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MECHANISM AND THERAPY OF HEREDITARY ANGIOEDEMA TYPE III AND ROLE OF THE CONTACT SYSTEM IN INFLAMMATORY DISEASES

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Mechanism and Therapy of Hereditary Angioedema Type III and Role of the Contact System in Inflammatory Diseases

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ABSTRACT

Combinations of proinflammatory and procoagulant reactions are associated with a variety of disorders affecting the cardiovascular system. Vascular leakage contributes to the pathology of conditions such as, sepsis, allergy and anaphylactic reactions. Edema formation is the result of extravasated proteins and fluid and the peptide hormone bradykinin is considered to be one of the key mediators in the regulation of vascular leakage. Bradykinin is produced by the kallikrein-kinin system, which consists of factor XII, plasma prekallikrein, high molecular weight kininogen and C1 esterase inhibitor. Activated factor XII generates active prekallikrein (kallikrein), which cleaves kininogen, leading to the liberation of bradykinin. Activation of mast cells during allergic reactions mediates inflammatory responses, which cause increased vascular permeability. We report a new mechanism by which mast cell-released heparin increases vascular leakage. Upon allergen challenge mast cells release the negatively charged polysaccharide heparin that efficiently activates factor XII and initiates the kallikrein-kinin system. Heparin-driven kallikrein-kinin system activation culminates in bradykinin formation causing excessive vascular leakage in mice that are deficient in C1 esterase inhibitor, the major endogenous inhibitor of factor XII and kallikrein. These findings also have implications in anaphylactic and allergic diseases and we show that the factor XII–driven kallikrein-kinin system critically contributes to the pathogenesis of anaphylaxis in both murine models and human subjects. The data indicate that heparin-initiated bradykinin formation plays a fundamental role in mast cell mediated diseases.

Hereditary angioedema (HAE) is a rare inherited disease that is characterized by acute swelling that involves the skin, extremities and mucosa. HAE types I and II are caused by deficiency in or dysfunctional C1 esterase inhibitor. In contrast, a third HAE variant exists in patients that have normal C1 esterase inhibitor (HAE III). HAE III is associated with a single point mutation at residue Thr309 in factor XII. However, the mechanism of HAE III was unknown. This study characterizes the mechanism and therapy of HAE III. HAE III patient-plasma and recombinant Thr309 mutated factor XII result in a double band or in a band with a lower molecular weight than wild-type factor XII in Western blotting. This is the consequence of a loss of glycosylation. The mutation in factor XII causes excessive activation of the kallikrein-kinin system resulting in enhanced production of bradykinin. Addition of C1 esterase inhibitor dose-dependently blocked bradykinin production in HAE types I and II, but not in HAE III. We generated a fully humanized antibody (3F7) that specifically interferes with activated factor XII proteolytic activity. 3F7 inhibits activated factor XII-driven cleavage of high molecular weight kininogen in a dose dependent manner and interferes with aberrant kallikrein-kinin system-triggered bradykinin formation in HAE III plasma. We reconstituted factor XII deficient mice with recombinant human mutated factor XII and established an HAE III transgenic mouse that expresses human Thr309-mutated factor XII in the liver using Tet-off transgenic technology. Intravital confocal scanning microscopy and tracer extravasation-based methods show excessive bradykinin-mediated vascular leakage in both *F12*$^{-/-}$ mice reconstituted with mutated factor XII and in HAE III transgenic mice when challenged with factor XII-contact activator. Both a kallikrein inhibitor and 3F7 reduce edema in HAE III associated leakage in mice. This study characterizes the mechanism of HAE III and establishes factor XII inhibition as a novel therapeutic strategy to interfere with excessive vascular leakage in HAE III and potentially, other causes of edema.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following publications and manuscript:

I. **Björkqvist, J.**, Lecher, B., Maas, C., and Renné, T. Zinc-dependent contact system activation induces vascular leakage and hypotension in rodents. *Biological Chemistry,* 2013, 394, 1195-204.

II. Oschatz, C., Maas, C., Lecher, B., Jansen, T., **Björkqvist, J.**, Tradler, T., Sedlmeier, R., Burfeind, P., Cichon, S., Hammerschmidt, S., Müller-Esterl, W., Wuillemin, WA., Nilsson, G., and Renné, T. Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. *Immunity*, 2011, 34, 258-68.

III. Sala-Cunill, A., **Björkqvist, J.**, Senter, R., Guilarte, M., Cardona, V., Labrador, M., Nickel, KF., Butler, L., Luengo, O., Kumar, P., Labberton, L., Long, A., Di Gennaro, A., Kenne, E., Jämsä, A., Krieger, T., Schlüter, H., Fuchs, T., Flohr, S., Hassiepen, U., Cumin, F., McCrea, K., Maas, C., Stavrou, E., and Renné, T. Plasma contact system activation drives anaphylaxis in severe mast cell–mediated allergic reactions. *Journal of Allergy and Clinical Immunology*, 2014, doi: 10.1016/j.jaci.2014.07.057

IV. **Björkqvist, J.**, Oschatz, C., Lewandrowski, U., Schönig, K., Noethen, M., Sickmann, A., Panousis, C., Maas, C., and Renné, T. Defective glycosylation of coagulation factor XII causes hereditary angioedema type III. *Submitted Manuscript.*

V. Larsson, M., Rayzman, V., Nolte, MW., Nickel, KF., **Björkqvist, J.**, Jämsä, A., Hardy, MP., Fries, M., Schmidbauer, S., Hedenqvist, P., Broomé, M., Pragst, I., Dickneite, G., Wilson, MJ., Nash, AD., Panousis, C., and Renné, T. A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. *Science Translational Medicine*, 2014, 6, 222ra17

Publications by the author, which are not included in the thesis

Björkqvist, J., Jämsä, A., and Renné, T. Plasma kallikrein: the bradykinin-producing enzyme. *Thrombosis and Haemostasis*, 2013, 110, 399-407

Björkqvist, J., Sala-Cunill, A., and Renné, T. Hereditary angioedema: a bradykinin-mediated swelling disorder. *Thrombosis and Haemostasis,* 2013, 109, 368-74

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Hansson, KM., **Björkqvist, J.**, Deinum, J. Addition of prothrombin to plasma can result in a paradoxical increase in activated partial thromboplastin time. *Blood Coagulation and Fibrinolysis,* 2014, doi: 10.1097/MBC.0000000000000161.

Hansson, KM., **Björkqvist, J.**, Deinum, J. The Effect of Recombinant and Plasma Derived Prothrombin on Prothrombin Time (PT) in Human Plasma. *International Journal of Laboratory Hematology,* 2014, doi: 10.1111/ijlh.12293.

CONTENTS

LIST OF ABBREVIATIONS

A1	Apple domain 1
A2	Apple domain 2
A3	Apple domain 3
A4	Apple domain 4
ACE	Angiotensin converting enzyme
ACEI-AAE	Acquired angioedema related to angiotensin converting enzyme inhibitors
aPTT	Activated partial thromboplastin time
B1R	B1 bradykinin receptor
B2R	B2 bradykinin receptor
BK	Bradykinin
bw	Body weight
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium concentration
C1INH	C1 esterase inhibitor
C1INH-AAE	Acquired angioedema with C1 esterase inhibitor deficiency
CPB	Cardiopulmonary bypass
CTI	Corn trypsin inhibitor
DNP-HSA	Dinitrophenyl-human serum albumin conjugate
a-DNP-IgE	IgE against dinitrophenyl
Dox	Doxycycline
DXS	Dextran sulfate
EA	Ellagic acid
ECMO	Extracorporeal membrane oxygenation
EGF I	Epidermal growth factor like domain I
EGF II	Epidermal growth factor like domain II
$FeCl_3$	Ferric chloride
Fib I	Fibronectin domain type I
Fib II	Fibronectin domain type II
FITC-dextran	Fluorescein isothiocyanate-dextran
FXI	Plasma coagulation factor XI
FXII	Plasma coagulation factor XII

FXIIa	Activated plasma coagulation factor XII
a-FXIIa	Activated plasma coagulation factor XII two-chain molecule
b-FXIIa	Activated plasma coagulation factor XII fragment
HAE	Hereditary angioedema
HAE I	Hereditary angioedema type I
HAE II	Hereditary angioedema type II
HAE III	Hereditary angioedema type III
HK	High molecular weight kininogen
IgE	Immunoglobulin E
IH-AAE	Idiopathic histaminergic acquired angioedema
InH-AAE	Idiopathic non-histaminergic acquired angioedema
LC polyP	Long-chain polyphosphates
MABP	Mean arterial blood pressure
MC	Mast cells
NO	Nitric oxide
OSCS	Oversulfated chondroitin sulfate
PCK	H-D-Pro-Phe-Argchloromethylketone
PK	Plasma kallikrein
PPK	Plasma prekallikrein
PS polyP	Platelet-sized polyphosphates
rHA-infestin-4	Recombinant human albumin infestin-4
rtTA	Reverse tetracycline controlled transactivator
Serpin	Serine protease inhibitor
Tet-on	Tetracycline inducible expression system
Tet-off	Tetracycline controlled expression system
tTA	Tetracycline controlled transactivator
U	Units
U-HAE	Hereditary angioedema with normal C1 esterase inhibitor and of unknown origin
w/o	Without
WT	Wild-type
Zn^{2+}	Zinc

1 INTRODUCTION

Under physiological conditions blood circulates in a closed system. Hemostasis is a balanced interaction of blood cell, vasculature and plasma proteins. A perfect hemostasis is when there is no bleeding and no thrombosis. Blood coagulation in parallel with inflammatory and repair provides a protective mechanism of the organism in response to vascular injury and pathological conditions. An example that accelerates the interactions of the reactants in these systems is a result of angioedema that could be driven by the kallikrein-kinin system, which is part of the contact system (Marder et al., 2013). This thesis is focused on the role of the contact system in inflammatory diseases (**Figure 1**).

1.1 THE CONTACT SYSTEM

The contact system is an enzymatic cascade in blood that exerts procoagulant and proinflammatory activities by the ability to activate the intrinsic coagulation pathway and the kallikrein-kinin system (**Figure 1**). The proteins assemble on cell surface like heparan- and chondroitin sulfate type proteoglycans (Colman et al., 1997; Renne et al., 2000; Renne and Muller-Esterl, 2001). The proteins of the contact system circulate in the bloodstream or are bound to the surface of different cell types, including endothelium, platelets, leukocytes and bacteria (Itakura et al., 2011; Renne et al., 2000; Renne et al., 2005b; Nickel and Renne, 2012). The contact system comprises five components: three serine proteases, factor XII (FXII), plasma prekallikrein (PPK) and factor XI (FXI), the non-enzymatic cofactor, high molecular weight kininogen (HK) and the C1 esterase inhibitor (C1INH), which is the major inhibitor of activated FXII (FXIIa) and activated PPK (plasma kallikrein, PK) (Colman and Schmaier, 1997).

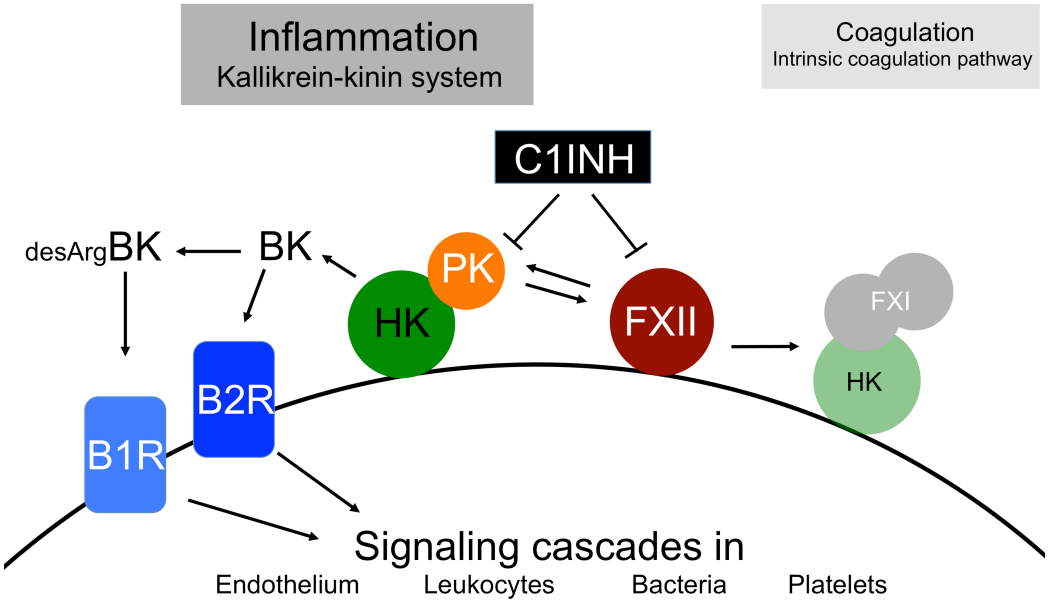

Figure 1: The FXII-driven contact system. The contact system could activate the kallikrein-kinin system and the intrinsic coagulation pathway. Contact with negatively charged surfaces activates FXII on endothelial cells, leukocytes, bacteria and thrombocytes initiating procoagulant and proinflammatory proteolytic reactions. Activated FXII triggers activation of prekallikrein by FXIIa leading to generation of the vasoactive peptide BK by PK-mediated cleavage of HK, the kallikrein-kinin system. FXIIa generates fibrin formation through the FXI-mediated intrinsic coagulation pathway.

FXII is the primary initiator of the contact system cascade and binding to negatively charged surfaces results in a conformational change in the FXII zymogen. The conformational change results in small amounts of FXIIa (auto-activation). FXIIa converts PPK into its active form PK. This proteolytically active PK can feedback to activate additional FXII (hetero-activation), which amplifies the initial signal and also cleaves HK to liberate BK through the kallikrein-kinin system (Colman and Schmaier, 1997). BK is a nonapeptide hormone that belongs to the kinin family and initiates signaling cascades that lead to vasodilation, increased vascular permeability and tissue swelling. FXIIa also initiates other cascades such as the intrinsic coagulation pathway via its substrate FXI (**Figure 1**). Furthermore, *in vitro* FXIIa also has the capacity to drive the classic complement system pathway, and the fibrinolytic system (Bjorkqvist et al., 2013a; Marder et al., 2013).

1.1.1 The intrinsic pathway of coagulation

Blood coagulation in parallel with inflammatory and repair reactions are protective mechanisms of the organism in response to vascular injury. The coagulation system reacts quickly to stop blood loss from a damaged vessel wall. It involves the primary hemostasis (vasoconstriction, platelet adhesion and aggregation), secondary hemostasis (activation of coagulation factors and formation of fibrin) and fibrinolysis (activation of fibrinolysis proteins and lysis of the clot) (Furie and Furie, 1992). Impaired or excessive coagulation activity leads to an increased risk of hemorrhage (bleeding) or thrombosis (clotting). Macfarlane (Macfarlane, 1964) and Davie and Ratnoff (Davie and Ratnoff, 1964) were the first to describe the classical coagulation cascade or waterfall model. They consist of two converging enzymatic pathways initiated either by exposure of blood to a damaged vessel wall (extrinsic pathway) or by blood-borne components of the vascular system (intrinsic pathway). The intrinsic pathway of coagulation is initiated by FXII. FXIIa cleaves its substrate FXI to form active FXI, which in turn promotes coagulation via Ca^{2+}-dependent activation of factor IX and with further activation resulting in conversion of prothrombin into thrombin. Thrombin cleaves fibrinogen to form fibrin and activates multiple pathways in the vascular system (Furie and Furie, 1992).

To inhibit thrombosis anticoagulants are needed. One of the most common anticoagulants is heparin. In extracorporeal membrane oxygenation (ECMO) the blood comes into contact with prothrombotic artificial surfaces (such as the tubing and oxygenator) and anticoagulant treatment is crucial for preventing clots. ECMO is a form of cardiopulmonary bypass (CPB) used in intensive care. ECMO provides both cardiac and respiratory support to patients with severe lung or heart failure. Currently, unfractionated heparin is used as an anticoagulant (Sanchez et al., 1998; Sniecinski and Chandler, 2011) but despite intensive monitoring as well as surgical and pharmacological hemostatic therapies, life-threatening bleeding remains the major threat to ECMO patients (Conrad et al., 2005).

1.1.2 The kallikrein-kinin system

The kallikrein–kinin system is a part of the contact system that results in a proinflammatory response via BK-generation. BK production is started when FXII becomes auto-activated or hetero-activated. FXIIa cleaves surface associated plasma PPK to generate PK, which in

turn reciprocally activates further FXII molecules, thereby amplifying the initial signal (Bjorkqvist et al., 2013c) (**Figure 1**).

1.1.3 Activation of the kallikrein-kinin system

Activation of the kallikrein-kinin system and generation of BK is a consequence of FXII activators. FXII is activated by binding to negatively charged macromolecules, and these molecules can be separated into two groups: artificial substances such as silica-based materials (kaolin), negatively charged polymers (dextran sulfate, DXS), polyphenolic compounds (ellagic acid, EA) and endogenous substances including certain polymers, nucleotides, sulfatides, misfolded proteins, and some types of collagen or glycosaminoglycans (Cochrane and Griffin, 1982; Maas et al., 2008; Maas et al., 2011; Muller et al., 2009; Oschatz et al., 2011; van der Meijden et al., 2009).

Recent studies have shown that misfolded protein aggregates only result in initiation of the kallikrein-kinin system without activating the intrinsic pathway of coagulation (Maas et al., 2008). Bacteria can also activate the kallikrein-kinin system when binding to the bacterial surface (Nickel and Renne, 2012). In addition, a molecule derived from platelets called polyphosphates (polyP) activates the contact system, resulting in the activation of both the kallikrein-kinin system and the intrinsic pathway of coagulation (Muller et al., 2009).

It has been shown that PPK could be triggered on endothelial cells independently of FXII activation (Motta et al., 1998). Intracellular chaperon heat shock protein 90 (Joseph et al., 2002), the mitochondrial prolylcarboxypeptidase (Shariat-Madar et al., 2002) have been described to activate PPK in cell culture systems, however their *in vivo* relevance remain a topic of future research. Extracellular carbonic anhydrase has shown to activate PPK *in vivo* (Gao et al., 2007) but more studies are necessary to elucidate the mechanism.

1.2 THE PROTEINS OF THE KALLIKREIN-KININ SYSTEM

1.2.1 Factor XII (FXII)

FXII is a serine protease and circulates in plasma as a single chain zymogen with molecular weight of 80 kDa. FXII has a concentration of 30-35 µg/ml (0.37 µM) and is primarily produced by hepatocytes with a half-life of 50 to 70 hours. FXII consists of 596 amino acids that code for the zymogen (Marder et al., 2013). The gene is composed of 13 introns and 14 exons and consists of an N-terminal fibronectin domain type II (Fib II, encoded by exon 3 and 4), an epidermal-growth-factor-like domain (EGF I, exon 5), a fibronectin domain type I (Fib I, exon 6), a second epidermal-growth-factor-like domain (EGF II, exon 7), a kringle domain (exon 8 and 9), a proline-rich region (55 amino acids, exon 9) and the catalytic domain (exon 10-14) (Cool and MacGillivray, 1987) (**Figure 2**). FXIIa consists of a disulfide-bond (Cys_{340}-Cys_{367}) linked heavy chain (52 kDa) and a light chain (28 kDa). The latter one mediates the enzymatic activity through the catalytic domain, which contains the catalytic triad Asp_{442}, His_{393}, and Ser_{554} (Colman and Schmaier, 1997).

FXII binds to negatively charged surfaces via its heavy chain. The surface-binding site of FXII is not precisely known, but several regions appear to contribute including: the distal N-terminal end (residue 1-28), the Fib II, the Fib I, the EGF II, the kringle domain and the proline-rich region (Marder et al., 2013).

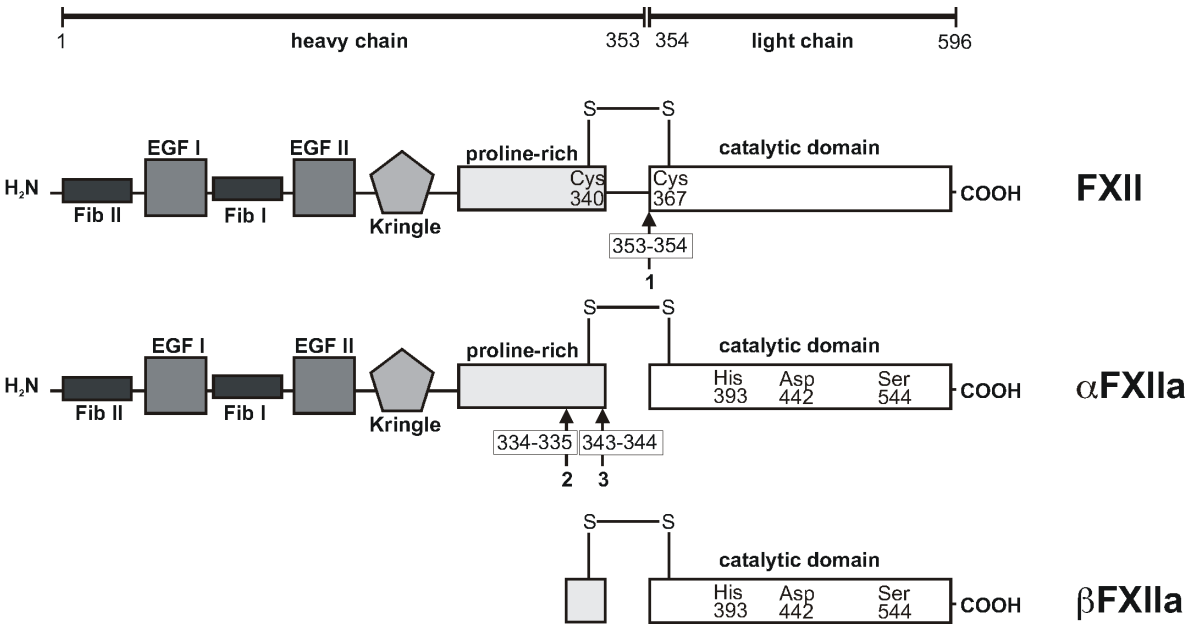

Figure 2. Domain structure of FXII forms. The heavy chain, which contains the surface-binding region of FXII, consists of five domains Fib II, EGF I, Fib I, EGF II, kringle domain and the proline-rich region. The light chain harbors the enzymatic site of the serine protease. The two chains are connected by a single disulfide-bond (Cys_{340} and Cys_{367}). Cleavage of the peptide bond Arg_{353}-Val_{354} (arrow 1) results in two-chain activated FXII (α-FXIIa). The catalytic triad of FXIIa consists of His_{393}, Asp_{442} and Ser_{544}. Further proteolysis of the peptide bonds Arg_{343}-Leu_{344} and Arg_{334}-Asn_{335} (arrows 2 and 3) by PK, results in activated FXII-fragment (β-FXIIa) (Bjorkqvist et al., 2014).

FXII zymogen is activated by limited proteolysis cleaving the bond connection Arg_{353}-Val_{354} and generating a two-chain molecule, FXIIa (α-FXIIa, **Figure 2**). FXII could be activated through two different principels. It is either activated when binding to negatively charged surfaces, which induce a conformational change (auto-activation) (Cochrane et al., 1973; Samuel et al., 1992) or activated by other proteases such as PK (hetero-activation). Zinc (Zn^{2+}) ions bind to FXIIa intermediate states and increase susceptibility for auto-activation by stabilizing conformations in the activation reaction (Bernardo et al., 1993). Further cleavages of the peptide bonds Arg_{334}-Asn_{335} and Arg_{343}-Leu_{344} of α-FXIIa by PK split the heavy from the light chain and result in activated FXII-fragment (β-FXIIa, M_w of 28-30 kDa, **Figure 2**). Although β-FXIIa retains its proteolytic ability to activate PPK it is no longer able to bind to negatively charged surfaces. β-FXIIa has lost its capacity to convert FXI to active FXI and could not promote thrombosis (Maas et al., 2011; Marder et al., 2013).

1.2.2 Plasma prekallikrein (PPK)

PPK is a glycoprotein that is predominantly synthesized in the liver but has also been found in the epithelial cells of the kidneys, adrenal glands and placenta. The calculated molecular weight for the protein product of the mRNA is 79.5 kDa and the apparent molecular weight is approximately 85-88 kDa, suggesting that it likely reflects two different glycosylation forms. PPK is secreted into the circulation as a single chain zymogen (Colman and Schmaier,

1997). PPK has a concentration of ~580 nM (50 µg/ml) in plasma and circulates mostly as a complex with HK (75-90%). PPK mRNA codes for a single chain polypeptide of 638 amino acid residues including an N-terminal signal peptide sequence of 19 residues. The mature PPK protein sequence of 619 residues is composed of five domains. The N-terminal portion consists of four tandem repeats of 84 to 85 residues each, called "apple" domains (designated A1, A2, A3 and A4). The four apple domains follow 248 residues that comprise the catalytic domain of the protein (amino acids 372 – 619, **Figure 3**). The PPK is stabilized by an arrangement of 18 disulfide bridges and is further modified by five N-linked complex-type oligosaccharides, two of which are located in the heavy chain portion and three on the light chain (Bjorkqvist et al., 2013a; Colman and Schmaier, 1997).

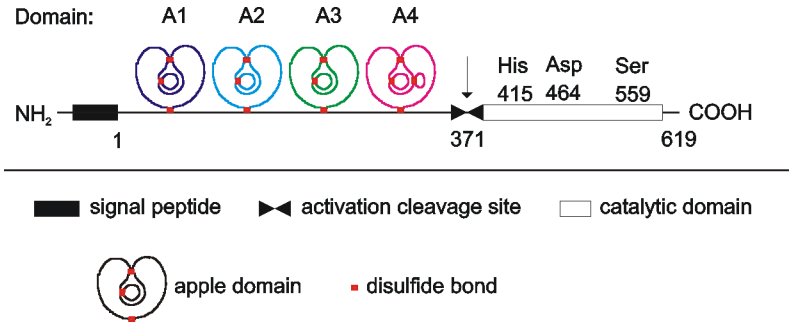

Figure 3: Domain structure of PPK. The primary translation product of 638 residues is cleaved at the NH_2-terminus by signal peptidase, which detaches the leader peptide consisting of 19 amino acid residues. The N-terminal portion consists of four tandem repeats, called "apple" domains (designated A1 to A4) and the catalytic domain. Arrows emphasize the cleavage sites for activating the protease. Activated FXII cleaves PPK at a single site to generate a heavy chain and a catalytic light chain connected by a single disulfide bridge. The relative positions of the residues of the catalytic triad are His_{415}, Asp_{464} and Ser_{559} (Bjorkqvist et al., 2013a).

PPK shares high homology with FXI and the proteases are 58% identical on the amino acid level, however they differ in their gross structures. PPK is a monomer whereas FXI is a homodimer. In contrast to PPK, FXI has a single free Cys residue in A4 (position 321) that forms a disulfide bond with the same residue in a second FXI polypeptide. Both PPK and FXI form tight 1:1 complexes with HK with K_Ds of 12 nM and 18 nM, respectively (Marder et al., 2013). In PPK, the apple domain A2 has been identified as the major binding segment for the HK domain 6, with domains A1 and A4 contributing to high- affinity binding (Herwald et al., 1993; Renne et al., 1999; Renne et al., 2002b). PPK is anchored to cell surfaces by binding to its substrate HK. PPK and PK bind to HK with similar affinity, indicating that proteolytic activation of PK does not interfere with the stability of the PPK/HK complex (Beaubien et al., 1991; Colman et al., 1985; Renne et al., 1999; Renne et al., 2002a).

The major physiological activator of PPK is FXIIa, which cleaves a single peptide bond (Arg_{371}-Ile_{372}) to generate a two-chain molecule with a heavy chain of 371 and a light chain of 248 residues held together by a disulfide bridge between Cys_{364} on A4 domain and Cys_{484} on the catalytic domain (Chung et al., 1986). Cleavage at Arg_{371}-Ile_{372} results in a conformational rearrangement of the light chain and generates the catalytic active serine protease.

1.2.3 C1 esterase inhibitor (C1INH)

C1INH is a member of the family of **ser**ine **p**roteinase **in**hibitors (serpins) and is a suicide inhibitor that inhibits proteases from the kallikrein-kinin system (FXIIa and PK) and also the complement proteases C1, the first complement of the complement system and MASP-1 and MASP-2 proteases in the lectin pathway. C1INH consists of 478 amino acids and contains an N-terminal domain (113 amino acids) and a serpin domain (365 amino acids). The domains are connected via two disulfide bonds (Bos et al., 2002). C1INH has a molecular weight of 105 kDa and is highly glycosylated (Zuraw and Curd, 1986). Serpins contain a reactive peptide loop, which is important for interaction with its target proteases. For the function of serpins, the most crucial amino acids in the loop are called P1–P1´ residues, and in C1INH it is position 444-445 (Bos et al., 2002). The protease cleaves the bond of P1–P1´ in the reactive center of the C1INH that induces a rearrangement of the protein and also forms a covalent binding between the P1 and the protease (such as FXIIa or PK) (Davis et al., 2008). The newly formed complex results in an inactive protease.

1.2.4 High molecular weight kininogen (HK)

HK is a 120 kDa α-globulin with a plasma concentration of 65-130 µg/ml. The heavy chain consists of domains 1 through 4 and domain 4 contains the BK region. The light chain consists of domain 5 and 6. The extreme C-terminal portion of domain 6 has the ability to form non-covalent complexes with either PPK/PK or FXI, and through domain 5 has the ability to interact with negatively charged surfaces with its histidine-rich region (Marder et al., 2013; Scott and Colman, 1980).

1.2.5 Bradykinin (BK)

Kininogens are the precursor of kinins, and one example of kinin is BK. BK is released by proteolysis of HK via PK and evokes inflammatory reactions. In the complex HK/PK, PK cleaves the HK peptide bond Arg_{371}-Ser_{372} (Colman and Schmaier, 1997) and this step produces a two-chain HK form where BK is still attached to HK heavy chain (Mori et al., 1981; Nishikawa et al., 1992). A subsequent second PK-mediated cleavage at Lys_{362}-Arg_{363} releases BK completely from HK. HK is then a two-chain protein that consists of a 65 kDa heavy chain and is linked via a disulfide bond to a 56 kDa light chain (Colman and Schmaier, 1997). The vasoactive proinflammatory BK has the amino acid sequence Arg_1-Pro_2-Pro_3-Gly_4-Phe_5-Ser_6-Pro_7-Phe_8-Arg_9. Free BK is rapidly degraded and by cleaving Arg at position 9 of BK the active metabolite desArg_9-BK is formed (Leeb-Lundberg et al., 2005).

1.3 SIGNALING PATHWAYS OF BK

1.3.1 B1- and B2- receptors (B1R and B2R)

BK acts through two distinct kinin receptors: B2-receptor (B2R) and B1-receptor (B1R). They are G protein-coupled receptors and are in most cells coupled to G_q and G_i (Leeb-Lundberg et al., 2005). These receptors mediate many pathophysiological functions of kinins, including edema formation, increased vascular permeability and capillary leakage, regulation of blood

pressure, hypotension, induction of fever, trans-endothelial cell migration and inflammation in different organs following injury (Leeb-Lundberg et al., 2005).

B1R is generally expressed at a low level and is largely up regulated by cytokines such as interleukin-1β (Kuhr et al., 2010; Leeb-Lundberg et al., 2005). Thus, B1R is exposed on cell surface in response to injury or inflammation. In contrast, B2R is constitutively expressed on various cell types, such as vascular smooth muscle cells, endothelial cells, and cardiomyocytes (Shukla et al., 2006). Kinins distinguish between these two receptors. BK primarily binds to B2R and des-Arg_9-BK (degradation product of BK) primarily binds to B1R (Bhoola et al., 1992; Leeb-Lundberg et al., 2005).

1.3.2 Signaling pathways

Kinin-binding to B1R or B2R increases intracellular calcium $[Ca^{2+}]_i$, which generates arachidonic acid release resulting in prostacyclin and nitric oxide (NO) production. These products diffuse from the endothelium to the smooth muscle, followed by further activation of secondary mediators resulting in relaxation, decreased blood pressure and increase in blood flow. BK stimulates B2R (G_q) causing the activation of phospholipase C (PLC) leading to the formation of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3) which increases $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ can activate multiple signaling cascades, including the phospholipase A2 pathway where arachidonic acid is released leading to production of prostacyclin (Leeb-Lundberg et al., 2005). $[Ca^{2+}]_i$ is also as a potent stimulator of endothelial nitric-oxide synthase (eNOS) and results in transient NO production. B1R interacts with G_q and G_i by desArg_9-BK stimulation in which it acts through many of the same signaling pathways as B2R. Even though B1R and B2R are coupled to similar cellular signaling pathways, there is a difference in pattern. In vascular smooth muscle cells, stimulation of B2R leads to transient production of NO, whereas B1R stimulation leads to high and sustained NO production (Kuhr et al., 2010; Leeb-Lundberg et al., 2005).

Additional downstream signaling effects of BK include activation of protein kinase C (PKC). PKC results in vasodilator-stimulated phosphoprotein (VASP)-mediated disassembly of cortical cytoskeletons (Benz et al., 2008) and of vascular endothelial (VE)-cadherin junctions in endothelial cells. Dependent on the cell type BK can induce proliferative and anti-proliferative responses and BK stimulation can also activate multiple transcription factors that are involved in tissue injury and inflammation (Leeb-Lundberg et al., 2005). NO and prostacyclin are thought to be major endothelium-derived vasodilators. However, all these factors have a role in BK-driven vasodilation and increased permeability (Shigematsu et al., 2002).

1.4 DEGRADATION OF BK

Non-receptor bound BK is rapidly degraded by multiple endo- and exopeptidases (kininases) (Skidgel, 1992) and has short half-life of <1 minute in plasma (Marder et al., 2013). One of the major kininases is kininase I (carboxypeptidase N) that removes the C-terminal Arg residue from BK. The resulting peptide des-Arg_9-BK looses its affinity for binding to B2R however mediates its effects via activation of B1R. The digestion of des-Arg_9-BK by kininase

II, also known as angiotensin converting-enzyme (ACE), results in release of the pentapeptide Arg-Pro-Pro-Gly-Phe and two dipeptides Ser6-Pro7 and Phe8-Arg9 (Bjorkqvist et al., 2013c; Sheikh and Kaplan, 1986). Other kinin-degrading enzymes are aminopeptidase P, dipeptidyl peptidase IV and neutral endopeptidase (Bjorkqvist et al., 2013c). Increased levels of BK in patients with various underlying diseases are difficult to monitor. There are, however, two conditions that are associated with high levels of BK namely hereditary angioedema (HAE, caused by a deficiency of C1INH) and severe infectious diseases (Cicardi et al., 2014; Frick et al., 2007).

1.5 MUTATION AND DEFICIENCY IN THE KALLIKREIN-KININ SYSTEM

1.5.1 Mutations in FXII

Defects in the FXII gene are known to cause loss of activity and/or deficiency of FXII. Mutations either reduce FXII plasma levels or affect FXII secretion (Schloesser et al., 1997). Some mutations reduce the enzymatic activity of FXIIa; these are located either in or close to the serine protease catalytic triad His_{393}-Asp_{442}–Ser_{544} (Schloesser et al., 1997). In 1955 the first patient, John Hageman, with FXII deficiency was described (Ratnoff and Margolius, 1955). Congenital deficiency in FXII (Hageman trait) is an autosomal recessive trait. Deficiency in FXII, similarly to the kallikrein-kinin system proteins (PPK and HK) deficiency, prolongs one of the most common diagnostic tests aPTT (a coagulation assay that depends on contact system activation) but the individuals with contact factors deficiency do not get spontaneous or injury-related increased bleeding. On the other hand, deficiency in other coagulation factors such as: tissue factor (Bugge et al., 1996), factors VII (Rosen et al., 1997), VIII and IX (Hemophilia A, B) results often in spontaneous bleedings (Colman et al., 1975; Ratnoff and Margolius, 1955; Sollo and Saleem, 1985). FXII knock out mice are not able to form stable thrombi and have suppressed BK levels, this suggest that FXII is important for thrombus formation and inflammation (Iwaki and Castellino, 2006; Kleinschnitz et al., 2006; Renne et al., 2005a).

One of many mutations in FXII is FXII Bern mutation, which results in a secreted but dysfunctional protein and PK-cleaved FXII Bern is not able to activate FXI and/or PPK (Wuillemin et al., 1991). Another mutation is FXII Washington DC which has a Cys_{571}-to-Ser_{571} substitution that leads to total loss of procoagulant activity *in vitro* (Miyata et al., 1989). FXII Locarno is also secreted but is dysfunctional due to an Arg_{353} substitution. The mutation alters the FXIIa/PK recognition site in FXII and abolishes zymogen activation by limited proteolysis (Hovinga et al., 1994). Mutations that are located outside the catalytic domain and result in FXII deficiency are in domain Fib II and EGF (Kanaji et al., 2001).

Beside non-functional FXII, there are other mutations resulting in edema formation. One of these mutations is in position Thr_{309}, which is located in the C-terminal proline-rich portion (residues 296-349) of the FXII heavy chain that mediates contact to other proteins and zymogen FXII surface binding (Citarella et al., 1996). This mutation is found in HAE III patients (described below). Moreover, a FXII gene deletion of 72 base pairs (starting at Lys_{305}) was identified in two unrelated families with edemas (Bork et al., 2014). Additionally, another novel FXII gene mutation in the proline-rich region of FXII is a duplication of 18 base

pairs. This duplication causes the repeated presence of 6 amino acids (p.298–303) (Kiss et al., 2013). So far there are only mutations in the proline-rich region that corresponds to edema.

1.5.2 Mutations in C1INH

Mutations in C1INH result in non-functional or non-secreted protein. Upon activation of FXII and PPK, mutated C1INH is dysfunctional or insufficient to inhibit these proteases and the kallikrein-kinin system becomes activated. Mutation in C1INH is related to the disease HAE (see below). C1INH is also the major inhibitor of C1 complex in the complement system, which is part of the immune system. Mutated C1INH allows for activation of the initial phase of the complement system (classical- and lectin- pathway) and results in a reduction of plasma levels of complementary component C4 (Bork, 2014).

1.6 GLYCOSYLATION

Most of all secreted proteins are glycosylated and changes in the glycosylation pattern could affect the properties in the protein. Glycosylation is a modification when glycans attach to proteins or lipids and is a form of post-translational modification. The addition of carbohydrates has several functions such as folding and stabilizing of the protein. There are five different types of glycosylations, and N-linked glycosylation is the most common one followed by O-linked. The difference between the two is that the N-linked type has a glycan attachment to a nitrogen atom of an Asn side-chain that is present as a part of Asn-X-Ser/Thr, where X could be any amino acid except for proline, while the O-linked type has a glycan attachment to oxygen of Thr or Ser side-chain. O-linked glycosylations do not need a specific consensus sequence to occur. Nevertheless, previous studies have shown that O-linked glycosylation of Ser or Thr is more common if Ser/Thr residues are present in clusters, or are located in areas rich in proline or alanine residues (Preston et al., 2013).

Congenital disorders affecting glycosylation are rare but almost 50 different have been identified (Preston et al., 2013). Mutations leading to the loss or gain of a single specific glycosylation site have been implicated in diseases. A mutation in C1INH (C1INH Ta, (Rosen et al., 1971) has a deletion of nucleotides encoding Lys_{251}. This amino acid deletion results in alteration of the sequence Asn-Lys-Ile to Asn-Ile-Thr thus creating a new potential N-linked glycosylation site and results in a non-functional C1INH (HAE II see below). The carbohydrate addition and/or the amino acid deletion result in altered conformation and function. Glycosylation leading to increased contact system activation is found for example in Lewis rats. They are more susceptible, than Buffalo and Fischer rats, to developing chronic intestinal and systemic inflammation by injection of group A streptococci (Sartor et al., 1996). Furthermore, in Lewis rats a B2R antagonist (Icatibant) attenuated experimental inflammatory arthritis (Uknis et al., 2001). The difference between the rat strains is a single nucleotide substitution from Ser_{511} to Asn_{511} in HK in Lewis rats, presumably due to an extra *N*-glycosylation and lack of an *O*-glycosylation. Lewis rats have an increased rate of HK cleavage by PK (Isordia-Salas et al., 2003). This shows that glycosylation has an important function regarding both increased and loss of protein function. The FXII protein contains 16.8% carbohydrate, including 4.2% hexose, 4.7% hexosamine, and 7.9% N-

acetylneuraminic acid (Fujikawa and Davie, 1981). The heavy chain contains a proline-rich region (33% of the sequence) and includes six of the eight carbohydrates chain linkages of FXII, all of which are O-linked (Cys_{276}-Arg_{334}) including Thr_{309} (McMullen and Fujikawa, 1985). Thr_{309} is mutated in HAE III (Cichon et al., 2006).

1.7 ALLERGY AND ANAPHYLAXIS

Allergy is a hypersensitive disorder of the immune system and anaphylaxis is the most severe form of allergic reactions. It is a serious allergic or hypersensitive reaction, which has a rapid onset and can sometimes be fatal. Anaphylactic reactions represent an immunologic response to certain allergens resulting in a sudden, systemic degranulation of mast cells (MC) and basophils (Brown et al., 2013; Galli and Tsai, 2012; Kemp and Lockey, 2002) (**Figure 4**). Some of the most common trigger factors are: food, medications, insect venoms and other allergens. Anaphylaxis affects as much as 1-15% of the population (Wood et al., 2014). The reaction typically manifests with a broad range of symptoms such as hypotension, vascular leakage, bronchial constriction, cardiac arrhythmias, as well as gastrointestinal and skin manifestations.

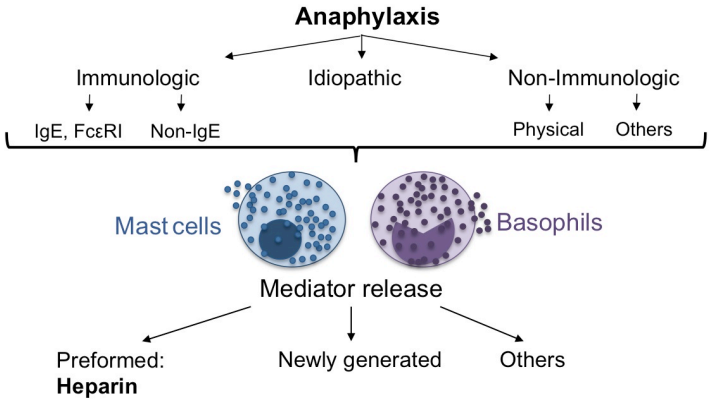

Figure 4. Activation of several cascades caused by anaphylaxis. Anaphylaxis is commonly mediated through immune IgE-dependent mechanisms. Additionally non-immunologic or idiopathic mechanisms exist that cumulatively lead to activation of MC and basophils with mediator release for example heparin, modified from (Sala-Cunill et al., 2014).

1.7.1 Mast cells (MC) released heparin

MC are part of the immune system and are localized in the connective tissue throughout the body. They are granulocytes, a type of leukocytes, containing granules in their cytoplasm that could be released upon stimuli and cause inflammation. MC can be activated and degranulate through different mechanisms: via toll-like receptors, which are important for direct pathogen recognition, through complement products such as C3a and C5a and via IgE antibody interaction, through the receptor FcεRI. The allergen/IgE complex initiates intracellular signaling in the MC with release mediators that are vasoactive, particularly histamine, serotonin and proteoglycans (Dawicki and Marshall, 2007; Marshall, 2004; Nilsson et al., 1996) (**Figure 4**). The mediators cause vasodilation, increase capillary leakage and can rapidly produce edema. Antihistamines (antagonists to histamine receptor) or corticosteroids are therapeutically used to treat allergic reactions and edema formation that are associated with aberrant MC activity (Theoharides and Kalogeromitros, 2006).

However, there are some patients that do not respond to these drugs.

MC secretory granules also contain highly sulfated proteoglycans, such as heparin. *In vivo* heparin is only synthesized in MC and contributes to the morphology and a storage capacity of their secretory granules. Heparin is a highly sulfated linear polysaccharide with repeating disaccharide units of 1-4-linked iduronic acid and glucosamine. Heparin is negatively charged and the average disaccharide unit has 2.7 sulfate groups (Capila and Linhardt, 2002).

1.8 ANGIOEDEMA

The definition of angioedema is edema localized in the subcutaneous and submucosal tissue, caused by local vasodilation and transient increase in vascular leakage due to release of vasoactive mediators. Urticaria is characterized by wheals, edema of the upper and mid-dermal skin layers, whereas angioedema is fluid leakage from deeper vessels (Bork, 2014). Urticaria and angioedema have many features in common; the edema is relatively short-lived in the skin and could involve other organs. Both belong to numerous disease entities and occur through various pathogenetic pathways. Quincke was the first who described angioedema as a separate entity in 1882 and named it angioneurotic edema that could still be referred to as Quincke edema (Quincke, 1882). Some years later Osler gave the first comprehensive description of angioedema where he called it “Hereditary angioneurotic edema” (Osler, 1888). The disease was later named HAE. Donaldson and Evens identified C1INH as the defective protein that is involved in the disease described by Osler (Donaldson and Evans, 1963). After that, angioedema research has been focused on revealing the patho-mechanism related to C1INH deficiency that eventually was shown to be a result of the release of BK (Cicardi et al., 2014).

Plasma levels of the peptide hormone BK are elevated during the swelling attacks in HAE patients. In acute episodes of HAE the mechanisms that result in increased vessel leakage is a result of excessive BK formation due to C1INH deficiency (Cugno et al., 2003; Joseph et al., 2008; Leeb-Lundberg et al., 2005; Schapira et al., 1983).

1.8.1 Hereditary angioedema (HAE)

Patients with HAE suffer from episodic swellings that can be fatal. The clinical symptoms include recurrent skin swelling, abdominal pain attacks, tongue swelling, and upper airway edema. The swelling typically lasts between 2–5 days (Davis, 2008; Zuraw, 2008). HAE is a rare inherited disease and has a prevalence of 1:10 000 to 1:100 000 and affects individuals from all races. Urticaria does not occur at any time in any of these patients (Cicardi and Agostoni, 1996; Longhurst and Cicardi, 2012). The increased vessel leakage is a result of excessive BK formation due to pathological activation of the FXII-driven plasma kallikrein-kinin system and plasma levels of BK are elevated during the swelling attacks (Cugno et al., 2003; Joseph et al., 2008; Schapira et al., 1983).

HAE is an autosomal dominant disorder and are present in different types. HAE I and II are affected by mutations in one of the two alleles in the *SERPING1* gene coding for the C1INH

protein, and there are almost 300 mutations registered, http://hae.enzim.hu/. HAE I is caused by mutations leading to deficiency of C1INH (quantitative defect) and the mutations are randomly distributed throughout the *SERPING*1 gene. The mutations are point mutations, large rearrangements, including partial deletions and duplications (Bowen et al., 2001; Kalmar et al., 2003; Stoppa-Lyonnet et al., 1987). The mutations in type II result in a secreted C1INH but with a misfolded formation causing a reduced enzymatic activity (functional defect). Most mutations in HAE II are clustered in exon 8 of the *SERPING1* gene, which encodes the active center (P1–P1´ residues) or hinge region of C1INH protein (Donaldson and Bissler, 1992). Despite having one normal allele, HAE I and II patients have lower than 50% of the physiological levels of C1INH. This is probably due to faster consumption of C1INH (Quastel et al., 1983). HAE has a high prevalence of *de novo* mutations, which account for around 25% of the cases (Pappalardo et al., 2000; Tosi, 1998).

1.8.2 Hereditary angioedema type III (HAE III)

HAE III does not differ clinically from HAE I or II, however, the patients have normal C1INH levels and functions (Bork et al., 2000). This type mostly affects women and was originally associated with estrogen intake. However a few male patients have been identified with HAE III questioning elevated estrogen levels to be causative for the disease (Charignon et al., 2014; Marcos et al., 2012; Riedl, 2013). Genome wide linkage analysis revealed a single point mutation in FXII. Two missense mutations at position Thr309 (1032C→A: Thr exchange to Lys, FXII_Thr309Lys, or 1032C→G: Thr exchange to Arg, FXII_Thr309Lys) have been identified (Dewald and Bork, 2006), which leads to increased FXII activity (Cichon et al., 2006). An *in vitro* study has shown that FXII_Thr309Lys mutation is a gain-of-function mutation, and that it has increased enzymatic activity however the FXII plasma levels are normal (Cichon et al., 2006). HAE III is also named FXII-HAE, however to be consistent with the original nomenclature (Bork et al., 2000; Cichon et al., 2006) the subtype of HAE with normal C1INH (Cicardi et al., 2014) that is associated with FXII mutations is here referred to as HAE III.

Bork and coworkers reported trigger factors that resulted in edema in 35 patients with HAE III. The most common ones included: acute trauma or physical pressure on the affected area, and after dental procedures. Some patients also reported emotional stress and ingestion of various spices and herbs as suspected triggers (Bork et al., 2009b; Riedl, 2013). The swellings in HAE III patients are highly variable regarding triggering factors, severity, frequency, and localization but are mainly localized to the face or the extremities. Each episode lasts between 2-5 days. In some of the women the clinical symptoms were initiated by oral contraceptives, hormonal replacement therapy or pregnancy, but others were not affected by these conditions (Cicardi et al., 2014). HAE III patients should avoid triggers such as exogenous estrogen therapy and ACE inhibitors (described below) (Riedl, 2013).

Diagnosing HAE III is very challenging. HAE III is an autosomal dominant disease with low penetrance: asymptomatic carriers are >90% in male gender and around 40% in female (Bork et al., 2007; Marcos et al., 2012; Riedl, 2013; Vitrat-Hincky et al., 2010). Most HAE III patients originate from Europe mainly from Germany, Spain and France but patients have

also been reported from Canada, UK, Australia, Hungary and Morocco (Baeza et al., 2011; Bork et al., 2014). The criteria for HAE III are listed in table 1.

Table 1: Recommended diagnostic criteria for HAE III reproduced from Zuraw et al. (Zuraw et al., 2012)

• A history of recurrent angioedema in the absence of concomitant hives or concomitant use of a medication known to cause angioedema
• Documented normal or near normal C4, C1INH antigen, and C1INH function
• Plus one of the following: 1. Demonstration of a FXII mutation associated with the disease 2. A positive family history of angioedema and documented evidence of lack of efficacy of chronic high-dose antihistamine therapy (cetirizine at 40 mg/day or the equivalent, for at least 1 month and an interval expected to be associated with three or more attacks of angioedema)

There are no controlled investigations of acute or preventative treatments making the medical management challenging. However, there has been significant progress in the development of therapeutic targets for HAE I and II over the last decade. The novel drugs are recombinant or plasma derived C1INH (Zuraw et al., 2010b), plasma kallikrein inhibition (Ecallantide, DX-88) (Cicardi et al., 2010b) and B2R antagonists (Icatibant) (Cicardi et al., 2010a). Replacement therapy with intravenous infusions of C1INH concentrate shortened the length of and frequency of swelling attacks in HAE I and II patients (Zuraw et al., 2010b). In contrast to C1INH infusions, the drugs Icatibant and Ecallantide can be injected subcutaneously in an acute attack in HAE I and II patients, resulting in a relief the symptoms (Cicardi et al., 2010a; Cicardi et al., 2010b). In comparison to established therapies in HAE type I and II not much is known about treatment of type III but according to published case reports inhibition of kallikrein-kinin system and BK formation have shown to be beneficial. Although published experience is limited, the B2R antagonist Icatibant has been reported to be efficient for the treatment of acute symptoms in several patients (Boccon-Gibod and Bouillet, 2012; Bouillet et al., 2009; Marcos et al., 2012). Ecallantide has also been reported successful in treating angioedema attacks in HAE III patients (Cronin and Maples, 2012). Interestingly, despite normal measured C1INH quantity and function, the use of plasma-derived human C1INH during acute attacks has been reported effective in some cases, although reports of inefficacy also have been described. (Bork et al., 2009b; Bouillet et al., 2007; Marcos et al., 2012; Vitrat-Hincky et al., 2010). For long-term prophylaxis, progesterone, danazol and tranexamic acid have been used to treat HAE III with variable success however the steroid drugs have significant side effects (Bork et al., 2000; Herrmann et al., 2004; Saule et al., 2013; Vitrat-Hincky et al., 2010).

1.8.3 Other forms of angioedema

There are other forms of angioedema which are: Idiopathic histaminergic acquired angioedema (IH-AAE), Idiopathic non-histaminergic acquired angioedema (InH-AAE), Acquired angioedema related to angiotensin converting enzyme inhibitors (ACEI-AAE), Acquired angioedema with C1 inhibitor deficiency (C1-INH-AAE) and Hereditary angioedema with normal C1 inhibitor and of unknown origin (U-HAE) (Cicardi et al., 2014).

IH-AAE is an acquired disease with no identified cause. The patients present angioedema

that can be defined as histaminergic and these patients respond to high dose of antihistamines used prophylactically on a daily basis.

InH-AAE is a type of angioedema, which is a nonhereditary form and could not be treated with antihistamines. BK is probably involved in InH-AAE, however the experimental evidence is still limited (Cicardi et al., 2014).

ACE is one of the major degradation proteins of BK. ACE inhibitor (a drug against hypertension) results in elevated plasma levels of BK, which is the consequence in ACEI-AAE. Analysis of large cohort of patients administered with ACE inhibitor shows that angioedema occur in <0.5%. However, because of the large number of people taking these medications, ACE inhibitors are the leading causes of drug-induced angioedema. It has also been shown that genomic and plasma variability of the degradation proteins interferes with the BK catabolism and results in angioedema (Cicardi et al., 2014).

C1-INH-AAE is an acquired disease, which has no mutations in C1INH or any family history of angioedema. Studies from patient plasma indicate consumption of C1INH, some of the complement components (C1 and C4) and activation of the kallikrein-kinin system. C1-INH-AAE can be associated with C1INH deficiency caused by autoantibodies that neutralizing C1INH function. (Cicardi et al., 2014)

U-HAE patients have no mutations in C1INH or FXII genes and no other genetic defects that have be identified. U-HAE is inherited and during attacks they do not respond to corticosteroids or antihistamines (Cicardi et al., 2014).

1.9 INHIBITORS OF THE KALLIKREIN-KININ SYSTEM

1.9.1 FXII inhibitors

The recombinant FXIIa inhibitor rHA-infestin-4 is based on the fourth domain of the nonclassic Kazal-type serine protease inhibitor from the midgut of the insect *Triatoma infestans* fused to human albumin (Hagedorn et al., 2010). Intravenous infusion of the inhibitor prior to the challenge protects mice and rats from $FeCl_3$-induced arterial thrombus formation, ischemic stroke (transient middle cerebral artery occlusion model) (Hagedorn et al., 2010), silent brain ischemia (Chen et al., 2012) and lethal pulmonary embolism (Muller et al., 2009). At high concentrations rHA-infestin-4 also inhibits plasmin and modestly factor Xa (Xu et al., 2014).

Another recombinant inhibitor Ixodes ricinus Contact Phase Inhibitor (Ir-CPI), a Kunitz-type protein from the salivary gland of *Ixodes ricinus* inhibits FXIIa, PK, and FXIa and provides protection from venous and arterial thrombus formation in mice (Decrem et al., 2009).

In murine models H-D-Pro-Phe-Arg-chloromethylketone (PCK) irreversibly inhibits the amidolytic activity of FXIIa and PK, and provided similar protection from ischemic stroke as in *F12*$^{-/-}$ without an increase in therapy-associated bleeding (Kleinschnitz et al., 2006). Pretreatment with PCK markedly reduced cerebral infarction in the transient middle cerebral artery occlusion model (Kleinschnitz et al., 2006). Furthermore, PCK protected mice from platelet polyP-induced edema formation (Muller et al., 2009).

The specific FXIIa inhibitor Corn trypsin inhibitor (CTI) attenuated the prothrombotic properties of catheters in rabbits (Yau et al., 2012).

Other protein inhibitors, such as cabbage seed protease inhibitor (Carter et al., 1990), pumpkin seed inhibitor CMTI-V (Hojima et al., 1982) and Ecotin (Ulmer et al., 1995) block FXIIa in plasma, but also inhibit other proteases including thrombin, activated factor Xa, or XI, PK and plasmin.

1.9.2 FXII inhibitory antibodies

The monoclonal anti-FXII zymogen antibody 15H8 reduced fibrin formation and platelet accumulation in a collagen-coated vascular graft in baboons (Matafonov et al., 2014) and is directed to FXII zymogen. However, the antibody binding characteristics, its epitope and mechanisms of inhibition are not currently known.

Another antibody is 3F7, which is a recombinant fully human antibody that binds with high affinity (K_D=6.2 ± 0.2 and 4.0 ± 0.1 nM for human and rabbit FXIIa, respectively) into the enzymatic pocket of FXIIa and specifically inhibits FXIIa activity and FXIIa-driven coagulation/inflammation in human, mouse and rabbit plasma (Larsson et al., 2014).

1.9.3 PK inhibitors

The drug Aprotinin (Trasylol) is an antifibrinolytic agent that was primarily used in complex and/or redo cardiac surgery as an addition to decrease postoperative bleeding and to reduce organ damage caused by hypotension. Aprotinin, a bovine pancreatic trypsin inhibitor, is a small protein, which inhibits several serine proteases including PK. However in 2008 the safety of Aprotinin was question because of a clinical trial showed a higher rate of death in patients receiving Aprotinin compared to other antifibrinolytic agents. The data in the clinical trail (Blood Conservation Using Antifibrinolytics in a Randomized Trial, BART) were not reproduced by independent trials and were only slightly above the statistical significance level but the drug was withdrawn from the market (Fergusson et al., 2008).

Ecallantide (DX-88, Kalibitor) is a novel recombinant PK inhibitor identified in a phage display-based screen containing the first Kunitz domain of human tissue factor pathway inhibitor variants. Ecallantide is a recombinant 60 amino acids protein that was selected on the basis of its affinity and specificity for PK (Williams and Baird, 2003). In a double-blind trail with HAE patients presenting with acute attacks Ecallantide was reported to give a significantly better outcome than placebo with regards to rapid relief and severity of symptoms. Ecallantide is injected subcutaneously and is now a drug for HAE patients used in acute attacks and is only approved by FDA (Cicardi et al., 2010b).

1.9.4 PK inhibitory antibody

DX-2930 was developed using phage display to select a potent and highly specific human antibody inhibitor of PK. Antibody therapeutics givens the potential for target specificity and they have a long serum half-life. Moreover, preclinical studies reveal DX-2930 to exhibit a

prolonged half-life in circulation (16-21 days), and that this translates into a prolonged capacity to inhibit the kallikrein-kinin system. Data support that DX-2930 can be used as a prophylactic inhibitor of PK in kallikrein-kinin system-mediated edema *in vivo* (Kenniston et al., 2014). DX-2930 has just completed a phase I study showing no toxicity symptoms in humans. The pharmacokinetic and pharmacodynamic data showed evidence for a long-acting biological effect and could be relevant to long-term prophylaxis for HAE patients (Chyung et al., 2014).

1.9.5 B2R antagonist

Icatibant (Firazyr) is a potent, specific and selective competitive B2R antagonist that is similar in structure to BK. It is short acting, and is composed of 10 amino acids, where five are synthetic and are resistant to fast degradation. In clinical studies administration of Icatibant was associated with symptomatic relief and is now used in acute attacks in HAE (Cicardi et al., 2010a; Cruden and Newby, 2008).

1.9.6 Recombinant and plasma derived C1INH

There are three different C1INH concentrates on the marked; recombinant C1INH (Rhucin), plasma derived C1INH (Berinert) and nano-filtered plasma derived C1INH (Cinryze) and some of these have been approved in Europe for decades but recently from the FDA. Intravenous infusion of recombinant or plasma derived C1INH concentrate shortens the extent and duration of acute swelling attacks. Additionally when used for prophylaxis the concentrate reduced the frequency of acute attacks (Zuraw et al., 2010a).

2 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the role of the contact system in inflammatory diseases with a focus on the mechanism and therapy of Hereditary angioedema type III *in vitro* and *in vivo*. More specifically, the studies can be divided into the following parts:

I) Establishment of methods for analyzing FXII-driven kallikrein-kinin activation

II) Activation and role of the plasma contact system in mast cell-mediated anaphylactic reactions

III) The mechanism and therapy of hereditary angioedema type III

3 EXPERIMENTAL PROCEDURES

3.1 *IN VITRO* METHODOLOGY

3.1.1 Kallikrein-kinin activation assay (paper I, II, III, IV and V)

Plasma was supplemented with increasing concentrations of the following triggers: dextran sulfate 500 000 Da (DXS, 1 pg/ml-100 µg/ml in 75 mM Tris pH 7.4, 10 µM $ZnCl_2$), MC-derived heparins (1-1000 µg/ml) Ellagic acid (EA, 1.5 µg/ml), long- or short- chain polyphosphates (LC- SC- polyP, 10 µg/ml). Following incubation for 30 min at 37°C, the reaction was stopped by addition of Laemmli sample buffer, and 0.2-0.25 µl plasma was separated by polyacrylamide gel and analyzed by Western blotting using primary antibodies against FXII, PPK, HK, C1INH and FXI and horseradish peroxidase–coupled secondary antibodies. For some experiments FXII-deficient plasma was used.

3.1.2 Chromogenic assay (paper I, IV and V)

The enzymatic activity of FXIIa was photometrically measured during dextran, low molecular weight dextran sulfate 5 000 Da, DXS, EA, heparin, LC and PS polyP-induced activation using the chromogenic substrate S-2302 (D-Pro-Phe-Arg-p-nitroanilide, 1 mM) at an absorbance wavelength of 405 nm. The chromogenic substrate contains the peptide-*p*-nitroaniline and upon hydrolysis it is released and changes in color. The change in absorbance (ΔA/min) is directly proportional to the enzymatic activity.

3.1.3 Coagulation assay (paper II, III, IV and V)

The aPTT was measured on a Blood Coagulation System (BCS) or on a coagulometer by adding 50 µl of plasma samples, 50 µl Pathromtin SL, followed by incubation for 120 s at 37°C. Subsequently, 50 µl of a calcium chloride solution (25 mM) was added to start the reaction and the time was recorded.

3.1.4 Expression of Thr309Lys- and Thr309Arg-mutated and wild-type FXII (paper IV)

To generate FXII_Thr309Lys and FXII_Thr309Arg Site-directed mutagenesis was performed on human FXII cDNA (MIM ID: 610619) with the following primer:

5`-CCGAAGCCTCAGCCCAAGACCCGGACCCCGCCTCAG-3´ and

5`-CCGAAGCCTCAGCCCAGGACCCGGACCCCGCCTCAG-3´

resulting in the exchange of C at position 1032 to A (encoding for FXII_Thr309Lys) or G (encoding for FXII_Thr309Arg), in a pcDNA3 vector. Transient transfection of wild-type FXII and the mutants into HEK293 (human embryonic kidney) cells was done using Lipofectamine 2000 according to the manufacturer's instructions. The supernatant was collected after 48 h and concentrated with Amicon Ultra centrifugal filters (30K).

3.1.5 Inducible FXII_Thr309Lys expression in cells (paper IV)

FXII_Thr309Lys was expressed using the reverse tetracycline-induced expression system ("Tet-on" System). For expression using Tet-on, a transactivator plasmid and a responder plasmid is needed. The transactivator plasmid expresses the reverse tetracycline-controlled transactivator (rtTA) under control of a CMV promotor and the responder plasmid expresses pTRE-Tight_FXII_Thr309Lys. In the presence of doxycycline (an analog to tetracycline) the transactivator could bind to the responder plasmid's tetracycline-responsive element and allows for the expression of the mutated FXII (Thr309Lys). The transactivator plasmid and FXII_Thr309Lys were transfected into HEK293 cells using Lipofectamine 2000 in the presence of different concentrations of doxycycline (6.4-0.1 µg/ml). The supernatant was collected 48 h after transfection and concentrated with Amicon Ultra centrifugal filters (30K) and Western blotting was performed.

3.1.6 Generation of HAE III transgenic mice using Tet-off system (paper IV)

HAE III transgene mice were generated using a transgenic mouse system with liver-specific inducible expression using the tetracycline-inducible expression system (Tet-Off system, **Figure 5 A**). This binary transgenic system involves two transgenic mice. The first component is a mouse that expresses the tetracycline-controlled transactivator (tTA) under control of LAP promotor (Kistner et al., 1996) on a C57BL/6 background and was purchased from Jackson Laboratories (Maine, USA Tg(Cebpb-tTA)5Bjd). The second component is the responder mouse line that expresses mutant FXII (Thr309Lys) under the control of a tetracycline-responsive-element-driven minimal CMV promoter. The responder mouse was generated via DNA microinjection of fertilized oocytes with the pTRE-Tight_FXII_Thr309Lys plasmid after linearization with *Bsp*HI (5´) and *Bsp*LU11I (3`). Genotyping of all mice was performed by PCR using the forward primer 5´cgtatgtcgaggtaggcgtg 3´ and reverse primer 5´cacaaatgtacccacaagggccggc 3´. Crossing of the two lines generates a mouse line that expresses mutated FXII specifically in hepatocytes (where the LAP promotor is active) and only in the absence of tetracycline. We have bred the double-transgenic mice with *F12*$^{-/-}$ mice (**Figure 5 B**). The result is an animal with inducible mutant human FXII expression and 50% of endogenous FXII levels, identical to the FXII state in HAE type III patients. Doxycycline diet was supplemented (final concentration 100 mg/kg) to the standard rodent chow. Doxycycline was removed from mice 10 days before experiments were done.

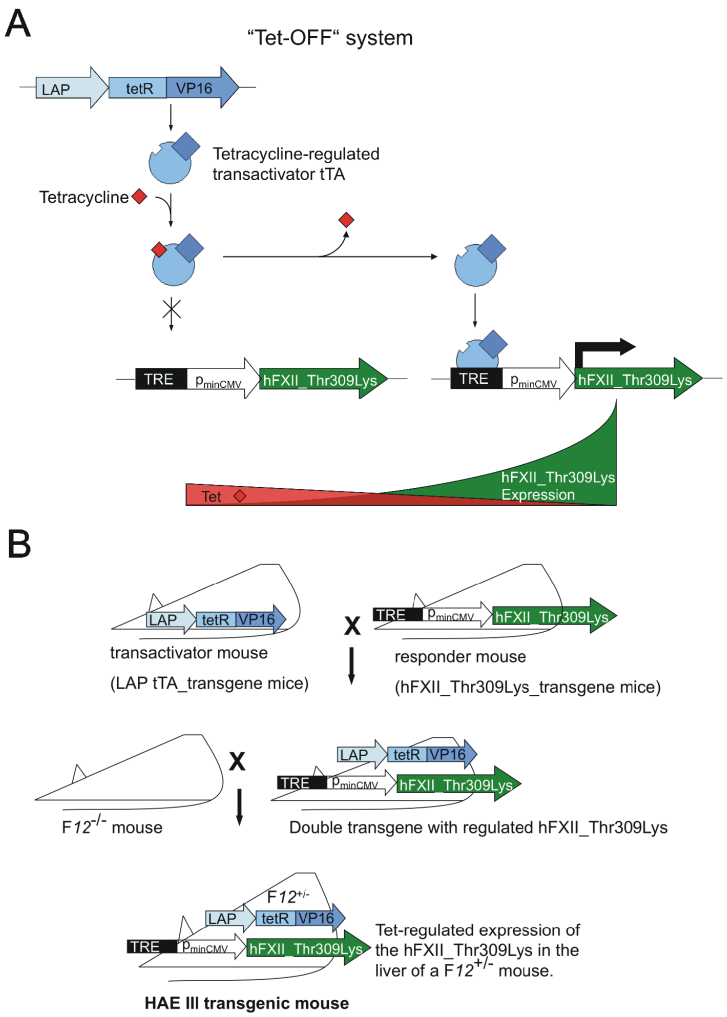

Figure 5. Liver specific inducible expression of hFXII_Thr309Lys by using the regulated Tet-off system. (A) The LAP promoter restricts expression of the regulatory protein tTA to the liver. In the absence of tetracycline, tTA binds to TRE-sequence and activates the expression of hFXII_Thr309Lys. (B) Generation of a mouse model for HAE III transgenic mice. LAP-tTA mouse expressing the tTA specifically in the liver was breed with the responder mouse to generate a double transgenic mouse with regulated hFXII_Thr309Lys expression. *F12*$^{-/-}$ mouse was bred with the double transgenic mouse and the pups were heterozygous for *F12* (*F12*$^{+/-}$), LAP-tTA and hFXII_Thr309Lys transgenic resulting in the HAE III transgenic mouse.

3.2 *IN VIVO* METHODOLOGY

3.2.1 Skin vascular leakage model (paper I, II and IV)

Vascular leakage in real time was analyzed using intravital laser scanning microscopy (Eriksson et al., 2001) and was described in (Oschatz et al., 2011) and (Bjorkqvist et al., 2013b). Mice were anesthetized by intraperitoneal injection of Avertin (2,2,2-tribromoethanol and 2-methyl- 2-butanol, diluted in 0.9% NaCl solution, 33 mg/ml, 15 µl/g body weight, bw). 5 ng/g bw FITC-dextran (150 kDa) was injected into the retro-orbital as tracer. In some of the *F12*$^{-/-}$ mice were pre-administered recombinant FXII, FXII_Thr309Lys or FXII_Thr309Arg (5 mg/kg bw). A ventral skin window was excised and the skin was fixed. The experiments started after topical application of DXS (20 µl of a 1 mg/ml solution) and after 1 min the drop was removed from skin. Extravasation of tracer from microvessels (35–60 µm diameter) was visualized using a Nikon Eclipse E600 microscope equipped with a C1 confocal scanning head and a 10x objective (**Figure 6**). Leakage was assessed for 5 min before and 20 min after topical application of DXS. Tissue scans were performed at 1 min intervals. Images

were analyzed by EZ-C1 2.10 software (Nikon) and the intensity of extravasated tracer was quantified using ImageJ 1.34 NIH software.

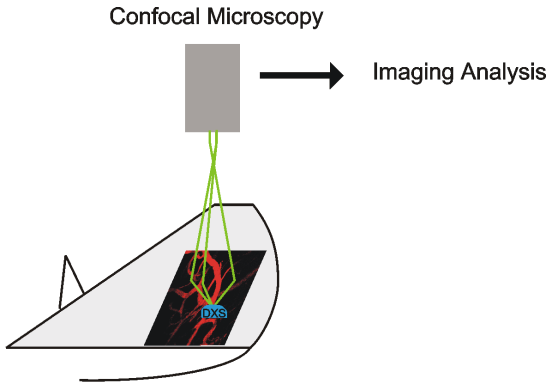

Figure 6 Scheme of our intravital laser-scanning microscope. To analyze microvascular leakage in murine skin microvessels in real time a Nikon Eclipse E600 microscope was used. The microscope was equipped with a C1 laser scanning head and a 10 x objective and was used to monitor extravasation of FITC-labeled tracer following by topical application of DXS on a skin lobe (Bjorkqvist et al., 2013b).

3.2.2 Miles edema model (paper II and IV)

Mice were anesthetized by intraperitoneal injection of Avertin (33 mg/ml, 15 µl/g bw). Sterile-filtered 0.25 % Evan's blue dissolved in sterile saline (0.9 % NaCl) 10 µl/g bw was injected retro-orbitally. Dorsal skin edema was induced 5 min later by intradermal injections, using a 23 G needle. The needle tip was bent to a 45 ° angle and 50 µl of DXS, NaCl (negative control) or BK (positive control, 100 µM) was injected into the skin (Miles and Miles, 1952). After 30 min, the mice were sacrificed and the skins were removed and photographed. All edema sites were excised and cut into smaller pieces. The Evan's blue dye was extracted by incubation in 700 µl N,N-dimethylformamide overnight at 55°C (Donelan et al., 2006). The following day, the Evan's blue absorbance from individual skin samples was quantified photometrically at wavelength of 620 nm. Subtracting the background signal normalized the concentration of Evan's blue in the sample. All samples was dividing with the value of NaCl-induced signal in WT mice. The method is described in (Oschatz et al., 2011).

To induce passive cutaneous anaphylaxis, mice were injected intradermally with anti-dinitrophenyl (DNP) IgE antibody (1.5 µg/ml), histamine (100 µM) or 50 µl sterile 0.9% NaCl in the dorsal skin. After 20 hours, the mice were challenged by retro-orbital injection of DNP-human serum albumin (7.5 mg/kg bw, DNP-HSA) together with 10 µl/g bw of 0.25% Evan's Blue. Tracer extravasation was measured as described above (Oschatz et al., 2011).

3.2.3 $FeCl_3$-induced arterial thrombosis model (paper IV)

Mice were anesthetized by intraperitoneal injection of Avertin (33 mg/ml, 15 µl/g bw). A midline incision was made from the sternum to the mouth and the fat glands were separated to reach the trachea. Fat was removed to prepare a clean carotid artery. The probe was inserted around the carotid artery. Topical application of a filter paper (1-2 mm) saturated with 5% $FeCl_3$ for 3 min, arterioles were monitored for 40 min or until complete occlusion (blood flow stopped for >1 min) occurred (Renne et al., 2005a).

4 RESULTS AND DISCUSSION

4.1 ESTABLISHMENT OF METHODS FOR ANALYZING FXII-DRIVEN KALLIKREIN-KININ ACTIVATION (PAPER I)

Compounds containing negatively charged surfaces initiate auto-activation of FXII, which further activates the kallikrein-kinin system. Recent studies indicate that polymer-induced auto-activation of FXII has a role in allergy-related vascular leakage and angioedema (Muller et al., 2009). In the present study, we characterize the effects of the negatively charged synthetic polysaccharide dextran sulfate (DXS) in human plasma and in rodent models.

Human plasma was supplemented with DXS or dextran (1 µg/ml). The production of FXIIa was measured, DXS with an average mass of 500 000 Da (equivalent with a chain length of >2000 glucose monomers) exceeded the threshold to initiate FXII activity. While low molecular weight DXS (average molecular mass of 5000 Da, <50 chain length) and dextran (average molecular mass of 200,000 Da, >1000 chain length), with identical polysaccharide backbones, did not activate FXII (**Figure 7 A**). DXS locally assembles FXII molecules with about 165–192 FXII binding sites on a single chain (Samuel et al., 1992). Surface-assisted local accumulation of kallikrein-kinin proteins is a crucial requirement for efficient BK formation on endothelial cells (Renne et al., 2000). Dextran is not negatively charged and therefore does not have the capacity to induce auto-activation of FXII. Additionally, low molecular weight DXS is negatively charged but does not activate FXII, indicating that a sufficient length of the polysaccharide molecule is also requirement for FXII activation.

DXS (500 000 Da) was used in further experiments and we tested whether it induced HK cleavage in human plasma. Small amounts of DXS (1 µg/ml) triggered complete HK cleavage. To analyze the mechanism and kinetic of DXS-driven BK formation, we incubated plasma with 0.1 and 1 µg/ml DXS and followed the cleavage of zymogens FXII, PPK and HK proteolysis (**Figure 7 B and C**). FXII, PPK and HK were completely activated after 2 min by 1 µg/ml DXS, supporting the FXIIa-initiated and PK-mediated HK cleavage cascade.

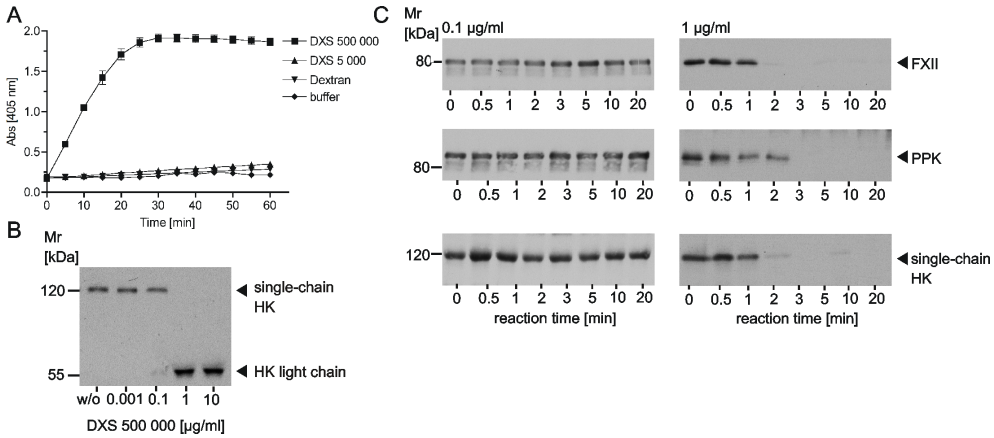

Figure 7: DXS-initiated kallikrein-kinin activation in plasma. (A) Human plasma was supplemented with 10 µg/ml of DXS 500 000 Da, low molecular weight DXS 5 000 Da, dextran 200 000 Da and tris-buffer and the kinetics of FXII activation were analyzed. FXII activity was measured using hydrolysis of the chromogenic FXIIa substrate S-2302 (B) Concentration-dependence of DXS-initiated HK cleavage. Human plasma was incubated with increasing concentrations of DXS incubated for 30 min at 37°C and analyzed by Western blotting using polyclonal HK antibody. (C) Kinetics of DXS-triggered contact activation. Human plasma was incubated with 0.1 µg/ml (left) and 1 µg/ml (right) DXS. At the indicated time-points aliquots were taken and analyzed by Western blotting using antibodies to FXII (upper panel), PPK (second panel), single-chain HK (uncleaved, lower panel).

Studies of C1INH deficient mice (HAE I) have demonstrated that vascular leakage is formed due to kallikrein-kinin activation (Han et al., 2002). Genetic ablation of C1INH results in excessive BK production and excess BK signaling, which increases vascular permeability in humans (Cugno et al., 1997) and mice (Han et al., 2002). In contrast, in mice with combined deficiency in C1INH and B2R the vascular leakage was normal (Han et al., 2002). Comprehensive studies have identified BK as the principal mediator of vascular leakage in HAE-related swelling attacks in patients (Cicardi et al., 2014). We established an intravital confocal scanning microscopy technique for analyzing DXS-mediated leakage in mice. Macromolecular FITC-dextran (150 kDa) was intravenously injected as a tracer to visualize microvascular fluid efflux from skin microvessels (**Figure 6**). FITC-dextran is a tracer for plasma albumin extravasation as occurs during attacks of HAE or in allergic reactions. No basal tracer extravasation was detectable for 5 min prior to stimulation, indicating intact vessel barriers. Topical DXS application (time point 0 min) provoked leakage from capillaries in wild-type (WT) mice within 20 min (**Figure 8**). We examined DXS as a possible trigger for edema formation in C1INH$^{-/-}$ mice. DXS provoked excessive vascular leakage in C1INH$^{-/-}$ compared to WT mice shown by intravital microscopy. This indicated that our DXS-triggered vascular leakage model is sensitive to genetic deficiencies associated with development of HAE attacks.

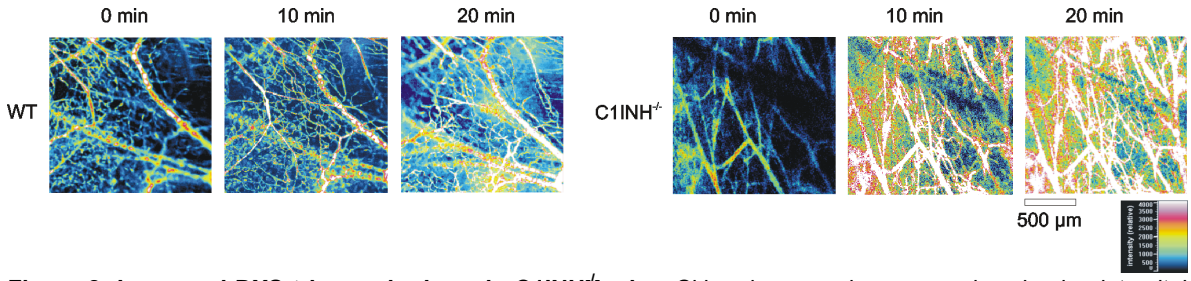

Figure 8: Increased DXS triggered edema in C1INH$^{-/-}$ mice. Skin microvessels were analyzed using intravital laser scanning microscopy in real time. Extravasation of FITC-labeled tracer after topical application of DXS (1 mg/ml) on a skin lobe of WT and C1INH$^{-/-}$ mice. Laser scanning images were taken at 10 and 20 min after stimulation at time point 0 min and are shown in false colors. White represents the highest tracer intensity and the scale is shown at the lower right. The scale bar represents 500 µm. Representative of a series of n = 5 mice per genotype is shown.

In conclusion, we show that DXS is a potential trigger for *in vitro* and *in vivo* studies for FXII-initiated BK formation with implications for vascular permeability.

4.2 ACTIVATION AND THE ROLE OF THE PLASMA CONTACT SYSTEM IN MC-MEDIATED ANAPHYLACTIC REACTIONS (PAPER II AND III)

The kallikrein-kinin system has been suggested to contribute to asthmatic disease for decades and BK levels are elevated in allergic airway disease (Proud and Kaplan, 1988) and allergic rhinitis (Turner et al., 2001). However, due to lack of technology for reliable measurement of kallikrein-kinin system activation *in vivo*, conclusive evidence for its involvement was missing. We analyzed MC derived heparin and their role in: the FXII-driven contact system, vascular leakage and anaphylactic reactions in human plasma samples and in genetically modified mice.

IgE/antigen-activated MC release the negatively charged polysaccharide heparin that is exclusively found in MC granules (Humphries et al., 1999). Human plasma was incubated with heparin and its function as a FXII contact activator and initiator of the contact system (both kallikrein-kinin system and intrinsic pathway of coagulation) was analyzed (**Figure 9**). Efficient activation depends on the charge density of the negatively charged polysaccharide (Bjorkqvist et al., 2013b). Heparin and DXS have almost the same amount of sulfate residues per disaccharide (2.7 and 2.3, respectively). In plasma, heparin activates the FXII-driven kallikrein-kinin system but does not initiate FXI activation indicating that the intrinsic pathway of coagulation is not triggered.

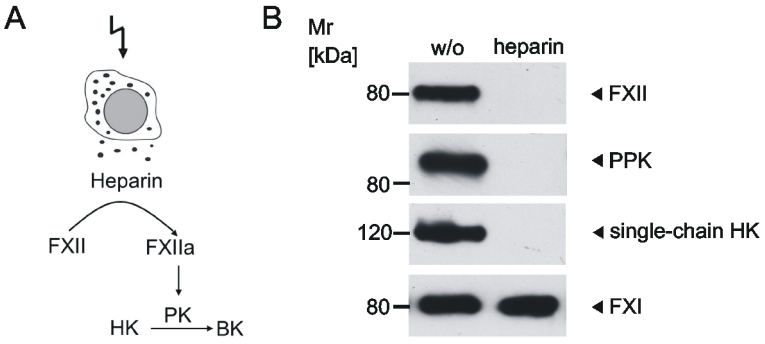

Figure 9: MC-derived heparin initiates the kallikrein-kinin system in plasma. (A) Schematic drawing of heparin induced activation of the FXII-driven kallikrein-kinin system. (B) Human plasma was incubated with heparin or w/o (buffer) and was probed for FXII, PPK, single-chain HK and FXI by Western blotting.

We used C1INH$^{-/-}$ mice to characterize the activation of the kallikrein-kinin system by MC-released heparin *in vivo*. A passive cutaneous anaphylaxis model in mice was established to characterize the edema formation. Mice were infused with IgE against dinitrophenyl (a-DNP-IgE) and 20 h later challenged intravenously with the corresponding antigen dinitrophenyl-human serum albumin (DNP-HSA). Allergen-stimulated MC triggered excessive IgE-mediated edema in C1INH$^{-/-}$ mice compared to WT mice (**Figure 10**). IgE-mediated edema was largely reduced in *F12*$^{-/-}$ and B2R$^{-/-}$ mice (data not shown) indicating that the kallikrein-kinin system is involved in allergic reactions.

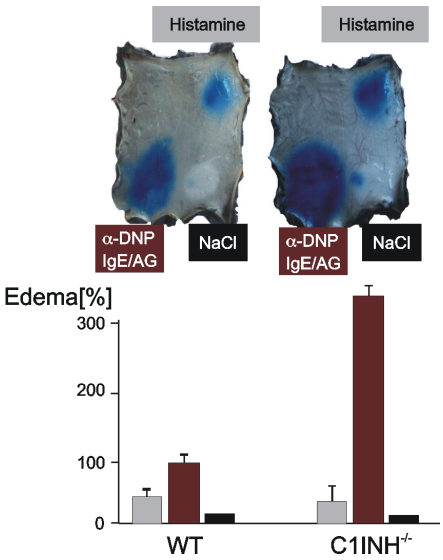

Figure 10: **Allergen activated MC initiate edema in C1INH$^{-/-}$ mice.** MC in mice were activated by aDNP-IgE or saline and challenged 20 h later by intravenous injection of the corresponding antigen DNP-HSA and Evan's blue tracer. Histamine was used as a control. Extravasated tracer was quantified 30 min after challenged.

To further analyze the IgE-mediated response on the cardiovascular system the mean arterial blood pressure (MABP) was measured at baseline, before and after injection of antigen DNP-HSA (**Figure 11**). First mice were injected intravenously with a-DNP-IgE and then challenged 24 hours later with DNP-HSA. When BK binds to its receptor B2R it initiates a cascade leading to increased vascular leakage and vasodilation. BK dependent vasodilation leads to a decrease in the total peripheral resistance and thereby a drop in MABP. Therefore, generation of BK and concurrent vasodilation results in a MABP drop.

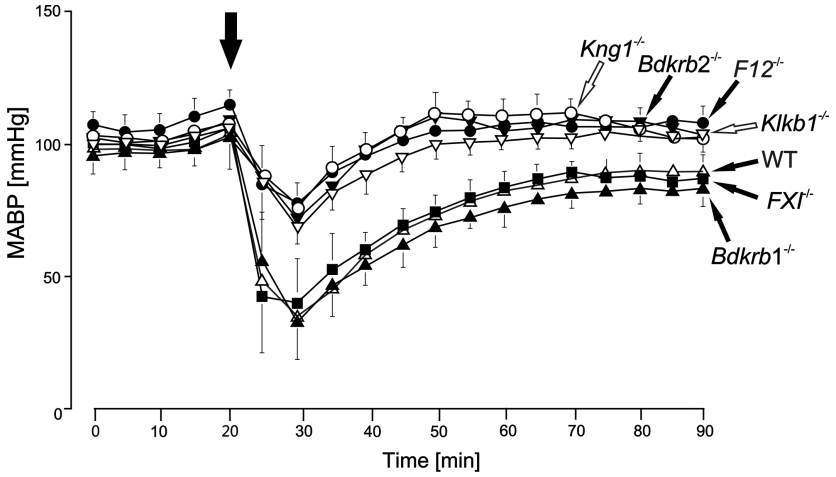

Figure 11: Measurement of the MABP in the contact system deficient mice during IgE/antigen-induced systemic anaphylaxis. MABP was measured in the left carotid artery in *F12*$^{-/-}$ (solid diamonds), FXI$^{-/-}$ (solid squares), *Klkb*$^{-/-}$ (PPK$^{-/-}$, inverted open triangles), *Kng1*$^{-/-}$ (HK$^{-/-}$, open circles), *Bdkrb1*$^{-/-}$ (B1R$^{-/-}$, solid triangles), *Bdkrb2*$^{-/-}$ (B2R$^{-/-}$, solid circles), and WT (open triangles) mice. The arrow indicates the time point when DNP-HSA was added.

Circulating BK is cleaved by carboxypeptidase N generating the metabolite des-Arg$_9$-BK, which is the principal ligand for B1R. des-Arg$_9$-BK activates the B1R and induces vasodilation similar to the effects seen with B2R signaling (Leeb-Lundberg et al., 2005; Prado et al., 2002). However, IgE/antigen challenge in *Bdkrb1*$^{-/-}$ (B1R$^{-/-}$) mice induced a strong hypotonic reaction that was similar to that seen in WT control animals, indicating that BK, but not des-Arg$_9$-BK, is a mediator of MC immune-mediated hypotensive responses (**Figure 11**). However, in sepsis and inflammation, induced by lipopolysaccharide (LPS) injection, the blood pressure decreased markedly in WT mice and *Bdkrb2*$^{-/-}$ mice, and only

moderately in *Bdkrb1*$^{-/-}$ mice, which indicates that the hypotensive response in systemic inflammation is predominantly mediated through B1R signaling (Cayla et al., 2007; Huang et al., 1999b; Pesquero et al., 2000). B2R is constitutively expressed by various vascular cell types: endothelial cells, vascular smooth muscle cells, and cardiac myocytes (Maurer et al., 2011). In contrast, B1R expression is inducible and upregulated during inflammation by cytokines such as interleukin-1b and therefore B1R plays a more important role during chronic inflammatory states (Huang et al., 1999a) yet has a minor contribution in acute hypotensive reactions, such as those observed during anaphylaxis. However, B1R antagonists reduce vessel leakage in a rat model using HAE I plasma (Bossi et al., 2009), indicating that B1R signaling could be involved in longer lasting edema formation.

These findings have implications for anaphylactic and allergic diseases, which contribute to the understanding of mechanisms of MC-mediated edema formation. For example, cutaneous edema in HAE patients is known to occur after allergen exposure or physical stress (Bork et al., 2009b), which is associated with MC degranulation (Seidel et al., 2011). As MC degranulation is a hallmark of anaphylaxis, we used Western blot to quantify the degree of HK cleavage to assess kallikrein-kinin system activation in anaphylactic patients' plasma (**Figure 12**). During anaphylaxis HK was largely reduced in all patients samples compared to HK levels at basal conditions (remission phase).

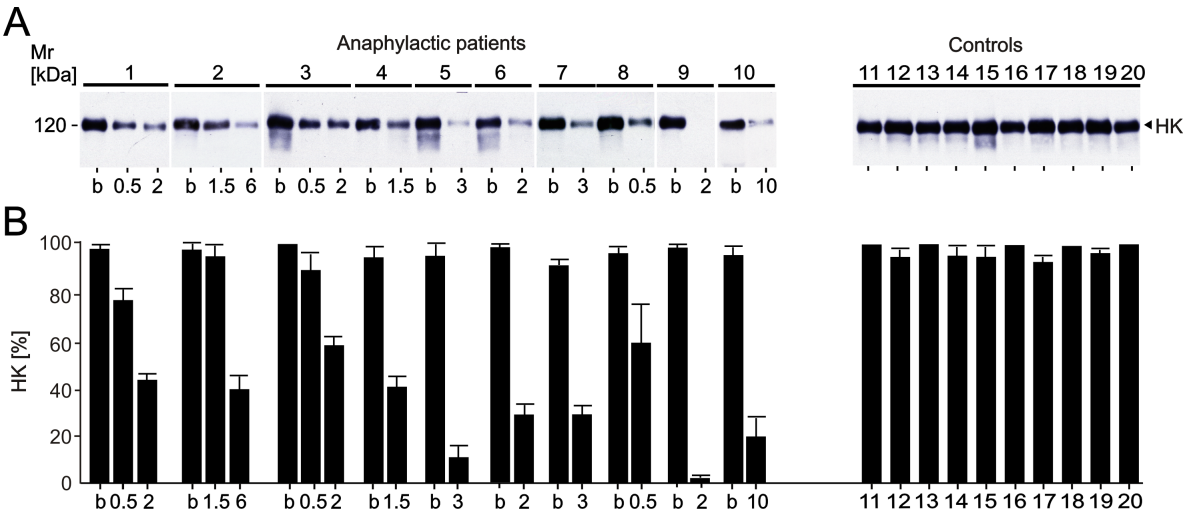

Figure 12: HK is consumed during anaphylaxis in patients but not in remission phase. (A) Plasma samples were collected from 10 patients with different grades of anaphylaxis at indicated time points after the onset of symptoms and at baseline (>14 days after anaphylaxis episode). For patients 1-3 two consecutive samples at an early and later time-point from the onset of anaphylaxis were obtained. Plasma samples from 10 age- and sex-matched healthy subjects were collected and served as controls. Plasma samples were resolved under reducing conditions and probed for HK by Western blotting using HK antibody. Time points of sample collection are given below the photographic film. Samples collected in basal conditions are designated with "b". A representative film of n=5 is shown. (B) HK signal intensity was assessed by densitometric scans.

Increased kallikrein-kinin system activity in anaphylactic patients is only seen during attacks, not during the remission phase. In contrast, HAE I patients have a spontaneous activation of the kallikrein-kinin system and an even more pronounced activity during attacks (Suffritti et al., 2014). HAE I and II patients have low levels or non-functional C1INH and therefore could be more prone to activate the kallikrein-kinin system at basal conditions. The BK degradation enzymes could likely metabolize BK and prevent edema to occur. However, the trigger factor that is responsible for the spontaneous activation of the system is not yet known.

Anaphylaxis is a severe allergic reaction and the activity of the kallikrein-kinin system is most probably due to the high release of trigger factors (such as heparin) and endogenous inhibitors do not have the capacity to stop the activation of the cascade. The phenomenon has also been seen in patients receiving oversulfated chondroitin sulfate (OSCS), contaminated unfractionated heparin (Kishimoto et al., 2008). Severe anaphylactic reaction or death was a consequence of OSCS after intravenously injected. OSCS-contaminated heparin has a greatly increased potency for activating FXII and triggering PK-mediated BK formation (Kishimoto et al., 2008).

Heparin-driven BK signaling increases vascular permeability, induces hypotension, and causes leukocyte-endothelium adhesion *in vivo*. Supporting a role for heparin as an *in vivo* kallikrein-kinin system activator, HK, PPK and FXII, but not FXI are consumed, in an experimental MC-driven anaphylaxis model in mice. Another trigger factor that only activates the kallikrein-kinin system, but not the intrinsic pathway of coagulation are misfolded protein aggregates (Maas et al., 2008). These agents do not induce thrombosis, which support the concept of selective activation of FXII-driven kallikrein-kinin pathway. Anaphylactic and HAE patients suffer from swellings but the edema attacks are not associated with an increased thrombotic risk, and the FXI-serpin complex is not formed in HAE patients (Nielsen et al., 1996). However, HAE patients have increased thrombotic and fibrinolytic parameters during acute attacks (Nielsen et al., 1996; van Geffen et al., 2012). Furthermore, PK and BK have the capacity to directly or indirectly activate plasminogen to plasmin (Bjorkqvist et al., 2013a; Colman, 1969) and plasmin is able to activate FXII-driven BK formation as well (van Geffen et al., 2012). The anti-fibrinolytic agent tranexamic acid is used in patients with high risk of hemorrhage due to its ability to inhibit plasmin formation. This agent is also used as a prophylaxis for patients with HAE. The treatment does not always leads to reduced edema formation in patients (only in 15%), but it shows that the fibrinolytic system might play a role in the disease. FXI and PPK have highly homologous structures and they both form a complex with cell bound HK (Bjorkqvist et al., 2013a). There may be mechanisms that activate the kallikrein-kinin system and/or the intrinsic clotting pathway that involve different FXII-activation mechanisms and different forms of FXIIa (Schmaier, 2008). There might be other yet unknown regulators of the kallikrein-kinin system that are activated during anaphylactic and HAE attacks.

4.3 MECHANISM AND THERAPY OF HAE III (PAPER IV AND V)

HAE III patients have a mutation in FXII (Thr309Lys or Thr309Arg) and they have normal levels and fully functional C1INH, but suffer from angioedema nonetheless. The pathophysiology of HAE III remains unidentified. Dysregulation of the kallikrein-kinin system is strongly suspected since there are several pieces indicating indirect evidence. HAE III symptoms are similar to type I and II (C1INH deficiency); the absence of urticaria and the lack of response to corticosteroid and/or antihistamine therapy support a nonhistaminergic disease (Zuraw et al., 2012). We aim to understand the role of FXII in HAE III and to develop a potential therapy.

HAE III patient and healthy plasma from 5 independent families (France, Germany and Spain) were analyzed by Western blotting and probed for FXII. The analysis showed that all patients with the Thr309Lys mutation have a double band of FXII (**Figure 13 A**). HAE III patients have a point mutation in FXII in one of the two alleles, position 309. FXII is glycosylated at multiple sites and three of the O-linked glycosylation sites are located in the proline rich region where Thr309 is one of them (McMullen and Fujikawa, 1985). This led us to hypothesize that the Thr309 mutation interferes with posttranslational protein modifications. Mass spectrometry confirmed a mucin-type HexHexNAcNeuAc glycan attached to the FXII fragment peptide Leu292-Arg311 in plasma from healthy individuals (**Figure 13 B**).

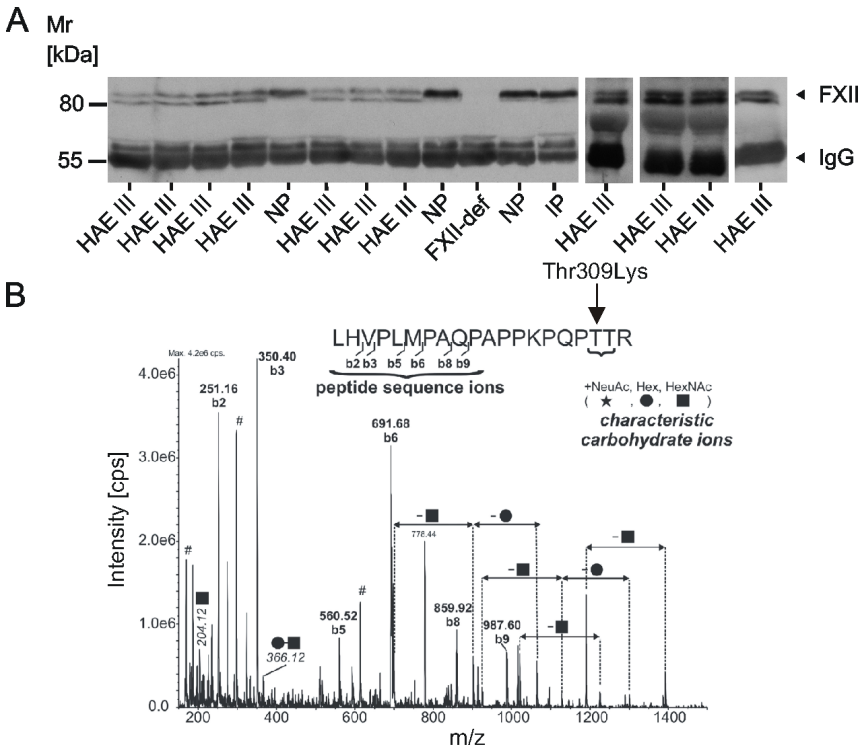

Figure 13: HAE III patients express a FXII protein that lacks glycosylation at Thr309. (A) FXII in HAE III patient plasma samples migrates as a doublet. Plasma from a French family with both healthy and HAE III members were analyzed as well as plasma from 4 other unrelated family's members carrying the HAE III mutation (France, Spain and Germany). Plasma from healthy individual (IP), pooled normal plasma (NP), and FXII deficient plasma (FXII-def). (B) Fragment mass spectrum of peptide Leu292-Arg311 being glycosylated with a HexHexNAcNeuAc glycan. A b-ion series from b2 to b9 and several y-ions (*) that correspond to the peptide moiety without the carbohydrate part are identified. The N-acetylneuraminic acid (NeuAc) is readily lost under tandem-Mass spectrometry conditions and consecutive loss of hexoses and N-acetylhexosamines is observed within the y-ion series. FXII mutation Thr309Lys is indicated in the peptide sequence.

The exact glycosylation site could not be determined from the Mass spectrometry data, due to the peptide fragmentation properties, but it was narrowed down to Thr309 or Thr310 (the only amino acid that could be O-linked glycosylated in the peptide). The position Thr309 is located in the C-terminal proline-rich portion of the FXII heavy chain (residues 1-353) that mediates contact to other proteins and zymogen FXII surface binding. The function of the mutation is unknown but changes in glycosylation could lead to increased kallikrein-kinin system activation, which has been found in Lewis rats. As a consequence of lack of *O*-glycosylation they are more susceptible, than other rats, to develop systemic inflammation (Sartor et al., 1996).

We cloned and produced non-mutated FXII and FXII containing the Thr309Lys and Thr309Arg as well as the combined FXII_Thr309Lys+Thr310Lys mutations in HEK239 cells. Their apparent molecular weight was compared to each other using Western blotting. Recombinant Thr309-mutated and Thr309Lys+Thr310Lys FXII variants migrated with the same but lower apparent molecular mass than wild-type protein in Western blot, which is consistent with the doublet migration pattern of FXII in HAE III patient plasma (**Figure 14 A**). The data confirm that it is only Thr309 that is glycosylated because the mutants migrate with the same apparent molecular weight.

The amount of FXIIa produced, by activation with DXS (100 ng/ml or 100 µg/ml), from the mutated and wild-type forms of FXII were analyzed by reconstituting human FXII deficient plasma. We utilized chromogenic assays and Western blot to quantify FXII-driven proteolytic processes (**Figure 14**). In the chromogenic assay the conversion of the substrate was similar in the activation potential of mutant and wild-type FXII and were indistinguishable at high DXS concentrations (100 µg/ml) (**Figure 14 B**). However, FXII_Thr309Lys and FXII_Thr309Arg exceeded wild-type FXII using lower DXS concentrations (**Figure 14 C,** 100 ng/ml DXS). When FXII deficient plasma, either reconstituted with FXII_Thr309Lys or FXII_Thr309Arg, was incubated with a low amount of DXS (100 ng/ml), HK was completely cleaved after 4 min. In contrast, plasma that was reconstituted with wild-type FXII failed to support HK cleavage within 32 min, under the same conditions (**Figure 14 D and E**). This indicates that the Thr309-mutants of FXII have an enhanced potential to initiate contact-activation.

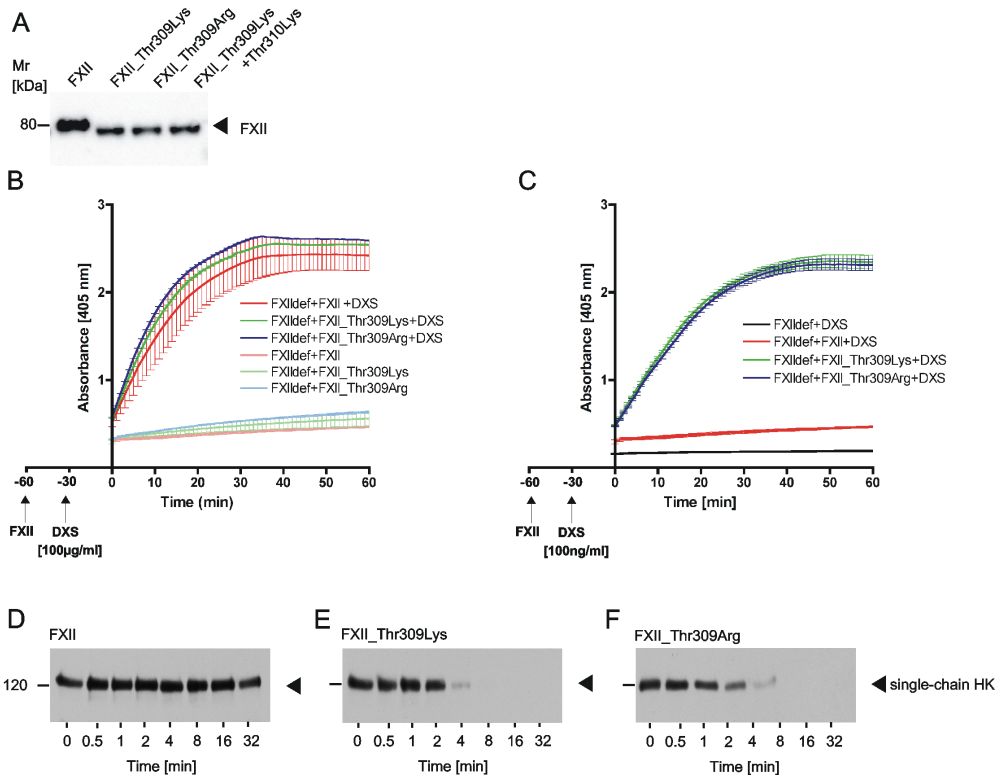

Figure 14: Thr309-mutations in FXII enhance contact activation. (A) Recombinant wild-type FXII and mutated FXII_Thr309Lys, FXII_Thr309Arg and FXII_Thr309Lys+Thr310Lys variants were analyzed by Western blotting using anti-FXII antibody (B-C) FXII deficient human plasma was reconstituted with FXII, FXII_Thr309Lys, FXII_Thr309Arg, or buffer and incubated with (B) 100 µg/ml and (C) 100 ng/ml DXS. Activity of FXIIa was measured by conversion of the chromogenic substrate S-2302. Means ± SEM. (D-F) FXII-deficient human plasma was reconstituted with wild-type FXII or Thr309-mutated FXII and activated with 100 ng/ml DXS. Contact-activated plasma samples were taken into reducing sample buffer at indicated time points and analyzed for cleavage of single chain HK by Western blotting.

To further address the mechanism of edema formation in HAE III we analyzed kallikrein-kinin system activation in plasma from HAE III patients and healthy controls. Samples were incubated for 30 min with DXS (1 pg/ml - 100 µg/ml) and then analyzed for zymogen FXII and PPK activation, formation of FXIIa and FXIIa-C1INH complexes, and HK cleavage (**Figure 15**). At lower concentration of DXS, FXIIa proteolytic activity is increased in HAE III patient plasma compared to healthy controls, although the disease-associated FXII mutation is outside the enzymatic domain. This lack of glycosylation in HAE III may influence folding (increase susceptibility for auto-activation), function (ability to activate PPK or hetero-activation), inhibition (decreased binding of C1INH-FXII complex) or clearance of FXII.

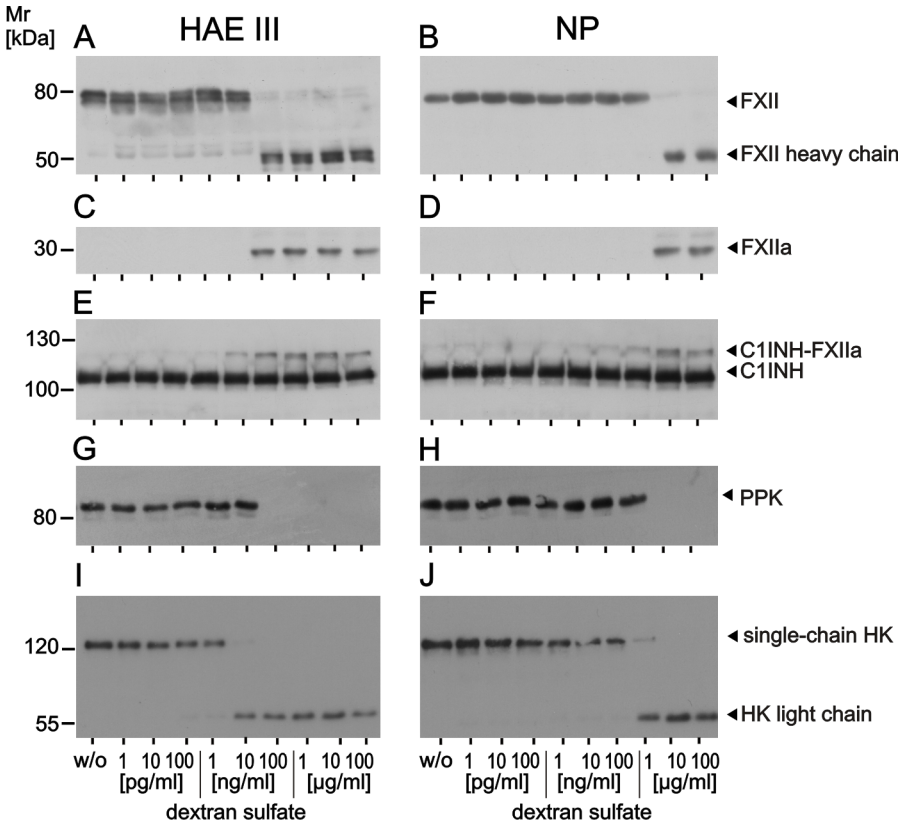

Figure 15: Increased kallikrein-kinin system activation potential in HAE III patients' plasma. (A-J) Plasma samples from HAE III patients (left panel) and healthy controls (right panel) were incubated with a concentration series of DXS (1 pg/ml-100 µg/ml) or buffer (w/o) for 30 minutes at 37°C. Plasma samples were analyzed by Western blotting with antibodies directed to various kallikrein-kinin system proteins: (A, B) anti-FXII, (C, D) anti-FXIIa, (E, F) anti-C1INH, (G, H) anti-PPK and (I, J) anti-HK.

We showed that the mutation results in an increased susceptibility for contact activation both in HAE III plasma and in the recombinant expressed mutated proteins, however the antigen levels are normal (aPTT levels are in the normal range, data not shown). Others also show that FXII plasma antigen levels and their total capacity to generate enzymatic activity after full activation is not increased in HAE III over healthy individuals (Bork et al., 2009a; Cichon et al., 2006). The aPTT-clotting assay measures total FXII activity levels triggered by prolonged incubation of excess amounts of the strong contact activator silica. The ability of DXS to trigger FXIIa-formation and activate the kallikrein-kinin system at a lower concentration in HAE III plasma than in healthy controls (**Figure 15**) supports the conclusive role of susceptibility for contact-driven FXII activation for edema in HAE III.

C1INH transfusion is the standard treatment for acute edema in HAE I and II. We have compared dose-dependent effects of the inhibitor for interference with the kallikrein-kinin system-mediated BK formation. We found that C1INH infusion blocks BK formation in HAE I and II plasma, whereas the drug had essentially no effect on BK generation in HAE III plasma (**Figure 16**).

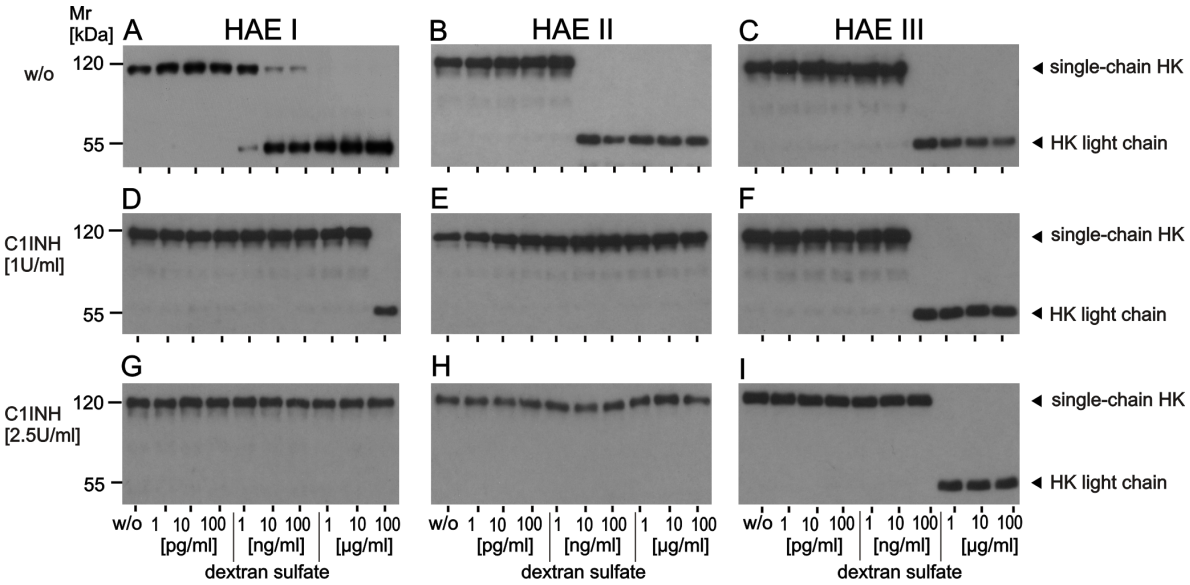

Figure 16: C1INH does not interfere with BK formation in HAE III. Plasma of HAE I, II and III patients was incubated with (A-C) buffer, 1 U/ml (D-F) or 2.5 U/ml (G-I) C1INH, with increasing concentrations of DXS and probed for HK and cleaved (light chain) HK by Western blotting using polyclonal anti-HK antibody.

Low efficiency of C1INH for dose-dependent inhibition of HK cleavage in HAE III motivated us to investigate alternative inhibitors. To inhibit FXII activation we screened for antibodies against FXIIa using phage display and demonstrated that the recombinant fully human antibody 3F7 binds into the FXIIa enzymatic pocket. 3F7 interfered with FXII-mediated HK cleavage in human plasma (**Figure 17 A-C**), coagulation and thrombus formation in mice and rabbits (data not shown). Furthermore, we analyzed the anti-FXIIa 3F7 antibody for its anti-inflammatory activities in ECMO. ECMO is a form of cardiopulmonary bypass (CPB) and patients treated in ECMO could develop edema (Cugno et al., 2001; Pretorius et al., 2005). The data from ECMO treated rabbit plasma suggest that 3F7 interferes with BK generation (**Figure 17 D and E**).

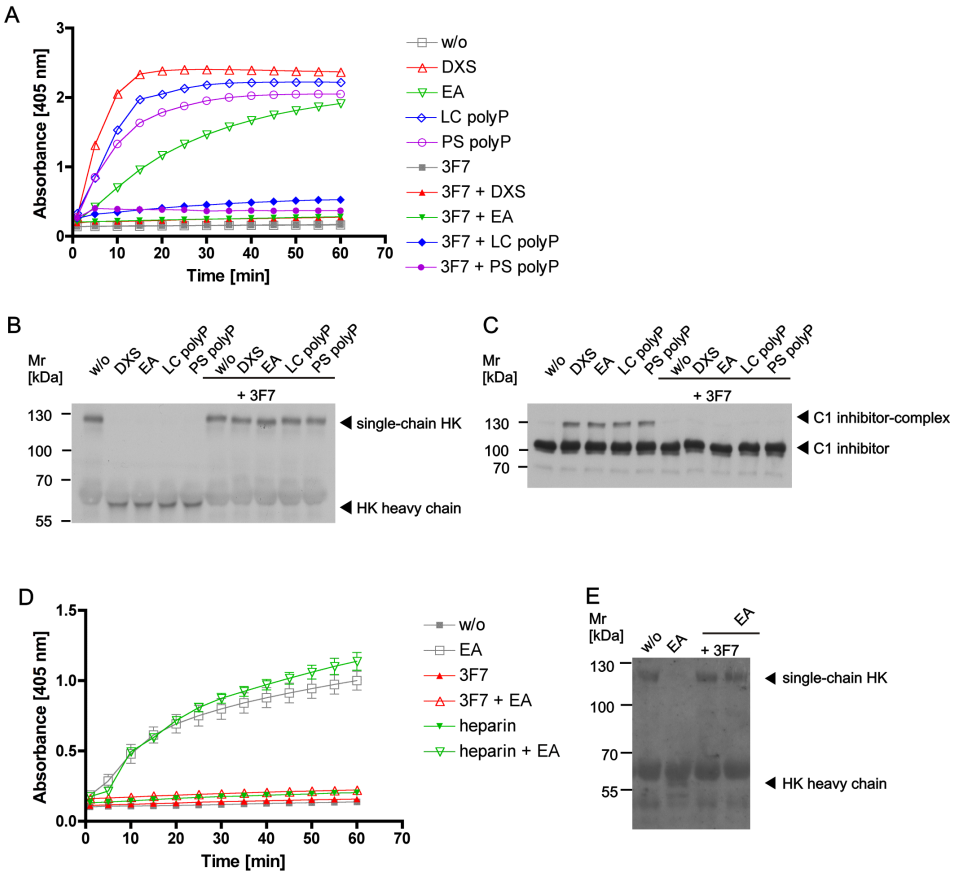

Figure 17: 3F7 interferes with FXIIa-driven kallikrein-kinin system activation in human plasma and in ECMO pretreated rabbit plasma. (A-C) Human plasma was incubated with contact activators: DXS (10 µg/ml), ellagic acid (EA, 1.5 µg/ml), long chain- and platelet size- polyP (LC polyP, PS polyP; 10 µg/ml) in the absence (open symbols) or presence of 3F7 antibody (100 µg/ml; filled symbols). (A) FXIIa was measured using hydrolysis of the chromogenic FXIIa substrate S-2302. Means ± SD of *n*=3. (B and C) Human plasma treated with different contact activators was separated by reducing laemmli sample buffer analyzed by Western blot for (B) HK cleavage and (C) formation of C1 inhibitor–FXIIa complexes. (D) FXIIa was measured as above in plasma of rabbits that underwent ECMO treatment. FXIIa was measured in plasma of 3F7- and heparin-treated animals at 5 h ECMO and in saline-treated rabbit plasma at 3 min. For control, plasma of the 3 animal groups was spiked with the contact activator EA (1.5 µg/mL). Means ± SD of *n*=3. (E) Rabbit plasma was incubated with EA (1.5 µg/ml) or buffer (w/o), and HK cleavage dependent on 3F7 addition (100 µg/ml) was analyzed.

We further analyzed clinically available drugs such as the PK inhibitor DX-88 and Aprotinin and pre-clinical/experimental FXIIa inhibitors such as PCK, rHA-infestin-4, CTI and 3F7 for their inhibitory effect on BK generation in HAE III patient plasma. We found that all of them except Aprotinin inhibit BK formation (**Figure 18**).

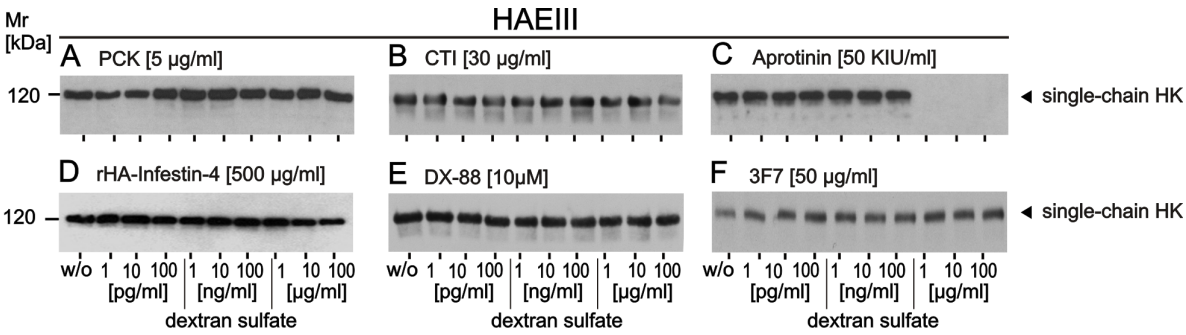

Figure 18: **FXII-inhibitors interfere with BK formation in HAE III.** HAE III patient plasma was incubated with different inhibitors (A) PCK, (B) CTI, (C) Aprotinin, (D) rHA-Infestin-4, (E) DX-88 and (F) 3F7 and supplemented with increasing concentrations of DXS and probed for HK.

Based on all the *ex vivo* results we studied the function of mutated FXII for vascular leakage and edema formation *in vivo* using genetically altered mice. Vascular leakage was analyzed using two different edema models that quantify vascular leakage using tracer extravasation. In the first model, we reconstituted *F12*$^{-/-}$ mice with recombinant wild-type or mutated FXII and studied the ability of these proteins to provoke edema by application of DXS using intravital microscopy (**Figure 19**).

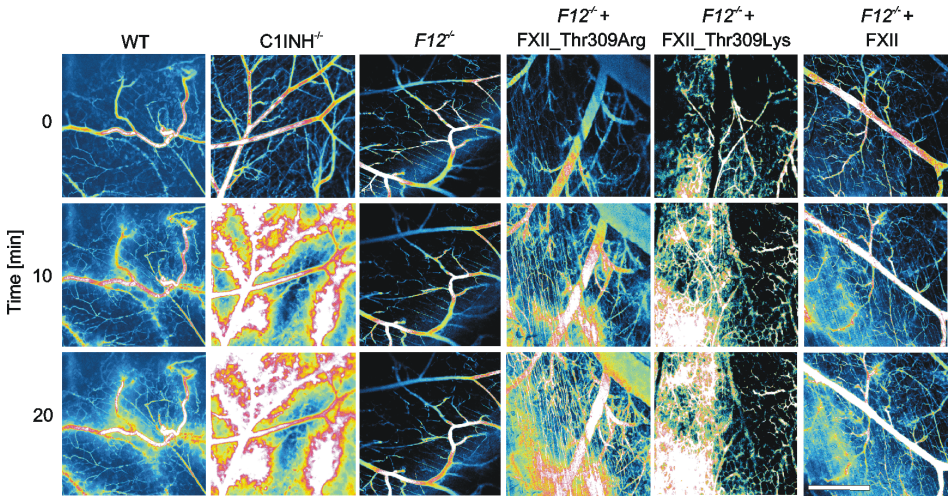

Figure 19: Thr309-mutated FXII increases microvascular permeability. Intravital laser scanning microscopy in real time recorded extravasation of fluorescencent FITC-dextran tracer from murine dorsal skin microvessels. The FXII activator DXS was topically applied to the inverted skin of WT, C1INH$^{-/-}$ (a mouse model for HAE I), *F12*$^{-/-}$ and *F12*$^{-/-}$ reconstituted with human FXII_Thr309Lys (*F12*$^{-/-}$ + FXII_Thr309Lys), with FXII_Thr309Arg (*F12*$^{-/-}$ + FXII_Thr309Arg) or with wild-type FXII (*F12*$^{-/-}$ + FXII) mice. Laser scanning images are shown in false colors. White represents the highest and black the lowest tracer intensity, respectively. The scale bar represents 500 µm.

To confirm our *in vivo* results, we generated a new double transgenic mouse system, which regulates the mutated human FXII (Thr309Lys) expression using the tetracycline-inducible system in the liver (**Figure 6 A**, Tet-off system). Using this system, it is possible to turn on or off the production of the human mutated FXII. These double-transgenic mice were bred with a FXII homozygous-deficient mouse (**Figure 6 B**). The generated mouse model (HAE III) mimics the situation in HAE III patients and allows analyzing the dose-dependent effects of mutant FXII *in vivo*. The expression of Thr309Lys-mutated FXII was confirmed using doxycycline (an analog of tetracycline) induced expression of mutant protein in transfected HEK293 cells and Western blotting (using a Tet-on system, **Figure 20 A**). We confirmed by Western blot the expression of FXII in the HAE III mice in the absence of doxycycline (**Figure 20 B**).

Since activation of FXII is associated with pathological thrombosis formation, we investigated the risk of thrombosis in the HAE III mice we used $FeCl_3$ model on the carotid artery. The HAE III mice form arterial thrombosis in a similar manner as WT, however the HAE III mice - dox, which do not express FXII_Thr309Lys (with a *F12*$^{+/-}$ background), have a prolonged occlusion time (**Figure 20 C**). The data indicate that HAE III mice are not associated with an increased risk for thrombosis.

The Miles edema model was used to investigate the contribution of FXII_Thr309Lys to vascular leakage in mice (Miles and Miles, 1952) (**Figure 20 D-H**). Mice were injected with Evan's blue dye and spectrophotometric quantification was used to determine the amount of extravasated dye in skin that was stimulated with different triggers. Basal vascular

permeability in saline-injected skin, was low in all mice. Dye extravasation was expressed relative to the saline induced signal in WT mice (WT set to 1.0, **Figure 20 I**). Intradermal injection of DXS induced leakage in WT mice and was strongly augmented in HAE III, with vascular permeability increasing >3-fold compared to non-expressing control animals (+dox). Since B2R$^{-/-}$ mice provide protection from kallikrein-kinin system mediated edema (Han et al., 2002), we tested the therapeutic potential of inhibitors of PK (DX-88) and FXIIa (3F7) in the HAE III mice. Infusion of DX-88 or 3F7 largely reduced DXS-induced edema in HAE III mice (**Figure 20 G-H**). Leakage in inhibitor-pretreated HAE III mice was below that in challenged WT, suggesting that inhibitors interfered with mutant and endogenous FXIIa-driven increases in vascular permeability. Cumulatively, the data show that FXII_Thr309Lys mediates increased contact-initiated vascular leakage *in vivo*.

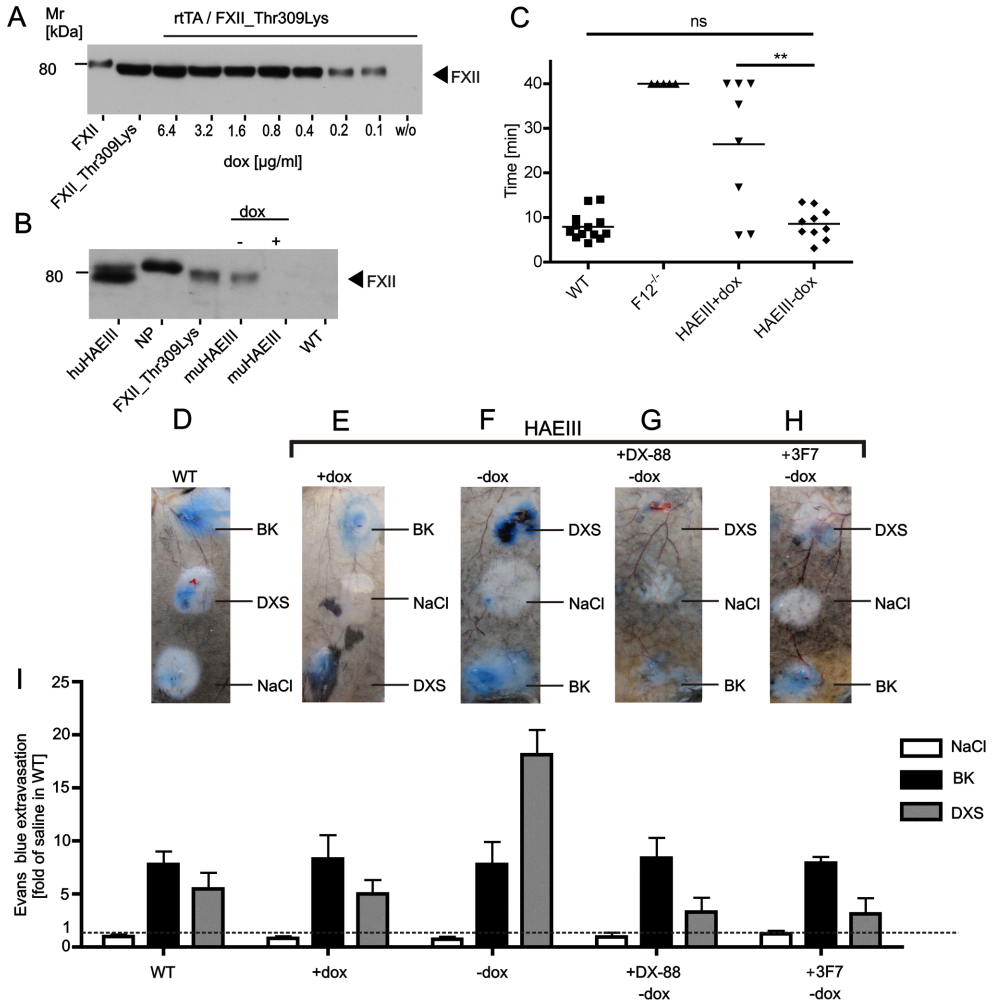

Figure 20: Increased vascular leakage but no thrombosis in HAE III mice. (A) Inducible expression of FXII_Thr309Lys in HEK293 cells using Tet-on system. The expression was induced using doxycycline (dox; 6.4-0.1 µg/ml), negative control buffer (w/o). The supernatant was analyzed by Western blotting after 48h of induction. Plasma-derived FXII and FXII_Thr309Lys were loaded as controls. (B) The HAE III mice induced FXII_Thr309Lys expression by dox withdrawal (-) or suppressed by dox application (+). Plasma from induced (-dox) and non-induced (+dox) HAE III mice were analyzed by Western blot probing for FXII that does not cross-react with the mouse ortholog. HAE III patient plasma (huHAEIII), healthy individuals (NP) and HEK293 cell-expressed FXII_Thr309Lys were loaded for comparison. (C) Thrombosis was induced in the carotid artery in WT, FXII$^{-/-}$, HAE III +dox and HAE III -dox mice after topical application of 5% $FeCl_3$ for 3 min. Time to complete occlusion after injury was monitored. The experiment was stopped after 40 min. (D-H) Evan's blue was intravenously infused as a tracer into (D) WT, (E) suppressed HAE III (+dox), (F) induced HAE III (-dox) mice, (G, H) Induced HAE III mice treated with DX-88 (430 µg/kg bw, -dox +DX-88) or 3F7 (7 mg/kg bw, -dox +3F7). Skin edema formation was induced by intradermal injection of 50 µl BK (100 µM), DXS (80 mg/ml), or saline (NaCl) as control, and visualized by tracer extravasation after 30 min. (I) Spots with extravasated tracer were excised entirely and dye was extracted and quantified. Tracer extravasation is plotted relative to leakage in WT mice stimulated with NaCl. Columns show means ± SEM, *n=4* per group. Representative mouse hides are shown in D-H.

DX-88 (Ecallantide) and Icatibant are used to treat acute attacks in HAE I and II and C1INH concentrate is one of the most common treatment for acute and prophylaxis. C1INH concentrate could not inhibit the activation in HAE III plasma (**Figure 16**). However, four patients with HAE III had a <50% decrease of C1INH function during attack (Marcos et al., 2012). This may suggest consumption of C1INH by activation of enzymes in the kallikrein-kinin or complement system during swelling episodes although reports of inefficacy C1INH have also been described. C1INH complexing might be to slow too form and inhibit the reaction in these patients (Riedl, 2013). However, humanized antibodies have been effectively introduced as patient therapy on numerous occasions (Mayforth and Quintans, 1990). A fully humanized antibody against PK (DX-2930) has just finished a placebo-controlled, dose-escalation Phase 1 trial without toxicity (Chyung et al., 2014). DX-2930 is applied subcutaneously and has a high bioavailability (66%) and long half-life (20 days) and interferes with BK formation in monkeys (Kenniston et al., 2014). DX-2930 could be used as a long-term prophylaxis for HAE patients including HAE III. An alternative prophylactic treatment could be the fully humanized antibody 3F7 (Larsson et al., 2013), which has shown to inhibit the kallikrein-system both *in vitro* and *in vivo* and may be useful both in the prophylaxis and acute settings of HAE.

Women with HAE III are more likely to be symptomatic than men and the vast majority of HAE III patients are females (Charignon et al., 2014; Marcos et al., 2012). Notably HAE III was originally believed to be X-chromosomal linked and exclusively affecting women (Bork et al., 2000). However the genetic defect is equally distributed among men and women (chromosome 5), indicating that poorly defined triggers induce swellings preferentially in women. HAE III has been believed to be associated with estrogen levels but so far clear experimental or clinical evidence for this hypothesis is missing. Nevertheless some cases of HAE III seem to be associated with intake of hormonal contraceptives but there are also reports describing swelling episodes in young children, in women after menopause and in men (Martin et al., 2007; Riedl, 2013). Estrogen has been shown to increase FXII levels and to decrease C1INH levels, (Gordon et al., 1980) as well as to suppress aminopeptidase P and ACE (Binkley, 2010). However, the effect on BK levels is most probably not sufficient to cause the severe episodes of angioedema. Furthermore, FXII levels are not elevated in HAE III patients (Cichon et al., 2006). This indicates that yet unidentified genetic and/or environmental factors account for the gender specificity of HAE III and that estrogen rather is a strong cofactor for phenotype expression.

Various genetic polymorphisms in BK degradation enzymes (Duan et al., 2009) as well as reduced level of these enzymes (Charignon et al., 2014) have been described in patients with HAE III. However, the non-carries in the family also presented with reduced protein levels compared to the healthy controls and the relevance of these polymorphisms to the clinical phenotype is unknown.

In summary, this study reveals knowledge of the mechanism of HAE III-driven BK generation and edema formation and the FXIIa function-blocking antibody 3F7 as a promising drug to block edema.

5 CONCLUDING REMARKS

Allergen-challenged MC release the charged polysaccharide heparin, which initiates FXII-driven kallikrein-kinin activation. Heparin-triggered kallikrein-kinin system activation culminates in BK-formation and increases vascular permeability. It is one of the links between MC-driven pro-inflammatory reactions and the kallikrein-kinin system, and have been shown to be activated during anaphylaxis in patients before a return to basal levels.

HAE III patients have a point mutation in FXII and this mutation results in a loss of an O-linked glycosylation. The mutated FXII has an increased susceptibility to become activated and to trigger the kallikrein-kinin system. C1INH concentrate is the standard treatment for acute edema in HAE I and II patients, however, C1INH is not inhibiting the activation in HAE III patient plasma. We have generated the fully humanized FXIIa-inhibitory antibody (3F7) and shown that 3F7 inhibits kallikrein-kinin system activation. PK and FXIIa inhibitors are able to inhibit HK cleavage in HAE III patient plasma and the inhibitors reduce vascular leakage and edema formation in HAE III transgenic mice. Using this knowledge the correct drugs could be used to target the edema in HAE III.

The FXII-driven kallikrein-kinin system provides an excellent platform to test principles of inflammation with new perspectives to improve diagnostics and therapies of inflammatory, infectious and allergic diseases. Learning more about FXII is an opportunity to move knowledge forward, as well as potentially creating a unique agent to target vascular thrombosis and inflammation. All projects have a strong translational aspect: since all FXII systems are well conserved between mice and humans. The experimental data from animal can be easily applied to human pathophysiology and will significantly shorten the time to an efficient therapy. Therefore, in light of the findings it is of interest to further investigate the role of FXII and the kallikrein-kinin system.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Den vanligaste orsaken till inflammation beror på en infektion, men det kan också finnas andra orsaker: skador på kroppen, allergi (aktivering av mastceller), anafylax (överkänslighetsreaktion) samt att en del sjukdomar kan utlösa en inflammation. Vid ett inflammerat området vidgas blodkärlen och dess väggar blir mer genomsläppliga än vanligt. Det sker då en transport av vätska från blodet till de omgivande vävnaderna som leder till ett lokalt ödem och en svullnad. Samverkan mellan inflammatoriska reaktioner tillsammans med koagulerande reaktioner, är förknippade med en rad olika sjukdomar som påverkar hjärt- och kärlsystemet.

Faktor XII (FXII) är ett protein som tillsammans med andra proteiner utgör kontakt systemet, vilket framförallt är involverat i två olika system: koagulationssystemet och kallikrein-kinin systemet. Vid aktivering av FXII i koagulationssystemet bildas tromboser och koagulering av blodet. Vid aktivering av FXII och kallikrein-kinin systemet frisätts substanser som leder till att de små blodkärlen läcker vätska till intilliggande vävnader (ödem). Experimenten i den här avhandlingen var utformade för att förstå hur FXII kan aktiveras i kroppen och dess roll i ödem samt mekanismen och behandling av den ärftligt sjukdomen angioödem (HAE) typ III.

Mastceller är en celltyp i immunförsvaret som hjälper till att försvara kroppen. När mastceller aktiveras utsöndrar de många olika substanser, bland annat histamin och heparin. Det har sedan länge varit känt att histamin är en orsak till svullnader men det finns patienter som fortfarande har svullnader även om man blockerar histamin. Heparin är en polymerisk kolhydrat som utsöndras från mastceller. Resultaten från experimenten visade att heparin från mastceller kan aktivera FXII och kallikrein-kinin systemet som leder till ödembildning. Medfödd brist och farmakologisk inhibering av FXII skyddar från heparin inducerat hudödem och lågt blodtryck hos råttor och möss som orsakas av aktiverade mastceller eller anafylaktisk reaktioner. Musmodeller visade att heparin ökade läckaget i kärl hos möss. Detta tyder på att heparin som utsöndrat från mastcell och initierat FXII och kallikrein-kinin systemet spelar en grundläggande roll för funktionen i kärlväggen och kan leda till inflammation, lågt blodtryck och ödem.

Det finns förändringar (mutationer) i FXII som kan leda till ett icke funktionellt protein och det finns en specifik mutation i FXII som leder det till den ovanliga sjukdomen, HAE typ III. HAE är en sällsynt, ärftlig sjukdom som karaktäriseras av episodiska anfall av smärtsamma svullnader i huden och mag-tarmkanalens slemhinnor samt i armar och ben. Anfallen kan också vara livshotande då de uppstå i strupen som kan svullna igen. Attackerna bildas vanligen med några veckors eller månaders intervaller och svullnaderna varar i ca 2-5 dagar. Anledningen till anfallen kan vara: skador, slag, tryck på hud och slemhinnor, operationer, tandläkarbehandlingar, infektioner, stress, allergi eller utan någon tydlig orsak. Man vet inte vilken substans som initierar ödemen hos HAE patienter. Sjukdomen har sedan länge varit känd i två olika former: HAE typ I och II och för några år sedan beskrevs även en tredje form, HAE typ III. Mekanismen av HAE typ III är inte känt och för att studera den har vi analyserat muterat FXII. Mekanismen har karaktäriserats genom att undersöka FXII och kallikrein-kinin systemet i plasma (hos patienter som är diagnostiserade med HAE typ III), med syntetiskt uttryckt FXII (normalt och muterat) och i ödemmodeller med hjälp av genetiskt modifierade möss. Läckage i olika djurmodeller har undersökts samt hur olika

substanser kan blockerar FXII så inga svullnader uppstår. Resultaten från experimenten visade att HAE typ III har ett överaktivt FXII och därför aktiveras kallikrein-kinin systemet som leder till ödemen. C1-inhibitor är ett protein som inhiberar FXII men det är för långsamt när väl aktiveringen sker och därför behövs andra substanser som kan blockerar FXII eller något av de andra proteinerna i kallikrein-kinin systemet. Vi har framställt en FXII hämmare kallad 3F7, där vi har visa att 3F7 inhiberar FXII medierat ödem.

För att kunna motverka svullnader är det viktigt att förstå mekanismen bakom. Resultaten i den här avhandlingen kan förhoppningsvis bidra till att utveckla läkemedel som kan användas både vid allergiska, anafylaktiska reaktioner samt i HAE typ III för att förbättra sjukdomstillståndet för dessa patienter.

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