.00, 3797.00) | 2670.00 (1547.00, 3906.00) | 0.602 | 290.20 (143.89, 1194.00) | 286.22 (148.81, 552.27) | 0.754 |
| GDF-15 | 74.40 (63.95, 90.59) | 67.05 (5.00, 87.12) | 0.175 | 63.56 (54.61, 75.17) | 65.11 (53.44, 70.15) | 0.602 |
| GM-CSF | 69.86 (66.07, 71.70) | 66.07 (64.13, 73.53) | 0.828 | 58.09 (55.99, 64.13) | 60.14 (58.09, 66.07) | 0.589 |
| Granzyme B | 37.78 (35.60, 37.78) | 35.60 (35.60, 37.78) | 0.221 | 35.60 (33.41, 38.87) | 35.60 (33.41, 410.62) | 0.665 |
| ICAM-1/CD54 | 17516.00 (16459.00, 22130.00) | 19276.00 (15555.00, 20653.00) | 0.917 | 18982.00 (13178.00, 27804.00) | 19600.00 (12365.00, 54392.00) | 0.754 |
| IFN-γ | 10.82 (10.46, 11.54) | 12.26 (10.46, 12.44) | 0.111 | 10.64 (7.57, 11.00) | 9.74 (8.83, 28.54) | 0.834 |

(continued)

| | Plasma 4 hours reperfusion | | | Plasma 4 weeks reperfusion | | |
|---|---|---|---|---|---|---|
| | 4h Ctrl (N=5) | 4h Riva (N=5) | p value | 4w Ctrl (N=5) | 4w Riva (N=5) | p value |
| IL-10 | 38.74 (35.63, 46.02) | 40.81 (38.74, 46.02) | 0.523 | 34.60 (33.57, 39.78) | 34.60 (30.47, 46.02) | 0.753 |
| IL-12(P40) | 29.14 (22.81, 32.97) | 20.30 (13.18, 31.69) | 0.175 | 24.07 (15.33, 25.97) | 25.97 (22.81, 563.33) | 0.143 |
| IL-12(P70) | 56.33 (53.12, 58.46) | 54.19 (52.04, 60.58) | 1.000 | 47.69 (45.50, 49.87) | 47.69 (41.06, 60.58) | 0.747 |
| IL-13 | 27.55 (24.90, 33.36) | 27.02 (23.84, 33.36) | 0.671 | 20.66 (17.48, 22.78) | 19.60 (12.18, 37.57) | 0.753 |
| IL-15 | 243.32 (228.83, 279.21) | 250.53 (236.08, 335.81) | 0.461 | 214.27 (206.95, 250.53) | 236.08 (221.56, 381.15) | ***0.074*** |
| IL-17 | 3.78 (3.34, 4.11) | 3.67 (3.34, 4.00) | 0.459 | 3.00 (2.67, 3.34) | 3.34 (2.34, 4.45) | 0.528 |
| IL-17E/IL-25 | 296.91 (266.93, 296.91) | 296.91 (281.94, 311.83) | 0.288 | 296.91 (266.93, 311.83) | 296.91 (281.94, 304.37) | 0.747 |
| IL-1a | 50.61 (47.36, 53.81) | 48.99 (44.06, 56.96) | 0.752 | 43.23 (39.02, 45.72) | 45.72 (40.71, 348.28) | 0.401 |
| IL-1b | 21.56 (21.56, 23.11) | 21.56 (20.00, 24.67) | 0.313 | 20.00 (18.44, 21.56) | 18.44 (16.88, 20.00) | 0.100 |
| IL-2 | 2.24 (2.24, 2.43) | 2.24 (2.00, 2.81) | 0.911 | 2.04 (2.00, 2.24) | 2.04 (2.04, 2.24) | 0.262 |
| IL-27 | 110.96 (96.07, 117.67) | 107.07 (101.12, 117.67) | 1.000 | 109.02 (108.05, 114.81) | 109.02 (99.11, 367.01) | 0.738 |
| IL-3 | 13.06 (10.30, 13.57) | 12.81 (11.56, 13.57) | 0.830 | 13.06 (11.56, 13.57) | 12.06 (11.56, 13.06) | 0.197 |
| IL-33 | 417.57 (395.79, 439.25) | 428.43 (406.70, 444.66) | 0.206 | 406.70 (379.37, 428.43) | 417.57 (417.57, 417.57) | 0.238 |
| IL-4 | 1.54 (1.54, 1.67) | 1.59 (1.54, 1.67) | 0.343 | 1.54 (1.54, 1.55) | 1.54 (1.54, 1.83) | 0.368 |
| IL-5 | 20.92 (16.66, 26.76) | 18.96 (13.71, 32.85) | 0.675 | 7.11 (5.78, 8.09) | 7.77 (6.45, 9.06) | 0.207 |
| IL-6 | 108.05 (84.91, 253.83) | 124.45 (90.61, 310.29) | 0.347 | 5.67 (4.96, 27.60) | 4.43 (3.82, 13.50) | 0.249 |
| IL-6R alpha | 13196.00 (12276.00, 16629.00) | 13645.00 (12495.00, 14402.00) | 0.917 | 11912.00 (10525.00, 12568.00) | 11642.00 (10385.00, 12330.00) | 0.347 |
| IL-7 | 32.34 (28.36, 34.33) | 34.33 (31.84, 40.36) | 0.172 | 27.86 (27.37, 30.34) | 29.35 (26.38, 44.40) | 0.833 |

**(continued)**

| | Plasma 4 hours reperfusion | | | Plasma 4 weeks reperfusion | | |
|---|---|---|---|---|---|---|
| | 4h Ctrl (N=5) | 4h Riva (N=5) | p value | 4w Ctrl (N=5) | 4w Riva (N=5) | p value |
| IL-9 | 159.26 (144.02, 166.67) | 159.26 (159.26, 177.54) | 0.180 | 166.67 (151.71, 188.15) | 159.26 (151.71, 241.06) | 0.747 |
| IP-10 | 68.30 (56.58, 89.75) | 75.48 (50.00, 92.30) | 0.917 | 60.60 (42.88, 98.73) | 79.13 (38.52, 116.94) | 0.465 |
| KC | 246.63 (154.84, 414.55) | 373.34 (175.11, 689.95) | 0.251 | 31.36 (25.04, 50.43) | 49.24 (16.37, 71.64) | 0.347 |
| LIX | 122.29 (97.38, 179.43) | 108.30 (99.23, 150.27) | 0.602 | 99.23 (91.76, 111.86) | 124.00 (102.89, 204.13) | ***0.028*** |
| MCP-1 | 73.77 (71.05, 79.07) | 71.05 (65.48, 79.07) | 0.262 | 62.62 (59.69, 71.05) | 62.62 (53.66, 76.44) | 0.738 |
| MIP-1a | 198.17 (184.16, 202.65) | 198.17 (184.16, 239.68) | 0.671 | 174.29 (163.94, 188.93) | 174.29 (174.29, 193.60) | 0.347 |
| MIP-1b | 95.76 (93.04, 100.99) | 102.26 (95.76, 104.77) | 0.068 | 82.95 (76.69, 93.04) | 85.94 (75.06, 135.39) | 0.834 |
| MIP-2 | 169.40 (162.85, 181.82) | 169.40 (169.40, 187.72) | 0.655 | 162.85 (141.50, 169.40) | 156.03 (156.03, 1259.00) | 0.665 |
| MMP-12 | 63.20 (47.78, 74.87) | 77.32 (55.81, 89.58) | 0.117 | 205.51 (131.25, 210.78) | 168.13 (103.67, 381.85) | 0.754 |
| MMP-8 | 274331.00 (211463.00, 349846.00) | 337422.00 (276463.00, 345712.00) | 0.249 | 182506.00 (155235.00, 270061.00) | 198167.00 (132028.00, 445336.00) | 0.602 |
| PIGF-2 | 75.66 (65.47, 80.73) | 78.20 (65.47, 84.12) | 0.599 | 73.96 (68.87, 78.20) | 73.96 (60.37, 79.04) | 0.597 |
| RANTES | 13.69 (12.18, 17.71) | 15.44 (12.79, 15.73) | 0.917 | 10.94 (5.59, 14.86) | 14.28 (8.02, 16.73) | 0.293 |
| TIMP-1 | 3818.00 (3481.00, 5407.00) | 3453.00 (2978.00, 5824.00) | 0.251 | 1240.00 (962.04, 3541.00) | 1437.00 (1053.00, 3706.00) | 0.465 |
| TIMP-4 | 617.00 (574.51, 683.61) | 670.66 (657.53, 696.40) | 0.168 | 683.61 (657.53, 758.06) | 670.66 (610.06, 683.61) | 0.206 |
| TNFa | 15.96 (15.27, 17.97) | 15.96 (13.14, 19.28) | 0.596 | 12.41 (11.67, 14.57) | 13.86 (9.38, 17.31) | 0.673 |
| uPAR | 5565.00 (5286.00, 5730.00) | 5620.00 (4826.00, 5675.00) | 1 | 5509.00 (5172.00, 5565.00) | 5620.00 (5286.00, 5839.00) | ***0.071*** |

Supplemental table 1. This table provides an overview of all biomarkers that were measured in plasma and left ventricle homogenates. The biomarkers are described in pg/ml and median(IRQ); per group, 5 samples were measured. A p value <0.05 was considered statistically significant.

Chapter 8

# Hypercoagulability as predictor of progression of atrial fibrilation

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In preparation

## ABSTRACT

**Background**

Atrial fibrillation (AF), the most common cardiac arrythmia, affects approximately 2 % of the Western population and is associated with hypercoagulability associated risk of thromboembolic stroke. Coagulation proteases such as factor Xa (FXa) and thrombin have pleiotropic functions through protease activated receptors. Experimental work suggested that hypercoagulability can serve as a substrate for AF and potentially contributes to AF progression. We hypothesized that a hypercoagulable state promotes AF progression in humans.

**Methods and Results**

In this registry, 369 patients (56.6% male) with self terminating AF were followed-up for a median of 2.2 years. Primary endpoint was progression of AF towards non self terminating permanent AF. Markers of hypercoagulability were measured in plasma by respectively in-house developed ELISA's directed against activated coagulation factors in complex with their physiological inhibitors. Markers of cardiovascular health were measured by Olink cardiovascular panel III. During the follow-up time, AF progressed in 42 subjects (11.4%). In these patients, coagulation factors of the common pathway and factors downstream of the extrinsic pathway, were independently predictive of AF progression (FXa, thrombin and FIXa), whereas the intrinsic route of coagulation was negatively correlated to AF progression. In line, tissue factor pathway inhibitor negatively predicted AF progression. NTproBNP, PGLYRP1 and SPON1 were among the proteins with the highest positive predictive value of AF progression. TFPI, MCP1 and COL1A1 were among the proteins with the highest negative predictive value AF progression.

**Conclusion**

This interim analysis supports preclinical work by showing that patients with a hypercoagulable state more often progress from self terminating paroxysmal AF towards permanent non self terminating AF.

## INTRODUCTION

Atrial fibrillation (AF) is the most common cardiac arythmia affecting approximately 2% of the western population, and this is expected to double in the next decade (1,2). AF is associated with a two-fold increased risk of all-cause mortality and a three- to five-fold increased risk of stroke, and therefore brings about a great burden to society (2,3). Current treatment options are not adequately directed against the progressive nature of AF, but are focused on rhythm control as well as preventing thromboembolic stroke with oral anticoagulants (4).

The term "AF begets AF" was introduced after the identification of multiple factors that turn AF into a self-sustainable and progressive pathology (5–8). Electrical remodeling after episodes of AF, inflammation and fibrosis contribute to AF progression (5–10)).

In addition, coagulation proteases including FXa and thrombin promoted AF development and progression via protease activated receptors (PARs) in experimental animal models (11). Coagulation proteases are generated at higher levels in blood, already early in the course of AF, as shown in young subjects with lone paroxysmal AF and a CHA2DS2-VASc ≤ 2, that had increased plasma levels of activated FIX (12).

Whether increased activity of the coagulation system also promotes AF in humans is currently unknown. In this study, we therefore aimed to determine whether markers of hypercoagulability are associated with AF progression.

## METHODS



### Participants

The RACE V study is a Dutch multicenter, prospective, investigator-initiated, observational study (Clinicaltrials.gov identifier NCT02726698). The study was performed in compliance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board. All subjects included gave written informed consent.

This study is part of a registry aimed to include 750 patients with self terminating paroxysmal AF, as previously described (13). Briefly, the selection criteria included AF patients > 18 years of age, with ≤ 10 years of paroxysmal, self terminating AF, as documented by an electrocardiogram (ECG), $CHA_2DS_2$-VASc ≤ 5 and no other indication for anticoagulant therapy. Patients had to be willing to receive the Medtronic Reveal LINQ ® implantable loop recorder. Patients who already had a Medtronic pacemaker could be included when the atrial high-rate episodes were >190 BPM, lasting >6 minutes, and were classified as AF episodes.

Exclusion criteria were the presence of persistent AF, trigger-induced AF (such as infection or postoperative), history of congenital heart disease,

prior pulmonary vein isolation (PVI) or intended PVI, implantable cardioverter defibrillator or cardiac resynchronization therapy, refusing to temporarily stop (N) OAC for coagulation phenotyping (in patients already on (N)OAC before inclusion in this study), with the exception for patients with a history of ischemic stroke/ transient ischemic attack, expected to start with, or currently using amiodarone, pregnancy, pacemaker that is not a Medtronic pacemaker, ventricular pacing >50% in patients with a Medtronic pacemaker, life expectancy of less than 2.5 years.

**Data acquisition**

After consent, participant baseline characteristics were collected from the RACE V database, including but not limited to age, gender, body mass index, hypertension, diabetes mellitus, hypercholesterolemia, clinical coronary artery disease, ischemic stroke, peripheral vascular disease, chronic renal failure, aortic valve stenosis, Agatston score and pulmonary embolism. Anticoagulant medication was reported in the database, but was not taken into account in this analysis. Anticoagulation was withdrawn 2 days prior to each blood drawing.

**AF Progression**

During a median follow up of 2.2 years, the primary endpoint AF progression was assessed and diagnosed. A custom-made software, using Microsoft Visual Basic, was used to visualize AF episodes. AF progression in a consecutive 30-day window, throughout the whole follow-up per participant was calculated.

AF progression was classified as follows: 1. No AF recurrence during follow up; 2. AF recurrence without progression; 3. AF progression without persistent or permanent AF; 4. AF progression with persistent or permanent AF. Groups 1 and 2 were classified as no progression, whereas group 3 and 4 were classified as progression. Patients without clear signs of persistent AF reevaluated by 4 physicians. AF progression was validated by a mathematical formula of AF burden: AF episodes that occurred in the early follow-up phase weighed less than AF episodes occurring at the end of the follow-up. Progression over time equaled the weighted AF burden minus the actual AF burden over time (determined as percentage). AF progression was present if AF progressed >3% during the follow-up per individual, or if AF progressed >3% in the first half of the follow-up.

**Proteomic and coagulation profiling**

Venous blood was drawn at baseline in anticoagulant 3.2%(w/v) (0.109 M) sodium citrate and Ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was prepared through double centrifugation at room temperature (5 min at 2,300 g and 10 min at 10,000 g) and stored at -80 °C until further use. Activated coagulation proteases of the intrinsic and extrinsic cascade, in complex with their

physiological inhibitors, were measured by in-house developed enzyme-linked immunosorbent assay (ELISA) as previously described (14,15). In brief, coagulation FXIIa in complexes with antithrombin (FXIIa-AT) or C1 esterase inhibitor (FXIIa-C1Inh) were assessed in EDTA plasma. Coagulation FXIa in complexes with antithrombin (FXIa-AT), C1-esterase inhibitor (FXIa-C1Inh) or alpha-1-antitrypsin (FXIa-α1AT), kallikrein in complex with C1-esterase inhibitor (PKa-C1Inh), FIXa, FXa, thrombin and FVIIa in complex with antithrombin (FIXa-AT, FXa-AT, TAT, FVIIa-AT, respectively) were assessed in citrated plasma.

The proximity extension assay (PEA) technology (Olink Proteomics) cardiovascular panel III was used to quantify 92 plasma cardiovascular disease related biomarkers (16).

**Statistical analysis**

The main study endpoint in this interim-analysis was AF progression, and data was stratified accordingly. Participant baseline characteristics were reported as mean ± SD for normally distributed data, median ± IQR for skewed variables, and categorical data were described as absolute frequencies (%). Data were tested with the student's *t* test, Mann-Whitney U or Chi Square test. Missing values were imputed to prevent a loss of statistical precision and to reduce the likelihood of selection bias, using random forest imputation implemented in the R package MissForest (17).

To identify AF predictive biomarkers, all proteomic markers were modeled with Least Absolute Shrinkage and Selection Operator (LASSO) logistic regression analysis using the glmnet and caret R packages with progression as a dependent variable (18,19). In addition to protein biomarkers, a set of baseline characteristics (*Table 1)* were also included in the model. To generalize the model, a 5-fold cross-validation (CV) was used to select the penalty term λ. The optimal regularization parameter λ was chosen based on the best CV-AUC (Supplemental Figure 1). The selected variables were ranked according to the standardized regression coefficient(20), a scale describing the robustness of AF prediction; values close to zero were the least important independent predictors, and values close to 1 were the most important predictors of AF progression. The direction of the association was color labeled according to the model coefficients. Lasso logistic regression analysis removes variables without explanatory value. When markers are highly correlated, the most predictive value was included in the model, removing the others. Pearson's correlation test was therefore used to find highly correlated variables that were removed from the model due to redundancy. Statistical analyses were conducted in R statistical software (version 4.0.2).

## RESULTS

Of the 750 patients registered from the nationwide centers, 369 patients finished a median follow-up time of 2.2 years and were included in this interim-analysis (Table 1). Mean age was 67.1 ±10.5 years, 160 (43.4%) were women. Patients were stratified by AF progression; of 369 patients, 42 (11.4%) were diagnosed with AF progression. Patients with AF progression suffered more frequently from hypertension (40 [95.2%] vs. 259 [79.2%], clinical coronary artery disease (10 [23.8%] vs. 34 [10.4%]), peripheral vascular disease (2 [4.8%] vs. 1[0.8%]) and had a higher coronary artery calcium score (186 [IQR: 4.2 - 674.8] vs. 24 [IQR: 0 - 204.9]).

Hundred-fourteen variables, of which 13 baseline characteristics with a potential confounding effect (Table 1), were included in the LASSO regression model. Thirty-seven variables were classified as having an independent predictive value in AF progression after LASSO-regularized logistic regression modeling with the best fitted λ penalty term. The 37 variables identified had the highest discriminatory ability (Simple AUC= 0.93; CV-AUC=0.672). Hypertension, ischemic stroke, peripheral vascular disease, and gender were independent confounding predictors of AF progression. The complete list of all the variables selected is provided in Supplemental table S1.

The biomarkers with the highest positive association included N-terminal pro-Brain Natriuretic Peptide (NTproBNP), Peptidoglycan recognition protein 1 (PGLYRP1), Spondin1 (SPON1), Cathepsin D (CTSD) and Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) (Figure 2).

Conversely, Tissue Factor Pathway Inhibitor (TFPI) and Monocyte Chemotactic Protein 1 (MCP-1), Collagen Type I α1 Chain (Col1A1), Epithelial cell adhesion molecule (EpCam) and Suppression Of Tumorigenicity 2 (ST2) were among the top most negatively associated proteins in the progression group. Patients with AF progression had decreased activity levels of proteins from the intrinsic coagulation cascade, as reflected by lower FXIa-α1AT. However, proteins associated with an active extrinsic and common coagulation pathway (increased TAT and FXa-AT, and decreased TFPI) were positively associated with AF progression (Figure 2).

**Table 1 Baseline characteristics**

| Variables: | All (369) | Non Progression (327) | Progression (42) | p |
|---|---|---|---|---|
| Gender (male) | 56.6% (209) | 55.0% (180) | 69.0% (29) | 0.099 |
| Age | 67.1±10.5 | 66.8±10.4 | 69.8±10.5 | 0.085 |
| BMI (kg/m2) | 27.7±4.8 | 27.7±4.7 | 27.8±5.2 | 0.89 |
| Hypertension (yes) | 81.0% (299) | 79.2% (259) | 95.2% (40) | **0.011** |
| Diabetes mellitus (yes) | 8.7% (32) | 8.3% (27) | 11.9% (5) | 0.39 |
| Hypercholesterelomia (yes) | 49.6% (183) | 48.0% (157) | 61.9% (26) | 0.1 |
| Clinical coronary artery disease (yes) | 11.9% (44) | 10.4% (34) | 23.8% (10) | **0.02** |
| Ischemic stroke (yes) | 4.3% (16) | 4.6% (15) | 2.4% (1) | 1 |
| Peripheral vascular disease (yes) | 0.8% (3) | 0.3% (1) | 4.8% (2) | **0.035** |
| Chronic renal failure (yes) | 7.0% (26) | 6.7% (22) | 9.5% (4) | 0.52 |
| Aortic valve stenosis | | | | |
| 0 | 95.2% (340) | 95.9% (304) | 90.0% (36) | |
| -1 | 3.9% (14) | 3.2% (10) | 10.0% (4) | |
| -2 | 0.6% (2) | 0.6% (2) | 0% (0) | |
| -3 | 0.3% (1) | 0.3% (1) | 0% (0) | |
| Agatson coronary arteries | 27.0 (0/258.3) | 24.20 (0/204.9) | 186.0 (4.2/674.8) | **0.0039** |
| Pulmonary embolism (yes) | 1.1% (4) | 1.2% (4) | 0% (0) | 1 |

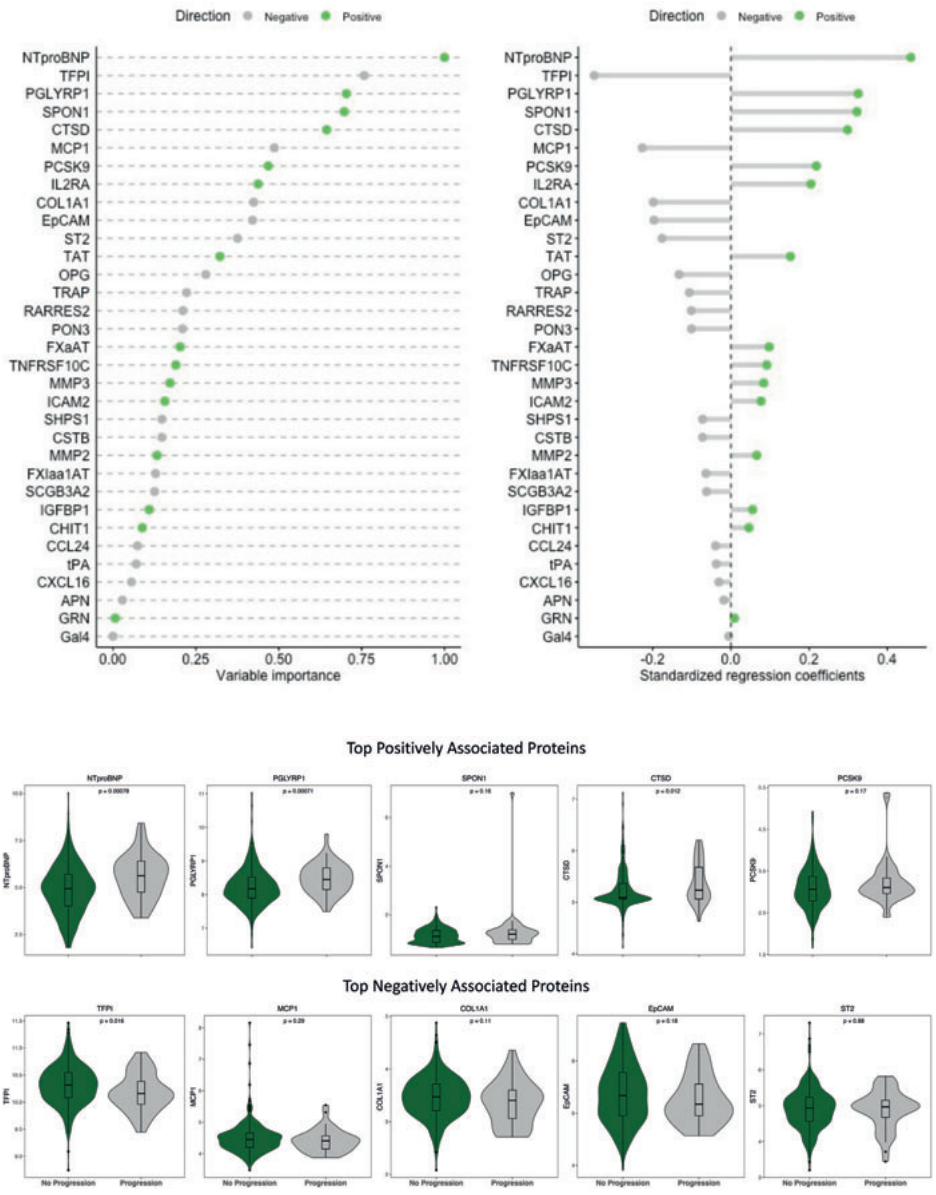


**Figure 2. Top AF Progression-Associated Proteins**
**A.** Biomarkers displayed in the figure are independently correlated with AF progression: a variable importance of 1 represents the highest predictive value for AF progression, while an importance close to 0 represents the lowest independent predictive value. **B.** Standardized regression coefficients of the top AF progression-associated proteins. Proteins marked green are positively associated with AF progression group, while markers in gray are negatively associated with AF progression. **C.** Violin plots of the top 5 positively associated proteins. **D.** Violin plots of the top 5 negatively associated proteins.

Pearon's correlation matrix revealed that FXIa-α1AT were significantly and positively correlated with XIIa-AT (0.946, p < 0.0001, Figure 3), FXIIa-C1inh (0.892, p < 0.0001), FXIa-AT (0.944, p < 0.0001) and FXIa-C1inh (0.260, p < 0.0001). FXa-AT highly correlated with TAT (0.434, p < 0.0001) and FIXa-AT (0.962, p < 0.0001) (Figure 3).

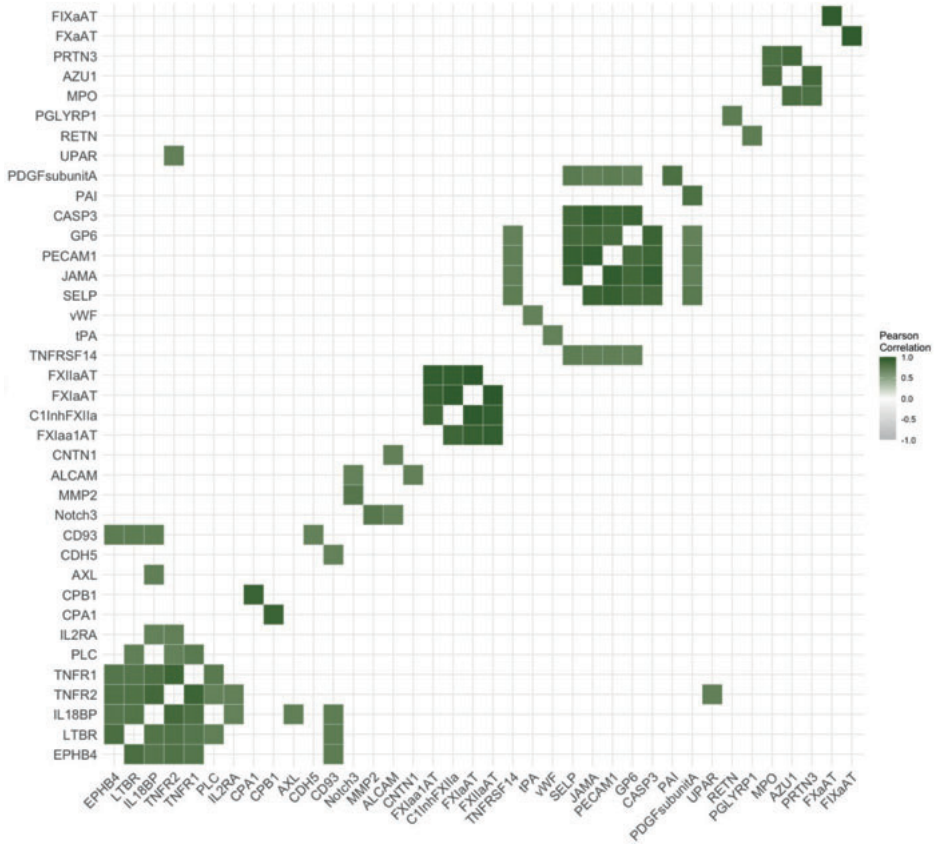


**Figure 3 Pearson's Correlation Matrix**
Pearson's correlation coefficients of the biomarkers with values < -0.7 and > 0.7 were visualized. Correlations reaching a value of 1 were removed from the graph. No negatively associated biomarkers reached the chosen cut-off value of -0.7.

## DISCUSSION

In the present study, we showed that patients with AF, who were diagnosed with progression during the 2.2 years follow-up time, had a different plasma biomarker signature at baseline compared to patients without AF progression. Several cardiovascular disease related markers were associated with the progression of AF. Moreover, the activity of the extrinsic coagulation cascade was more often increased at baseline in patients with AF progression, as reflected

by enhanced activity of FIXa, FXa and thrombin. This was in line with decreased expression of TFPI and decreased intrinsic coagulation activity. TFPI inhibitor is the most potent natural inhibitor of the TF/FVIIa/FXa pathway, and is mostly produced by megakaryocytes and endothelial cells (21).

The role of TFPI in atrial fibrillation is not well understood. As circulating TFPI levels may reflect atherosclerosis associated endothelial cell activation (22–24), one might have anticipated higher TFPI levels in those with AF progression, as these subjects had overall more evidence of coronary artery disease and risk factors like hypertension. Since this was not the case, other scenarios need to be considered. Rapid atrial pacing in a paroxysmal rat AF model decreased endocardial TFPI expression, thereby potentially inducing a local dis-balance in hemostasis (25). The exact mechanisms behind the AF-induced reduction of endocardial TFPI expression was not further studied. Giovanni et al. showed that TFPI was degraded in endothelial cells exposed to several reactive oxygen species (ROS) (26). Since ROS has emerged as a potential player in the pathogenesis of AF (27–29), AF-induced production of ROS might also contribute to reduced TFPI in AF progression.

TF is the activator of the extrinsic route of coagulation and is highly expressed in the sub-endothelial layer (30–32). TF expression was found to be upregulated in endocardial tissue of patients with AF, specifically in regions with high inflammatory cell content (33). In addition, activated monocytes are a rich source of TF, and its expression can be upregulated by cytokines and chemokines including interleukin-6 (IL-6) (34,35). Marcus et al. showed that in a cohort of coronary artery disease patients, elevated levels of IL-6 were positively associated with AF (36). Although AF progression was not monitored, it provides a possible link between IL-6 and TF expression in AF.

The extrinsic route of coagulation is initiated upon binding of FVII to cellular TF. The activated TF/FVIIa complex then activates FX, which subsequently generates thrombin, the central enzyme in coagulation that cleaves fibrinogen into fibrin. Furthermore, TF/FVIIa also activates FIX, termed the "Josso loop", which is then able to activate FX. In line with a lower plasma concentrations of the natural inhibitor of the extrinsic route, TFPI, our data reflect an increased activity of the common pathway, likely resulting from TF driven extrinsic coagulation activity, in those with AF progression. Interestingly, markers of the contact and intrinsic coagulation pathways were negatively linked to AF progression; at this stage we do not have an explanation for this effect, other than that apparently, there is little influence of any potential drivers of contact activation, including neutrophil activation/NETosis, or polyphosphates in these otherwise stable subjects.

Proteases of the extrinsic and common routes have been linked to inflammation and fibrosis, two pathways known to be associated with AF progression. TF/FVIIa, FXa and thrombin can activate different PARs. PARs

comprise a family of four G-protein coupled receptors (PAR1-4) and are expressed on the membrane of multiple cell types including, endothelial cells, vascular smooth muscle cells, fibroblasts and macrophages. Thrombin is the main activator of PAR-1, 3 and 4, TF/FVIIa activates PAR-1 and 2, whereas FXa mainly activates PAR-2. In earlier studies, we showed that hypercoagulable mice were more prone to develop AF and had increased atrial collagen deposition compared to control animals. Furthermore, goats with induced AF, who were treated with the indirect FXa and thrombin inhibitor nadroparin, were better protected from AF(11). Enhanced cardiac remodeling, as reflected by increased atrial collagen deposition, was attributed to thrombin-mediated PAR-1 activation of cardiac fibroblasts(11,37). Furthermore, FXa has been shown to induce a pro-inflammatory remodeling response in atrial tissue, which was further enhanced by tachyarrhythmia. Interestingly, FXa induced up-regulation of PAR-2 mRNA, while no effect was seen on PAR-1 mRNA. In contrast, tachyarrhythmia increased PAR-1 mRNA while no effect was seen on PAR-2 (38). The synergistic effects of FXa and tachyarrhythmia on PARs suggest that coagulation proteases potentially enhance their AF progressive effects via multiple pathways, such as the thrombin-PAR-1 and FXa-PAR2 axis.

**Limitations**

Observed associations cannot provide insight in causality, and biomarkers can therefore still be indirectly linked to the complex pathology of AF progression. Also, all biomarkers were the result of selection based on assumed mechanisms, so other, relevant markers may be missed.

Biomarkers of coagulation were independently linked to progression of AF. However, the coefficients corresponding to the LASSO logistic regression analysis were rather weak. This can be explained by the skewed distribution of the complex data. Additionally, small changes in systemic coagulation activity may well reflect locally elevated concentrations of such markers, in a context of hypercoagulability driven AF, which may also comprise locally expressed and activated coagulation proteins. Ongoing studies of locally sampled blood specimens in the atria may provide further insight in this process.



## CONCLUSION

In summary, increased activity of the extrinsic and common route of coagulation can predict progression of AF. Whether a highly active extrinsic and common route of coagulation simply reflects a diseased heart or directly promotes AF progression has to be established.

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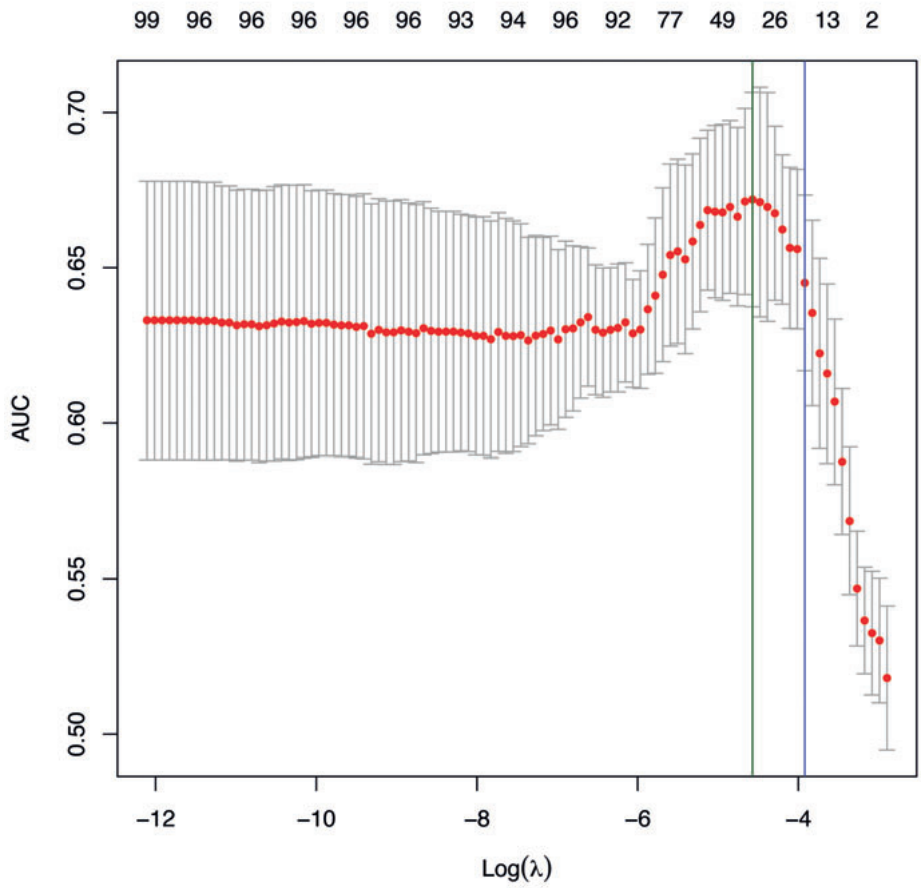
## SUPPLEMENTS

**Supplemental Table 1 Regression coefficients of biomarkers determined by LASSO regression analysis**

| Positive Association | | | Negative Association | | |
|---|---|---|---|---|---|
| Protein | beta | Relative Importance | Protein | beta | Relative Importance |
| Peripheral vascular disease | 1.7420 | 4.8626 | TFPI | -0.9561 | 3.6784 |
| CTSD | 0.8165 | 3.6784 | COL1A1 | -0.5052 | 2.7826 |
| SPON1 | 0.7545 | 4.0370 | MCP1 | -0.4826 | 2.5354 |
| PGLYRP1 | 0.6045 | 4.4306 | OPG | -0.3367 | 1.4508 |
| Hypertension | 0.5650 | 3.6784 | Ischemic stroke | -0.3241 | 1.4508 |
| IL2RA | 0.4516 | 2.5354 | ST2 | -0.3209 | 1.9179 |
| PCSK9 | 0.4437 | 2.7826 | RARRES2 | -0.3101 | 1.7475 |
| Gender | 0.3894 | 3.0539 | TRAP | -0.2840 | 1.9179 |
| NTproBNP | 0.3503 | 5.3367 | SHPS1 | -0.1798 | 1.4508 |
| ICAM2 | 0.1867 | 1.3219 | EpCAM | -0.1776 | 2.3101 |
| MMP2 | 0.1740 | 1.2045 | PON3 | -0.1456 | 4.4306 |
| TNFRSF10C | 0.1737 | 1.7475 | CSTB | -0.1137 | 1.3219 |
| MMP3 | 0.1250 | 2.1049 | CXCL16 | -0.0937 | 1.2045 |
| IGFBP1 | 0.0442 | 1.4508 | SCGB3A2 | -0.0786 | 1.3219 |
| CHIT1 | 0.0335 | 1.5923 | APN | -0.0541 | 1.2045 |
| GRN | 0.0275 | 1.0975 | CCL24 | -0.0440 | 1.2045 |
| TAT | 0.0170 | 2.3101 | tPA | -0.0277 | 1.2045 |
| FXaAT | 0.0001 | 4.0370 | Gal4 | -0.0098 | 1.0975 |
| | | | FXIaa1AT | -0.0001 | 1.3219 |




**Supplemental Figure 1. Cross-validation plot penalty term**
Fitted model of a 5-fold cross-validation in which the top x-axis represents the number of variables used in the model with the corresponding log(λ) on the bottom x-axis; in the top left corner, all predictors are included and in the top right only one predictor. The area under the curve (AUC) is plotted against the log(λ), the tuning parameter. The error bars represent the s.e., whereas the verticle lines visualize the optimal range of included variables (the green line is the minimum criteria, and the blue line the 1 - s.e.).

Chapter 9

# Summary

Hemostasis is a sophisticated and well-orchestrated interplay between platelets and the coagulation system to prevent blood loss after injury. A dis-balance in this system can lead to thrombosis or bleedings. However, over the past two decades experimental work has shown that proteins of the coagulation system also modulate various other processes beyond their role in hemostasis. In this thesis, I describe the role of factor Xa (FXa; one of the central enzymes in coagulation) in atherosclerosis, myocardial infarction, and atrial fibrillation. To study FXa I often used the clinically available anticoagulant rivaroxaban which is a direct inhibitor of FXa.

*Chapters 2 and 3* provide general background information regarding the pleiotropic actions of coagulation proteins. *Chapter 2* dives deeper into the signaling functions of the two central enzymes of coagulation, FXa and thrombin, via protease activated receptors, and how activation of these receptors by coagulation proteins can alter multiple biological processes.

*Chapter 3* focuses on the role of coagulation FXa in multiple cardiovascular diseases that have an inflammatory component since FXa is known to promote inflammation. This chapter provides an overview of the downstream pathways of FXa that are potentially indirectly targeted by rivaroxaban.

*Chapter 4* elaborates on the role of FXa. In this chapter we intervened with atherogenesis by inhibiting FXa with rivaroxaban. In a prevention study we found that the addition of this anticoagulant to atherosclerosis prone mice, decreased atherosclerotic burden and stabilized the plaque. In an intervention study with atherosclerotic mice, we even showed that FXa inhibition promoted the regression of highly progressed atherosclerotic lesions. Given the lack of optimal atherosclerotic treatment options, this finding potentially has clinical implications.

Given the importance of elucidating the mechanisms that drive FXa inhibition mediated atheroprotection, we unbiasedly studied gene expression after rivaroxaban treatment in atherosclerosis prone mice. In *chapter 5* we show that FXa inhibition influences a large set of different genes related to cellular metabolism: genes belonging to pathways of fatty acid metabolism were mostly upregulated after rivaroxaban treatment. We also show that different processes play a role in early versus late atherosclerosis and that FXa inhibition can have different outcomes depending on the stage of the disease.

*Chapter 6* provides four biomarkers that can predict cardiovascular events in patients suffering from peripheral arterial disease. IL-6 was among the predictive markers of cardiovascular events and is a known predictor. Novel predictors of cardiovascular events and mortality included PAR-1, Gal-9 and TNFRSF11a.

*In chapter 7* we tested the role of FXa during myocardial ischemia reperfusion injury. Just two bolus injections of the FXa inhibitor rivaroxaban attenuated myocardial cell death and induced long-lasting changes in protein expression.

During ischemia, a timely reperfusion of the tissue is needed to circumvent cell death, and therefore biologically, the formation of new blood vessels is imminently needed. We showed that FXa inhibition promotes a long-lasting increase in proteins involved in the formation of new blood vessels and thereby potentially contributes to cellular protection during ischemia reperfusion injury.

*Chapter 8* tested the role of increased coagulation activation in the progression of atrial fibrillation. To study this, a large subpopulation of patients with atrial fibrillation had been followed for 2.5 years with continuous heart rhythm monitoring. We could show that hypercoagulability can predict progression of AF and that mainly the extrinsic route of coagulation, being tissue factor in complex with FVIIa, is responsible for enhanced FXa and thrombin activity during atrial fibrillation progression.

*Chapter 9 and 10* contain the thesis summary and general discussion. Here, I put the experimental findings in context and look ahead for avenues of further research and translation.

# Chapter 10

# General discussion

It is well-established that several proteases of the coagulation cascade have roles beyond the general aim of hemostasis: prevention of blood loss after injury (1–5). The most direct protease-to-cell communication is being conveyed by protease activated receptors (PARs), as discussed. However, other downstream proteins can also induce cell signaling. In humans, FXa is the main activator of PAR2, while thrombin activates PAR1, 3 and 4. Activation of these receptors can modulate multiple processes, such as cell migration, cell survival, and inflammation (1). Although the implications of coagulation signaling and its inhibition are not fully understood in humans, this does not withhold physicians to prescribe to patients anticoagulants directed against FXa and thrombin readily on a large scale, for prevention or treatment of thrombosis. In this thesis I studied the complexity of the coagulation proteins and their function in system biology, with a focus on cardiovascular diseases. I showed that Factor Xa (Fxa) inhibition protects the heart after a myocardial infarction and decreases atherogenesis. Furthermore, in patients with atrial fibrillation (AF), hypercoagulability predicted progression of AF. This thesis spans multiple cardiovascular diseases, but has FXa as a common denominator. I will therefore not discuss the diseases separately in detail but will provide evidence for a common ground.

FXa inhibition affects multiple downstream proteins, including thrombin generation and platelet activation (1). When simply discussing hemostasis, one would not assume that direct FXa or direct thrombin inhibition would have different effects on hemostasis, since there is no evidence of a relevant bypass to either FXa or thrombin towards fibrin formation. Furthermore, thrombin inhibits FXa as well, because inhibition of thrombin also attenuates thrombin's positive feedback loop towards FXa (via FXIa) (6–9). Therefore, it can also be argued that FXa inhibition alters thrombin signaling and vice versa. Recently it was shown that FXa inhibition might alter different downstream pathways than thrombin inhibition, but this goes beyond the scope of this discussion (10,11). The data in my thesis supports an overall positive effect of FXa inhibition on cardiovascular pathologies. However, due to the complex interactions of FXa, the involved pathways could not yet be fully exposed.

Multiple studies highlighted the role of coagulation proteases FXa and thrombin, and their receptors PAR1 and PAR2 in cardiovascular disease, but none, including the studies in this thesis, were able to directly correlate coagulation-mediated activation of PARs to the observed phenotype (12–23). The complex nature of the coagulation cascade and PARs make interpretation challenging. First, thrombin is indirectly inhibited by FXa inhibition (and vice versa) and is known for its role in cardiovascular diseases. Second, the targets of thrombin, including fibrin, platelets, PAR1 and activated protein C (APC), also modulate cardiovascular diseases (24–29). Platelets can be highly inflammatory once activated, and fibrin attracts leukocytes (30). Thrombin mediated activation of PAR1 generally promotes inflammation, whereas APC-mediated PAR1 activation

has protective effects (1). Thrombin thereby adds a second layer of complexity to FXa inhibition.

Despite the highly similar cardiovascular phenotypes found between PAR1- and PAR2-deficient models and inhibition of coagulation, robust evidence of an active coagulation-PAR signaling route in cardiovascular diseases does not exist. PARs can be activated by multiple proteases, such as matrix metallo proteases, trypsin, and deficiency of PAR also diminishes signaling by those proteases (31). Furthermore, deficiency also interferes with the immunomodulating role of PAR2, for example, that does not require direct activation (32,33). To fully elucidate the complex interactions between coagulation proteases and signaling functions, novel PAR mutants, such as the G37I (34), must be implemented into traditional models of cardiovascular disease. The G37I PAR2 mouse mutant is insensitive to FXa-mediated PAR2 activation but the receptor can still be activated by other proteases. Furthermore, FXa would still be able to exert its pleiotropic and hemostatic functions but is not able to activate PAR2. Thus, the G37I PAR 2 mutant resembles FXa inhibition without interfering with downstream hemostatic actions.

Although this thesis does not fully elucidate which mediators of FXa inhibition lead to the protective downstream effects, it offers novel directions for future research. With bulk RNA sequencing I showed that early and late atherosclerosis can be different entities. This must be taken into account in drug discovery because treatment might have to be tailored to atherosclerotic patients depending on their disease state. Additionally, I showed that FXa inhibition affects cellular metabolism by enhancing the expression of genes related to fatty acid metabolism. Through which mechanisms this affects protein levels and cell function has to be established. Nevertheless, it has been demonstrated that pro-inflammatory cells rely more on glycolytic metabolism in contrast to pro-resolving inflammatory cells relying more on fatty acid metabolism (35). It is tempting to speculate that FXa inhibition changes the local environment (or directly the cellular phenotype) at the site of injury to promote resolution and thereby brings about a better balance in pro-inflammatory and pro-resolving cells. FXa inhibition would then result in a better outcome after cardiovascular events. Indeed, macrophages can display different phenotypes ranging from pro-inflammatory to proresolving phenotypes and everything in-between. Macrophages express TF, FVII, and when activated also FX, thereby forming a local FX activation complex. It has been shown that oxLDL, but also other inflammatory triggers, induce a long-lasting pro-inflammatory memory in macrophages, termed trained immunity (36–38). A “trained macrophage” has enhanced tissue destructive, pro-inflammatory characteristics. Furthermore, locally synthesized FXa can activate macrophage PAR2 and thereby modulates their phenotype, a phenomenon disabled in the G37I PAR2 mutant or inhibited by rivaroxaban (39). Since rivaroxaban can penetrate tissues, local FXa inhibition

potentially alters the macrophage phenotype towards a less pro-inflammatory state more capable of repairing injured tissues. The clinical relevance of FXa inhibition on non-hemostatic pathways has yet to be established.

It has been suggested that long-term anticoagulant treatment with direct FXa or thrombin inhibitors in patients with AF, reduces the risk of dementia potentially by decreasing stroke risk (40–44). However, recent data showed a decreased risk of dementia independent of stroke risk (45,46). Although undiagnosed microemboli and silent brain infarcts can underlie this association, other factors might be in play (47). Wingo et al. found a strong association between cerebral atherosclerosis and dementia in a proteome-wide association study with dorsolateral prefrontal cortices (48). Since the studies in this thesis and other preclinical work robustly show that anticoagulants attenuate atherogenesis, anticoagulant treatment in AF might protect the cerebral vasculature and thereby decrease the risk of dementia.

The concept of vascular protection by anticoagulant treatment is supported by results from the COMPASS trial (49). Patients with stable coronary artery disease treated with a low dose of the FXa inhibitor rivaroxaban on top of traditional anti-platelet therapy (aspirin), had reduced secondary cardiac events and mortality rates (49). Given the low dose rivaroxaban used in these patients, the additive anti-thrombotic effect might be relatively low, suggesting that other vascular protective effects might be in play (50). Given the potential long-term interference with coagulation signaling by drugs like rivaroxaban, we urgently need to fully elucidate signaling functions of these coagulation proteases. In addition, it would be of great interest to find druggable targets downstream of FXa-PAR signaling that would convey protective effects at a lower cost of bleeding, consistently associated with the use of any of the central acting anticoagulants.

In summary, in this thesis I showed that FXa inhibition attenuates cardiovascular diseases in experimental models. In recent years there has been growing evidence that anticoagulant treatment in humans also affects the outcome of cardiovascular diseases. Finding the targets involved in these beneficial effects would be of great interest, since current anticoagulants come at the cost of significant bleedings and can therefore not be widely used for their potential cardiovascular protective effects.

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# Chapter 11

# Impact Paragraph

For decades, patients have been prescribed anticoagulants to prevent and treat thrombosis. Anticoagulants inhibit enzymes of the coagulation cascade, and thereby prevent clot formation. Heparin was one of the first anticoagulants introduced already early in the previous century. In the same period, warfarin was introduced and became the number one oral agent to prevent and treat thrombosis. After almost 70 years of warfarin treatment, it became appreciated that warfarin therapy has effects beyond prevention and treatment of thrombosis, including the calcification of large blood vessels. Although warfarin is currently still being used as an anticoagulant, new direct oral anticoagulants (DOACs), such as dabigatran and rivaroxaban (I used the latter anticoagulant extensively in my thesis to study the pleiotropic effects of coagulation), are now the preferred agents in thrombosis management. However, in the past two decades, it became evident that through administration of these anticoagulants, biological and cellular processes beyond coagulation can be modulated. Although long-term effects on biological processes in humans remain largely unknown, preclinical data suggest that DOAC treatment might have protective effects on the cardiovascular system.

Since increasing numbers of patients will be administered DOACs oftentimes lifelong, it is of significant importance to fully elucidate the pleiotropic effects of coagulation proteins and the long-term biological consequence of their inhibition.

In chapter 4 I showed that the anticoagulant drug rivaroxaban, inhibited the development of atherosclerosis, and more importantly promoted regression of highly advanced atherosclerotic lesions. I attempted to further elucidate the biological processes that explain anticoagulant-mediated atheroprotection. In doing so, I revealed that inhibition of the coagulation system by rivaroxaban modulated biology at the genetic level, thereby affecting cellular metabolism. If we can fully understand the pleiotropic actions of inhibition of the coagulation system, we can turn this knowledge into new treatment options for atherosclerosis, without the bleeding risk associated with DOACs. Recent clinical trial data supports the concept of atheroprotection by DOACs: mortality rates significantly decreased in patients with stable coronary artery disease treated with a low dose rivaroxaban (in combination with aspirin) when compared to their control receiving traditional treatment. The studies described in this thesis can help create a stronger foundation for the wider use of a low dose anticoagulant in poly vascular atherosclerotic diseased patients, provided that the benefit risk (bleeding) ratio allows it. More efficient treatment of atherosclerosis will further diminish the burden on society and cost-effectiveness studies demonstrate the potential gain in subsets of patients with atherosclerosis, when treated with the dual pathway inhibition strategy.

Coronary atherosclerosis is the main cause of most myocardial infarctions and combined with stroke, responsible for the majority of deaths in Western

society. The treatment of an acute myocardial infarction is directed towards fast restoration of blood flow to the ischemic heart, termed reperfusion. However, reperfusion is considered a double-edged sword because it further enhances the damage to the infarcted site. Chapter xx describes how inhibition of coagulation by anticoagulants can potentially protect the heart from reperfusion injury, and can thereby provide a better long-term outcome for the patients.

In 2017, treatment of coronary atherosclerotic disease accounted for 22% of the total cardiovascular expenses (and 2.6% of the total healthcare expenses) amounting to 2.3 billion euros in the Netherlands alone. Since no optimal treatment for atherosclerotic disease exists to date, and prevalence rates are expected to rise significantly, expenses can become unsustainable in the next decade. Besides improved patient care, the data from this thesis can aid in decreasing the global economic burden that atherosclerotic disease and myocardial infarction have on society by guiding future treatments strategies.

In conclusion, in the short-term this thesis supports a stronger foundation for wider use of specific anticoagulants and in the long-term it aids in guiding future treatment strategies.

# Appendices

# Curriculum Vitae
# Publicaties en presentaties
# Dankwoord

## CURRICULUM VITAE

Jens Posma was born on June 16, 1986, in Heerlen, The Netherlands. After he finished his high school education (MAVO), he studied at CIOS Sittard, where he found out his ambitions lie elsewhere. He studied Physical Rehabilitation for 1 year at Hoge School Zuyd (Heerlen) as a way station on the way back to high school. During a second round of high school, he has followed the VWO courses Mathematics, Physics, Biology and Chemistry. At VAVO Maastricht he combined these 4 courses (years 4-6) and graduated in 1 year, only to lose the admissions lottery to study for medicine. He then went to Maastricht University where he studied Biomedical Sciences, with a focus on Molecular Sciences. Three years later he graduated and pursued his academic adventure by doing a Master Biomedical Sciences at the Transnational University Limburg, a collaboration between Maastricht University and Hasselt University. During his first year he studied dose volume metrics in treatment planning strategies for head and neck cancers at the Maastro Clinic in Maastricht. In his second and final year of his master he specialized in cardiovascular diseases and wrote his thesis about factor Xa inhibition and atherosclerosis, under the supervision of Dr. H.M.H. Spronk at the department of Biochemistry. In the same group, supervised by Prof. Dr. H. ten Cate, he took the opportunity to start his academic career as a PhD-candidate. He was appointed to study the role of coagulation in Atrial Fibrillation. However, during his 5 years as a PhD-candidate he has studied coagulation and cardiovascular diseases in a broader sense. In 2018, he visited the laboratory of Prof N. Mackman, Chapel Hill, North Carolina, USA, where he further explored the role of coagulation Factor Xa in atherosclerosis. He has also worked for short periods in the laboratories of Prof. Dr. A.J. van Zonneveld (Leiden, Netherlands), Prof. Dr. Ruf and Prof. Dr. P Wenzel (Mainz, Germany), and Dr. S. Heitmeier (Wuppertal, Germany). The highlights of his doctoral work are presented in this thesis.

# PUBLICATIES EN PRESENTATIES

**Publications**

*Grover SP, Coughlin T, Fleifil SM,* ***Posma JJN****, Spronk HHM, Heitmeier S, Owens AP 3rd, Mackman N*. Effect of combining aspirin and rivaroxaban on atherosclerosis in mice. Atherosclerosis **2022** Mar; 345:7-14

*Mattila N, Hisada Y, Przybyla B,* ***Posma JJN****, Jouppila A, Haglund C, Seppänen H, Mackman N, Lassila R*. Levels of the cancer biomarker CA 19-9 are associated with thrombin generation in plasma from treatment naïve pancreatic cancer patients. Thrombosis Research **2021** Mar;199:21-31

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