Unraveling the role of factor XI and plasma prekallikrein in coagulation

Citation for published version (APA):

Visser, M. (2021). *Unraveling the role of factor XI and plasma prekallikrein in coagulation*. [Doctoral Thesis, Maastricht University]. ProefschriftMaken. <https://doi.org/10.26481/dis.20210630mv>

Document status and date:

Published: 01/01/2021

DOI:

[10.26481/dis.20210630mv](https://doi.org/10.26481/dis.20210630mv)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Unraveling the role of factor XI and plasma prekallikrein in coagulation

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Unraveling the role of factor XI and plasma prekallikrein in coagulation

Thesis Maastricht University

ISBN: 978-94-6423-268-4

Cover: Christine Huber

Printing: ProefschriftMaken || www.proefschriftmaken.nl

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Unraveling the role of factor XI and plasma prekallikrein in coagulation

DISSERTATION

to obtain the degree of Doctor at the Maastricht University,
on the authority of the Rector Magnificus,
Prof.dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public on
Wednesday 30th of June 2021 at 16:00 hours

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Table of content

Chapter 1	General introduction	7
Chapter 2	Role of factor XIa and plasma kallikrein in arterial and venous thrombosis	19
Chapter 3	Plasma kallikrein contributes to coagulation in the absence of factor XI by activating factor XI	43
Chapter 4	Increased plasma kallikrein activity in coagulation factor XI-deficient individuals	67
Chapter 5	Evaluation of in vitro systems to determine factor XI and plasma prekallikrein activation and to assess their role in patients with acute VTE	85
Chapter 6	Complement, neutrophils, extracellular histones, and contact activation as potential drivers of Covid-19	103
Chapter 7	General discussion	119
Chapter 8	Summary	133
	Samenvatting	139
	Impact paragraph	145
	Curriculum Vitae	151
	List of publications and presentations	155
	Acknowledgement	159

Chapter 1

General introduction

Arterial and venous thrombosis

Thrombosis as the underlying event in ischemic stroke, myocardial infarction and venous thromboembolism (VTE), is the leading cause of mortality and morbidity worldwide. An estimated one in four deaths worldwide is caused by thrombotic conditions and this number is likely to increase in the aging population.¹ Thrombosis describes the partial or complete occlusion of blood vessels and can be divided into arterial thrombosis and venous thrombosis, depending on whether an artery or a vein is affected. Arterial and venous thrombosis were considered separate diseases resulting from distinct pathogenic causes. However, there are certain factors such as age and obesity that pose a risk for both diseases.² Interestingly, several studies demonstrated that patients with arterial thrombosis have an increased risk of venous thrombosis and vice versa.³⁻⁷

The underlying pathologic disorder causing arterial thrombosis is atherosclerosis. This chronic inflammatory condition is characterized by endothelial dysfunction, lipid retention and recruitment of immune cells.^{8,9} Monocytes, recruited to the affected site by activated endothelial cells, differentiate into macrophages and internalization of oxidized lipoproteins leads to foam cell formation that induces, amongst others, the formation of a fibrous cap.¹⁰ Once the fibrous cap of the atherosclerotic plaque ruptures, tissue factor and collagen are exposed to the blood stream leading to initiation of the extrinsic pathway of coagulation and activation of platelets, respectively.¹⁰ In addition, physiologic negatively charged surfaces such as polyphosphates or DNA and RNA released from activated platelets and damaged cells, respectively, have been shown to initiate the intrinsic pathway of coagulation by activation of Factor (F)XII.^{11, 12} Neutrophil extracellular traps (NETS) excreted by activated neutrophils, which are involved in arterial thrombus formation, may also activate FXII.¹³ Plaque rupture and subsequent thrombus formation is referred to as atherothrombosis and results in partial or complete occlusion of the affected artery. Since platelets play a crucial role in the development of atherothrombosis, arterial thrombi are platelet-rich and are often referred to as "white" thrombi.¹⁴ Cross-linked fibrin as a result of the activated coagulation cascade stabilizes the thrombus. Clinically, atherothrombosis causes myocardial infarction, ischemic stroke and peripheral artery disease. Main risk factors for arterial thrombosis are hyperlipidemia, smoking, diabetes, hypertension and obesity.²

Venous thrombosis involves the formation of blood clots in the deep veins of the legs (less frequently in the arms, rarely in other locations), referred to as deep vein thrombosis (DVT). These thrombi may dislodge and migrate to the pulmonary arteries resulting in occlusion of these vessels, referred to as pulmonary embolism (PE). The

underlying trigger for venous thrombotic diseases is the presence of one or a combination of the so-called Virchow triad factors: hypercoagulability, venous blood flow stasis or endothelial dysfunction.¹⁵ Epidemiological studies in humans and animal models demonstrated that components of the intrinsic, extrinsic and common pathway of coagulation as well as, amongst others, endothelial cells, red blood cells, platelets and cell-derived microvesicles are involved in VTE. In addition, TF and NETs released from recruited leukocytes may drive venous thrombosis.¹⁶ Compared with arterial thrombus formation, venous thrombi slowly form under low shear rate ($10\text{--}100\text{ s}^{-1}$) and are referred to as "red" thrombi as they mainly contain red blood cells and fibrin, and, to a lesser extent, platelets.¹⁴ Risk factors for VTE include genetic factors such as factor V Leiden, congenital deficiencies of antithrombin, protein C or protein S, or non-O- blood group, and non-genetic factors for instance surgery, immobilization, age or pregnancy.^{2, 16}

The coagulation cascade

Coagulation is a complex process in which multiple prothrombotic, antithrombotic and cellular mechanisms are involved. Hemostasis is essential at sites of vascular injury and is important for the prevention of bleeding. Excessive or pathological coagulation, however, leads to arterial or venous thrombosis, resulting in serious complications such as stroke, myocardial infarction and pulmonary embolism. Therefore, a balance between pro-and anticoagulant components is essential.

The coagulation cascade involves the sequential proteolytic activation of various zymogens to form proteases (Figure 1).^{17, 18} Upon vascular injury, TF, a transmembrane protein, is exposed to the blood stream where it activates and forms a complex with FVIIa.¹⁹ This represents the extrinsic pathway and merges in the common pathway of coagulation, in which FX is activated to FXa by TF-FVIIa. Finally, the cleavage of prothrombin by the prothrombinase complex, composed of FXa and its cofactor FVa, produces thrombin, which is considered the key enzyme of coagulation. This process results only in a small amount of thrombin, since FXa and the TF-FVIIa complex are rapidly inhibited by tissue factor pathway inhibitor (TFPI).²⁰ However, TF-FVIIa also activates FIX to FIXa, a component of the intrinsic pathway of coagulation, to enhance thrombin formation.^{21, 22} FIXa forms the intrinsic tenase complex with its cofactor FVIIIa, which activates FX to FXa, leading to thrombin formation. Thrombin amplifies its own generation by activating FV, FVIII and FXI, a component of the intrinsic pathway and an activator of FIX.²³⁻²⁵

The intrinsic pathway of coagulation is initiated by autoactivation of FXII on anionic polymers (e.g. kaolin, ellagic acid, DNA, polyphosphates) (Figure 1). This

process involves a conformational change of FXII upon binding to the surface resulting in limited proteolytic activity.²⁶ Autoactivation of FXII, which is enhanced by Zn^{2+} ,^{27, 28} leads to formation of a two-chain molecule connected by a disulfide bond, referred to as α FXIIa. α FXIIa activates plasma prekallikrein (PK) to plasma kallikrein (PKa), which in turn cleaves more FXII, thereby amplifying its own generation and formation of α FXIIa.^{26, 29} PKa additionally cleaves α FXIIa into β FXIIa, a potent activator of PK.²⁶ α FXIIa binds to negatively charged surfaces via the heavy chain and activates FXI, eventually leading to thrombin generation, while β FXIIa lacks both abilities.³⁰ In the final step of the coagulation cascade, thrombin converts fibrinogen to fibrin monomers and activates FXIII that stabilizes and crosslinks fibrin.²⁴

Not all coagulation factors are equally important for hemostasis. The extrinsic pathway is essential for hemostasis as it is activated upon vascular injury. FVII-deficient mice are not viable since they bleed to death during the neonatal period. FIX

Figure 1. The coagulation cascade. The coagulation cascade involves the sequential proteolytic activation of various zymogens to form proteases. Autoactivation of FXII to FXIIa on surfaces is the primary step of the contact activation coagulation pathway, leading to subsequent activation of PK, FXI, FIX, FX and prothrombin. FXII in turn is reciprocally activated by PKa. FVII activation by TF is the initial step of the extrinsic pathway. Zymogens are indicated in roman numerals while proteases are additionally presented with an „a“.

and FVIII are also essential for hemostasis; FIX or FVIII deficiency in humans causes the bleeding disorders hemophilia B and A, respectively.³¹ In contrast, the proteases of the contact system do not appear to play a role in hemostasis, which has been shown in several animal studies. FXII- and PK-deficient animals do not have a bleeding phenotype, but interestingly, they have reduced thrombus formation compared to WT animals.^{32, 33} Epidemiological studies have shown that individuals with FXII or PK deficiency do not have abnormal bleeding.^{34, 35} FXI also appears to play a minor role in hemostasis, although this is not quite as clear as for FXII and PK. Some individuals with FXI deficiency have a higher risk of bleeding, others do not. The risk of bleeding however does not appear to be related to FXI levels. Interestingly, FXI-deficient individuals have a lower risk of DVT and ischemic stroke,³⁶⁻³⁸ suggesting that this enzyme plays a role in thrombosis.

Regulation of coagulation

Many regulatory mechanisms exist to avoid excessive coagulation resulting in thrombosis. Activated coagulation factors are rapidly inhibited by various endogenous inhibitors, which are essential to keep the balance between blood clotting and bleeding. C1-inhibitor (C1inh) belongs to the serpin family and represents, with a plasma concentration of 240 µg/mL, one of the most abundant protease inhibitors in the blood circulation. C1inh is primarily a regulator of the complement system by inhibiting C1s and C1r.³⁹ It is also the most important inhibitor of FXIIa and, in addition to α2-macroglobulin, the major inhibitor of PKa.^{40, 41} C1inh is therefore considered as the key regulator of the contact system of coagulation. Like all serpins, C1inh forms stable, covalent complexes with the proteases in a 1:1 stoichiometric manner and is a so called suicide inhibitor.⁴² However, the inhibitory potential of C1inh towards the proteases is low as indicated by a rather slow association constant compared with other serpins.⁴³ In addition, it has been demonstrated that C1inh is a less effective inhibitor in the presence of endothelial cells as compared with its inhibitory potential in the fluid phase.³⁹

The activity of FXIa is regulated by C1inh, α1-antitrypsin (α1-AT), α2-antiplasmin (α2-AP) and antithrombin III (AT).⁴⁴ α1-AT and α2-AP, which are the main inhibitors of elastase released by leukocytes and plasmin, respectively,^{45, 46} and AT are members of the serpin superfamily of serine protease inhibitors. It has been shown, that C1inh is not only the major inhibitor of FXIIa and PKa but also of FXIa as demonstrated by its contribution to 47% inactivation of FXIa in human plasma, followed by α2AP (24.5%), α1AT (23.5%), and AT (5%).⁴⁴ A similar distribution of these inhibitors contributing to the inactivation of FXIa was observed *in vivo* with C1inh being

the predominant inhibitor. The half-life time of clearance of the FXIa:inhibitor complexes, however, was longest for FXIa: α 1AT indicating that this parameter may be most suitable to determine FXI activation in patient cohorts.⁴⁷

AT is the most important regulator of coagulation as it inhibits thrombin, FXa, FIXa⁴⁸ and also to a lesser extent other proteases such as FXIIa⁴⁰, FXIa⁴⁴ and FVIIa⁴⁹. The role of AT in regulating blood clotting is illustrated by the fact that individuals with congenital or acquired AT deficiency are predisposed to thrombotic diseases.⁴⁸ Physiologically, AT is a rather slow inhibitor. However, its inhibitory potential towards serine proteases can be enhanced by heparin, to which it binds with high affinity. Under normal physiological conditions, heparin sulfate, localized on the endothelium, and heparin released from endothelium-associated mast cell granules support the antithrombotic effect of AT.⁴⁸ In addition, the therapeutically used anticoagulant heparin exerts its effect in patients by potentiating its antithrombotic mode of action through endogenous AT.⁴⁸

In vitro assays to assess coagulation in plasma

In order to gain insights into coagulation of individuals and patients, various methods have been developed to investigate the potential and activation of coagulation. One method that has been widely described and used to study coagulation and to determine the efficacy of anticoagulant approaches is the thrombin generation assay.⁵⁰⁻⁵² Thrombin generation assay reflects the temporal interaction of almost all coagulation factors in the coagulation cascade and offers the possibility of detecting pro- and anticoagulant processes. This assay determines the amount of thrombin formed in clotting plasma over time by detecting the conversion of a low affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC). Both the contribution of the extrinsic pathway and the intrinsic pathway to thrombin generation can be analyzed by initiating the coagulation cascade via TF and a trigger of the contact system (e.g. ellagic acid), respectively. In order to correct for inner-filter effects and substrate consumption, all tested plasma samples are calibrated against a particular amount of thrombin- α 2-macroglobulin complex.⁵⁰

Following parameters are calculated by the Thrombinoscope software:

- Lag Time (describes the time from activation of coagulation to formation of the first thrombin)
- Thrombin Peak (indicates maximum thrombin activity during coagulation)
- Time to Peak (describes the time from activation of coagulation to reaching the thrombin peak)

- Velocity Index (defines the slope and thus the velocity of the thrombin generation curve)
- Endogenous Thrombin Potential (describes the total amount of thrombin formed)

While the thrombin generation assay detects the formation of a single enzyme in the coagulation cascade, namely thrombin, enzyme-linked immunosorbent assays (ELISA) may be valuable approaches to reflect the activation of other enzymes of the coagulation cascade. Since activated coagulation factors are rapidly inhibited by their endogenous inhibitors,⁴⁸ the measurement of these enzyme-inhibitor complexes can be a useful tool to investigate the activation of specific coagulation factors in individuals or different patient cohorts, thereby assessing the role of coagulation in various diseases. For instance, analysis of thrombin-antithrombin complexes is an accepted biomarker of coagulation. Enzyme-inhibitor complex ELISAs have also been used to detect and assess the activation of components of the contact system in cardiovascular disease^{53, 54} as well as sepsis^{55, 56} and dengue fever⁵⁷. The underlying principle of the ELISA used is a Sandwich ELISA. The activated coagulation factor is bound to the bottom of a 96-well plate by a specific antibody. A specific biotinylated secondary antibody recognizes the endogenous inhibitor. The enzyme-inhibitor complex is detected via the conversion of a substrate by streptavidin-labeled horseradish peroxidase, which is coupled to the secondary antibody via biotin-streptavidin binding.

Outline and aims of the thesis

The research in this thesis aims to gain further insights in the role of the contact activation pathway in coagulation, particularly of factor XI and plasma kallikrein, and to investigate their contribution to thrombotic diseases.

Chapter 2 provides an overview about the role of plasma kallikrein and factor XI in arterial and venous thrombosis. The contact activation system of coagulation has gained in importance in recent years as data from animal models demonstrated that inhibition or lowering the levels of PK(a), FXI(a) and FXII(a) reduced the risk of thrombosis while hemostasis is not affected. This chapter also includes a summary of therapeutic strategies to prevent and treat thrombotic diseases.

Chapter 3 examines the contribution of PKa to intrinsic coagulation in the absence of FXI. For this purpose, commercially available human FXI-deficient plasma and *F11^{-/-}* mice were used to investigate thrombin generation and FIXa:AT complex formation in the presence or absence of a PKa-specific small molecule inhibitor.

Chapter 4 aims to find reasons behind the different bleeding phenotype of FXI-deficient individuals. To address this, plasma from 21 FXI-deficient individuals from Israel was characterized by determining the coagulation factor levels, analyzing the coagulation potential and estimating the individual endogenous coagulation status.

Chapter 5 describes the role of the intrinsic coagulation cascade, in particular of FXI and PK, in patients with acute VTE. This study was implemented to gain insights about the extent of FXI and PK activation and their relation to the clinical outcome of acute VTE.

Chapter 6 investigates the inflammatory response and thrombotic complications in patients with COVID-19 infection to be able to provide novel targets for improved therapy. The main focus was on intrinsic coagulation, complement and neutrophil activation.

Chapter 7 briefly summarizes the main results and critically discusses the key findings in relation to the literature.

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Chapter 2

Role of factor XIa and plasma kallikrein in
arterial and venous thrombosis

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Thrombosis and Haemostasis 2020; 120(6):883-993.

Abstract

Cardiovascular disease, including stroke, myocardial infarction and venous thromboembolism, is one of the leading causes of morbidity and mortality worldwide. Excessive coagulation may cause vascular occlusion in arteries and veins eventually leading to thrombotic diseases. Studies in recent years suggest that coagulation factors are involved in these pathological mechanisms. Factors XIa (FXIa), XIIa (FXIIa) and plasma kallikrein (PKa) of the contact system of coagulation appear to contribute to thrombosis, while playing a limited role in normal hemostasis. Contact activation is initiated upon auto-activation of FXII on negatively charged surfaces. FXIIa activates plasma prekallikrein (PK) to PKa, which in turn activates FXII and initiates the kallikrein-kinin pathway. FXI is also activated by FXIIa, leading to activation of FIX and finally to thrombin formation, which in turn activates FXI in an amplification loop. Animal studies have shown that arterial and venous thrombosis can be reduced by the inhibition of FXI(a) or PKa. Furthermore, data from human studies suggest that these enzymes may be valuable targets to reduce thrombosis risk. In this review, we discuss the structure and function of FXI(a) and PK(a), their involvement in the development of venous and arterial thrombosis in animal models and human studies, and current therapeutic strategies.

Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality worldwide. An estimated 422.7 million cases of CVD occurred in 2015, of which an estimated 17.9 million people died. Among cardiovascular diseases, myocardial infarction and stroke have been the two major causes of CVD-related health loss worldwide.¹ While normal blood clotting is essential to stop bleeding at sites of vascular injury, excessive coagulation can result in vascular occlusions in arteries or veins eventually leading to thrombotic diseases.²

The coagulation cascade is based on a waterfall model, described by Macfarlane³ and Davie & Ratnoff⁴ in 1964, that involves the sequential activation of different zymogens to active enzymes. Blood coagulation can be initiated by exposure of tissue factor (TF) to the blood stream or by activation of FXII through negatively charged surfaces which can be either artificial (e.g. ellagic acid, kaolin) or of natural origin (e.g. polyphosphates, collagen)^{5,6}. In the extrinsic pathway, complex formation of TF and FVIIa leads to activation of FX and subsequently to thrombin generation.⁷ The conversion of FXII to FXIIa is the primary step of the intrinsic coagulation pathway, leading to subsequent activation of FXI, FIX, FX and prothrombin. FXIIa also activates PK, thereby initiating the kallikrein-kinin pathway and amplifying the formation of FXIIa through the reciprocal activation of FXII by PKa.⁸ Thrombin converts fibrinogen to fibrin in the common pathway.

Inhibition of coagulation factors is an obvious approach for antithrombotic therapies since excessive coagulation is a potential cause for thrombotic diseases. However, not all coagulation factors are equally suitable targets as some coagulation factors are essential for hemostasis and inhibition of these factors might increase the bleeding risk. For instance, FIX or FVIII-deficient patients suffer from spontaneous bleedings, whereas FXI-deficient individuals have none to a mild bleeding phenotype.⁹ In this review, we discuss the role of FXI(a) and its homolog PK(a) in thrombosis, taking into account results from arterial and venous thrombosis animal models and reports from human studies and deficiencies.

FXI(a) structure

FXI is a 160 kDa zymogen that circulates in plasma at a concentration of 30 nM¹⁰ and is mostly bound in a non-covalent complex to high molecular weight kininogen (HK), a 120 kDa plasma protein possibly functioning as an adaptor for FXI to bind to negatively charged surfaces.^{11, 12} FXI is mainly synthesized in hepatocytes¹⁰ and regulated by the transcription factor hepatocyte nuclear factor-4 α .¹³ Recently, FXI

pre-mRNA could be identified in platelets by Zucker et al. They showed that FXI pre-mRNA is spliced into mature mRNA upon platelet activation by either thrombin or ADP. The resulting protein has similar properties as plasmatic FXI when analyzed by western blot or an activity assay.¹⁴ The structure of FXI is fundamentally different from other coagulation factors since it forms a dimeric structure and unlike vitamin K-dependent coagulation factors lacks a Gla domain (Figure 1). Papagrorgoriou et al provided a crystal structure of full-length FXI purified from human plasma.¹⁵ FXI consists of two identical subunits of 607 amino acids, which are linked to each other via an interchain disulfide bond at Cys321. In addition, hydrophobic interactions and

Structure of FXI

Structure of PK

Figure 1. Structure of human factor XI and plasma prekallikrein. The domain structure of both zymogens comprises four tandem repeats called apples domains. The catalytically active serine protease domain (CD) is situated at the C-terminus. The activation of FXI and PK occurs through cleavage of the Arg369-Ile370 and the Arg371-Ile372 bond, respectively. While FXI comprises two identical subunits linked to each other via the A4-domains, PK does not form a dimeric structure. The apple domains contain binding sites for various proteins and enzymes.

salt bridges stabilize this dimeric structure.¹⁵ The activation of the zymogen monomers occurs through cleavage of the Arg369 – Ile370 bond (Figure 1) generating a 47 kDa heavy chain and a 33 kDa light chain linked by a disulfide bond.¹⁶

Each monomer comprises four tandem repeats of 90-91 amino acids each. These so-called apple domains A1 to A4 at the N-terminus of the heavy chain contain seven antiparallel β -strands and an α -helix. The serine protease domain in the light chain is situated at the C-terminus and connected to the apple 4 domain.^{15, 17} This linkage generates a so-called “cup and saucer” conformation with the catalytic domain located on the disk-like arrangement of the apple domains.¹⁵ Each apple domain contains binding sites for various proteins and enzymes (Figure 1). Thrombin and HK interact with FXI via the A1 and A2 domains,^{18, 19} respectively, whereas the A3 domain has binding sites for FIX,²⁰ the platelet receptor GPIb²¹ and heparin.²² FXIIa seems to bind to the A4 domain of FXI, which also carries the linkage domain for dimerization.^{23, 24}

FXI activation and function

FXI is mainly activated via two enzymes, namely FXIIa²⁵ in the contact activation pathway and thrombin^{26, 27} as part of an amplification loop (Figure 2). Upon vessel injury, subendothelial tissue factor is exposed to the peripheral blood stream. Together with FVIIa it activates FX to FXa and FIX to FIXa. In complex with the cofactor FVa, FXa converts prothrombin to thrombin,⁷ which is considered the key enzyme at the end of the coagulation cascade triggering various (patho)physiological pathways, including fibrin formation and stability, platelet activation, and initiating inflammatory processes. Thrombin is inactivated via antithrombin,²⁸ the TF-FVIIa-FXa complex by TFPI,⁷ quickly. Larger amounts of thrombin are generated during thrombus stabilization and propagation in positive feedback loops including amongst others (FV, FVIII) the activation of FXI by thrombin.²⁸

Besides the activation by thrombin, FXIIa converts FXI to FXIa in the contact activation pathway. Here, the autoactivation of FXII on negatively charged surfaces, which can be either artificial (e.g. ellagic acid, kaolin) or of natural origin (e.g. polyphosphates, collagen, nucleic acids)^{5, 6, 29} represents the initial step and results in sequential activation of FXI, followed by FIX, FX and prothrombin.⁸ In summary, FXI activation plays a critical role in both, the propagation phase of thrombin generation and during contact activation.

Figure 2. Coagulation cascade The coagulation cascade is based on a waterfall model that involves the sequential activation of different zymogens to active enzymes. The conversion of FXII to FXIIa is the primary step of the intrinsic coagulation pathway, leading to subsequent activation of FXI, FIX, FX and prothrombin. FXIIa also activates PK, which reciprocally activates FXII, initiates the kallikrein-kinin pathway and, in the absence of FXI, activates FIX (dashed line). The FVIIa-TF complex of the extrinsic pathway activates FIX and FX, leading to thrombin generation. Zymogens are indicated in roman numerals and their activated forms end with an „a“. Cofactors within the coagulation cascade are shown in red.

This activation does not happen in a single step but through formation of an intermediate with one activated subunit ($\frac{1}{2}$ -FXIa). It can be distinguished from fully activated FXIa on SDS PAGE under reducing conditions.³⁰ The natural substrate for FXIa is FIX, a 57 kDa single-chain glycoprotein that binds to the A3 domain of FXIa, but does not interact with the zymogen FXI, since the binding site on A3 for FIX is only exposed after a conformational change upon FXI activation.³¹ FIX seems to interact with FXIa via its Gla domain.³¹

In vitro data of several groups suggest that FXIa might activate other proteins in the coagulation cascade as well, including FV, FVIII and FX. The sites cleaved by FXIa on FV and FVIII appear to be similar to those observed during their activation by thrombin, which is still viewed as the main activator of both cofactors, while FV activation by FXIa occurs at a slower rate.³² Matafonov et al described that FX can be activated by FXIa, but to a lesser extent than the main FXIa substrate FIX. While FIX activation via FXIa is calcium-dependent, FX and FV activation are apparently not.³³ It is also reported that tissue factor pathway inhibitor (TFPI), an inhibitor of the TF/FVIIa/FXa complex, can be inactivated by FXIa through proteolytic cleavage of TFPI between the Kunitz 1 and 2 domains.³⁴ Because lack of functional TPFI may lead to prolonged activity of the TF/FVIIa/FXa complex, this may have an impact on the thrombin concentration in the early amplification phase.

Recent data suggest a role for FXI as a molecular linker of coagulation and inflammation. Ge et al demonstrated in their studies that prochemerin (chem163S) can be cleaved in plasma by contact phase-activated FXIa to a partly active intermediate, which is subsequently completely activated by plasma carboxypeptidases to chemerin, an adipokine and chemoattractant. The formation of the intermediates via FXIa could be enhanced by the addition of phospholipids to plasma or in the presence of platelets.³⁵ The interaction of FXI and the GPIIb- receptor on platelets and its linkage to inflammation and coagulation has also been investigated in previous studies, in which an impact of thrombin-activated FXI bound to platelets on arterial hypertension was demonstrated.³⁶

In vivo data show the contribution of FXIa to intrinsic coagulation. Infusion of purified FXIa into chimpanzees resulted in activation of the factors IX, X, and prothrombin demonstrating that FXIa is an important factor in the coagulation cascade.³⁷ In addition, it has also been shown that inhibition of FXIa caused an increase of fibrinolysis in a thrombosis model in rabbits due to decreased activation of TAFI by thrombin possibly contributing to the mild bleeding phenotype in some FXI-deficient individuals.³⁸ Recently, Mohammed et al also provided insights in the role of FXIa acting downstream of FIX *in vivo*, by showing that infusion of FXIa or increasing the plasmatic FXI level up to 200% reduced the bleeding time in a saphenous vein bleeding model in FIX^{-/-} mice indicating an involvement of FXI pathways independent of FIX.³⁹ Further investigations are needed to determine the role of the possible alternative pathways in hemostasis or thrombosis.

FXIa is inhibited by binding of several proteins. *In vivo*, C1 inhibitor is the dominant inhibitor (68% of FXIa in complex with the inhibitor), followed by alpha 2-antiplasmin (13%), alpha 1-antitrypsin (α 1AT, 10%) and antithrombin III (9%).⁴⁰ Almost

all FXIa-inhibitor complexes had a half-life of 95-104 minutes while the half-life of the FXIa- α 1AT complexes was 349 minutes.⁴⁰ Recently, it was demonstrated that FXIa can also form a complex with endothelial plasminogen activator inhibitor-1 (PAI-1), thereby inhibiting FXIa activity. These complexes were also observed in a baboon model of *Staphylococcus aureus*, suggesting that the complex formation with PAI-1 leads to clearance of FXIa.⁴¹

PK(a) structure

PK is the 85 - 88 kDa precursor of the serine protease PKa and circulates in plasma at a concentration of ~580 nM. It is mainly expressed in hepatocytes.⁴² However, mRNA of PK was also found in nonhepatic cells such as endothelial cells, fibroblasts, leukocytes and in extrahepatic tissues. PK expressed in nonhepatic cells and tissues might contribute to local actions, but its physiological role is not completely understood.^{43, 44} The amino acid sequence of PK is 58% identical to that of FXI. The zymogen contains, like FXI, the characteristic four apple domains that are comprised of 90-91 amino acids (Figure 1) each, but there is a major structural difference between both zymogens. While FXI comprises two identical subunits linked to each other, PK only has one subunit and does not form a dimeric structure.^{42, 45} PK is mostly bound to HK in a noncovalent complex.⁴⁶ HK facilitates the activation of PK by FXIIa and, on the other hand, serves as a natural substrate for PKa which cleaves HK to liberate the potent pro-inflammatory peptide bradykinin.^{12, 42} The complex with HK also allows PK to interact with cells such as endothelial cells,⁴⁷ platelets⁴⁸ and neutrophils.⁴⁹ The HK binding sites in PK are situated in the domains A1, A2 and A4 whereby the A2 domain seems to be most important for complex formation with the D6H domain of HK (Figure 1). The A3 domain is, however, not involved.⁵⁰

PK is activated by cleavage of the Arg371 – Ile372 bond (Figure 1). The active enzyme PKa consists of a heavy chain (371 residues) and a light chain (248 residues) that are connected to each other via disulfide bond.⁴² A recent study provided a full-length crystal structure for PKa.⁵¹ Compared to the dimeric FXI structure, there is a conformational difference in the apple 4 domain that may explain the monomeric structure of PKa. There is also a large conformational difference in the A3 domain and a 180° rearrangement of the apple domains relative to the protease domain compared to the FXI structure indicating that a conformational change occurs upon activation of PK.⁵¹ However, this still needs to be confirmed by comparison with a crystal structure of PK.

PK activation and function

The plasma contact activation system comprises a group of proteins including FXII, PK, FXI and HK which are activated upon binding of FXII to negatively charged surfaces. FXII undergoes a conformational change during binding, causing the protein to autoactivate to FXIIa. Besides activating FXI, as discussed before, FXIIa also cleaves PK to form the enzyme PKa (Figure 2). PKa in turn activates FXII thereby amplifying the initiation of the FXIIa mediated intrinsic coagulation cascade. PK can also be activated to PKa independently of FXII. Prolylcarboxypeptidase expressed by endothelial cells for instance was found to activate PK when bound to cells^{52, 53} and also heat shock protein 90 has been considered as an activator of PK, possibly by enhancing its autoactivation.⁵⁴

Recently, we demonstrated that PKa contributes to coagulation in a FXI-independent manner. In the absence of FXI, activation of FXII on ellagic acid or long-chain polyphosphates led to thrombin generation in human plasma and in a mouse model via FIX activation by PKa.⁵⁵ PKa is also involved in inflammatory processes mostly via release of the pro-inflammatory peptide bradykinin (BK) out of HK. By binding to its G-protein coupled B1 or B2 receptors, BK causes vasodilation and increased vascular permeability (Figure 2).⁵⁶ Besides its role in coagulation and inflammation, PKa acts on the fibrinolytic system by activating pro-urokinase-type plasminogen activator (pro-uPA) to uPA and on the renin-angiotensin system by activating prorenin.^{57, 58} In addition, the complement pathway can be initiated through cleavage of the central complement component C3 by PKa.⁵⁹

Given the fact that PKa is involved in so many pathways it is not surprising that this protease is associated with various diseases. Hereditary angioedema (HAE) is in many cases primarily a consequence of a C1 inhibitor deficiency, which results in hyperactivity of the kallikrein-kinin signaling pathway.⁶⁰ In recent studies, it has been shown that the events in HAE patients can be reduced in number and severity by the inhibition of PKa.^{61, 62} In addition, increased PKa activity could be detected in diabetes patients⁶³ and the protease appears to play a role in the development of diabetic retinopathy.⁶⁴ Recent data suggest a role for PKa in processes inducing neuroinflammation. Göbel et al demonstrated that PK levels were increased in the central nervous system lesions of multiple sclerosis patients and that PKa might amplify leukocyte trafficking by modulating the blood-brain barrier in a PAR2-dependent manner.⁶⁵ Furthermore, it has been shown that PKa potentiates ADP-initiated platelet activation by acting through PAR1 receptors.⁶⁶ PKa in plasma is mainly inhibited by endogenous C1 inhibitor (52%) and α 2-macroglobulin (48%).⁶⁷

Animal models to study thrombosis and bleeding

Both, arterial and venous thrombosis can be studied in various animal models. Knockout animal models are widely used to investigate the contribution of coagulation factors to thrombosis risk (Table 1). *F11*^{-/-} mice, first described by Gailani et al,⁶⁸ are viable and have a normal reproductive capacity. Although the activated partial thromboplastin time (aPTT) is significantly prolonged, the bleeding times of *F11*^{-/-} mice are comparable to wild type mice and they do not exhibit spontaneous bleedings.⁶⁸

The role of FXI in arterial thrombus formation was initially investigated by Rosen et al using *F11*^{-/-} mice in a ferric chloride (FeCl₃)-induced carotid artery injury model.⁶⁹ Compared to wild type mice, thrombus formation was markedly reduced in *F11*^{-/-} mice and infusion of human FXI led to vessel occlusion times similar to control animals, indicating that FXI contributes to arterial thrombus formation.⁶⁹ This was confirmed by a comparative study, in which the effects of FIX and FXI deficiencies on arterial thrombosis were investigated in a FeCl₃-induced carotid artery injury model in mice using different concentrations of FeCl₃.⁷⁰ While the vessels in wildtype mice exposed to 3.5% FeCl₃ occluded within 10 min, *F9*^{-/-} and *F11*^{-/-} mice were fully protected from occlusion at 5% FeCl₃ and partially protected at 7.5% FeCl₃. Remarkably, the bleeding time in *F11*^{-/-} mice was similar to that of wildtype mice, whereas it was significantly prolonged in *F9*^{-/-} mice.⁷⁰ Interestingly, a recent study showed that FXI, but not FIX deficiency in mice with low levels of TF is associated with increased blood pool size in the placenta, suggesting a tissue-specific role for FXI in mice.⁷¹ Postnatal survival of low TF mice was however dependent on FIX levels.⁷¹ Similar antithrombotic effects of FXI deficiency could be shown in a FeCl₃-induced vena cava thrombosis mouse model.⁷²

Likewise, the role of FXI in development of thrombosis has been studied in higher species. A human FXI monoclonal antibody was used to prevent vascular graft occlusion and to reduce thrombus formation in a primate thrombosis model.⁷³ In addition, FXI ASO inhibited thrombus formation without increasing bleeding risk in a primate model.⁷⁴

To determine the role of PK in the development of thrombosis, Bird et al used PK-deficient mice showing normal blood pressure and heart rate.⁷⁵ Compared to wild-type mice, PK-deficient mice were completely protected from occlusion in a 3.5% FeCl₃-induced carotid artery injury model and partially protected at 5% FeCl₃, indicating that PK contributes to arterial thrombosis. In addition, thrombus weight in a venous thrombosis model induced by 3.5% FeCl₃ was significantly reduced in PK-deficient mice compared to wildtype mice. In contrast to *F11*^{-/-} mice, PK-deficient mice had a slightly increased tail bleeding time but a similar renal bleeding time compared

Table 1. Contribution of FXI and PK to thrombosis in animal models.

	Arterial thrombosis model	Literature	Venous thrombosis model	Literature
<i>F11</i> ^{-/-}	↓ FeCl ₃ induced thrombus formation (mouse)	69, 70	↓ FeCl ₃ induced thrombus formation (mouse)	72
<i>Klk1</i> ^{-/-}	↓ FeCl ₃ induced thrombus formation (mouse) ↓ Middle cerebral artery occlusion induced intracerebral thrombosis (mouse)	75,76,77	↓ FeCl ₃ induced thrombus formation (mouse)	75
FXI antibody	↓ Thrombus formation in a vascular graft occlusion model (baboon)	73	↓ FeCl ₃ induced thrombus formation (mouse)	105
FXI ASO	↓ Thrombus formation in a vascular graft occlusion model (baboon)	74	↓ FeCl ₃ induced thrombus formation (mouse) ↓ FeCl ₃ induced mesenteric vein thrombosis (mouse) ↓ Stenosis induced thrombosis (mouse)	108
PK ASO	↓ FeCl ₃ induced mesenteric arterial thrombosis (mouse)	79	↓ FeCl ₃ induced thrombus formation (mouse) ↓ Stenosis induced thrombosis (mouse)	79

ASO, antisense oligonucleotide; FXI, factor XI; PK, prekallikrein.

to wildtype mice.⁷⁵ The antithrombotic effect of PK deficiency in arterial thrombosis was also demonstrated in a FeCl₃-induced carotid artery injury mouse model by Kokoye et al.⁷⁶ Given the fact that PKa is an important activator of FXII and vice versa it is not surprising that FXII-deficient mice were also protected from carotid artery occlusion.⁷⁶

Since PK is not only involved in contact activation, but also induces the kinin-pathway, Göb et al investigated whether PKa contributes to thromboinflammation. In transient and permanent models of ischemic stroke PK-deficient mice had reduced intracerebral thrombosis and improved cerebral blood flow compared to wild-type mice, while infarct-associated hemorrhage was not increased.⁷⁷ A similar outcome could be observed using a PK-specific antibody.⁷⁷

In addition, there is evidence that PK also contributes to arterial thrombosis independently of FXII. It has been shown that PK deficiency in mice resulted in increased generation of prostacyclin leading to reduced vascular TF expression.⁷⁸ Selective depletion of PK by ASO was also found to be thrombo-protective in arterial and venous thrombosis mouse models without increasing the bleeding risk.⁷⁹ However,

contradictory results exist indicating a pro-thrombotic effect in PK-deficient mice, for instance when, prolylcarboxypeptidase as activator of PK is depleted simultaneously.⁸⁰

The results of these animal studies (Table 1) strongly support the hypothesis that both, FXI and PK, play a role in the development of thrombosis and that the zymogens or their activated forms could be suitable drug targets to reduce the risk of thrombosis. Whether their respective impact might differ quantitatively remains to be elucidated.

Results from human studies and deficiencies

FXI deficiency in humans is a rare disorder and was first described in 1953 by Rosenthal et al.⁸¹ This disorder, inherited as an autosomal recessive trait, is more frequently observed in Ashkenazi Jews. In contrast to FIX or FVIII deficiency, FXI deficiency, which is defined by levels below 20 IU/dL (severe deficiency), is associated with a mild bleeding phenotype with very rare spontaneous bleedings. Bleeding in FXI-deficient subjects is more likely to occur following trauma or surgery, especially if tissues with high fibrinolytic activity are affected.⁹ The bleeding tendency is, different from FIX or FVIII deficiency, not correlated with the FXI level.^{9, 82} Therefore, other hemostatic abnormalities including low levels of von Willebrand factor have been suggested to contribute to the bleeding risk in FXI-deficient subjects.⁸³

There are several studies that examine the role of FXI in thrombosis in humans. High levels of FXI within the general population have been identified as risk factor for ischemic stroke⁸⁴ and deep vein thrombosis (DVT).⁸⁵ In addition, results from the Risk of Arterial Thrombosis in Relation to Oral Contraceptives (RATIO) Case-Control Study showed an association between high levels of FXIa:C1inh and FXIa:AT inhibitor complexes and ischemic stroke in young women.⁸⁶ A study with 115 severe FXI-deficient subjects demonstrated that FXI deficiency might be protective against ischemic stroke.⁸⁷ Furthermore, the incidence of DVT is reduced in FXI-deficient individuals compared to the incidence in individuals with normal activity. In the study of Salomon et al no cases of DVT could be reported in 219 individuals with severe FXI deficiency.⁸⁸ A lower risk for DVT in FXI-deficient subjects could be confirmed by a comparative study, in which a protective effect of FXI deficiency against cardiovascular events (composite of myocardial infarction, stroke, and transient ischemic attack) was observed.⁸⁹ A recent study investigating the association between FXI and thrombosis risk in a cohort of patients with a first unprovoked venous thromboembolism demonstrated that lower levels of FXI reduced the risk of recurrent venous thrombosis.⁹⁰ Taken together, the results from these epidemiology studies strongly support an association between FXI and ischemic stroke and DVT, respectively, in

humans. The contribution of FXI to myocardial infarction (MI) is less clear. Some data suggest that individuals with FXI deficiency are not protected from MI,⁹¹ while another study observed an association between FXI and MI.⁹² In addition, Minnema et al found a significant increase of FXI activity in patients with MI demonstrated by detection of FXIa:C1inhibitor complexes in 24% of the patients with an acute myocardial infarction (AMI).⁹³ Other studies also showed an increase in FXI activation during the acute phase in AMI patients, either by detection of FXIa-C1 complex levels or by a thrombin generation-based FXIa assay.^{94, 95} These studies show that FXI plays an important role in thrombosis and might be a valuable drug target to prevent thrombosis (Table 2).

While FXI deficiency has been described relatively well in literature, there are fewer studies on PK deficiency in humans (Table 2). PK deficiency, also known as “Fletcher trait”, was first described in a family by Hathaway et al back in 1965.⁹⁶ At least 80 reported cases of severe PK deficiency (levels below 15% of normal), inherited as an autosomal recessive trait, can be found in literature. However, most cases of PK deficiency may be undetected, since it is clinically asymptomatic and not associated with an increased bleeding tendency, although individuals with this disorder exhibit a prolonged aPTT.⁹⁷ The role of PK as risk factor for thrombosis in humans is therefore difficult to estimate. In 2011 Girolami et al hypothesized that deficiency of one of the contact system proteins may not protect against thrombosis⁹⁸ and in 2018 it was stated

Table 2. Contribution of FXI and PK to thrombosis risk in humans.

	Findings		Literature	
High levels of FXI	↑ Ischemic stroke ↑ Deep vein thrombosis		84, 85	
Lower levels of FXI	↓ Recurrent venous thrombosis		90	
FXI deficiency	↓ Ischemic stroke ↓ Deep vein thrombosis		87, 88, 89	
FXI	Association with MI	No association with MI	92, 93, 94, 95	91
High PKa/PK levels	↑ Ischemic stroke in young women ↑ Incidence of arterial vascular disease → VTE risk in general population		86, 100, 101	
PK deficiency	→ Thrombosis Associated with cardiovascular disease		98, 99	

FXI, factor XI; MI, myocardial infarction; PK, prekallikrein; PKa, plasma kallikrein; VTE, venous thromboembolism.

that PK deficiency often seems to be associated with cardiovascular diseases.⁹⁹ The RATIO Case-Control study provides data suggesting that increased PKa levels is associated with ischemic stroke in young women. In this study it was demonstrated that high levels of PKa-C1 inhibitor complexes led to a 4-fold increase in ischemic stroke.⁸⁶ The association of increased PK levels with a higher incidence of arterial vascular disease such as myocardial infarction has also been shown in another study.¹⁰⁰ However, there is also evidence that PK levels cannot be associated with a higher VTE risk in the general population.¹⁰¹ Overall, more human studies have to be carried out to determine if PKa may be associated with thrombosis risk.

Therapeutic strategies

The development of direct oral anticoagulants (DOACs) has improved the treatment of patients in need for anticoagulation, which was previously dominated by vitamin K antagonists as the only available oral drug class. In contrast to warfarin, no routine monitoring is required.¹⁰² However, the general risk of bleeding remains - with a focus on gastrointestinal bleeding¹⁰² and other types of mucosa-related bleeds.

There are several therapeutic approaches to target FXI by inhibiting antibodies, its synthesis by antisense oligonucleotides (ASO), or FXIa by antibodies and small molecule inhibitors (summarized in table 3). Antibodies are characterized by a rapid onset of action, which depends on the site of application (intravenous vs. subcutaneous), and usually have a long half-life, requiring follow-up treatment only after weeks or months. First human data on safety, pharmacodynamics and pharmacokinetics of antibodies directed against the active site of FXIa were derived from a phase 1 study. Compared to controls, the aPTT was prolonged and FXIa activity was reduced in cohorts receiving the anti-FXIa antibody osocimab (BAY 1213790).¹⁰³ Administration of MAA868, an antibody against FXI and FXIa, also diminished FXIa activity and increased aPTT in a phase 1 study¹⁰⁴ and is currently investigated in a phase 2 trial (NCT04213807). Anti-FXI antibodies reduced thrombus formation in a primate model⁷³ and decreased thrombus size in FXI-deficient mice administered with human FXI.¹⁰⁵

ASOs bind to the RNA of the target protein, thereby preventing its expression and finally lowering its plasma concentration.¹⁰⁶ Since the impact on FXI levels via synthesis inhibition by FXI ASO is rather slow, this approach is not feasible as standalone therapy for early secondary prevention after an event or when urgent protection is needed, but might be more suitable for primary prevention or during elective procedures. A FXI-directed ASO (IONIS 416858) has been tested in patients undergoing total knee arthroplasty in a phase 2 trial, in which ASO treated patients

Table 3. Pharmacological agents targeting FXI(a) or PKa in clinical trials.

	Mode of action	Site of action	Reported clinical trial	Literature
BAY 1213790	Antibody	Adjacent to the FXIa active site	Phase 2	115 NCT03276143 NCT0378368
MAA868	Antibody	Catalytic domain of FXI and FXIa	Phase 2	104 NCT04213807
IONIS 416858	Antisense	FXI mRNA	Phase 2	107
BMS-962212	Small molecule	FXIa inhibitor	Phase 1	109
ONO-7684	Small molecule	FXIa inhibitor	Phase 1	NCT03919890
BMS-986177	Small molecule	FXIa inhibitor	Phase 2	NCT03891524 NCT03766581
BAY 2433334	Small molecule	FXIa inhibitor	Phase 2	NCT04218266
Ecaltantide	Recombinant protein	PKa inhibitor	Registered for HAE	114
Lanadelumab	Antibody	PKa active site	Registered for HAE	62
BCX7353	Small molecule	PKa inhibitor	Phase 3	61 NCT03485911

FXI, factor XI; HAE, hereditary angioedema; PKa, plasma kallikrein.

were compared to patients receiving enoxaparin instead. Lowering FXI levels with 300 mg ASO reduced the incidence of venous thromboembolism after surgery and the size of the clots without increasing the bleeding risk.¹⁰⁷ These data confirmed the results obtained from studies in primates⁷⁴ and mice¹⁰⁸, in which thrombus formation was reduced without increasing bleeding risk.

Synthetic small molecules can be administered either parenterally or orally and are suitable for situations, in which a rapid antithrombotic effect is required. First data on safety, pharmacodynamics and pharmacokinetics of a small molecule inhibitor (BMS-962212) was reported in a phase 1 study. Intravenous administration of the inhibitor to healthy individuals resulted in prolongation of the aPTT and reduction of FXI activity.¹⁰⁹ Several oral FXIa inhibitors are reported to be at different development stages: While ONO-7684 is reported to be in phase 1 (NCT03919890),¹¹⁰ BMS-986177 (NCT03766581, NCT03891524)^{111, 112} and BAY 2433334 (NCT04218266)¹¹³ are investigated in phase 2 studies.

The data on PKa as a therapeutic approach in thrombotic diseases are rather limited compared to FXI. There are some preclinical data on the effect of ASO specific

to PKa. The efficacy of ASO was investigated in a mouse model showing that selective depletion of PKa is thrombo-protective in arterial and venous thrombosis.⁷⁹ Ecallantide, a peptidic inhibitor of PKa,¹¹⁴ and Lanadelumab (DX-2930) are registered for HAE, while active site small molecules inhibitors of PKa (e.g. BCX7353) demonstrated that this approach might be effective in patients with HAE.^{61, 62} However, these inhibitors were not tested for their antithrombotic effect in clinical studies.

The main advantage of FXI/FXIa inhibition over currently used antithrombotic strategies is most likely in the area of safety. Animal studies^{68, 70} and recent clinical trials^{107, 115} indicate that inhibition of FXIa or lower FXI levels reduce the risk of venous thrombosis without major impairment of hemostasis. In the previously conducted FOXTROT trial the highest dose of osocimab was superior to enoxaparin in reduction of asymptomatic DVT. In this phase 2 study a very low number of relevant bleeding events was found in all groups.¹¹⁵ Thus, further safety assessment of osocimab will have to be conducted in future studies. Inhibition of PKa may also be a safe antithrombotic strategy, since PK-deficient animals did not show any impairment in hemostasis as compared with WT animals.⁷⁵ While limited data exist on the antithrombotic effect of PKa inhibitors the additional anti-inflammatory properties render this very interesting approach to treat thromboinflammatory diseases.⁷⁷

Conclusion

Since cardiovascular disease is still one of the most common causes of morbidity and mortality worldwide, it remains important to develop new antithrombotic therapies. However, antithrombotic efficacy is consistently linked to bleeding. Based on risk associations in epidemiologic studies, both with venous and arterial thromboembolism, recent years spurred an interest in targeting FXI(a) to reduce the risk of thrombosis. This development has now proceeded towards extensive proof of concept clinical testing as demonstrated by several FXI(a) inhibitors entering or completing phase 2 trials in preventing venous thrombosis. There is potential to expand towards other indications, including prevention of ischemic stroke, myocardial infarction or prevention of clotting in extracorporeal devices including extracorporeal membrane oxygenation as these artificial surfaces may lead to contact activation. The premise of FXI(a) inhibitors as effective and safe anticoagulants still needs to be established for a broader range of indications.

The contribution of the contact system protein PK to the development of thrombosis is not quite as clear. Although results from animal studies indicate that depletion of PK decreases thrombosis risk, there is only limited and somewhat contradictory clinical data of human studies showing possible protective effects in

humans. More clinical studies in populations at risk of thrombosis, in PK-deficient individuals or with compounds registered or developed for HAE are necessary to investigate the contribution of PK(a) and its inhibition to thrombosis, its prevention and the impact on bleeding risk. Considering the role of the kallikrein kinin pathway in inflammatory responses, targeting PKa might be a valuable approach in reducing thromboinflammation,

Sources of funding

HtC was a Fellow of the Gutenberg Research Foundation and is Adjunct Professor at the Center for Thrombosis and Hemostasis, Gutenberg University, Mainz, Germany. HS and HtC receive support from the Netherlands Heart Foundation: CVON2014-09, Reappraisal of Atrial Fibrillation: Interaction between HyperCoagulability, Electrical Remodeling, and Vascular Destabilisation in the Progression of Atrial Fibrillation (RACE V).

Disclosures

SH is an employee of Bayer AG, Germany. MV, HtC and HS have nothing to disclose.

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Chapter 3

Plasma kallikrein contributes to
coagulation in the absence of factor XI by
activating factor IX

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Arteriosclerosis, Thrombosis, and Vascular Biology 2020; 40(1):103-111;
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Abstract

Objectives: Factor XIa (FXIa) induces clot formation, and human congenital FXI deficiency protects against venous thromboembolism and stroke. In contrast, the role of FXI in hemostasis is rather small, especially compared to FIX deficiency. Little is known about the cause of the difference in phenotypes associated with FIX deficiency and FXI deficiency. We speculated that activation of FIX via the intrinsic coagulation is not solely dependent on FXI(a) and aimed at identifying a FXI-independent FIX activation pathway.

Approach and results: We observed that ellagic acid and long-chain polyphosphates activated the coagulation system in FXI-deficient plasma, as could be demonstrated by measurement of thrombin generation, FIXa-antithrombin (AT) and FXa-AT complex levels, suggesting a FXI bypass route of FIX activation. Addition of a specific plasma kallikrein (PKa) inhibitor to FXI-deficient plasma decreased thrombin generation, prolonged aPTT and diminished FIXa-AT and FXa-AT complex formation, indicating that PKa plays a role in the FXI bypass route of FIX activation. In addition, FIXa-AT complex formation was significantly increased in *F11^{-/-}* mice treated with ellagic acid or long-chain polyphosphates compared to controls and this increase was significantly reduced by inhibition of PKa.

Conclusions: We demonstrated that activation of FXII leads to thrombin generation via FIX activation by PKa in the absence of FXI. These findings may, in part, explain the different phenotypes associated with FIX and FXI deficiencies.

Introduction

The contact activation system is initiated upon binding of factor XII to negatively charged surfaces,¹ which can be either artificial chemicals, like kaolin or ellagic acid, or of natural origin, such as polyphosphates (polyP)², collagen³ and nucleic acids⁴. Activated factor XII (FXIIa) transforms the zymogens FXI, that circulates in plasma at a concentration of 30-60 nM,⁵ and plasma prekallikrein into their activated forms FXIa and plasma kallikrein (PKa) in the presence of high molecular weight kininogen (HK).^{1,6} While FXIa contributes to coagulation by activating FIX,⁵ PKa is involved in several processes mostly via the generation of bradykinin.⁷ In addition, it activates FXII in a so-called amplification loop.^{1,7} Its precursor plasma prekallikrein circulates in plasma at a concentration of around 580 nM and its amino acid sequence shares 58% with the sequence of FXI.⁸

FXI deficiency was first described in humans by Rosenthal et al. in 1953⁹ and is defined by levels below 20 IU/dL (severe deficiency).¹⁰ In contrast to other coagulation factor deficiencies like FVIII or FIX bleedings are very rare and occur more likely perioperatively.¹⁰⁻¹² Since this likelihood is not related to FXI levels it is thought that other hemostatic abnormalities may contribute to the bleeding risk in FXI-deficient individuals.¹³

However, the question about the cause of the different phenotypes associated with FXI deficiency and FIX or FVIII deficiency still remains. We recently demonstrated that FIX can be activated by tissue factor (TF)-FVIIa (Josso-Loop) in mice (Grover et al., ISTH 2019). This, however, would not explain why the activated partial thromboplastin time (aPTT) in FXII-deficient plasma is much longer than in FXI-deficient plasma upon contact activation (Figure I Supplement). This suggests an alternative FXI-independent pathway from FXIIa down to thrombin generation.

Plasma prekallikrein has a high homology to FXI^{8,14} especially in the active site, suggesting that it could potentially activate FIX as well. The first experimental evidence for this was provided by Osterud et al. in 1978,¹⁵ demonstrating that PKa can activate FIX under the conditions of an artificial, purified system.

Our goal was to evaluate whether PKa contributes to the FXIa-independent activation of FIX in human plasma and in mice.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Characterization of plasma kallikrein inhibitors

Selectivity of plasma kallikrein inhibitors (PKa-i) used in the *in vitro* and *in vivo* experiments was determined using purified systems. The assays were based on detection of aminomethylcoumarine (AMC), released from the fluorogenic peptidic protease substrates upon protease catalyzed cleavage.

PKa-i were serially diluted and mixed with the human purified enzyme (thrombin, FXa, FXIIa, FXIa, plasmin, tissue-type plasminogen activator, and trypsin, Enzyme Research Laboratories, IN) and its specific substrate (Bachem, Switzerland). Fluorescence (excitation: 360 nm, emission: 465 nm) was measured using a microtiter plate fluorescence reader (Tecan infinite M1000Pro) for 40 min at RT. IC₅₀ values were determined by plotting logarithmic values of the test compound concentration against the relative protease activity. The effect of PKa-i on FVIIa and FIXa was determined in coupled FVIIa/FX and FIXa/FX assays, respectively.

The inhibitory potencies of the PKa-i on PKa were additionally determined in pooled human plasma collected from 10 healthy donors. PKa-i were serially diluted and added to human plasma. Contact activation was induced by addition of a kaolin-cephalin suspension (C.K. Prest®, Diagnostica Stago). After a 3 min incubation at 37°C, a kallikrein-specific substrate (50 µM, I-1295, Bachem) was added and fluorescence was measured for 40 min at 37°C (excitation: 360 nm; emission: 460 nm). The relative PKa inhibition by the inhibitors was calculated and IC₅₀ values were determined as described above.

aPTT measurements

Factor XII-initiated coagulation and fibrin formation were assessed using the aPTT assay. To characterize the role of PKa in different plasma types, a PKa-i (0.185-45 µM, Bayer AG, Germany) was added to either pooled normal plasma (10 healthy donors), plasma prekallikrein immunodepleted plasma (Sekisui Diagnostics, KY), or congenital FXI-deficient plasma (< 1% FXI, George King Bio-Medical Inc., KS), respectively. Contact activation was initiated by addition of kaolin-cephalin suspension (C.K. Prest®, Diagnostica Stago). Measurements were performed on a semi-automated ball coagulometer (MC 10 Plus, MERLINmedical, ABW Medizin und Technik GmbH, Germany).

Thrombin generation assay

Thrombin generation measurement by means of the Calibrated Automated Thrombogram (Stago, France) was performed as described previously.¹⁶ In brief, the

contact activation system in congenital FXI-deficient plasma (George King Bio-Medical Inc.) was induced by addition of 100 µg/mL ellagic acid (Sigma-Aldrich, MO), 4 µM phospholipids at 20:20:60 mol% PS:PE:PC and calcium chloride. To investigate the role of PKa as a possible activator for prothrombin, FVII, FIX or FX, PKa-i (0.45-150 µM, BAY-992, Bayer AG) alone or in combination with rivaroxaban (1.5 µM, Bayer AG) or active site-inhibited factor VIIa (45 nM, ASIS), which was sufficient to inhibit tissue factor-induced thrombin generation (data not shown), was added to the sample. Furthermore, an antibody directed against the active site of FXIa (Bayer AG, described previously)¹⁷ was used at a plasma concentration of 4.5 µM in order to verify FXI-deficiency of the used plasma. In comparison, thrombin generation was measured in normal pooled plasma in the presence or absence of PKa-i (45 µM).

To investigate if sufficient FXIIa is formed through ellagic acid in the presence of PKa-i, thrombin generation was determined in plasma with different FXI plasma levels, which was prepared by mixing normal plasma with FXI-deficient plasma. Thrombin generation was initiated as described above in the presence or absence of PKa-i (45 µM).

Long-chain polyphosphates (P700, Kerafast, MA) was used as physiologic trigger of contact activation. For these experiments, contact activation in congenital FXI-deficient plasma was initiated by 2.5 mM polyP, 4 µM phospholipids and calcium chloride in the presence or absence of PKa-i (0.45-4.5 µM).

Thrombin generation curves were recorded after 10 minutes (min) of preheating at 37 °C. The parameters lag time and peak height were calculated by the Thrombinoscope software (Thrombinoscope BV, The Netherlands).

Measurement of factor IXa-antithrombin and factor Xa-antithrombin complexes in plasma

In order to get a better understanding of FIXa and FXa generation, samples were taken from the earlier described thrombin generation experiments at certain time points followed by measurement of FIXa-antithrombin (AT) or FXa-AT complexes. 4.5 mg/mL Pefabloc® FG (Pentapharm, Switzerland) was added to each sample in order to prevent clot formation. 10 µL per sample was taken at 5, 10, 20, 30, 40, 50 and 60 min after initiation of contact activation and mixed with 10 µL 3.2% (w/v) sodium citrate to stop coagulation. Each citrated sample was further diluted in 40 µL buffer (25 mM Hepes, 175 mM NaCl, pH 7.7), snap frozen and stored at -80°C.

In-house FIXa-AT or FXa-AT inhibitor complex enzyme-linked immunosorbent assays (ELISA) were used to determine the amount of FIXa or FXa, as described previously.¹⁸ In brief, sheep anti-human FIX or sheep anti-human FX (2 µg/mL, Affinity

Biologicals, Canada) was used as capture antibody, whereas biotin-labeled sheep anti-human AT antibodies (1 µg/mL, Affinity Biologicals) were applied as detection antibody. Standard curves consisting of known concentrations of FIXa-AT complexes (0.34-32.4 pM) or FXa-AT complexes (0.35-34 pM) were used to assess the complex levels in individual samples.

Ellagic acid- and long-chain polyphosphate-induced contact activation in *F11^{-/-}* knock out mice

All animal experiments were conducted in accordance to the Guidelines of the European (Guideline 2010/63/EU) and National Legislation (dt. Tierschutzgesetz v. 04.07.2013) for the use and protection of animals for scientific purposes and approved by the institutional animal care office of Bayer AG and the competent regional authority (LANUV NRW, permit no. 84-02.05.40.17.12). *F11^{-/-}* mice, described by Gailani et al.¹⁹, were backcrossed to C57BL/6J from Charles River (Sulzfeld, Germany). Animals were kept under standard conditions (at 22°C room temperature, 50% humidity, 12-hour light/dark cycle) and had free access to pellet food and tap water.

The inhibitory potential of the PKa-i towards murine PKa was validated in a pilot experiment using plasma from FXI-deficient animals (*F11^{-/-}*). In brief, contact activation in murine FXI-deficient plasma was initiated by ellagic acid (100 µg/mL), phospholipids (4 µM) and calcium chloride in the presence or absence of the PKa-i (45 µM, BAY-077, Bayer AG). After 10 minutes, samples were taken to determine the levels of FIXa-AT complexes.

Nineteen *F11^{-/-}* mice (male and female, average body weight 29 g) were used for ellagic acid-induced contact activation and eighteen *F11^{-/-}* mice (male and female, average body weight 28 g) for polyP-induced contact activation. Mice were randomly divided into different groups (control, EA and EA+PKa-i group or control, polyP and polyP+PKa-i group) and anesthetized by intraperitoneal injection of 10 µL/g mixture of 11.1 mg/mL Ketamin (Ketaset®, Zoetis, NJ) and 0.11%(w/v) Xylazin (Rompun®, Bayer Vital, Germany). 30 mg/kg ellagic acid (EA, Sigma-Aldrich, concentration was selected based on Na et al.²⁰) and 9 mg/kg Pefabloc® FG (Pentapharm) with or without 20 mg/kg PKa-i (BAY-077, Bayer AG), were administered intravenously to animals in the EA or EA+PKa-i group. Animals of the polyP group were injected with 1 µL/g of 107 mM polyP (P700, Kerafast, concentration was selected based on Zilberman-Rudenko et al.²¹) and 9 mg/kg Pefabloc® FG with or without 10 mg/kg PKa-i. Animals of the control group received 0.9%(w/v) saline and 9 mg/kg Pefabloc® FG only. 30 min after administration, a midline abdominal incision was made to expose the vena cava and 4%(w/v) citrated blood was collected for plasma preparation as described previously.²²

Data were pooled for analysis to obtain a higher total number of animals in the groups, as the data showed no differences between male and female animals.

Statistical analysis

Data are presented as mean values $\pm$ standard deviation. Data were not tested for normality and equal variance. Comparison of groups of the *in vivo* experiment was performed using one-way ANOVA and the Holm-Sidak test for multiple comparisons in Prism 8 (Graphpad, CA). A value of $P < 0.05$ was considered statistically significant. All concentrations of the inhibitors used in *in vitro* experiments represent plasma concentrations.

Results

PKa inhibitors are highly selective

BAY-992 (Bayer AG; used for the *in vitro* experiments) and BAY-077 (Bayer AG, used in the *in vivo* experiments) are potent PKa inhibitors in buffer systems with IC_{50} values of 4.6 and 0.18 nM, respectively, and are selective versus various other proteases ($IC_{50} > 10 \mu M$ for the proteases thrombin, FXa, FXIIa, FIXa, FXIa, FVIIa, plasmin, tissue-type plasminogen activator, trypsin, tissue kallikrein). The IC_{50} values towards human PKa in human plasma were 1.3 μM for BAY-992 and 0.2 μM for BAY-077 (Figure II Supplement).

PKa is involved in the contact activation pathway in the absence of FXI

First, the contribution of PKa to the contact activation system was estimated using the aPTT assay in three different plasma types: normal human, plasma prekallikrein-depleted and FXI-deficient plasma, respectively. In order to determine the effect of PKa in the individual plasma types, PKa-i was added in different concentrations and contact activation was induced with kaolin. In both normal and plasma prekallikrein-depleted plasma, addition of a PKa-i prolonged the aPTT by a factor 1.2 (Figure 1A). In contrast, the aPTT in FXI-deficient plasma was clearly prolonged by a factor 2.4 (EC_{150} : 5 μM) in the presence of PKa-i (Figure 1A). The time to clot formation was prolonged to 380 s by the PKa-i (Figure III Supplement), which is similar to the clotting time in FXII-depleted plasma (Figure I Supplement).

PKa inhibition resulted in inhibition of fibrin formation in the absence of FXI, whereas PKa-i prolonged the lag time but did not reduce peak thrombin in plasma with 1% or 5% FXI level. In plasma with 25% FXI level hardly any effect of the PKa-i on thrombin generation was observed (Figure IV Supplement). Therefore, FXI-deficient

plasma was used in further *in vitro* experiments. Thrombin generation in FXI-deficient plasma could be initiated with ellagic acid (Figure 1B graph 2), kaolin (Figure VA Supplement) and long-chain polyP (Figure 1E), but not by silica (Figure VA Supplement; in line with the work of Rosen et al.²³). This suggests contact activation of coagulation independent of FXI. Addition of PKA-i reduced ellagic acid-triggered thrombin generation in the absence of FXI, with complete inhibition at a concentration of 150 μM PKA-i (Figure 1B-D), which was also observed in kaolin-triggered plasma (Figure VB Supplement).

Figure 1. PKA is involved in the contact activation pathway initiated by artificial surfaces or long-chain polyP in the absence of FXI. (A) PKA inhibition (PKA-i: 0.185–45 μM BAY-992) dose-dependently prolonged kaolin-triggered clotting time in human congenital FXI-deficient plasma but not in normal pooled or plasma prekallikrein depleted plasma. (B) Thrombin generation induced with 100 $\mu\text{g}/\text{mL}$ ellagic acid, 4 μM phospholipids and calcium chloride was not influenced by PKA-i in normal pooled plasma (graph 1) and was dose-dependently decreased by a PKA-i (0.45–150 μM BAY-992) in FXI-deficient plasma (graph 2) but not by a FXIa antibody (4.5 μM). (C) The lag time of ellagic acid-induced thrombin generation in FXI-deficient plasma was prolonged and (D) peak height was decreased by inhibition of PKA. (E) Thrombin generation in FXI-deficient plasma was initiated by 2.5 mM polyP, 4 μM phospholipids and calcium chloride in the presence or absence of PKA-i (0.45–4.5 μM BAY-992). Thrombin generation was dose-dependently decreased by PKA-i but not by a FXIa antibody (4.5 μM). (F) The lag time of polyP-induced thrombin generation in FXI-deficient plasma was prolonged and (G) peak height was decreased in the presence of PKA-i. Data are presented as the means $\pm$ SD ($n = 3$).

Moreover, polyP-induced contact activation in FXI-deficient plasma was dose-dependently inhibited by PKa-i (Figure 1E-G). Without addition of ellagic acid or polyP coagulation was not initiated and addition of a FXIa antibody had no effect on thrombin generation. In contrast to the results observed in FXI-deficient plasma, PKa-i did not influence ellagic acid-induced thrombin generation in normal pooled plasma (Figure 1B graph 1). Overall, these results suggest that, in the absence of FXI, PKa is involved in contact activation of coagulation.

PKa does not directly activate prothrombin or FVII

To investigate direct activation of prothrombin by PKa, ellagic acid-triggered thrombin generation was repeated in the absence of FXI and presence of the direct FXa inhibitor rivaroxaban. At a plasma concentration of 1.5 μ M rivaroxaban, thrombin generation was completely abolished (Figure 2A) independent of the presence of PKa-i, excluding the possibility of direct prothrombin activation by PKa. In line with this, direct activation of FVII by PKa was excluded by addition of active-site inhibited FVIIa (ASIS, 45 nM). In the absence of PKa-i, ellagic acid-triggered thrombin generation in FXI-deficient plasma was not influenced by inhibition of the TF-FVII pathway (Figure 2B). Taken together, these data suggest that, in the absence of FXI, PKa does not directly activate prothrombin or FVII.

FXIIa does not activate FIX directly

It was investigated if FXIIa activates FIX in plasma and if sufficient FXIIa is formed through ellagic acid in the presence of PKa-i. Ellagic acid-induced thrombin generation in FXI-deficient plasma was completely inhibited by PKa-i (45 μ M). However, at a FXI plasma concentration of 1%, PKa-i prolonged the lag time but did not reduce peak thrombin (Figure 2C). This suggests that despite the addition of PKa-i, sufficient FXIa is generated to activate FIX. FXIIa was unable to induce clotting in FXI-deficient plasma in the presence of PKa-i, excluding the possibility of FIX activation by FXIIa (Figure 2C). These results indicate that FIX is not activated by FXIIa and that sufficient FXII is formed by ellagic acid in the presence of PKa-i.

PKa induces FXa-AT complex formation

FX was activated upon ellagic acid-induced contact activation as demonstrated by subsampling and assessing the FXa-AT levels by in-house ELISA. Upon initiation of thrombin generation in FXI-deficient plasma, FXa-AT complex levels were immediately above the upper detection limit of 1.6 nM. Addition of PKa-i delayed FXa-AT complex formation for 20 and 50 min for the 15 and 150 μ M PKa-i plasma concentrations, respectively (Figure 2D). These data suggest that, in the absence of FXI, PKa may activate FX.

Figure 2. PKa does not enhance plasma thrombin generation, and is involved in FXa-AT complex formation. In addition, FXIIa does not activate FIX. Thrombin generation in FXI-deficient plasma was initiated with 100 μ g/mL ellagic acid, 4 μ M phospholipids and calcium chloride. (A) Thrombin generation was completely inhibited by rivaroxaban (1.5 μ M) alone or in combination with PKa-i (0.45–45 μ M BAY-992). (B) Thrombin generation was not influenced by ASIS (45 nM) alone, but was reduced in combination with PKa-i (0.45–45 μ M BAY-992). (C) Plasma with different FXI concentrations was prepared by mixing normal plasma with FXI-deficient plasma. Thrombin generation was initiated with 100 μ g/mL ellagic acid, 4 μ M phospholipids and calcium chloride. Thrombin generation in FXI-deficient plasma was completely inhibited by PKa-i (45 μ M BAY-992). In plasma with 1% FXI level, however, PKa-i prolonged the lag time but did not reduce peak thrombin. (D) FXa-AT complex formation in FXI-deficient plasma was initiated with 100 μ g/mL ellagic acid, 4 μ M phospholipids and calcium chloride in the presence or absence of the PKa-i BAY-992. Samples were taken at defined time points. Addition of the PKa-i (15 and 150 μ M) dose-dependently inhibited FXa-AT complex formation. Data are presented as the means $\pm$ SD (n = 3).

PKa induces FIXa-AT complex formation in the presence of calcium

Since formation of FXa-AT complexes could be observed in ellagic acid-induced contact activation in FXI-deficient plasma, we investigated if FIXa might be formed by using an in-house ELISA. Upon initiation of thrombin generation with ellagic acid, FIXa-AT complex levels instantly reached the upper detection limit of 1.6 nM. Addition of PKa-i dose-dependently delayed FIXa-AT complex formation for 20 min at an inhibitor concentration of 15 μ M, with complete inhibition at a concentration of 150 μ M PKa-i (Figure 3A). In contrast, addition of a FXIa-blocking antibody did not influence the formation of FIXa-AT complexes (Figure 3A). Without the addition of calcium chloride, no FIXa-AT complex formation could be observed (Figure 3B). Similar results were observed when contact activation was induced with long-chain polyP.

Figure 3. PKa is involved in FIXa-AT complex formation in presence of calcium ions in FXI-deficient plasma. (A) Contact activation in FXI-deficient plasma was induced with 100 μ g/mL ellagic acid, 4 μ M phospholipids and calcium chloride in the presence or absence of the PKa-i (BAY-992). Samples were taken at defined time points. FIXa-AT complex formation could be induced by ellagic acid and was dose-dependently inhibited by PKa-i (0.45-150 μ M), but not by a FXIa antibody (4.5 μ M). (B) In the absence of calcium chloride contact activation in FXI-deficient plasma was not initiated as no FIXa-AT complexes could be determined. (C) Contact activation in FXI-deficient plasma was induced with 2.5 mM long-chain polyP, 4 μ M phospholipids and calcium chloride. FIXa-AT complex was dose-dependently inhibited by PKa-i (0.45-4.5 μ M BAY-992), but not by a FXIa antibody (4.5 μ M). Data are presented as the means $\pm$ SD ($n = 3$).

FIXa-AT complex formation was slower than in ellagic acid-initiated contact activation, but the upper detection limit was also reached after 30 minutes. Addition of PKa-i dose-dependently inhibited FIXa-AT complex formation (Figure 3C). These results indicate that PKa may activate FIX in the absence of FXI and that calcium is required for the formation of FIXa-AT complexes.

Ellagic acid and long-chain polyphosphates induce FIXa-AT complex formation in $F11^{-/-}$ mice

In order to extend the *in vitro* data showing a role for PKa in FIX activation in human plasma, we investigated if similar results could be observed in mice. Since the *in vitro* experiments show that the effect of PKa inhibition on fibrin formation was more pronounced in the absence of FXI, $F11^{-/-}$ mice instead of wild type mice were used in the *in vivo* experiments. In an *in vitro* experiment, ellagic acid induced FIXa-AT complex formation in murine FXI-deficient plasma compared to non-activated plasma, which was inhibited by the addition of the PKa-i (Figure 4A). This confirms that the inhibitor is effective in murine plasma.

We observed a significant increase in FIXa-AT complex formation in $F11^{-/-}$ mice treated with 30 mg/kg ellagic acid (Figure 4B) or with 1 μ l/g of 107 mM polyP (Figure 4C), indicating that *in vivo* FIX might be activated in ellagic acid- or polyP-induced contact activation independently of FXI. In addition, a specific inhibitor against human PKa significantly reduced levels of FIXa-AT complexes in mice treated with ellagic acid (Figure 4B) or with polyP (Figure 4C). An average total plasma concentration of 20 μ M PKa-i and an unbound plasma concentration of 7 μ M were determined in mice treated with ellagic acid (Table I Supplement).

The data obtained from *in vivo* experiments suggest that PKa, in the absence of FXI, may also be involved in FIX activation *in vivo*.

Discussion

In this study, we demonstrated that PKa activates FIX in the absence of FXI and thus contributes to coagulation (Figure 5). While activation of FIX by PKa in a buffer system was described 40 years ago,¹⁵ we were now able to show that PKa might activate FIX under certain conditions in human plasma and in an *in vivo* mouse model.

To investigate the potential activation of FIX by PKa we conducted both *in vitro* and *in vivo* experiments. In FXI-deficient human plasma, we observed thrombin generation and formation of FIXa-AT and FXa-AT complexes, when contact activation was initiated with either an artificial surface (ellagic acid) or a physiological trigger (long- chain PolyP). This indicates a FXI bypass route of coagulation. Addition of a

Figure 4. Ellagic acid increases FIXa-AT complex formation in plasma from $F11^{-/-}$ mice, and ellagic acid and long-chain polyP induce FIXa-AT complex formation in $F11^{-/-}$ mice.

(A) Plasma obtained from $F11^{-/-}$ mice was activated with 100 μ g/mL ellagic acid, 4 μ M phospholipids and calcium chloride. FIXa-AT complex formation was increased by ellagic acid compared to control (plasma w/o ellagic acid) and this increase was prevented by PKa-i (45 μ M BAY-077). Data are presented as individual values. (B) Ellagic acid (30 mg/kg) or (C) long-chain polyP (1 μ l/g of 107 mM polyP) and Pefabloc® FG (9 mg/kg) with or without the PKa-i BAY-077 (20 mg/kg in EA group; 10 mg/kg in polyP group) were injected intravenously into $F11^{-/-}$ mice. The control group received 0.9%(w/v) saline and Pefabloc® FG (9 mg/kg) only. Citrated blood was taken 30 min after i.v. injection and plasma was prepared. Data are presented as the means $\pm$ SD. Groups were statistically analyzed by a one-way ANOVA test and a Holm-Sidak test for multiple comparisons ($n = 5-8$, $*P < 0.05$).

specific active-site inhibitor of PKa caused a dose-dependent reduction of thrombin generation, a prolongation of aPTT and a delay of FIX-AT and FX-AT complex formation in FXI-deficient plasma in both, artificial and physiological settings. This suggests that PKa plays a role in the FXI bypass route of FIX activation. In the absence of calcium, no FIXa-AT complex levels were measured, suggesting that calcium is either essential for the activation of FIX by PKa or for the measurement of FIXa-AT complexes.

Furthermore, $F11^{-/-}$ mice treated with ellagic acid or long-chain polyP to initiate contact activation, exhibited increased FIXa-AT levels compared to untreated animals, which was significantly attenuated by a PKa inhibitor. These results suggest that PKa plays a role in FIX activation in the absence of FXI *in vitro* and *in vivo*.

The initial evidence for FXIa-independent activation of FIX was reported in 1978. Using a buffer system, Osterud et al. showed that PKa activates FIX.¹⁵ In these experiments, purified PKa was incubated with purified FIX in the presence or absence of CaCl_2 , followed by subsequent analysis of the test sample in a FIXa-dependent clotting assay. In addition to that, the work of Puy and colleagues demonstrated FXIa independent *in vitro* thrombin generation upon enhanced FXII and plasma prekallikrein activation by long-chain polyphosphates.²⁴

The fact that FXIa and PKa activate the same substrate (in this case FIX), may stem from the close sequence homology of these two proteins, especially in the active site. FXI consists of two identical subunits each comprising four tandem repeats of each 90-91 amino acids, named apple domains (A1 to A4), and a trypsin-like catalytic domain.^{14, 25} The amino acid sequence of plasma prekallikrein is 58% identical to that of FXI and the zymogen contains the characteristic four apple domains.^{8, 26} Besides

Figure 5. Contact activation of coagulation. The contact pathway of coagulation is initiated upon activation of FXII to FXIIa on negatively charged surfaces. This leads to activation of FXI to FXIa, which in turn converts FIX to FIXa, eventually leading to thrombin generation and fibrin formation. Our data indicate that PKa can activate FIX in the absence of FXI. Zymogens are indicated in roman numerals and their activated forms end with an „a“.

the sequence homology, both proteins mostly circulate in a noncovalent complex with HK^{27, 28}, facilitating the activation of both zymogens by FXIIa.²⁹

In the past, it had been shown that enzymes of the coagulation cascade may act on several different zymogens or cofactors. For example, thrombin not only converts fibrinogen to fibrin but also activates FXI, FV, FVIII, and protein C (Figure 5) (reviewed by Lane et. al.).³⁰ The TF-FVIIa complex of the extrinsic pathway activates not only FX but also FIX in the so-called Josso loop (Figure 5).^{31, 32} FX has always been considered as the predominant enzyme of the common pathway merging the two coagulation pathways. Since FIX seems to be activated via the Josso loop in the extrinsic pathway, by FXI in the intrinsic pathway, and by PKa, the enzyme FIXa might be considered as a key regulatory coagulation protease.

Our *in vivo* data indicate that the FXI bypass pathway of FIX activation by PKa exists in mice. However, the existence and physiological role of the PKa-FIX pathway in humans still needs to be investigated. Compared to FIX or FVIII deficiency, FXI-deficient subjects exhibit in general a mild bleeding phenotype with very rare spontaneous bleedings.¹⁰ The likelihood of bleeding is increased after trauma or surgery, especially if tissues with high fibrinolytic activity are affected.^{10, 12} However, the bleeding tendency of FXI-deficient subjects is, unlike FIX or FVIII deficiency, not correlated with the FXI level.¹¹ It has been suggested that other hemostatic abnormalities including low von Willebrand factor level contribute to the bleeding risk in FXI-deficient subjects.¹³ We theorize that reductions in plasma prekallikrein levels or activity in FXI-deficient subjects might lead to a higher bleeding risk due to diminished activation of FIX. Conversely, plasma prekallikrein might play two roles in thrombin generation. First through amplifying the activation of FXII and second through activation of FIX in a FXI-independent manner. In support of this, plasma prekallikrein-deficient mice showed reduced thrombus formation in both arterial and venous thrombosis models.³³⁻³⁵

The role of FIX activation by PKa in the presence of FXI is not clear yet. However, FXIIa-activation of FXI to FXIa, which in turn activates FIX, is most likely the more dominant pathway, as specific inhibition of PKa did not inhibit FIXa generation in normal plasma compared to the attenuation observed in FXI-deficient plasma. In addition, the procoagulant function of PKa in plasma with 1% FXI is less visible compared to complete FXI deficiency and undetectable at FXI plasma levels > 1%. In line with this, Osterud et al. calculated that FXI is approximately 20,000 times more active than PKa in activating FIX under the conditions they used for their experiments.¹⁵ This, however, does not mean that the existence and contribution of the bypass pathway in the presence of FXI can be excluded.

High levels of FXI have been identified as risk factor for deep vein thrombosis³⁶ and ischemic stroke.³⁷ Salomon et al. demonstrated that the incidence of ischemic stroke and deep vein thrombosis are reduced in severe FXI-deficient patients.^{38, 39} These studies show that FXI plays a role in thrombosis and might be a valuable target to prevent thrombosis. FXI directed antisense oligonucleotide (ASO) has been tested in patients undergoing total knee arthroplasty. Patients receiving FXI ASO were compared to patients treated with enoxaparin, demonstrating that the incidence of venous thromboembolism after surgery was reduced by lowering FXI levels with 300 mg ASO without increasing the bleeding risk.⁴⁰ There are many other approaches and ongoing studies to minimize the risk of thrombosis through FXIa inhibition.⁴¹

In conclusion, we demonstrate that in the absence of FXI, activation of factor XII by ellagic acid or long-chain polyphosphates leads to thrombin generation via FIX activation by PKa. The contribution of this FXI-independent pathway to bleeding risk in FXI deficiency or to thrombosis risk needs to be elucidated.

Acknowledgements

- a) Acknowledgements: The authors kindly acknowledge D. Fens, J. Glunz, C. Luecker, A. Tersteegen and S. Zimmermann for excellent support and technical assistance.
- b) Sources of funding: HtC was a Fellow of the Gutenberg Research Foundation. HS and HtC receive support from the Netherlands Heart Foundation: CVON2014-09, Reappraisal of Atrial Fibrillation: Interaction between HyperCoagulability, Electrical Remodeling, and Vascular Destabilisation in the Progression of Atrial Fibrillation (RACE V).
- c) Disclosures: MV, VL, and SH are employees of Bayer AG, Germany, HtC and HS received research funding of Bayer AG, Germany. HtC received reimbursement for advisory boards from Pfizer, Boehringer, Leo farma and Daiichi. N.M. consults for Bayer AG and has received research funding from Bayer AG

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Supplementary Figures

Supplementary Figure I. aPTT measurement in different plasma types. Clotting times in normal plasma, FXII-depleted plasma, FXI-deficient plasma and PK-depleted plasma were determined in kaolin- and calcium chloride-induced contact activation. Compared to normal plasma, the time until clot formation in FXII-depleted, FXI-deficient plasma and PK-depleted was prolonged. Clotting times in FXII-depleted plasma were much longer than in FXI-deficient plasma or PK-depleted plasma. Data are presented as the means $\pm$ SD ($n = 3$).

Supplementary Figure II. Determination of the IC_{50} values of the PKa inhibitors in pooled human plasma. PKa inhibitors were serially diluted and mixed with human plasma and a kaolin-cephalin suspension. After 3 min incubation, a kallikrein-specific substrate ($50 \mu M$) was added and fluorescence was measured for 40 min at $37^\circ C$. IC_{50} values were determined by plotting logarithmic values of the test compound concentration against the relative percentage protease inhibition. Data are presented as the means $\pm$ SD ($n = 9$).

Supplementary Figure III. Inhibition of PKa prolonged aPTT in FXI-deficient plasma (absolute aPTT values). PKa inhibition (PKa-i: 0.185-45μM) dose dependently increased kaolin-induced clotting time in human congenital FXI-deficient plasma but not in normal pooled or plasma prekallikrein depleted plasma. Data are presented as the means ± SD (n = 3).

Supplementary Figure IV. The procoagulant function of PKa in plasma with 1% FXI is less visible compared to complete FXI deficiency and undetectable at FXI plasma levels > 1%. Plasma with different FXI concentrations were prepared by mixing normal plasma with FXI-deficient plasma. Thrombin generation was initiated with 100 μ g/mL ellagic acid, 4 μ M phospholipids and calcium chloride in the presence or absence of the PKa-i (4.5 and 45 μ M). (A) PKa-i reduced thrombin generation in FXI-deficient plasma. (B) In plasma with 1% FXI level, however, PKa-i prolonged the lag time but did not reduce peak thrombin. (C) The lag time in plasma with 5% FXI level was prolonged only at a high PKa-i concentration. (D) In plasma with 25% FXI level hardly any effect of the PKa-i on thrombin generation was observed.

Supplementary Figure V. PKa-i inhibits thrombin generation in kaolin-induced contact activation in FXI-deficient plasma. (A) Contact activation in FXI-deficient plasma was initiated with 100 µg/mL ellagic acid, 150 µg/ml or 150 µg/ml silica, 4 µM phospholipids and calcium chloride. Both ellagic acid and kaolin induced thrombin generation whereas silica did not initiate coagulation in FXI def. plasma. (B) Thrombin generation induced with 150 µg/mL kaolin, 4 µM phospholipids and calcium chloride was dose-dependently decreased by a PKa-i (0.15-4.5 µM).

Supplementary Table I. PKa-i plasma concentration in PKa-i treated mice. PKa-i plasma concentrations in mice treated with ellagic acid were determined by liquid-chromatography – mass-spectrometry.

PKa-i treated animal	Determined PKa-i plasma conc. (nM)	Determined Pka-i plasma conc. unbound (nM)
1	14000.8	5040.3
2	16545.1	5956.2
3	17923.2	6452.3
4	31213.8	11237.0
5	17858.1	6429.1
6	25412.1	9148.4
mean:	20492.3	7377.2

Chapter 4

Increased plasma kallikrein activity in
coagulation factor XI-deficient individuals

EMBARGOED

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In preparation

Chapter 5

Evaluation of in vitro assays to determine factor XI and plasma prekallikrein activation and to assess their role in patients with acute VTE

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Chapter 6

Complement, neutrophils, extracellular histones, and contact activation as potential drivers of Covid-19

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Based on: Neutrophils and Contact Activation of Coagulation as Potential Drivers of COVID-19. Circulation 2020; 142(18):1787-1790

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Abstract

Background: The spectrum of coronavirus disease 2019 (Covid-19) ranges from mild disease to the life-threatening acute respiratory distress syndrome, with hyperinflammation, vascular damage, and high rates of thrombotic events. The underlying mechanism is unclear. We hypothesized that activated neutrophils damage the endothelium and constantly trigger coagulation.

Methods: We prospectively analyzed biomarkers of inflammation and early coagulation in 228 consecutive patients with Covid-19. At presentation and during follow-up, complement C5a, histone H3, either citrullinated or not, formation of neutrophil extracellular traps (NETs), and activated factors of coagulation in complex with natural inhibitors were measured.

Results: Elevated C5a ($n/N=153/201$, 76%) was common, with highest levels in moderate and in progressive/severe patients. Histone H3 was found in 8 ($N=65$, 12%) and 15 ($N=102$, 15%) patients with moderate and progressive/severe disease, the prevalence of which increased to 5 ($N=23$, 24%) and 30 ($N=52$, 58%) during the course of disease ($P=0.008$). Histone H3 was citrullinated in 38 (73%) out of 52 patients with extracellular histones detected, confirming NETosis as the source. Serum from patients with progressive/severe Covid-19 induced *in vitro* NETosis. Elevated von Willebrand factor antigen ($n/N=207/217$, 95%) and plasma kallikrein ($n/N=194/217$, 89%), activated factor XI ($n/N=206/217$, 95%), activated factor IX ($n/N=145/217$, 67%), and thrombin ($n/N=131/217$, 60%) in complex with natural inhibitors were common, with highest levels in those with progressive/severe Covid-19.

Conclusion: We demonstrate that with increasing severity of disease, complement activation, NETosis, and activation of coagulation, and, in particular, the contact pathway, becomes more dominant, pointing to potential treatment targets.

Introduction

Ever since the first case of pneumonia related to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), known as coronavirus disease 2019 (Covid-19),¹ a pandemic has occurred, with a dramatic impact on global society. Covid-19 varies from a mild disease with full recovery to the life-threatening acute respiratory distress syndrome (ARDS). Mortality rates are high² and exceed 25% in patients with ARDS admitted to the intensive care unit.³ The pathogenesis underlying the rapid deterioration in a subset of patients remains poorly understood and, consequently, targeted treatment is lacking. It is important to distinguish targets of intervention in the early phase of disease progression, not only to clear the virus, but also to prevent irreversible organ damage and permanent disability. Critically ill patients typically show hyper-inflammation,^{2, 4} vascular damage, and coagulopathy,⁵ with high rates of thrombotic events. Unraveling the connection between these features can be the bridge to timely and targeted therapies in Covid-19.

We hypothesize that activated neutrophils damage the endothelium, activate platelets, and constantly trigger coagulation in patients with Covid-19, particularly in those patients with progressive/severe disease. This is in line with the neutrophil infiltration, capillaritis, and fibrin deposits found in lung tissue specimens from deceased Covid-19 patients.^{6, 7} We consider C5a to be a main neutrophil driving factor, neutrophil extracellular trap (NET) formation a key process, and the release of cytotoxic histones as downstream effectors for progressive/severe Covid-19 to occur. We studied this premise in a well-defined cohort of 228 patients with Covid-19 by measuring circulating biomarkers of inflammation and vascular damage, NETosis, and early activation of coagulation.

Methods

Patient population and sampling

Consecutive patients with Covid-19 who presented at the Maastricht University Medical Center, Maastricht, the Netherlands, from March 21 through April 29, 2020, were included. Covid-19 was diagnosed in patients with typical radiologic findings on computed tomography, such as diffuse ground glass opacities and/or bilateral consolidations,⁸ and confirmed by reverse transcriptase polymerase chain reaction of nasopharyngeal swab and/or sputum (i.e., SARS-CoV-2 RNA with a cycle threshold value <40).⁹ Disease severity was classified as mild in patients not admitted to the hospital, moderate in patients admitted to the general ward requiring supplemental oxygen via a nasal cannula (up to 5 l/min), progressive in patients requiring

supplemental oxygen via a face mask, and severe in patients admitted to the intensive care unit for invasive ventilation and/or those who died ≤ 7 days of admission.

At the time of presentation, blood samples were obtained as part of standard care and routine practice. Citrated platelet poor plasma was processed as quickly as possible at 4 degrees Celsius. Serum tubes were allowed to coagulate for 30 minutes at room temperature. Plasma and serum samples were aliquoted and stored at -80 degrees Celsius until testing. Follow-up samples, obtained every 5 (± 2) days, were used when available. In addition, sputum samples were collected in selected cases. This study was approved by the appropriate ethics committee (2020-1315), with a waiver of informed consent.

Soluble C5a

C5a was quantified in plasma by enzyme immunoassay (Quidel, San Diego, CA), according to the manufacturer's instructions; the assay measures the amount of desarginated C5a.

Histone H3 and citrullinated histone H3

Extracellular histone H3, either citrullinated (citH3) or not, was detected in plasma and sputum by Western blot.

Neutrophil extracellular trap formation

Neutrophils from healthy donors were prepared from 10 mL whole blood collected in EDTA tubes. Blood was fractionated by density gradient centrifugation using Lymphoprep (Stemcell, Vancouver, Canada) before lysing residual RBCs with 0.84% ammonium chloride. Neutrophils, labeled with PKH26 (Sigma-Aldrich, St. Louis, MA), were resuspended in medium (phenol red free RPMI, 2% inactivated FBS, and 10% penicillin/streptomycin), seeded on glass culture slides, and incubated with serum diluted in medium to undergo NETosis for 4 hours; pooled normal human serum and medium were used as a negative control. Neutrophils were fixed and blocked with 1% BSA, incubated with SYTOX green (Thermo Fisher, Waltham, MA) for 15 minutes and DAPI for 5 minutes. Also, neutrophils were incubated with rabbit anti-citH3 pAb (1:200; Abcam, Cambridge, United Kingdom), rabbit anti-neutrophil elastase pAb (1:100; Abcam), or rabbit anti-myeloperoxidase pAb (Dako, Glostrup, Denmark) for 2 hours, Alexa 488 labeled anti-rabbit Ab (1:100; Life Technologies, Carlsbad, CA) for 30 minutes, and DAPI for 5 minutes.

Coagulation activation markers

Plasma kallikrein in complex with C1 esterase inhibitor (PKa:C1inh), factor (F)XIa in complex with antithrombin (FXIa:AT) and α 1-antitrypsin (FXIa: α 1AT), FIXa in complex with AT (FIXa:AT), thrombin in complex with AT (TAT), and von Willebrand factor antigen (vWF:Ag) were quantified by in-house developed and validated (CLSI Guidelines, EP5 protocol) enzyme-linked immunosorbent assays.

Statistical analysis

Continuous variables were presented as mean ($\pm$ SD) or median (interquartile range) as appropriate; between-group differences were analyzed by unpaired sample *t* test, Mann Whitney U test, one-way ANOVA, or Kruskal Wallis. Differences in categorical variables were analyzed by Fisher's exact test. $P < 0.05$ was considered significant.

Results

Patient population

The baseline characteristics of 228 consecutive patients with Covid-19 are listed in Table 1. In total, 149 (65%) out of 228 patients were male; the mean age was 67 ($\pm$ 14) years. Patients presented with dyspnea and typical biochemical features of Covid-19, that is, elevated C reactive protein ($n/N=205/228$, 90%), lactate dehydrogenase ($n/N=169/228$, 74%), aspartate transaminase ($n/N=158/228$, 69%), and lymphopenia ($n/N=131/228$, 63%). Also, fever ($n/N=70/228$, 31%) was common. The median duration from onset of symptoms to presentation at our hospital was 7 (IQR, 5-12) days. Mild, moderate, and progressive/severe Covid-19 was classified in 54, 68, and 106 patients, respectively. Patients with progressive/severe Covid-19 had the highest levels of C reactive protein and lactate dehydrogenase. Importantly, neutrophil counts were higher as compared to those with mild disease. Of note, comorbidities did not differ between groups.

In total, 59 patients were admitted to the intensive care unit; these patients had elevated D-dimer ($n/N=51/53$, 96%) and fibrinogen ($n/N=49/54$, 91%), with median levels of 5,498 (1,623-10,000) μ g/L and 6.9 (5.8-8.0) g/L, respectively. Prolongation of the activated partial thromboplastin time (aPTT) and low platelet counts (i.e., $<100 \times 10^9/L$) were found in 13 ($N=55$, 24%) patients and 1 ($N=58$, 2%) case, respectively. None of the patients had disseminated intravascular coagulation.¹⁰

Table 1. Baseline characteristics of 228 patients with Covid-19.

	Mild	Moderate	Progressive or severe	Overall <i>P</i>
No.	54	68	106	
M/F	29/25	42/26	78/28 ^b	0.03
Age, yr.	62 (±16)	69 (±13) ^c	69 (±12) ^b	0.003
Days from illness onset	7 (5-11)	7 (5-14)	7 (5-14)	NS
SBP, mmHg	130 (±18)	138 (±22)	138 (±25)	NS
DBP, mmHg	80 (70-89)	83 (75-87)	80 (70-88)	NS
Heart rate, bpm	90 (±21)	89 (±18)	96 (±20)	NS
Body temperature, °C	37.6 (±0.8)	38.1 (±1.0) ^c	38.1 (±1.0) ^b	0.009
Fever, %	10, 19	24, 36	36, 40 ^b	0.03
SARS-CoV-2, Ct	31 (±4)	29 (±5)	29 (±5)	NS
Medical history				
Hypertension, %	16, 30	27, 40	35, 33	NS
Diabetes, %	12, 22	10, 15	25, 24	NS
CVA, %	7, 13	8, 12	15, 14	NS
Cardiac disease, %	15, 28	23, 34	32, 30	NS
COPD/asthma, %	9, 17	16, 24	13, 12	NS
Cancer, %	8, 15	11, 16	17, 16	NS
Immunocompromised, %	4, 7	3, 4	5, 5	NS
None, %	14, 26	16, 24	28, 26	NS
Platelets, ×10 ⁹ /L	225 (±97)	213 (±88)	209 (±66)	NS
Leukocytes, ×10 ⁹ /L	5.9 (5.1-8.5)	6.6 (4.7-9.0)	7.5 (5.8-10.1) ^b	0.006
Neutrophils, ×10 ⁹ /L	4.5 (3.5-6.3)	5.0 (3.4-7.4)	5.9 (4.6-8.1) ^b	0.02
Lymphocytes, ×10 ⁹ /L	1.2 (0.7-1.5)	0.8 (0.6-1.2) ^c	0.7 (0.5-1.1) ^b	0.0002
ALT, U/L	29 (20-47)	33 (25-53)	35 (24-51)	NS
AST, U/L	38 (27-55)	49 (36-64) ^c	54 (39-79) ^b	0.0001
LDH, U/L	273 (±91)	362 (±142) ^c	480 (±191) ^{a, b}	<0.0001
SCr, μmol/L	83 (61-110)	86 (71-112)	92 (71-121)	NS
Albumin, g/L	34 (31-38)	34 (30-36)	29 (26-32) ^{a, b}	<0.0001
CRP, mg/L	57 (17-96)	66 (39-123)	101 (56-179) ^{a, b}	<0.0001
High C5a t ₀ , n/N	29/46	50/56 ^c	71/96 ^b	0.006
C5a t ₀ , ng/mL	15.4 (9.0-25.4)	21.9 (16.5-29.7) ^c	22.1 (10.8-31.6) ^b	0.02
High histone H3 t ₀ , n/N	0/45	8/65	15/102	NS
High histone H3 t _n , n/N	N/a.	5/23	30/52	0.006
n/N	48/54	66/68	103/106	
vWF:Ag, %	327 (±139)	383 (±148)	440 (±141) ^{a, b}	<0.0001
TAT, ng/mL	4.2 (3.0-5.7)	5.4 (3.6-9.9)	7.0 (4.6-14.0) ^{a, b}	<0.0001
PKa:C1inh, nM	2.0 (0.8-3.9)	2.2 (0.9-4.8)	1.8 (1.0-3.5)	NS
FXIa:AT, pM	24.4 (17.4-36.2)	25.0 (19.7-35.8)	29.8 (22.0-61.3) ^{a, b}	0.002
FXIa:α1AT, pM	391 (309-625)	513 (395-751)	544 (402-774) ^b	0.007
FIXa:AT, pM	54 (33-81)	63 (43-89)	82 (61-114) ^{a, b}	<0.0001

Significant differences between patients groups: progressive/severe versus ^amoderate or ^bmild disease; moderate versus ^cmild disease.

α1AT, α1-antitrypsin; ALT, alanine transaminase; AST, aspartate transaminase; AT, antithrombin; COPD, chronic obstructive pulmonary disease; C1inh, C1 inhibitor; CRP, C-reactive protein; CVA, cerebrovascular accident; DBP, diastolic blood pressure; FIXa, activated factor IX; FXIa, activated factor XI; LDH, lactate dehydrogenase; NS, not significant; PKa, plasma kallikrein; SBP, systolic blood pressure; SCr, serum creatinine; TAT, thrombin in complex with antithrombin; vWF:Ag, von Willebrand factor antigen.

C5a, neutrophils, and NETosis

At presentation, the potent anaphylatoxin C5a, was elevated in 153 (76%) out of 201 patients with Covid-19 (Table 1); highest levels were found in patients admitted to the hospital as compared to those with mild Covid-19. Also, histone H3, a marker of NETosis, was found in 15 ($N=102$, 15%) patients with progressive/severe Covid-19 and 8 ($N=65$, 12%) patients with moderate disease. In none of the patients with mild disease extracellular histones were detected. During follow-up, histone H3 was found in 33 (58%) out of 57 patients with progressive/severe Covid-19, including 28 patients admitted to the intensive care unit and 2 patients with palliative care; histone H3 was also found in 6 (24%) out of 25 patients with moderate Covid-19 ($P=0.008$ versus patients with progressive/severe disease). Histone H3 was citrullinated in 38 (73%) out of 52 patients with extracellular histones detected (progressive/severe Covid-19, $n=28$; moderate disease, $n=10$), confirming NETosis as the origin of these histones.

Figure 1. Serum from patients admitted to the hospital and, in particular, those with progressive/severe Covid-19, induced excessive *in vitro* formation of neutrophil extracellular traps. The presence of citrullinated histone H3 indicates peptidylarginine deiminase 4 -dependent chromatin decondensation; original magnification, 400 $\times$.

Next, we evaluated histone H3 in sputum samples from 9 patients admitted to the intensive care unit; all samples contained histone H3 and, in particular, citH3. Serum from patients with progressive/severe and, to a lesser extent, moderate Covid-19 ($n=5$ samples from both groups) induced *in vitro* formation of NETs as indicated by extracellular fibers that contain DNA with citH3, neutrophil elastase, and myeloperoxidase (Figure 1). The presence of citH3 indicates that PAD4 was responsible for chromatin decondensation and NETosis to occur. Serum from patients with mild Covid-19 ($n=5$), normal human serum, and medium did not induce citrullination of histone H3.

Markers of coagulation

To better understand the link between NETosis, extracellular histones, and coagulation, we decided to delineate early activation of hemostasis by measuring vWF:Ag, as marker of endothelial activation, and activated factors of coagulation in complex with natural inhibitors (Figure 2, Table 1). Most patients had elevated levels of vWF:Ag (i.e., $>160\%$; $n/N=207/217$, 95%), PKa:C1inh (i.e., ≥ 0.3 nM; $n/N=194/217$, 89%), FXIa: α 1AT (i.e., ≥ 248 pM; $n/N=206/217$, 95%), and TAT (i.e., ≥ 5 ng/mL; $n/N=131/217$, 60%); elevated levels were found respectively in 100 (97%), 93 (90%), 102 (99%), and 77 (75%) out of 103 patients with progressive/severe Covid-19. Elevated FIXa:AT (i.e., ≥ 56 ; $n/N=145/217$, 67%) and FXIa:AT (i.e., ≥ 42 pM, $n/N=60/217$, 28%) were common; elevated levels were respectively found in 83 (81%) and 40 (39%) out of 103 patients with progressive/severe Covid-19. Notably, vWF:Ag, FXIa:AT, FIXa:AT, and TAT levels were highest in patients with progressive/severe Covid-19; also, FXIa: α 1AT was higher as compared to those with mild disease. PKa:C1inh did not differ between patient groups.

During the course of disease, markers of coagulation remained elevated with highest levels in patients with progressive/severe Covid-19 (Table 2). FXI can be activated *via* contact activation and upon activation of the extrinsic pathway through the positive feedback loop of thrombin. Since low molecular weight heparin (LMWH) accelerates the inactivation of FXa and thrombin we tested whether LMWH affects the activation of FXI. Ninety (85%) out of 106 patients with progressive/severe Covid-19 were treated with LMWH (therapeutic dose, $n=7$); 12 (11%) patients not treated with LMWH continued vitamin K antagonists (VKA) or non-VKA oral anticoagulants. Neither FXIa: α 1AT nor FXIa:AT decreased despite LMWH (data not shown), suggesting, at least partially, activation of FXI through contact activation by FXIIa.

Figure 2. Markers of coagulation at the time of presentation. Mild, moderate, and progressive/severe Covid-19 have been depicted in light blue, green, and orange.

Table 2. Markers of coagulation in moderate as well as progressive/severe Covid-19 during admission.

	Moderate	Progressive or severe	<i>P</i> value
Patients			
No. at t ₀	65	103	
No. at t ₁	25	53	
No. at t ₂	8	40	
vWF:Ag, %			
Level at t ₀	383 (±18)	462 (±16)	0.01
Level at t ₁	391 (±29)	497 (±22)	0.005
Level at t ₂	342 (±47)	520 (±23)	0.003
TAT, ng/mL			
Level at t ₀	5.5 (3.7-10.2)	8.7 (4.9-21.6)	0.005
Level at t ₁	4.3 (2.8-9.3)	7.6 (5.3-22.2)	0.009
Level at t ₂	4.6 (3.8-5.2)	6.7 (4.8-10.9)	0.03
PKa:C1inh			
Level at t ₀	2.2 (0.9-4.8)	1.9 (1.0-3.5)	NS
Level at t ₁	1.9 (1.0-5.0)	2.3 (1.0-12.5)	NS
Level at t ₂	2.0 (1.7-5.6)	2.7 (1.1-9.4)	NS
FXIa:AT, pM			
Level at t ₀	25.3 (19.8-36.4)	30.0 (22.2-65.2)	0.004
Level at t ₁	21.4 (19.4-31.2)	39.7 (25.0-75.9)	0.005
Level at t ₂	20.6 (14.3-28.8)	33.2 (22.4-62.0)	0.02
FXIa:α1AT, pM			
Level at t ₀	513 (395-751)	547 (403-809)	NS
Level at t ₁	399 (340-569)	635 (483-1,027)	0.001
Level at t ₂	385 (294-614)	604 (468-937)	0.01
FIXa:AT, pM			
Level at t ₀	63.7 (43.4-91.4)	85.8 (61.7-126.7)	0.006
Level at t ₁	66.4 (49.9-89.7)	92.0 (73.7-127.2)	0.004
Level at t ₂	61.7 (39.1-78.4)	75.4 (58.8-107.4)	NS

t₀, t₁, and t₂ indicate baseline, day 5 (±2), and day 10 (±2), respectively.

α1AT, α1-antitrypsin; AT, antithrombin; C1inh, C1 inhibitor; FIXa, activated factor IX; FXIa, activated factor XI; NS, not significant; PKa, plasma kallikrein; TAT, thrombin in complex with antithrombin; vWF:Ag, von Willebrand factor antigen.

Disease course of Covid-19

Most of the 174 admitted patients were treated with antibiotics ($n=161$, 93%), chloroquine ($n=134$, 77%), and anticoagulation ($n=155$, 89%; therapeutic dose, $n=29$) in addition to oxygen support. The median follow-up of these patients was 8 (IQR, 4-18) days; the median time of hospitalization was 6 (IQR, 4-9) days in patients with moderate Covid-19 and 9 (IQR, 4-19) days in patients progressive/severe disease. Fifty-nine patients were admitted to the intensive care unit, with a median stay of 15 (IQR, 7-21) days and invasive ventilation for 14 (IQR, 5-20) days.

Thrombotic events were noted in 23 (22%; pulmonary embolism, $n=19$) out of 106 patients with progressive/severe Covid-19, 3 (4%) out of 68 patients with moderate disease, and 2 (4%; pulmonary embolism, $n=2$) out of 54 with mild disease; most events were noted in the patients with progressive/severe Covid-19 ($P < 0.01$). Markers of coagulation did not differ between patients with and those without thrombotic events. Fifty-seven (25%) out of 228 patients died after a median of 6 (IQR, 3-14) days.

Discussion

Here, we provide evidence for the pathogenic link between C5a, neutrophils, endothelial cell damage, and coagulation, particularly *via* the contact pathway, in a large and well-defined cohort of patients with Covid-19. At presentation, despite comparable disease duration, there is already a striking difference in markers of inflammation and early coagulation between patients with mild Covid-19 and those admitted to the hospital, ranging from moderate to progressive/severe disease. C5a is highest in patients admitted to the hospital, reflecting its role as a main neutrophil driving factor. The high cumulative incidence of histone H3, and in particular citH3, in plasma and sputum from patients with progressive/severe Covid-19, in contrast to none in patients with mild disease, supports the pathogenic role of NETosis in Covid-19 progression. In line with this hypothesis, serum from patients with progressive/severe Covid-19 but not from those with mild disease induced *in vitro* NETosis, containing citH3. Downstream effects on vessel wall and the contact pathway of coagulation are more pronounced in patients with moderate and, even more so, in patients with progressive /severe Covid-19 as compared to those with mild disease. This explains the finding that thrombosis is more prevalent among patients with progressive/severe Covid-19.

The clinical spectrum of Covid-19 ranges from mild manifestations to a progressive/severe disease, often characterized by a rapid deterioration. Disease severity in our cohort was not related to comorbidities. Baseline parameters were quite balanced between the groups, although patients with progressive/severe disease tended to be older. Neutrophilia is common and may predict worse outcomes.¹¹ We demonstrate elevated C5a, a potent anaphylatoxin, in most patients with Covid-19, with highest levels in patients with moderate and those with progressive/severe disease. Murine data have shown that complement activation contributes to severe disease caused by viruses that share genetic homology with SARS-CoV-2, that is, SARS-CoV-1 and Middle East respiratory syndrome-CoV.^{12, 13} C5a can prime and activate neutrophils, causing more generation of C5a *via* the alternative pathway of complement and thus, a feedback amplification of neutrophil activation.¹⁴ Neutrophils,

when incubated with serum from admitted patients and, in particular, those with progressive/severe Covid-19, release NETs, corroborating Zuo *et al.*¹⁵ NETs have been linked to disease severity in ARDS.¹⁶

Extracellular histones, released during the process of NETosis, exhibit cytotoxic and activating effects on the endothelium.¹⁷ Histones injected into mice cause death, with neutrophil margination to the lungs, alveolar deposition of fibrin, and microvascular thrombosis;¹⁸ identical lesions have been found on lung tissue specimens from deceased Covid-19 patients.^{6, 19} Histones, although often absent at presentation, were most prevalent during the course of disease in patients with progressive/severe Covid-19. The presence of citH3 in sputum of patients admitted to the intensive care unit indicates localized NETosis within the lungs. Moreover, vWF:Ag levels were highest in patients with progressive/severe Covid-19, reflecting more severe histone-induced activation of endothelium.²⁰

The cumulative incidence of thrombotic manifestations and, in particular, pulmonary embolism, is ~40% in patients admitted to the intensive care unit.^{5, 21} Thrombosis occurred in 22% of our patients with progressive/severe Covid-19 despite the use of LMWH. We show that with increasing severity of disease, activation of the contact pathway becomes more dominant as reflected by high levels of FXIa:α1AT, FXIa:AT, and FIXa:AT that persist during the course of disease. FXI can be activated both *via* the contact and extrinsic/common pathway. Elevated PKa:C1inh was found in almost all patients, indicating activation of the contact pathway. LMWHs are known to have only mild effects on PKa and/or FXIIa, both key for contact activation to occur. LMWHs did not affect FXIa:AT and FXIa:α1AT, confirming that activation of FXI occurred through PKa and/or FXIIa. NETosis and the release of DNA can initiate this pathway of coagulation through activation of FXII and FXI.²² NETs contained citH3, indicating PAD4 induced chromatin decondensation. Mice deficient for PAD4 are protected from thrombosis to occur.²³ Thus, we postulate that NETosis is key for so-called Covid-19 associated coagulopathy to occur.

Previous studies on Covid-19 associated coagulopathy documented elevated D-dimer and, although less common, prolonged aPTT.^{9, 24} D-dimer was elevated in patients admitted to the intensive care unit, often accompanied by fibrinogen concentrations above the upper limit of normal. Of note, D-dimer reflects plasmin activity, which can further enhance thrombo-inflammation.²⁵ The prolonged aPTT did not reflect disseminated intravascular coagulation in our cohort and has recently been linked to lupus anticoagulant, either with FXII deficiency or not.²⁶ Lupus anticoagulant, however, was not linked to thrombosis and its role in Covid-19 associated coagulopathy, if any, remains to be determined.

The so-called “hyper-inflammation” in severe Covid-19, characterized by high levels of ferritin and interleukin-6 among other cytokines,²¹ can also be triggered by NETosis. DNA can promote localization of Toll-like receptor 4 to endosomes containing histones in human monocytes, inducing release of pro-inflammatory cytokines.²⁷ Bronchoalveolar lavage fluid from patients with severe Covid-19, indeed, showed a high proportion of pro-inflammatory monocyte-derived macrophages and neutrophils.²⁸ In conclusion, we analyzed complement, neutrophils, and coagulation in a large and well-defined cohort of patients with Covid-19, providing a deeper understanding of the intricate link between these factors. The feedforward and feedback loops intricately connecting innate immunity and hemostasis can amplify a hypercoagulable state. These findings provide a rationale for novel therapies in Covid-19 patients, such as inhibition of complement and neutralizing extracellular histones.

Acknowledgements

HtC and HMHS receive grant support by the Netherlands Heart Foundation (CVON2014-09, Reappraisal of Atrial Fibrillation: Interaction between HyperCoagulability, Electrical Remodeling, and Vascular Destabilisation in the Progression of Atrial Fibrillation (RACE V), and from REG-MED XB: Cardiovascular Moonshot. HtC was supported by a fellowship of the Gutenberg University Mainz. MN is supported by grant funding from REG-MED XB: Cardiovascular Moonshot. MV is supported in part by funding from a Health-Holland project/partnership grant (LSHM 9111). HtC, HMHS, BvB are members of the Dutch Coalition on Thrombosis in Covid-19 (DCTC), which is supported by the National Science Foundation (ZON-MW) and the Dutch Thrombosis Foundation (TSN).

Conflicts of Interest

HtC and HMHS received funding for research from Bayer and Pfizer; they are stakeholder in Coagulation Profile; HTC is consultant for Alveron and has served at advisory boards for Bayer, Pfizer, Daiichi, Leo, Gilead. CPR and GAFN are co-inventors of a patent describing use of low anticoagulant heparins in sepsis and owned by Maastricht University. CPR is scientific consultant for Matisse Pharmaceuticals and Annexin Pharmaceuticals.

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Chapter 7

General discussion

The risk of thrombotic diseases is a globally growing challenge and a leading cause of mortality worldwide. A variety of cellular, prothrombotic, and antithrombotic factors are involved in blood clotting which, if unbalanced, may lead to thrombotic complications. Among the factors contributing to thrombosis, the contact activation system of coagulation has gained in importance in recent years. This thesis focuses on new aspects of the contact system of coagulation in order to better understand how the factors of the contact system, in particular factor XI and plasma prekallikrein, play a role in thrombosis as a disease in itself or as a concomitant of other diseases. The aims of the thesis were to identify new pathways contributing to intrinsic coagulation, to characterize the phenotype of FXI-deficient individuals, to gain insights in the activation status of FXI and PK in a VTE cohort and to study the role of the intrinsic coagulation cascade in patients with COVID-19 infection.

Revised model of coagulation: novel role of plasma kallikrein in intrinsic coagulation

The contact system of coagulation, consisting of the factors FXII, PK and HK, is induced when negatively charged polymers such as polyphosphates, collagen or NETs are exposed to the blood.¹⁻³ This results in formation of FXIIa, which activates both PK and FXI, thereby initiating the kallikrein-kinin pathway and the intrinsic pathway, respectively (chapter 2). In the kallikrein-kinin pathway, PKa cleaves HK to release bradykinin, which induces vasodilation via its bradykinin B2 receptor by stimulating the generation of nitric oxide and prostacyclin.⁴ Other physiological activators of PK include prolylcarboxypeptidase, an endothelial cell membrane serine protease, and heat shock protein 90. The well-known role of PK in intrinsic coagulation is to amplify the generation of FXIIa in a reciprocal manner. This results in thrombin generation via activation of FXI, FIX and FX (chapter 2). However, results from thrombin generation experiments and aPTT measurements indicated a FXI-independent coagulation pathway towards thrombin generation. Previous studies suggested that PKa may be involved in this alternative pathway.^{5, 6}

Chapter 3 describes the study to examine the novel role for PKa in intrinsic coagulation by activating FIX. Thrombin generation, clot formation and FIXa:AT complex formation were observed in ellagic acid- or long-chain polyP-induced contact activation in FXI-deficient plasma, which was inhibited in the presence of a PKa inhibitor. FIXa:AT complex formation in *F11^{-/-}* mice, caused by injection of ellagic acid or polyP, was also decreased by a PKa inhibitor. These results indicate, that in the absence of FXI, PKa contributes to intrinsic coagulation by activating FIX. The question remains, if this is also true when FXI is present in plasma as these results were

obtained in FXI-deficient plasma. However, this might be challenging to assess, as it is difficult to differentiate between FXIa and PKa as an activator of FIX. Data generated by other research groups support the outcome of our study that PKa activates FIX.^{6, 7} FXIa-independent *in vitro* thrombin generation was observed upon initiation of the intrinsic coagulation cascade by long-chain polyphosphates. It was suggested that PKa might activate FIX, but this could not be clearly demonstrated.⁶ A recent study confirmed that red cell-derived microvesicles propagate coagulation⁸ but added that the effects are mediated directly by activating PK to PKa, which then activates FIX in an FXI-independent manner.⁷ This indicates that red blood cell transfusion may lead to FIX activation via two independent pathways potentially resulting in thrombotic complications. Furthermore, a direct interaction of PKa with FIX was recently demonstrated.⁹

The physiological role of this alternative pathway of intrinsic coagulation is not yet entirely clear. To this end, further studies and investigations are needed to determine its role within the coagulation cascade. However, it could provide explanations for the cause of the different phenotypes of FIX deficiency and FXI deficiency, as FIX can be activated not only by FXI but also by PKa. The pathway could also possibly contribute to the different bleeding phenotypes of FXI-deficient individuals as the risk of bleeding does not appear to be dependent on the FXI level itself.

Overall, the discovery of the alternative pathway could lead to a revised model of coagulation. FIX instead of FX might be considered now as the key regulatory coagulation protease merging the extrinsic, intrinsic and contact pathways into the common pathway, as it can be activated by FVII, FXI and PKa.

Increased PKa activity was determined in FXI-deficient individuals compared with healthy donors

Coagulation involves a complex interplay of various cellular components, enzymes and proteins. If there is an imbalance of the blood coagulation system due to increased or decreased activity of individual factors, this will affect coagulation and can lead to serious complications. Hemophilia A and B, which are characterized by reduced FVIII or FIX levels, respectively, are hemostatic disorders that are associated with an increased bleeding phenotype in affected patients.¹⁰ In contrast, FXI deficiency, also known as hemophilia C, is characterized by a mild bleeding phenotype, although this disorder is classified as a hemophilic disease. Spontaneous bleedings are rare and mostly trauma related.^{11, 12} However, the prediction of the bleeding risk in FXI-deficient individuals is challenging as it is, unlike FIX or FVIII deficiency, not related to the FXI

level.¹³ Hence, it is thought that additional hemostatic abnormalities such as low von Willebrand factor level may be associated with the bleeding risk in FXI-deficient individuals.¹⁴

In the study described in chapter 3, we showed that PKa directly activates FIX in the absence of FXI and therefore contributes to intrinsic coagulation. Since this alternative coagulation pathway was identified in commercially available FXI-deficient plasma, we were interested in investigating if it is also present in several FXI-deficient individuals. We also wanted to examine whether PKa has a greater impact on coagulation in FXI-deficient individuals and whether this may be linked to the risk of bleeding. For this purpose plasma from FXI-deficient individuals from Israel was used, as this otherwise very rare disorder is predominantly observed in Jewish population of Ashkenazi descent.¹⁵

In chapter 4 plasma from FXI-deficient individuals was characterized by determining the coagulation factor levels, analyzing the coagulation potential and estimating the individual endogenous coagulation status. Variations in PKa:C1inh complex formation and PKa activity were found in FXI-deficient individuals upon contact activation which were, however, overall increased in FXI-deficient individuals compared with healthy donors. This suggests that PKa may play a greater role in intrinsic coagulation when compared with plasma from healthy donors. Inhibition of PKa prevented FIXa:AT complex formation in plasma with very low FXI levels indicating that the alternative pathway from PKa to FIX activation described in chapter 3 is present in plasma of FXI-deficient individuals. Furthermore, we demonstrated that thrombin may be the main activator of FXI in plasma with very low FXI levels as no FXIa-inhibitor complexes were detected in the absence of calcium chloride in plasma with less than 11% FXI. In addition, FXIa:C1inh complexes were hardly detected in the plasma samples of FXI-deficient individuals, indicating that C1inh may not be a major inhibitor of FXIa under the conditions used in our study.

Several studies have been conducted to better reflect and predict the bleeding phenotype in FXI-deficient individuals.¹⁶ While most studies failed,¹⁶ some investigators were able to discriminate between bleeders and non-bleeders^{17, 18} However, the question about the underlying cause of the different bleeding phenotype of FXI deficiency remains. In our study described in chapter 4 we demonstrated variations in PKa activity in FXI-deficient individuals. Whether this contributes to the bleeding phenotype of FXI deficiency needs to be further investigated.

Enzyme:inhibitor complex ELISA as potential tool to unravel the contribution of coagulation factors to different diseases

The status of blood coagulation can be examined by measuring various biomarkers. If thrombosis or increased coagulation is suspected in the clinic, D-dimer levels and on occasion thrombin-antithrombin complexes, are routinely measured in plasma.¹⁹ D-dimers arise as a plasmin-initiated degradation product of fibrin and the measured amount of D-dimers is associated with fibrin formation and thus coagulation activity in the respective patient.²⁰ Thrombin is considered to be the key enzyme of coagulation by activating platelets, amplifying its own generation and transforming fibrinogen to fibrin.²¹ However, it is not possible to differentiate whether the extrinsic or the intrinsic pathway of coagulation is activated or even both, since D-dimer and thrombin represent the common steps of the coagulation cascade. In order to gain insights about the role of the various coagulation factors in thrombosis, additional enzyme:inhibitor complex assays were developed. These were used for instance to determine the predominant inhibitor of FXIa²² or to assess the activation of components of the contact system in cardiovascular disease.^{23, 24}

In chapter 5 enzyme:inhibitor complex ELISAs were used to assess the role of FXI and PK in patients with acute VTE. In addition, FXI activation in these patients was investigated using a thrombin generation-based FXIa assay²⁵ in which, however, no essential differences in the FXIa levels between the case and the control group were determined. An explanation for this result could be that most of the patients were treated with anticoagulants, which interfere with this assay.

Using enzyme:inhibitor complex ELISAs, we demonstrated significantly elevated PKa:C1inh complex levels in patients compared with controls. Importantly, elevated FXIa:C1inh complex formation was associated with an increased risk of mortality while a trend was observed for increased PKa:C1inh complex formation in relation with death and recurrent VTE or death. These results indicate a contributing role of the contact system, in particular the generation of FXIa and PKa, in acute VTE. This is in line with earlier studies in which an increased risk of ischemic stroke and DVT in the general population was associated with elevated FXI levels.^{26, 27} In addition, elevated PKa levels have been associated with an increased risk of ischemic stroke in young women. However, there is a lack of clinical studies to support a contributing role of PKa in thrombosis.²⁸

Although the introduction of direct oral anticoagulants (DOACs) has improved the treatment of thrombotic disease in patients, the general risk of bleeding remained.²⁹ As animal studies have shown that inhibitors of the contact activation system prevent thrombosis but do not increase the risk of bleeding,³⁰⁻³² these inhibitors could represent

novel and potentially safe approaches for the treatment of VTE. First clinical studies have provided proof of concept for the prevention of venous thrombosis by lowering FXI levels or inhibiting FXIa.^{33, 34}

Enzyme:inhibitor complex ELISAs may be helpful tools to assess the activation status of coagulation factors in other diseases. For instance they have been used to study *in vivo* contact activation in patients with sepsis^{35, 36} or dengue fever³⁷. There is evidence that inflammation, in particular the complement pathway, is associated with coagulation. Both are distinct systems but also share several common characteristics and even affect each other. PKa has been reported to cleave the complement component C3 directly or via activation of factor B.³⁸ In addition, thrombin has been shown to activate complement C3 and C5, which can also be cleaved by the coagulation factors FXIa, FXa, FIXa, and plasmin.^{38, 39} On the other hand it was demonstrated that the complement system stimulates the coagulation cascade through cleavage of prothrombin to thrombin by MASP-2, which is formed in the lectin pathway of the complement system.³⁹

Given these interplays between inflammation and coagulation, it is likely that inflammatory diseases are often associated with thrombotic complications. This also applies to severe acute-respiratory syndrome coronavirus 2 (SARS-Cov-2), also known as COVID-19,⁴⁰ which is currently a growing challenge worldwide. Characteristics of COVID-19 infection include severe inflammation,^{41, 42} vascular damage and coagulopathy⁴³ resulting in hypercoagulation and thrombosis. Elevated coagulation biomarkers, i.e. D-dimer and fibrinogen levels have been associated with the outcome of the disease,⁴⁴ however the exact underlying mechanism remains unclear.

Chapter 6 describes a prospective analysis of biomarkers of coagulation and inflammation in plasma from consecutive patients with COVID-19. Elevated levels of complement C5a and citrullinated extracellular H3 have been identified indicating activation of complement system and neutrophils in COVID-19 patients, respectively. NETs as a result of NETosis from activated neutrophils have been reported to activate FXII of the contact system.³ Indeed, elevated levels of PKa:C1inh, FXIa:α1AT, and TAT were observed in most patients and increased levels of FIXa:AT and FXIa:AT were common. Furthermore, elevated levels of vWF antigen were determined in most patients, indicating endothelial activation in COVID-19 patients. Importantly, downstream signaling of contact activation (FXIa, FIXa, thrombin) is associated with the severity of COVID-19 as the highest number of enzyme:inhibitor complexes was observed in the severe group. This obviously points to a major role of coagulation especially of the contact system, as could be shown by elevated levels of PKa:C1inh,

in COVID-19 patients which is in line with the findings of severe thrombotic complications.⁴³

Overall, the outcome of the study suggests a close interaction between neutrophils, coagulation and complement in COVID-19 disease and may provide novel treatment approaches for COVID-19. Components of the contact system (FXIIa, PKa, FXIa) may be valuable targets to reduce severe complications caused by a COVID-19 infection, as proposed by others as well.^{45, 46} In addition, agents that neutralize extracellular histones or inhibitors of C5a may be promising candidates.

In conclusion chapter 5 and chapter 6 both demonstrate a contributing role of the contact activation pathway to thrombotic diseases and also highlight that the measurement of coagulation factors in complex with their natural inhibitors represents a valuable approach to assess activation of coagulation in different patient cohorts.

Concluding remark

This thesis aimed to gain insights in the role of FXIa and PKa in coagulation and to examine their contribution to thrombosis. Key findings included the discovery of a novel coagulation pathway from PKa to FIX activation, thereby bypassing FXI, and the identification of this alternative pathway in FXI-deficient individuals from Israel. In addition, variations in PKa activity were observed in FXI-deficient individuals. The contribution of FXIa and PKa to thrombotic diseases was demonstrated using enzyme:inhibitor complex ELISAs. Elevated PKa:C1inh complex levels were detected in patients with acute VTE and increased FXIa:C1inh complex formation was associated with increased mortality. In addition, the contact activation pathway, in close interaction with neutrophils and the complement system, was identified as potential driver of hypercoagulability in COVID-19 infection.

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Chapter 8

Summary

Samenvatting

Impact paragraph

Curriculum Vitae

List of publications and presentations

Acknowledgement

Summary

Summary

My thesis aimed to investigate the role of the contact activation pathway in coagulation with a special focus on factor XI (FXI) and plasma prekallikrein (PK) and their contribution to thrombotic diseases.

Chapter 1 provides background information about the underlying processes and risk factors for arterial and venous thrombosis. In addition, it gives an overview about the coagulation cascade consisting of the intrinsic, extrinsic and common pathway which involve sequential proteolytic activation of various zymogens. This chapter also introduces the importance of endogenous inhibitors in regulating the coagulation cascade to ensure the balance between blood clotting and bleeding. Furthermore, two techniques employed in this thesis are presented. While the Calibrated Automated Thrombogram (CAT) assay investigates the potential of coagulation by monitoring thrombin generation, ELISAs measuring activated coagulation factors in complex with their natural inhibitors reflect the activation status of coagulation in different plasma samples.

A detailed overview about the structure and function of factor XI and plasma prekallikrein, and their contribution to arterial and venous thrombosis is given in **Chapter 2**. Both proteins belong to the contact activation pathway of coagulation and are activated by FXIIa that is formed upon contact with negatively charged polymers. PK is also linked to inflammatory processes through its ability to release bradykinin from kininogen. Animal studies, as well as epidemiological studies, described in detail in this chapter, indicate that components of the contact activation system contribute to thrombosis while having only a limited effect on hemostasis. Therefore this chapter also emphasizes the clinical importance of FXI(a) and plasma kallikrein (PKa) inhibitors, presents current therapeutic strategies and discusses future perspectives.

A novel coagulation pathway contributing to intrinsic coagulation is described in **Chapter 3**. Measuring thrombin generation and FIXa:AT as well as FXa:AT complexes in FXI-deficient plasma, activation of coagulation was observed, when triggered with negatively charged artificial (ellagic acid) or physiologic (long-chain polyphosphates) polymers. This indicates the presence of an alternative coagulation pathway bypassing FXI. Given the sequence homology of FXI and PK, especially in the catalytic domain, the aim was to find out if FXIa and PKa may have the same substrate, namely FIX. For this purpose a small molecule inhibitor specific to PKa was used. Inhibition of PKa resulted in decreased thrombin generation, prolonged aPTT and, interstingly, diminished FIXa:AT complex formation in FXI-deficient plasma, suggesting that PKa directly activates FIX. The presence of the alternative pathway was also demonstrated in animal studies using *F11^{-/-}* mice, in which ellagic acid- or long-chain polyphosphates-

induced FIXa:AT complex formation was significantly reduced by inhibition of PKa. These findings may, in part, help to dissect the bleeding phenotype in FXI-deficient individuals.

In **Chapter 4**, plasma of FXI-deficient individuals from Israel was characterized to identify potential parameters, which may provide reasons for the bleeding phenotype and enable a prediction of the bleeding risk. Given that PKa directly activates FIX (chapter 3), particular emphasis has been placed on examining the role of PKa in FXI-deficiency. Inhibition of PKa reduced FIXa:AT complex formation upon contact activation, proving the presence of the alternative pathway in FXI-deficient individuals with very low FXI levels. Furthermore, variations in PKa:C1inh complex formation and PKa activity were observed among the FXI-deficient individuals upon contact activation. These two parameters were overall increased indicating that PKa may have a greater impact on coagulation in FXI-deficient individuals as compared with healthy donors. In addition, the variations in PKa activity may provide initial indication about the cause of the different bleeding phenotype in FXI-deficient individuals. Furthermore, it was demonstrated that FXIIa alone was insufficient to activate FXI in plasma with low FXI levels, as evidenced by the lack of FXIa:inhibitor complexes upon contact activation. This suggests that thrombin has a greater impact on FXI activation in plasma with low FXI levels.

Components of the contact activation system have been shown to contribute to thrombosis. Therefore, the role of FXIa and PKa in patients with acute venous thromboembolism (VTE) was assessed in **Chapter 5** by measuring the extent of FXI and PK activation in comparison with healthy controls using enzyme:inhibitor complex ELISAs and a thrombin generation-based FXIa assay. Significantly elevated PKa:C1inh complex levels were detected in patients compared with controls. In addition, increased FXIa:C1inh complex formation was associated with an increased risk of mortality while a trend was observed for elevated PKa:C1inh levels in relation with death and recurrent VTE or death. CAT:FXIa could not be associated with any of the analysed clinical outcomes of acute VTE which could be due to the treatment of the patients with anticoagulants. Overall, this study points towards a contributing role of FXI and PK to acute VTE and provides novel and possibly safe clinical approaches for the treatment of VTE.

Given the high incidence of thrombotic complications associated with COVID-19 infection, prothrombotic and inflammatory processes occurring in infected patients were examined in **Chapter 6**. Elevated complexes of activated coagulation factors and their natural inhibitors were found in patients with COVID-19 infection, which in part were associated with the severity of the disease. Increased PKa:C1inh and

FXIa:inhibitor complexes point towards the contact system as a potential driver of hypercoagulability. In addition, increased NET formation, a mediator of contact activation, and complement activation, shown by elevated C5a levels, were detected. This indicates a complex interplay of contact activation, complement system and neutrophils contributing to the severe complications of COVID-19 infection. These results may offer new clinical approaches for improved therapy. Coagulation factors of the contact system (FXIa or PKa), C5a of the complement system and histones or a combination of these may be valuable targets to treat COVID-19 infection.

Finally, the key findings of the thesis are critically discussed in context to the literature in **Chapter 7**.

Samenvatting

Samenvatting

Mijn thesis richtte zich op het onderzoeken van de rol van de contactactivatie-cascade in stolling met een bijzondere focus op factor XI (FXI), plasma prekallikrein (PK) en hun bijdrage aan trombotische ziekten.

Hoofdstuk 1 biedt achtergrondinformatie over de onderliggende processen en risicofactoren voor arteriële en veneuze trombose. Bovendien geeft het een overzicht van de stollingscascade, bestaande uit de intrinsieke, extrinsieke en gemeenschappelijke cascade, waarbij een aaneenschakeling van proteolytische activatie van verschillende zymogenen plaatsvindt. Dit hoofdstuk introduceert ook het belang van endogene remmers in de regulatie van de stollingscascade, hetgeen zorgt voor een balans tussen bloedstolling en bloedingen. Verder worden twee technieken gepresenteerd die gebruikt werden in deze thesis. Terwijl de Calibrated Automated Thrombogram (CAT)-bepaling het potentiaal aan stolling onderzoekt door de trombinegeneratie te monitoren, reflecteren de ELISAs van geactiveerde stollingsfactoren in complex met hun endogene remmers juist de activatiestatus van de stolling in verschillende plasmamonsters.

Een gedetailleerd overzicht van de structuur en functie van factor XI en plasma prekallikrein, en hun bijdrage aan arteriële en veneuze trombose wordt gegeven in **Hoofdstuk 2**. Beide proteïnen behoren tot de contactactivatie-cascade van de stolling en worden geactiveerd door FXIIa, dat gevormd wordt bij contact met negatief geladen polymeren. PK is ook betrokken bij inflammatoire processen door het vermogen om bradykinine van kininogeen vrij te laten. Zowel dierenstudies als epidemiologische studies, die in detail worden besproken in dit hoofdstuk, tonen aan dat componenten van het contactactivatie-systeem bijdragen aan trombose terwijl ze slechts een beperkt effect hebben op de hemostase. Bijgevolg benadrukt dit hoofdstuk het klinische belang van FXI(a)- en plasma kallikrein (PKa)-remmers, legt huidige therapeutische strategieën voor en bespreekt toekomstperspectieven.

Een nieuwe stollings-cascade die bijdraagt aan de intrinsieke coagulatie wordt beschreven in **Hoofdstuk 3**. Bij de meting van trombinegeneratie en zowel FIXa:AT- als FXa:AT-complexen in FXI- deficiënt plasma werd stollingsactivatie geobserveerd, indien het op gang gebracht werd met negatief geladen artificiële (ellaginezuur) of fysiologische (lange-keten polyfosfaten) polymeren. Dit wijst op de aanwezigheid van een alternatieve stollings-cascade die FXI omzeilt. Vanwege de sequentiehomologie tussen FXI en PK, met name in het katalytische domein, werd het doel gesteld om te achterhalen of FXIa en PKa een gemeenschappelijk substraat hebben, namelijk FIX. Hiervoor werd een kleine molecuul-remmer specifiek voor PKa gebruikt. Remming van PKa resulteerde in afgenomen trombinegeneratie, verlengde aPTT en, interessant

genoeg, verminderde FIXa:AT-complexvorming in FXI-deficiënt plasma, hetgeen suggereert dat PKa rechtstreeks FIX activeert. De aanwezigheid van deze alternatieve cascade werd ook aangetoond in een dierenstudie met *F11^{-/-}* muizen, waarin ellaginezuur- of lange-keten polyfosfaat-geïnduceerde FIXa:AT-complexvorming significant was afgenomen bij remming van PKa. Deze bevindingen kunnen, gedeeltelijk, helpen in het ontleden van het bleedingsfenotype bij FXI-deficiënte individuen.

In **Hoofdstuk 4** werd plasma van FXI-deficiënte individuen uit Israël gekarakteriseerd om potentiële parameters te identificeren, welke mogelijk redenen voor het bleedingsfenotype kunnen bieden en het voorspellen van het bleedingsrisico mogelijk maken. Aangezien PKa rechtstreeks factor FIX activeert (Hoofdstuk 3), werd de nadruk gelegd op het onderzoeken van de rol van PKa in FXI-deficiëntie. Remming van PKa vermindert FIXa:AT-complexvorming bij contactactivatie, hetgeen de aanwezigheid van een alternatieve cascade bewijst in FXI-deficiënte individuen met erg lage FXI-concentraties. Bovendien werden variaties in PKa:C1inh-complexvorming en PKa-activiteit geobserveerd onder FXI-deficiënte individuen bij contactactivatie. Deze twee parameters waren over het geheel toegenomen, hetgeen erop wijst dat PKa een grotere impact op de stolling bij FXI-deficiënte individuen zou kunnen hebben dan bij gezonde donoren. Voorts zouden de variaties in PKa-activiteit een initiële indicatie kunnen geven over de oorzaak voor de verschillende bleedingsfenotypes in FXI-deficiënte individuen. Daarnaast werd aangetoond dat alleen FXIIa onvoldoende was om FXI in plasma te activeren bij lage FXI-concentraties, zoals bewezen werd door het gebrek aan FXIa:remmer-complexen bij contactactivatie. Dit suggereert dat trombine een grotere invloed heeft op FXI-activatie in plasma bij lage FXI-concentraties.

Van componenten van het contactactivatie-systeem werd aangetoond dat ze bijdragen aan trombose. Bijgevolg werd in **Hoofdstuk 5** de rol van FXIa en PKa in patiënten met acute veneuze tromboembolie (VTE) geëvalueerd door de mate van FXI- en PK-activatie te meten in vergelijking met gezonde controles, gebruikmakend van enzym:remmer-complex ELISAs en een trombinegeneratie-gebaseerde FXIa-bepaling. Significante toegenomen PKa:C1inh-complexconcentraties werden gedetecteerd in patiënten vergeleken met controles. Bovendien was toegenomen FXIa:C1inh-complexvorming geassocieerd met een toegenomen mortaliteit, terwijl een trend van verhoogde PKa:C1inh-concentraties werd geobserveerd in relatie tot overlijden en een VTE-recidief of overlijden. De CAT:FXIa kon met geen enkele van de geanalyseerde klinische uitkomsten van acute VTE geassocieerd worden, hetgeen mogelijk verklaard wordt door de behandeling van de patiënten met

antistollingsmedicatie. Over het geheel genomen wijst deze studie naar een bijdragende rol van FXIa en PKa in acute VTE en biedt nieuwe en mogelijk veiligere klinische benaderingen voor de behandeling van VTE.

Vanwege de hoge incidentie van trombotische complicaties geassocieerd met COVID-19-infectie, werden protrombotische en inflammatoire processen die plaatsvinden in geïnfecteerde patiënten bestudeerd in **Hoofdstuk 6**. Verhoogde complexen van geactiveerde stollingsfactoren met hun natuurlijke remmers werden gevonden in patiënten met COVID-19-infectie, hetgeen deels geassocieerd was met de ernst van de ziekte. Verhoogde PKa:C1inh- en FXIa:inh-complexen wijzen op het contactactivatie-systeem als een mogelijke drijfveer van hypercoagulabiliteit. Verder werd verhoogde NET-vorming, een mediator van contactactivatie, en complementactivatie, middels verhoogde C5a-concentraties, aangetoond. Dit geeft aan dat een complex samenspel van contactactivatie, het complementsysteem en neutrofielen bijdraagt aan de ernstige complicaties van COVID-19-infectie. Deze resultaten kunnen mogelijk nieuwe klinische benaderingen bieden voor een betere behandeling. Stollingsfactoren van het contactsysteem (FXIa of PKa), C5a van het complementsysteem en histonen of een combinatie van deze, zouden waardevolle aangrijpingspunten in de behandeling van COVID-19-infectie kunnen bieden.

Ten slotte worden in **Hoofdstuk 7** de belangrijkste bevindingen van deze thesis kritisch besproken in de context van de literatuur.

Impact paragraph

Impact paragraph

Reflecting on the scientific work and establishing a connection between the results and the significance for the scientific and economic society have gained in importance. However, it is often challenging especially for a basic research scientist to clearly identify a direct link between the scientific outcomes and the social and economic impact. In contrast, translational research deals with the applicability of scientific findings to concrete, practice-relevant approaches that may have a more obvious impact on industrial and clinical applications.

This thesis combines work from basic research as well as from research with a more translational perspective to unravel the role of the contact activation pathway, particularly of FXI and PK, in coagulation and to investigate their role in thrombotic diseases.

Thrombosis as the underlying event of myocardial infarction, ischemic stroke and venous thromboembolism is still the leading cause of mortality worldwide.¹ Coagulation involves various cellular and pro- and antithrombotic factors, which, if unbalanced, may cause thrombotic complications or severe bleedings. Scientific research has made great efforts and progress in the treatment of thrombosis in recent years. The clinical application of anticoagulants was initially dominated by vitamin K antagonists, which were largely replaced by the introduction of direct oral anticoagulants (DOACs).² While this has improved the treatment of thrombotic disease in patients, the general risk of major bleeding of about 2%/year, remained. Particularly in an aging population both the risks of thrombosis and bleeding increase, which makes long term anticoagulation a major challenge. In recent years interest in the factors of the contact activation system of coagulation as possible targets for the treatment of thrombosis has increased, since they were shown to play a role in thrombosis while having only little or no impact on hemostasis.^{3, 4}

Chapter 2 elaborates on the role of the contact activation system, in particular of FXI and PK, in arterial and venous thrombosis. The clinical applicability of FXI(a) inhibitors or FXI antisense oligonucleotides is promising, since inhibiting FXI reduced thrombus formation in animal studies without increasing the risk of bleeding.⁵ The impact of PK on thrombosis is not quite as clear. While some data from preclinical studies suggest that PK(a) could be a valuable target for the treatment of thrombosis, there is a lack of clinical studies to support this. Given its role as initiator of the kallikrein-kinin pathway that regulates inflammatory processes, inhibition of PK(a) may, however, be a useful approach to treat thrombo-inflammation.⁶

The well-known role of PKa, besides triggering the kallikrein-kinin pathway, is the reciprocal activation of FXII, a component of the contact activation pathway, to

FXIIa which in turn activates FXI.⁷ In **chapter 3** it was demonstrated that PKa contributes to intrinsic coagulation in an alternative pathway by directly activating FIX. This discovery could lead to a revised model of the coagulation cascade in which FIX could be considered as the key enzyme that merges the intrinsic and extrinsic in the common pathway. Furthermore, this could draw attention to PKa, which was previously viewed as an initiator of inflammatory processes rather than a component of the coagulation cascade, as a possible target in the treatment of thrombosis. In order to interpret the physiological role of this alternative pathway, further studies need to be conducted. However, the finding that FIX can be activated not only by FXI but also by PKa could help to reveal the reason for the variation in bleeding phenotypes of FXI-deficient individuals. Since the bleeding phenotype in FXI-deficient individuals does not necessarily correlate with the FXI level, it is difficult to predict future bleeding complications, which occur most likely after surgery or trauma.⁸ In **Chapter 4** variations in PKa:C1inh complex formation and PKa activity were found among the analyzed plasma samples upon initiation of the contact activation pathway. Highest PKa:C1inh complexes and PKa activity were most likely observed in FXI-deficient individuals indicating a greater role for PKa in coagulation in these individuals. Due to the ability of PKa directly activating FIX and thus contributing to intrinsic coagulation, variations in PKa activity could explain the cause for the different bleeding phenotypes. Ideally, testing the PKa activity may help to predict potential trauma related bleeding complications. Whether fluctuations in PKa activity can indeed be associated with the bleeding phenotype in FXI-deficient individuals requires further investigation.

Biomarker research is valuable for clinical applications as the obtained parameters allow a direct interpretation of a patient's disease status and thus enable rapid treatment. To address this, the development of methods to determine suitable biomarkers for a particular disease is required. Throughout this thesis, enzyme:inhibitor complex ELISAs were used to gain insights in the coagulation status of plasma from healthy donors and different patient cohorts. In **Chapter 5**, the aim was to assess the extent of FXI and PK activation in patients with acute VTE. Elevated PKa:C1inh complex levels were determined in patients as compared with controls and increased FXIa:C1inh complex formation was associated with an increased risk of mortality. These results indicate a contributing role of FXI and PK to VTE and points towards novel treatment approaches for thrombotic diseases.

Chapter 6 is another example of how biomarker research can be used to gain further insights into a disease, in this case the globally challenging disease COVID-19, and to identify potential targets for improved therapy. Elevated levels of activated coagulation factors in complex with their natural inhibitors and increased complement

and neutrophil activation were determined in patients with COVID-19 infection indicating a close interplay between neutrophils, coagulation and complement. Inhibitors of components of the contact activation system or of C5a of the complement system could find clinical use to reduce or prevent severe complications caused by COVID-19 infection.

Taken together the finding that PKa directly activates FIX may prompt scientists to look at the coagulation cascade from a different angle and may provide novel treatment options in thrombosis. In addition, it may help to explain the cause of the variations in the bleeding manifestations in FXI-deficient individuals that have been shown to have variations in PKa activity upon contact activation. The finding that PKa:C1inh complexes are elevated in patients with acute VTE and that FXIa:C1inh complexes are associated with the clinical outcome of death, may lead to novel approaches for the treatment of acute VTE. Moreover, the cause of severe complications in COVID-19 infection suggests an interplay between neutrophils, coagulation and complement which may be valuable targets to improve the treatment of the disease.

From a treatment perspective, the availability of anticoagulants with preserved antithrombotic activity but reduced bleeding risk, such as might be the case with inhibitors of FXIa and/or PKa, would be another step forward for society. Currently several clinical trials with such inhibitors are ongoing.^{4, 9} In translational terms, the identification of the involvement of specific procoagulant elements in human disease, like in Covid-19, VTE or other cardiovascular diseases, is a crucial process pointing to targets for intervention that were previously unknown. Hence, for long term treatment of aging patients with (recurrent) VTE, in addition to FXI(a) inhibitors the use of a PKa inhibitor may become an alternative for a DOAC (aimed at FXa or thrombin), to further reduce bleeding risk. Alternatively, in settings with a high atherothrombosis risk, but a similarly increased bleeding risk, a combination of PKa inhibitor and aspirin may provide a safer combination therapy as compared to currently registered combination of rivaroxaban and aspirin. Since safer platelet inhibitors are also being developed, the array of antithrombotic agents and possible combinations of agents further increases. As it becomes impossible to study all such combinations in dedicated clinical trials, new strategies will be required to use biomarkers for risk stratification and to address benefit/risk ratios in other trial designs than the thus far obligatory randomized trials requiring thousands of subjects.

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Curriculum Vitae

Curriculum Vitae

Mayken Visser was born on November 8th 1989 in Burghausen, Germany. She graduated from secondary school at the Aventinus Gymnasium Burghausen in 2009. Afterwards she started the shared bachelor study program at the University of Salzburg and the University of Linz, Austria, and received the bachelor's degree in Molecular Biosciences with honor in 2012. In the same year she continued her education by attending the master study program of Molecular Biomedicine at the University of Münster, Germany, from which she graduated with honor in 2015. Subsequently, she conducted an internship at the Institute of Cardiovascular Research at Bayer AG under supervision of Dr. Stefan Heitmeier. In 2016, she started her PhD trajectory at the department of Biochemistry at the Cardiovascular Research Institute Maastricht, under supervision of Prof. Hugo ten Cate, Dr. Henri Spronk and Dr. Stefan Heitmeier. During that time she studied the role of factor XI and plasma prekallikrein in coagulation and performed her research from 2016 to 2019 mainly at Bayer AG in Wuppertal and from 2019 to 2020 at the Cardiovascular Research Institute Maastricht. She currently works as a postdoctoral researcher at the department of Hematology/Transfusion Medicine at the Paul-Ehrlich-Institute in Germany.

List of publications and presentations

Publications

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Acknowledgement

Acknowledgement

“Alone we can do so little, together we can do so much” (Helen Keller)

This applies very well to a doctoral thesis that cannot be achieved alone and without the support of others. Therefore, I would like to thank the people who supported me during my PhD.

First of all, I would like to express my gratitude to my supervisory team, Prof. ten Cate, Dr. Spronk and Dr. Heitmeier, who played an important role during my PhD trajectory. Thank you for your support and for the opportunities for me to develop as a researcher. Dear Hugo, thank you for giving me the opportunity to join your research group. I really appreciate your patience and the fact that you always take the time for discussions, reading drafts and valuable feedback, from which I learned a lot. Thank you for always leaving your door open for questions.

Dear Henri, I really appreciate your positive thinking even on a day full of experiments with odd results. Thank you for the many discussions, which I enjoyed very much, and your valuable feedback. These have always been of great help to me, and I have learned a lot about research, planning of experiments, and writing publications.

Dear Stefan, despite your busy schedule, you always took the time for discussions, which often resulted in very crazy figures on your board. I learned a lot from our discussions, not only about research but also about how ideas can be structured and implemented in experiments. Thank you for your valuable advice and for the great time at congresses.

I would like to thank the members of the reading committee Prof. Tilman Hackeng, Prof. Erik Beckers, Prof. Joost Meijers, Prof. Judith Sluimer and Prof. Jeff Weitz for reviewing my thesis.

A big thank you to my two paranympths, Magdi and Jens. Magdi, at the end of my PhD, we spent a lot of time together in the lab, which is why we were called M&Ms after a while. Thank you for the pleasant time, the nice conversations and your support and help both inside and outside the lab. I really appreciate the fact that I could always count on you and come to you for advice. Jens, I still remember the fun time at the beginning of my PhD, when we practiced the reperfusion model together in Wuppertal. With your positive nature, you always ensure “gezelligheid” (and singing) in the lab. Thank you for your support and the nice atmosphere in the lab and in our office. Bram and Aaron, my other roommates, I enjoyed the time in our office very much. Thank you for the nice conversations and coffee breaks, and the discussions during

our Journal Club. Special thanks to Aaron for translating my summary into Dutch. Rene van Oerle, I really enjoyed the discussions with you and learned a lot in terms of planning and performing experiments. Thank you for always leaving your door open for questions. Diane, Stefanie, Patricia and Simone, you really rock the lab. A big thank you for all your help and support, even if I came to you with a lot of samples (which happened quite frequently). All the other members of the CTH group Paola, Arina, Elisa, Billy, Renske, Yvonne, Peter, Marieke, Gaukhar, Marcel, thank you for your help and the valuable discussions.

I would also like to thank all the other employees in the biochemistry department for the nice atmosphere. Special thanks to Trees and Lidewij for helping me with the paperwork regarding the PhD.

I would also like to thank the people at Bayer in Wuppertal who supported me during my PhD. Ein großer Dank geht an Anja, Michaela, Verena und Claudia. Ihr habt mich vom Anfang als Praktikantin bis zum Ende meiner Doktorarbeit begleitet. Vielen Dank für eure Unterstützung, die angenehme Atmosphäre und die lustigen Restaurant-Besuche. Anja, meine Labor-, Büro- und Lunchpartnerin, es hat Spaß gemacht mit dir zu arbeiten und ich danke dir für die angenehme Zeit. Bei Fragen konnte ich immer zu dir kommen und ich habe deine Erfahrungen im Labor immer sehr geschätzt. Michaela, danke für die Hilfsbereitschaft bei Fragen und Experimenten und die netten Gespräche, die ich immer sehr genossen habe. Verena und Claudia, euch gibt es nur im Doppelpack. Danke für die netten Gespräche, bei welchen wir oft die Zeit vergessen haben, und eure Hilfe.

Zudem möchte ich Caro für die Unterstützung und Geduld bei den Mäuseversuchen danken, welche ich ohne dich nicht hätte durchführen können. Julia, auch dir möchte ich danken für die Hilfe bei den Versuchen, und auch für die netten Gespräche und schöne Zeit auf Kongressen. Volker, vielen Dank für die Möglichkeit, meine Dissertation in der Abteilung Cardiovascular 1 anzufertigen.

Auch den anderen Kolleginnen und Kollegen der Abteilung Cardiovascular 1 möchte ich für die nette Atmosphäre und Hilfe danken.

Neben den Kollegen spielten zudem meine Freunde eine wichtige Rolle bei dieser Dissertation. Ich danke euch für eure Unterstützung und die willkommenen Abwechslungen während meiner Doktorarbeitszeit. Egal wo wir alle verteilt sind, für ein Wochenende an der Mosel, im Harz, in Basel, im Disneyland oder einfach in der Heimat kommen wir immer wieder zusammen. Es ist wirklich schön solche Freunde

zu haben, auf die man sich immer verlassen kann. Ein spezielles Dankeschön geht an Chrissy, die das wunderschöne Cover zu dieser Arbeit gezeichnet hat.

Am wichtigsten ist jedoch meine Familie, die immer hinter mir stand und steht.

Lieve oma Lies, bedankt voor je oprechte interesse in mijn onderzoek en je hulp en steun tijdens mijn promotie maar ook in mijn gehele leven. Ik wil ook opa Ko, oma Riet en opa Gerard, die deze dag helaas niet meer mogen beleven, bedanken voor de steun die ze mij altijd hebben gegeven.

Mein Bruder Jappe und Caro, meine Schwester Femke und Niko, danke, dass ihr immer für Abwechslung und Ablenkung mit viel Lachen und guter Stimmung gesorgt habt. Es ist schön, euch an meiner Seite und eure Unterstützung zu haben.

Meine Eltern, Mama und Papa, euch gilt mein größter Dank, welcher kaum in Worte auszudrücken ist. Ohne eure Unterstützung und euren Zuspruch wäre diese Arbeit niemals zustande gekommen. Ihr steht mir in allen Lebenslagen zur Seite und habt immer und für alles ein offenes Ohr. Ich bin froh euch als Eltern zu haben.

Andi, ich kann gar nicht beschreiben wie dankbar ich dir bin. Du stehst mir immer mit Rat und Tat zur Seite, baust mich mit deiner positiven Art auf und unterstützt mich in allem was ich mache. Ich kann mich glücklich schätzen, dich schon seit so vielen Jahren an meiner Seite zu haben.

Thank you/ Danke/ Bedankt!!