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Lopatko Fagerström, Ingrid

2025

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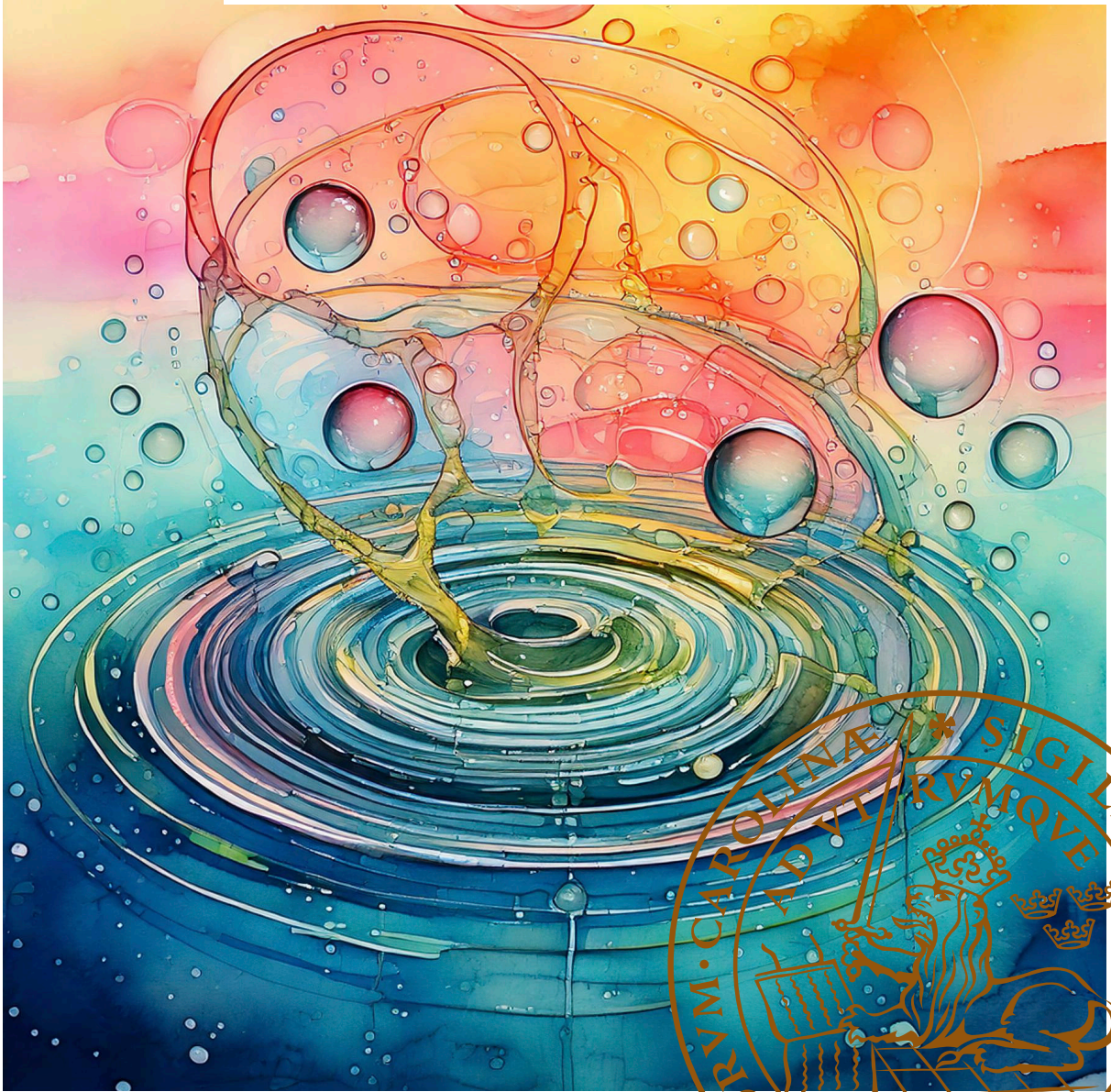

Printed by Media-Tryck, Lund 2025 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences Lund
Pediatrics

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2025:31
ISBN 978-91-8021-684-5
ISSN 1652-8220

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended in Belfragesalen, D15, Biomedicinskt Center (BMC)

Friday April 4th, 2025, at 1:00 pm.

Faculty opponent

Professor Peter Zipfel,

Infection biology, Friedrich-Schiller-University Jena

Organization: LUND UNIVERSITY

Document name: Doctoral dissertation **Date of issue** April 4th 2025

Author(s): Ingrid Lopatko Fagerström **Sponsoring organization:**

Title and subtitle: Interactions between the kallikrein-kinin system and the complement system in inflammation

Abstract:

The aim of this thesis was to investigate the interactions between the kallikrein-kinin system (KKS) and the complement system during vascular inflammation and the contribution of extracellular vesicles (EVs). Vasculitis, in patients and in experimental models, was studied as a prototype inflammatory disease of the vasculature.

Papers I and II addressed the impact KKS signalling has on complement activation. In **Paper I** vasculitis patient plasma was shown to have increased levels of endothelial-derived EVs bearing deposits of complement C3 and C9. Patient plasma induced complement deposition on endothelial EVs in a perfusion system. Kinin B1 and B2 receptor antagonists or C1 inhibitor reduced complement deposition. Similar effects were found using C1 inhibitor-depleted plasma in which the KKS is activated. In **Paper II** the effect of KKS activation, by kaolin or factor XIIa, was studied directly on ADP-preactivated glomerular endothelial cells showing that complement factors C3a, Ba, C5b-9 were released from or deposited on cell lysates. These effects were inhibited by a B1 receptor antagonist. The B1 receptor is a G-protein coupled receptor, and we could show that the effects were mediated via the intracellular inositol triphosphate receptor. Furthermore, in **Papers I and II** mice were treated with a B1 receptor antagonist, or lacked the B1 and B2 receptor, and both papers showed that complement deposition in glomeruli was reduced under these conditions suggesting that signaling via these kinin receptors contributes to complement activation.

In **Paper III** we found that vasculitis patients have high levels of leukocyte-derived EVs bearing the B1 receptor. The receptor was shown to be transferred between cells by EVs. This was demonstrated using transfected cells but also native leukocyte-derived EVs. Importantly, the transferred B1 receptor was functional. In patient biopsies we identified leukocyte-derived B1 receptor-positive EVs docking onto glomerular endothelial cells, suggesting that the transfer of the B1 receptor to glomerular endothelial cells by EVs may occur in vivo and thereby induce kidney inflammation.

In **Paper IV** we show that kallikrein cleaves all components of C5b-9 in pure form, in the absence of plasma. In plasma, particularly C1 inhibitor-depleted plasma, kallikrein cleaved C5b, C6 and C7. The physiological significance of kallikrein activity was reduced formation of the membrane attack complex on rabbit red blood cells, and thus less hemolysis, and decreased deposition of C5b-9 on glomerular endothelial cells. Specificity of the enzymatic effect was demonstrated by using a monoclonal antibody against kallikrein, lanadelumab.

We demonstrate both activating and inhibitory interactions between the KKS and the complement system. In addition, circulating EVs are increased in patients with vasculitis, and they bear both complement components and the B1R. These may be critical features during inflammation. The interactions between the KKS and complement could be of importance for treatment of inflammatory disorders.

Key words: inflammation, kallikrein-kinin system, complement, kallikrein, extracellular vesicles

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **Number of pages**: 96

ISSN and key title: 1652-8220

ISBN: 978-91-8021-684-5

Recipient's notes Price Security classification

Signature Date 2025-02-24

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Cover photo: AI generated using Adobe firefly

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ISBN 978-91-8021-684-5
ISSN 1652-8220

Tryckt i Sverige av Media-Tryck, Lunds universitet
Lund 2025

To my family.

Table of contents

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Lopatko Fagerström I**, Ståhl AL, Mossberg M, Tati, R, Kristoffersson AC, Kahn R, Bascands JL, Klein J, Schanstra JP, Segelmark M, Karpman D. Blockade of the kallikrein-kinin system reduces endothelial complement activation in vascular inflammation. *EBioMedicine,* 2019; 47: 319–328

II. **Lopatko Fagerström I**, Gerogianni A, Wendler M, Arvidsson I, Tontanahal A, Kristoffersson AC, Qadri F, Bader M, Karpman D. Bradykinin B1 receptor signaling triggers complement activation on endothelial cells. *Frontiers in Immunology,* 2025; 16:1527065

III. Kahn R, Mossberg M, Ståhl A, Johansson K, **Lopatko Lindman I**, Heijl C, Segelmark M, Mörgelin M, Leeb-Lundberg LMF, Karpman D. Microvesicle transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis. *Kidney International*, 2017; 91:96-105

IV. **Lopatko Fagerström I**, Kristoffersson AC, Karpman D. Kallikrein cleaves all components of C5b-9 reducing terminal complement complex function on cell surfaces. Manuscript.

The following review was published but not included in this thesis:

Bekassy Z, **Lopatko Fagerström I**, Bader M, Karpman D. Crosstalk between the renin-angiotensin, complement and kallikrein-kinin systems in inflammation. *Nature Reviews Immunology*, 2022;22(7):411-428

Abbreviations

AAV	Anti-neutrophil cytoplasmic antibody-associated vasculitis
ACE	Angiotensin converting enzyme
ANCA	Anti-neutrophil cytoplasmic antibody
B1R	Bradykinin 1 receptor
B2R	Bradykinin 2 receptor
EGPA	Eosinophilic granulomatosis with polyangiitis
EVs	Extracellular vesicles
FIX	Factor IX
FXI	Factor XI
FXII	Factor XII
FXIIa	Activated factor XII
GPA	Granulomatosis with polyangiitis
HAE	Hereditary angioedema
HK	High molecular weight kininogen
KKS	The kallikrein-kinin system
NETs	Neutrophil extracellular traps
MAC	Membrane attack complex
MPA	Microscopic polyangiitis

Abstract

The aim of this thesis was to investigate the interactions between the kallikrein-kinin system (KKS) and the complement system during vascular inflammation and the contribution of extracellular vesicles (EVs). Vasculitis, in patients and in experimental models, was studied as a prototype inflammatory disease of the vasculature.

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We demonstrate both activating and inhibitory interactions between the KKS and the complement system. In addition, circulating EVs are increased in patients with vasculitis, and they bear both complement components and the B1 receptor. These may be critical features during inflammation. The interactions between the KKS and complement could be of importance for treatment of inflammatory disorders.

Introduction

Vascular inflammation is a common feature in many different disorders, such as sepsis,[1] COVID-19 infection[2] and trauma.[3] Additionally, vascular inflammation is the cardinal symptom in vasculitides[4] and the genetic disorder hereditary angioedema (HAE).[5]

HAE is driven by excessive kallikrein-kinin system (KKS) activation,[6] most often caused by a decreased or dysfunctional C1 inhibitor. C1 inhibitor is the most important inhibitor of KKS and the classical pathway of complement. HAE is characterized by recurrent swelling of skin and mucosal tissues.

The hallmark of vasculitis is inflammation in and around the vessel wall, causing a weakened vessel wall with reduced blood flow and increased vascular permeability. Both the KKS[7] and the complement system[8] have been shown to be activated during vasculitis.

Extracellular vesicles (EVs) are released from cells in the resting state, and even more so during stress or inflammation.[9] They are vital for intercellular communication[10] as well as for the cells ability to rid themselves of unwanted components. They can transport cargo from one cell to another, either in close proximity or longer distances. Levels of EVs have been shown to be increased during acute vasculitis.[11]

The KKS is found at the interface of coagulation and inflammation. Activation of the KKS results in the release of a vasoactive nonapeptide called bradykinin, which can be further cleaved to desArg^9-bradykinin. Both kinins bind to the kinin receptors and induce increased vascular permeability, vascular relaxation and cytokine release.[12] In addition, FXIIa activates FXI of the intrinsic pathway of coagulation.

The complement system is an important part of innate immunity and yields metabolites that can induce inflammation, by neutrophil recruitment[13] and anaphylatoxin effects. In addition, complement has an important function in the eradication of intruding pathogens.

The aim of this thesis was to investigate how the KKS and the complement system interact during vascular inflammation, and if EVs could contribute to these effects in inflammation. In addition, inhibitors of the KKS were used and their effect on complement activation was assessed. This could be of importance when developing and evaluating new therapeutics targeting the KKS and complement.

The kallikrein-kinin system

The kallikrein-kinin system (KKS) has a prominent role in coagulation and inflammation. The KKS is abundant in both plasma and in tissues, with important differences. This thesis will focus on the plasma KKS, which consists of Factor XII (FXII), plasma prekallikrein/kallikrein and high molecular weight kininogen (HK). C1 inhibitor is the most important inhibitor of the KKS, inhibiting both activated FXII (FXIIa) and kallikrein.

Factor XII

FXII is a zymogen form of a serine protease mainly produced in the liver,[14] although studies have suggested it may also be produced in neutrophils[15] and fibroblasts.[16] It has a molecular weight of 80 kDa and the plasma concentration is approximately 40 µg/mL.[17-18] Although described as a zymogen, a recent study demonstrated that FXII has some proteolytic activity, and the ability to cleave and activate prekallikrein.[19] FXII circulates in plasma but can bind to endothelial cells. There are three known binding sites, the gC1q receptor (gC1qR),[20] cytokeratin-1 (CK-1)[21] and plasminogen activator receptor (uPAR),[22] forming bimolecular receptor complexes.[23] FXII preferentially binds to the receptor complex consisting of uPAR and CK-1 on endothelial cells.[24] Studies have shown that FXII in its zymogen form stimulates the endothelium to induce cell growth and angiogenesis,[25] activates vascular smooth muscle cells[26] and induce chemotaxis and stimulate neutrophils to produce neutrophil extracellular traps (NETs).[15] Upon activation FXII undergoes a conformational change to yield the enzyme FXIIa. In addition to activating prekallikrein and thereby downstream components of the KKS, FXIIa cleaves and activates Factor XI, initiating the activation of the intrinsic pathway of coagulation. *In vitro* FXIIa has also been shown to activate C1r from the classical pathway of complement, thereby participating in host defense (Figure 1).[27]

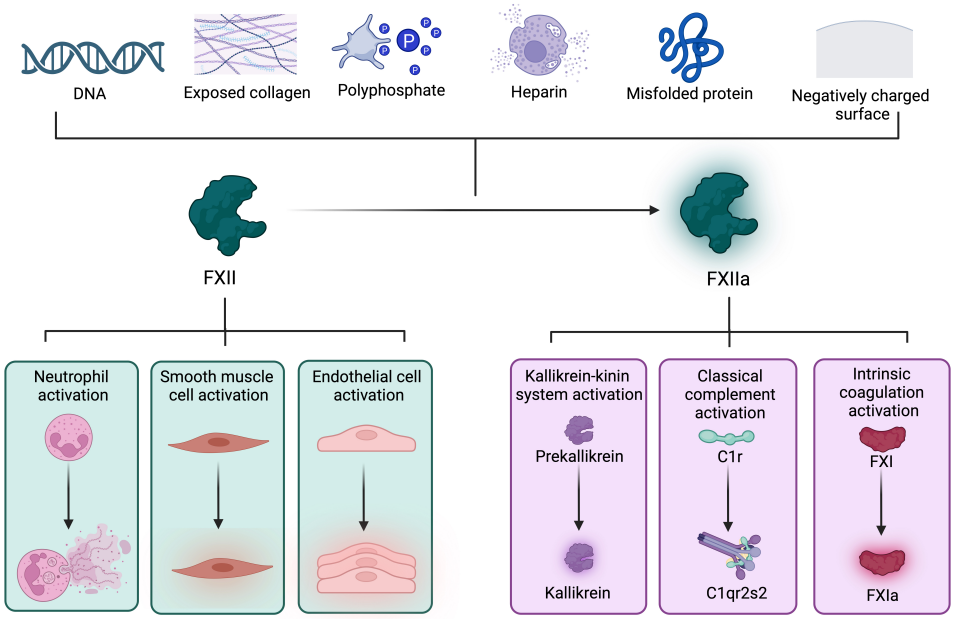

Figure 1. Activation and function of factor XII and FXIIa
Factor XII (FXII) is the zymogen form of factor XIIa (FXIIa), circulating in plasma. It is activated by a number of endogenous substances and negatively charged surfaces. FXII: Factor XII, FXI: Factor XI. Created with BioRender.

Kallikrein

Kallikrein is a serine protease with a plasma concentration ranging from 35-50 µg/mL.[28] The molecular weight is 86-88 kDa.[29] Prekallikrein is the precursor form of kallikrein, circulating in the bloodstream bound to its co-factor and substrate HK. Prekallikrein can also bind to the endothelium together with HK.[30] Activation of prekallikrein occurs either by FXIIa or by cell-bound prolylcarboxypeptidase (PRCP) yielding kallikrein. Kallikrein in turn cleaves HK, releasing bradykinin (described in detail below). *In vitro*, kallikrein has also been shown to cleave multiple components of the complement system, which will be described below. In addition, kallikrein has been shown to stimulate monocytes to produce cytokines and to upregulate the C5a receptor,[31] induce chemotaxis[32] and elastase production in neutrophils,[33] and to cleave plasminogen to plasmin initiating the fibrinolytic pathway.[34] Kallikrein has also been shown to directly cleave coagulation factor IX yielding FIXa, thereby bypassing FXII in activating the intrinsic pathway of coagulation.[35]

High molecular weight kininogen

HK is a glycoprotein consisting of six functional domains. The heavy chain consists of domain 1-3 and the light chain of domain 5-6. Domain 4 links the heavy chain to the light chain and contain the nonapeptide bradykinin, which is released upon cleavage by FXIIa or kallikrein.[36] Domains 1,[37] 2 and 3[38] have cysteine protease inhibiting effects. HK binds to one of the same bimolecular receptor complexes on endothelial cells described for FXII binding, CK1 in complex with gC1qR, binding occurs via domains 3 and 5.[39] Prekallikrein and FXI bind to domain 6.[40] HK has a molecular weight of 120 kDa and a plasma concentration of approximately 70 µg/mL.[36] In plasma it circulates bound to prekallikrein. The carrier function is believed to be important, as HK deficiency is associated with low plasma levels of prekallikein.[41]

Activation of the kallikrein-kinin system

Historically, activation of the KKS was believed to only occur when FXII encountered negatively charged surfaces, hence the term 'contact system'. These activating surfaces could be both endogenous and exogenous. Collagen,[42] heparin,[43] misfolded proteins,[44] RNA[45] and polyphosphate,[46] are endogenous FXII-activating surfaces, whereas LPS,[47] kaolin[48] and dextran sulfate[49] are well-known exogenous activating surfaces (Figure 1). Binding to a surface induces a conformational change in FXII leading to the formation of the enzymatically active FXIIa. FXIIa cleaves prekallikrein yielding kallikrein, an important enzyme of the KKS (Figure 2). The process of autoactivation of FXII is a relatively slow process, and a more effective way of activation of the KKS has been established. Prekallikrein bound to endothelial cells via HK can be activated by cell-bound prolylcarboxypeptidase (PRCP) (Figure 3).[50] Kallikrein can then activate FXII to FXIIa, which reciprocally cleaves prekallikrein to release more kallikrein. Kallikrein cleaves HK, releasing the active nonapeptide bradykinin. Bradykinin can be further processed to desArg^9-bradykinin by carboxypeptidases.[51-52] Bradykinin and desArg^9-bradykinin bind to the bradykinin 2 (B2R) and bradykinin 1 (B1R) receptors, respectively. Both bradykinin and desArg^9-bradykinin are degraded by angiotensin converting enzyme (ACE)[53] to fragments with varying biological activities, independent of B1R and B2R.[54]

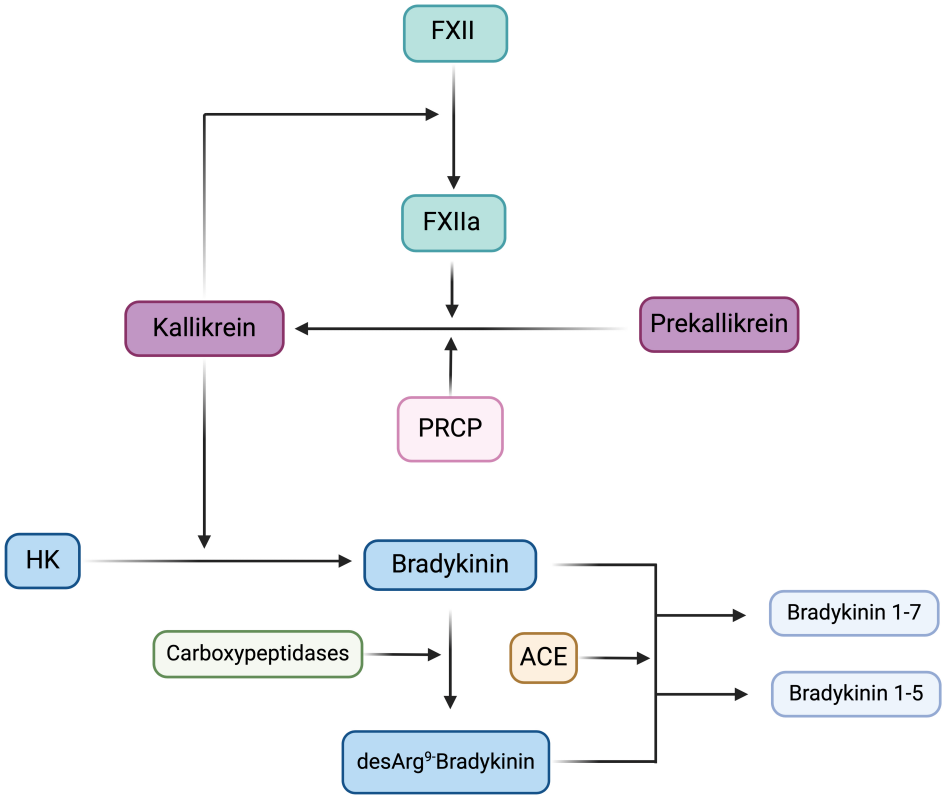

Figure 2. Schematic figure of the activation of the kallikrein-kinin system. PRCP: Prolylcarboxypeptidase, FXII: Factor XII, HK: high molecular weight kininogen, ACE: angiotensin convering enzyme. Created with BioRender.

Bradykinin

Bradykinin is a nine amino acid long peptide, liberated from HK when cleaved by kallikrein. The half-life of bradykinin is estimated to be approximately 30 sec in plasma, which is prolonged when ACE inhibitor is administered as this decreases its degradation.[55] Bradykinin signals via the B2R on various cell types. On endothelial cells, bradykinin induces production and release of nitric oxide,[56-57] which will cause vasodilation and hypotension by relaxation of smooth muscle cells. In addition, binding of bradykinin to endothelial cells will cause changes to the cell membrane,[58] leading to capillary leakage, production of prostacyclin,[59-60] as well as release of superoxide.[61] When binding to the B2R on fibroblasts, bradykinin will

induce the release of IL-1ß,[62] IL-6 and IL-8.[12] Bradykinin has been shown to induce histamine-release from mast cells[63] and inflammatory pain when binding to sensory neurons.[64] In the blood brain barrier (BBB), binding of bradykinin will loosen the tight junctions between endothelial cells and increase permeability of the BBB.[65-66]

DesArg^9-bradykinin

DesArg^9-bradykinin shares many of the same properties of bradykinin and arises when arginine is cleaved off of the carboxyterminal of bradykinin. It has a half-life of approximately 600 sec.[67] In the vasculature, desArg^9-bradykinin has various properties. It causes vasodilation by inducing nitric oxide release from fibroblasts and endothelial cells,[68-69] as well as a non-endothelial mediated effect on smooth muscle cells causing them to contract.[70] *In vivo* experiments have shown that mice treated with a B1R antagonist present with an increase in blood pressure, which was reduced when injected with a B1R agonist.[71] In addition, it also causes bronchoconstriction and constriction of smooth muscle cells of the intestines. Indirect evidence suggests that desArg^9-bradykinin, via its receptor B1R, has a stimulating effect on neutrophil activation and recruitment.[72-73] On the other hand, a recent publication showed that B1R KO mice with sepsis exhibit an increased neutrophil migration, lower bacterial load and improved survival rate compared to controls, suggesting that desArg^9-bradykinin may have an inhibitory effect on neutrophil migration and bacterial clearance. In addition, treating wild-type mice with a B1R antagonist in addition to antibiotics, improved survival rates compared to antibiotics alone.[74] As further proof of the double nature of desArg^9-bradykinin, it has been shown to increase prostacyclin production in endothelial cells,[75-76] but it inhibits prostacyclin production in neural glia cells.[77]

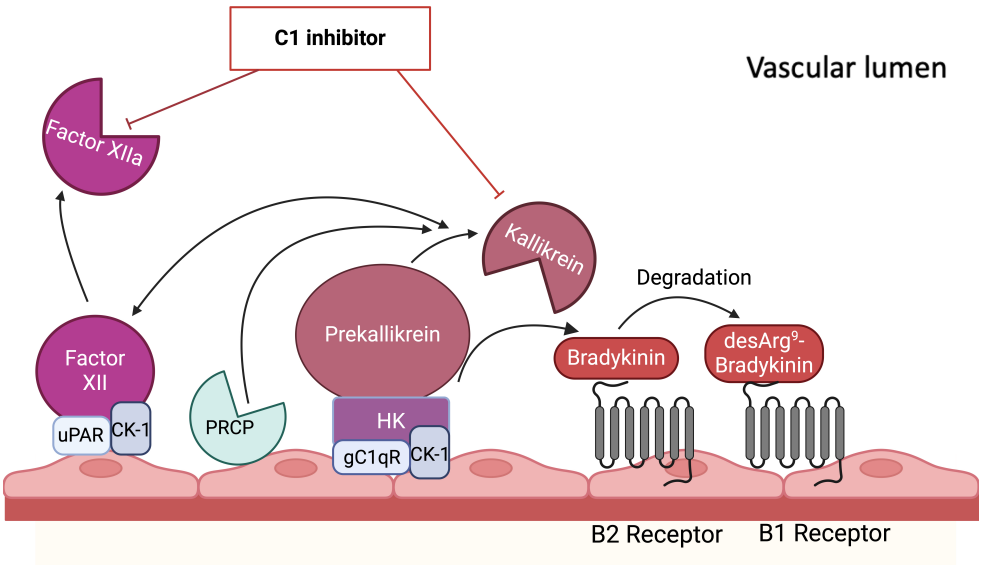

Figure 3. Activation of the kallikrein-kinin system Activation of the kallikrein-kinin system occurs both on the surface of endothelial cells and in plasma. CK-1; Cytokeratin-1, uPAR; urokinase plasminogen activator receptor, gC1qR; gC1q-receptor, PRCP; Prolylcarboxypeptidase, HK; high molecular weight kininogen. Figure originally created by Alexandra Gerogianni using BioRender software, with minor modifications.

Bradykinin 1-7 and bradykinin 1-5

The half-life of bradykinin and desArg^9-bradykinin is short, and degradation renders bradykinin 1-7 (BK1-7) and bradykinin 1-5 (BK1-5). BK1-5 has been suggested to be the most stable form of degradation product.[78] Until recently, degradation products from bradykinin and desArg^9-bradykinin were considered inactive. In 2022, Souza-Silva et al. published data showing that BK1-7 and BK1-5 can induce nitric oxide production and vasodilation both *in vivo* and *in vitro*. The presence of a B1R or B2R antagonist did not affect nitric oxide production *in vitro*, suggesting that this process was not mediated by the two known bradykinin-receptors. No effect was seen on vascular permeability, and only mild effects were seen on nociception.[54]

The kinin receptors

The kinin receptors are expressed on endothelial cells, leukocytes, fibroblasts, smooth muscle cells, neural cells and epithelial cells.[79] Both receptors are G-protein coupled receptors, with seven transmembrane domains whereby the extracellular N-terminal interacts with its ligand, and the intracellular C-terminal is linked to a G-

protein.[79] Ligand binding activates intracellular phospholipase C to generate inositol triphosphate (IP3),[80] which in turn binds to the inositol triphosphate receptor (IP3 receptor) on the endoplasmic reticulum. Activation of the IP3 receptor leads to release of intracellular Ca^{2+}.[81] Ligand binding also activates the phospholipase A_2 signaling pathway, resulting in arachidonic acid production.[82-83] Another known effect of activation of both kinin receptors is activation of endothelial nitric oxide synthase (eNOS)[68] and increased inducible nitric oxide synthase (iNOS),[84] both resulting in nitric oxide production.

The bradykinin 1 receptor

In the healthy state, the B1R is not present on the cell membrane, but stored in the endoplasmic reticulum.[85] Upon stimulation, the B1R is upregulated[86] and transported to the cell membrane.[87] Stimulating mediators can be lipopolysaccharide (LPS),[88] interleukins[89] and ischemia.[90] In addition, B1R has been shown to be induced at the mRNA and protein level in the absence of B2R.[91] Known ligands are $desArg^9$-bradykinin,[79] $desArg^9$-kallidin[79] and PR3-kinin, a 13 amino acid peptide generated by neutrophil-derived proteinase 3 cleaving HK.[92] Ligand binding increases B1R expression and delays its internalization. Since the B1R is not desensitized and internalized upon ligand binding, it produces a prolonged and sustained signal. The B1R is therefore important during chronic inflammation.

The bradykinin 2 receptor

The B2R is constitutively and widely expressed. Bradykinin and kallidin are its agonists. When a ligand binds to the B2R it is desensitized and internalized, generating a transient signal. Activation of the B2R causes a temporary increase of Ca^{2+} [93] and generates a response similar to that described for the B1R, albeit short-lived.

Tissue kallikrein-kinin system

Tissue kallikrein is a member of a gene family consisting of 15 homologous serine proteases. Only one of them (tissue kallikrein, KLK1) has any significant capacity to cleave kininogens.[94] Tissue kallikrein is abundant in most tissues in its inactive form, prokallikrein, and can be activated by plasmin or plasma kallikrein. Activated tissue kallikrein cleaves light molecular weight kininogen, releasing kallidin. Kallidin can be further processed to $desArg^9$-kallidin. In addition, kallidin can be

cleaved into bradykinin by removing a Lys residue from its N-terminal. Kallidin and desArg^{9}-kallidin share affinity for the same receptors as their plasma counterparts, B2R and B1R, respectively. Tissue kallikrein is inhibited by kallistatin.[95]

Inhibitors of the kallikrein-kinin system

C1 inhibitor is the most prominent inhibitor of the kallikrein-kinin system, and it is discussed below. Another important inhibitor is alpha-2 macroglobulin, inhibiting kallikrein and plasmin.[96]

C1 inhibitor

C1 inhibitor is a serine protease inhibitor, and the most important inhibitor of the KKS inhibiting both FXIIa[97] and kallikrein (Figure 4).[98] In addition, it prevents activation of the classical pathway of complement, by inhibiting the activity of C1r and C1s[99] and the lectin pathway by blocking the activity of MASP-1 and MASP-2.[100] In the coagulation system, C1 inhibitor inhibits factor XI[101] and has been shown to both inhibit and enhance generation of thrombin.[102-103] C1 inhibitor also interacts with plasmin of the fibrinolytic system, some report that C1 inhibitor blocks activation of plasmin,[104] and others state that C1 inhibitor forms complexes with plasmin,[105] leading to cleavage of C1 inhibitor, hence C1 inhibitor inactivation. Any inhibitory action by C1 inhibitor leads to its consumption.

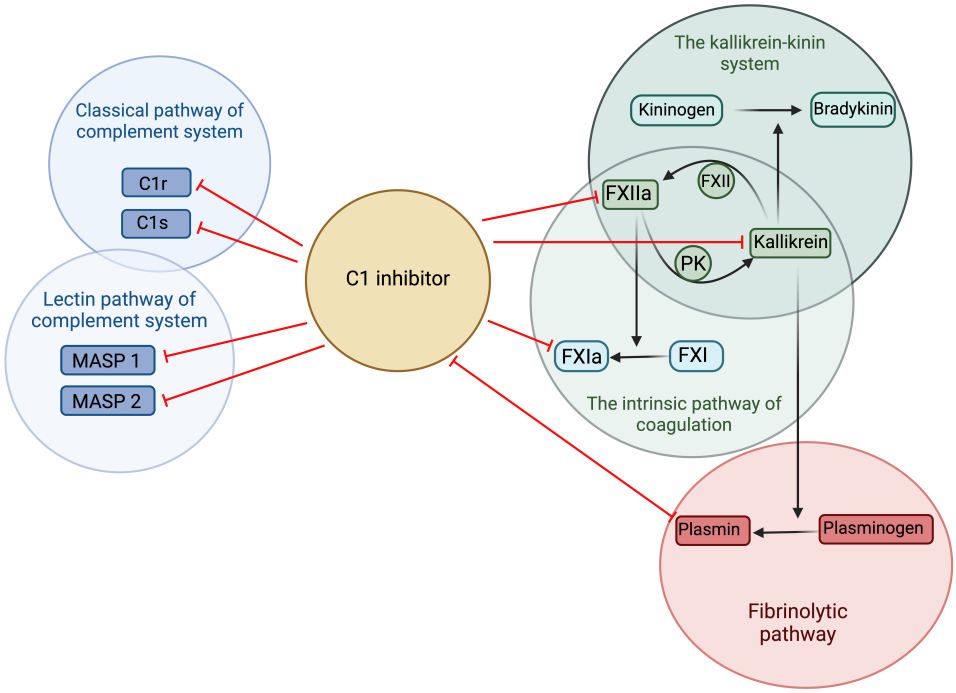

Figure 4. C1 inhibitor and its effects
C1 inhibitor is a serine protease inhibitor circulating in plasma. It inhibits the classical pathway of complement by inhibiting the activity of C1r and C1s, and inhibits the kallikrein-kinin system, in which it blocks the activity of both kallikrein and factor XIIa. It reduces the activity of FXIa in the intrinsic pathway of coagulation, plasmin of the fibrinolytic pathway and MASP 1 as well as MASP 2 of the lectin pathway of complement. Created with BioReder.

The kallikrein-kinin system in health

The KKS plays an important part in the homeostasis of the vascular environment. The system helps control blood pressure and mediate inflammatory reactions, through its ability to cause vasodilation and increase vascular permeability, and its ability to induce prostacyclin production.[75] The kallikrein-kinin system is also implicated in nociception.[106]

The kallikrein-kinin system in disease

An imbalance between kallikrein-kinin system activation and its natural plasma inhibitors may result in an uncontrolled activation and excessive release of

bradykinin and its metabolites. Increased KKS-activation has been described for hereditary and autoimmune diseases such as hereditary angioedema,[6] inflammatory bowel disease,[107] systemic lupus erythematosus,[108] vasculitis[109] (further described below), rheumatoid arthritis[110] and sickle cell anemia.[111] KKS-activation is also seen in traumatic injury,[112] sepsis,[113] severe COVID-19 infection[114] and the development of acute respiratory distress syndrome (ARDS).[115]

During myocardial or renal ischemia[116] the KKS is activated and can exert a protective effect.[117] The actions providing tissue protection include dilation of collateral blood vessels, lowered oxidative stress[118] and angiogenesis.[119] Kinins have been suggested to reduce the infarct size during acute cardiac infarction,[117, 120] and to have a preventive effect on cardiac dysfunction and eventually death in the aftermath.[91]

Treatments

There are many commercially available modulators of the KKS on the market, see Table 1. All but one (Human urinary kallidinogenase, only approved on the Chinese market) are inhibitors of the system. Most are used for treatment of hereditary angioedema, as described below, but Ulinastatin is approved for treatment of sepsis[121] as well as pancreatitis[122-123] and Aprotinin is used during surgery to reduce blood loss.[94] There are many ongoing studies, both on new drug candidates, but also to expand the indication of already approved treatments.

Synthetic bradykinin-analog peptides

Synthetic bradykinin-like peptides are typically designed to be more stable and resist metabolism in plasma and tissues. A few are being tested in preclinical settings. Labradimil is a peptide agonist of the B2R, which was developed to weaken the blood brain barrier to increase efficiency of chemotherapy in primary brain tumors. It was tested in a clinical trial but discontinued.[124] To this day no B1R or B2R agonist has been approved for clinical use.

Table 1. Approved treatments targeting the kallikrein-kinin system

DRUG	CLASSIFICATION	INDICATION	REF
C1 inhibitor			
Human C1 inhibitor	Inhibitor replacement	HAE (prophylaxis and acute phase)	125
Conestat alfa	Recombinant inhibitor replacement	HAE (acute phase)	126
Factor XII inhibitor			
Garadacimab	Monoclonal antibody	HAE (prophylaxis)	127
Kallikrein modulator			
Ecallantide	Specific inhibitor	HAE (acute phase)	128
Lanadelumab	Monoclonal antibody	HAE (prophylaxis)	129
Berotralstat	Specific small molecule inhibitor	HAE (prophylaxis)	130
Aprotinin	Serine protease inhibitor	Reduction of perioperative bleeding	123
Ulinastatin	Serine protease inhibitor	Sepsis, pancreatitis	121
Human urinary kallidinogenase	Replacement therapy	Acute ischemic stroke	131
Bradykinin 2 receptor antagonist			
Icatibant	Antagonist	HAE (acute phase)	132

HAE: Hereditary angioedema

The complement system

The complement system is a cascade system of plasma proteins and an important part of the innate immune system. The main functions of the system are opsonization, chemotaxis and cell lysis, contributing to local inflammation and removal of pathogens or unwanted cells.

The complement system can be activated through three different pathways, the classical, the lectin and the alternative pathway. Although the initiating stimuli will vary, all pathways converge at the amplification loop of the alternative pathway and form a common C5 convertase. Formation of the C5 convertase will activate the terminal pathway with the formation of the membrane attack complex (MAC, C5b-9) (Figure 5).

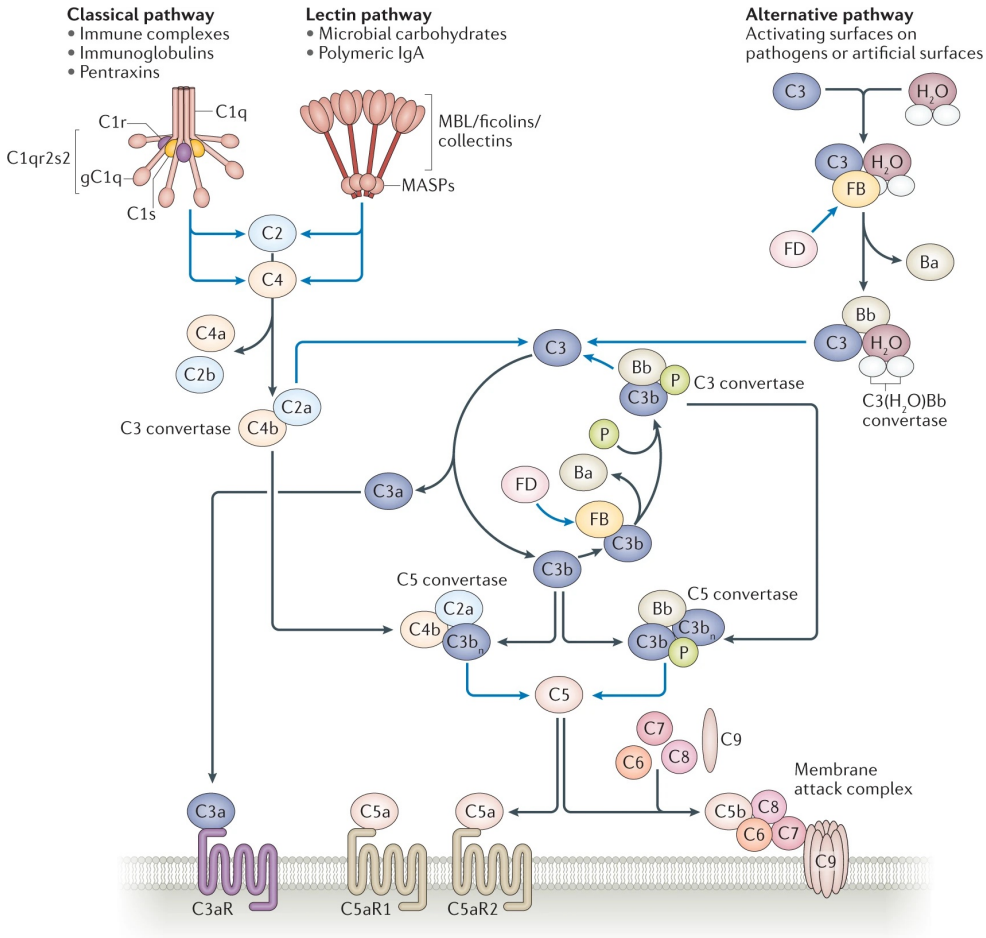

Figure 5. The complement system

The complement system is activated through one of the initiating pathways: classical, lectin or alternative. All three pathways converge at the terminal pathway and ultimately lead to C5b-9 formation. In the process anaphylatoxins C3a and C5a are released, inducing an inflammatory effect by binding to their respective receptors; the C3a receptor (C3aR) and the C5a receptors 1 and 2 (C5aR1 and C5aR2). Blue arrows indicate enzymatic activity, and black arrows show binding. Figure from reference [133]

The classical pathway

The classical pathway of complement is activated when the globular head of C1q binds to the Fc-region of an antibody-antigen complex or to an acute-phase protein, such as C-reactive protein (CRP).[134] C1q associates with 2 molecules of C1r and of C1s, forming C1qr2s2 (Figure 5). C1r and C1s cleave C2 into C2a and C2b and C4 into C4a and C4b. C4b and C2a form the C3 convertase of the classical pathway,

C4bC2a, cleaving C3 into C3a and C3b. The cleavage of C3 is amplified by the C3 amplification loop. When additional C3b molecules bind to the C3 convertase, the C5 convertase ($C4bC2aC3b_n$) is formed. The C5 convertase cleaves C5 into C5a and C5b, and the terminal pathway is initiated.

The lectin pathway

The lectin pathway is activated when mannose-binding lectin (MBL)/collectins[135] or ficolins[136] interact with pathogen-specific carbohydrates, activating MBL-associated serine proteases (MASPs) -1 and -2. MASP-1 and -2 cleave C4 and C2, and the lectin pathway continues in a similar fashion to that of the classical pathway (Figure 5).

The alternative pathway

The alternative pathway is spontaneously activated on surfaces when C3 is hydrolyzed to $C3(H_2O)$, which can bind to factor B. When bound to C3 factor B is cleaved by factor D into Bb and Ba. Bb forms a complex with $C3(H_2O)$, creating the initial C3 convertase, $C3(H_2O)Bb$. This C3 convertase cleaves C3 into C3a and C3b. C3b binds to factor B, which again is cleaved into Ba and Bb, and the C3 convertase (C3bBb) is formed. C3bBb has a short half-life but is stabilized by properdin.[137] C3 convertase cleaves C3 into C3a and C3b continuously via the amplification loop, and when one or more additional C3b molecules are bound to the C3 convertase, the C5 convertase is formed.

The terminal pathway

The terminal pathway is initiated when the C5 convertase is formed. C5 convertase cleaves C5 into C5a and C5b. C5b will bind to C6 and C7. This will change the complex from hydrophilic to amphiphilic and C7 will serve as the anchor attaching the complex to the recipient cell.[138] C5b-7 will bind C8 and a multimer of C9s forming a pore through the cell membrane (Figure 4), hence the name membrane attack complex (MAC). The membrane bound C5b-9 will cause osmotic lysis of the cell if a sufficient number of complexes are inserted.[139] If sublytical amounts of C5b-9 are incorporated into the membrane, it will cause cell activation.[140]

Functions of the complement system

The most well-known functions of the complement system include opsonization of target cells, recruitment and activation of leukocytes and mast cells by anaphylatoxins and cell lysis by C5b-9 formation (Figure 6). These properties are all essential for pathogen eradication. In addition to being a first line defense against microbes, emerging evidence suggests that complement has an important function in cellular homeostasis, which will be discussed below.

Opsonization

In addition to contributing to the formation of the C3 convertase, C3b opsonizes foreign or harmful substances, such as pathogens, immune complexes and apoptotic cells. C3b deposition serves as a first-line defense leading to rapid eradication of harmful elements. C3b can be further processed to iC3b. C3b binds primarily to complement receptor 1 whereas iC3b has a stronger affinity for complement receptor 3, both present on neutrophils and both leading to phagocytosis.[141-142]

Anaphylatoxins

C3a and C5a are potent anaphylatoxins produced when the complement cascade is activated. They exert their effect by binding to their respective receptors, the C3a receptor (C3aR) and the C5a receptors 1 and 2 (C5aR1 and C5aR2). Both the C3aR and C5aR1 are G-protein couple receptors and lead to Ca^{2+}-mobilization in the target cell,[143] whereas the C5aR2 lacks the ability to couple a G-protein and signals thru β arrestin.[144]

The C3a receptor is present on activated T-cells,[145] neutrophils,[146] monocytes[146] and mast cells.[147] In addition to being present on immune cells, the C3aR has been demonstrated in epithelial and smooth muscle cells of the lungs[148-149] and in neurons.[126] The C5aR1 is present on leukocytes,[150] mast cells,[151] macrophages[152] and granulocytes, as well as hepatic cells,[153] lung epithelial[153] and endothelial cells.[154] Ligand-binding induces chemotaxis,[147, 155] degranulation of neutrophils,[156] eosinophils[157] and mast cells[158] and cytokine production by monocytes.[156] In addition, both C3a and C5a (via C5aR1) have been shown to stimulate neutrophils to produce neutrophil extracellular traps (NETs).[159-160]

The C5aR2 is less well studied than the C5aR1, but has been found to coexist with the C5aR1, albeit at lower levels.[161] It has been suggested to function as a decoy receptor for C5a, removing free C5a from the extracellular space.[162]

Soluble C5b-9

Soluble C5b-9 (sC5b-9), which has not been incorporated into the cell membrane, functions as a biomarker of complement activation. It has been shown to be elevated during vasculitis,[163] sepsis[164] and atypical hemolytic uremic syndrome (aHUS).[165] Levels of sC5b-9 do not correlate with deposition of C5b-9 in kidneys.[166] sC5b-9 leads to activation of a variety of human cells, such as lymphocytes, endothelial cells and smooth muscle cells.[167] Microglia have been demonstrated to be activated when incubated with the components of C5b-9 leading to cytokine release.[168] In endothelial cells, sC5b-9 induce cellular proliferation and migration, promoting angiogenesis.[169]

Cell lysis

When C5b-9 is incorporated into the cell membrane of a pathogen or a host cell it can cause the recipient cell to lyse. Cell lysis occurs when the cell fails to maintain its osmotic pressure. Human cells are normally protected from damage caused by complement through multiple complement regulators, discussed below. Erythrocytes, however, are relatively susceptible to lysis induced by C5b-9.

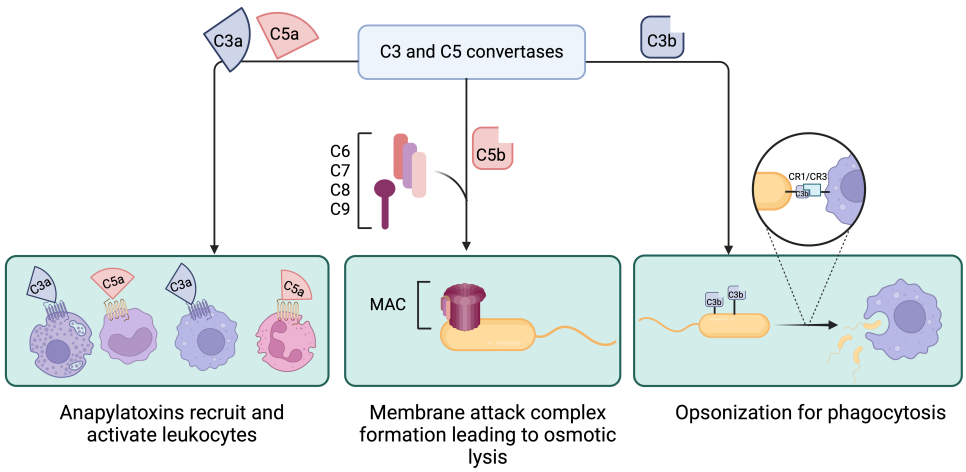

Figure 6. Functions of the complement system.
Activation of the complement system ultimately leads to inflammation and abolishment of pathogens.These effects are achieved by metabolites of C3 and C5 cleavage, i.e. the anaphylatoxins C3a and C5a, the opsonin C3b and C5b. MAC: membrane attack complex, CR1: complement receptor 1, CR3: complement receptor 3. Created with BioRender.

Complement regulators

Excessive activation of the complement system can cause harm to host cells. To avoid this, the system is tightly regulated by several cell-bound and fluid phase complement inhibitors, presented in Table 2. There is only one positive regulator of the complement system, properdin, which binds to the C3 convertase of the alternative pathway and stabilizes it.

Table 2. Regulators of the complement system

REGULATOR	PATHWAY	LOCALIZATION	FUNCTION	REF
Properdin	Alternative	Soluble	Binds to and stabilizes C3bBb	170
C1 inhibitor	Classical	Soluble	Binds to C1r and C1s, inhibiting their complex formation, binds MASP 1 and 2	104
Factor H	Alternative	Soluble	Dissociates C3bBb, cofactor for factor I, participates in host cell recognition	171
C4 binding protein	Classical and Lectin	Soluble	Promotes dissociation of C4 convertase, cofactor for factor I	172
Factor I	Classical, Lectin and Alternative	Soluble	Inactivates C3b and C4b together with cofactors	173
MCP (CD46)	Classical, Lectin and Alternative	Cell-bound	Cofactor for factor I	174
DAF (CD55)	Classical, Lectin and Alternative	Cell-bound	Supports dissociation of C3 and C5 convertases	175
Complement receptor 1 (CR1, CD35)	Classical, Lectin and Alternative	Soluble and cell bound	Regulates C3 degradation and cofactor for factor I	176
Clusterin	Terminal	Soluble	Inhibits MAC formation	177
Vitronectin	Terminal	Soluble	Inhibits MAC formation	178
Protectin (CD59)	Terminal	Cell-bound	Inhibits MAC formation	179

MCP: membrane cofactor protein, DAF: decay-accelerating factor

Intracellular complement – the complosome

Historically, complement proteins were considered to defend the host from intruding pathogens in the circulation. Recently, however, the complement system was also found to take part in basic cellular functions. It has been shown to contribute to cell metabolism,[180] gene transcription[181] and autophagy seen in pancreatic ß-cells.[182] Many cell types produce complement proteins in addition to hepatocytes, such as neutrophils,[183] monocytes,[184] epithelial cells,[185] T-cells,[186]

adipocytes[187] and endothelial cells.[188] In response to cytokine stimulation, C3 has been found to be produced and secreted by respiratory epithelial cells, as well as internalized from the extracellular compartment, and in the same study internalized C3 protected the cell from stress-induced death.[185] In contrast to this, $CD4^+$ T cells have been shown to relocate intracellular C3 to their surface in response to activation, engaging the C3aR. In the kidney, glomerular epithelial, mesangial and endothelial cells and renal tubular cells have been shown to produce complement components.[188]

Complement activation in disease

An imbalance between complement activation and regulation can cause disease. The imbalance can be due to deficiencies or dysfunctional complement components, or an excessive trigger such as severe tissue damage. Complement activation is seen in atypical hemolytic uremic syndrome (aHUS),[189] C3 glomerulopathy,[190] age-related macular degeneration,[191] SLE[192] and vasculitis.[193] In addition, increased complement consumption is seen in sepsis,[194] transplant rejection[195] and COVID-19 infection.[196]

Treatment

In 2007, Eculizumab was the first approved treatment specifically inhibiting the complement system. During the following years several more treatments have been approved for clinical use, see Table 3. Most drugs were approved for treatment of atypical hemolytic uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH), but ongoing studies are investigating complement modulation in COVID-19 infection,[197-198] age-related macular degeneration (AMD)[199] and C3 glomerulopathy.[200] Avacopan is the only commercially available C5aR1-inhibitor and has been found to improve time to remission and renal outcomes in patients with antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis.[201-202] Both C3 and C5[203] inhibition have been tested for treatment of amyotrophic lateral sclerosis (ALS) in clinical trials, but failed to slow functional decline. As several severe diseases are associated with an excessive complement activation, it is likely that additional complement altering drugs will be approved for other diseases.

C5 antibodies, which block the terminal pathway, are the most well-studied among complement-modulators, but they are very expensive and expose the patient for an increased risk of encapsulated bacterial infection. Therefore, there is still a need for

additional treatments, targeting other components of the system and/or their activation.

Table 3. Approved drugs targeting the complement system.

DRUG	CLASSIFICATION	INDICATION	REF
C1 inhibitor			
Human C1 inhibitor (multiple producers))	Inhibitor replacement	HAE (prophylaxis and acute phase)	125
Conestat alfa	Recombinant inhibitor replacement	HAE (acute phase)	126
C1s inhibitor			
Sutimlimab	Antibody	Reduced need for transfusion in patients with cold agglutinin disease	204
C3 inhibitor			
Pegcetacoplan	Peptide	PNH and geographic atrophy	205
Factor B inhibitor			
Iptacopan	Small molecule	PNH	206
Factor D inhibitor			
Danicopan	Small molecule	PNH	207
C5 inhibitor			
Eculizumab	Antibody	PNH, aHUS, MG, NMOSD	208
Ravulizumab	Antibody	PNH, aHUS	209
Pozelimab	Antibody	PLE	210
Crovalimab	Antibody	PNH	211
Zilucoplan	Peptide	MG	212
Avacincaptad pegol	Aptamer	Geographic atrophy	213
C5aR1 antagonist			
Avacopan	Small molecule	ANCA-associated vasculitis	214

PNH: Paroxysmal nocturnal hemoglobinuria, aHUS: atypical hemolytic uremic syndrome, MG: Myasthenia gravis, NMOSD; neuromyelitis optica spectrum disorder, PLE: CD55-deficient protein-losing enteropathy, ANCA; Anti-neutrophil cytoplasmic auto-antibody

Interactions between the complement and the kallikrein-kinin system

Traditionally, the different cascade systems controlling the homeostasis of the vasculature have been viewed as separated compartments. Slowly, this conception has been challenged and more interactions between the different systems are being elucidated. It has long been known that the classical pathway of complement and the KKS share the same inhibitor, C1 inhibitor.[215] Kallikrein has been shown to cleave both C3 and factor B to yield active metabolites, with the potential of forming functional C3 convertases.[216-217] Kallikrein has also been suggested to cleave C5, generating a C5a-like fragment.[218-219] These are all actions with an activating potential of the complement system. On the other hand, kallikrein cleaves C5b[220] and iC3b,[221] which could have a suppressive effect on complement actions, reducing the C5 convertase formation and complement opsonization.

FXIIa is known to activate the classical pathway of complement by activating C1r as well as C1s.[222] C1q of the classical pathway of complement bind to the same receptor as HK on endothelial cells, namely gC1qR. Most studies demonstrate the KKS altering the activation of complement, and not the other way around. However, Bossi et al. showed that sC5b-9 induced vascular permeability both *in vitro* and *in vivo*, mediated through bradykinin-release.[223] Taken together, the two systems seem to be tightly linked, and there are probably many more interactions that have not yet been revealed. Some novel aspects are addressed in this thesis.

The kidney and vasculature in inflammatory diseases

The kidneys are two bean-shaped organs located on each side of the back of the abdomen. They are responsible for filtrating blood and removing waste into the urine, maintaining the pH-balance as well as the water and electrolyte balance. In addition, the kidney produces the hormones renin and erythropoietin, increasing blood pressure and stimulating production of red blood cells.

The human kidney consists of approximately 1 million nephrons. The nephron is the functional unit of the kidney where blood is filtered, reabsorbed, secreted and excreted, resulting in the production of urine. The kidney is one of the most well-vascularized organs in the body and is therefore susceptible to vascular disease.

This thesis will focus on the kidney in inflammatory diseases.

Functions of the healthy kidney

The filtration of blood takes place in the glomerulus, consisting of a network of small capillaries, surrounded by the Bowman's capsule. This unit is called the renal corpuscle. The capillaries of the glomerulus consist of fenestrated endothelial cells, separated from the podocytes by a shared basement membrane, composing the filtration barrier. Water, small molecules and ions can pass through the filtration barrier into the Bowman's space. From here the fluid enters the tubule, where reabsorption of glucose, water and ions takes place. The tubule is surrounded by a network of capillaries. This is also where the secretion occurs, releasing molecules from the peritubular capillaries into the tubular lumen. The final step is the excretion, where the fluid of the tubule enters the collecting duct system, further to the ureter and the urinary bladder. This is how urine is generated.

The vasculature

The heart, arteries, capillaries and veins constitute the cardiovascular system (Figure 7), which transports blood and its content to organs, delivering oxygen and other nutrients. Oxygenated blood enters the heart from the pulmonary circulation and is transported from the heart into the aorta. The aorta divides into large arteries, further dividing into smaller and smaller vessels. The finest vessels are called capillaries, creating a capillary network which delivers oxygen and nutrients to organs. Capillaries then converge into veins, transporting the oxygen-poor blood back to the lungs. In the lungs, the blood is reoxygenated before it re-enters the heart.

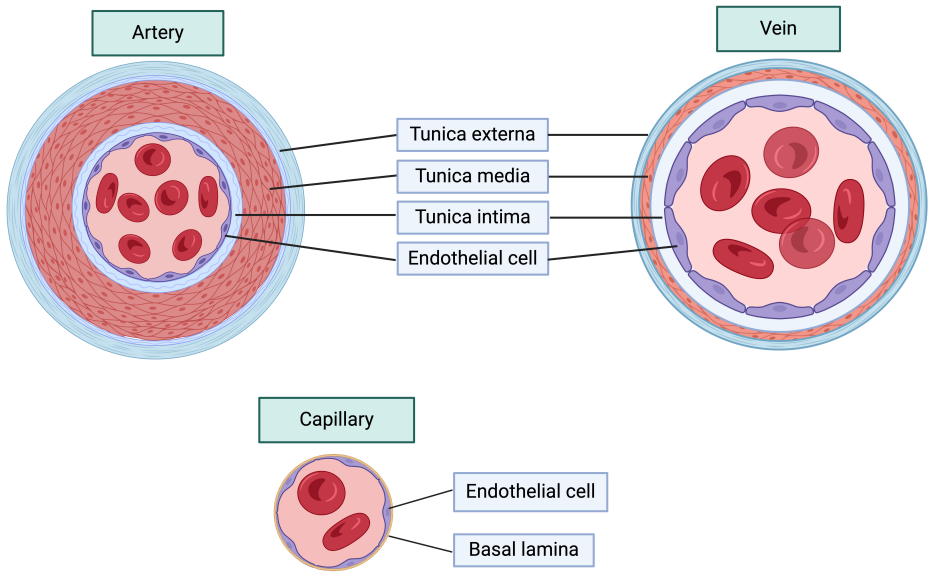

Figure 7. Schematic picture of an artery, vein and capillary.
All vessels have an inner lining of endothelial cells, in direct contact with the vascular lumen. Below the endothelial layer is the tunica intima, tunica media and tunica externa of arteries and veins. Tunica media consists of smooth muscle cells that can contract to alter the diameter of the vascular lumen, hence affecting the blood flow. The smooth muscle layer is markedly thicker in arteries compared to veins, mirroring their ability to contract. Created with BioRender.

The endothelial cell

The endothelium constitutes the innermost layer of the blood vessel. Endothelial cells possess the function to proliferate and migrate, making them essential in the

repair of vascular damage. In addition, these functions are essential for angiogenesis. Angiogenesis is stimulated by vascular endothelial growth factor (VEGF) released from hypoxic cells[224] or activated platelets.[225]

The endothelium functions as a barrier between the blood and underlying tissues and has the capacity to respond to external stimuli. The cells can change in number, size and arrangements. Rearrangement of the endothelial cell layer can increase vascular permeability, causing migration of cells, fluids, ions and molecules to the extravascular space. Activated endothelial cells release cytokines promoting inflammation,[226] express E-selectin causing leukocyte attachment and transportation[227] and release nitric oxide[228] causing smooth muscle cells to relax, hence inducing vasodilation. Furthermore, activated endothelial cells promote thrombus formation by releasing von Willebrand factor mediating platelet adhesion and aggregation,[229] and by expressing P-selectin, causing platelet activation and transportation[230] as well as leukocyte rolling.[231] P-selectin has also been shown to activate complement on endothelial cells during hemolysis.[232]

Extracellular vesicles

Extracellular vesicles (EVs) are small vesicles released from the membrane of a variety of cells, including endothelial cells, platelets, erythrocytes and leukocytes. They carry content from their parent cell, such as membrane receptors, proteins, mRNA and enzymes. Extracellular vesicles are released from resting cells, but more so upon cellular activation. Their content reflects the state of their parent cell as well as the factor triggering their release.

The release of EVs was initially viewed as a process of eliminating unwanted content from the cell but are now considered an important part of intercellular communication.

EVs can be divided into different groups depending on size and pattern of release. The smallest are exosomes (30-100 nm in diameter), followed by microvesicles (0.1-1 μm), which will be termed extracellular vesicles (EVs) throughout this thesis. Some studies include apoptotic bodies (1-5 μm). However, newer studies suggest that there is a significant overlap in size,[233] and a more correct way of subdividing them may be through the different mechanisms by which they are released.

Release

Exosomes are released through exocytosis, whereby an early endosome through inward budding of its membrane generates several exosomes, creating a

multivesicular body. The multivesicular body fuses with the cell membrane and releases its content to the extracellular space (Figure 8).[234]

The EV is created through ectocytosis, by budding of the cell membrane of the parent cell. This is a complex process consisting of extracellular stimuli, cell membrane dynamics, intracellular changes in Ca^{2+} concentration and reformation of the cytoskeleton.[235] An increase in Ca^{2+} will activate intracellular enzymes which will rearrange the cell membrane, exposing phosphatidylserine on its external surface, and rearrange the cytoskeleton. This will cause the cell membrane to bend and subsequently EVs will bud off (Figure 8).[236]

Apoptotic bodies are released from the cell during the late process of programmed cell death.

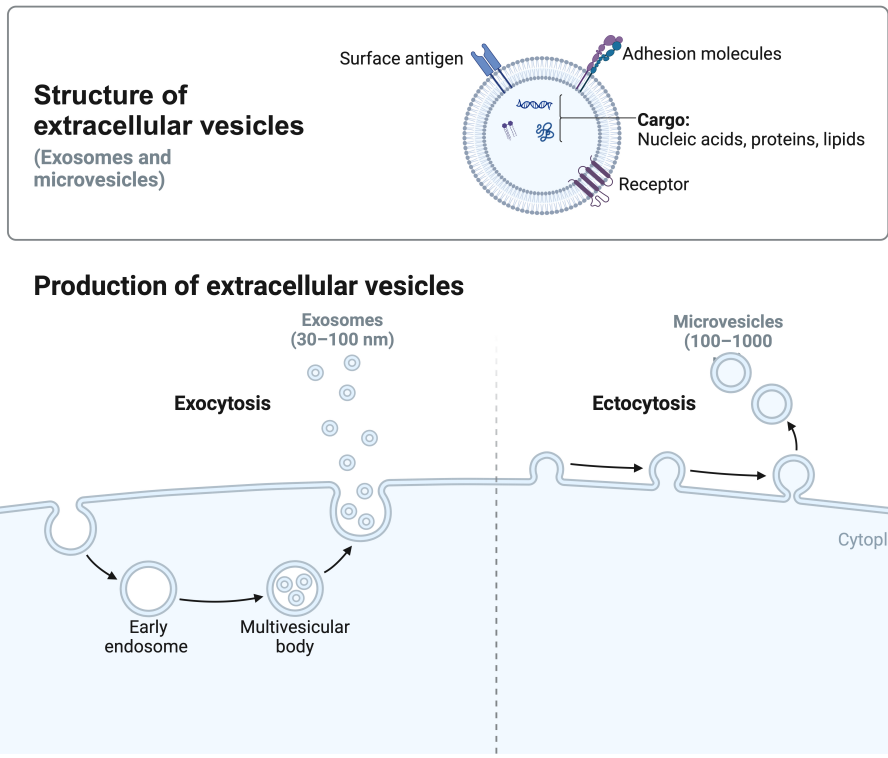

Figure 8. The release of extracellular vesicles. Reprinted from BioRender.com

Extracellular vesicles in inflammation

Circulating EVs are increased during inflammatory diseases such as vasculitis,[11] systemic lupus erythromatosus[237] and rheumatoid arthritis.[238] Metabolic disorders

such as acute coronary syndrome,[239] hypertension[240] and diabetes[241] also display increased release of EVs.

EVs have the potential to deliver their cargo to neighboring cells as well as to more distant places. EVs have been found to contain inflammatory mediators such as tumor necrosis factor, interleukin-1ß, C-X-C ligand (CXCL)-2 and -8.[242] In addition, B cell-derived EVs have been shown to transport major histocompatibility complex (MHC),[243] suggesting a role in the antigen specific immune response. Leukocyte-derived EVs have been shown to stimulate endothelial cells to release interleukin-6 and -8,[244] as well as upregulate the expression of E-selectin,[245] promoting leukocyte adhesion and transportation. In addition, platelet-derived EVs induce the expression of intracellular cell adhesion molecule-1 (ICAM-1) on endothelial cells, further promoting cell adhesion to the endothelium.[246] In addition, EVs have been shown to transfer the chemokine receptor CCR5 between cells,[247] important for the internalization of HIV-1. EVs are also well-studied in cancer, and transfer of the oncogenic epidermal growth factor receptor (EGFR)vIII was shown between glioma cells, promoting oncogenic actions.[248]

In sepsis, EVs have been demonstrated to have both protective and damaging effects to host cells. For example, EVs from platelets of sepsis patients were shown to generate reactive oxygen species, inducing cell death in endothelial cells,[249] whereas EVs released from neutrophils exhibit antibacterial properties.[250]

Potentially, EVs could be used to treat various diseases.

Vasculitis

Vasculitis is a group of autoimmune diseases, characterized by inflammation in and around the vessel wall causing a narrowed lumen and decreased blood flow as well as thinning of the wall leading to increased vascular leakage and possibly wall rupture with hemorrhage as a result.[251] Vasculitis may be systemic and can cause damage to many different organs, such as the kidneys,[252] joints,[253] skin[254] and lungs.[255] The symptoms can be mild and brief, or severe and life-threatening, causing destruction of the organs involved.[251] The etiology is multifactorial, and genetic, autoimmune and environmental aspects as well as infectious triggers are of importance for the development of disease.[256-257] Vasculitis can affect both children and adults. It is classified based on the size of the affected vessel (Figure 9). In this thesis small vessel vasculitis, particularly IgA vasculitis, granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) will be discussed.

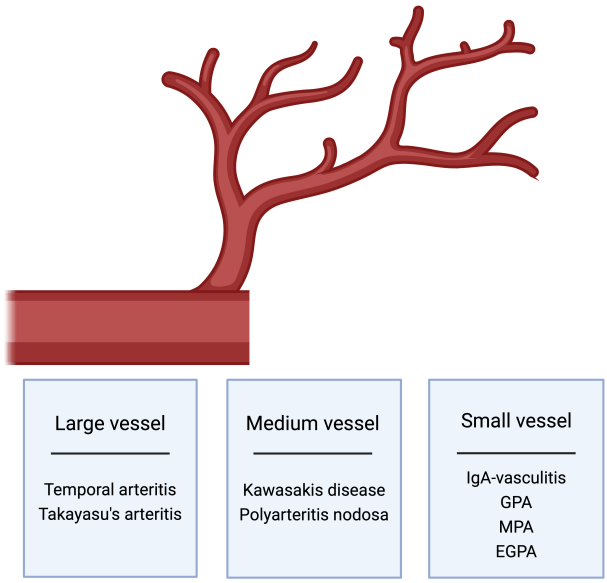

Figure 9. Classification of vasculitides depending on the size of the predominantely affected vessel. GPA: granulomatosis with polyangiitis, MPA: microscopic polyangiitis, EGPA: Eosinophilic granulomatosis with polyangiitis. Created with BioRender.

IgA vasculitis

IgA vasculitis, formerly known as Henoch-Schönlein's purpura, is the most common form of vasculitis affecting children. The incidence of IgA vasculitis is seasonal, peaking during periods of respiratory and gastrointestinal infections. Typically, IgA vasculitis debuts shortly after an upper respiratory tract infection,[258] further suggesting that infections may trigger the development. Patients presents with cutaneous purpura predominantly on the lower extremities and one or more of the following findings: abdominal pain/bleeding, arthritis/arthralgia, renal dysfunction and/or immune deposits of IgA around small vessels.[259]

Pathology

The histological examination of skin biopsies of IgA vasculitis, exhibit deposition of IgA, a complex of C4+C3c+C3d and C3c in the vessel wall.[260] In addition, the vessel wall is thickened, with infiltrates and partly degraded leukocytes, referred to as leukocytoclastic vasculitis, and the surrounding tissue contains erythrocytes constituting purpura.[261]

In the kidneys, mesangial proliferation and leukocyte infiltration is seen. In more severe cases, crescent formation can be present.[262] In addition, IgA-deposits in the mesangium can be visualized by immunofluorescence.[263]

Complement deposition of C3,[264] properdin[264] and C5b-9[265-266] can also be present in glomerular mesangium. Properdin deposition suggests that the alternative pathway of complement is involved. In addition, the lectin pathway is considered to be activated, demonstrated by the presence of deposits of C4d (in the absence of C1q)[267] and MBL as well as MASP-1.[268]

KKS has also been shown to be activated in patients with IgA vasculitis, demonstrated by increased HK-degradation, high plasma bradykinin-levels and the presence of kinin peptides in kidney biopsies.[7]

Prognosis

IgA vasculitis is often mild and self-limiting but some patients, most commonly adults, may develop severe glomerulonephritis.[269] Relapse is common, especially within the first year.

Treatment

Most patients with IgA vasculitis do not require treatment to recover. In more severe cases with renal involvement, corticosteroids, immunosuppressants and antihypertensive treatment is administered, even though the evidence is weak.[270]

Anti-neutrophil cytoplasmic antibody associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a rare group of diseases, preferentially affecting adults. It can be subdivided into granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA) with varying manifestations.

Many patients presents with flu-like symptoms, such as fever, headache and arthralgia.[271] Symptoms originate from the affected organ, commonly kidneys, skin, lungs and gastrointestinal tract. GPA commonly affects the upper airways, lungs and kidneys, whereas MPA does not typically affect the upper airways.[271]

Pathogenesis

Most patients with GPA and MPA are positive for ANCA in blood samples, and about 30% of patients with EGPA are positive. ANCA are antibodies directed against antigens found in the cytoplasm of neutrophils, either around the nucleus or spread throughout cytoplasm. There are two different types of ANCA, ANCA directed against proteinase 3 (PR3) or against myeloperoxidase (MPO). GPA is mostly associated with PR3-ANCA,[272] whereas MPA and EGPA are predominantly positive for MPO-ANCA.[273]

Neutrophils are believed to be essential for the pathogenesis of AAV. Neutrophils pre-stimulated with C5a,[274] LPS or tumor necrosis factor[275] relocate PR3 or MPO to their cell membrane, making them available for circulating ANCAs.[276] ANCA

binding to neutrophils have been shown to activate the cell, causing them to degranulate,[275] release reactive oxygen species,[275] induce NETosis[277] and attach to endothelial cells. The degranulation of neutrophils is harmful for endothelial cells.

The importance of neutrophils in the pathogenesis of AAV was further established in 2005 when Xiao et al. published that a mouse model depleted of neutrophils was completely protected from developing AAV.[278]

Complement is activated during AAV, and plasma levels of C3a, Bb, C5a and C5b-9 are elevated during active AAV.[163] The presence of Bb in plasma indicates that the alternative pathway is activated. C3, factor B and C5b-9 are deposited in glomeruli of patients with renal involvement.[193] ANCA-activated neutrophils relocate C5aR1 to their cell membrane. In addition, ANCA-activated neutrophils activates the complement system,[274] releasing more C5a. C5a has the ability to both recruit neutrophils through its chemoattractant properties, and to prime them to relocate of MPO or PR3 to their cell membrane,[274] hence creating an amplification loop of inflammation. *In vivo*, mice receiving bone marrow depleted of the C5a receptor were protected from developing AAV.[274]

Similar to IgA vasculitis, HK degradation and increased levels of bradykinin in plasma were reported in patients with AAV.[7] A kidney biopsy from a patient with GPA was examined for the presence for kinins and exhibited positive staining.[7] Klein et al. found an upregulation of the B1R in kidney biopsies from patients with AAV and IgA vasculitis. In addition, a mouse model with nephrotoxic serum-induced glomerulonephritis, treated with a B1R-antagonist, was protected against renal dysfunction and exhibited reduced renal pathology.[279] Taken together, these results indicate that the KKS is activated during AAV.

Histopathology

Vascular lesions are typically characterized by necrotizing inflammation and are described as pauci-immune, as they lack immune complex deposits. Leukocytoclasia and neutrophil extravasation is commonly seen. In kidney biopsies, necrotizing crescentic glomerulonephritis is a key finding. In addition, interstitial fibrosis and tubular atrophy are common findings. In GPA and EGPA granulomas, consisting of aggregations of immune cells are present. Neutrophils are typically present in the granulomas.

Prognosis

The 5-year survival rate in adults with AAV receiving adequate treatment in Sweden was established to 70%, with no difference between groups positive for MPO- or PR3-ANCA.[280] The same study described that 38% of the patients with positive MPO-ANCA developed end stage renal disease (ESRD) compared to 15% positive for PR3-ANCA. Without adequate treatment the mortality of AAV was shown to be approximately 60% in 6 months, and 80% in 1 year.[281]

Treatment

The goal of treatment is to achieve remission. This can be achieved by different regimes. Traditionally, cyclophosphamide in combination with high dose corticosteroids were used, and this regime has been shown to reduce mortality rates, although the risk of relapse remains high.[282] Rituximab, a monoclonal antibody targeting B cells, has been shown to be successful in treating AAV.[283] Avacopan is the newest member of approved treatments for AAV, and the only approved drug targeting the complement system. In a randomized controlled trial, avacopan proved to be non-inferior regarding inducing remission and superior in sustaining remission at 1 year follow-up compared to prednisolone treatment.[284] In addition, it has been proved to improve renal function[285] and reduce the need for adjuvant corticosteroids.[284]

Hereditary angioedema

Hereditary angioedema (HAE) is an autosomal dominant genetic disease, approximately 50% of the offspring of an affected individual will inherit the disease. It is characterized by episodes of recurrent swelling of subcutaneous and mucosal tissues. Symptoms vary in severity, and in the most severe cases swelling of the larynx may occur. This is a life-threatening event and can require acute intervention, such as rushed administration of drugs and in worst cases the need to secure the airway by invasive procedures.

Most common presentations include swelling of the gastrointestinal (GI) tract and skin. The patient with GI-manifestations will present with nausea, pain, vomiting and diarrhea. Skin edema often affects the face. The symptoms develop over a couple of hours and last 2-5 days.[286]

Pathogenesis

Most often, HAE is caused by low levels of, or a dysfunctional, C1 inhibitor, due to a gene mutation in the *SERPING1* gene encoding C1 inhibitor.[287] This causes an imbalance between the KKS and its main inhibitor. Other mutations has been described, affecting FXII (*F12*),[288] plasminogen (*PLG*)[289] and angiopoietin 1 (*angpt1*).[290-291] Recently, a mutation to the kininogen gene (*KNG1*),[292] changing the cleavage site of bradykinin, and a mutation to the gene coding for Myoferlin (*MYOF*)[293] were detected. Mutations affecting the genes encoding C1 inhibitor, FXII and plasminogen all causes an increased production of bradykinin. Mutations to the *KNG1* have also been also suggested to cause increased bradykinin-levels, not due to increased production but due to prolonged half-life of the mutated bradykinin. Mutations to angiopoietin 1 and myoferlin do not affect the KKS, but the endothelial cells leading to increased vascular permeability.

Typically, some form of trigger is required to induce symptoms. A trigger could be mild trauma, infection or stress. The trigger causes activation of the KKS and an increased production of bradykinin. Bradykinin will bind to B2R and induce increased vascular permeability, vascular leakage and edema, cardinal symptoms of HAE.[294] C1 inhibitor is also an inhibitor of the classical and lectin pathways of complement and patients typically present with low plasma levels of C4 and after an attack C2 is also diminished. Despite that, HAE is viewed as an KKS-driven disease.[6] Patients exhibit prekallikrein and HK depletion in plasma after an attack[295] and plasma levels of bradykinin are increased.[296] A mouse model of HAE was effectively treated by target disruption of the *B2R* gene.[297]

Prognosis

HAE is a life-long disease. Some patients have attacks seldom while others are affected multiple times a month.

Treatment

Treatments include KKS inhibition, as mentioned before. Recommended treatment during an attack is C1 inhibitor, Icatibant (B2R antagonist) or ecallantide (kallikrein inhibitor). Patients with frequent and/or severe attacks can benefit from prophylactic treatments. First line prophylactic treatments are lanadelumab (monoclonal kallikrein antibody), berotralstat (kallikrein inhibitor) and C1 inhibitor. In addition, Garadacimab (blocking FXII) is a new preventative treatment of HAE[298] awaiting approval by European Medicines Agency (EMA). It has already been approved for use in the United Kingdom.

Present investigation

Aims:

- To study if activation of the kallikrein-kinin system during vascular inflammation induces the release of complement-coated extracellular vesicles from the glomerular endothelium.
- To elucidate if activation of the kallikrein-kinin system and signaling via kinin receptors induces complement activation on glomerular endothelial cells.
- To investigate if leukocyte-derived extracellular vesicles from patients with vasculitis bear the kinin B1 receptor and have the capacity to transfer the functional receptor to recipient cells.
- To demonstrate a direct interaction between kallikrein and components of C5b-9, in pure form and in plasma, thereby affecting the function of C5b-9.

Methods

In the following section, a brief summary of the methods used in this thesis is presented. For a more detailed description the reader is referred to **Papers I-IV**.

Ethical considerations

In **Paper I, III and IV** patient samples were included. In all papers human plasma or serum samples were used. In **Paper III** kidney biopsies from patients (n=2) were obtained. Written informed consent was obtained from all included patients, their parents and the healthy volunteers. The studies were performed with the approval of the Swedish Ethical Review Authority (approval number: 2021-04438 and 2019-01623) or the Regional Ethical Review Board in Lund (approval number: 2004/731) and Linköping Universities.

Animal models were used in **Papers I** and **II**. Animal experiments in **Paper I** were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local Animal Care and Use Committee of Toulouse, France. Animal experiments in **Paper II** were approved by the regional Animal Ethics Committee in Lund (approval number 17452-20) and performed in accordance with the Swedish Board of Agriculture and the European Directive on the protection of animals used for scientific purposes.

Cells

In all papers primary glomerular endothelial cells (PGECs) were used. These cells were chosen as they are primary and not immortalized thus resembling the cell lining of glomerular capillaries. In **Paper III**, two forms of human embryonic kidney (HEK) cell were cultured, wild-type HEK that do not express the Bradykinin 1 receptor (B1R), and HEK cells transfected a B1R with a FLAG tag.[85] These cells were used for determining if EVs could transfer the B1R. Detection was carried out with an anti-FLAG antibody.

Blood cells

Leukocytes were isolated from whole blood and separated depending on their origin. They were analyzed for the presence of the B1R, to investigate if the cells and EVs carry the same receptors. Cell specific antibodies were used, and cells were analyzed using flow cytometry.

Isolation of extracellular vesicles

To isolate EVs in **Paper I and III**, a protocol previously described was used.[299] Serial centrifugation was performed. To obtain platelet-free plasma, samples were centrifuged at 9800 x g for 5 min, followed by washing in HBSS without Ca^{2+} and centrifuged at 20800 x g for 10 min. The supernatant was discarded, and the pellet washed twice at the same conditions as above. This results in an EV-enriched suspension, which was used in experiments described below.

Perfusion system

Cultured PGECs were exposed to plasma from patients and controls under shear stress in a microfluidic system (Cellic, Dublin Ireland). Vena8 Endothelial+ biochip capillaries were seeded with the cells and exposed to plasma perfused through the capillaries with a pump to mimic renal glomerular shear stress.

Samples were collected after perfusion and analyzed for the presence of complement-positive endothelial-derived extracellular vesicles (EVs). To assess the release of endothelial EVs induced by perfusion, the number of EVs from the pre-perfused sample was subtracted from the post-perfused sample. This experiment was carried out in **Paper I**.

Flow cytometry

In **Papers I and III** flow cytometry were used to assess the presence and the identity of EVs. Flow cytometry is a well-established method, sorting particles by size and composition. It is often used to analyze EVs.[300] In flow cytometry, each particle passes through a laser beam and is analyzed for its ability to scatter light. Both forward scatter, dependent on the particle size, and side scatter, dependent on the particle granularity, are analyzed. This reduces the risk of analyzing cellular debris. To further specify the population of interest, specific antibodies were used.

Transmission electron microscopy

In **Paper III** transmission electron microscopy (TEM) of kidney biopsies from vasculitis patients (n=2) were performed. This was used to detect the presence of EVs in glomerular capillaries. Nanogold coupled antibodies against the B1R and CD66 (present on the cell membrane of neutrophils) were used.

Calcium influx

Calcium influx on cells transferred with the B1R was performed to investigate if the receptor was functional. If a ligand binds to a functional B1R, this will produce inositol triphosphate (IP3),[80] activating the IP3 receptor. Ca^{2+} will be redistributed from the ER to the cytosol, leading to an increased intracellular Ca^{2+} concentration.[81]

To this end, cells were incubated with EVs, together with Fluo-4. Fluo-4 is a cell permeable calcium indicator, which is cleaved and fluorescent upon binding to Ca^{2+}. Cells were incubated with a B1R agonist. Agonist binding to a functional B1R would be detected as increased fluorescence. These experiments were performed in **Paper III.**

Kallikrein-kinin system activation on primary glomerular endothelial cells

In **Paper II**, experiments with PGECs were designed to determine if KKS activation leads to complement deposition. To this end cells were preactivated with adenosine diphosphate (ADP), known to activate endothelial cells,[301]and stimulated with kaolin and activated FXIIa for 30 min. Kaolin is known to activate the KKS,[302] whereas FXIIa is one of the initiating step of KKS activation.[303] For some experiments, inhibitors of the B1R or its intracellular signaling pathway, were added (Figure 10) to investigate the contribution these receptors to complement activation. Cell supernatants and lysates were collected for further analysis.

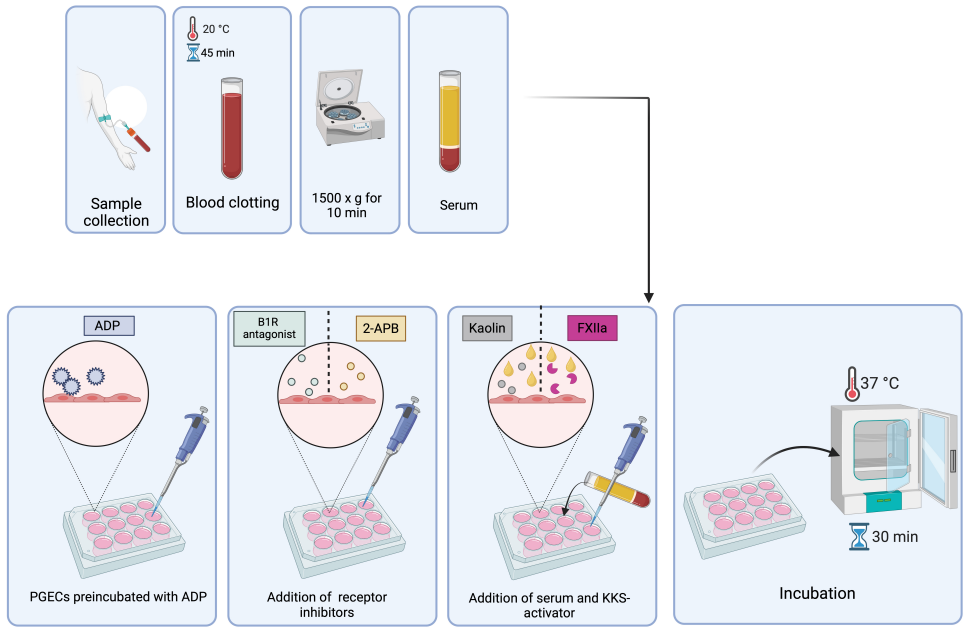

Figure 10. Schematic presentation of cell experiments, activating the kallikrein kinin system on primary glomerular endothelial cells. ADP: adenosine diphosphate, B1R, bradykinin 1 receptor, 2-APB: 2-aminoethoxydiphenyl borate, FXIIa: activated factor XII, KKS: kallikrein-kinin system. Created with BioRender.

In **Paper IV**, the deposition of C5b-9 on PGECs in the presence of kallikrein was investigated. To this end, PGECs were incubated with all the components of C5b-9, in the presence and absence of kallikrein. Cells were stained C5b-9 and visualized using a microscope.

Incubation of kallikrein and components of C5b-9

In **Paper IV**, to investigate if kallikrein interacts with the components of C5b-9, activated kallikrein was incubated with C5b-6, C6, C7, C8 and C9. In certain experiments lanadelumab was preincubated with kallikrein to determine if the effect of kallikrein was inhibited. Samples were further analyzed by immunoblotting to determine cleavage.

To investigate if interactions between kallikrein and the components of C5b-9 could take place under physiological conditions, normal plasma, C1 inhibitor-depleted plasma as well as plasma from a patient with HAE were incubated with activated kallikrein under the same circumstances as above. Some experiments were performed after preincubation of kallikrein and lanadelumab. Effects of kallikrein

was determined by running plasma samples on western blot (described below), using antibodies against C5, C6, C7, C8 and C9.

Detection of complement components and their degradation

Detection of complement components was either performed by flow cytometry (**Paper I**), immunofluorescence (**Paper I, II and IV**), ELISA (**Paper II**) or immunoblotting (**Paper II and IV**).

ELISA

The presence of complement activation products C3a (in supernatants), Ba (supernatants) and C5b-9 (cell lysates and supernatants) were analyzed using commercially available ELISA kits.

Immunoblotting

C3a in cell lysates (**Paper II**) and degradation of the components of the terminal complement complex (**Paper IV**) was assessed by immunoblot. Using specific antibodies against C3, C5, C6, C7, C8 and C9, cleavage could be detected.

Hemolytic assay

To investigate if kallikrein alters the function of C5b-9, a hemolytic assay in the presence and absence of kallikrein was performed. This was performed in **Paper IV**. Rabbit erythrocytes were used, as they are sensitive to human C5b-9.[304] This is due to species specific membrane bound complement regulators.[305-306] In certain experiments kallikrein was preincubated with lanadelumab.

Kallikrein activity assay

Kallikrein activity was measured in supernatants from cell experiments in **Paper II** and to verify that lanadelumab effectively inhibited kallikrein activity at the used concentrations in **Paper IV**. Kallikrein activity were analyzed using a chromogenic substrate PNAPEP, specifically cleaved by kallikrein.

Mouse models

Two mice models were used in this thesis. In **Paper I**, a mice model with nephrotoxic serum-induced glomerulonephritis (a known vasculitis model)[279] was studied. Mice were orally treated with a B1R antagonist or vehicle 2 weeks after induction of disease (Figure 11). The treatment or vehicle were administered every

other day until week 6. After sacrifice, renal sections were assessed for the presence of C3 deposition by immunofluorescence, described below.

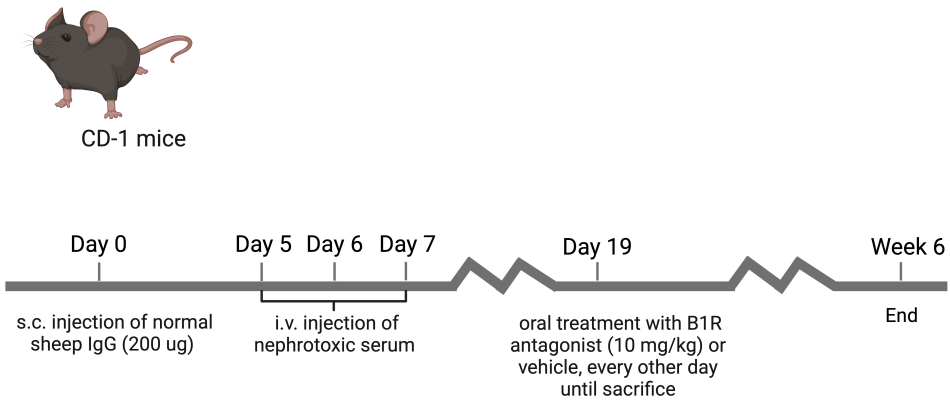

Figure 11. Schematic presentation of the mouse model of nephrotoxic serum-induced glomerulonephritis, and its treatment. Created with BioRender.

In **Paper II**, a mouse knockout model, deficient in the B1R and the bradykinin 2 receptor (B2R)[307] was used to investigate if the absence of kinin receptors affected complement activation *in vivo*. B1/B2 receptor double knockout (B1/B2 KO) mice were used, as depleting only the B1R has been shown to cause upregulation of the B2R,[91] which could potentially interfere with interpretation.

B1/B2 KO mice and their corresponding wild-type was injected with 2 mg/kg lipopolysaccharide (LPS) from *Escherichia coli* B5:O55 or PBS. This LPS was chosen as it has been shown to cause an upregulation of the B1R, suggesting that it activates the KKS.[308]

Plasma samples were prepared as described above, and kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. After sectioning, kidneys were assessed as described below.

Light microscopy

In **Paper II**, renal tissues from mice were stained with hematoxylin and eosin to evaluate histopathological changes. All kidney sections were evaluated in a blinded fashion.

Immunofluorescence

Immunofluorescence was performed in **Papers I and II** to detect complement deposition in glomeruli. Specific antibodies against C3 (**Paper I and II**) and C5b-9 (**Paper II**) were used. Sections were stained and assessed in a blinded fashion. To determine the level of positive staining, each glomerulus of a kidney section was counted and given a grade between 0 and 3 (0=no staining, 1=mild staining, 2=moderate staining, 3=severe staining) dependent on the degree of fluorescence. To assess total fluorescent in the kidney the sum of all fluorescence in that section was calculated, which was presented as total fluorescence in **Paper I**. In **Paper II**, fluorescence was presented as mean fluorescence intensity (MFI)/glomerulus, hence the total fluorescence of a section was divided by the number of glomeruli in that section.

Urea levels in murine plasma

Blood urea nitrogen levels were measured in mouse plasma in **Paper II**, as a measurement of kidney function. This was performed using a QuantiChrome Urea assay kit.

Prekallikrein in murine plasma

In **Paper II**, to assess KKS activation, residual prekallikrein in mouse plasma was measured.[309] These experiments were designed as kallikrein has a short half-life in plasma,[310] and is expected to be consumed within 24 h of kallikrein activation. To determine residual prekallikrein, samples were incubated with and without kaolin, before adding PNAPEP and measuring absorbance at time 0 and after 1 h, as described above. The difference between the kaolin-stimulated sample at time 1 h and the non-stimulated sample at time 0 was determined residual prekallikrein in plasma. A lower prekallikrein value was indicative of kallikrein consumption *in vivo*.

Results

The following is a brief summary of the results presented in this thesis. For a more detailed presentation, see the results section of each respective paper.

Paper I

The aim of **Paper I** was to investigate if plasma with active vascular inflammation, such as in vasculitis, could trigger the release of endothelial EVs (termed EMVs in the paper) bearing complement components, and if this release could be modulated by inhibiting the kallikrein-kinin system. Additionally, an *in vivo* mouse model of vasculitis and glomerulonephritis, treated with or without an oral B1R antagonist, was examined for complement deposition in the glomerulus.

Vasculitis plasma (n=13) contained more endothelial-derived EVs than controls (n=17), and significantly more endothelial vesicles were positive for C3 and C9.

When perfused over PGECs, patient plasma (n=6) induced excessive release of endothelial EVs bearing complement, compared to normal plasma (n=6). Adding C1 inhibitor or a B1R antagonist in combination with a B2R antagonist to patient plasma prior to perfusion significantly reduced the total EVs released, as well as EVs with C3 and C9 deposition. Perfusion with C1 inhibitor-depleted plasma also induced release of EVs bearing complement. Adding C1 inhibitor, B1R/B2R antagonists or the B2R antagonist alone decreased the release of EVs and the EVs bearing C3 and C9. B1R antagonist alone reduced the release of EVs carrying C3, but not C9 or total release of EVs. Kininogen-depleted plasma did not induce release of EVs.

Kidney sections from the mouse model described above (Figure 11) treated with a B1R antagonist (n=5) exhibited significantly less C3 deposition in glomeruli compared to mice treated with the vehicle (n=3). Taken together, this paper shows that vasculitis patients exhibit increased levels of endothelial-derived EVs positive for complement, which could induce an augmented release of EVs from PGECs. Blocking the KKS reduced the release of complement bearing EVs as well as complement deposition in glomeruli in a mouse model.

Paper II

Paper I addressed complement deposition on EVs from glomerular endothelial cells. In **Paper II** we assessed the interactions of the KKS and the complement system directly on endothelial cells, and the importance of intracellular signaling for this interaction to occur.

Supernatants from cell experiments activated with kaolin or FXIIa showed KKS activation, demonstrated by HK cleavage and increased kallikrein activity. These supernatants exhibited complement activation, as demonstrated by increased levels of Ba and C5b-9. As for kaolin, C3a levels were also increased. In cell lysates both C3a and C5b-9 were deposited in the presence of kaolin or FXIIa. When adding the B1R antagonist Ba and C5b-9 levels in supernatants were significantly decreased and both C3a and C5b-9 in cell lysates were likewise reduced. When preincubating cells with an IP3 receptor inhibitor both Ba and C5b-9 in supernatants were reduced. This suggests that the effects of the KKS activators are mediated via G protein coupled receptors as the IP3 receptor is a downstream signaling receptor of the G protein/phospholipase C activating pathway.[311]

In this paper another mouse model was utilized to stimulate KKS activation. Mice were injected with LPS. Wild-type mice were compared with B1R/B2R knockout mice. In the kidneys, LPS-treated wild-type mice (n=7) presented with more tubular epithelial cell desquamation and increased deposition of both C3 and C5b-9 in glomeruli compared to B1/B2 receptor knockout mice (n=7). In summary, this paper shows that activation of the KKS causes complement activation, and that this activation is at least in part due to signaling through the B1R.

Paper III

In **Paper III** we investigated if leukocyte-derived EVs bear the B1R, and if the receptor could be transferred from the vesicle to a recipient cell, as a mode of spreading inflammation.

Plasma from vasculitis patients (n=9) exhibit increased levels of leukocyte-derived EVs compared to controls (n=9), and a larger portion of these EVs carried the B1R. The majority of EVs originated from neutrophils and monocytes. EVs derived from B cells were also slightly positive for B1R.

In renal biopsies (n=2) from patients with GPA, leukocyte-derived EVs bearing the B1R could be visualized docking onto glomerular endothelial cells by electron microscopy.

EVs obtained from leukocytes and HEK cells transfected with the B1R transferred the receptor to wild-type HEK cells and PGECs. The transferred receptor was shown to be functionally active, as demonstrated by measuring calcium influx after incubation with the B1R agonist. Incubation with a B1R antagonist abolished calcium influx, demonstrating the specificity of the receptor.

In conclusion, this paper demonstrated that patients with vasculitis exhibit increased levels of B1R-positive leukocyte-derived EVs, and that a functional B1R can be transferred via EVs to a recipient cell.

Paper IV

In **Paper IV** we studied direct interactions between kallikrein and components of the terminal pathway of complement, and its effect on C5b-9 function.

In a pure system (i.e. without plasma), kallikrein cleaved all the components of C5b-9. When preincubating kallikrein with lanadelumab, a specific kallikrein inhibitor, cleavage was prevented.

In C1 inhibitor-depleted plasma, C5, C6 and C7 were cleaved by added kallikrein. A similar cleavage of C5 and C7 was observed in one HAE patient with C1 inhibitor-defiency. The effect of kallikrein was inhibited by lanadelumab. Likewise, when using normal plasma C5, C6 and C7 were cleaved in the presence of kallikrein, albeit to a lesser extent. Cleavage of C8 and C9 could not be detected.

The effect of kallikrein on complement-mediated hemolysis was investigated. Rabbit red blood cells were sequentially exposed to components of C5b-9, or normal human plasma. Significantly less hemolysis was demonstrated when kallikrein was added and this effect was abolished in the presence lanadelumab.

PGECs incubated with the separate components of C5b-9, without kallikrein, exhibited C5b-9 deposition. In the presence of kallikrein, significantly less C5b-9 staining was detected.

This paper suggests that kallikrein directly interacts with the components of C5b-9, disrupting the formation and hence the function of C5b-9.

Discussion

In this thesis I have investigated how the kallikrein-kinin system and the complement system interact and contribute to inflammation. Traditionally, the different cascade systems securing homeostasis and first line defense in our vessels have been viewed as separate compartments, but evidence is emerging to reconsider this division. In this thesis I present additional evidence for the interactions between the KKS and complement system.

Both the complement[163] and the KKS[7] are activated during vasculitis, and levels of EVs in patient plasma are increased.[11] We demonstrate that endothelial EVs in patient plasma bear complement C3 and C9, and that these EVs stimulate further release of EVs from endothelial cells exposed to patient plasma. The shedding of EVs bearing complement components may be of importance for cell protection, to rid the cell of harmful substances, and/or be a manner of intercellular communication, spreading inflammation. EVs bearing the B1R have been shown to induce neutrophil chemotaxis, an important feature of vascular inflammation.[72] Furthermore, a novel function of B1R-positive EVs was demonstrated as these have

the capacity to deliver a functional B1R to a recipient cell. The transfer of B1R from one cell to another may cause an increased inflammatory response. This could be of importance as EVs are abundant in plasma during inflammation.

The findings have physiological relevance as patients with vasculitis have increased levels of leukocyte-derived EVs bearing the B1R, indicating that these findings could have an importance in the systemic development of vasculitis. Importantly, EVs positive for the B1R were found at sites of inflammation in kidney sections from vasculitis patients. Ligand-binding to the B1R results in a sustained inflammatory response and the receptor has been demonstrated to be upregulated during vasculitis.[279]

In **Paper I** and **II** we demonstrate that activation of the kallikrein-kinin system can cause complement system activation on glomerular endothelial cells and their EVs. Blocking FXIIa and/or kallikrein with C1 inhibitor or blocking the two kinin receptors using specific antagonists resulted in less deposition of complement on endothelial cells, their supernatants and EVs. This indicates that activation of the KKS, with the release of bradykinin and/or desArg^9-bradykinin, contributes to complement activation. Blocking the intracellular signaling pathway of kinin receptors using an IP3-receptor inhibitor reduced complement activation products Ba and C5b-9 as well.

Others have shown that kallikrein has the potential to activate the complement system by cleaving C3, yielding active metabolites,[216] as well as Factor B,[217] and C5.[218] C3 and Factor B cleavage was demonstrated in low concentrations of plasma or in pure systems, and if it can occur under physiological circumstances is yet to be elucidated. One could argue that our findings of KKS activation leading to increased levels of C3a, Ba, C5b-9 is simply due to kallikrein cleavage of these components. However, the inhibitory effect by the B1R antagonist and IP3R inhibitor *in vitro* suggest that the endothelial cell is involved. In addition, in serum in the absence of cells, addition of kaolin or FXIIa did not induce C3a release. This could possibly be due to the presence of C1 inhibitor in serum. It has been proposed that binding of kallikrein to endothelial cells protects it from inactivation by C1 inhibitor,[312] which could contribute to the complement activation seen in the presence of endothelial cells.

To evaluate if KKS activates complement *in vivo*, two different mice models were used, as described in **Paper I** and **II**. Importantly, *in vivo* blocking of the KKS ability to signal through cells, either by a B1R antagonist or by knocking out the B1R and the B2R, significantly reduced complement deposition on glomerular endothelial cells. These findings suggest that the KKS can contribute to complement activation *in vivo*, which can be off importance when designing new drugs aiming to treat complement-mediated diseases.

The number of patients and controls are low in my studies which is a limitation. Experiments carried out using the perfusion system consume large volumes of

plasma, making it impractical to perform with patient plasma, since there is only a limited amount available. For some patients' kidney biopsies were available, for other only plasma samples.

Since the synergistic effects between the KKS and complement system is relatively well studied, the findings of **Paper IV** surprised us. However, kallikrein has been suggested to have a possible inhibitory effect of complement activation, as seen in the cleavage of C5b and iC3b (Figure 12). We demonstrate for the first time that kallikrein interferes with the function of C5b-9, by preventing hemolysis and cell deposition. This indicates an inhibitory effect on the complement system by kallikrein. Cleavage of C5, C6, C7 could be demonstrated in C1 inhibitor-depleted plasma and to a minor extent in normal plasma, albeit at high concentrations of kallikrein. In addition, C5 and C7 were cleaved in plasma from a patient with hereditary angioedema (HAE) when incubated with kallikrein. This indicates that these actions of kallikrein could be exerted *in vivo*. It is likely that these actions of kallikrein are dose-dependent, and requires high concentrations of kallikrein, and are therefore most likely to occur locally. These actions of kallikrein could be important for patients with HAE. To our knowledge, there are no reports of an increased risk of meningococcal infections in untreated HAE-patients. Possibly, this could be due to an increased activation of the classical pathway of complement, generating sufficient C5b-9 to protect the patient from invading microbes. Additionally, kallikrein is not constantly activated in HAE-patients, but activates in response to a trigger.

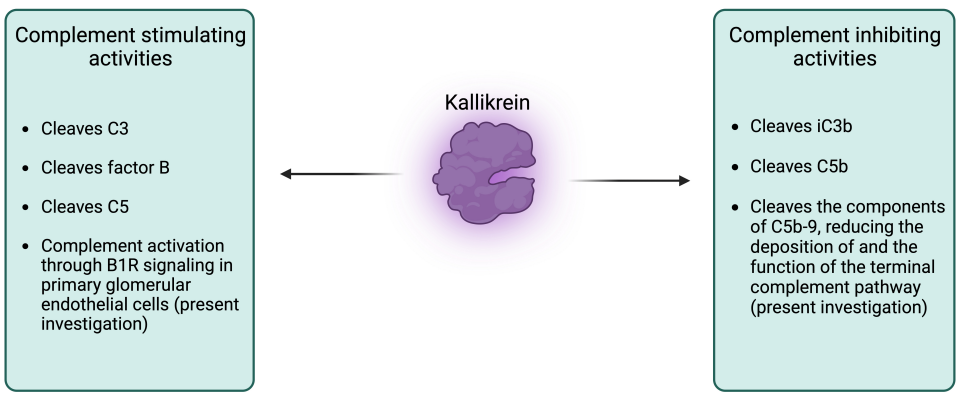

Figure 12. The dual effects of kallikrein on complement system activation and inhibition. Created with BioRender.

Interestingly, addition of lanadelumab together with kallikrein protected erythrocytes from hemolysis and prevented cleavage of complement components

both in plasma and in a pure system. This could be of significance for HAE-patients receiving this, and other kallikrein-inhibiting treatments.

In this thesis we present two completely different actions of the KKS, one that engages and activates the complement system, and one that inhibits the formation of the final step of complement, the formation of C5b-9. It is important to remember that the different experiments were performed under completely different settings. In **Paper I** and **II**, no extra kallikrein were added, and cells were only incubated with plasma or serum for a relatively short amount of time. In **Paper IV**, kallikrein was added in a relatively high concentration, and incubated in plasma or with its substrate for a comparatively long time.

Taken together we present evidence suggesting that the interactions between the kallikrein-kinin system and the complement system during vascular inflammation is more comprehensive than previously believed. These actions do not only occur under experimental conditions but seem to have an importance *in vivo* as well. Kallikrein can function both as an activator and as an inhibitor of complement, possibly dependent on concentrations of both enzyme and substrates in the local environment. It is known that kallikrein has counterbalancing purposes in other parts of the vascular homeostasis. Kallikrein can for example initiate the intrinsic pathway of coagulation either by activating FXII or FIX,[35] as well as activating the fibrinolytic pathway by cleaving plasminogen into plasmin,[313] hence elicit both protrombotic and fibrinolytic functions. In addition, kallikrein cleaves HK, releasing the vasodilator bradykinin, but it is also known to cleave prorenin to yield renin,[314] which leads to the liberation of angiotensin II, a vasoconstrictor. With this knowledge in mind, it is plausible that kallikrein has both activating and inhibiting properties of the lytic pathway of complement.

There are many available treatments targeting the kallikrein-kinin system and complement system. EV-release is often suggested as a potential target when discussing new drugs. But it is important to remember that when you target one of these systems, you also affect the others.

The findings of this thesis may seem incongruous, but in my opinion, they reflect the complexity of the human physiology. An enzyme can be both an activator and an inhibitor, both good and evil. And with this thesis I have provided evidence supporting the famous quote of Albert Einstein:

"The more I learn, the more I realize how much I don't know."

Conclusions

- Patients with vasculitis have increased levels of endothelial- and leukocyte-derived EVs, positive for complement and the B1R.
- Leukocyte-derived EVs transferred functional B1R to recipient cells, specifically glomerular endothelial cells.
- Activating the KKS leads to complement activation on endothelial cells and endothelial cell-derived EVs. Inhibition of the B1R/B2R or kallikrein decreases complement activation.
- Kallikrein cleaves the components of C5b-9, inhibiting C5b-9 formation and its ability to deposit on and ultimately cause damage to cells.

Populärvetenskaplig sammanfattning

Inflammation är kroppens akuta svar på en oväntad och potentiellt farlig händelse. Det kan handla om en skada i organ eller kärlvägg, ett inkräktande virus eller skadade celler. Kännetecknen för inflammation är rodnad, värmeökning, svullnad, smärta och nedsatt funktion. Dessa symtom förklaras av en ökad genomtränglighet i kärlväggen som tillåter vätska och celler träda ut i omgivande vävnad. En sjukdomsgrupp som kännetecknas av inflammation i just kärlväggen är vaskuliter. Eftersom kärl finns i alla kroppens kroppsdelar, kan vaskuliter drabba olika organ, vanligast är lungor, hud och njurar. Vaskuliter drabbar vuxna och barn och kan variera i svårighetsgrad från lätta och relativt snabbt övergående, till livslånga och potentiellt livshotande.

Vid vaskulit är många inflammatoriska system aktiverade i kroppen, två av dessa är kallikrein-kininsystemet och komplementsystemet. En viktig spelare i kallikrein-kininsystemet är kallikrein. Kallikrein är ett enzym, alltså ett protein som kan bryta andra protein, som efter aktivering leder till frisättning av kininer. Kininer kan påverka celler genom att binda till receptorer. Bindning av kininer till kärlväggens celler leder till ökad genomtränglighet, och därför ökat utsläpp av vätska och celler.

Komplementsystemet är en viktig del av immunförsvaret och hjälper kroppen att avlägsna främmande ämnen från blodbanan. Detta sker genom att delar av komplementsystemet kan binda till det främmande ämnet och antingen orsaka ett hål i dess vägg så att den går sönder, eller genom att locka till sig andra celler från immunförsvaret som kan äta upp det främmande ämnet. Komplementaktivering leder också till ökad inflammation, då det har en förmåga att kalla till sig celler från immunförsvaret.

I vår blodbana finns också mikrovesikler. Dessa är små cellblåsor som friges från celler, och innehåller olika byggstenar från ursprungscellerna, tex DNA eller receptorer. En ökad frisättning av cellblåsor ses vid vaskulit och andra inflammatoriska tillstånd, möjligtvis för att skydda cellen från oönskade ämnen som fäst sig på cellen, eller för att kunna kommunicera med andra celler längre bort i blodbanan.

Dessa system är kraftfulla och behöver kontrolleras noggrant. I friska individer sker detta genom olika regulatorer som finns i blodbana och bundet till celler. Men emellanåt sker en obalans i reglering och aktivering, och då kan individen drabbas av en inflammatorisk sjukdom, såsom vaskulit.

I artikel I visar vi att patienter med vaskulit har en ökad mängd mikrovesikler i blodbanan, och att dessa mikrovesikler bär på delar av komplementsystemet. Dessa komplementbärande mikrovesikler ökade också till en ökad frisättning av nya mikrovesikler från friska celler. Genom att blockera aktiviteten av kallikrein-kininsystemet, lyckades vi förhindra att nya mikrovesikler frisattes från de friska cellerna. Dessutom behandlades en vaskulitmusmodell med en blockerare av kininreceptorer, och uppvisade mindre komplement i sina njurar, ett tecken på minskad sjukdomsaktivitet.

I artikel II utforskade vi sambandet mellan dessa två system ytterligare. Genom att aktivera kallikrein-kininsystemet på celler sågs ökad komplementaktivering på celler och i omgivande cellvätska. Genom att blockera kininreceptorerna på cellerna minskades komplementaktiveringen. En musmodell som saknar kininreceptorer uppvisade mindre komplementaktivering i njurar, efter att de behandlats med ett gift som frisläpps av bakterier.

I artikel III mätte vi mängden mikrovesikler frisläppta från vita blodkroppar i blodet från patienter med vaskulit. Dessa patienter hade fler mikrovesikler från vita blodkroppar, och en större andel av dessa bar på kininreceptorer. Dessutom lyckades vi flytta en kininreceptor från en mikrovesikel till en mottagande cell, en helt nyupptäckt funktion.

I artikel IV utforskade vi om kallikrein genom klyvning av komplementproteiner kan leda till minskad komplementfunktion. Vi upptäckte att kallikrein kan klyva de sista delarna av komplementsystemet, och därigenom förhindra att hål bildas på ytan av celler eller invaderande bakterier.

Att förstå hur olika system samverkar i kroppen är viktigt för kartläggningen av hur en sjukdom utvecklas. Dessutom är det viktigt att ha kännedom om när nya mediciner designas. I nuläget används många mediciner som påverkar ett av dessa system för att behandla inflammatoriska sjukdomar. Då är det extra viktigt att få insyn i hur de andra systemen i kroppen ändras av den behandling man ger.

Acknowledgements

Diana, thank you for sharing your love and enthusiasm for science with me. Thank you for always believing in me, even when I have doubts. Thank you for everything that you have taught me and for giving me the opportunity to evolve during my many years as a Phd-student.

Zivile, my co-supervisor. Thank you for your kindness and willingness to help.

Ann-Charlotte, your love for science is contagious. Thank you for all your questions, even the hard ones. Thank you for your warmth and sense of humour, and for setting the positive tone in the lab.

Ida, thank you for always being willing to share your knowledge. And special thanks for being such a good friend and colleague, always ready to share a laugh by the lab bench.

Anne-lie, thank you for your support, and for having my back. I wish you all the best!

Alexandra, I really appreciate your kindness and knowledge in the field. You are a great asset to the lab, and my final year as a Phd-student would not have been the same without you. A special thanks for always checking in on me, asking how it's going, and more importantly how I am feeling. Thank you!

Niklas, my roomie. Thank you for being such a joyous person to be around. Always kind, calm and helpful. I really appreciate you.

Markus, you are great! Thank you for sharing the early mornings with me. I wish you all the best and remember: you can do this!

Sunna, thank you for always spreading happiness around you. I have missed you this last year in the lab, but I am grateful for all the times we have spent together.

Annie, my friend and co-lab rat. Thank you for always being supportive and a true friend, both in the lab and on the outside.

Kalle, Sebastian, Milan and Ashmita. My years in research would not have been the same without you. You are all great people and amazing researchers.

Robin, my first hands-on supervisor, thank you for introducing me to science, and sharing your knowledge and enthusiasm for the field.

Maria, without you I would not have been here today. You are an extraordinary person. Thank you for introducing me to the lab, taking me under your wings and sharing both work and life events with me. And **Andreas,** congratulations for finding Maria, and thank you for being the best cousin one could imagine. Thank you for always taking your time to check in, even when life is hectic. You are the new glue to our family.

Anna, Jan och David. Mina chefer på kliniken. Tack för att ni gett mig tid och möjlighet att disputera. Utan er förståelse hade jag aldrig blivit färdig.

Ulrika Pahlm. Tack för att du trodde på mig och lärde mig vad som är viktigt på riktigt i livet. Tack.

Nicolina och Emelie, ni är otroliga. Tack för ert stöd, och för att ni fixar och trixar och löser alla mina problem. Jag är evigt tacksam.

Mamma och pappa, tack för att ni alltid försett mitt liv med trygghet. Tack för att ni alltid ställer upp och hjälper till på alla sätt möjliga. Tack för att ni är de bästa mormor och morfar mina barn någonsin kunnat önska sig.

Jacob och Karin, vi följs genom livet och jag vet att ni alltid finns där, oavsett hur många mil vi har emellan. Vi lyckas nästan alltid göra samma val i livet, men nu, för en gångs skull, fick jag vara först.

Ett stort tack till hela förlängda familjen **Fagerström**, som gjort mig till en del av sin.

Victor, the love of my life, min person. Aldrig hade jag klarat detta utan ditt stöd. Jag älskar dig.

Liv, Elise och Harry. Jag älskar er till månen och tillbaka, ni är allt.

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