Factor XII in community-acquired pneumonia
Sex specific differences and correlation between clinical parameters

Inaugural dissertation
for the degree of Doctor of Medicine
of the Faculty of Medicine
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Presented by Ehrlich, Kristin
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Tag der Disputation: 25.02.2025

I Table of contents

1 Introduction

1.1 Pneumonia

1.1.1 Definition and Epidemiology

Pneumonia is an inflammatory disease affecting the alveolar epithelium and/ or the interstitium of the lung[1]. The infection is mainly caused by bacteria like *Streptococcus pneumoniae* or *Hemophilus influenzae*; by viruses like Adenovirus or Influenza virus and to a lesser extent by fungi or parasites[1]. It occurs most notably in small children and people above the age of 60[2]. Pneumonia is found amongst the ten main causes of death not only in Germany[3] but also worldwide[4]. Furthermore, it is the seventh most common reason for hospitalization in Germany[5] and ranks third on the list of the global burden of disease estimates in 2016 published by the World Health Organization (WHO)[6]. The incidence of community-acquired pneumonia in Europe was found to be around 1,6 per 1000 inhabitants per year and increased with age (14 per 1000 person-years in people aged ≥65 years)[7]. Generally, men are more likely to be affected and to be hospitalized compared to women[7].

1.1.2 Classification

There are many ways to characterize different types of pneumonia. The definition based on the place of acquisition has been proven favorable in the context of clinical practice as is has direct consequences on the therapeutic strategy[1]. According to this definition 3 groups of pneumonia can be differentiated: community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP) and the pneumonia in the immunosuppressed host. Pneumonia in patients who develop symptoms before admission to the hospital or within 48 h after hospitalization is defined as community-acquired. Pneumonia in patients who show first symptoms 48 h after admission to the hospital up to 3 months after discharge from the hospital is by definition hospital-acquired. CAP and HAP have to be distinguished from the pneumonia in the immunosuppressed host. Irrespective of the environment in which the infection is acquired, all immunosuppressed patients are summed up in an independent group. The immune deficiency can be the result of many different causes like for example Acquired Immune Deficiency Syndrome (AIDS), neutropenia or stem cell transplantation[8].

Table 1-1 Criteria for classification of pneumonia

Type of pneumonia	Place of acquisition	Immune status
[1]CAP (community-acquired pneumonia)	Outside the hospital	Immunocompetent
[2]HAP (hospital-acquired pneumonia)	In the hospital (> 48 h after hospitalization)	Immunocompetent
Pneumonia in the immunosuppressed host	In- or outside the hospital	Highly immunodeficient

[1]CAP, Community-acquired pneumonia; [2]HAP, Hospital-acquired pneumonia; adapted from S. Ewig et al.[8]

1.1.3 Etiology

The main pathogens causing CAP are gram-positive bacteria like *Streptococcus pneumoniae* and *Staphylococcus aureus*; gram-negative bacteria like *Haemophilus influenzae*, *Chlamydophila pneumoniae* or *Legionella species*.; atypical bacteria *like Mycoplasma pneumoniae* or viruses like Influenza A and B, adenovirus, respiratory syncytial virus, parainfluenza and corona virus[9,10]. In 70 to 90 % bacteria are the main cause for CAP, with *Streptococcus pneumoniae* being responsible for about 40% of bacterial infection[10]. Recent studies detected viruses in nasopharyngeal swab samples in about 30 % of adults with CAP[10–12]. A co-infection due to bacterial and viral pathogen was observed in one-fifth of adults with CAP and associated with a more serious disease progression[12].

First, these pathogens have to enter the alveoli, most likely by inhalation of contaminated respiratory droplets or small volume aspiration of bacteria for example while sleeping or in people with dysphagia. Rarely a direct inoculation, as seen after thoracic trauma, is the reason for a pulmonary infection. An extending adjacent infectious focus can also lead to pneumonia either via contiguous or hematogenous or spread, which is mostly exceptional[12].

Normally an infection of the lower respiratory system is prevented by mechanical obstacles and supported by the innate und acquired immune system, which seems to be altered in CAP patients. Several risk factors for CAP have been identified, comprising regular consumption of alcohol as well as smoking, age over 65 years, male sex, crowded living conditions and close contact with children, decreased body weight and poor dental hygiene[12,13]. There has also been an association with chronical conditions like respiratory compromise, cardiovascular diseases, Parkinson's disease, epilepsy,

dementia, dysphagia, and chronic renal or liver disease. These raised the risk of CAP by twofold to fourfold[7,13].

1.1.4 Pathophysiology

Although it has been and still is the subject of intense research the underlying pathomechanism of pneumonia appears to be very complex and remains mostly elusive. Assuming that not only the type and the number of pathogens, but also the individual host immunity are responsible for the outcome of the infection it is to underline that an excessive immune response mainly determines lung failure and fatal outcome of the disease[14]. The balance between sufficient eradication of the pathogen on the one hand and preservation of lung function on the other hand is delicate.

In a healthy organism pulmonary defense mechanisms prevent an infection of the lower respiratory system. The former consisting of mechanical barriers in the form of nasal hairs and turbinates, intact gag and cough reflexes as well as the ciliary respiratory epithelium. Additionally cellular compounds like neutrophils, macrophages and immunoglobulins complete the airway defense mechanism[12,15]. Analogous to the microbiome of the intestine, the microbial flora of the upper airways contributes to balance a healthy microbiome by disposing pathogens[12].

If a pathogen still manages to overcome all these barriers it will face surfactant. Consisting of phospholipids and glycosaminoglycans it exerts diverse functions. Via surfactant protein A and D it is also part of the innate immunity. They can interact with surface antigens of bacteria and viruses to make them accessible for phagocytosis[12,16,17]. When the infectious pressure persists nonetheless, an individual inflammatory reaction is triggered, being responsible for most of the CAP symptoms[12]. S. Hippenstiel et al.[14] have summarized the process as follows: Pathogens can secret various molecules like proteases or toxins that can directly inactivate host defense mechanisms and lead to an epithelial barrier damage. Moreover, the binding of pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS) by multiple transmembrane or cytosolic receptors like the “toll-like”-receptors (TLR) initiates intracellular signal cascades leading to the activation of the cellular immune response of the epi-and endothelium as well as macrophages and neutrophil granulocytes. The subsequent liberation of chemo- and cytokines, toxic radicals and digestive enzymes plays an important role in the defense against pathogens but can cause further structural damages. As a consequence, hyperpermeability, lung edema and pulmonal arterial vasoconstriction can develop and result in an impairment of the lung function.

Bacteria mainly cause lobar pneumonia which effects the alveoles, in contrast to atypicial pneumonia, where the interstitium is affected. In the following I will focus on the stages of lobar pneumonia, as it accounts for the majority of CAP.

The first phase is congestion. The alveoles fill with serous exudate, bacteria and erythrocytes leading to an impairment of oxygen diffusion[15]. During consolidation stage neutrophils, erythrocytes and fibrin aggregate in the fluid. With hematoxylin and eosin staining the lung resembles the liver, which is why it is also called red hepatization.

As the red blood cells are degraded and lysed, fibrin deposition and neutrophils prevail in the phase of gray hepatization. At last, macrophages dominate the picture by resolving the debris.

The phase of red hepatization mainly is where factor XII (FXII) comes into play. The role of FXII in inflammation is going to be displayed in one of the following sections.

1.1.5 Symptoms, diagnosis and treatment of community-acquired pneumonia

As the cohort studied in this work consists of patients suffering from CAP, the following description will only approach symptoms, diagnosis and treatment of CAP according to the 2021 updated version of the German S3 guideline: “Management of Adult Community-acquired Pneumonia and Prevention“ published by the German Respiratory Society (DGP), the Paul-Ehrlich-Society for Chemotherapy (PEG), the German Society for Infectious Diseases (DGI), the German Society of Medical Intensive Care and Emergency Medicine (DGIIN), the German Viological Society (DGV), the Competence Network CAPNETZ, the German Society of General Practitioneers (DEGAM), the German Society for geriatric medicine (DGG), the German Palliative Society (DGP), the Austrian Society of Pneumology Society (ÖGP), the Austrian Society for Infectious and Tropical Diseases (ÖGIT), the Swiss Respiratory Society (SGP) and the Swiss Society for Infectious Diseases Society (SSI)[8].

1.1.5.1 Symptoms and diagnosis of CAP

People can display various unspecific symptoms, that can also be found in other lower respiratory infections, like dyspnea, cough with or without purulent expectoration, breath-dependent thoracic pain, malaise, fever or hypothermia, myalgia, arthralgia, cephalgia and confusion. During the clinical examination an elevated breathing rate, tachycardia and a low blood pressure level as well as crackles on auscultation might be observed. In order to prove the assumption of pneumonia further diagnostic steps have to be taken. An x-ray of the chest in 2 planes to reveal newly arisen infiltrates is considered the main criterion for the diagnosis of pneumonia[1]. In addition, a blood analysis could show

elevated levels of leucocytes and c-reactive protein (CRP), a marker for infections. For identification of the underlying pathogen blood, sputum, or bronchoalveolar lavage fluid (BALF) should be collected and cultured.

In synopsis with the findings the tentative diagnosis of pneumonia can be made. The decision on outpatient treatment or admission to the hospital is often based on the CRB-65 score. The CRB-65 score serves as a risk stratification tool for mortality and helps to establish a risk-appropriate therapeutic strategy. The acronym CRB-65 stands for: Confusion; Respiratory rate ≥ 30/min; Blood pressure: diastolic ≤ 60 mmHg; systolic < 90 mmHg and 65 age: ≥ 65 years. Every symptom equates one point so that the final score can range from 0 to 4 points. Patients with 0 points show a low risk mortality and are suited for an outpatient treatment. Hospitalization should be seriously taken into account from 1 point and is highly recommended from 2 points on as a score of 1-2 equals a moderate and 3-4 points equal a high-risk mortality[18,19].

1.1.5.2 Treatment of CAP

A calculated antimicrobial therapy adjusted to the severity of pneumonia and possible comorbidities is the basis of the treatment. Mainly aminopenicillins without and with beta-lactamase-inhibitors, fluoroquinolones, cephalosporines and macrolides are prescribed. A detailed description of the recommended antibiotics can be found in the German S3 guidelines for CAP[8]. The mode of administration can be oral or intravenous depending on the severity of the illness.

In addition, the patients should avoid strenuous physical activity, do breathing exercises and inhale sodium chloride to support secretolysis. In that regard it is also important to mind a sufficient liquid intake.

Patients suffering from severe pneumonia displaying respiratory insufficiency and/ or signs of sepsis must be transferred to an intensive care unit (ICU) to ensure extended monitoring and an intensified therapy including ventilation treatment, administration of catecholamines and/ or blood transfusion.

1.2 Factor XII

1.2.1 Introduction

Factor XII (FXII), also known as Hageman factor (HF), was first described in 1955 as a coagulation factor[20]. Its role in initiating the intrinsic coagulation pathway was proposed and proven a decade later[21,22]. Strikingly, people who suffer from a FXII deficiency do not display major hemorrhagic symptoms[20,23], indicating that FXII is dispensable for hemostasis. That is why further functions of FXII remain the subject of current research.

The gene encoding FXII (*F12*) was detected on chromosome 5. *F12* comprises 12 kilobases (kb) organized in 14 exons and 13 introns[24,25]. FXII is mainly synthesized in the liver. Its expression is predominantly regulated by the transcription factor hepatocyte nuclear factor 4 α (HNF4α) in response to estrogen[26–28].

In human plasma FXII circulates with a mean concentration of 30 μg/ml within a reference range of 15 to 47 μg/ml. The concentration of FXII measured in the same individuals at different time points shows low fluctuations of ± 5 μg/ml and is therefore considered to be relatively stable[29].

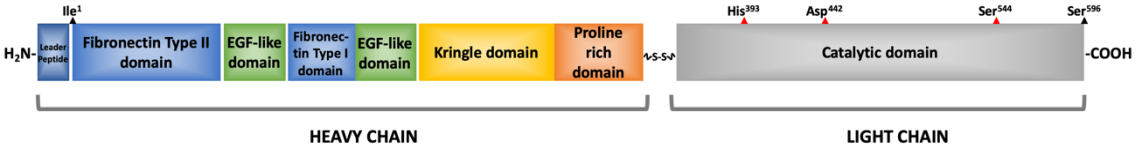
1.2.2 Structure

FXII is produced as a zymogen[30] with a molecular weight of about 80 kilodaltons (kDa)[29]. It consists of a heavy chain (353 residues) and a light chain (243 residues) that are connected with each other by a disulfide bond[31,32]. FXII contains multiple structural domains. Starting from the N-terminus the heavy chain comprises a leader peptide, followed by a fibronectin domain type II, an epidermal-growth-factor (EGF)-like domain, a fibronectin domain type I, a second EGF-like domain, a kringle domain and a proline-rich region. The light chain on the other hand contains the catalytic domain[33]. So far, the functions of the respective domains have only been described partially[33,34].

In the heavy chain, zinc ion binding sites have been proposed to be in the fibronectin type II domain (residues 40-44; 78-82) and in the first EGF-like domain (residues 176-180) and they were proposed to enhance FXII surface binding. Putative surface binding sites have been identified in the fibronectin type I (residues 134-153)[35] and type II domains (residues 1-28)[36], in the second EGF-like domain or the kringle domain and in the proline-rich region[37]. While the fibronectin type II domain also displays the ability to interact with factor XI (residues 3-19)[38,39] and binds to endothelial cells (residues 39-47)[40,41] and neutrophils[42], the fibronectin type I domain interacts with fibrin and heparin[43]. Although EGF is demonstrably responsible for mitogenic effects on various cell types[44] it is not yet proven that the EGF-like domains of FXII mediate similar mitogenic activities[33]. Except for the proline-rich region, the other domains of FXII share structural homologies with respective domains found in other serine proteases like tissue plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA)[24]. That is why the proline-rich region is considered to be unique for FXII[45].

The light chain comprises the catalytic triad[24] with its amino acids His^{393}, Asp^{442} and Ser^{544}, allowing activated FXII to exert proteolytic functions and classifying it as serine protease[46].

Figure 1-1 Structural domains of FXII



Amino acids -19-1: leader peptide, 1-88: fibronectin type II domain, 94-131: EGF-like domain, 133-173: fibronectin type I domain, 174-210: EGF-like domain, 215-295 kringle domain, 296-349: proline-rich region, 354-596: catalytic domain with catalytic triad (red triangles). Adapted from Stavrou and Schmaier[33]

1.2.3 Activation and inhibition

After cleavage between residue Arg[353] and Val[354] FXII is activated to α-FXIIa[32,47]. α-FXIIa is a two-chain polypeptide consisting of the light-chain (28 kDa) and the heavy-chain (52 kDa), which are held together by a disulfide bond[31,32,48]. Further cleavage of α-FXIIa after residue Arg[334] produces β-FXIIa with a molecular weight of 30 kDa, a polypeptide consisting of the light-chain (28 kDa) and a nonapeptide, which are linked by a disulfide bond[31,32]. The activation can either be triggered by proteinases like plasma kallikrein (PKa) or upon autoactivation[47,49]. Autoactivation upon contact with negatively charged surfaces like glass and kaolin[40] has been identified to activate the coagulation pathway *in vitro.* That is why it is also referred to as contact activation system (CAS*).* Meanwhile the question of physiologic activators of the contact system was left unanswered. Several physiologic activators like dextran sulfates and sulfatides[50], phospholipids[51], endotoxins[52] and urate crystals[53] have been proposed. Yet their ability to activate FXII was only shown in *in vitro* experiments. Nowadays new insights into the activation of FXII allow the proposal of DNA[54], RNA[55], aggregated proteins[56], collagen[57], polyphosphates[58] (polyP) and neutrophil extracellular traps[54] (NETs) as physiologic or pathophysiologic activators of the contact system. The reciprocal activation of FXII and plasma prekallikrein (PK) culminates in an amplification of FXIIa, PKa and its downstream products and is therefore essential for the activation of the system[33].

The most potent physiological inhibitor of FXIIa is C1 esterase inhibitor (C1INH) [59–62]. C1INH is an important regulator of the CAS, the KKS and the complement system as it also inhibits PKa, plasmin, tPA, and complement system proteases[63]. C1INH is a serine protease inhibitor belonging to the family of serpins. In SDS-PAGE the highly glycosylated[64] C1INH shows a molecular weight of 105 kDa, while the calculated weight is about 76 kDa[65,66]. In the presence of polyanionic surfaces like kaolin, sulfatides, and heparin the inhibition of α-FXIIa by C1INH is attenuated[67], indicating that α-FXIIa is protected from inactivation when bound to surfaces. In line with these findings is the

observation that this effect does not apply to β-FXIIa as is lacks surface binding sites. Further physiological inhibitors of FXIIa are plasminogen activator inhibitor-1[68], α-1-antitrypsin[69], antithrombin III[70,71], α-2-macroglobulin[72] and α-2-antiplasmin[73], although to a lesser extent than C1INH.

1.2.4 Function

1.2.4.1 Contact Activation System

In 1964 Ratnoff and Davie proposed α-FXIIa, in the following referred to as FXIIa, as activator of the intrinsic coagulation pathway by introducing their theory on the waterfall sequence for intrinsic coagulation[21]. They hypothesized that FXII was activated into FXIIa after contact with negatively charged surfaces such as glass. Which is why it is often referred to as CAS. This assumption was supported by other *in vitro* studies, which showed that FXIIa indeed activates factor XI (FXI) leading to a cascade of reactions that culminate in the production of a fibrin clot[74–77]. Activated FXI (FXIa) converts factor IX (FIX) into activated FIX (FIXa). In the presence of Ca^{2+} and activated factor VIII (FVIIIa) factor X (FX) is activated to FXa by FIXa, which in return leads to cleavage of prothrombin to thrombin. The latter results in fibrin generation and thus clot formation[78]. The CAS is therefore considered as a major part of the physiological surface host defense mechanism[26,79].

In contrast with these findings, it is to mention that FXII deficiency is not accompanied by a bleeding disorder of any kind[23]. In parallel, the extrinsic coagulation pathway was discovered. It was shown that tissue factor (TF) liberated by damaged endothelial cells acts as cofactor of factor VII (FVII). The formatted complex of TF and activated FVII (FVIIa) then activates FX, culminating in the common pathway of coagulation[78,80]. Soon more studies supported the assumption that the extrinsic coagulation pathway is the physiological initiator of coagulation, which nowadays is widely agreed on. This raised doubts as to whether FXIIa really plays a role in coagulation. On the other hand, in 1984 R. Colman collected evidence that people lacking FXII might display a higher risk for developing thrombosis[79], rising new interests on FXII in thrombosis and hemostasis, which is going to be highlighted in a further section.

1.2.4.2 Kallikrein-kinin system

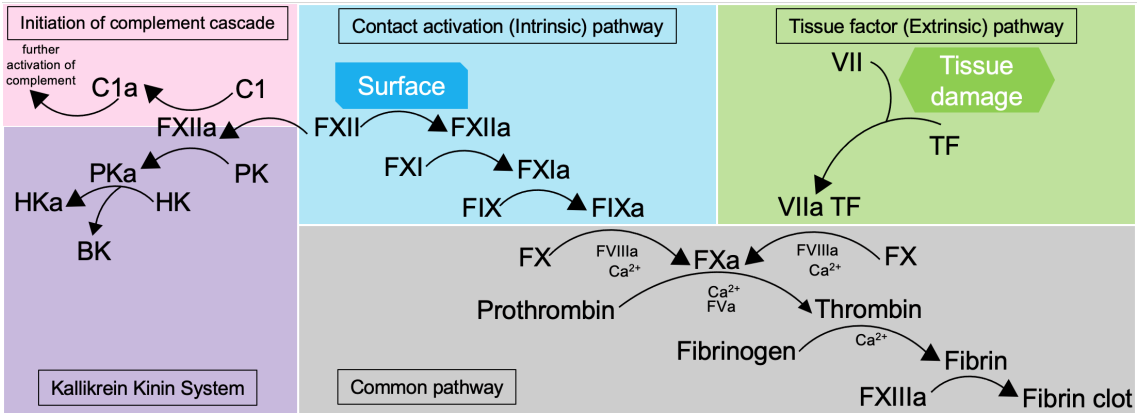
Via activation of PK, FXIIa liberates bradykinin (BK) from high molecular weight kininogen (HK)[81,82], which is also referred to as kallikrein kinin system (KKS). The nonapeptide BK[83] binds to the G-protein coupled receptors B1[84] and B2[85]. While B1 receptor is upregulated in response to inflammation, B2 receptor is expressed constitutively[86]. The intracellular signal transduction causes vasodilatation[87] and edema

formation[88,89] making BK a main effector of vascular inflammation[86]. Moreover, it was shown that FXIIa triggers fibrinolysis via the activation of plasminogen to plasmin[90] by PKa[91]. However, FXIIa-mediated plasmin activation seems to have a small impact in comparison to the classical fibrinolytic pathway[90].

1.2.4.3 Complement System

There is evidence for the activation of the classical pathway of complement by FXIIa[92,93]. The complement system is an ancient defense mechanism in vertebrates. Triggered by antibodies bound to foreign fragments, the complement system initiates various immunological responses like release of proinflammatory molecules via degranulation of mast cells and basophils, opsonization of bacteria and immune complexes, and phagocytosis of pathogens[94]. The activation of the first component of complement (C1) by FXIIa leads to conformational changes within the C1 macromolecule followed by autoactivation of the subunits C1r and C1s, which in turn trigger the powerful proteolytic complement cascade[92–94]. β-FXIIa is also able to activate PK and the complement system, but the lack of the heavy chain with its surface binding properties, prevents β-FXIIa from activating the CAS.

Figure 1-2 Overview of pathways involving FXII



In the blue square the intrinsic pathway of coagulation is shown. After contact with surfaces FXII is activated into FXIIa which is shown by an arrow. FXIIa then activates FXI into FXIa, which itself activates FIX into FIXa.

In the green square the extrinsic pathway of coagulation is shown. Tissue damage leads to the exposure of tissue factor, which activates VII into VIIa.

In the grey square the culmination of both pathways, referred to as common pathway, is shown. In the presence of FVIIIa and Calcium Ions (Ca^{2+}) FIXa activates FX into FXa. VIIa and TF also activate FX into FXa in the presence of FVIIIa und Ca^{2+}. FXa triggers the formation of thrombin form prothrombin in the presence of Ca^{2+} and FVa. Thrombin converts fibrinogen to fibrin. After activation by FXIIIa the latter results in the formatting of a fibrin clot.

In the rosa square the initiation of the complement cascade is shown. FXIIa leads to the activation of C1 into C1a, which in turn leads to further activation of the complement cascade.

In the purple square the activation of the KKS is shown. FXIIa activates PK into PKa. PKa leads to the liberation of BK from HK and to the formation of HKa from HK.

1.2.4.4 FXII in thrombosis and hemostasis

The role of FXII in coagulation was highly questioned for several reasons. First, FXII deficient people are free of hemorrhagic symptoms[20]. Second, a persuasive physiologic surface was not identified[95] and lastly the discovery of the TF- and FVII-dependent extrinsic coagulation pathway provided proof of an independent and potent clotting pathway *in vivo*[96,97]. In 2009 Spronk et al. confirmed that TF is the physiologic activator of coagulation. They found that TF-deficient mice (low TF) that were crossed with FXI- and FIX-null embryos were not viable due to heavy bleedings in utero, while low TF and FXII deficiency was compatible with live. They concluded that *in vivo* thrombin formation occurs independently of FXII and that FXII is hence dispensable for hemostasis[33,98].

However, recent findings have shown, that *f12*-knockout ($f12^{-/-}$) mice are protected from thrombosis caused by adrenaline/collagen or $FeCl_3$, raising the question whether FXII might be important for pathological thrombus formation. The fact that infusion of FXII deficient mice with human FXII reconstituted thrombus formation, supports the

hypothesis of FXII being responsible for thrombus formation and stabilization[99]. In line with these findings, another study demonstrated that mice benefit from FXII deficiency after cerebral ischemia. In comparison to wild-type mice, $f12^{-/-}$ mice showed less infarcted brain volume without additional infarct-associated bleeding. Arterial occlusion due to thrombus formation was restored after supplementing the mice with human FXII. Similar effects were observed in FXI deficient mice, indicating that FXII triggers pathologic thrombus formation via the initiation of the CAS[100]. If these effects could be translated to humans, FXII would be a possible target in reducing arterial thrombosis without an increased bleeding risk and would therefore be of an immense medical interest.

Yet, contradictory results in various studies leave more questions than answers to the role of FXII in thrombosis. Doggen et al. claimed that lower FXIIa levels were associated with higher risk for myocardial infarction (MI)[101], as well as Govers-Riemslag et al., who saw similar links between levels of C1INH-FXIIa complexes and the risk of coronary heart disease[102]. Opposed to that, elevated FXIIa levels were detected to correlate with long-term mortality after an episode of chest pain[103] and could predict acute coronary syndromes (ACS) after MI[104]. Eventually, Konings et al. could not see changes in C1INH-FXIIa levels at admission in patients with MI compared to controls[105]. All in all, these conflicting results, emphasize the necessity to analyze the role of FXII in human thrombosis more reliably.

1.2.4.5 FXII/FXIIa as growth factor

As a growing body of evidence suggests that FXII zymogen also exerts functional properties, these will be discussed in the following.

FXII and FXIIa both contain two EGF-like domains, giving rise to the question whether they also exert EGF-like functions. First a stimulation of mitogenesis in Hep-G2 cells in the presence of FXII or FXIIa was observed[106]. Analogously this observation was made with further EGF-responsive cells like endothelial cells, rat aortic smooth muscle cells, human epitheloid carcinoma cells, fetal hepatocytes and fetal alveolar type II (ATII) cells[107].

Moreover, it was shown that FXII effects growth, angiogenesis and proliferation of human umbilical vein endothelial cells in an urokinase plasminogen activator receptor (uPAR), β-Integrin and epidermal growth factor receptor (EGFR) dependent manner[108].

All in all, FXII/FXIIa overexpression could result in pathological cell growth that can be observed in the formation of malignant tumors[34].

1.2.4.6 FXII in inflammation

Originally the role of FXII in inflammation was only associated with the liberation of BK from HK by PKa. The formation of BK leads to vasodilatation via release of nitric oxide and prostaglandins which causes edema and inflammation consequently[86–89]. Moreover, a growing body of evidence suggests that bradykinin also plays an important role in migration and apoptosis of neutrophils via the B1-receptor[109,110]. B1-receptor deficient mice showed a diminished influx of neutrophiles to inflammatory sites and prolonged survival of neutrophils, proposing that the KKS might be of great interest in modifying inflammatory diseases[109].

Similarly, FXII, FXI and PK were immunolocalized to the surface of neutrophils, indicating that they play a direct role in regulating neutrophil function[42]. This was supported by the finding that FXII and FXIIa influence neutrophil aggregation and degranulation[111]. In line with these findings, it was observed that f12 knock out mice ($f12^{-/-}$) benefitted from accelerated wound healing due to a reduced activation of neutrophils. Reversely, stimulation of neutrophils with FXII zymogen favored neutrophil migration, cell adhesion and release of NETs[112].

Furthermore, FXII seems to be involved in the production and release of proinflammatory cytokines[113]. Via uPAR FXII provokes a cytokine shift in dendritic cells leading to cerebral inflammation[114]. In acute respiratory distress syndrome (ARDS), higher levels of FXII were associated with increased amounts of cytokines causing inflammation of the lung[115].

After treatment of mice with antisense oligonucleotide (ASO) directed against FVII or FXII, liver synthesis of FVII and FXII was significantly reduced. When these mice were infected with *Klebsiella pneumoniae* only the inhibition of FVII had a protective effect while the absence of FXII did not play a significant role in the outcome of gram-negative sepsis. On the other hand, it was shown that levels of FXII in the lung where independent of the ASO treatment indicating that there is a specific role of FXII expressed in the lung tissue which needs further investigation[116].

Binding of FXII to the surface of viruses, fungi and bacteria[117] was also observed. As mentioned before the activation of the CAS can be triggered by bacterial polyP[58] from gram-negative *Escherichia coli* or *Salmonella* for example[118,119]. The formed fibrin may entrap bacteria and prevent bacterial spreading as part of the host defense mechanism[120]. On the one hand it seems like FXII displays antibacterial properties. The inhibition of FXIIa and PKa in mice suffering from an infection with *Streptococcus pyogenes* led to an increase of bacteria in the spleen[121]. In FXI-deficient mice ($f11^{-/-}$) infected with *Klebsiella pneumoniae* or *Streptococcus pneumoniae* increased bacterial load in the spleen, lung and blood followed by an enormous influx of neutrophils into the

lung was observed. On the other hand, overactivation of the CAS can lead to an excessive inflammatory response including edema formation and hypotension culminating in organ failure[121]. This was supported by a study showing that inhibition of FXII diminished hypotension and mortality in baboons infected with *Escherichia coli*[122]. Additionally, *f12*$^{-/-}$ mice challenged with *Escherichia coli* LPS showed reduced hypotension[123]. Further, *f12*$^{-/-}$ mice infected with *Klebsiella pneumoniae* displayed ameliorated survival and reduced bacterial load while an infection with *Streptococcus pneumoniae* did not evoke beneficial effects in *f12*$^{-/-}$ mice[124]. When patients with systemic inflammatory response syndrome were treated with a BK antagonist no overall effect on the risk-adjusted 28-day survival was observed, yet, a subgroup of patients with gram-negative bacteremia presented a statistically significant improvement[125]. Conclusively, the role of FXII in inflammation is connected to the type of the invading bacteria and needs to be enlightened by further studies.

1.3 Objectives

As mentioned above there are contradictory results on the function of FXII during inflammation. Moreover, most studies are based on mouse models, this makes translation of the findings on the role of FXII in pneumonia to the situation in humans difficult. With this study I would like to shed a light on the levels of FXII and FXIIa in human plasma during CAP and try to find an answer to the following questions:

Is there a difference in plasma levels of FXII and or FXIIa in healthy donors compared to patients suffering from CAP?

Can sex-specific differences be detected?

Do differences in plasma levels of FXII or FXIIa correlate with clinical parameters like CRB-65 score, CRP-levels and mortality?

2 Materials and methods

2.1 Materials

2.1.1 Apparatuses and Equipment

Name	Provided by
CELLSTAR® plastic pipettes	Greiner Bio-One GmbH, Frickenhausen, Germany
Cellstar® tubes, 15 ml, 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Centrifuge MIKRO 185	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Centrifuge Rotina 35, type: 1705	Andreas Hettich GmbH und Co. KG, Tuttlingen, Germany
ChemiDoc^{tm} Touch digital imager	Bio-Rad Laboratories, Inc., Hercules, California, USA
Combitips advanced®, 1 ml	Eppendorf AG, Hamburg, Germany
Coverslips, Thickness: 1, 24 x 60 mm	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
DM IL LED Microscope	Leica Microsystems GmbH, Wetzlar, Germany
Electrophoresis chamber	Bio-Rad Laboratories, Inc., Hercules, California, USA
Glass pipettes 5 ml, 10 ml, 25 ml	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Heating Plate	Leica Microsystems GmbH, Wetzlar, Germany
HI1210, Water bath for paraffin section	Leica Microsystems GmbH, Wetzlar, Germany
HI1220, Flattening table for clinical histopathology	Leica Microsystems GmbH, Wetzlar, Germany
Holten LaminAir laminar flow hood, type S-2010	Heto-Holten A/S, Allerød, Denmark
Maxisorp plates F96	Thermo Fisher Scientific, Roskilde, Denmark
MCO-17AIC CO_2 Incubator	Sanyo Electric Co. Ltd., Osaka, Japan

MicroAmp® Fast 96-Well Reaction Plate (0,1mL)	Applied biosystems® by life technologies™, Carlsbad, CA, USA
Microcentrifuge, model: 5415 R	Eppendorf AG, Hamburg, Germany
Microscope	Müller Optronics, Erfurt, Germany
Microscope slide Superfrost® L 76 x W 26 mm, thickness 1 mm	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Microwell plate 269620, 96-well	Thermo Fisher Scientific, Roskilde, Denmark
Modular tissue embedding center EG1150	Leica Microsystems GmbH, Wetzlar, Germany
Multipipette® plus	Eppendorf AG, Hamburg, Germany
Optically Clear Adhesive Seal Sheets	Thermo Fisher Scientific, Waltham, MA, USA
PerfectSpin P, Plate centrifuge	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Pipetboy 2	INTEGRA Biosciences GmbH, Biebertal, Germany
Pipets	Gilson, Middleton, WI, USA
Pipette tips	Greiner Bio-One GmbH, Frickenhausen, Germany
Platinum® SYBR® Green qPCR SuperMix-UDG	Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA
PowerPac™ HC High-Current Power Supply	Bio-Rad Laboratories, Inc., Hercules, California, USA
Rotamax 120 platform shaker	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Roti®-Polyvinylidene difluoride (PVDF) membrane	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
ROTILABO® embedding cassettes	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
SafeSeal Reaction tubes 0,5 ml; 1,0 ml; 1,5 ml; 2,0 ml	Sarstedt AG & Co. KG, Nümbrecht, Germany
SM2000 R, Sliding microtome	Leica Microsystems GmbH, Wetzlar, Germany
SpectraMax® 190 Microplate Reader	Molecular Devices, LLC, San José, California, USA

SRT9 roller mixer	Stuart equipment, Cole-Parmer, Stone, Staffordshire, UK
StepOnePlus™ Real Time PCR System	Life technologies™, Carlsbad, CA, USA
T-Gradient Thermoblock	Biometra GmbH, Göttingen, Germany
TC Dish 100, Standard	Sarstedt AG & Co. KG, Nümbrecht, Germany
TC Plate 12 Well, Standard, F	Sarstedt AG & Co. KG, Nümbrecht, Germany
TC Plate 6 Well, Standard, F	Sarstedt AG & Co. KG, Nümbrecht, Germany
Thriller® Thermoshaker Incubator	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Vortex-Genie® 2 vortex mixer	Scientific Industries, Inc., Bohemia, New York, USA
Water bath	Leica Microsystems GmbH, Wetzlar, Germany
Water bath, Type 1004	Gesellschaft für Labortechnik mbH, Burgwedel, Germany

2.1.2 Reagents

Name	Provided by
10 x RT Buffer	Applied Biosystems by Thermo Scientific, Vilnius, Lithuania
10 x RT Random Primers	Applied Biosystems by Thermo Scientific, Vilnius, Lithuania
17-β-Estradiol	Calbiochem, Merck KGaA, Darmstadt, Germany
2-Mercaptoethanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
2-Methyl-1-Propanol, 99.6% (Isopropanol)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acetic acid, 99.6%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acrylamide	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ammonium persulfate (APS)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ampuwa® (RNase free water)	Fresenius Kabi GmbH, Bad Homburg, Deutschland
Antibody diluent, cat. no.: ZUC025-500	Zytomed Systems, Berlin, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Bromophenol blue	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
CAT Hematoxylin, cat. no.: CATHE	Biocare Medical, Zytomed Systems GmbH, Berlin, Germany
Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets	Roche Diagnostics GmbH, Mannheim, Germany
D-Phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK), cat. no.: FPRCK-01	CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany
Dako REAL™ Proteinase K Diluent, code: S2032	Dako, an Agilent Technologies Company, Santa Clara, California, USA

Dako REAL™ Proteinase K, code: S2019	Dako, an Agilent Technologies Company, Santa Clara, California, USA
Deoxynucleotide Triphosphate (d-NTP)	Applied Biosystems by Thermo Scientific, Vilnius, Lithuania
Disodium hydrogen phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt, Germany
Dulbecco's modified eagle's medium (DMEM), high glucose, pyruvate	Gibco™, Life Technologies Ltd, Paisley, UK
Endothelial Cell Medium (Glucose and Phenol Red Free), cat. no.: GPF1168	Cell Biologics, Chicago, IL, USA
Eosin	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethanol, 99.6%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Fetal bovine serum (FBS)	Gibco™, Life Technologies Ltd, Carlsbad, CA, USA
Fibronectin, powder cat. no.: F0635	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Glycine	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Guanidine thiocyanate	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hemalum solution acidic according to Mayer, cat. no.: T865	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hydrochloric acid, 32%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hydrogen peroxide, 30%	Merck KGaA, Darmstadt, Germany
Methanol, 99.6%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
MultiScribe Reverse Transcriptase (50 U/µl)	Applied Biosystems by Thermo Scientific, Vilnius, Lithuania
N-Lauroylsarcosine	Sigma-Aldrich, St. Louis, MO, USA

N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	Merck KGaA, Darmstadt, Germany
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Paraformaldehyde (PFA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Penicillin - Streptomycin	Gibco™, Life Technologies Ltd, Carlsbad, CA, USA
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich, St. Louis, MO, USA
Pierce™ ECL Western Blotting Substrate	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Potassium chloride (KCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
RiboLock RNase Inhibitor 40 U/µl	Thermo Scientific, Vilnius, Lithuania
ROTI® Histokitt	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Skim milk powder	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium azide (NaN_3)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium citrate	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium deoxycholate	Sigma-Aldrich, St. Louis, MO, USA
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium orthovanadate	Sigma-Aldrich, St. Louis, MO, USA
Sulfuric acid (H_2SO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tris	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Triton-X-100	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Trypsin-EDTA (0.5%), no phenol red, cat. no.: 15400054	Gibco™, Life Technologies Ltd, Carlsbad, CA, USA
Tween 20	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Xylene	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

2.1.3 Kits

Name	Provided by
F12 (Human) ELISA Kit, cat. no.: KA1037	Abnova, Taiwan, China
Goat-on-Rodent AP-Polymer Kit, cat. no.: GAP514H	Biocare Medical, Zytomed Systems GmbH, Berlin, Germany
peqGOLD Total RNA Kit	Peqlab, Erlangen, Germany
Permanent AP Red Kit, cat. no.: ZUC001-125	Biocare Medical, Zytomed Systems GmbH, Berlin, Germany
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
Pierce™ TMB Substrate Kit	Thermo Fisher Scientific, Waltham, MA, USA
Warp Red™ Chromogen Kit	Biocare Medical, Zytomed Systems GmbH, Berlin, Germany
Zytochem-Plus AP Polymer Kit, cat. no.: POLAP-006	Biocare Medical, Zytomed Systems GmbH, Berlin, Germany

2.1.4 Antibodies

Antibody	Provided by
Goat anti-FXII antibody, cat. no.: 206-0056	Zytomed Systems, Berlin, Germany
Goat anti-mouse immunoglobulins conjugated to HRP, cat. no.: P0447	Dako, an Agilent Technologies Company, Santa Clara, California, USA
Goat anti-rabbit immunoglobulins conjugated to HRP, cat. no.: P0448	Dako, an Agilent Technologies Company, Santa Clara, California, USA
Mouse anti-C1 esterase inhibitor antibody (KOK 12)	Dr. Sacha Zeerleder, Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Medical Center, University of Amsterdam, Netherlands
Mouse anti-C1 esterase inhibitor antibody (RII)	Dr. Sacha Zeerleder, Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Medical Center, University of Amsterdam, Netherlands
Mouse anti-CD68 antibody, cat. no.: DLN-09267	DIANOVA GmbH, Hamburg, Germany
Rabbit anti-albumin antibody, cat. no.: A 0001	Dako, an Agilent Technologies Company, Santa Clara, California, USA
Rabbit anti-goat immunoglobulins conjugated to HRP, cat. no.: P0449	Dako, an Agilent Technologies Company, Santa Clara, California, USA
Rabbit anti-high molecular weight kininogen (HK) antibody, cat. no.: ab35105	Abcam plc., Cambridge, UK
Rabbit anti-kallikrein antibody, cat. no.: A12-7118	Zytomed Systems, Berlin, Germany
Rabbit anti-prosurfactant protein C antibody, cat. no.: AB3786	Millipore, Merck KGaA, Darmstadt, Germany
Rabbit anti-von Willebrand Factor antibody, cat. no.: A 0082	Dako, an Agilent Technologies Company, Santa Clara, California, USA

2.2 Methods

2.2.1 Test samples

Human blood plasma, human bronchoalveolar lavage fluid (BALF) and human lung tissue of healthy donors and CAP patients served as test samples for the present work. The collection and use of the respective samples was approved with the informed consent of donors and patients or their next of kin and authorized by the corresponding vote of the ethics committee.

2.2.1.1 Plasma samples

Plasma was collected from blood that was mixed with the anticoagulant substance ethylenediaminetetraacetic acid (EDTA) instantly after the blood was drawn. After centrifugation for 10 min at 2000 g the blood is separated into 3 layers. Erythrocytes are situated at the bottom. Leukocytes and platelets make the intermediate buffy coat and plasma which consists of the fluid phase containing proteins, coagulation factors, electrolytes etc. is found on top. Plasma was transferred into a fresh tube and used directly or stored at -80°C.

2.2.1.1.1 CAP cohort

EDTA-plasma samples from 140 CAP patients were obtained from the CAPNETZ (application form no.: 2017-08-17-KPB8) consortium. The collection and use of the respective samples was approved with the informed consent of patients or their next of kin and authorized as part of the Transregional Collaborative Research Centre SFB-TR84 funded project “A02” by the corresponding vote of the Ethics Committee of Charité – University Medicine Berlin, approval no.: EA2/079/13.

2.2.1.1.1.1 Inclusion criteria

All CAPNETZ samples were collected during a timespan from February 2007 to March 2017. Inclusion criteria consisted of a proven pulmonary infection due to *Streptococcus pneumoniae*, *Klebsiella pneumoniae* or Influenza virus.

2.2.1.1.1.2 Demographic and clinical data

The average age of the entire group was 70 years (SD ± 11.6) with a median of 71 years. The youngest patients were 46 years old, while the eldest aged 92 years. The average age for the female was 70 years (SD ± 11.9) with a median of 71 years. For the male division the average age was 70 years (SD ± 11.4) with a median of 71 years.

The CRB-65 score, ranging from minimal 0 to maximal 4 points, averaged at 1 point (SD ± 0.9) within the entire group. The median score was 1 point also. Consistent with these data, the largest group of 61 people achieved a CRB-65 score of 1 point, followed by 29 patients with a score of 2 points. One fifth showed a CRB-65 score of 0 points. The smallest group consisted of 20 people with a score of 3 points, while a score of 4 points was only reached by 1 person. For 3 of the 140 patients the CRB-65 score was not evaluated. That is why they were excluded from the analysis.

2.2.1.1.2 Control cohort

EDTA-plasma samples of 50 healthy blood donors were provided by the Department of Clinical Immunology and Transfusion Medicine at the Justus-Liebig-University Giessen, Germany with kind authorization of Prof. Dr. Gregor Bein. The collection and use of the respective samples was approved with the informed consent of donors and authorized by the corresponding vote of the Ethics Committee of Justus–Liebig-University Giessen, approval no.: 05/00.

Another 10 samples of healthy blood donors were obtained by the Institute of Physiology - Charité - University Medicine Berlin, Germany with generous permission of Prof. Dr. Wolfgang Kübler. The collection and use of the respective samples was approved with the informed consent of donors and authorized as part of the Transregional Collaborative Research Centre SFB-TR84 funded project “A02” by the corresponding vote of the Ethics Committee of Charité – University Medicine Berlin, approval no.: EA2/079/13.

2.2.1.1.2.1 Demographic data

The average age of the entire group was 65 years (SD ± 4.7) with a median of 65 years. The youngest donors were 57 years old, while the eldest aged 76 years. The average age for the female was 64 years (SD ± 4.4) with a median of 63 years. For the male group the average age was 66 years (SD ± 4.7) with a median of 66 years.

Figure 2-1 Age and sex distribution within the cohorts

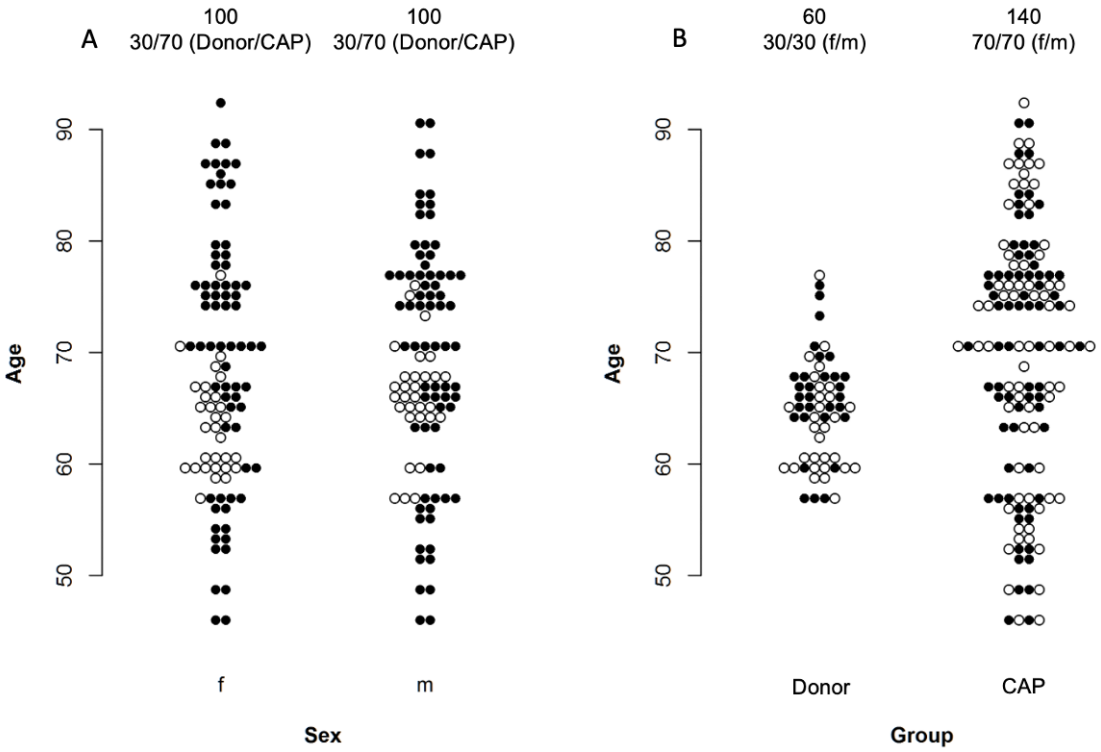


Figure 2-1A) The age distribution within the total female and male group is shown. Full circle represents CAP patient. Open circle represents healthy donor.

Figure 2-1B) The age distribution within the donor and the CAP group is shown. Full circle represents male sex. Open circle represents female sex.

At the top the total number of people per group is presented. In parentheses the distribution within the group is shown. f, female; m, male.

Table 2-1 Demographic and clinical data of CAP patients and healthy donors (Plasma samples)

	CAP	**Healthy donor**
Subjects n (%)	140 (100)	60 (100)
Sex, male/female n (%)	70/70 (50/50)	30/30 (50/50)
Age, in years		
mean ([1]SD)	70 (± 11.6)	65 (± 4.7)
median	71	65
range	46 - 92	57 - 76
Hospitalization in days		
mean (SD)	16 (± 13)	
median	12	
range	4 - 79	
[2]CRB-65 score n (%)		
0	27 (19.5)	
1	61 (44.2)	
2	29 (21)	
3	20 (14.5)	
4	1 (0.7)	

[1]SD, standard deviation; [2]CRB-65 score, confusion, respiratory rate ≥ 30/min, blood pressure: diastolic ≤ 60 mmHg, systolic < 90 mmHg and 65 age: ≥ 65

2.2.1.1.2.2 Inclusion criteria

The samples were collected from February till March 2019. Healthy blood donors and CAP patients were age- and sex-matched.

2.2.1.2 BALF samples

BALF was obtained by use of a fiberoptic bronchoscope at day 1 (within 24 h following diagnosis) from all patients. One segment of the lingula or the right middle lobe was lavaged with a total volume of 200 ml of sterile 0.9% NaCl in 10 aliquots with a fluid recovery ranging between 50 and 70%. The fractions of the BALF were pooled, filtered through sterile gauze and centrifuged at 200 g for 10 min at 4°C to remove cells and membranous debris.

2.2.1.2.1 CAP cohort

The collection and use of the respective samples was approved with the informed consent of donors and and authorized as part of the Transregional Collaborative Research Centre SFB-TR84 funded project “A02” by the corresponding vote of the Ethics Committee of Charité – University Medicine Berlin, approval no.: EA2/079/13.

2.2.1.2.1.1 Inclusion criteria

BALF was obtained from 10 CAP patients that were admitted to the Intensive Care Unit of the Department of Internal Medicine, Pneumology, and Intensive Care Medicine at the University of Giessen Lung Center, Germany. All patients were 18 or more years old and required mechanical ventilation. Their respective therapy consisted of intravenous volume substitution, low-dose heparin application, parenteral nutrition, antibiotic drug therapy and administration of vasoactive or inotropic drugs, when indicated.

2.2.1.2.2 Control cohort

The collection and use of the respective samples was approved with the informed consent of donors and authorized as part of the Transregional Collaborative Research Centre SFB-TR84 funded project “A02” by the corresponding vote of the Ethics Committee of Charité – University Medicine Berlin, approval no.: EA2/079/13.

2.2.1.2.2.1 Inclusion criteria

As controls, 20 spontaneously breathing healthy volunteers without any history of cardiac or lung disease and with normal pulmonary function (medical students and staff at the Medical School of the Justus-Liebig-University Giessen, Germany) were chosen.

2.2.1.3 Human lung tissue

Paraffin blocks containing post mortem sections of human lung tissue of three CAP patients were provided by the Institute of Pathology of the Justus-Liebig-University Giessen, Germany. The collection and use of the respective samples was approved with the informed consent of donors and authorized by the corresponding vote of the Ethics Committee of Justus-Liebig-University Giessen, approval no.: 31/93.

2.2.2 Enzyme-linked immunosorbent assay (ELISA) for human FXII

For quantification of FXII levels in the plasma samples a commercially available enzyme-linked immunosorbent assay (ELISA) purchased from Abnova (Taiwan, China) was used. All steps were executed according to the manufacturer’s instruction. First all reagents were diluted and brought to room temperature. Standard (100 ng/ml - 0.024 ng/ml) was generated by preparing serial dilutions of FXII with the supplied MIX diluent.

The ideal dilution factor of the samples was determined in preliminary experiments and found to be 1:20 000. Plasma samples were also diluted with MIX diluent. A volume of 50 µl of the standard or the sample were added to the wells of the plate pre-coated with a murine anti-FXII antibody. After 2 h, the plate was washed 5× manually by rinsing the wells with the supplied washing buffer and hitting the plate 4-5× onto absorbent paper cloths to thoroughly remove the liquid from the wells. Next, 50 µl of a biotinylated anti-FXII antibody were pipetted into each well and the plate was incubated for 1 h. Before 50 µl of a streptavidin-peroxidase (SP) conjugate were added to the wells, the plate was washed as described previously. After 30 min of incubation with the SP conjugate, the plate was washed. Then 50 µl of a peroxidase enzyme substrate were applied and as soon as the color development had reached the optimal blue color density, the reaction was stopped by the addition of 50 µl 0.5 M HCl. Directly the absorbance was read in a microplate reader (SpectraMax® 190, Molecular Devices, LLC, San José, CA, USA) at a wavelength of 405 nm and 570 nm. Absorbance at 570 nm was subtracted from readings at 405 nm to correct optical imperfections.

2.2.3 ELISA for human FXIIa

The B7 nanobody ELISA to capture FXIIa was performed, with slight modifications, according to the paper published in 2013 by S. De Maat et. al. in the third issue of the Journal of Thrombosis and Hemostasis[93]. A volume of 50 µl per well of 4 µg/ml B7 nanobodies in PBS (21 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) was immobilized overnight at 4°C onto a 96-well maxisorp plate (Thermo Fisher Scientific, Roskilde, Denmark). Afterwards the plate was washed with PBS and blocked with 1% (w/v) bovine serum albumin (BSA) in Tris buffered saline (TBS) (25 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 h at room temperature. After washing with PBS, 50 µl of standard (FXIIa, American Diagnostica Inc., Stamford, CT, USA, 1000 ng/ml - 31.25 ng/ml diluted in 0.9% NaCl) or plasma samples (diluted 1:100; 1:500; 1:1000; 1:2000 in 0.9 % NaCl) containing 25 mM D-Phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) (CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany) were added to the plate and incubated for 2 h at room temperature while shaking. Wells were rinsed with PBS-T [PBS, 0.05% (v/v) Tween-20] and incubated with 50 µl of the goat polyclonal anti-FXII antibody (Zytomed Systems, Berlin, Germany, cat. no.: 206-0056, dilution 1:2000 in 0.1% (w/v) BSA in PBS) for 1 h at room temperature. Thereafter the plate was washed with PBS-T and 50 µl of the rabbit anti-goat immunoglobulin conjugated to horse radish peroxidase (HRP) [Dako, an Agilent Technologies Company, Santa Clara, CA, USA, dilution 1:5000 in 0.1% (w/v) BSA in PBS] were added and incubated for 1 h at room temperature. The wells were rinsed with PBS-T and stained with 100 µl

tetramethylbenzidine (TMB) substrate (Pierce™ TMB Substrate Kit, Thermo Fisher Scientific, Waltham, MA, USA). The reaction was stopped by the addition of 50 µl 0.3 M H_2SO_4. Absorbance was determined at 450 nm. B7 nanobodies were provided by and used with generous permission of Dr. Coen Maas, Department for Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, the Netherlands.

2.2.4 Western Blot

Human plasma samples were analyzed by Western blotting in order to detect and demonstrate differences in levels of FXII along with related proteins of the contact activation pathway.

2.2.4.1 Preparation of the samples for gel electrophoresis

2.2.4.1.1 Plasma samples

Plasma samples were stored at -80°C prior use. First, plasma was thawed at 37°C in a water bath (Type 1004, Gesellschaft für Labortechnik mbH, Burgwedel, Germany). Thawed plasma was kept at room temperature for the next steps. Plasma was diluted 1:20 in 0.9% NaCl and mixed properly (Vortex-Genie® 2 Shaker, Scientific Industries, NY, USA). A volume of 5 µl of 5× concentrated Laemmli buffer [0.2 M Tris, pH 6.8; 10% (w/v) sodium dodecyl sulfate (SDS); 20% (v/v) glycerol; 10% (v/v) β-mercaptoethanol; 0.05% (w/v) bromophenol blue] were added to 20 µl of diluted plasma. Then samples were boiled at 99°C (Thriller Thermoshaker Incubator, PEQLAB, Erlangen, Germany) for 10 min and shortly centrifuged (MIKRO 185 centrifuge, Hettich GmbH, Tuttlingen, Germany) afterwards.

2.2.4.1.2 BALF samples

BALF was stored at -80°C and slowly thawed on ice before usage. After the samples were vigorously vortexed, 20 µl of undiluted BALF was mixed with 5 µl 5x concentrated Laemmli loading buffer, boiled and centrifuged as illustrated above.

2.2.4.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for separation of proteins by molecular weight. Prior to electrophoresis a polyacrylamide gel, consisting of a lower separating gel [7.5% separating gel: 375 mM Tris, pH 8.8, 0.1% (w/v) SDS, 7.5% (v/v) acrylamide, 0.2% (v/v) bis-acrylamide, 0.1% (v/v) tetramethyl-ethylenediamine (TEMED), 0.1% (w/v) ammonium persulfate (APS)] and an upper stacking gel [5% stacking gel: 250 mM Tris, pH 6.8, 0.1% (w/v) SDS, 5% (v/v) acrylamide, 0.1% (w/v) APS, 0.1% (v/v) TEMED] was prepared. The gel was loaded with 25 µl of the prepared samples as well as with 5 µl of a molecular weight size marker

(Page Ruler Prestained Protein Ladder, Thermo Fisher Scientific, Waltham, MA, USA). The proteins were then separated in an electrophoresis chamber filled with SDS-PAGE-running buffer [25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS] at a voltage of 100 V (PowerPac™ HC High-Current Power Supply, Bio-Rad Laboratories, Inc., Hercules, CA, USA) for about 2 h.

2.2.4.3 Immunoblotting

To make the separated proteins attainable for treatment with antibodies, they were transferred from the gel onto a 0.45 µm polyvinylidene difluoride-membrane (Roti®-Polyvinylidene difluoride (PVDF), Carl Roth GmbH + Co. KG, Karlsruhe, Germany), that was activated with 99.6% methanol. Transfer was achieved via electroblotting in blotting buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] for 1 h at a voltage of 100 V on ice. Hereinafter the membrane was incubated with 5% (w/v) skim milk powder in TBS with Tween 20 [TBS-T (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20)] for 1 h at room temperature while shaking (Rotamax 120 platform shaker, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) to block non-specific binding sites of the membrane. After the membrane was washed 3× for 10 min in TBS-T, it was incubated with one of the following antibodies: goat anti-FXII (Zytomed Systems, cat. no.: 206-0056, dilution 1:1000); rabbit anti-HK (Abcam plc., Cambridge, UK, cat. no.: ab35105, dilution 1:1000); rabbit anti-PKa (Zytomed Systems, Berlin, Germany, cat. no.: A12-7118, dilution 1:1000), mouse anti-C1INH RII (dilution 1:1000, this antibody detects free C1INH and C1INH in complexes with proteases); mouse anti-C1INH KOK 12 (dilution 1:1000, this antibody recognizes C1INH in complexes with proteases). RII and KOK 12 antibodies were provided by and used with generous authorization of Dr. Sacha Zeerleder from the Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Medical Center, University of Amsterdam, Netherlands. The incubation was performed in 50 ml falcons, rolling (SRT9 roller mixer, Stuart equipment, Cole-Parmer, Stone, Staffordshire, UK) overnight at 4°C. All primary antibodies were diluted in 1% (w/v) BSA in TBS-T containing 0.01% (v/v) sodium azide. The following day, the membrane was washed 3× for 10 min in TBS-T and incubated with a HRP-conjugated secondary antibody (all from Dako, an Agilent Technologies Company, Santa Clara, CA, USA, dilution 1:3000 in 5% (w/v) skim milk powder in TBS-T) for 1 h at room temperature while rolling. Before proceeding, the membrane was washed as described above. Finally, protein bands were uncovered via chemiluminescent development of the membrane (ChemiDoc^{tm} Touch, Bio-Rad Laboratories, Inc., Hercules, CA, USA) by applying an enhanced chemiluminescent (ECL) substrate for detection of HRP (Pierce^{TM} ECL Western Blotting Substrate, Thermo Fisher Scientific, Waltham, MA, USA). To

evaluate the amount of protein loaded on the gel, the membrane was treated with a rabbit anti-albumin antibody (Dako, an Agilent Technologies Company, Santa Clara, CA, USA, 1:1000). This step was preceded by the incubation of the membrane with a 50°C warm stripping solution [100 mM glycine, 0.32% (v/v) HCl] 2× for 15 min each to remove the antibody previously bound to the membrane.

2.2.5 Histological staining

2.2.5.1 Tissue slicing

The paraffin embedded tissue was cut to 3 µm thin slices with a sliding microtome (SM2000 R, Leica Microsystems GmbH, Wetzlar, Germany). The slices were immobilized to microscope slides (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and dried at 37 °C overnight (HI1220, Leica Microsystems GmbH, Wetzlar, Germany).

2.2.5.2 HE-Staining

The slides were heated at 59°C for 1 h to ease the deparaffinization. The slides were then deparaffinized 3× for 5 min in xylene (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). In the following steps the slides were hydrated in series of graded ethanol (99.6%, 96%, 70%, 50%) to distilled water. Thereafter they were placed in Mayer's hemalum solution (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 1 min and blued in tap water for 10 min. Afterwards they were immersed in distilled water and stained in eosin solution [0.5% (w/v) eosin, 0.5% (v/v) acetic acid] for 2 min while shaking. Finally, the slides were dehydrated in series of graded ethanol (99.6%, 96%, 70%, 50%) shortly cleared in xylene and sealed with a coverslip (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) utilizing Roti Histokitt (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.2.5.3 Immunohistochemistry for FXII

The staining was performed at room temperature. Before the slides were deparaffinized 3× for 10 min in xylene, they were heated at 59°C for 1 h. In the following steps the slides were hydrated in series of graded ethanol (99.6%, 99.6%, 96%, 70%) to distilled water. Next, the slides were placed in sodium citrate buffer (10 mM sodium citrate, pH 6) and heated at 95°C for 30 min. After cooling down to room temperature, the slides were washed shortly in distilled water and TBS (50 mM Tris, 150 mM NaCl, pH 7.2). In addition, antigen retrieval was performed with proteinase K (Dako, an Agilent Technologies Company, Santa Clara, CA, USA) for 5 min at room temperature, followed by a further washing step with TBS. Subsequently, the slides were placed in a freshly made hydrogen peroxide methanol solution (15% (v/v) H_20_2, 50% (v/v) CH_3OH) for 20 min. Afterwards they were washed 2× for 5 min in distilled water and then 2× for 5 min

in TBS while shaking. Thereafter the slides were blocked with 10% (w/v) BSA in TBS for 1 h and then washed 4× in TBS. The primary goat-anti-FXII antibody (Zytomed Systems, Berlin, Germany, cat. no.: 206-0056, dilution 1:500) was diluted in antibody diluent (Zytomed Systems, Berlin, Germany) and applied overnight at 4°C. Next, the slides were washed 4× for 5 min in TBS while shaking and then 3× for 20 min in TBS. About 5 drops of Goat Probe (Goat-on-Rodent AP-Polymer, Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) were applied on every slide for 15 min and washed in TBS for 5 min subsequently. Similarly, the goat-on-rodent AP-polymer (Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) was utilized. One drop of warp red chromogen (Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) was mixed with 2.5 ml of warp red buffer (Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) before 200 µl of the mix were added to the slides. The staining intensity's development was observed under the microscope (Müller Optronic, Erfurt, Germany) and stopped after 3-7 min, when the specific staining reached its optimum. To stop the reaction the slides were immersed in distilled water and washed in TBS for 1 min. Counterstaining was performed with hematoxylin (Biocare Medical, Zytomed Systems GmbH, Berlin, Germany, dilution 1:10 in distilled water) for 30 s. After bluing in tap water for 10 min, the slides were dehydrated in isopropanol (99.6%) and cleared in xylene. The slides were sealed with a coverslip (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) using Roti Histokitt (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.2.5.4 Immunohistochemical staining for macrophages

Staining for macrophages was performed, with slight adjustments, similarly to the FXII-staining. Instead of citrate buffer, the slides were heated in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 9). Treatment with proteinase K was not required. Before applying the antibody, the slides were incubated with blocking solution (Zytochem-Plus AP Polymer Kit (#POLAP-006), Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) for 5 min at room temperature and then washed 4× with TBS. About 3-5 drops of mouse anti-CD68-antibody (DIANOVA GmbH, Hamburg, Germany, cat. no.: DLN-09267) were applied and the slides were incubated overnight at 4°C. After washing in TBS, the post block reagent (Zytochem-Plus AP Polymer Kit (#POLAP-006), Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) was administered for 20 min. Thereafter, the slides were washed 3× in TBS for 5 min and the AP-Polymer (Zytochem-Plus AP Polymer Kit (#POLAP-006), Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) was applied for 30 min. Slides were rinsed with TBS 3× for 2 min before the AP-Red Chromogen (Permanent AP Red Kit, Zytomed Systems GmbH, Berlin, Germany) was mixed with the AP-Red buffer (Permanent AP Red Kit, Zytomed Systems

GmbH, Berlin, Germany) according to the instruction provided by the supplier and applied upon the slides. The following steps were executed as for the FXII-staining.

2.2.5.5 Immunohistochemical staining for alveolar type II cells

Except for the antibody used, staining for lung epithelial cells was performed similarly to the staining for macrophages. The rabbit anti-pro-surfactant protein-C (pro-SP-C) antibody (Millipore, Merck KGaA, Darmstadt, Germany, cat. no.: AB3786) was diluted 1:700 in antibody diluent.

2.2.5.6 Immunohistochemical staining for endothelium

Deparaffinization and hydration was executed as described above followed by digestion with proteinase K for 10 min at room temperature (see FXII-staining). The slides were rinsed in distilled water and incubated with Blocking reagent (Zytochem-Plus AP Polymer Kit (#POLAP-006), Biocare Medical, Zytomed Systems GmbH, Berlin, Germany) for 5 min. After washing 4× in TBS for 5 min the antibody was applied. The rabbit anti-von Willebrand Factor (vWF) antibody (Dako, an Agilent Technologies Company, Santa Clara, California, USA, cat. no.: A 0082) was diluted 1:400 in antibody diluent and incubated on the slides for 30 min at room temperature. The next steps were performed as described for the staining for macrophages.

2.2.6 Statistics

Densitometric analysis was performed using Image Lab software[126]. To analyze ELISA and Western blot data from experiments with plasma the program R4.0.0[127] was used. Log concentrations of FXII and log ratios to albumin of FXIIa- C1INH and HK were used in statistical analyses. Western blot data were adjusted for differences between membranes using a general mixed model with disease group (CAP/donor), sex, group: sex interaction, and a natural spline with 3 degrees of freedom for age as fixed effects and a random intercept by membrane ID. Adjustment was done using the sum of the model prediction without random effects plus the model residuals, effectively removing any variation attributable to the random effects (i.e., to the different membranes), accounting for differences in group, sex, and age. Correlations with group, sex, and age were analyzed using linear models. CBR-65 score was modeled depending on the FXII/FXIIa-C1INH/HK amounts using an ordered logistic regression model. Survival was modeled with a binary logistic regression model including a natural spline with 3 degrees of freedom for age. Probability values (p-values) were obtained from likelihood-ratio tests[128]. For analysis of data resulting from experiments with BALF GraphPad Prism software[129] was used.

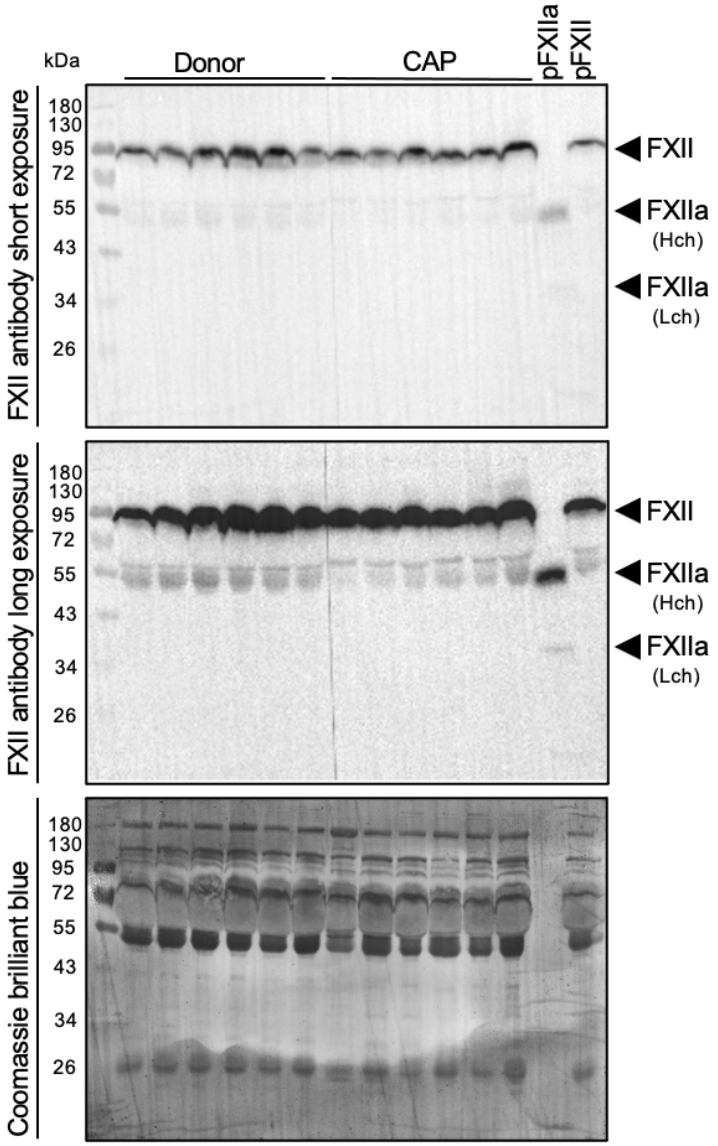
3 Results

3.1 No differences in levels of FXII between healthy donors and CAP patients measured by western blot

To investigate the expression of FXII and FXIIa in CAP, western blot analysis of human plasma samples was performed. A group of CAP patients was compared to an age- and sex-matched group consisting of healthy donors. The Zytomed anti-FXII antibody detects the FXII zymogen at around 80 kDa, the heavy chain (Hch) at around 50 kDa and the light chain (Lch) at around 30 kDa as shown in Figure 3-1.

Comparing the FXII levels in the healthy donors and in CAP patients, there is no visible difference between the groups. This is why we decided to perform an ELISA for better quantification.

Figure 3-1 No differences in levels of FXII between healthy donors and CAP patients measured by western blot

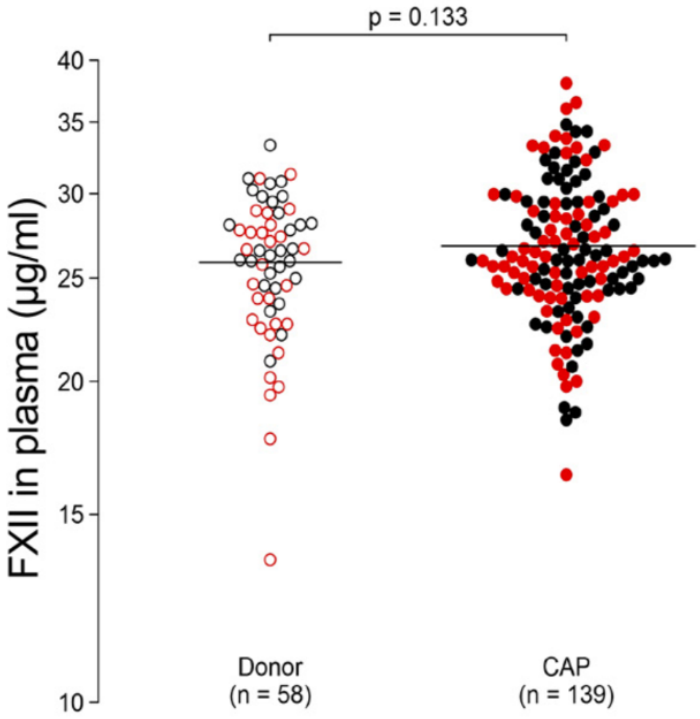


Western blot showing FXII and FXIIa protein levels in plasma of CAP patients and healthy donors. Here, 6 out of 138 CAP patients and 6 out of 58 healthy donors are displayed. Purified FXII (pFXII) and FXIIa (pFXIIa) were added to the blot to show the specificity of the antibody used as shown in the top and middle blot. At the bottom the Coomassie brilliant blue staining of the respective blot is displayed in order to show that equal amounts of proteins in the samples were used. kDa, kilodaltons; Hch, heavy chain; Lch, light chain.

3.2 No differences in levels of FXII between healthy donors and CAP patients measured by ELISA

With the commercially available Abnova ELISA for detection of FXII, 139 plasma samples of CAP patients and 58 plasma samples of sex- and age-matched donors were analyzed. As shown in Figure 3-2 the mean concentration of FXII in donors was about 26 μg/ml compared to 27 μg/ml in CAP patients. All in all, no significant difference between these groups was detected.

Figure 3-2 No differences in levels of FXII between healthy donors and CAP patients measured by ELISA

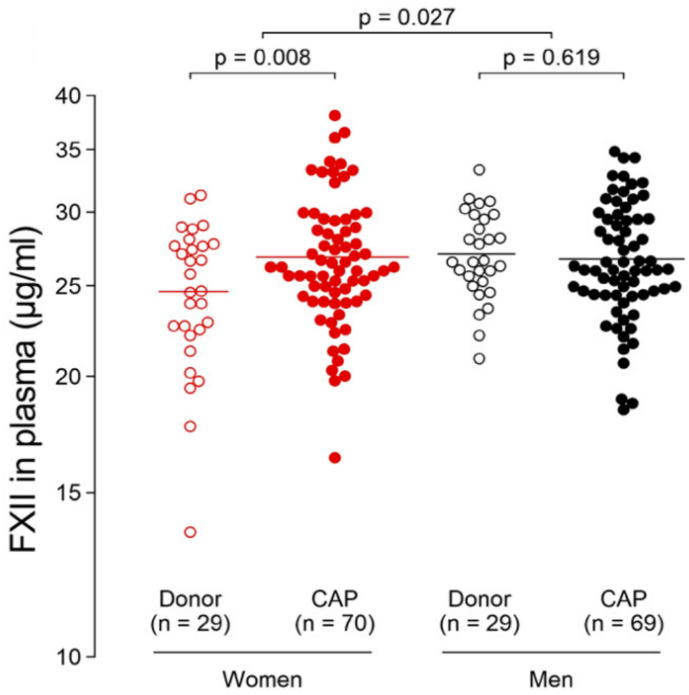


FXII concentration in plasma as measured by ELISA in healthy donors and CAP patients is shown. The data were analyzed with a two-way ANOVA model (log-transformed response). Black circles represent men. Red circles represent women. Full circles represent CAP patients. Open circles represent healthy donors. n, number; p, p-value.

3.3 Lower levels of FXII in healthy women than in female CAP patients

With regard to sex-specific differences the results of the performed ELISA show lower levels of FXII in healthy women than in healthy men (see Figure 3-3). There was no difference detected comparing female and male CAP patients. Within the male cohort there was also no difference between healthy donors and CAP patients. Compared to female CAP patients, healthy women showed lower levels of FXII.

Figure 3-3 Lower levels of FXII in healthy women than in female CAP patients

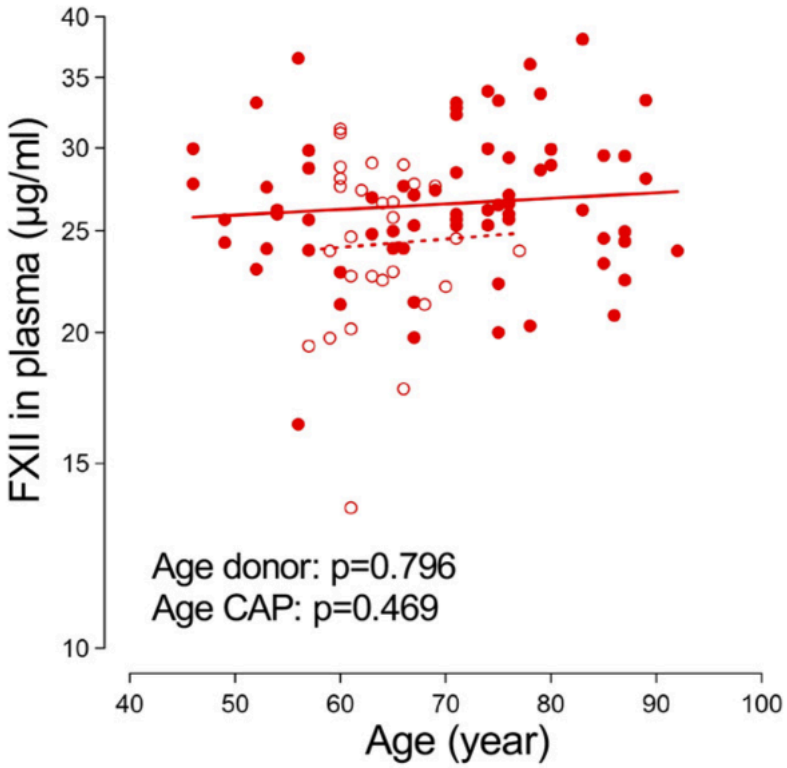


FXII concentration in plasma as measured by ELISA in women and men in healthy donors and CAP patients is shown. The data were analyzed with a two-way ANOVA model (log-transformed response). Black circles represent men. Red circles represent women. Full circles represent CAP patients. Open circles represent healthy donors. n, number; p, p-value.

3.4 No correlation between FXII levels and age of female subjects

As we know that FXII synthesis is stimulated by estradiol[27,130] and that estradiol concentrations decline during postmenopause[131], I wanted to examine the association between FXII levels and age of female subjects. According to my results as displayed in Figure 3-4 there is no correlation between age and FXII levels neither in female patients nor in female donors.

Figure 3-4 No correlation between FXII levels and age of female subjects



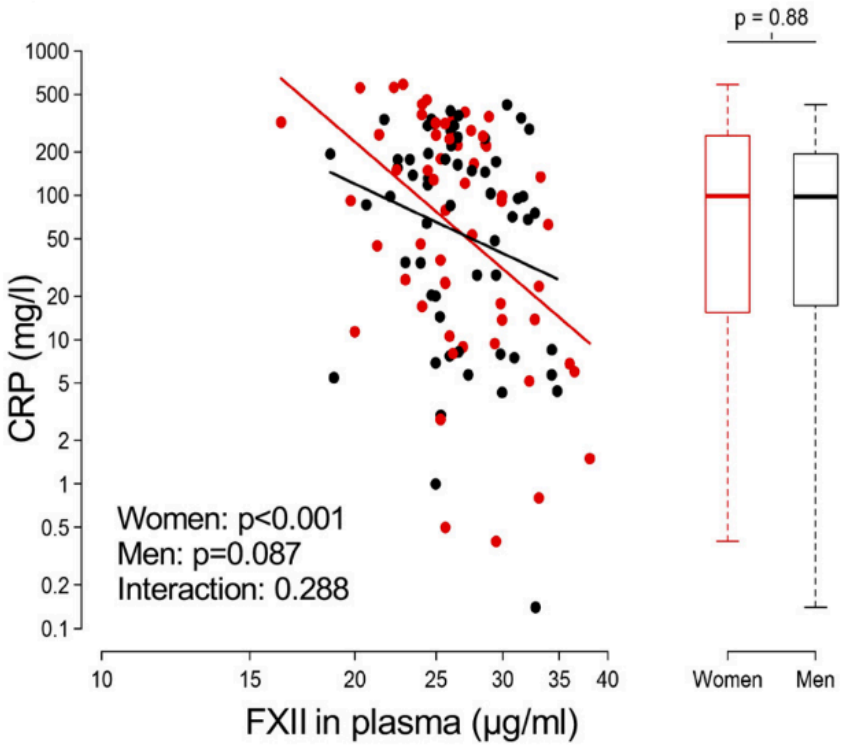
Correlation between FXII plasma levels and age of female donors and of females with CAP is shown. The data were analyzed with an ANCOVA model (log-transformed variables). Full circles represent female CAP patients. Open circles represent female donors. p, p-value.

3.5 Negative correlation between CRP levels and FXII levels in female CAP patients

Plasma levels of CRP increase during infection and inflammation[132]. In the clinical context CRP serves as a parameter to evaluate the severity of an inflammation as higher CRP concentrations correlate with higher mortality[133,134].

During my analysis I saw that there is a negative correlation of CRP and FXII levels in female patients, whilst there is no evident correlation in male patients. The respective results are displayed in Figure 3-5.

Figure 3-5 Negative correlation between CRP levels and FXII levels in female CAP patients



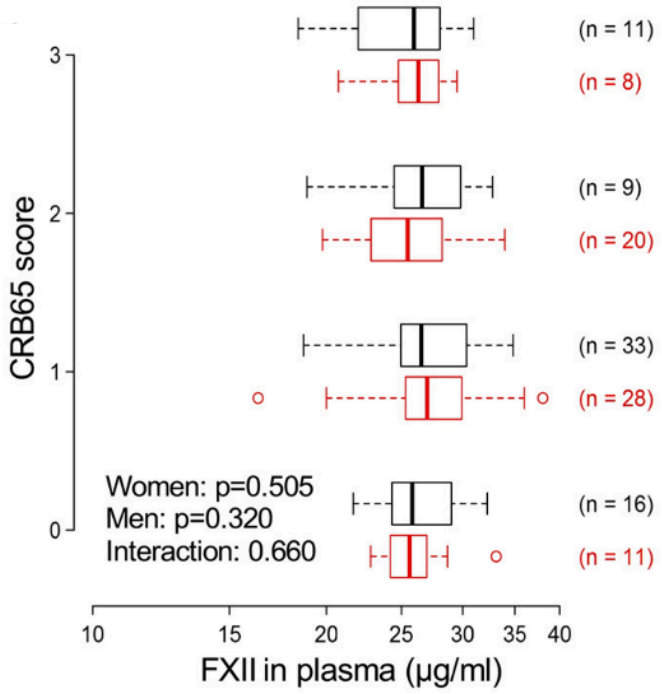
On the left the correlation between levels of FXII and the concentration of CRP on admission in plasma of patients with CAP is shown. The data were analyzed with an ANCOVA model (log-transformed variables). On the right CRP values in CAP in women and in men are shown. Red circles represent women. Black circles represent men. p, p-value.

3.6 No correlation of FXII levels with other clinical parameters like CRB-65 score or mortality of CAP patients

As outlined in the objectives of this work I wanted to examine correlations of FXII levels with clinical parameters. Exemplarily shown are the CRB-65 score and the 28-day probability of survival (see Figure 3-6 and Figure 3-7). As mentioned above the CRB-65 score is a score used initially to decide on an outpatient setting or an admission to the hospital. A higher CRB-65 score correlates with a higher mortality[18,19].

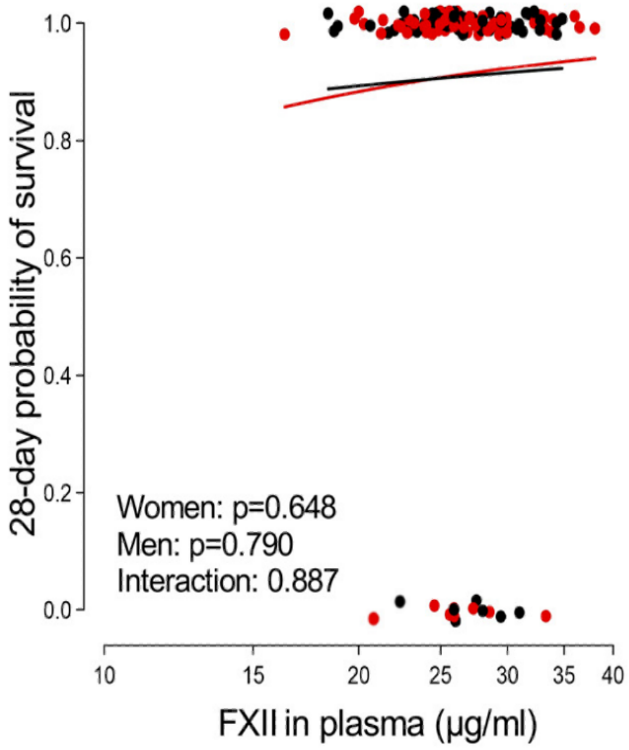
Apart from negative correlations between CRP and FXII levels there were no further correlations detectable.

Figure 3-6 No correlation between CRB-65 score an FXII levels in CAP patients



FXII plasma levels an CRB-65 score values on admission in patients suffering from CAP are shown. The data were analyzed with an ordered logistic model of score vs. log concentration. Red squares represent women. Black squares represent men. n, number; p, p-value.

Figure 3-7 No correlation between 28-d probability of survival and FXII levels in CAP patients



The 28-day survival probability in dependency of FXII levels in the plasma of patients with CAP is shown. The data were analyzed with a binomial model adjusted for age (cubic spline with 3 degrees of freedom). Red circles represent women. Black circles represent men. p, p-value.

3.7 FXIIa levels in CAP patients are decreased in comparison to healthy donors

With regard to activated FXII (FXIIa) the middle blot in Figure 3-1 shows that there is a weaker signal of FXIIa. in CAP patients than in sex- and age-matched healthy donors, indicating that an activation of FXII takes place during infection.

As the signal for FXIIa was rather light even after long exposure time, we decided to perform an ELISA for better quantification of FXIIa levels in plasma.

3.8 FXIIa concentration in plasma samples could not be detected by ELISA for human FXIIa

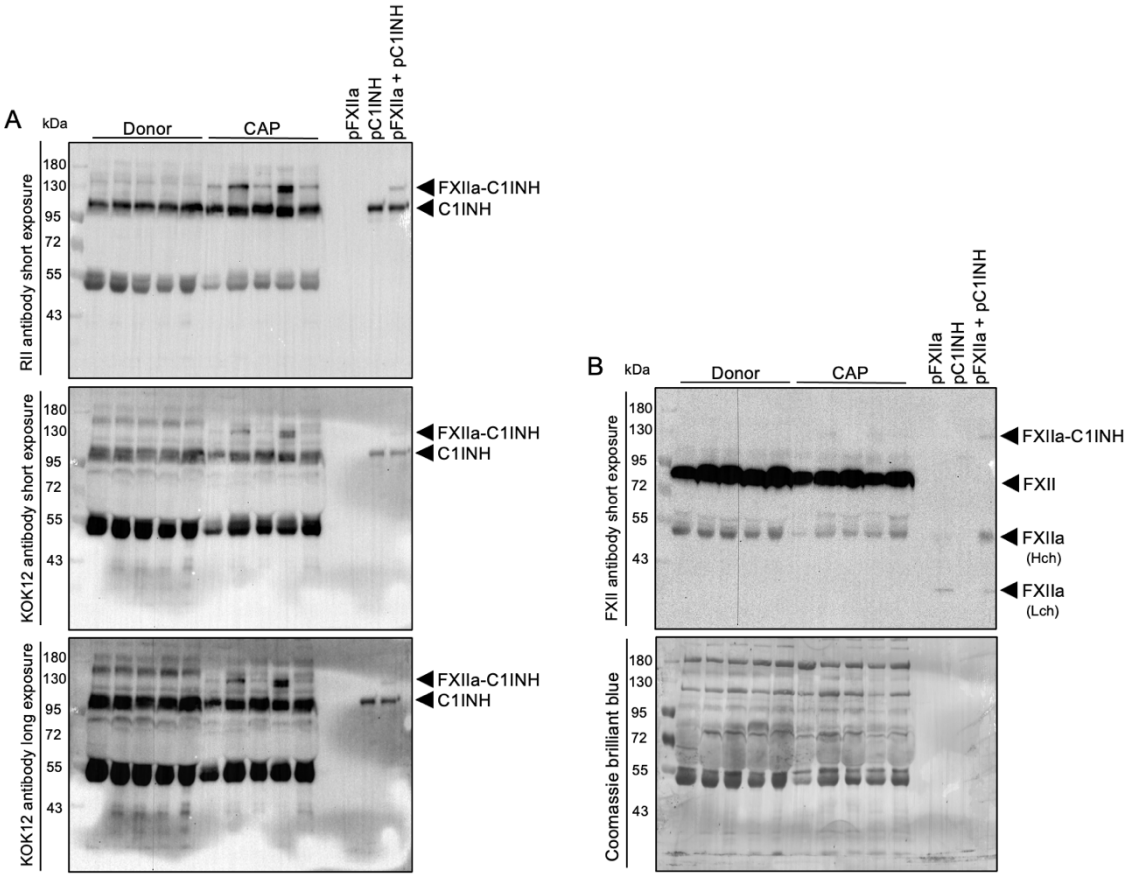
We performed a B7 nanobody based ELISA for measurement of FXIIa in plasma samples following the instructions of Coen Maas' publication[47]. Our positive control consisting of purified FXIIa in different dilutions could always be detected proofing that

the ELISA itself works. Unfortunately, FXIIa levels in our samples were always below the detection limit. The addition of D-Phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) also did not stabilize FXIIa in the present samples. Therefore, we decided to perform a modified western blot analysis for the detection of FXIIa in our samples.

3.9 FXIIa-C1INH/albumin ratio is increased in plasma of CAP patients

Activated FXII (FXIIa) is rapidly inhibited through the binding to C1INH. Together they form a complex, which is very stable[62]. The FXIIa-C1INH complex can be detected by the specific antibodies. As the signal for FXIIa with the used FXII antibody was weak (see Figure 3-1 and Figure 3-8B), we decided to perform a western blot using the C1INH antibody RII, which detects not only the C1INH but also its complexes with FXIIa (see Figure 3-8A) to quantify the amount of FXII activation in CAP. The FXIIa-C1INH complex generated in vitro served as a positive control (see Figure 3-8A). As the signal for FXIIa-C1INH complexes with the KOK 12 antibody was lighter than the signal with the RII antibody (see Figure 3-8A) even after long exposure, we decided to perform the following analysis based on the RII antibody. Plasma samples of 140 CAP patients were tested and compared to 56 sex- and age-matched donors. To minimize the effect of irregular sample application, I used albumin as loading control. Albumin is the most abundant protein in human plasma[135]. Its molecular weight is around 66 kDa[135,136]. Using densitometric analysis via Image Lab Software[126] a ratio of FXIIa-C1INH and albumin was calculated. CAP patients showed higher FXIIa-C1INH/albumin ratios than healthy donors. The difference could be observed both in female and male cohorts. The respective results are shown in Figure 3-9, Figure 3-10 and Figure 3-11.

Figure 3-8 The RII und the KOK12 antibody bind to FXIIa-C1INH complexes that cannot be detected using the FXII antibody

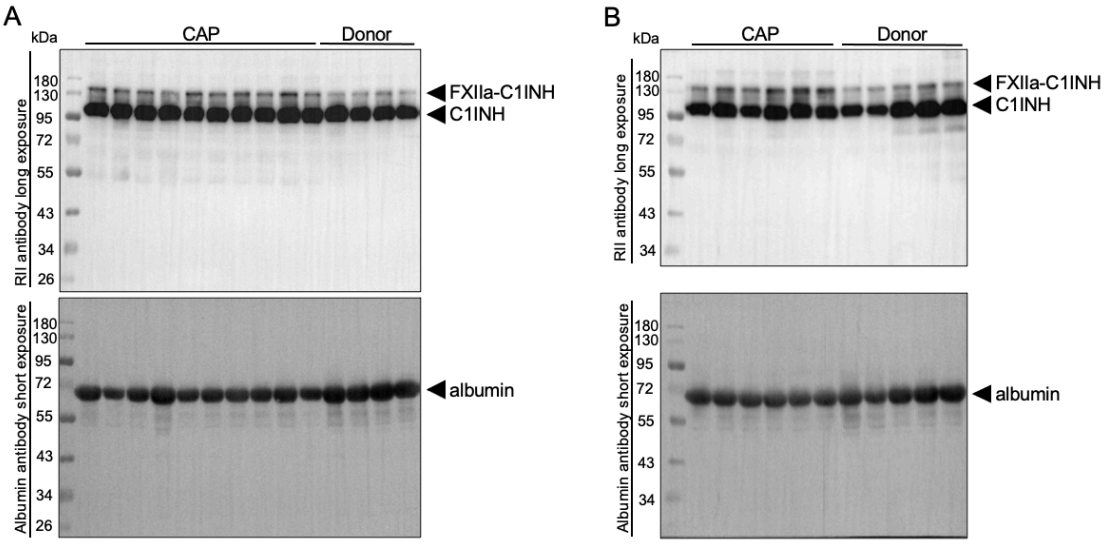


A) Western blot showing FXIIa-C1INH-complexes in plasma of CAP patients and healthy donors detected with the RII and the KOK12 antibody, which detect C1INH at 100 kDa and its complexes with FXIIa at 130 kDa. For KOK12 short and long exposure time are shown.

B) Top panel shows FXII in plasma of CAP patients and healthy donors. Bottom panel shows coomassie brilliant blue staining of the respective blot, displayed to demonstrate equal amounts of proteins in the plasma samples used.

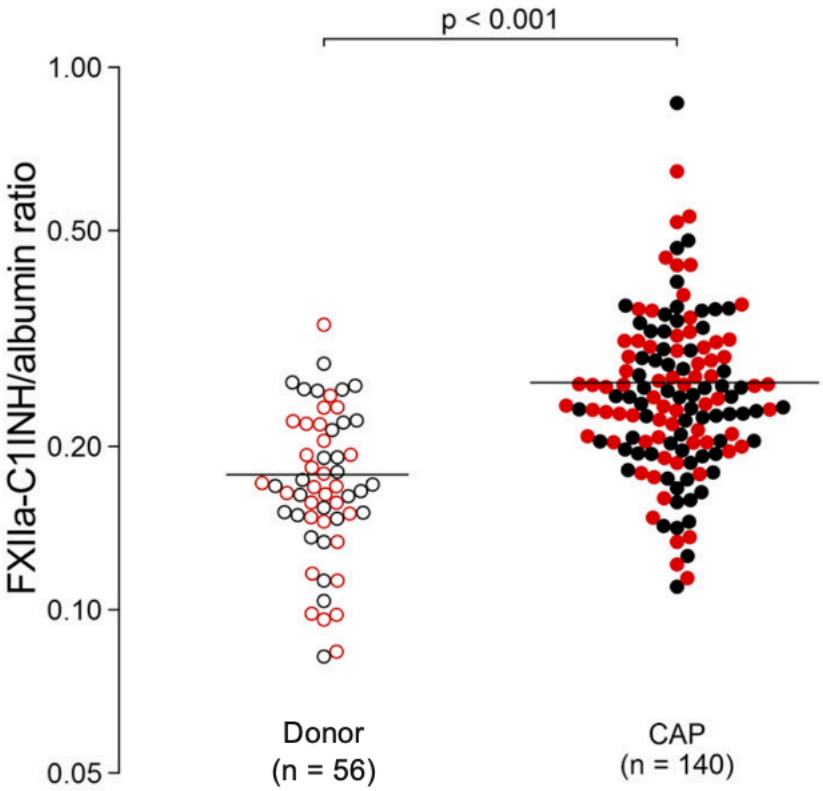
A + B) 5 out of 140 CAP patients and 5 out of 56 healthy donors are displayed. Purified FXIIa (pFXIIa) and purified C1INH (pC1INH) were added to the blot to show the specificity of the antibodies used. kDa, kilodaltons; Hch, heavy chain; Lch, light chain.

Figure 3-9 Levels of FXIIa-C1INH complexes are increased in plasma of CAP patients



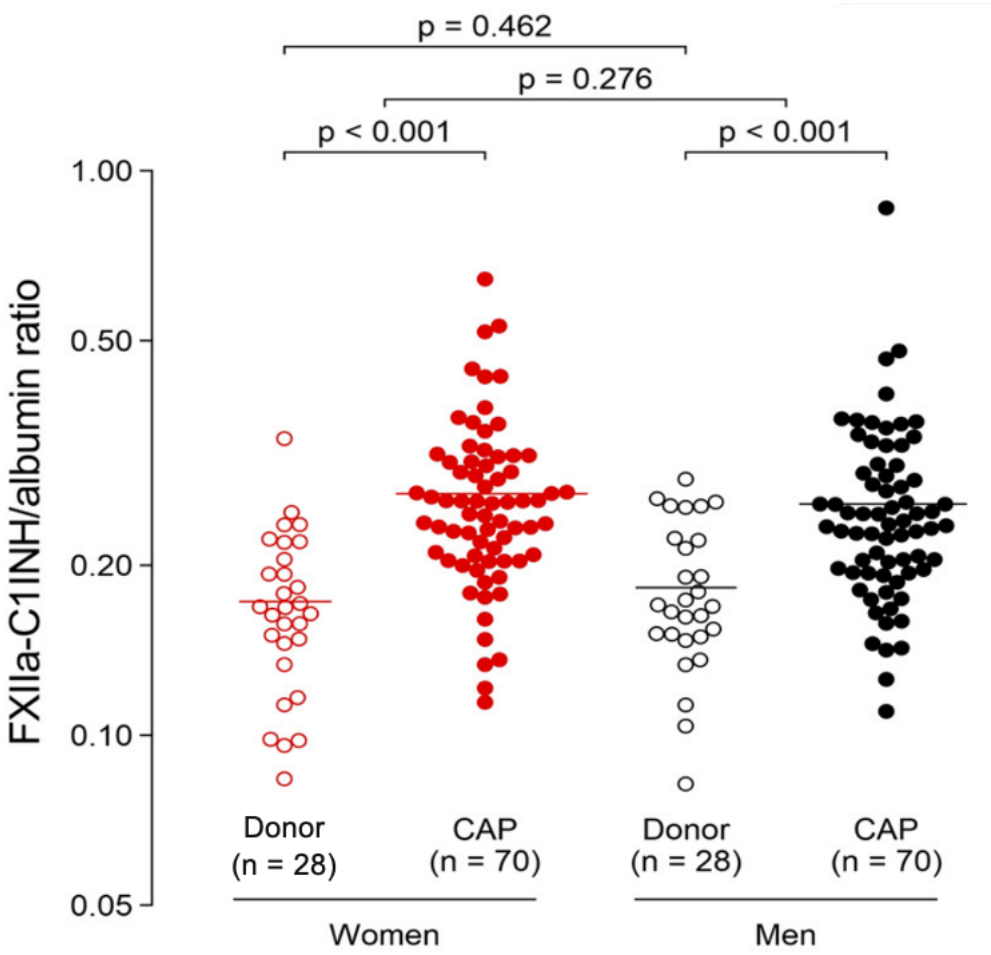
A + B) Top panel shows western blot of FXIIa-C1INH-complexes in plasma of CAP patients and healthy donors detected with the RII antibody. Bottom panel shows signal for albumin used as a loading control for visualization of total protein content. 16 out of 140 CAP patients and 9 out of 56 healthy donors are shown. kDa, kilodaltons.

Figure 3-10 Levels of FXIIa-C1INH complexes are increased in plasma of CAP patients



FXIIa-C1INH ratio as obtained via densitometric analysis of western blots in healthy donors compared to CAP patients is shown. The data were analyzed with a two-way ANOVA model (log-transformed response). Full circles represent CAP patients. Open circles represent healthy donors. Red circles represent women. Black circles represent men. n, number; p, p-value.

Figure 3-11 Levels of FXIIa-C1INH complexes are increased in plasma of CAP patients in the sex specific subgroup analysis

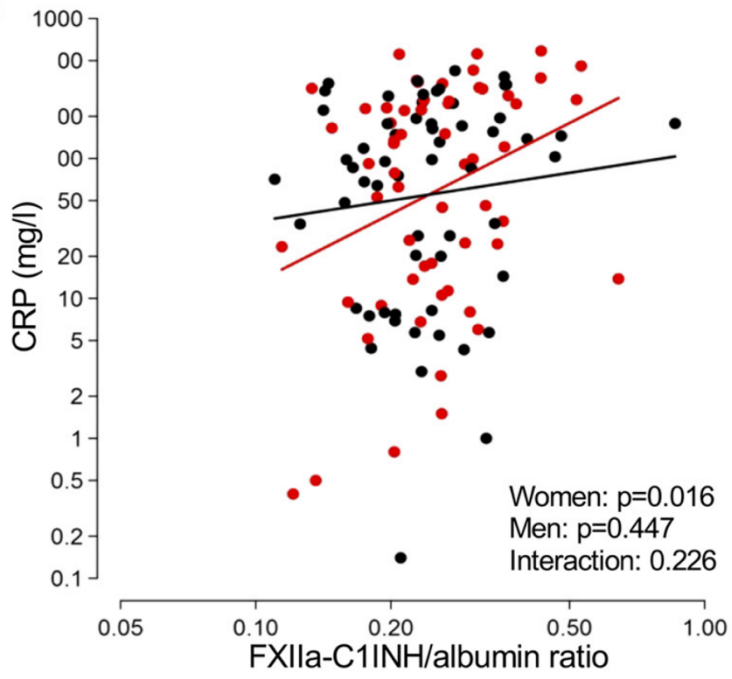


FXIIa-C1INH ratio in CAP patients and healthy donors stratified for sex is shown. The data were analyzed with a two-way ANOVA model (log-transformed response). Full circles represent CAP patients. Open circles represent healthy donors. Red circles represent women. Black circles represent men. n, number; p, p-value.

3.10 CRP levels in female CAP patients correlate with FXIIa-C1INH/albumin ratios

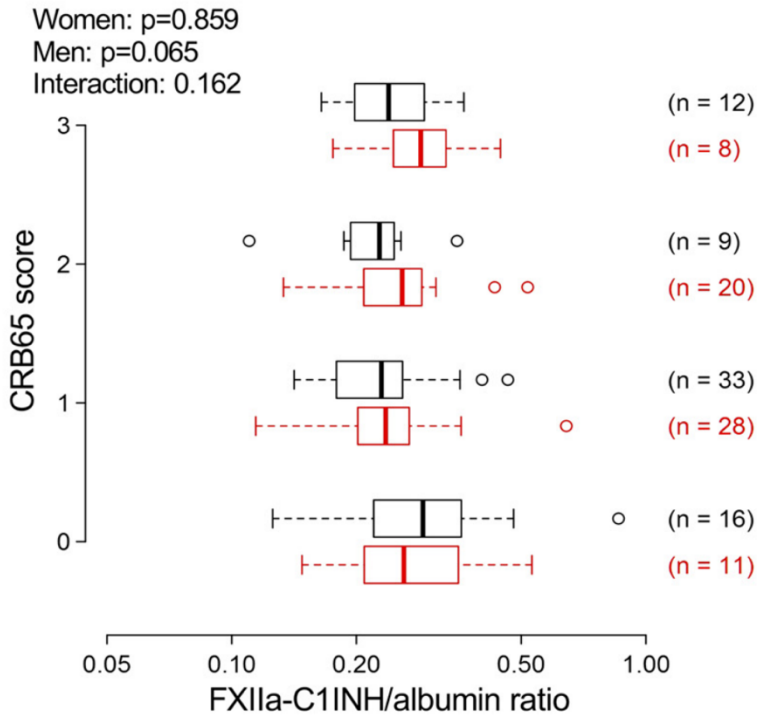
As shown in Figure 3-12 a positive correlation between FXIIa-C1INH/albumin ratios and CRP levels in female CAP patients could be detected. As there has been a negative correlation between FXII levels and CRP levels in female CAP patients the results indeed indicate an activation of FXII in severe CAP. On the other hand, no correlation between CRB-65 score or mortality could be detected (see Figure 3-13 and Figure 3-14). Also, for male patients there were no correlations between FXIIa-C1INH/albumin ratios and clinical parameters.

Figure 3-12 CRP levels in female CAP patients correlate with FXIIa-C1INH/albumin ratios



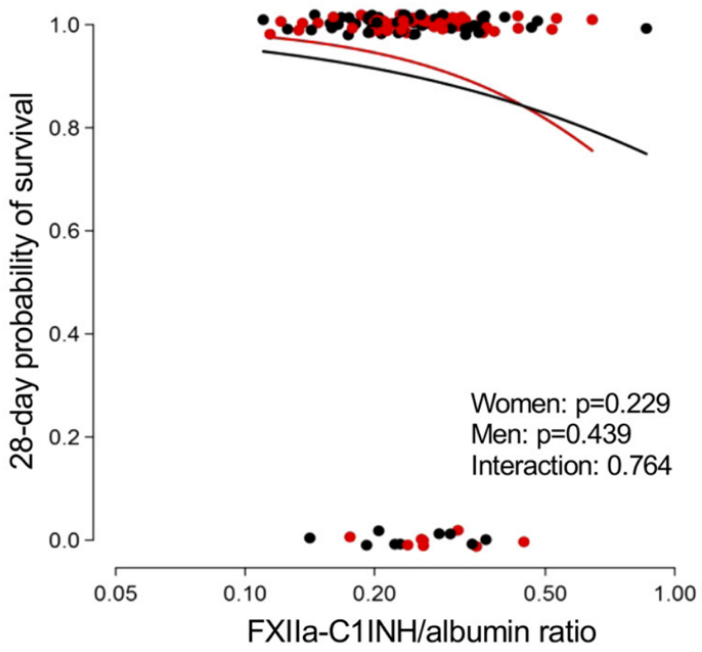
Correlation between the levels of FXIIa-C1INH ratio and the concentration of CRP in plasma on admission in patients with CAP is shown. The data were analyzed with an ANCOVA model (log-transformed variables). Red circles represent women. Black circles represent men. p, p-value.

Figure 3-13 No correlation between FXIIa-C1INH/albumin ratios and CRB-65 score in CAP patients



FXIIa-C1INH ratio and CRB-65 score values on admission in patients with CAP are shown. The data were analyzed with an ordered logistic model of score vs. log concentration. Red squares represent women. Black squares represent men. n, number; p, p-value.

Figure 3-14 No correlation between FXIIa-C1INH/albumin ratios and 28-d probability of survival of CAP patients

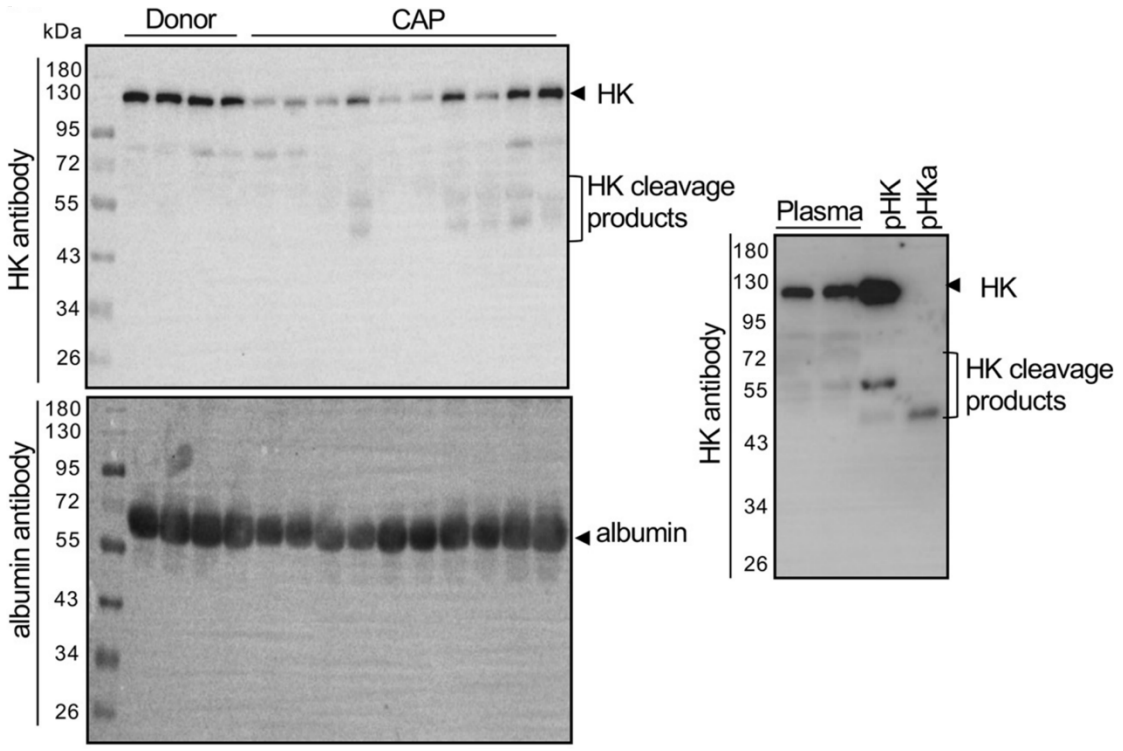


28-day survival probability in dependency of the levels of FXII-C1INH ratio in plasma of patients with CAP is shown. The data were analyzed with a binomial model adjusted for age (cubic spline with 3 degrees of freedom). Red circles represent women. Black circles represent men. p, p-value.

3.11 Decreased HK/albumin ratios in female CAP patients compared to healthy women

Densitometric analysis of western blots detecting high molecular weight kininogen (HK) as part of the kallikrein kinin system (KKS) in plasma samples of CAP patients compared to plasma samples of healthy donors showed that the signal of HK in plasma of CAP patients is weaker than in healthy donors (see Figure 3-15). Additionally, HK cleavage products (see Figure 3-15 bands at 56 kDA and 43 kDa) in plasma of CAP patients became visible. All in all, these observations support the hypothesis of an activation of the KKS as an indicator for FXII activation in CAP. As cleavage products of HK are relatively unstable due to fast degradation it is difficult to measure them[137]. For this reason, we decided to measure HK as an indicator for contact phase and FXII activation. A number of 138 CAP patients and 58 donors was tested (see Figure 3-16). Albumin served as loading control leading to a HK/albumin ratio after densitometric analysis. With regard to sex specific differences, we saw that a difference between healthy donors and patients was only visible in women (see Figure 3-17). Compared to healthy men, healthy women showed higher HK/albumin ratios. HK/albumin ratios were not different comparing male groups. Furthermore, a correlation between the CRP levels and the HK/albumin ratios in CAP patients was not found. There was also no correlation found between the mortality and HK/albumin ratios in CAP patients (see Figure 3-18 and Figure 3-19).

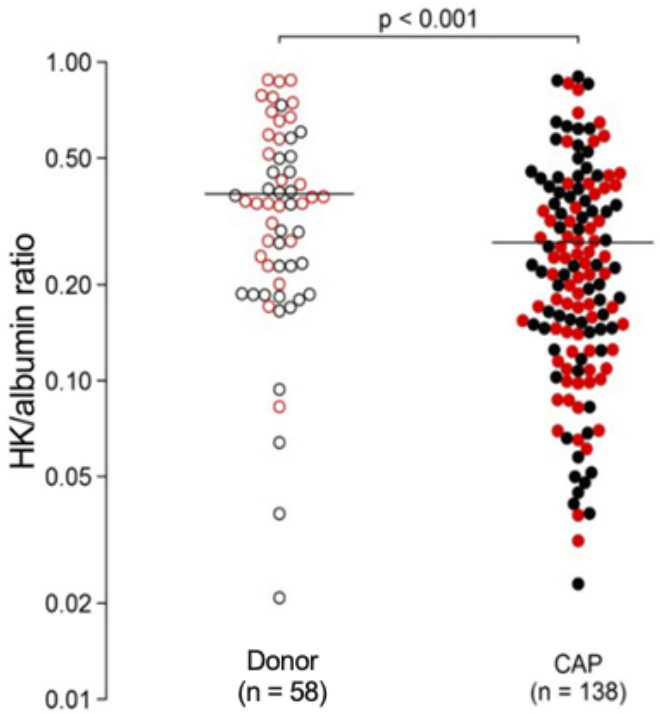
Figure 3-15 Levels of HK are lower in CAP patients than in healthy donors



Left panel shows western blot analysis of HK in plasma from patients with CAP and donors. Albumin was used as a loading control. 10 out of 138 patients with CAP and 4 out of 58 donors are demonstrated.

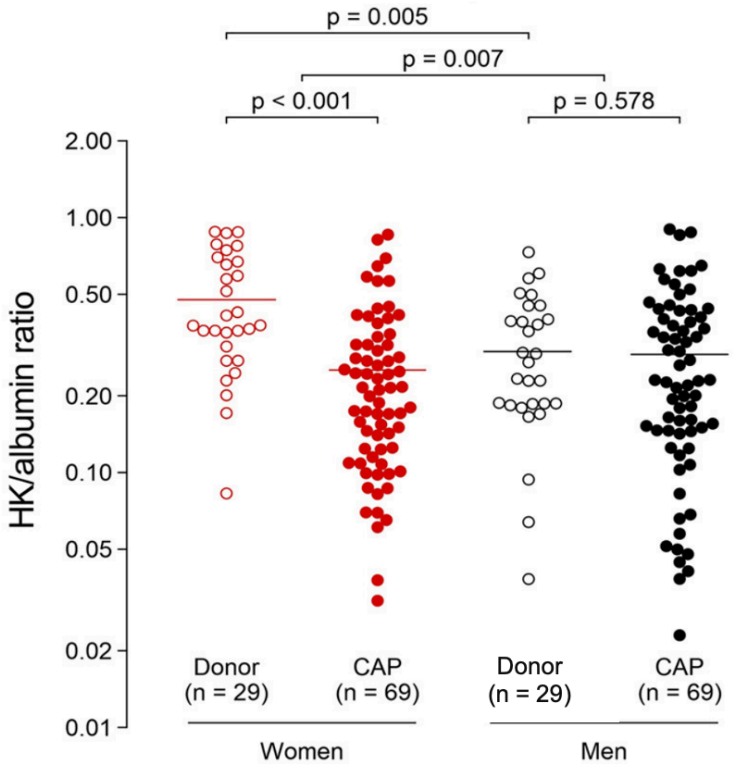
Right panel displays the western blot demonstrating the specificity of the detected bands. Purified HK (pHK) and purified HKa (pHKa) were added as positive controls. kDa, kilodaltons.

Figure 3-16 HK/albumin ratio is decreased in CAP as compared to healthy donors



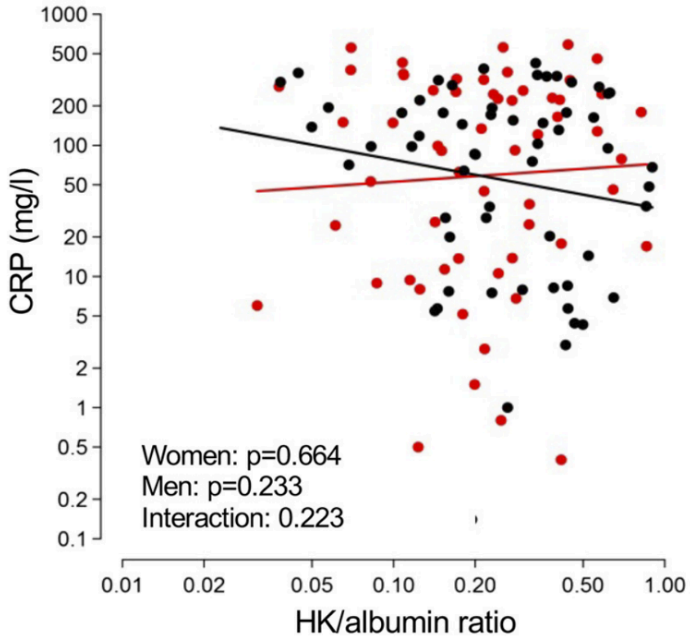
HK/albumin ratio in plasma of donors and patients with CAP as assessed by densitometric analysis is shown. The data were analyzed with a two-way ANOVA model (log-transformed response). Red circles represent women. Black circles represent men. Full circles represent CAP patients. Open circles represent healthy donors. n, number; p, p-value

Figure 3-17 HK/albumin ratio is increased in healthy women as compared to women with CAP



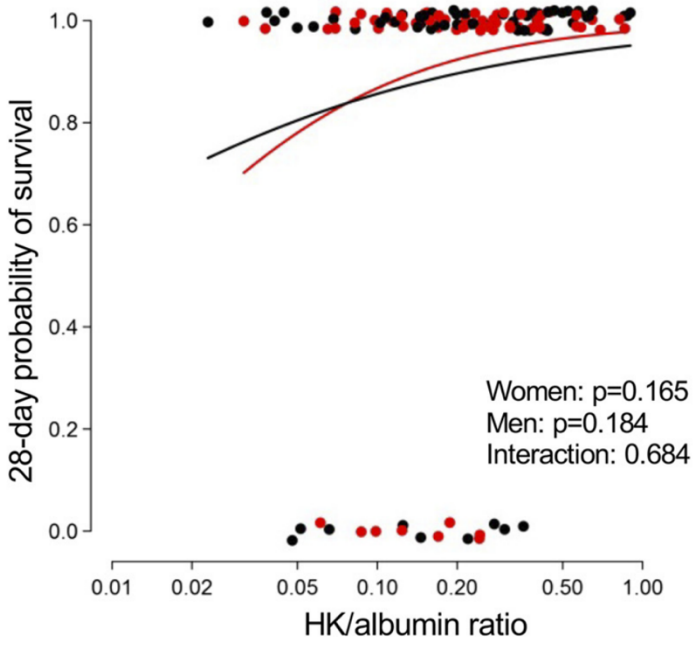
HK/albumin ratio in plasma of donors and CAP patients is depicted in sex specific subgroups. The data were analyzed with a two-way ANOVA model (log-transformed response). Red circles represent women. Black circles represent men. Full circles represent CAP patients. Open circles represent healthy donors. n, number; p, p-value.

Figure 3-18 No correlation between HK/albumin ratio and CRP levels in CAP



The correlation between HK/albumin ratio and the concentration of CRP in plasma on admission in patients with CAP is shown. Analyzed with an ANCOVA model (log-transformed variables). Red circles represent women. Black circles represent men. p, p-value.

Figure 3-19 No correlation between HK/albumin ratio and 28-days probability of survival in CAP

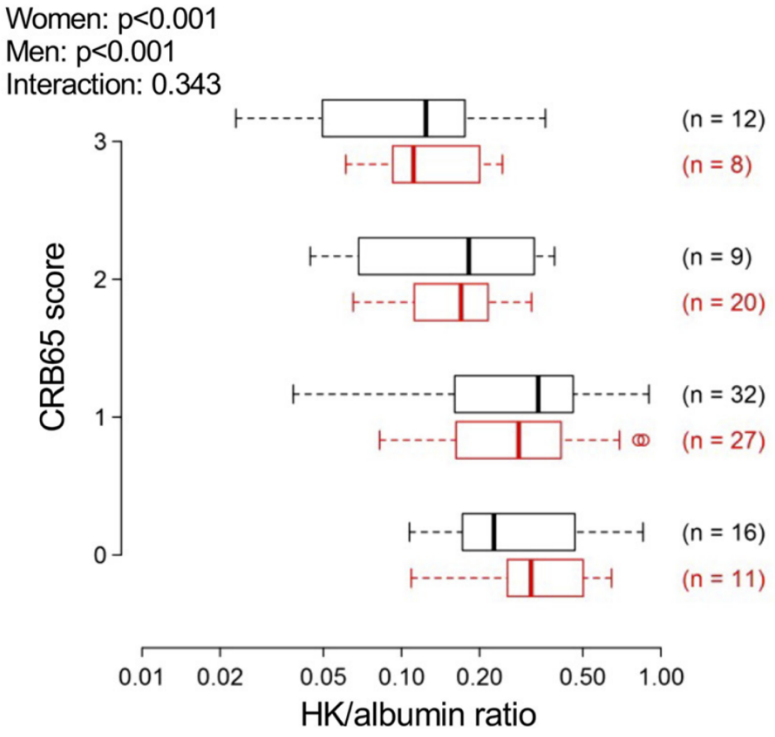


HK/albumin ratio in dependency of the 28-day survival probability in patients with CAP is shown. The data were analyzed with a binomial model adjusted for age (cubic spline with 3 degrees of freedom). Red circles represent women. Black circles represent men. p, p-value.

3.12 Negative correlation between HK/albumin ratio and CRB-65 score

Interestingly a negative correlation between the CRB-65 score and HK/albumin ratio in CAP patients for women as well as for men was detected (see Figure 3-20).

Figure 3-20 Negative correlation between the CRB-65 score and HK/albumin ratio in CAP patients

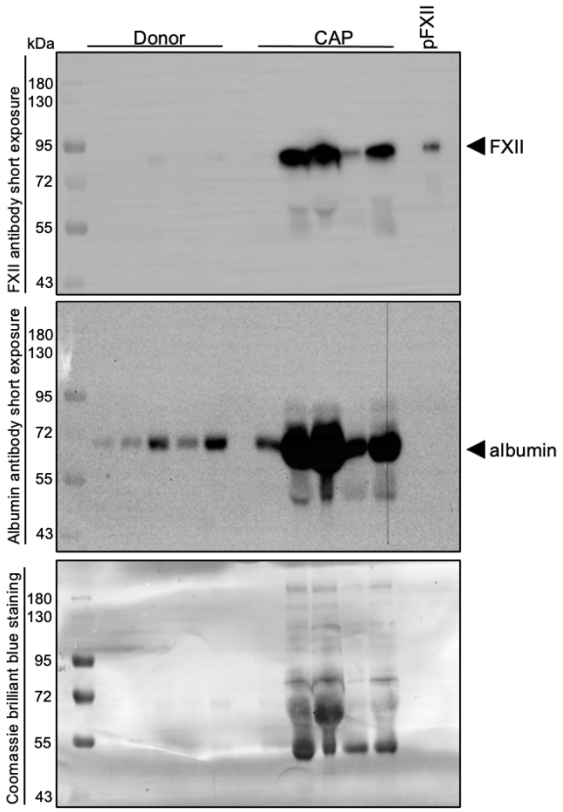


HK/albumin ratio and CRB-65 score values on admission in patients with CAP are shown. The data were analyzed with an ordered logistic model of score vs. log concentration. Red boxes represent women. Black boxes represent men. n, number; p, p-value.

3.13 Concentration of FXII in BALF is higher in CAP patients than in healthy donors

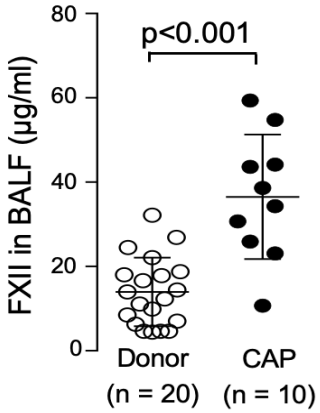
Figure 3-21 displays the results of a western blot, were the expression of FXII in BALF from healthy donors and CAP patients was assessed. Purified FXII served as a positive control. The depicted blot shows a strong signal of FXII in BALF of CAP patients, whilst nearly no signal of FXII in healthy donors can be detected. Albumin served as a loading control. Interestingly, there is also a stronger signal for albumin in BALF of CAP patients, indicating plasma protein leakage to the alveolar compartment. For more precise quantification of FXII levels in BALF, FXII ELISA was used. FXII levels in BALF of CAP patients were markedly elevated as compared to healthy donors (see Figure 3-22).

Figure 3-21 FXII levels in BALF are elevated in CAP patients



Protein levels of FXII in BALF of CAP patients and healthy donors as measured with western blot are shown. Exemplarily 5 healthy donors and 5 CAP patients are shown. Purified FXII (pFXII) served as positive control to show the specificity of the antibody. Albumin and Coomassie were used as loading control. kDa, kilodaltons.

Figure 3-22 Levels of FXII are elevated in BALF of CAP patients measured by ELISA

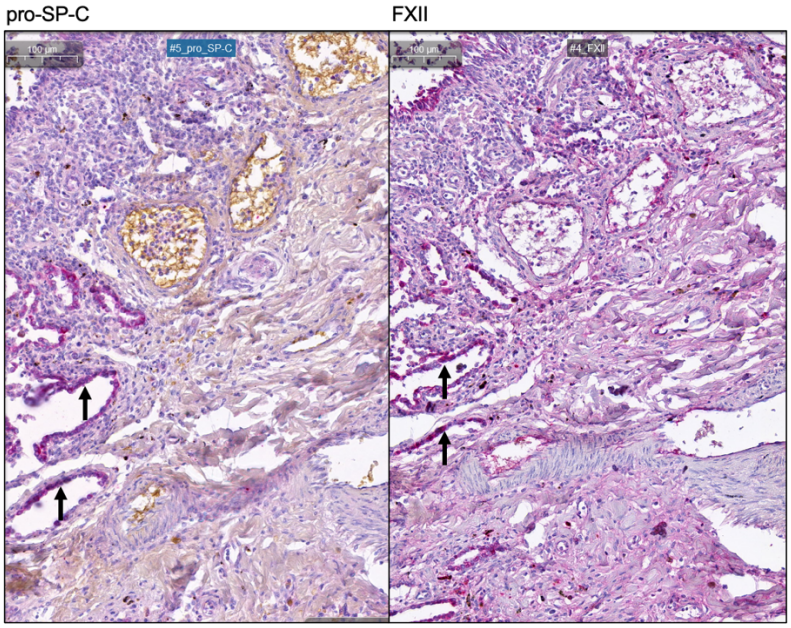


Levels of FXII in BALF of healthy donors and CAP patients as measured by ELISA are shown. Full circles represent CAP patients. Open circles represent healthy donors. n, number; p, p-value.

3.14 FXII is immunolocalized to endothelium, ATII cells and macrophages in CAP lungs

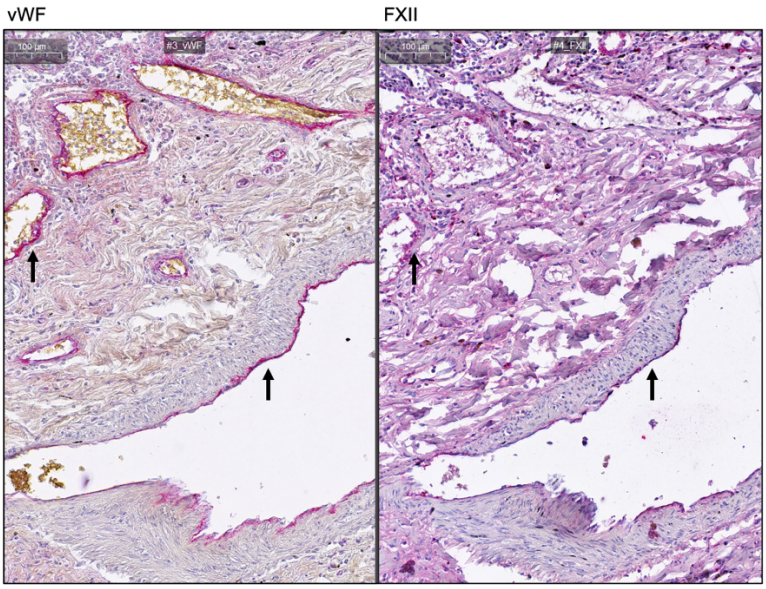
Immunohistochemical stainings with markers for endothelium (vWF), alveolar epithelium type II cells (pro-SP-C), macrophages (CD 68) and FXII were performed. FXII was detected in CAP lungs and partially overlap with alveolar type II cells (see Figure 3-23), endothelium (see Figure 3-24) and macrophages (see Figure 3-25). Lung sections of three different CAP patients have been stained. Of note, lung tissue used for analysis was collected post mortem.

Figure 3-23 Immunolocalization of FXII to AT II cells in CAP lungs



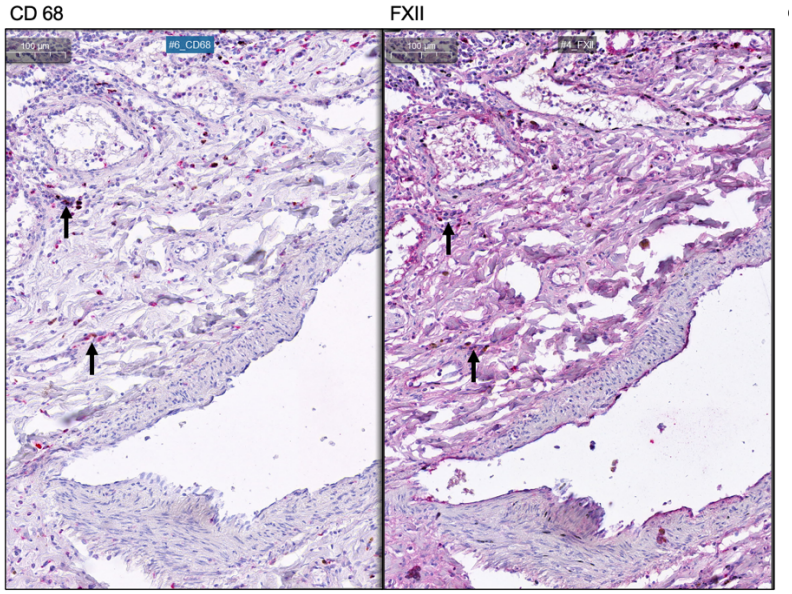
To identify FXII positive cells immunostaining for pro-SP-C on the serial section was performed. Arrows demonstrate positive signal for FXII and pro-SP-C. Here, a section from one out of three lungs that have been stained is shown.

Figure 3-24 Immunolocalization of FXII to endothelium in CAP lungs



To identify FXII positive cells immunostaining for vWF on the serial section was performed. Arrows demonstrate positive signal for FXII and vWF. Here, a section from one out of three lungs that have been stained is shown.

Figure 3-25 Immunolocalization of FXII to macrophages in CAP lungs



To identify FXII positive cells immunostaining for CD 68 on the serial section was performed. Arrows demonstrate positive signal for FXII and CD 68. Here, a section from one out of three lungs that have been stained is shown.

4 Discussion

Growing evidence supports the theory that FXII, as part of the contact phase system, influences processes connected to inflammation and thrombosis and thereby is playing an important role for innate host defense against infection. Multiple sources showed that FXII does not only lead to fibrin formation but also to production and release of proinflammatory cytokines as well as neutrophil aggregation, migration and degranulation[96,112,113,138]. Neutrophil function can be directly affected by FXII but also by downstream products of FXII activation like PKa and BK[34,115,139].

In patients with ARDS reduced levels of FXII, PK and HK as a result of CAS activation have been described[140]. Furthermore, a link between PK levels in patients suffering from ARDS and their static thoracic compliance has been reported[141]. Ultimately, a significant decrease in FXII, PK, and HK levels has been described during fatal episodes of bacterial shock as compared to nonfatal episodes and uncomplicated bacteremia[142].

ARDS is a common complication of CAP[143]. But although there is well-documented evidence of CAS activation in plasma of ARDS patients, corresponding data investigating the role of FXII and the downstream products of its activation during CAP are currently lacking. In this study I show that there is no significant difference in FXII or HK levels in men with CAP compared to sex- and age-matched donors, while FXII and HK levels in female CAP patients were altered in comparison to age-matched female donors. Interestingly, FXII amounts in healthy men and the male CAP cohort were the same as in the female CAP cohort. These findings could indicate that healthy women have the ability to regulate FXII levels and that this ability might be impacted by infection. As it was already shown that expression of FXII is regulated by estradiol[26–28] it is a plausible idea to assume that the levels of FXII are linked to estradiol levels. Subsequent to my initial findings, our group therefore performed further experiments investigating estradiol levels in CAP patients and donors and found that FXII levels indeed correlate with estradiol levels[128]. As the median age of my study population was rather high with a median age of 71 years in CAP patients and 65 years in the donor cohort, one could state, that lower FXII levels in healthy women are due to postmenopausal decrease in estradiol[131]. Interestingly, elderly men show higher levels of estradiol than postmenopausal women[144]. That could partially explain why the men in this cohort showed higher levels of FXII than healthy women. Nonetheless, that does not explain why women suffering from CAP show higher levels of FXII when compared to healthy women of the same age. These observations let me speculate that changes of FXII plasma levels may be influenced by alterations of estradiol levels in the healthy elderly population as well as in the context of pulmonary infection. Concordantly, further

investigations displayed, that plasma levels of FXII in ovariectomized rats treated with 17b estradiol were increased as well as steady-state hepatic FXII mRNA levels[145]. The same was observed in women taking oral contraceptive drugs[146]. All in all, further studies investigating the correlation between estradiol and other sex hormone levels and the expression of FXII in the elderly population suffering from pneumonia are needed.

Generally, epidemiological data show that male individuals are more prone to infectious diseases than female individuals. The difference in susceptibility to infections can already be observed in newborns and maintains until the old age[147]. In the clinical context it has been noticed that men suffering from CAP need to be hospitalized more often than affected women of the same age. Moreover, during the course of the infection, male patients are more likely to undergo treatment at an intensive care unit, indicating a higher morbidity linked to CAP for men than for women. Consecutively, men with pneumonia show a higher mortality than women suffering from pneumonia[148]. These sex-specific differences in the susceptibility to but also the outcome of pulmonary infection are hypothesized to originate from sex-hormone-dependent but also sex-hormone-independent variations of innate and adaptive immune responses. Earlier studies already showed that the biologic response to CAP is different among men and women, with higher circulating concentrations of antithrombin III and factor IX in men, and increased D-dimer, indicating higher coagulative activity in male patients. There are also differences in levels of pro-and anti-inflammatory biomarkers between men and women[148]. Recently, whole blood transcriptome studies in healthy genetically diverse adults exposed that sex and age strongly influence gene expression profiles. For example, it was discovered, that there are sex-dependent differences in the expression of gene networks associated with pathogen clearance as well as T- and B-cell immunity. While the male blood-based molecular signature was more related to modulation of the innate immunity, genes with different expression in women were connected to adaptive immune responses[137,147]. Interestingly, women develop higher antibody titers following vaccination, but also present more adverse events as a reaction to vaccines than men[149].

As part of the CAS, FXII and the downstream products of its activation play a major role for different immune responses. The results of the work presented add further evidence for the existence of sex-specific differences in the immune status of the healthy elderly population compared to elderly individuals suffering from CAP. The underlying pathomechanisms need to be further investigated to understand its impact on the predisposition to pulmonary infections, the outcome of the disease and to detect future treatment options for CAP.

Interestingly, in this study I show that female donors in comparison to female CAP patients display lower levels of FXII and increased levels of HK while no difference of

FXII or HK levels between male CAP patients and male donors was detected. As women are more prone to thromboembolic events and higher levels of estrogens have been shown to correlate with the incidence of thrombosis[150] I would like to discuss an imaginable association of HK and FXII levels with thromboembolic events. In vitro studies showed that thrombin induced platelet aggregation may be inhibited by HK[151] and the function of the plasminogen activator inhibitor-1 is reduced by the kinin-free form of HK[152]. In line with these findings plasma levels of HK were observed to negatively correlate with the risk of venous thromboembolism within the general population[153]. This could indicate a protective role of HK in the context of thrombosis. As the difference is only seen in women one could delineate, that healthy women can regulate HK levels and that this function is disturbed during CAP. Also, it would be interesting to investigate whether estradiol levels influence HK levels to explain the sex-specific differences in HK levels during CAP. As discussed previously, lower FXII levels in healthy women in the cohort studied could be due to a postmenopausal decrease in estradiol. Therefore, I would expect a negative correlation of estradiol levels and HK in the cohort studied. All in all, further studies have to be performed to understand the role of HK in the context of thrombosis.

The influence of FXII in thromboembolic events appears to be even more intricate as contradictive results have been published. Low activity of FXII was found to correlate with a higher risk for myocardial infarction in men[154]. In contrast to that, it was shown that higher plasma levels of FXIIa serve as a predictor for long-term all-cause cardiac mortality[155]. Different FXII and HK plasma levels at the beginning of the disease probably influence different disease outcomes. For example, I observed that a negative correlation between HK/albumin ratio and CRB-65 score in CAP patients exists. Furthermore, there is a negative correlation between FXII and CRP levels in women with CAP. Concordantly, FXIIa-C1INH complexes in female CAP patients were shown to have a positive correlation with CRP levels. On the other hand, I could not reveal an association between HK/albumin ratio and CRP levels or plasma levels of FXII and CRB-65 score in CAP patients or FXIIa-C1INH complexes and CRB-65 score in CAP patients. These results are striking as I would have expected more correlations to clinical parameters as the levels of FXII and HK are interdependent. One imaginable explanation to the correlation of HK and CRB-65 score could be that HK via the release of BK leads to vasodilatation and thus to a certain extent influences the blood pressure, which is part of the CRB-65 score. As the CRB-65 score excludes components, that are directly linked to FXII levels, the lacking relationship between these parameters might be explained partially. The association between CRP and FXII amounts and FXIIa-C1INH-complexes respectively, might be due to the dependency of FXII activation on the presence of

diverse inflammatory mediators in plasma of female CAP patients. All in all, the weak correlations to CRP and CRB-65 score imply a supplementary, rather than a substitutive role of HK and FXII in the prediction of disease outcome of CAP.

After activation FXII is converted into FXIIa, which is rapidly bound to C1INH in plasma. Preceding studies showed that levels of FXII and its inhibitor decline simultaneously during infection, hypothesizing a progressive consumption of both proteins via formation of a complex[156]. On the contrary, the results of the work presented display an increase of FXIIa-C1INH/albumin ratio in plasma of CAP patients, while no decrease of FXII levels was observed at the same time. That could be a sign of either inhibited degradation of FXII or of a stimulation of production or secretion of FXII. For example, it has been previously reported that neutrophils treated with N-Formylmethionyl-leucyl-phenylalanine (fMLF) stimulate secretion of FXII in the context of inflammation[112]. The same has been noticed for cold-induced urticarial autoinflammatory syndrome where neutrophils have been identified as a local source of FXII[157]. Furthermore, it has been demonstrated that estrogens play a supporting role in neutrophilic inflammation[158]. As the results of the present work show lower levels of FXII in healthy women compared to FXII levels in female CAP patients, the association between estrogen levels, neutrophil function and FXII in pneumonia should be investigated more profoundly in the future.

The analysis of BALF plays an important role in the clinical context when it comes to differentiate the origin of inflammatory pulmonary processes. Previous studies have already shown increased FXII levels in BALF of ARDS patients as well as a positive correlation with mortality. Moreover, it was reported that FXII stimulated the production and release of different proinflammatory chemokines[115]. Despite that, corresponding data for the role of FXII in BALF of CAP patients is currently missing. In the present study I show that FXII levels in BALF of CAP patients are significantly higher than in donors. Due to the small sample size a subgroup analysis for gender differences was not possible. Still, the presented data can support the theory of proinflammatory potential of FXII. On the one hand, an increase of FXII in BALF during CAP could be caused by a capillary leak during the phase of congestion, which is characterized by massive exudation[159]. That would also explain the high amounts of albumin in BALF of CAP patients. On the other hand, it is well known that pulmonary bacterial infections are strongly correlated with a high proportion of neutrophils (up to 50%) in BALF[160]. The present study might provide more evidence for an association of FXII activation/secretion and neutrophil stimulation[112], as already discussed above. Still, the origin of FXII in BALF of CAP patients and its potential influence on disease outcome has to undergo further investigations.

Although the source of FXII in BALF of CAP patients has to remain the object of further studies, I would like to discuss the results of the immunohistochemical staining performed in this study. It was shown that FXII can be partially immunolocalized to endothelium, macrophages and alveolar epithelium type II cells. Previous studies showed that FXII binds to uPAR on endothelial cells in the presence of zinc ions released by platelets[41,161]. In bovine newborn aortic cells, murine aortic endothelial cells and human umbilical vein endothelial cells, the FXII-uPAR-triggered-signalling, can activate the cells and lead to proliferation and angiogenesis[41,161–163]. Furthermore, the binding of FXII promotes FXIIa-driven activation of the KKS[161,164] leading to vasodilatation[87] and edema formation[88,89] causing vascular inflammation[86]. Further studies displayed an impairment of leukocyte recruitment to the endothelium in uPAR-deficient mice[165], whereas the lack of uPA-uPAR interaction conflicted plasminogen activation leading to fibrin-associated inflammation in older animals[161,166]. On the one hand, binding of FXII to endothelial cells seems to have a protective effect during inflammation. On the other hand, an extensive activation of the KKS by FXIIa after binding of FXII to endothelial can result in vascular inflammation. The present study adds further evidence of a probable binding of FXII to endothelial cells during pulmonary inflammation, whereas the resulting effects on inflammatory processes should be investigated further in the future.

Previous studies revealed that FXII binds to human macrophage-like cells[167]. In line with these findings in the present study FXII could be immunolocalized to the surface of macrophages in human lungs during CAP. Precedent studies reported that macrophages are capable of directly activating FXII, with FXIIa being able to stimulate macrophages to secrete pro-inflammatory cytokines, which can lead to formation of atherosclerosis in mice[168]. In the context of COVID-19 a dysregulation of macrophages is seen to contribute extensively to hyperinflammation and fatal disease outcome[169]. Together this lets me speculate that the binding of FXII to macrophages in the context of pneumonia can cause secretion of pro-inflammatory cytokines and lead to extensive inflammatory activity and thereby influence disease outcome. To proof this assumption further research on that topic is required.

Finally, FXII was also partially immunolocalized to ATII cells in human lungs facing CAP. ATII cells have been reported to be responsible for synthesis and secretion of surfactant and, among other functions, for the regeneration of the alveolar epithelium following lung injury[170]. The alveolar-capillary barrier mainly exists of surfactant together with epithelial and endothelial cells[171]. Following infection, the barrier is disrupted, resulting in influx of neutrophils, protein leakage to alveolar space, edema, cell apoptosis and necrosis, and the inhibition of pulmonary surfactant as typically seen in ARDS[171], which is a frequent complication of CAP. Therefore, a profound understanding of the pathomechanism that

leads to barrier disruption is needed in order to delineate probable therapeutic targets. In the present study I showed a possible binding of FXII to ATII cells during CAP. Validation of that hypothesis and an answer to the question in how far FXII can influence the function of ATII cells in the context of pulmonary infection should be achieved by further studies.

In the following, I would like to discuss several limitations of the study presented. As only one patient achieved four points at the CRB-65 score and the majority of the population had a score of one point, severe cases of CAP are underrepresented in this study. Therefore, probable correlations to CAP severity and disease outcome might be missed. Furthermore, donor plasma samples were collected from volunteers, who defined themselves as non-smokers. Consequently, an imaginable impact of smoking and comorbidities on the levels of FXII/ FXIIa and HK in donors cannot be eliminated. Especially as precedent work has shown that activation of the CAS can be linked to diabetes, hypertension or hyperlipidemia[34] this study lacks thorough information considering this question. Nonetheless, I can state, that an exclusion of CAP patients with aforementioned comorbidities did not change the results. Moreover, the present cohort is predominantly Caucasian on a Western diet and lifestyle. Thus, it remains uncertain whether the current observations can be transferred to other ethnicities and lifestyles. Eventually, the levels of FXIIa-C1INH complexes and HK in plasma were quantified by calculating the density of bands on a western blot. Even though the volumes of all samples were adjusted to albumin, the relationship between sample loading and band intensity was evaluated and calibrated to determine the linear range of detection for the assay, and western blot data were adjusted for differences between membranes, the results should be confirmed using a method for measuring absolute values of these proteins in plasma. Sample sizes of BALF and human lung tissue were small due to the fact, that BALF is not regularly collected from CAP patients as well as lung tissue. Therefore, subgroup analysis was not possible and probable associations might have been left undiscovered. Validation of the performed study with larger sample sizes might add complementary information.

5 Conclusion

After TF was discovered to be the main activator for coagulation *in vivo* and as it was shown that a deficiency in FXII is not associated with a bleeding predisposition, the role of FXII for hemostasis was highly questioned. In the present study I add valuable information on levels of FXII, FXIIa and HK in plasma and levels of FXII in BALF of CAP patients compared to healthy donors. The presented results were put into perspective and compared to results from multiple studies investigating the role of FXII in the context of inflammation proposing different possible effects of FXII and HK in mediating inflammatory processes during pulmonary infection and associated coagulopathy. The intriguing findings of the present study are 1) that women with CAP show higher levels of FXII than sex-matched donors, 2) and HK levels in women with CAP are decreased in comparison to sex-matched donors, 3) while FXIIa-C1INH/albumin ratios are elevated in CAP patients in comparison to donors, irrespective of sex. 4) Moreover, an increase of FXII in BALF of CAP patients was observed and 5) FXII could be partially immunolocalized to the surface of macrophages, ATII cells and endothelium.

To fully understand and explain these results, the underlying pathomechanisms have to be uncovered. Therefore, further research is essential. Nevertheless, I would like to conclude by proposing several probable explanations on how components of the CAS may modulate infectious burden and inflammatory responses. First, it was reported that binding of FXII to bacteria, viruses and NET leads to autoactivation of FXII. Apart from the effects of the activation of the KKS, FXIIa also directly leads to activation of inflammatory cells and the secretion of proinflammatory mediators[34,54,96,172]. The ability to stimulate inflammatory mediators has also been attributed to FXII[115].

Next, FXIIa can lead to immunothrombosis via thrombin generation[54,173]. On the one hand, immunothrombosis is considered to support pathogen recognition and thereby host defense. On the other hand, an extended activation of the immune system may cause disseminated intravascular coagulopathy, a life-threatening complication[54,173].

With regard to COVID-19, where massive destruction of endothelial cells and maintained activation of FXII cause immense microvascular thrombosis[174,175], the impact of FXII in inflammation of the lung is highlighted even more.

Finally, sex-specific differences and effects in disease and health have to be recognized and studied more thoroughly, not only in the context of immune responses and coagulation in CAP but generally. Only then we will be able to found an understanding of individual disease outcome and probable personalized treatment options.

6 Summary

Pneumonia accounts for one of the ten main causes of death worldwide with highest incident rates in small children and elderly people. Generally, men are more susceptible to an infection and show elevated rates of hospitalization.

The role of FXII as a coagulation factor has been highly questioned as people deficient in FXII do not show any kind of bleeding disorder and with the discovery of a TF-dependent coagulation pathway *in vivo*, FXII seemed to be dispensable for hemostasis. Therefore, current studies focus on alternative roles of FXII. A growing number of evidence supports the theory that FXII is directly and indirectly associated with inflammatory processes and thrombosis and thereby is playing an important role for innate host defense against infection. Multiple sources showed that FXII does not only lead to fibrin formation but also to production and release of proinflammatory cytokines and stimulation of neutrophils.

Although previous studies highlighted the role of FXII and its downstream products during ARDS, a common complication of pulmonary infection, corresponding data for FXII in the context of pneumonia is missing. The present study investigated levels of FXII, FXIIa and HK in plasma of 140 CAP patients and 60 sex- and age-matched healthy donors by performing analysis of data collected via western blot and ELISA. The results were further analyzed to determine sex-specific differences and correlations with CRP levels, CRB-65-score and mortality. Additionally, FXII levels in BALF of 20 CAP patients and 10 donors was measured by western blot and ELISA. Finally, immunohistochemical stainings for FXII and pro-SP-C, CD-68 and vWF in human lung tissue of 3 CAP patients were performed.

Previous studies showed that levels of FXII and its inhibitor decline simultaneously during infection, hypothesizing a progressive consumption of both. In contrast to that I found elevated levels of FXIIa-C1INH-complexes in plasma of CAP patients, while no parallel decrease of FXII levels was observed. That could be a sign of either inhibited degradation of FXII or stimulation of production and secretion of FXII. Multiple studies suggest neutrophils as a source of FXII and show a proinflammatory influence of estrogen on neutrophil function.

In line with these findings the present work shows sex specific differences with regard to FXII and HK levels in CAP patients. Female CAP patients showed higher amounts of FXII than sex-matched donors, while HK levels in women suffering from CAP were decreased in comparison to sex-matched donors. As estradiol is known to stimulate FXII production and studies have shown an association between estradiol levels and FXII levels in plasma, these observations let me speculate that changes of FXII plasma levels

may be influenced by alterations of estradiol levels in the healthy elderly population as well as in the context of pulmonary infection. Epidemiological data show that male individuals are more prone to infectious diseases than female individuals. These sex-specific differences in the susceptibility to but also the outcome of pulmonary infection are hypothesized to originate from sex-hormone-dependent but also sex-hormone-independent variations of innate and adaptive immune responses. The results presented add further evidence to sex specific differences in the context of pulmonary infection and emphasize the need to support further research on that matter.

Moreover, the sex-specific differences in FXII and HK levels let me speculate about the role of FXII and its downstream products with regard to immunothrombosis as women are more prone to thrombosis and elevated levels of estrogens are related to an increased risk of thromboembolic events. Currently contradictory results have been published, leaving the question of a rather protective or a harmful role of FXII and its downstream products in the context of inflammation and related coagulopathy unanswered. That is why further studies regarding this question need to be performed.

Although I would have expected more correlations of FXII and its downstream products with clinical parameters, only a negative correlation of CRP levels and FXII levels in women with CAP, as well as a corresponding positive correlation with CRP levels and FXIIa-C1INH complexes in female CAP patients was detected. Furthermore, a negative correlation of HK levels and the CRB-65 score in female CAP patients was found. The weak correlations to CRP and CRB-65 score imply a supplementary, rather than a substitutive role of FXII, FXIIa and HK in the prediction of disease outcome of CAP.

Finally, elevated levels of FXII in BALF of CAP patients were shown, indicating local production or accumulation of plasma-derived FXII in CAP lungs.

Via immunolocalization of FXII to endothelial cells a binding of FXII to endothelial cells has been proposed in this study. While FXII-uPAR-associated signalling seems to have a rather protective role in the context of inflammation the consequent activation of the KKS by FXIIa can result in extensive vascular inflammation.

Immunolocalization of FXII to the surface of macrophages implies a binding of FXII to macrophages with a probable consequent activation of FXII into FXIIa leading to a secretion of pro-inflammatory cytokines and fatal disease outcome.

Moreover, FXII was immunolocalized to ATII cells which, together with endothelial cells, play an important role in maintaining a functioning capillary-alveolar barrier, which is essential for host defense. In this study a possible association of FXII and the regulation of ATII cell function is proposed.

All in all, the work presented shows altered levels of FXII, FXIIa and HK during CAP with sex-specific differences, indicating that FXII plays an important role in the context of

pulmonary inflammation and associated coagulopathy. Not only the role of FXII as an effector of host defense but especially sex-specific effects of FXII should be investigated further in the future. Only then we will be able to draw conclusions on individual disease outcome and can develop personalized therapeutic strategies.

7 Zusammenfassung

Pneumonien zählen zu den zehn häufigsten Todesursachen weltweit, wobei hauptsächlich kleine Kinder und ältere Menschen betroffen sind. Grundsätzlich sind Männer anfälliger für eine Infektion und zeigen höhere Hospitalisierungsraten.

Die Rolle von FXII als Gerinnungsfaktor wurde stark in Frage gestellt, da Personen mit einer Faktor-XII-Defizienz keine Blutungskomplikationen aufweisen und die Entdeckung von Gewebsthromboplastin, als Initiator der plasmatischen Gerinnung *in vivo,* FXII als Gerinnungsfaktor überflüssig machte.

Daher fokussieren aktuelle Studien sich auf alternative Funktionen von FXII. Es bestehen zunehmend Beweise dafür, dass FXII direkt und indirekt mit inflammatorischen Prozessen und Thrombose assoziiert ist und dadurch eine wichtige Rolle für die angeborene Immunabwehr gegen Infektionen darstellt. Mehrere Studien zeigten, dass FXII nicht nur zu Fibrinproduktion sondern auch zur Entstehung und Freisetzung profinflammatorischer Zytokine und Stimulation von Neutrophilen führt.

Obwohl vorherige Studien die Rolle von FXII und seiner Folgeprodukte während akutem Lungenversagen (ARDS), einer häufigen Komplikation pulmonaler Infektionen, hervorgehoben haben, fehlen entsprechende Daten für die Rolle von FXII im Kontext von Pneumonien. Die vorliegende Arbeit untersuchte Plasmaspiegel von FXII, FXIIa und HK bei 140 Patienten mit ambulant erworbener Pneumonie und 60 gesunden Probanden gleichen Geschlechts und Alters, indem mittels Western Blot und ELISA generierte Daten analysiert wurden. Die Ergebnisse wurden noch weiter auf geschlechtsspezifische Unterschiede und Korrelationen mit CRP, CRB-65 Risiko-Stratifizierung und Sterblichkeit untersucht. Zusätzlich wurden FXII-Spiegel in BALF von 20 Pneumoniepatienten und 10 Gesunden mittels Western Blot und ELISA gemessen. Schließlich wurden immunhistochemische Färbungen an menschlichem Lungengewebe von Pneumoniepatienten für FXII, pro-SP-C, CD-68 und vWF durchgeführt.

Vorherige Studien zeigten, dass die Spiegel von FXII und der jeweiligen Inhibitoren während einer Infektion gleichzeitig sinken, sodass ein progredienter Verbrauch beider angenommen wurde. Im Gegensatz dazu stellte ich erhöhte Spiegel von FXIIa-C1INH-Komplexen in Plasma von Pneumoniepatienten fest, ohne einen gleichzeitigen Abfall von FXII-Spiegeln. Das könnte einerseits Zeichen eines inhibierten Abbaus von FXII oder einer Stimulation der FXII-Produktion/-Sekretion sein. Mehrere Studien bringen Neutrophile als Quelle von FXII an und zeigen außerdem einen proinflammatorischen Einfluss von Östrogenen auf die Funktion von Neutrophilen. Im Einklang mit den eben genannten Ergebnissen zeigt die vorliegende Arbeit geschlechtsspezifische Unterschiede von FXII- und HK-Spiegeln bei Pneumoniepatienten. Weibliche Patienten

zeigten höhere FXII Spiegel als Gesunde gleichen Geschlechts, während HK-Spiegel bei Patientinnen mit Pneumonie, im Vergleich zu Gesunden des gleichen Geschlechts, erniedrigt waren. Da Östrogene bekannt dafür sind die Produktion von FXII zu stimulieren und es Studien gibt, die die Assoziation von Östrogenspiegeln und FXII-Spiegeln in Plasma aufzeigten, lassen mich diese Beobachtungen spekulieren, dass Veränderungen von FXII-Spiegeln im Plasma durch Veränderungen von Östrogen-Spiegeln in der gesunden älteren Population aber auch im Kontext von Lungenentzündungen beeinflusst werden könnten.

Epidemiologische Daten zeigen, dass männliche Individuen anfälliger für infektiöse Erkrankungen sind. Es wird angenommen, dass diese geschlechtsspezifischen Unterschiede in der Anfälligkeit für eine Lungeninfektion, aber auch in deren Verlauf auf geschlechtshormonabhängige, aber auch geschlechtshormonunabhängige Variationen der angeborenen und adaptiven Immunantwort zurückzuführen sind. Die vorgestellten Ergebnisse liefern weitere Belege für geschlechtsspezifische Unterschiede im Zusammenhang mit pulmonalen Infektionen und unterstreichen die Notwendigkeit weitere Forschung zu diesem Thema zu unterstützen. Darüber hinaus lassen die geschlechtsspezifischen Unterschiede in den FXII- und HK-Spiegeln Spekulationen über die Rolle von FXII und seinen Folgeprodukten im Hinblick auf die Immunthrombose zu, da Frauen anfälliger für Thrombosen sind und erhöhte Östrogenspiegel mit einem erhöhten Risiko für thromboembolische Ereignisse in Verbindung gebracht werden. Derzeit sind widersprüchliche Ergebnisse veröffentlicht worden, sodass die Frage nach einer eher schützenden oder schädigenden Rolle von FXII und seinen Folgeprodukten im Zusammenhang mit Entzündungen und der damit verbundenen Koagulopathie noch unbeantwortet ist. Deshalb müssen weitere Studien zu dieser Frage durchgeführt werden.

Obwohl ich mehr Korrelationen von FXII und seinen Folgeprodukten mit klinischen Parametern erwartet hätte, wurde nur eine negative Korrelation von CRP-Spiegeln und FXII-Spiegeln bei Frauen mit Pneumonie sowie eine entsprechende positive Korrelation von CRP-Spiegeln und FXIIa-C1INH-Spiegeln bei Pneumoniepatientinnen festgestellt.

Außerdem wurde eine negative Korrelation zwischen den HK-Werten und dem CRB-65-Score bei weiblichen Pneumoniepatienten festgestellt. Die schwachen Korrelationen zu CRP und CRB-65-Score deuten darauf hin, dass FXII, FXIIa und HK bei der Vorhersage des Krankheitsverlaufs einer Pneumonie eher eine ergänzende als eine substantielle Rolle spielen.

Schließlich wurden erhöhte Werte von FXII in der BALF von Pneumoniepatienten nachgewiesen, was auf eine lokale Produktion von FXII oder eine Akkumulation von FXII aus Blutplasma während einer Pneumonie hinweist.

Durch die Immunlokalisierung von FXII auf Endothelzellen wurde in dieser Studie eine Bindung von FXII an Endothelzellen vermutet. Während die FXII-uPAR-assoziierte Signalgebung im Kontext von Entzündungen eher eine schützende Rolle zu spielen scheint, kann die konsequente Aktivierung des KKS durch FXIIa zu einer ausgedehnten Gefäßentzündung führen.

Die Immunlokalisierung von FXII auf der Oberfläche von Makrophagen deutet auf eine Bindung von FXII an Makrophagen hin, die wahrscheinlich zu einer Aktivierung von FXII zu FXIIa führt, was die Ausschüttung von pro-inflammatorischen Zytokinen und einen fatalen Krankheitsverlauf zur Folge haben könnte.

Darüber hinaus wurde FXII in ATII-Zellen immunolokalisiert, die zusammen mit Endothelzellen eine wichtige Rolle bei der Aufrechterhaltung einer funktionierenden kapillar-alveolären Barriere spielen, die für die Wirtsabwehr unerlässlich ist. In dieser Studie wird ein möglicher Zusammenhang zwischen FXII und der Regulierung der ATII-Zellfunktion vermutet.

Insgesamt zeigt die vorgestellte Arbeit veränderte Konzentrationen von FXII, FXIIa und HK während ambulant erworbener Pneumonie mit geschlechtsspezifischen Unterschieden, was darauf hindeutet, dass FXII eine wichtige Rolle im Kontext von Lungenentzündung und der damit verbundenen Koagulopathie spielt. Nicht nur die Rolle von FXII als Effektor der Wirtsabwehr, sondern insbesondere die geschlechtsspezifischen Effekte von FXII sollten in Zukunft weiter untersucht werden. Nur dann werden wir in der Lage sein, Rückschlüsse auf den individuellen Krankheitsverlauf zu ziehen und personalisierte Therapiestrategien zu entwickeln.

8 List of abbreviations

°C	Degree Celsius
ACS	Acute coronary syndrome
AIDS	Acquired immune deficiency syndrome
AP	Alkaline phosphatase
APS	Ammonium persulfate
ATII	alveolar type II
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C1	First component of complement
C1INH	C1 esterase inhibitor
C1r	Subunit of C1
C1s	Subunit of C1
CAP	Community-acquired pneumonia
CAS	Contact activation system
CD68	Cluster of Differentiation 68
cDNA	Complementary DNA
CRB-65 score	C= Confusion, R= Respiratory Rate, B= Blood pressure, 65: age in years
CRP	C-reactive protein
C_t	Cycle threshold
d-NTP	Deoxynucleotide Triphosphate
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ECGS	Endothelial cell growth supplement
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
F12	Gene encoding FXII
f12	Gene encoding murine FXII
f12-/-	*f12* knockout
FBS	Fetal bovine serum
fig.	Figure
fMLF	N-Formylmethionyl-leucyl-phenylalanine
FVII	Factor VII

FVIIa	Activated FVII
FIX	Factor IX
FIXa	Activated FIX
FX	Factor X
FXa	Activated FX
FXI	Factor XI
FXIa	Activated FXII
FXII	Factor XII
FXIIa	Activated FXII
GTC	Guanidine thiocyanate
HAP	Hospital-acquired pneumonia
Hch	Heavy chain
HF	Hageman factor
HK	High molcecular weight kininogen
HKa	activated high molcecular weight kininogen
HMVEC	Human microvascular endothelial cells
HNF4α	Hepatocyte nuclear factor 4 α
HRP	Horseradish peroxidase
i.e.	Id est
ICU	Intensive care unit
kb	Kilobases
kDa	Kilodaltons
KKS	Kallikrein kinin system
Lch	Light chain
LPS	Lipopolysaccharides
MI	Myocardial infarction
n	Sample size
NETs	Neutrophil extracellular traps
p-value/s	Probability value/s
PAMPs	Pathogen-associated molecular patterns
PBGD	Gene encoding porphobilinogen deaminase
PBS	Phosphate-buffered saline
PBS-T	PBS + Tween 20
pC1INH	purified C1 esterase inhibitor
pFXII	purified factor XII
pFXIIa	purified activated factor XII

pFXIIa-C1INH	purified activated factor XII in complex with C1 esterase inhibitor
pHK	purified high molcecular weight kininogen
pHKa	purified activated high molcecular weight kininogen
PK	Plasma prekallikrein
PKa	Plasma Kallikrein
PMSF	Phenylmethylsulphonyl fluoride
polyP	Polyphosphate
PPACK	D-Phenylalanyl-prolyl-arginyl chloromethyl ketone
pro-SP-C	Pro-surfactant protein-C
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT	Reverse transcriptase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	Streptavidin-peroxidase
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline + Tween 20
TEMED	Tetramethyl-ethylenediamine
TF	Tissue factor
TLR	“Toll-like”-receptor(s)
TMB	Tetramethylbenzidin
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
V	Volt
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor
w/v	Weight per volume
WHO	World Health Organization

9 List of figures

10 List of tables

11 References

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12 Publication list

1) Sex-specific differences in plasma levels of FXII, HK, and FXIIa-C1-esterase inhibitor complexes in community-acquired pneumonia published in American Journal of Physiology – Lung cellular and Molecular Physiology 321: L764-744 (2021) by Ehrlich, Kristin; Wilhelm, Jochen; Markart, Philipp; Weisser, Heike; Wolff, Jens Christian; Bein, Gregor; Pak, Oleg; Barreto, Guillermo; Weissmann, Norbert; Schramm, Fabian; Seeger, Werner; Schaefer, Liliana; Kuebler, Wolfgang M. and Wygrecka, Malgorzata

13 Declaration of intent

“I hereby declare that I have completed this work independently and without inadmissible assistance or the use of other than the resources quoted. All texts that have been quoted verbatim or by analogy from published and non-published writings and all details based on verbal information have been identified as such. In the analyses that I have conducted and to which I refer in this thesis, I have followed the principles of good scientific practice, as stated in the Statute of Justus Liebig University Giessen for Ensuring Good Scientific Practice, as well as ethical principles and those governing data protection and animal welfare. I give my assurance that third parties have not received from me, either directly or indirectly, any financial remuneration for work in connection with the content of this doctoral thesis and that the work presented has not been submitted in the same or a similar form to another assessment authority in Germany or elsewhere for the purpose of being awarded a doctorate or another assessment procedure. All material taken from other sources and other persons and used in this thesis or to which direct reference is made has been identified as such. In particular, all those who took part directly and indirectly in the production of this study have been named. I agree to my thesis being subjected to scrutiny by plagiarism detection software or by an internet-based software program.”

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14 Acknowledgements

First and foremost, I would like to express my heartfelt thanks to Prof. Dr. Malgorzata Wygrecka. This work would not have been possible without your support. I am truly grateful for the opportunity to work in your group. Your ideas, explanations, motivation, and patience have been invaluable to me. I deeply admire your scientific knowledge and creativity. You are a wonderful role model for young scientists, and I believe the world needs more women like you.

I would also like to extend my gratitude to Simone Kegel, Carola Rudolph and Dr. Miroslava Didiasova. Thank you for welcoming me into your team and for teaching me the necessary laboratory skills. I have always enjoyed working with you, and I cherish the fond memories of our time together in the lab.

Last but not least, I want to express a huge thank you to my family and my husband. You have always believed in me, and without your support, I would not have found the strength to finish this monography. I am especially grateful to my Papa for always encouraging and motivating me. I will never forget how you told me to always try to reach for the stars. “Per aspera ad astra.”