BRADYKININ DRIVEN INFLAMMATION

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Bradykinin driven inflammation

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BRADYKININE GEDREVEN ONTSTEKING

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General introduction

BRADYKININ

Bradykinin is a peptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) that is released in circulation upon activation of the contact system[1]. Interaction of bradykinin with its receptors on the vascular wall increases vascular permeability and vasodilation[2,3]. Moreover, bradykinin acts as a nociceptive upon interaction with afferent neurons and supports the immune response via interaction with neutrophils[4,5], monocytes[6] and dendritic cells[7]. Due to its contribution to edema, pain and cellular immunity, bradykinin is considered a proinflammatory peptide. Although the combined actions of bradykinin can be beneficial for healthy vascular tone, preventing tissue damage and fighting of pathogens excessive bradykinin release comes with a price. Bradykinin is notorious for causing extreme tissue swelling[8]. Therefore, release of this inflammatory peptide is tightly controlled. Within seconds after its generation bradykinin is degraded by kininases, such as angiotensin converting enzyme (ACE)[9], and the contact system is kept in check by C1-esterase inhibitor (C1-INH)[8].

C1-ESTERASE INHIBITOR AND THE CONTACT SYSTEM

C1-INH is a serine protease inhibitor and is the main inhibitor of the contact system[8]. The contact system comprises of factor XII (FXII), plasma prekallikrein (PK) and high molecular weight kininogen (HK). FXII is known to auto-activate upon contact with negatively charged surfaces, hence the name contact system[10]. Active FXII (FXIIa) will activate PK into plasma kallikrein (PKa) that will cleave bradykinin from HK (Figure 1). C1-INH controls bradykinin release by inhibiting FXIIa and PKa. In the extravascular space, a Lys-bradykinin variant is generated from low molecular weight kininogen by tissue kallikreins[2]. The mechanism of action of the various tissue kallikreins is largely unrevealed and beyond the scope of this thesis that will mainly focus on bradykinin in circulation.

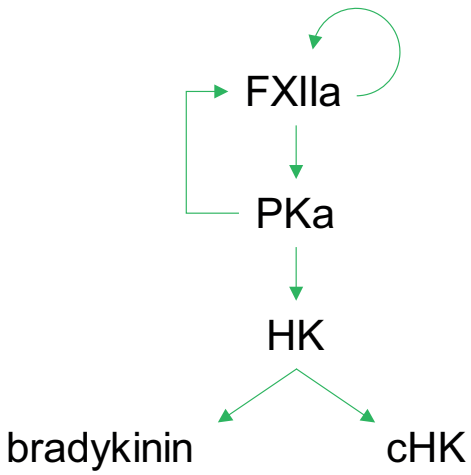

Figure 1. The contact system. FXIIa: active Factor XII, PKa: plasma kallikrein, HK: high molecular weight kininogen, cHK: cleaved HK.

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C1-ESTERASE INHIBITOR AND HEREDITARY ANGIOEDEMA

Insight into the pathophysiological mechanism of bradykinin was gained from the discovery of its involvement in hereditary angioedema (HAE)[11]. HAE is an autosomal dominant disease with an estimated prevalence of 1:50 000[8]. The majority of HAE patients have a mutation in the SERPING1 gene, coding for C1-INH. This gene contains a high density of Alu elements[12,13] which, together with some other structural features[14,15] make SERPING1 susceptible to mutations. Up until today 486 HAE related SERPING1 mutations have been described[16] (HGMD database, accessed June 2019). In ~85% of patients their mutation causes C1-INH deficiency (HAE-1) and in ~15% C1-INH dysfunction (HAE-2)[8]. HAE-1 and 2 are clinically indistinguishable and both lead to insufficient control of bradykinin production.

HAE patients experience episodes with swelling of subcutaneous or submucosal tissue (Figure 2). These angioedema attacks last for hours up to days and result in temporarily dysfunction of swollen limbs, disfigurement of the face, painful bowel obstruction and dyspnea or asphyxiation due to swelling of the upper airways[8,17]. Knowledge on the pathophysiological mechanism causing HAE led to the development of several successful therapies. These include, C1-INH supplementation, PKa inhibition with small molecules or targeted antibodies and a bradykinin receptor antagonist[18].

OTHER FORMS OF ANGIOEDEMA

Over the past decades, major advances in understanding and treating HAE-1 and 2 were made. However, most people that experience angioedema, do not have HAE. As much as 7% of all people self-reported to have experienced angioedema at least once in their lifetime[19]. Some of these other forms of angioedema are also strongly associated with bradykinin release. These include acquired C1-INH deficiency caused by increased consumption of C1-INH or the presence of autoantibodies against C1-INH; ACE inhibitor induced angioedema, as ACE breaks down bradykinin its inhibition causes increased bradykinin levels; and lastly

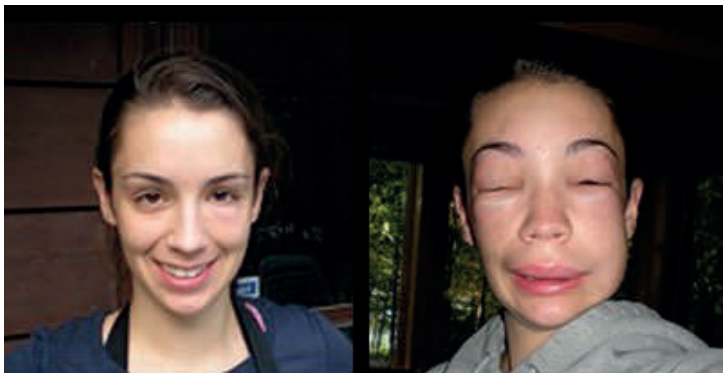

Figure 2. A patient with angioedema of the face during remission and angioedema attack. Image downloaded from: https://www.haea.org/page/disease.

HAE based on mutations in the f12 gene coding for FXII. Over the past three years, novel HAE related mutations have been described, even though the pathophysiological mechanisms behind these mutations are currently being investigated, symptoms in carriers are suspected to be bradykinin induced[20,21].

Angioedema can also be part of an allergic response; these swellings are induced by mast-cell activation with histamine being one of the vasoactive peptides hold responsible for tissue swelling. Likewise, angioedema seen in the mast-cell driven disease chronic spontaneous urticaria (CSU) is believed to be caused by histamine. CSU patients suffer from spontaneously occurring, recurrent episodes with itchy wheals and/or angioedema[22]. Lastly, patients are diagnosed with idiopathic angioedema when no underlying cause for the disease can be identified. Often, idiopathic angioedema is subdivided into idiopathic histaminergic and idiopathic non-histaminergic angioedema based upon clinical response to antihistamine therapy. Table 1 gives an overview of the various angioedema types based on the latest HAE guideline consensus paper[18] with some minor additions to give a more complete overview.

THESIS OUTLINE: BRADYKININ DRIVEN INFLAMMATION

Although a lot was learned from HAE, the role of bradykinin as an inflammatory mediator beyond HAE remains largely unexplored. *With this thesis I aimed to gain more insight in the mechanisms of bradykinin production in order to increase our understanding of HAE and extend this knowledge to potential bradykinin driven diseases beyond HAE.*

Table 1. Classification of angioedema*

Bradykinin-induced AE				**Mast-cell mediator induced AE**		
C1-INH deficiency/ dysfunction		**C1-INH normal**		**IgE mediated**	**Non-IgE mediated**	**Unknown mediator**
Inhereted	**Acquired**	**Inhereted**	**Acquired**	AE with anaphylaxis	AE with urticaria/CSU	Idiopathic angioedema**
HAE-1 HAE-2	AAE-C1-INH	HAE nC1-INH (HAE-FXII, HAE-ANGPT1, HAE-PLG, HAE-KNG, HAE-UNK)	ACEI-AE			

AE: angioedema, C1-INH: C1-esterase inhibitor, HAE: hereditary angioedema, HAE-1: HAE due to C1-INH deficiency, HAE-2: HAE due to C1-INH dysfunction, AAE-C1-INH: acquired angioedema due to C1-INH deficiency, HAE nC1-INH: HAE with normal C1-INH function either due to a mutation of factor XII, angiopoietin-1, plasminogen, kininogen or unknown (HAE-FXII, HAE-ANGPT1, HAE-PLG, HAE-KNG, HAE-UNK), CSU: chronic spontaneous urticaria.

* Altered version of "Table 4 classification of angioedema". Maurer *et al*. 2018. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. Accessed: 17-8-2019. [https://onlinelibrary-wiley-com.proxy.library.uu.nl/doi/full/10.1111/all.13384]

** Based on clinical response to antihistamines subdivided into idiopathic histaminergic-angioedema and idiopathic non-histaminergic angioedema

Continuing this introduction, **chapter 2** reviews the role of FXII in coagulation and inflammation and discusses possible routes of contact activation as it is uncertain what regulates FXII activation and bradykinin production *ixn vivo*. We give an overview of biomarkers associated with bradykinin production and discuss how these studies in mechanisms of contact activation and biomarker research may help point out bradykinin mediated diseases beyond HAE.

Part I of this thesis will focus on HAE, trying to further extend our knowledge on bradykinin driven inflammation. **Chapter 3** describes the observation that c-reactive protein, a broad marker of inflammation, is elevated in HAE and discusses what the clinical implications of this finding may be on bradykinin receptor regulation. **Chapter 4** describes our observation that HAE patients often suffer from swellings at multiple locations during one episode with angioedema. In **chapter 5** we review on bradykinin and its interaction with bradykinin receptors. We postulate the hypothesis that the onset and location of swelling in HAE may be regulated by upregulation of the bradykinin B1 receptor.

As direct measurement of bradykinin is complicated by its rapid degradation, cleaved HK (cHK), the byproduct of bradykinin production, is considered a valid biomarker for bradykinin release. **Chapter 6** describes the development of a nanobody-based cHK ELISA and its validation by demonstrating increased cHK levels in two cohorts of HAE-1 and 2 patients. **Chapter 7** demonstrates the use of this cHK ELISA to accompany HAE drug development, as we show effectivity of a novel kallikrein inhibiting compound in a phase I single ascending dose study by *ex vivo* generation and detection of cHK.

Part II of this thesis explorers the implications of bradykinin in diseases beyond HAE. In **chapter 8** we report on the occurrence of antihistamine resistance in patients with idiopathic angioedema and found a high incidence of antihistamine resistance in idiopathic angioedema underlining the need for additional treatment. **Chapter 9** describes preliminary data of a pilot study exploring the use of recombinant C1-INH as prophylactic therapy in idiopathic non-histaminergic angioedema.

Chapter 10 describes the observation of increased cHK levels in patients with CSU. The pathophysiological mechanism of CSU is not fully understood but is believed to driven by mast-cells and histamine release. Although increased cHK levels may be an 'innocent bystander' in CSU we cautiously suggest a contribution of bradykinin to pathology.

Very recently a novel point mutation in factor 12 gene was found resulting in FXII-W268R[23]. FXII-W268R carriers experience cold-induced episodes with wheals, fatigue, arthralgia and headaches. In collaboration with the discovers of this novel mutation we demonstrated excessive contact activation in patient plasma[24]. In **chapter 11** we investigate the biomolecular mechanism behind the FXII-W268R mutation and show that FXII-W268R enhances FXII (auto)activation. FXII is an enzyme that requires cleavage at a specific position in an amino acid sequence that is called the activation loop. We propose that in FXII-W268R, unlike normal, the activation loop is uncovered explaining accelerated activation. Learning from this new bradykinin mediated disease, we further explore the mechanism behind regulation of FXII by shielding of the activation loop in **chapter 12.**

Finally, **Chapter 13** describes the main findings of this thesis in discusses what was learned on the mechanisms of bradykinin release and its implications in HAE and bradykinin mediated disease beyond HAE.

REFERENCES

1. Nussberger J, Cugno M, Cicardi M, Agostoni A. Local bradykinin generation in hereditary angioedema. *J Allergy Clin Immunol*. 1999;104(6):1321-1322. doi:10.1016/S0091-6749(99)70030-8
2. Leeb-Lundberg LMF, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev*. 2005;57(1):27-77. doi:10.1124/pr.57.1.2
3. Mesquita TRR, Campos-Mota GP, Lemos VS, et al. Vascular Kinin B1 and B2 Receptors Determine Endothelial Dysfunction through Neuronal Nitric Oxide Synthase. *Front Physiol*. 2017;8:228. doi:10.3389/fphys.2017.00228
4. Ehrenfeld P, Millan C, Matus CE, et al. Activation of kinin B 1 receptors induces chemotaxis of human neutrophils Abstract : Kinins are biologically active peptides that are powerful mediators of cellular inflammation . inducing vasodilatation and by increasing vascular. *J Leukoc Biol*. 2006;80(July):117-124. doi:10.1189/jlb.1205744
5. Veszeli N, Csuka D, Zotter Z, et al. Neutrophil activation during attacks in patients with hereditary angioedema due to C1-inhibitor deficiency. *Orphanet J Rare Dis*. 2015:1-8. doi:10.1186/s13023-015-0374-y
6. Bertram CM, Baltic S, Misso NL, et al. Expression of kinin B1 and B2 receptors in immature, monocyte-derived dendritic cells and bradykinin-mediated increase in intracellular Ca2+ and cell migration. *J Leukoc Biol*. 2007;81(6):1445-1454. doi:10.1189/jlb.0106055
7. Monteiro AC, Schmitz V, Morrot A, et al. Bradykinin B2 Receptors of dendritic cells, acting as sensors of kinins proteolytically released by Trypanosoma cruzi, are critical for the development of protective type-1 responses. *PLoS Pathog*. 2007;3(11):e185. doi:10.1371/journal.ppat.0030185
8. Longhurst H, Cicardi M. Hereditary angio-oedema. *Lancet (London, England)*. 2012;379(9814):474-481. doi:10.1016/S0140-6736(11)60935-5
9. Beaudouin E, Defendi F, Picaud J, Drouet C, Ponard D, Moneret-Vautrin DA. Iatrogenic angioedema associated with ACEi, sitagliptin, and deficiency of 3 enzymes catabolizing bradykinin. *Eur Ann Allergy Clin Immunol*. 2014;46(3):119-122. http://www.ncbi.nlm.nih.gov/pubmed/24853572.
10. de Maat S, Maas C. Factor XII: form determines function. *J Thromb Haemost*. 2016;14(8):1498-1506. doi:10.1111/jth.13383
11. Fields T, Ghebrehiwet B, Kaplan AP. Kinin formation in hereditary angioedema plasma: evidence against kinin derivation from C2 and in support of "spontaneous" formation of bradykinin. *J Allergy Clin Immunol*. 1983;72(1):54-60. doi:10.1016/0091-6749(83)90052-0
12. Nicolicht P, Faria DOS, Martins-Silva L, et al. Gene mapping strategy for Alu elements rearrangements: Detection of new large deletions in the SERPING1 gene causing hereditary angioedema in Brazilian families. *Gene*. 2019;685:179-185. doi:10.1016/j.gene.2018.10.084
13. Stoppa-Lyonnet D, Carter PE, Meo T, Tosi M. Clusters of intragenic Alu repeats predispose the human C1 inhibitor locus to deleterious rearrangements. *Proc Natl Acad Sci U S A*. 1990;87(4):1551-1555. doi:10.1073/pnas.87.4.1551
14. Bissler JJ, Cicardi M, Donaldson VH, et al. A cluster of mutations within a short triplet repeat in the C1 inhibitor gene. *Proc Natl Acad Sci U S A*. 1994;91(20):9622-9625. doi:10.1073/pnas.91.20.9622
15. Skriver K, Radziejewska E, Silbermann JA, Donaldson VH, Bock SC. CpG mutations in the reactive site of human C1 inhibitor. *J Biol Chem*. 1989;264(6):3066-3071. http://www.ncbi.nlm.nih.gov/pubmed/2563376.
16. Stenson PD, Mort M, Ball E V, et al. The Human Gene Mutation Database: 2008 update. *Genome Med*. 2009;1(1):13. doi:10.1186/gm13

17. Bork K, Barnstedt S-E. Laryngeal edema and death from asphyxiation after tooth extraction in four patients with hereditary angioedema. *J Am Dent Assoc*. 2003;134(8):1088-1094. http://www.ncbi.nlm.nih.gov/pubmed/12956349.

18. Maurer M, Magerl M, Ansotegui I, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. *Allergy*. 2018;73(8):1575-1596. doi:10.1111/all.13384

19. Madsen F, Attermann J, Linneberg A. Epidemiology of Non-hereditary Angioedema. *Acta Derm Venereol*. 2012;92(5):475-479. doi:10.2340/00015555-1389

20. Bork K, Wulff K, Rossmann H, et al. Hereditary angioedema cosegregating with a novel kininogen 1 gene mutation changing the N-terminal cleavage site of bradykinin. *Allergy*. May 2019. doi:10.1111/all.13869

21. Bork K, Wulff K, Steinmüller-Magin L, et al. Hereditary angioedema with a mutation in the plasminogen gene. *Allergy*. 2018;73(2):442-450. doi:10.1111/all.13270

22. Zuberbier T, Aberer W, Asero R, et al. The EAACI/GA2LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: The 2013 revision and update. *Allergy Eur J Allergy Clin Immunol*. 2014;69(7):868-887. doi:10.1111/all.12313

23. Mahnke NA, Scheffel J, Wu J, et al. A novel urticarial and Factor XII mutation-related autoinflammatory syndrome. *Exp Dermatol*. 2018;27(3):e2-e106. doi:10.1111/exd.13486

24. Hofman ZLM, Krause K, Mahnke NA, et al. Human Factor XII Mutation W268R Causes Urticarial Autoinflammatory Syndrome through Intracellular Misprocessing. *Res Pract Thromb Haemost*. 2018;2:1-368. doi:10.1002/rth2.12125

2

Bradykinin: Inflammatory Product of the Coagulation System

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ABSTRACT

Episodic and recurrent local cutaneous or mucosal swelling are key features of angioedema. The vasoactive agents *histamine* and *bradykinin*, are highly implicated as mediators of these swelling attacks. It is challenging to assess the contribution of *bradykinin* to the clinical expression of angioedema, as accurate biomarkers for the generation of this vasoactive peptide are still lacking. In this review, we will describe the mechanisms that are responsible for *bradykinin* production in hereditary angioedema (HAE) and the central role that the coagulation Factor XII (FXII) plays in it.

Evidently, several plasma parameters of coagulation change during attacks of HAE and may prove valuable biomarkers for disease activity. We propose that these changes are secondary to vascular leakage, rather than a direct consequence of FXII activation. Furthermore, biomarkers for fibrinolytic system activation (i.e. *plasminogen* activation) also change during attacks of HAE. These changes may reflect triggering of the *bradykinin*-forming mechanisms by *plasmin*.

Finally, multiple lines of evidence suggest that neutrophil activation and mast-cell activation are functionally linked to *bradykinin* production. We put forward the paradigm that FXII functions as a 'sensor molecule' to detect conditions that require *bradykinin* release via crosstalk with cell-derived enzymes. Understanding the mechanisms that drive *bradykinin* generation may help to identify angioedema patients that have *bradykinin*-mediated disease and could benefit from a targeted treatment.

INTRODUCTION

Angioedema is characterized by local, non-itchy, cutaneous or mucosal edema that lasts for hours up to a few days. Angioedema is by definition a patho-physiologic process involving increased permeability of the vascular-endothelial lining of small blood vessels (mostly post-capillary venules). The clinical picture of angioedema is well-defined but the underlying mechanisms responsible for the swellings are not fully understood. Two vasoactive agents are implicated in mediating swelling attacks in angioedema. The first is *histamine*, released by mast cells or basophils. *Histamine* is the main suspect mediator in allergic reactions, since angioedema can be seen in anaphylaxis [1] or as a concurrent symptom of the mast-cell driven diseases like chronic spontaneous urticaria [2]. For angioedema with unknown aetiology (idiopathic angioedema), histamine receptor antagonists are clinically applied on a trial-and-error basis, sometimes with higher than recommended doses [2,3]. Approximately one in six patients with idiopathic angioedema remains unresponsive to antihistamines [4,5]. In such cases the involvement of other mediators should be considered.

The second suspect mediator of angioedema is *bradykinin*. This vasoactive peptide was first identified as a mediator for angioedema in patients with hereditary angioedema (HAE) [6-8]. Bradykinin is the end product of the contact activation system. This enzymatic cascade circulates in the plasma and consists of FXII, plasma *prekallikrein* (PPK) and *high molecular weight kininogen* (HK). This system is linked to the intrinsic coagulation system via factor XI (FXI). Classically, the contact activation system is considered the most redundant part of the blood coagulation system. *In vitro*, FXII auto-activates when it binds to negatively charged surfaces such as glass or kaolin, hence the name 'contact system'. Active FXII (FXIIa) activates PPK (Figure 1). When activated, *plasma kallikrein* (PK) liberates *bradykinin* from HK by cleavage. At present time, it is unknown how *bradykinin* is produced in the human body. Several studies suggested potential natural activators of FXII [9-13], but thus far none of these have been definitively established to induce activation of the contact system during angioedema *in vivo*.

Here, we will discuss the role of *bradykinin* in angioedema, the link to the coagulation system and how *bradykinin* may be produced *in vivo*.

BRADYKININ-MEDIATED ANGIOEDEMA

The available genetic evidence of HAE-related mutations clearly point towards a central role of the plasma contact system in this disease. Most HAE patients either have SERPING1 gene mutations (encoding for *C1-INH* production) [14,15] while a small minority have mutations in FXII, with normal *C1-INH* activity [16-20].

Hereditary angioedema (HAE) is hallmarked by recurrent attacks of angioedema. Attacks can be life-threatening when swelling compromises the airways, and extremely painful when located in the intestine [21,22]. Therapy targeting the contact system has been successful in HAE, strongly supporting the concept that angioedema is mediated via *bradykinin* production [23-25]. Evidence for *bradykinin* involvement in angioedema is not limited

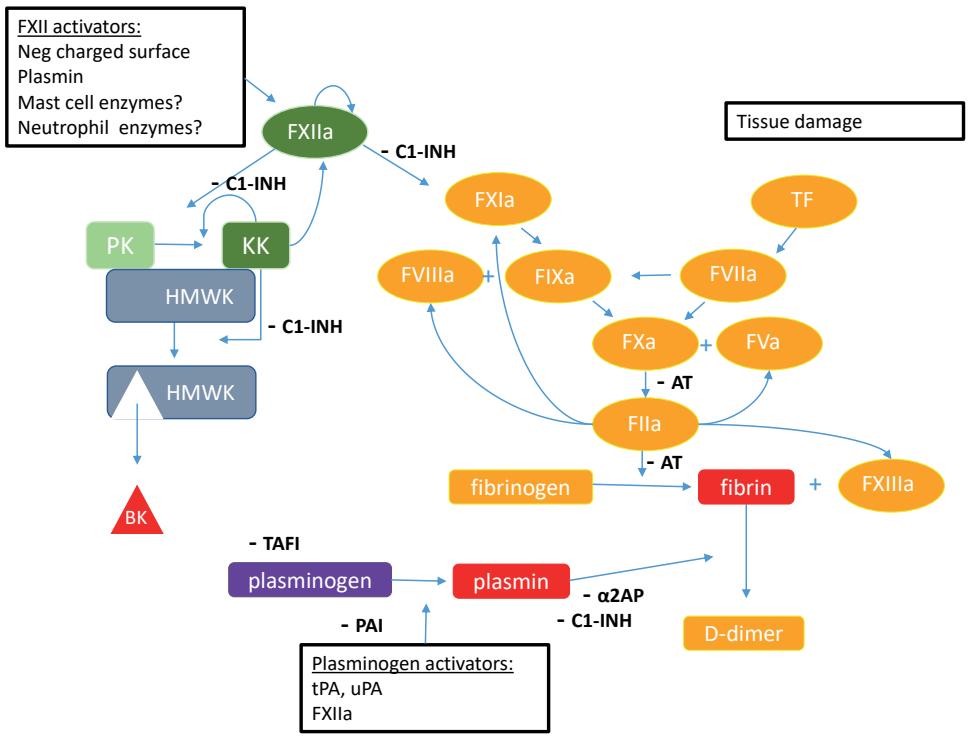

Figure 1. Overview of Coagulation, Contact Activation and Fibrinolysis. The coagulation cascade is initiated by either tissue factor (TF) or FXIIa. Positive feedback by *thrombin* (FIIa) accelerates coagulation. The end-product of coagulation is *fibrin*, cross-linked by factor XIII. *Fibrin* is degraded by *plasmin*. During this process D-dimer, an important clinical biomarker for thrombosis, is generated. Fibrinolysis is started when tPA bound to *fibrin*, or uPA expressed on the endothelium, converts *plasminogen* into *plasmin*. Contact activation starts with the activation of FXII that will eventually lead to *bradykinin* release and vascular leakage. *C1-INH* is the most important inhibitor of contact activation and a weak inhibitor of *plasmin*. *Antithrombin* (AT) inhibits coagulation and *α2-antiplasmin* inhibits *plasmin*, these inhibitors bound to their target enzymes can be measured in plasma as biomarkers for contact activation, coagulation and fibrinolysis. PAI-1 inhibits *plasminogen* activation via inhibition of tPA and uPA. TAFI modulates plasminogen to prevent activation.
Abbreviations*TF: tissue factor. FXIIa: active factor XII. PK: plasma prekallikrein. KK: kallikrein. HMWK: high molecular weight kininogen. BK: bradykinin. C1-INH: C1 inhibitor. AT: anti-thrombin. α2AP: α2-anti-plasmin. TAFI: thrombin activatable fibrinolysis inhibitor. PAI: plasminogen activator inhibitor. tPA: tissue plasminogen activator. uPA: urokinase plasminogen activator.*

to HAE. First, a comparable phenotype can be observed in patients that have acquired C1-INH deficiency due to underlying auto-immune or lymphoproliferative disease [26,27]. Second, anti-hypertensive drugs that inhibit *bradykinin* breakdown, such as *angiotensin converting enzyme* (ACE), *dipeptidyl peptidase IV* (DPPIV) and the novel anti-hypertensive drug neprilysin (NEP), can induce angioedema. During clinical trials of neprilysin inhibitors [28] up to 2.17% of patients and 0.2-0.65% of patients prescribed ACE-inhibitors have developed angioedema [29,30].

Evidently, contact activation is closely linked to the coagulation system [31-33]. Activation of coagulation and fibrinolysis during HAE attacks has been repeatedly reported [34-47]. Yet, HAE patients present with swellings but not with thrombotic tendency [37]. Combined genetic and clinical findings suggest that a subset of coagulation factors are actively involved in angioedema attacks.

BLOOD COAGULATION

Coagulation factors are readily available throughout the blood circulation to initiate *fibrin* formation and reinforce platelet plugs at sites of injury. These interactions are essential to ensure a properly functioning hemostatic system (Figure 1). This system consists of a set of precursor proteins (zymogens) that circulate in the blood and has to be activated to become biologically active. The key initiator of the coagulation system, *tissue factor* (TF), is normally not present in the circulation. Cells that surround the vessel wall express TF so that only when the endothelial layer is compromised locally active coagulation takes place [48,49]. After binding to TF, activated coagulation factor VII (FVII) of the extrinsic pathway activates *factor X* (FX), and to a lesser extent *factor IX* (FIX) [50]. Activated FX next triggers the formation of a small amount of active *factor II (thrombin)*. *Thrombin* accelerates coagulation via positive feedback mechanisms. FVIII, activated by *thrombin*, forms a complex together with FIX that strongly increases additional FXa and thrombin generation. *Thrombin* activates FV that, in a similar manner, contributes to thrombin formation in complex with FXa. Furthermore, FXI of the intrinsic pathway is also activated by *thrombin* [51], and additional FIX is being amplified by *thrombin* activation. Ultimately, *thrombin* converts *fibrinogen* into fibrin. *Fibrin* strands are reinforced through crosslinking by activated FXIII (also activated by thrombin). The *fibrin* lattice, together with platelet aggregates and trapped red blood cells, form a thrombus to seal the damaged area and prevent further haemorrhage. The initiator of this cascade of events TF, is of vital importance since TF deficiency is lethal and incompatible with normal embryonic development or may cause early death in the perinatal period [52].

FACTOR XII AS A COAGULATION FACTOR

When blood comes into contact with negatively charged surfaces, FXII activates spontaneously. FXIIa subsequently activates FXI, thereby providing an alternative trigger for coagulation that is independent of TF- the intrinsic pathway. FXII does not take part in the positive feedback mechanism of thrombin. As a result of the discovery of this *in vitro* mechanism, FXII is generally regarded as a coagulation factor.

Mysteriously, FXII deficiency is a seemingly asymptomatic condition in both mice and human (Hageman's disease), as it is not associated with bleeding tendency, unlike deficiencies of other coagulation factors [53]. The same holds for the other components of the contact system cascade; their deficiency does not result in bleeding [54]. This makes the physiological relevance of FXII and the contact system for physiological hemostasis debatable. This logic raises a pertinent question: should FXII be regarded as a coagulation factor, as far as angioedema is concerned? It has been demonstrated that the bradykinin-

producing contact system machinery can be fully activated in plasma without evidence of coagulation [10,11,55]. This might be explained by the physical properties of FXII(a): in a sequence of cleavage events, FXIIa is progressively fragmented. The fragment that remains after multiple cleavages (βFXIIa or FXIIf) has lost its potential for coagulation while its bradykinin-forming potential remains intact.

FACTOR XII AS A BRADYKININ-GENERATING FACTOR

Although the relevance of FXII for coagulation and hemostasis seems to be limited to *in vitro* experiments, this is certainly not the case for bradykinin production. Low levels of *bradykinin* are continuously formed and present in the bloodstream. Murine models of FXII deficiency indicate that the basal production of bradykinin in plasma is approximately 50% dependent on FXII [56]. This suggests the presence of a second pathway generating *bradykinin in vivo*. Several alternative, FXII-independent, routes for initiation of bradykinin generation have been identified. The endothelial-cell derived factors *heat shock protein 90 (HSP90)* and *prolyl-carboxy-peptidase* may facilitate FXII-independent PPK activation on endothelial cell surface [57]. Moreover, *bradykinin* can be directly released from HK by other enzymes than PPK, this was recently demonstrated for *Mannose Binding Serine Protease 1* [58]. The relevance of these FXII-independent pathways of *bradykinin* generation needs to be established. It is imaginable that during endothelial cell activation or damage, small amounts of PPK are activated by these mechanisms, which boost up FXII activation as a result.

The clinical importance of FXII for bradykinin generation was already demonstrated in HAE patients. Genome-wide screening of a subset of HAE patients with normal *C1-INH* level and function (formerly called: Type III HAE), resulted in the discovery of disease-related mutations in the F12 gene (named: FXII-HAE). Since then, several mutations in HAE patients have been described, (mostly) located in the *proline* rich region of FXII (according to mature amino acid sequence: Thr309Arg, Thr309Lys, Ala324Pro, 72-bp deletion at c971_1018þ24 and a 18bp duplication c894_911, and c1681-1G/A in intron 13) [16-20,59]. Additionally, isolated cases of normal C1-INH with FXII mutation has been successfully treated with the bradykinin-receptor antagonist- icatibant.

PLASMINOGEN ACTIVATION AND FIBRINOLYSIS

After a thrombus has fulfilled its hemostatic function, it has to be cleared. This natural process is mediated mainly by the breakdown of *fibrin*: fibrinolysis (Figure 1). *Plasmin*, the central enzyme of the fibrinolytic system, cleaves the *fibrin* lattice into smaller *fibrin* degradation products (FDP's) including D-dimers, which are regarded as a valuable biomarker for thrombosis [60]. *Plasmin* is generated from its zymogenic precursor plasminogen by either *tissue plasminogen activator* (tPA) or *urokinase plasminogen activator* (uPA) [61]. *Fibrin* provides a platform for its own degradation by binding and potentiating both t-PA and plasminogen. The fibrinolytic system is amenable to inhibition at several stages: *plasmin* is directly inhibited by *α2-antiplasmin*, while specific inhibitors of *plasminogen activators* (PAI-1 and

PAI-2) control tPA and uPA [61,62]. Activation of *plasminogen* on the *fibrin* lattice is indirectly prevented by *thrombin*-activatable fibrinolysis inhibitor, via removal of C-terminal lysines that are needed for the binding of tPA and *plasminogen* to *fibrin*. These Lysin-binding sites are the target of drugs that are being used in HAE (i.e. Tranexamic Acid and Epsilon amino-caproic Acid). Intriguingly, *plasminogen* activation on the endothelium can take place in the absence of *fibrin* when the uPA-receptor is expressed [63]. This amongst others occurs during tissue injury and hypoxia, and makes it attractive to speculate that *plasmin* may have additional functions beyond fibrinolysis.

A FUNCTIONAL LINK BETWEEN PLASMINOGEN ACTIVATION AND CONTACT SYSTEM ACTIVATION

As formerly discussed, the most important function of FXII appears to be activation of PPK. Besides that, FXII is also capable of activation of *plasminogen* [33,64,65]. Compared to tPA and uPA, FXIIa is a relatively weak *plasminogen* activator. However, population studies have proposed that FXIIa may protect against cardiovascular disease via *plasminogen* activation [66,67]. This may be attributable to additional interactions between the contact system and the fibrinolytic system: FXIIa can enzymatically inactivate PAI-1 [68], whereas PK stimulates uPA activation on the endothelium [65].

EVIDENCE FOR PLASMIN AS NATURAL FXII ACTIVATOR

There is also evidence that the fibrinolytic system triggers the activation of the contact system. It has been demonstrated that *plasmin* can induce FXII activation *in vitro* [69]. In line with this finding, patients with myocardial infarction treated with therapeutic *plasminogen*-activating agents, such as streptokinase or recombinant tPA (r-tPA), showed increased plasma levels of *cleaved HK* (a surrogate marker for bradykinin release) [69] and elevated plasma levels of FXIIa [70]. Other clinical observations also support the importance of *plasmin* as a natural FXII activator. Neurologists repeatedly reported angioedema, which is presumably mediated via *bradykinin*, as a side-effect of *plasminogen* activators given to patients with ischaemic stroke [71-82]. Up to 8% of stroke patients receiving r-tPA develop angioedema, often located in the oral cavity and lingual region, and contralateral to the infarction site [83]. Similar observations were made with other thrombolytic agents [84-86]. A study with 42 post-r-tPA angioedema cases reported that 5 patients required emergency intubation or crico-thyroidotomy due to laryngeal swelling, with fatal outcome in two cases. Notably, concurrent use of ACE-inhibitors is also reported in patients who developed angioedema during r-tPA treatment [78,79,83]. Evidence for *plasmin*-dependent bradykinin generation as a cause of angioedema during treatment with fibrinolytic agents is accumulating. However, the majority of these adverse reactions are still treated as a *histamine*-driven hypersensitivity reaction [86]. Future studies should determine if targeting the contact system is beneficial for treatment of angioedema as a side effect of fibrinolytic therapy. Putting these data together makes a strong case for *plasmin*-dependent activation of FXII *in vivo*.

THE INVOLVEMENT OF PLASMIN IN HAE ATTACKS

We recently investigated three subtypes of FXII-HAE patients with F12 gene mutations (de Maat *et al.* article accepted for publication). Unexpectedly, these patients plasma did not enhance FXII-dependent coagulation after contact with a negatively charged compound *in vitro* (i.e. kaolin). This is in good correspondence with the normal clotting times that were reported in FXII-HAE patients [18]. These patients plasma also did not become unusually active upon cleavage by plasma *kallikrein*. Detailed biochemical studies showed that these mutations introduce new cleavage sites in the FXII molecule. The mutated sites are collectively sensitive to cleavage by *plasmin*, resulting in enhanced susceptibility for activation. Analyses of plasma from two FXII-HAE patients who carry the F12 mutation T309K, showed that the *plasmin*-forming potential correlated with disease activity. These findings underscore the clinical relevance of *plasmin* as a FXII activator.

Empiric body of evidence for the importance of *plasmin* in the pathology of HAE has been presented in the last four decades. Anti-fibrinolytic therapy, mainly tranexamic acid, has been used as prophylactic therapy for HAE attacks since the 1970's [4,87,88]. Tranexamic acid is a *lysine*-derivate (analogue) that binds to *lysine* binding sites (LBS) of *plasminogen* and thereby prevent its binding to *fibrin* and subsequent activation by tPA or uPA [89]. Moreover, HAE patients with normal *C1-INH* levels (i.e. FXII-HAE), have decreased levels of PAI-2 during remission compared to patients with HAE due to *C1-INH* deficiency [36]. This suggests that their angioedema episodes might have originated from inadequate inhibition of *plasminogen* activation and *bradykinin* formation downstream activated FXII. These findings together, point out that *plasmin* formation and contact system initiation might be linked.

BIOMARKERS OF CONTACT ACTIVATION, FIBRINOLYTIC ACTIVITY AND COAGULATION IN ANGIOEDEMA

Detection of Contact system Activation

As *bradykinin* was already proven to be the pivotal mediator of angioedema, biomarkers for its production or predecessors are highly sought after, as they may help in diagnosis, monitoring disease activity and response to treatment. *Bradykinin* is only present in the circulation for a few seconds after it is released from HK, due to rapid degradation by *kininases* [90] which complicates its detection. Activation products of contact system activation in plasma are valuable biomarkers as they may reflect recent *bradykinin* production. However, due to pre-analytical *in vitro* activation, accurate measurements have proved to be challenging and relatively labour-intensive (see Farkas et al, Chapter 7 in this issue).

Circulating *cleaved HK* seems among the most suitable biomarkers for contact activation since *cleaved HK* levels have been shown to correlate with attack frequency [91]. Currently, *cleaved HK* can be detected by immunoblotting [91]. This assay often shows profound cleavage of HK in citrated plasma from patients with *C1-INH* deficiency. A diagnostic tool that could specifically measure cleaved HK, only after it has released bradykinin, would be compelling instrument for HAE diagnosis and follow-up, but is currently lacking.

BIOMARKERS OF COAGULATION AND PLASMINOGEN ACTIVATION

There is a large base of evidence that contact activation during angioedema attacks are accompanied by changes in fibrinolytic and coagulation system that could serve as disease biomarkers [34-47,92-94]. The understanding that the contact system and fibrinolytic system are functionally linked sheds a new light on the repeatedly reported occurrence of increased levels of complexes of plasmin and its main inhibitor *α2-antiplasmin* (PAP), and decreased levels of *PAI-1* measured during HAE attacks [34-36,39,40,43,44]. Fibrinolytic biomarkers may also be proven helpful in identifying *bradykinin*-mediated angioedema. The presence of these biomarkers in any patient that presents with angioedema in the absence of apparent evidence for thromboembolic event should raise the suspicion that the contact system is involved. Interestingly, parameters of coagulation are repeatedly reported to be increased in HAE. D-dimer levels are elevated during remission periods and markedly increase during attacks [34,37,39,40,92]. Also, *thrombin-antithrombin* complexes and *prothrombin fragments F1+F2* increase during attacks [34,39,40,42,43,92].

THE PARADOX OF FIBRINOLYSIS IN HAE

Strikingly, HAE is not associated with increased risk of thrombotic disease. It is therefore hard to imagine that the coagulation parameters measured reflect intravascular fibrin formation. Since HAE is associated with extreme vascular leakage, this may offer an alternative explanation for this phenomena. We would like to propose that when plasma coagulation factors move into the extra-vascular space, FVIIa may complex with TF. This can trigger coagulation in the absence of vascular injury or intravascular thrombi. Evidence for extrinsic pathway coagulation was demonstrated by a significant increase of FVIIa during angioedema attacks in 14 patients, by Cugno *et al*, [42]. It would be attractive to hypothesize that increased coagulation parameters in HAE are secondary to massive vascular leakage (Figure 2). Even though biomarkers for coagulation, such as D-dimer, will not directly reflect *bradykinin* production, their strong association with HAE may make them helpful as biomarkers for monitoring disease activity.

FUTURE PERSPECTIVES IN ANGIOEDEMA - NATURAL CONTACT SYSTEM ACTIVATION AND BRADYKININ PRODUCTION

Efforts to identify the initial spark of contact activation focused on the presence of *in vivo* negatively charged compounds (i.e. glycosaminoglycans, misfolded protein aggregates *etc*.). The discovery that *plasmin* can enzymatically activate FXII, in the absence of a surface, provides an alternative mechanism for this activation process. The importance is underscored by observations in HAE and patients receiving r-tPA therapy. Under physiological circumstances, this mechanism may take place at sites of vascular obstruction, or slow perfusion, where the tissues is endangered by hypoxia. Alternatively, minor tissue trauma or infection may provide signals to the vascular endothelium, resulting in local sequestration and activation of the contact system. We postulate that FXII functions as a **'sensor molecule'**

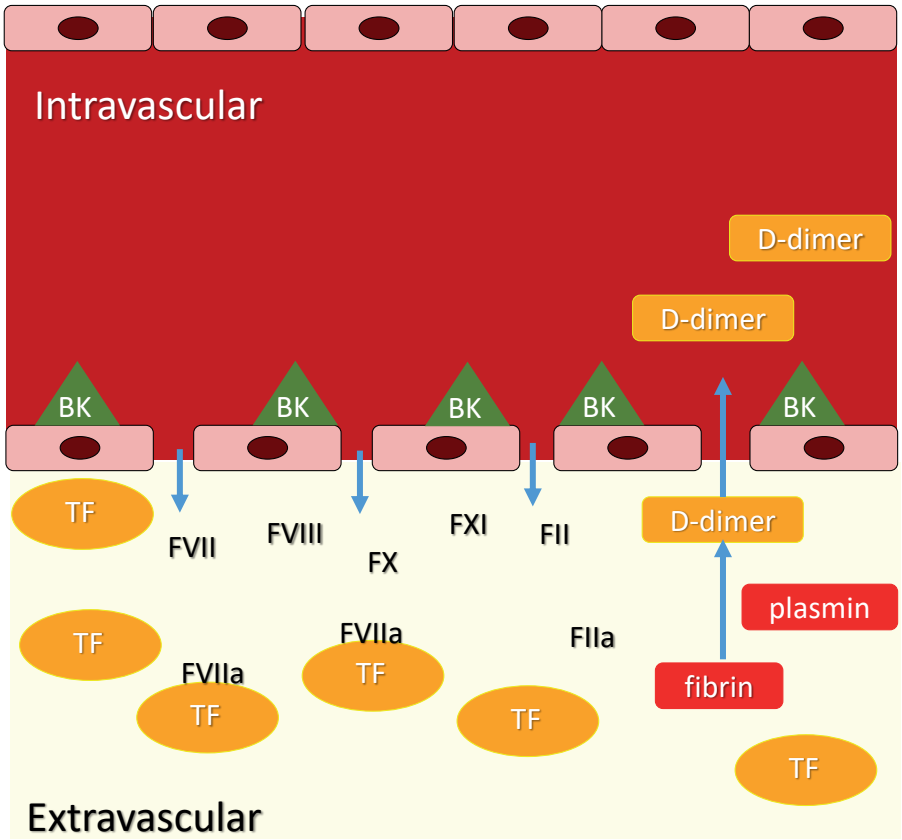

Figure 2. Proposed Model: Increased Plasma Coagulation Parameters Secondary to Increased Vascular Permeability and Extravascular Coagulation. *Bradykinin* binds to its receptors on endothelial cells. Increased vascular permeability allows extravasation of coagulation factors. Tissue factor expressed in the extravascular space can initiate coagulation. In the absence of any injury, the forming *fibrin* lattice is continuously degraded by *plasmin*. Subsequently, *D-dimer* formed in the extra vascular space dissipate into the blood stream.

that interacts with the environment to detect conditions where increased vasopermeability is required. Excitingly, it is possible that a variety of enzymes other than *plasmin* can fulfil a similar role and contribute to FXII activation.

NEUTROPHIL ACTIVATION AND CONTACT SYSTEM ACTIVATION ARE FUNCTIONALLY LINKED

Recent work shows that HAE attacks are associated with increasing neutrophil counts and neutrophil elastase levels [95]. Neutrophils are important cells for the innate immunity. They migrate out of the bloodstream within hours after a pathogen is detected. Migration of neutrophils to sites of inflammation is orchestrated by several mechanisms, such as

chemotaxis by interleukins and complement factors, and interaction of neutrophils with endothelial cell receptors [96]. It can be envisioned that neutrophils interact with the contact system to boost neutrophil extravasation by *bradykinin*-mediated vasodilatation. First of all, neutrophil elastase, released by active neutrophils, inactivates *C1-INH* thereby allowing contact activation to take place [97]. Second, *in vitro* studies show that the *bradykinin B1 receptor* (BKB1R) regulates neutrophil trafficking [98,99]. Third, both *kallikrein* and FXIIa can induce neutrophil degranulation [100]. Finally, neutrophil extracellular traps (NETs) have been shown to activate FXII. NETs exist on DNA, and the negative charge of DNA is believed to induce auto-activation of FXII [101]. At the very least, NETs can sequester FXII and present it for activated cleavage. NETs are a binding site for antimicrobial proteins such as histones, neutrophil elastase and cathepsins [98]. To what extent the proteins loaded on NETs contribute to neutrophil-induced FXII activation is not yet elucidated. The negative charge of NETs, in combination with potential FXII-activating enzymes would make neutrophils a plausible platform for contact activation. Further research to neutrophil and FXII activation may help understand its possible relevance for HAE patients.

MAST CELL ACTIVATION IS LINKED TO BRADYKININ GENERATION

Angioedema as a symptom of anaphylaxis and chronic spontaneous urticaria are likely be mediated by mast cell/basophil activation. *Histamine* is the central chemical mediator released by mast cells, inducing hyperpermeability and vasodilatation, and is therefore held responsible for the angioedema seen in allergic responses. Indeed, anti-*histamine* therapy reduces the occurrence of angioedema in urticaria patients [2]. It should be noted that plasma *bradykinin* levels were not found elevated in four patients with an acute attack of anti-*histamine* sensitive angioedema [102]. Yet, evidence accumulates that angioedema in allergic reactions is also accompanied by *bradykinin* release. *FXIIa-C1-INH* complexes and *kallikrein-C1-INH* complexes were increased up to tenfold, within minutes after experimental insect stings in six allergic patients who developed shock or angioedema [1]. In contrast patients who only developed urticarial rash in reaction to the venom showed a non-significant increase in *C1-INH* complexes. In line with these results, it was shown that in the patients with angioedema or shock a third up to half of the total pool of plasma HK was cleaved within minutes [1]. These results were confirmed in another study where HK was analysed in patients with an anaphylactic reaction (mainly induced by food allergens) [103]. In this study, even patients with a mild reaction (i.e gastro-intestinal complaints) showed 60% cleavage of their HK pool. Evidence of the clinical relevance of *bradykinin* during anaphylaxis with hypotension, was further demonstrated in animal models. Hypotension after IgE-mediated, antigen-induced anaphylaxis, was reduced in FXII-deficient mice compared to wild-type mice [103]. The same protection toward IgE-mediated hypotension was reported in BKB2R knock-out mice, and HK and PPK-deficient mice [104]. Although direct release of *bradykinin* from HK by tryptase could also contribute to these findings, it would be reasonable to propose that *bradykinin* production during anaphylaxis is for a major part FXII-driven.

Moreover, Mast cell degranulation may trigger FXII activation via the release of *heparin*, which activates FXII *in vitro* and induces FXII-dependent hypotension in mice models [10]. *Heparin*-induced vascular leakage in mice was diminished by BKB2R antagonist (icatibant) and exaggerated in *C1-INH* deficient mice [10]. *Heparin* is a negatively charged compound, capable of inducing FXII auto-activation. However, like the NETs of neutrophils, heparin binds a large variety of proteins [105]. Contrary to this, a study of 14 HAE patients showed that tryptase levels do not increase during HAE attacks [106]. It might be helpful to keep in mind that in HAE patients that also suffer from allergies, attacks may be aggravated by an allergic trigger. What do these studies mean for HAE? Angioedema attacks in HAE are currently not believed to be mast cell driven ("allergic") and anti-*histamine* therapy has no effect on symptoms. So far there is no solid evidence for an association between allergic responses and angioedema attacks. Additionally, *Heparin*-protein interactions are usually studied in an isolated *in-vitro* manner. However, the possibility of protein-protein interactions on *heparin* extracellular matrix may be of importance. In the case of FXII both the net charge of *heparin* and nearby proteins might synergistically activate FXII. Further research into mast cell and FXII interactions may help to understand the pathological mechanisms behind *in-vivo* FXII activation and angioedema.

The contribution of *bradykinin* to angioedema with normal levels of *C1-INH* (i.e chronic spontaneous urticaria with angioedema, IH-AAE, InH-AAE, U-HAE etc.) is still uncertain. It might be speculated that since FXII activation can result from mast cell degranulation, *bradykinin* may also play a supportive role in forms of angioedema that are currently classified as 'histaminergic', based on the presence of wheals, pruritus or (partial) response to anti-*histamine* therapy. Current studies were unable so far to demonstrate *bradykinin* or an increased HK cleaving in such patients [102,103,107]. Considering the strong evidence of interaction between mast cells and *bradykinin* production, patients with idiopathic angioedema and recurrent swellings who fail to respond to high-dose anti-histamine treatment might benefit from therapy targeting the contact activation.

CONCLUSIONS

- FXII is an important player in bradykinin production during HAE attacks. However, It is currently unknown how FXII activation occurs *in vivo*.
- Coagulation parameters change during attacks. This is possibly secondary to vascular leakage, rather than a consequence of FXII activation. However biomarkers of coagulation may reflect disease activity.
- Fibrinolytic parameters change during attacks. These may reflect a role for plasminogen activation in *bradykinin* production of (via *plasmin*).
- Multiple lines of evidence demonstrate that the cellular system, i.e. neutrophil and mast cell activation are functionally linked to contact system activation and bradykinin production.
- It is possible that *bradykinin* plays a role in other forms of angioedema with normal *C1-INH* activity, including those that are currently classified as 'histaminergic'.

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REFERENCES

1. van der Linden PW, Hack CE, Eerenberg AJ, Struyvenberg A, and van der Zwan JK (1993) Activation of the contact system in insect-sting anaphylaxis: association with the development of angioedema and shock. Blood 82: 1732–1739
2. Zuberbier T, Aberer W, Asero R, Bindslev-Jensen C, Brzoza Z, Canonica GW et al (2014) The EAACI/GA(2) LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: the 2013 revision and update. Allergy 69:868–887
3. Cicardi M, Aberer W, Banerji A, Bas M, Bernstein JA, Bork K et al (2014) Classification, diagnosis, and approach to treatment for angioedema: consensus report from the Hereditary Angioedema International Working Group. Allergy 69:602–616
4. Mansi M, Zanichelli A, Coerezza A, Suffritti C, Wu MA, Vacchini R et al (2015) Presentation, diagnosis and treatment of angioedema without wheals: a retrospective analysis of a cohort of 1058 patients. J Intern Med 277: 585–593
5. Zingale LC, Beltrami L, Zanichelli A, Maggioni L, Pappalardo E, Cicardi B et al (2006) Angioedema without urticaria: a large clinical survey. Can Med Assoc J 175: 1065–1070
6. Fields T, Ghebrehiwet B, Kaplan AP (1983) Kinin formation in hereditary angioedema plasma: evidence against kinin derivation from C2 and in support of "spontaneous" formation of bradykinin. J Allergy Clin Immunol 72: 54–60
7. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A (1998) Plasma bradykinin in angio-oedema. Lancet 351: 1693–1697
8. Nussberger J, Cugno M, Cicardi M, Agostoni A (1999) Local bradykinin generation in hereditary angioedema. J Allergy Clin Immunol 104: 1321–1322
9. Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM et al (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139: 1143–1156
10. Oschatz C, Maas C, Lecher B, Jansen T, Björkqvist J, Tradler T et al (2011) Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. Immunity 34: 258–268
11. Maas C, Govers-Riemslag JWP, Bouma B, Schiks B, Hazenberg BPC, Lokhorst HM et al (2008) Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. J Clin Invest 118: 3208–3218
12. White-Adams TC, Berny MA, Patel IA, Tucker EI, Gailani D, Gruber A et al (2010) Laminin promotes coagulation and thrombus formation in a factor XII-dependent manner. J Thromb Haemost 8: 1295–1301
13. van der Meijden PEJ, Munnix ICA, Auger JM, Govers-Riemslag JWP, Cosemans JMEM, Kuijpers MJE et al (2009) Dual role of collagen in factor XII-dependent thrombus formation. Blood 114: 881–890
14. Kaplan AP (2010) Enzymatic pathways in the pathogenesis of hereditary angioedema: the role of C1 inhibitor therapy. J Allergy Clin Immunol 126: 918–925
15. Gösswein T, Kocot A, Emmert G, Kreuz W, Martinez-Saguer I, Aygören-Pürsün E et al (2008) Mutational spectrum of the C1INH (SERPING1) gene in patients with hereditary angioedema. Cytogenet Genome Res 121: 181–188
16. Dewald G and Bork K (2006) Missense mutations in the coagulation factor XII (Hageman factor) gene in hereditary angioedema with normal C1 inhibitor. Biochem Biophys Res Commun 343: 1286–1289
17. Cichon S, Martin L, Hennies HC, Müller F, Van Driessche K, Karpushova A et al (2006) Increased activity of coagulation factor XII (Hageman factor) causes hereditary angioedema type III. Am J Hum Genet 79: 1098–1104
18. 18. Bork K, Wulff K, Meinke P, Wagner N, Hardt J, Witzke G (2011) A novel mutation in the coagulation factor 12 gene in subjects with hereditary angioedema and normal C1-inhibitor. Clin Immunol 141: 31–35

19. Grumach AS, Stieber C, Veronez CL, Cagini N, Constantino-Silva RN, Cordeiro E et al (2016) Homozygosity for a factor XII mutation in one female and one male patient with hereditary angio-oedema. Allergy 71: 119–123
20. Gelincik A, Demir S, Olgaç M, Karaman V, Toksoy G, Çolakoğlu B et al (2015) Idiopathic angioedema with F12 mutation: is it a new entity? Ann Allergy Asthma Immunol 114: 154–156
21. Longhurst H, Cicardi M (2012) Hereditary angio-oedema. Lancet 379: 474–481
22. Hofman ZLM, Relan A, Hack CE (2014) Hereditary Angioedema Attacks: Local Swelling at Multiple Sites. Clin Rev Allergy Immunol 50: 34-40
23. Bygum A, Andersen KE, Mikkelsen CS (2009) Self-administration of intravenous C1-inhibitor therapy for hereditary angioedema and associated quality of life benefits. Eur J Dermatol 19: 147–151
24. Cicardi M, Levy RJ, McNeil DL, Li HH, Sheffer AL, Campion M et al (2010) Ecallantide for the treatment of acute attacks in hereditary angioedema. N Engl J Med 363: 523–531
25. Cicardi M, Banerji A, Bracho F, Malbrán A, Rosenkranz B, Riedl M et al (2010) Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema. N Engl J Med 363: 532–541
26. Wu MA and Castelli R (2016) The Janus faces of acquired angioedema: C1-inhibitor deficiency, lymphoproliferation and autoimmunity. Clin Chem Lab Med 54: 207–214
27. Sinclair D, Smith A, Cranfield T, Lock RJ (2004) Acquired C1 esterase inhibitor deficiency or serendipity? The chance finding of a paraprotein after an apparently low C1 esterase inhibitor concentration. J Clin Pathol 57: 445–447
28. McMurray JJV, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR et al (2014) Angiotensin-Neprilysin Inhibition versus Enalapril in Heart Failure. N Engl J Med 371: 993-1004
29. Miller DR, Oliveria SA, Berlowitz DR, Fincke BG, Stang P, Lillienfeld DE (2008) Angioedema incidence in US veterans initiating angiotensin-converting enzyme inhibitors. Hypertension 51: 1624–1630
30. Kostis JB, Kim HJ, Rusnak J, Casale T, Kaplan A, Corren J et al (2005) Incidence and characteristics of angioedema associated with enalapril. Archives of internal medicine 165: 1637–1642
31. Maas C (2013) The Protease Storm of Angioedema. The Journal of Angioedema 1: 18–27
32. Levi M, Hack CE, de Boer JP, Brandjes DP, Büller HR, Cate ten JW (1991) Reduction of contact activation related fibrinolytic activity in factor XII deficient patients. Further evidence for the role of the contact system in fibrinolysis in vivo. J Clin Invest 88: 1155–1160
33. Braat EA, Dooijewaard G, Rijken DC (1999) Fibrinolytic properties of activated FXII. Eur J Biochem 263: 904–911
34. Csuka D, Veszeli N, Imreh É, Zotter Z, Skopál J, Prohászka Z et al (2015) Comprehensive study into the activation of the plasma enzyme systems during attacks of hereditary angioedema due to C1-inhibitor deficiency. Orphanet J Rare Dis 10: 132
35. Bork K, Wulff K, Witzke G, Hardt J (2015) Hereditary angioedema with normal C1-INH with versus without specific F12 gene mutations. Allergy 70: 1004–1012
36. Joseph K, Tholanikunnel BG, Wolf B, Bork K, Kaplan AP (2015) Deficiency of plasminogen activator inhibitor 2 in plasma of patients with hereditary angioedema with normal C1 inhibitor levels. J Allergy Clin Immunol. doi: 10.1016/j.jaci.2015.07.041
37. Reshef A, Zanichelli A, Longhurst H, Relan A, Hack CE (2015) Elevated D-dimers in attacks of hereditary angioedema are not associated with increased thrombotic risk. Allergy 70: 506–513
38. Bork K, Kleist R, Hardt J, Witzke G (2009) Kallikrein-kinin system and fibrinolysis in hereditary angioedema due to factor XII gene mutation Thr309Lys. Blood Coagul. Fibrinolysis 20: 325–332
39. van Geffen M, Cugno M, Lap P, Loof A, Cicardi M, van Heerde W (2012) Alterations of coagulation and fibrinolysis in patients with angioedema due to C1-inhibitor deficiency. Clin Exp Immunol 167: 472–478

40. Relan A, Bakhtiari K, van Amersfoort ES, Meijers JCM, Hack CE (2012) Recombinant C1-inhibitor: effects on coagulation and fibrinolysis in patients with hereditary angioedema. BioDrugs 26: 43–52
41. Joseph K, Tholanikunnel TE, Kaplan AP (2010) Treatment of episodes of hereditary angioedema with C1 inhibitor: serial assessment of observed abnormalities of the plasma bradykinin-forming pathway and fibrinolysis. Ann. Allergy Asthma Immunol 104: 50–54
42. Cugno M, Cicardi M, Bottasso B, Coppola R, Paonessa R, Mannucci PM et al (1997) Activation of the coagulation cascade in C1-inhibitor deficiencies. Blood 89: 3213–3218
43. Nielsen EW, Johansen HT, Høgåsen K, Wuillemin W, Hack CE, Mollnes TE (1996) Activation of the complement, coagulation, fibrinolytic and kallikrein-kinin systems during attacks of hereditary angioedema. Scand J Immunol 44: 185–192
44. Cugno M, Hack CE, de Boer JP, Eerenberg AJ, Agostoni A, Cicardi M (1993) Generation of plasmin during acute attacks of hereditary angioedema. J Lab Clin Med 121: 38–43
45. Donaldson VH (1993) Plasminogen activation in hereditary angioneurotic edema. J Lab Clin Med 121: 13–14
46. Cugno M, Bergamaschini L, Uziel L, Cicardi M, Agostoni A, Jie AF, et al (1988) Haemostasis contact system and fibrinolysis in hereditary angioedema (C1-inhibitor deficiency). J Clin Chem Clin Biochem 26: 423–427
47. Cullmann W, Kövary PM, Müller N, Dick W (1982) Complement, coagulation and fibrinolytic parameters in hereditary angioedema (HAE). Clin Exp Immunol 49: 618–622
48. Mackman N (2009) The role of tissue factor and factor VIIa in hemostasis. Anesth Analg 108: 1447–1452
49. Fleck RA, Rao LV, Rapaport SI, Varki N (1990) Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. Thromb Res 59: 421–437
50. Vadivel K, Bajaj SP (2012) Structural biology of factor VIIa/tissue factor initiated coagulation. Front Biosci (Landmark Ed) 17: 2476–2494
51. Maas C, Meijers JCM, Marquart JA, Bakhtiari K, Weeterings C, de Groot PG et al (2010) Activated factor V is a cofactor for the activation of factor XI by thrombin in plasma. Proc Natl Acad Sci U.S.A. 107: 9083–9087
52. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I et al (1996) Role of tissue factor in embryonic blood vessel development. Nature 383: 73–75
53. Ratnoff OD, Colopy JE (1955) A Familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma. J Clin investig 34: 602–613
54. Giangrande PLF (2003) Six characters in search of an author: the history of the nomenclature of coagulation factors. Br J Haematol 121: 703–712
55. de Maat S, van Dooremalen S, de Groot PG, Maas C (2013) A nanobody-based method for tracking factor XII activation in plasma. Thromb Haemost 109: 458-68
56. Iwaki T, Castellino FJ (2006) Plasma levels of bradykinin are suppressed in factor XII-deficient mice. Thromb Haemost 95: 1003–1010
57. Joseph K, Tholanikunnel BG, Bygum A, Ghebrehiwet B, Kaplan AP (2013) Factor XII-independent activation of the bradykinin-forming cascade: Implications for the pathogenesis of hereditary angioedema types I and II. J Allergy Clin Immunol 132: 470–475
58. Dobó J, Major B, Kékesi KA, Szabó I, Megyeri M, Hajela K et al (2011) Cleavage of kininogen and subsequent bradykinin release by the complement component: mannose-binding lectin-associated serine protease (MASP)-1 PloS one 6: e20036

59. Kiss N, Barabás E, Várnai K, Halász A, Varga LÁ, Prohászka Z et al (2013) Novel duplication in the F12 gene in a patient with recurrent angioedema. Clin Immunol 149: 142-145
60. Olson JD (2015) D-dimer: An Overview of Hemostasis and Fibrinolysis, Assays, and Clinical Applications. Adv Clin Chem 69: 1–46
61. Cesarman-Maus G, Hajjar KA (2005) Molecular mechanisms of fibrinolysis. Br J Haematol 129: 307–321
62. Declerck PJ, Gils A (2013) Three decades of research on plasminogen activator inhibitor-1: a multifaceted serpin. Semin Thromb Hemost 39: 356–364
63. Ellis V, Behrendt N, Danø K (1991) Plasminogen activation by receptor-bound urokinase. A kinetic study with both cell-associated and isolated receptor. J Biol Chem 266: 12752–12758
64. Goldsmith GH, Saito H, Ratnoff OS (1978) The activation of plasminogen by Hageman factor (Factor XII) and Hageman factor fragments. J Clin Invest 62: 54–60
65. Lenich C, Pannell R, Gurewich V (1995) Assembly and activation of the intrinsic fibrinolytic pathway on the surface of human endothelial cells in culture. Thromb Haemost 74: 698–703
66. Pedersen OD, Munkvad S, Gram J, Kluft C, Jespersen J (1993) Depression of factor XII-dependent fibrinolytic activity in survivors of acute myocardial infarction at risk of reinfarction. Eur Heart J 14:785–789
67. Govers-Riemslag JWP, Smid M, Cooper JA, Bauer KA, Rosenberg RD, Hack CE et al (2007) The plasma kallikrein-kinin system and risk of cardiovascular disease in men. J Thromb Haemost 5: 1896–1903
68. Tanaka A, Suzuki Y, Sugihara K, Kanayama N, Urano T (2009) Inactivation of plasminogen activator inhibitor type 1 by activated factor XII plays a role in the enhancement of fibrinolysis by contact factors in-vitro. Life Sci 85: 220–225
69. Ewald GA, Eisenberg PR (1995) Plasmin-mediated activation of contact system in response to pharmacological thrombolysis. Circulation 91: 28–36
70. Pönitz V, Pritchard D, Grundt H, Nilsen DWT (2006) Specific types of activated Factor XII increase following thrombolytic therapy with tenecteplase. J Thromb Thrombolysis 22: 199–203
71. Bozkurt S, Arslan ED, Köse A, Ayrık C, Yılmaz A, Dündar GA (2015) Lingual angioedema after alteplase treatment in a patient with acute ischemic stroke. World J Emerg Med 6: 74–76
72. Madden B, Chebl RB (2015) Hemi orolingual angioedema after tPA administration for acute ischemic stroke. West J Emerg Med 16: 175–177
73. Correia AS, Matias G, Calado S, Lourenço A, Viana-Baptista M (2015) Orolingual angiodema associated with alteplase treatment of acute stroke: a reappraisal. J Stroke Cerebrovasc Dis 24: 31–40
74. Wallon D, Girardie P, Bombois S, Lucas C (2010) Angioneurotic orolingual oedema following thrombolysis in acute ischaemic stroke. BMJ Case Rep doi: 10.1136/bcr.06.2010.3094
75. Sonny A, Avitsian R, Hussain MS, Elsharkawy H (2015) Angioedema in the neurointerventional suite. J Clin Anesth 27: 170–174
76. Scialò C, Strada L, Finocchi C, Gandolfo C, Balestrino M (2015) Angioedema after rt-PA treatment in acute ischemic stroke may be attended by shock and worsening of stroke outcome. Neurol Sci 36: 795–796
77. Wu TY, Wei DY, Baker Y, Sharkey B, Frith RW (2014) Asymmetric oro-facial angioedema following alteplase for acute ischaemic stroke. J Clin Neurosci 21: 2004–2006
78. Lin SY, Tang S-C, Tsai L-K, Yeh S-J, Hsiao Y-J, Chen Y-W et al (2014) Orolingual angioedema after alteplase therapy of acute ischaemic stroke: incidence and risk of prior angiotensin-converting enzyme inhibitor use. EurJ Neurol 21: 1285–1291

79. Lekoubou A, Philippeau F, Derex L, Olaru A, Gouttard M, Vieillart A et al (2014) Audit report and systematic review of orolingual angioedema in post-acute stroke thrombolysis. Neurol Res 36: 687–694
80. Yayan J (2013) Onset of Orolingual Angioedema After Treatment of Acute Brain Ischemia with Alteplase Depends on the Site of Brain Ischemia: A Meta-analysis. N Am J Med Sci 5: 589–593
81. Foster-Goldman A, McCarthy D (2013) Angioedema from recombinant TPA administration: case report and pathophysiology review. Am J Ther 20: 691–693
82. Yayan J (2012) Lingual angioedema with macroglossia during the treatment of acute ischemic stroke with alteplase. Int J Gen Med 5: 183–186
83. Hurford R, Rezvani S, Kreimei M, Herbert A, Vail A, Parry-Jones AR, et al (2015) Incidence, predictors and clinical characteristics of orolingual angio-oedema complicating thrombolysis with tissue plasminogen activator for ischaemic stroke. J Neurol Neurosurg Psychiatr 86: 520–523
84. Oliveira DC de, Coelho OR, Paraschin K, Ferraroni NR, Zolner R de L (2005) Angioedema related to the use of streptokinase. Arq Bras Cardiol 85: 131–134
85. Cooper JP, Quarry DP, Beale DJ, Chappell AG (1994) Life-threatening, localized angio-oedema associated with streptokinase. Postgrad Med J 70: 592–593
86. Pechlaner C, Knapp E, Wiedermann CJ (2001) Hypersensitivity reactions associated with recombinant tissue-type plasminogen activator and urokinase. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis 12: 491–494
87. Frank MM, Sergent JS, Kane MA, Alling DW (1972) Epsilon aminocaproic acid therapy of hereditary angioneurotic edema. A double-blind study. N Engl J Med 286: 808–812
88. Blohmé G (1972) Treatment of hereditary angioneurotic oedema with tranexamic acid. A random double-blind cross-over study. Acta Med Scand 192: 293–298
89. Tengborn L, Blombäck M, Berntorp E (2015) Tranexamic acid--an old drug still going strong and making a revival. Thromb Res 135: 231–242
90. Murphey LJ, Hachey DL, Oates JA, Morrow JD, Brown NJ (2000) Metabolism of bradykinin In vivo in humans: identification of BK1-5 as a stable plasma peptide metabolite. J Pharmacol Exp Ther 294: 263–269
91. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M (2014) High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin mediated angioedema due to hereditary c1-inhibitor deficiency. Clin Exp Allergy 44: 1503-1514
92. Cugno M, Zanichelli A, Bellatorre AG, Griffini S, Cicardi M (2009) Plasma biomarkers of acute attacks in patients with angioedema due to C1-inhibitor deficiency. Allergy 64: 254–257
93. Nilsson T, Bäck O (1985) Elevated plasmin-alpha 2-antiplasmin complex levels in hereditary angioedema: evidence for the in vivo efficiency of the intrinsic fibrinolytic system. Thromb Res 40: 817–821
94. Bork K, Witzke G, Artmann K, Benes P, Böckers M, Kreuz W (1984) Interaction between C1-INA, coagulation, fibrinolysis and kinin system in hereditary angioneurotic edema (HANE) and urticaria. Arch Dermatol Res 276: 375–380
95. Veszeli N, Csuka D, Zotter Z, Imreh É, Józsi M, Benedek S et al (2015) Neutrophil activation during attacks in patients with hereditary angioedema due to C1-inhibitor deficiency. Orphanet J Rare Dis 10: 156
96. Nauseef WM, Borregaard N (2014) Neutrophils at work. Nat Immunol 15: 602–611
97. Brower MS, Harpel PC (1982) Proteolytic cleavage and inactivation of alpha 2-plasmin inhibitor and C1 inactivator by human polymorphonuclear leukocyte elastase. J Biol Chem 257: 9849–9854

98. Stuardo M, Gonzalez CB, Nualart F, Boric M, Corthorn J, Bhoola KD et al (2004) Stimulated human neutrophils form biologically active kinin peptides from high and low molecular weight kininogens. J Leukoc Biol 75: 631–640

99. Araújo RC, Kettritz R, Fichtner I, Paiva AC, Pesquero JB, Bader M (2001) Altered neutrophil homeostasis in kinin B1 receptor-deficient mice. Biol Chem 382: 91–95

100. Wachtfogel YT, Pixley RA, Kucich U, Abrams W, Weinbaum G, Schapira M et al (1986) Purified plasma factor XIIa aggregates human neutrophils and causes degranulation. Blood 67: 1731–1737

101. Oehmcke S, Mörgelin M, Herwald H (2009) Activation of the human contact system on neutrophil extracellular traps. J Innate Immun 1: 225–230

102. 102. Nussberger J, Cugno M, Cicardi M. Bradykinin-mediated angioedema (2002) Bradykinin-mediated angioedema. N Engl J Med 347: 621–622

103. Sala-Cunill A, Björkqvist J, Senter R, Guilarte M, Cardona V, Labrador M, et al (2014) Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions. J Allergy Clin Immunol 135:1031-43

104. Proud D, Siekierski ES, Bailey GS (1988) Identification of human lung mast cell kininogenase as tryptase and relevance of tryptase kininogenase activity. Biochem. Pharmacol 37: 1473–1480

105. 105. Meneghetti MCZ, Hughes AJ, Rudd TR, Nader HB, Powell AK, Yates EA et al (2015) Heparan sulfate and heparin interactions with proteins. J R Soc Interface 12: 0589

106. Obtułowicz A, Pirowska M, Dyga W, Czarnobilska E, Wojas-Pelc A (2015) Tryptase and histamine in patients with angioedema due to C1-inhibitor deficiency (Hereditary Angioedema, HAE) and in patients with mastocytosis. Alergia Astma Immunologia 20: 106-110

107. Lang DM, Sugimoto SL, Curd JG, Christiansen SC, Zuraw BL (1989) High-molecular weight kininogen is cleaved in active erythema multiforme. J Allergy Clin Immunol 83: 802–810

Insight in hereditary angioedema

3

C-reactive protein levels in Hereditary Angioedema

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ABSTRACT

Background

Hereditary Angioedema (HAE) patients experience recurrent episodes of angioedema attacks that can be painful, disfiguring and even life-threatening. The disorder results from a mutation in the gene that controls the synthesis of C1-inhibitor (C1INH). C1INH is a major regulator of activation of the contact system. It is often assumed that attacks result from uncontrolled, local activation of the contact system with subsequent formation of bradykinin.

Objective

To evaluate involvement of inflammatory reactions in HAE, we analyzed C-reactive protein (CRP) levels before, during, and after HAE attacks.

Methods

HAE patients included in a clinical database of recombinant human C1- inhibitor (rhC1INH) studies were evaluated. For the current study we analyzed CRP levels when patients were asymptomatic, during a clinical attack and in a follow up period and correlated these with the clinical manifestations of the attack.

Results

Data from 68 HAE patients were analyzed and included CRP levels at 273 occasions. While asymptomatic, 20% of the patients analyzed had increased CRP. At the onset of the attack ($p=0.049$) and over the next 24 hours CRP rose significantly ($p=.002$) in patients with an abdominal location, and post-attack levels were significantly higher in these patients than in patients with attacks at other locations ($p=.034$).

Conclusion

CRP levels are elevated in a substantial proportion of asymptomatic HAE patients. Levels of CRP significantly increase during an abdominal attack. These data suggest low-grade systemic inflammatory reactions in HAE patients as well as a triggering event for attacks that starts prior to symptom onset.

INTRODUCTION

Hereditary angioedema (HAE) results from a mutation in the gene coding for the plasma protein C1-inhibitor (C1INH).[1,2] HAE patients experience recurrent episodes of pain and edema that can be located at various sites in the body such as in the extremities and the uro-genital tract. Submucosal swelling in the upper airways can lead to life-threatening asphyxia, whereas abdominal attacks present as a functional bowel obstruction causing extreme pain.[3,4]

C1INH controls three intertwined cascade systems: the contact system, the classical and lectin complement pathways and the factor XII-dependent fibrinolytic cascade. Of these, the contact system is generally considered the most important for the pathogenesis of the angioedema attacks.[5,6]

Activation of the contact system generates bradykinin (BK), which can bind to receptors on endothelial cells and mediate vasodilatation and increased permeability. BK is cleaved off from high-molecular-weight kininogen (HK) by kallikrein, which is generated from prekallikrein by activated factor XII (factor XIIa). C1INH regulates the activity of kallikrein as well as that of factor XIIa.[7-9] Hence a lack of C1INH, as occurs in HAE, results in enhanced BK formation, which presumably explains why C1INH deficiency leads to angioedema.[1-3,5,6]

The effects of BK and its metabolites such as vasodilatation, increased vascular permeability, sensorial and sympathetic nerve stimulation and smooth muscle contraction are mediated by two types of G-protein coupled receptors, B1R and B2R. These receptors occur on vascular endothelium, primary sensory afferent neurons and vascular and nonvascular smooth muscle cells.[7-9] B2R is constitutively expressed while B1R is absent in most tissues under physiological conditions.[7,8] B1R is upregulated by cells upon exposure to bacterial endotoxins, tissue trauma and inflammation suggesting a role for B1R in pathophysiological processes.[10-12] Differential expression of these receptors explains the pharmacological effects of B1R and B2R antagonists in inflammatory conditions. Early in inflammation plasma-extravasation reacts to a B2R antagonist whereas later when inflammation becomes chronic, B1R antagonists are more efficacious.[13,14]

In HAE B2R is considered to be the main BK receptor involved in vasopermeability changes with a minor, if any, role for B1R[7,15], implying BK, the main agonist for B2R, is the main mediator of angioedema. However, *ex vivo* studies by Bossi and co-workers showed that prevention of vascular leakage across cultured endothelial cells induced by HAE (C1INH-deficient) plasma required blocking of both B2R as well as B1R, suggesting involvement of both receptors in an acute angioedema attack in HAE.[16] Expression of B1R by endothelial cells, however, requires stimulation by cytokines such as tumour necrosis factor (TNF)α or interleukin-1 (IL1)β[7,11-13], which are not known to be involved in HAE.

TNF and IL1 via induction of IL6 stimulate the synthesis of C-reactive protein (CRP) by the liver.[17-22] Hence, CRP levels reflect the release of these cytokines *in vivo*. In the present study we analyzed CRP levels in a cohort of HAE patients enrolled in randomized controlled trials with recombinant human C1INH.[23-26] As part of the clinical protocol CRP levels were collected from the patients at baseline and at various time points during and after an

attack. [26] We evaluated CRP levels before, during, and after HAE attacks and analyzed how levels were related to clinical manifestations of the attack.

MATERIALS AND METHODS

Patients

All patients included in this study were enrolled in two randomized, double-blind, placebo-controlled studies to evaluate recombinant human C1INH (rhC1INH) as a treatment for hereditary angioedema attacks. [24-26] Patients could participate when they were older than 12 years, had signed informed consent, and had a plasma level of functional C1INH <50% of normal to confirm the diagnosis. [24] They were instructed to come to a clinical center when they had onset of moderate to severe angioedema symptoms at any anatomical location. Severity of symptoms was determined using a set of Visual Analog Scale (VAS) scores. An angioedema attack was eligible when the overall severity of at least one location of the attack had been rated by the patients as ≥50 mm on a 100-mm VAS at presentation.

As part of the clinical protocol high sensitivity CRP tests and erythrocyte sedimentation rate (ESR) tests were performed with samples from the patients at screening when they were asymptomatic (this time-point is further referred to as baseline), 45 minutes before, and 24 hours (referred to as Day 1), 7 days and 22 days after study drug infusion (rhC1INH or saline). [26] Overall, 70 HAE patients with an eligible attack treated with saline or rhC1INH were included in the two RCTs (Figure 1). Two of these 70 patients (one with systemic lupus erythematosus, another with prostatitis and tendinitis) were excluded from the present study because they had an underlying disease that could impact on CRP levels.

CRP levels were measured by the local clinical chemistry laboratory using high-sensitivity assay with normal ranges varying from 0-0.3 to 0-1.0 mg/dL. For the analysis the absolute value was used without correction for differences between laboratories since CRP is a well defined biochemical parameter. To assess whether the actual level was elevated compared to normal controls, the normal range of the local laboratory was used. Blood counts and differentials of the patients were determined in the local laboratory.

Data Analysis

Data normally distributed are expressed as the mean and SD. Data not normally distributed are given as the median and the 25th and 75th percentile. Differences in CRP and ESR levels at the various time points in individual patients were calculated with the Wilcoxon paired sample test (2 sided). Differences in age, BMI and attack rate as well ass differences in CRP levels of attacks at different locations were analyzed with the Mann Whitney test (2 sided). Further, distribution of gender and use of prophylactic therapy of patients with attacks at different locations were analyzed with Fisher's exact test. Differences in CRP levels at all time points between patients with various forms of prophylactic therapy were analyzed with the Kruskal-Wallis test. Correlation between parameters was calculated with Spearman's correlation coefficient. A p-value ≤0.05 was considered to represent a significant difference. The analysis was done with Graph Pad Prism 5 and 6.

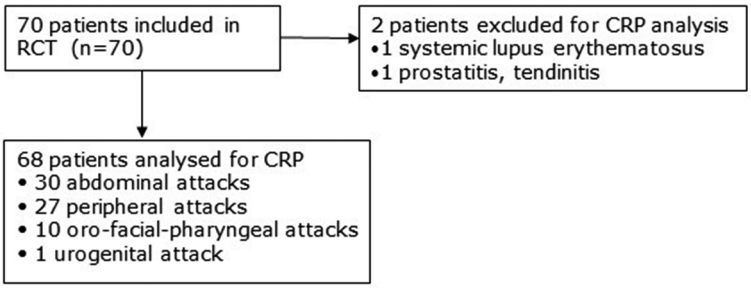

Figure 1. Overview of HAE patients included in the analysis of CRP levels. RCT is randomized controlled trial.

RESULTS

Patients included in the study

Of the 68 patients included 30 patients had an abdominal location of the attack when presenting at the clinical center, whereas the others had attacks at other locations, together referred to as non-abdominal locations (Figure 1). Thirteen patients had an attack at multiple locations. There were no differences in age, gender, BMI, use of prophylactic medication and self reported attack rate between patients with or without an abdominal location of the attack. The mean age, gender, BMI, average attacks per year and use of prophylactic medication are listed in Table 1. Other medication used as prophylactic therapy were C1INH (n= 19), plasma kalikrein inhibitor (n=5), fresh frozen plasma (n=5) and selective bradykinin B2 receptor antagonists (n=2).

CRP levels at 276 occasions during the clinical trial from 68 patients were available. Table 2 gives an overview of the CRP values available for analysis. Levels on 3 occasions (in 3 different patients) were excluded from analysis because of an intercurrent event that may have influenced CRP levels; one patients had tonsillitis on day 22; in one patient swelling around the iv site occurred and one patient developed erythema on the left wrist on Day 1 (Table 2).

CRP levels at baseline and during attacks

The majority of the patients (37 patients, 54%) had elevated CRP on one (16 patients, 24%) or multiple (21 patients, 31%) occasions. At baseline, 12 patients (20%) had elevated CRP levels. In the presence of an attack 23 (38%) patients had elevated CRP levels, whereas on Day 22 after the attack in the absence of symptoms 15 (28%) patients had elevated CRP. On Day 1 after onset of the attack mean CRP levels were significantly higher than in the absence of an attack at screening (p=0.002), Day 7 (p=0.004) and Day 22 after the attack (p=0.0003). CRP levels early during the attack at presentation in the clinical center, tended to be higher than at screening or at Days 7 and 22 after the attack, but this difference was not significant. Finally, CRP levels on Day 1 as well as on Days 7 and 22 did not significantly differ in patients that received saline compared to patients that received rhC1INH (p=0.516

Table 1. Overview of the HAE patients included in the analysis for CRP

Male (n; %)	23 (34%)
Female (n; %)	45 (66%)
Mean age (range) years	40 (17-71)
Mean BMI (range)	27 (18-45)
Mean number (range) of attacks/year	30 (0-101)
Mean number (range) of abdominal attacks/year	12 (0-50)
Number of patients (%) on prophylactic therapy	52 (76%)
Number of patients (%) using androgens	36 (53%)
Number of patients (%) using C1 inhibitor	19 (28%)
Number of patients (%) using antifibrinolytics	9 (13%)
Number of patients (%) with other therapy	15 (22%)

Table 2. CRP levels before, during and after an attack in 68 HAE patients

	Baseline		
	n[a]	**Missing[b]**	**Elevated CRP (%)**
Total	59	9	12 (20%)
Abdominal	26	4	4 (15%)
Non-abdominal	33	5	8 (24%)
	<5 Hours		
Total	63	5	18 (29%)
Abdominal	29	1	8 (28%)
Non-abdominal	34	4	10 (29%)
	Day 1		
Total	60[c]	6	23 (38%)
Abdominal	27	2	14 (52%)
Non-abdominal	33	4	9 (27%)
	Day 7		
Total	38	30	10 (26%)
Abdominal	19	11	6 (32%)
Non-abdominal	19	19	4 (21%)
	Day 22		
Total	53[d]	14	15 (28%)
Abdominal	22	8	5 (23%)
Non-abdominal	31	6	10 (32%)

a) number of patients with available CRP level at the indicated time-point; b) number of patients with no CRP value at the indicated time-point; c) 2 CRP values excluded because of drug reactions; d) 1 CRP value excluded because of tonsillitis in the patient.

for Day 1; p=0.267 for Day 7; and p=0.993 for Day 22, respectively). CRP levels in patients that used prophylactic therapy did not differ from those that did not at any given moment (any prophylaxis p=0.551, androgens p=0.502, C1INH=0.108). Thus, these data demonstrate that CRP levels in asymptomatic HAE patients can be elevated above the normal range, and that 24 hours after attack onset average CRP levels are higher than before the attack.

Comparison of CRP levels in abdominal and non abdominal attacks

Of the 68 patients, 30 had abdominal symptoms (Figure 1 and Table 2). In the patients with an abdominal location of the attack CRP levels within the first five hours after onset of symptoms were significantly higher than baseline levels (p=0.049; Figure 2). Levels further increased on Day 1 (Figure 2). In contrast, CRP levels in the patients with non-abdominal attacks did not change significantly over time (Figure 3). On Day 1 52% (14 of 27 patients) of the patients with an abdominal attack had elevated CRP versus only 27% (9 of 33 patients) of the other patients (Table 2). Moreover, levels on Day 1 were significantly higher (p=0.034) in the abdominal group compared to patients with a non-abdominal location.

A similar effect was observed for the ESR which was found to be increased in particular following abdominal attacks. Moreover, this increase was most marked on Day 7 and not on Day 1 (Figure 4) in agreement with the notion that the ESR increases more slowly then CRP.[27] The median ESR more then doubled compared to baseline (6.0 mm/h at baseline, 15 mm/h on Day 7) and 20 % (5 of 25) of tests were outside the reference range on Day 7.

CRP levels in patients with multiple locations

Thirteen patients had attacks at multiple locations during the same episode. CRP levels of these patients did not significantly differ from those in patients with a single location, except for levels on Day 22 after the attack, which were significantly higher in the patients with multiple locations (p=0.023). Also the mean CRP level during asymptomatic periods, which was defined as the mean of levels at baseline and on Days 7 and 22, was significantly higher in the population that reported attacks at multiple locations (p=0.034; Figure 5), suggesting a somewhat higher CRP level during asymptomatic periods in patients with attacks at multiple locations. Finally, we did not find a correlation between the attack frequency and the average CRP level during asymptomatic periods at baseline, and on Days 7 and 22 after the attack (p=0.516).

DISCUSSION

The synthesis of CRP in the liver is regulated by cytokines.[17-22] As the half-life of clearance from the circulation of CRP is about 19 hours[28] versus that of most cytokines of less than 1 hour, circulating levels of this acute phase protein provide a convenient, indirect but sensitive biomarker for the production of cytokines *in vivo*. In the present study we analyzed circulating CRP levels in HAE patients as an indirect parameter for *in vivo* cytokine production. Our study yielded some remarkable findings: 1) CRP is elevated in HAE patients

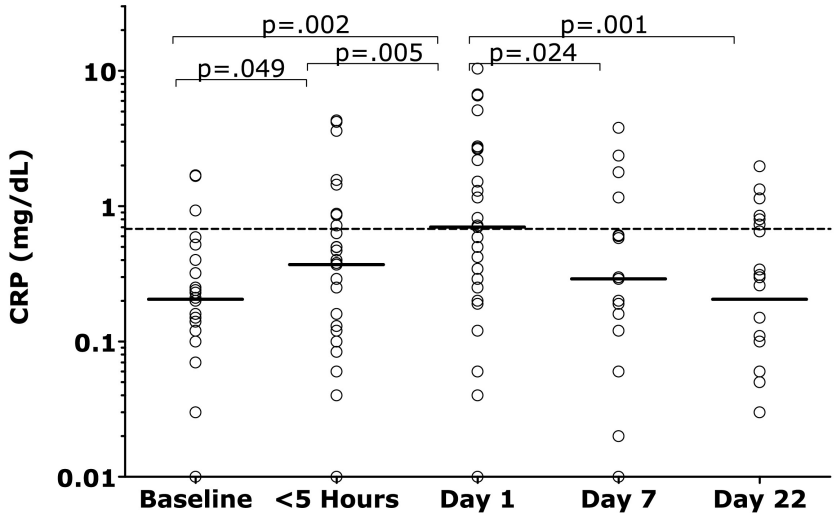

Figure 2. CRP levels before, during and after an attack in HAE patients with an abdominal location of the attack. Solid lines indicate the median in each group. The dotted line shows the mean upper level of normal as used by the laboratories which performed the determinations. The differences between levels at various time points were analyzed with Wilcoxon paired sample test (2-sided p-value)

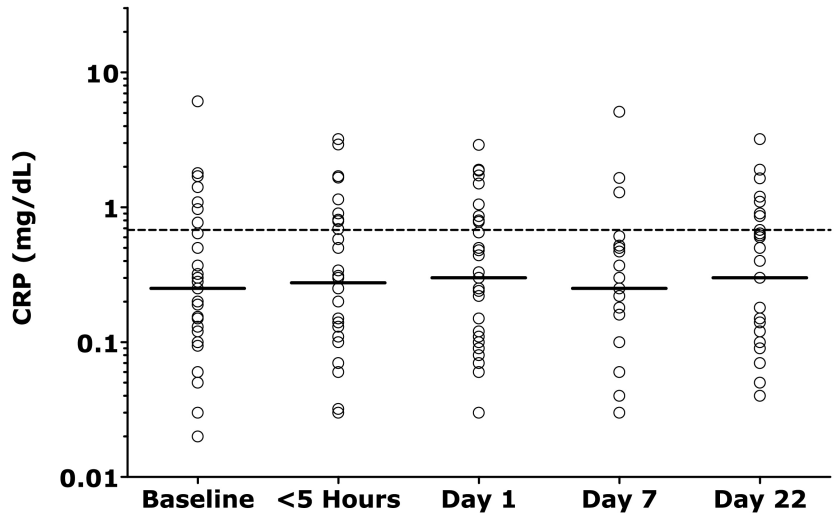

Figure 3. CRP levels before, during and after a non abdominal attack. Solid lines indicate the median in each group. The dotted line shows the mean upper level of normal as used by the laboratories which performed the determinations.

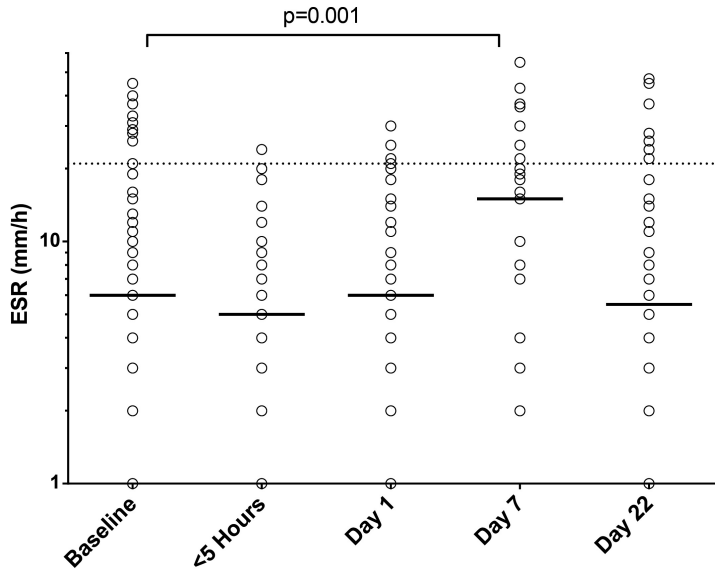

Figure 4. ESR levels before, during and after an HAE attack. Solid lines indicate the median in each group. The dotted line shows the mean upper level of normal as used by the laboratories which performed the determinations. The differences between levels at various time points were analyzed with Wilcoxon paired sample test (2-sided p-value)

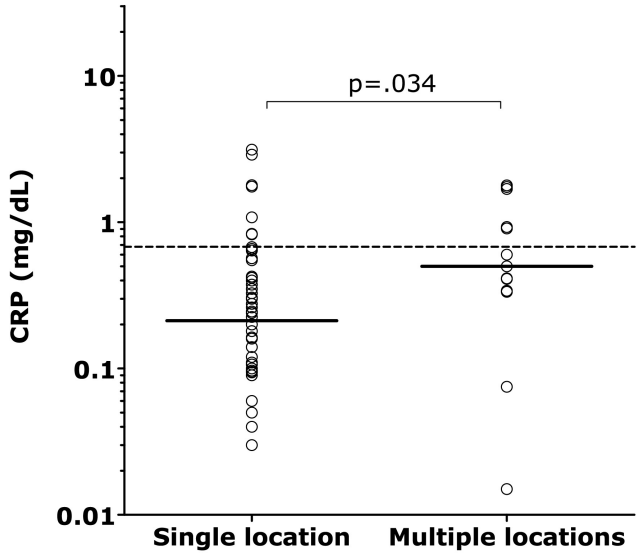

Figure 5. Mean CRP levels in patients with multiple symptomatic locations. Mean CRP levels at baseline, Day 7 and Day 22 are given for patients with single and multiple symptomatic locations. Solid lines indicate the median in each group. The dotted line shows the mean upper level of normal as used by the laboratories which performed the determinations. The difference between the groups was analyzed with the Mann Whitney test (2-sided p-value).

in the absence of an attack; 2) increases of CRP levels during an angioedema attack mainly occur in patients with abdominal locations; 3) the rise in CRP in patients with abdominal attacks occurs early in the course; and 4) CRP levels are somewhat higher in asymptomatic patients who develop attacks at multiple locations.

CRP levels reported in this study were determined in several laboratories, which due to inter-laboratory variation, may have influenced the results. However, last decade efforts have been made to improve the standardization of CRP assays, since levels in the normal range of individual patients are interpreted regarding cardiovascular risk using population-based cut-off points. Therefore, CRP levels determined in different laboratories in general are comparable. Nevertheless, results of the study may have been influenced by some inter-laboratory variation. To overcome this study limitation, we performed a sensitivity analysis of a subgroup of patients using the same CRP cut-off value. Similar results were found. Therefore, we do not think that the conclusions of this study have been influenced by inter-laboratory variations of CRP measurements. Furthermore, we did not use absolute levels of CRP for the analyses but rather focused on the CRP variations in individual patients over time and on levels relative to those of regional normal healthy controls. Moreover, our data of a low-grade inflammation in asymptomatic HAE patients are confirmed by a recent study on leukocytosis in HAE patients.[29] In that study 7 out of 13 HAE patients had CRP levels just above the normal level at inclusion when they were without symptoms. Finally, a low-grade inflammatory response in the HAE patients reported here was confirmed by the increase of ESR after an attack.

We can only speculate about the cause of increased CRP levels in asymptomatic HAE patients. A recent study with 17 HAE patients showed that various cytokine levels increase during an HAE attack. Interestingly, IL-17 was also elevated in the absence of symptoms.[30] This cytokine stimulates CRP expression in hepatocytes and smooth muscle cells.[31] Therefore, the presence of IL-17 during asymptomatic periods could be a possible explanation for CRP increase. Interestingly, IL-17 production can be induced by bradykinin.[32, 33] Therefore, we speculate that the slightly increased CRP concentrations in HAE actually reflect increased exposure of the liver to bradykinin in asymptomatic as well as during acute attacks due to poor control of contact activation in C1INH deficiency.

CRP levels did not change in patients suffering from a non-abdominal attack. Hence, these attacks are apparently not triggered by processes that elicit cytokine responses. One may therefore question the role of suggested triggers of attacks like minor trauma or inflammation[3], since these may induce cytokines.

Strikingly, patients suffering from abdominal attacks showed significant increases of CRP when presenting for treatment and in particular on Day 1 after the treatment (Figure 2). Since it takes CRP plasma levels 6-12 hours to increase after cytokine stimulation, and the symptoms had only been present for less than 5 hours when the patients came for treatment, these data suggest that the CRP increase preceded the patient's symptoms. [28], Moreover, it can be speculated that vasodilatation and edema in the intestinal mucosa

affect the epithelial barrier of the gastro-intestinal tract. Impaired barrier function may in its turn result in translocation of bacteria, fungi or endotoxins from the intestinal lumen into the bloodstream. [34,35] These agents trigger cytokine production and subsequently increase CRP production. This could be a possible explanation for the observed CRP increase following abdominal attacks. Further, it seems reasonable to speculate that this endotoxemia contributes to the severity of the clinical symptoms of an abdominal attack.

The finding that CRP is often elevated in asymptomatic HAE patients has intriguing mechanistic implications. Currently it is generally accepted that the main receptor involved in angioedema attacks is the bradykinin receptor B2R with a minor if any role for B1R. [7,16] A main reason for this notion is that, in contrast to B2R, B1R is not constitutively expressed but rather is exposed by endothelial cells upon stimulation with cytokines. [10-12] Yet, B2R stimulation by BK is notorious for induction of hypotension [36] and it remains unclear why in spite of detectable bradykinin levels in the circulation, hypotensive reactions are uncommon in symptomatic HAE patients. [37,38] We suggest that lack of hypotension in HAE attacks reflects rapid desensitization of B2R [39,40], raising doubts about a dominant role of this receptor in angioedema attacks. Our findings reported here leave room for a role of B1R as CRP increases in HAE patients point to increased exposure to inflammatory cytokines which can up-regulate B1R on the vessel endothelium. This hypothesis of B1R involvement during HAE attacks is strengthened by the study mentioned above showing that cytokine levels increase during an HAE attack.[29]

Interestingly, patients with multiple locations of attacks had higher CRP levels (Figure 5). One may speculate that this reflects higher cytokine production in these patients and therefore a higher chance of exposure of B1R at multiple sites. Strikingly, increases of CRP in the absence of leukocytosis have also been reported in patients with angioedema due to the use of angiotensin-converting-enzyme (ACE)-inhibitors, whereas patients on ACE-inhibitors without angioedema had normal CRP values. [41] This suggests that also in ACE-inhibitor associated angioedema B1R may be involved, either or not in addition to B2R. Ohsawa and colleagues measured CRP levels in 17 HAE patients with abdominal attacks. White blood cell counts were significantly higher compared to attacks located elsewhere, and consistent with our observation there was no further rise in CRP within the first hours after onset of symptoms. [29]

CONCLUSION

This study shows for the first time that increases in CRP often occur in HAE patients. Elevated CRP levels were frequently observed during angioedema attacks as well as during asymptomatic periods. We suggest that increased CRP levels point to increased production of cytokines in HAE patients. These data suggest a possible role for B1R in the pathophysiology HAE.

AUTHORS AND CONTRIBUTIONS

All authors participated in designing the study and devising a protocol for data analysis. Zonne Hofman carried out the analyses and prepared the first draft of the manuscript. Erik Hack and Anurag Relan supervised the work, contributed to the interpretation of the data, and revised the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

Anurag Relan is an employee of Pharming Technologies; Erik Hack has received consultancy fees from Pharming Technologies in the past.

REFERENCES

1. Zuraw BJ. Hereditary angioedema. *N Engl J Med*. 2008;**359**:1027-36.
2. Pappalardo E, Caccia S, Suffritti C, Tordai A, Zingale LC, Cicardi M. Mutation
3. screening of C1 inhibitor gene in 108 unrelated families with hereditary angioedema:functional and structural correlates. *Mol Immunol*. 2008;**45**:3536-44.
4. Nzeako UC, Frigas E, Tremaine WJ. Hereditary angioedema: a broad review
5. for clinicians. *Arch Intern Med*. 2001;**161**:2417-29.
6. Kusuma A, Relan A, Knulst AC, Moldovan D, Zuraw B, Cicardi M, Levy RJ, Nuijens JH, Hack CE. Clinical Impact of Peripheral Attacks in Hereditary Angioedema Patients. *Am J Med*. 2012;**125**;937:17-24.
7. Davis AE III. Mechanism of angioedema in first complement component inhibitor deficiency. *Immunol Allergy Clin North Am*. 2006;**26**:633-51.
8. Kaplan AP. Enzymatic pathways in the pathogenesis of hereditary angioedema: The role of C1 inhibitor therapy. *J Allergy Clin Immunol* 2010;**126**:918-925.
9. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev*. 2005;**57**:27–77.
10. Moreau ME, Garbacki N,Molinaro G, Brown NJ, Marceau F, Adam A. The Kallikrein-Kinin System: Current and Future Pharmacological Targets. *J Pharmacol Sci*. 2005;**99**:6-38.
11. Maurer M, Bader M, Bas M, Bossi F, Cicardi M, Cugno M, Howarth P, Kaplan A, Kojda G, Leeb-Lundberg F, Lotvall J, Magerl M. New topics in bradykinin research. *Allergy*.2011;**66**: 397–1406.
12. Phagoo SB, Yaqoob M, McIntyre P, Jones C, Burgess GM. Cytokines increase B1 bradykinin receptor mRNA and protein levels in human lung fibroblasts. *Biochem Soc Trans*. 1997;**25**:43S.
13. Zhou X, Polgar P, Taylor L. Roles for interleukin-1beta, phorbol ester and a post-transcriptional regulator in the control of bradykinin B1 receptor gene expression. *Biochem J*. 1998;**330**:361–366.
14. Phagoo SB, Yaqoob M, Herrera-Martinez E, McIntyre P, Jones C, Burgess GM. Regulation of bradykinin receptor gene expression in human lung fibroblasts. *Eur J Pharmacol*. 2000;**397**:237–246.
15. Decarie A, Adam A, Couture R. Effects of captopril and Icatibant on bradykinin (BK) and des [Arg9] BK in carrageenaninduced edema. *Peptides*. 1996;**17**:1009–1015.
16. Cruwys SC, Garrett NE, Perkins MN, Blake DR, Kidd BL. The role of bradykinin B1 receptors in the maintenance of intraarticular plasma extravasation in chronic antigen-induced arthritis. *Br J Pharmacol*. 1994;**113**:940–944.
17. Han ED, MacFarlane RC, Mulligan AN, Scafidi J, Davis AE 3rd. Increased vascular permeability inC1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. *J Clin Invest*. 2002;**109**:1057–63.
18. Bossi F, Fischetti F, Regoli D, Durigutto P, Frossi B, Gobeil F, Ghebrehiwet B, Peerschke EI, Cicardi M and Tedesco F. Novel pathogenic mechanism and therapeutic approaches to angioedema associated with C1 inhibitor deficiency. J Allergy Clin Immunol. 2009;**124**:1303-1310.
19. Toniatti C, Demartis A, Monaci P, Nicosia A, Ciliberto G. Synergistic transactivation of the human C-reactive protein promoter by transcription factor HNF-1 binding at two distinct sites. *EMBO J*. 1990; **9**: 4467–4475.
20. Majello B, Arcone R, Toniatti C, Ciliberto G. Constitutive and IL-6 induced nuclear factors that interact with the human C-reactive protein promoter. *EMBO J*. 1990; **9**: 457–465.
21. Taylor AW, Ku NO, Mortensen RF. Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-beta. *J Immunol*. 1990; **145**: 2507–2513.

22. Li SP, Goldmann ND. Regulation of human C-reactive protein gene expression by two synergistic IL-6 responsive elements. *Biochemistry*. 1996; **35**: 9060–9068.
23. 21 Zhang D, Sun M, Samols D, Kushner I. STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. *J Biol Chem*. 1996; **271**: 9503–9509.
24. Kleemann R, Gervois PP, Verschuren L, Staels B, Princen HM, Kooistra T. Fibrates downregulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFκB-C/EBP-β complex formation. *Blood*. 2003; **101**: 545–551.
25. van Doorn MB, Burggraaf J, van Dam T, Eerenberg A, Levi M, Hack CE, Schoemaker RC, Cohen AF, Nuijens J. A phase I study of recombinant human C1 inhibitor in asymptomatic patients with hereditary angioedema. *J Allergy Clin Immunol*. 2005;**116**:876-883.
26. Choi G, Soeters MR, Farkas H, Varga L, Obtulowicz K, Bilo B, Porebski G, Hack CE, Verdonk R, Nuijens J, Levi M. Recombinant human C1-inhibitor in the treatment of acute angioedema attacks. *Transfusion*. 2007;**47**:1028-1032.
27. Zuraw B, Cicardi M, Levy RJ, Nuijens JH, Relan A, Visscher S, Haase G, Kaufman L, Hack CE. Recombinant human C1-inhibitorfor the treatment of acute angioedema attacks in patients with hereditary angioedema. *J Allergy Clin Immunol*. 2010;**126**:821-827.
28. Pharming Technologies B.V. Efficacy, Safety and Immunogenicity Study of Recombinant Human C1 Inhibitor for the Treatment of Acute HAE Attacks. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2014 Jan 14]. Available from: http://clinicaltrials.gov/show/NCT01188564. NLM Identifier: NCT01188564.
29. Hussain TM, Kim DH. C-Reactive Protein and Erythrocyte Sedimentation Rate in Orthopaedics. The University of Pennsylvania Orthopaedic Journal. 2002:**15**; 13–16.
30. Pepys MB, Hirschfield GM.C-reactive protein: a critical update. *J Clin Invest*. 2003;**11**:1805-12.
31. Ohsawa I, Nagamachi S, Suzuki H, Honda D, Sato N, Ohi H, Horikoshi S, Tomino Y. Leukocytosis and high hematocrit levels during abdominal attacks of hereditary angioedema. *BMC Gastroenterology.* 2013;**13**:123-128.
32. Francesco Arcoleo , Marilisa Salemi , Antonio La Porta , Valentina Selvaggio a , Vitalba Mandalà ,Vito Muggeo , Gabriella Misiano , S alvatore Milano , Giuseppina Colonna Romano and Enrico Cillari. Upregulation of cytokines and IL-17 in patients with hereditary angioedema. Clin Chem Lab Med. Epub ahead of print 8 January 2014. DOI 10.1515/cclm-2013-1008 aop.
33. Devang N. Patel‡, Carter A. King‡, Steven R. Bailey‡, Jeffrey W. Holt‡, Kaliyamurthi Venkatachalam‡, Alok Agrawal§, Anthony J. Valente‡ and Bysani Chandrasekar. Interleukin-17 Stimulates C-reactive Protein Expression in Hepatocytes and Smooth Muscle Cells via p38 MAPK and ERK1/2-dependent NF-κB and C/EBPβ Activation. *J Biol Chem*. 2007:**282**;27229-27238.
34. Coelho dos Santos JS, Menezes CA, Villani FN, Magalh ã es LM, Scharfstein J, Gollob KJ, et al. Captopril increases the intensity of monocyte infection by Trypanosoma cruzi and induces humanT helper type 17 cells. Clin Exp Immunol 2010;**162**:528 – 36.
35. Monteiro AC, Scovino A, Raposo S, Gaze VM, Cruz C, Svensjö E, Narciso MS, Colombo AP, Pesquero JB, Feres-Filho E, Nguyen KA, Sroka A, Potempa J, Scharfstein J. Kinin danger signals proteolytically released by gingipain induce Fimbriae-specific IFN-gamma- and IL-17-producing T cells in mice infected intramucosally with Porphyromonas gingivalis. Journal of Immunology. 2009. 15;**183**:3700-11.
36. Brenchley JM and Douek DC. Microbial Translocation Across the GI Tract. *Annu Rev Immunol*. 2012 ; **30**: 149–173.
37. Balzan S, del Almeida Quadros , de Cleva R, Zilberstein B and Cecconello I. Bacterial translocation: Overview of mechanisms and clinical impact. *J Gastroenterol Hepatol*. 2007;**22**:464–471

38. Wang DZ, Chao L, Chao J. Hypotension in transgenic mice overexpressing human bradykinin B2 receptor. *Hypertension*. 1997;**29**:488-93.

39. Nussberger J, Cugno M, Cicardi M. Bradykinin-mediated angioedema. *N Engl J Med*. 2002;**347**:621-2.

40. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A. Plasma bradykinin in angio-oedema. *Lancet*. 1998;**351**:1693-7.

41. Blaukat A, Micke P, Kalatskaya I, Faussner A, Müller-Esterl W. Downregulation of bradykinin B2 receptor in human fibroblasts during prolonged agonist exposure. *Am J Physiol Heart Circ Physiol*. 2003;**284**:1909-16.

42. Hecquet C, Becker RP, Tan F, Erdös EG. Kallikreins when activating bradykinin B2 receptor induce its redistribution on plasma membrane. *Int Immunopharmacol*. 2002;**2**:1795-806.

43. Bas M, Hoffmann TK, Bier H, Kojda G. Increased C-reactive protein in ACE-inhibitor-induced angioedema. Br J Clin Pharmacol. 2005;**59**:233-8.

4

Hereditary Angioedema Attacks: Local Swelling at Multiple Sites

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ABSTRACT

Hereditary angioedema (HAE) patients experience recurrent local swelling in various parts of the body including painful swelling of the intestine and life-threatening laryngeal oedema. Most HAE literature is about attacks located in one anatomical site, though it is mentioned that HAE attacks may also involve multiple anatomical sites simultaneously. A detailed description of such multi-location attacks is currently lacking. This study investigated the occurrence, severity and clinical course of HAE attacks with multiple anatomical locations. HAE patients included in a clinical database of recombinant human C1-inhibitor (rhC1INH) studies were evaluated. Visual Analog Scale scores filled out by the patients for various symptoms at various locations and Investigator Symptoms Scores during the attack were analysed. Data of 219 eligible attacks in 119 patients was analysed. Thirty-three patients (28%) had symptoms at multiple locations in anatomically unrelated regions at the same time during their first attack. Up to five simultaneously affected locations were reported. The observation that severe HAE attacks often affect multiple sites in the body suggests that HAE symptoms result from a systemic rather than from a local process as is currently believed.

INTRODUCTION

Hereditary angioedema (HAE) was first described by William Osler in 1888 as a clinical triad of recurrent attacks of local swellings in various parts of the body, often associated with colicky abdominal pain and a dominant hereditary pattern.[1] In 1963 Donaldson and Evans discovered deficiency of the plasma protein C1-inhibitor (C1INH), a major regulator of the bradykinin-forming cascade, as the main pathological defect in patients with HAE[2].

Since its first description, the disease HAE is well described. Though attacks usually evolve at a single anatomical site, the simultaneous occurrence of symptoms at multiple sites have been mentioned[1,3-10]. In a recent study 186 HAE patients were interviewed about their last attack. One hundred sixty-four patients had an attack in the previous six months, 24% of them experienced an attack at multiple locations[6]. In another study 55% of patients with severe peripheral attacks reported at least 2 separate anatomical locations of the attack[8]. Likewise a large study evaluating 512 abdominal attacks reported that 51% of abdominal attacks were associated with symptoms elsewhere[9] Further, another study observed that symptoms at various anatomical sites can develop in a similar fashion and that over the course of an attack some symptoms can evolve while others emerge[10].

Thus, HAE attacks simultaneously occurring at multiple anatomical sites are frequently mentioned in the literature. However, a detailed description of the clinical manifestations and course of HAE attacks that occur at multiple anatomical locations, further referred to as multi-location attacks, is lacking. The aim of the present study is to evaluate the occurrence, severity, anatomical locations and course of multi-location attacks in a large group of HAE patients.

PATIENTS AND METHODS

Study design

This descriptive study is based on a secondary analysis of data from clinical trials with recombinant human C1-inhibitor (rhC1INH) by Pharming Technologies BV, Leiden, the Netherlands. During these trials an extensive clinical database of more than 100 HAE patients suffering from an acute attack, has been made.

Patients

All patients included in this analysis were enrolled in two randomized, double-blind, placebo-controlled trials (RCT) and their open label extensions to evaluate rhC1INH as a treatment for HAE attacks[11,12]. Patients were asked to fill out a set of Visual Analog Scale (VAS) scores to determine the severity of symptoms during the course of the attack. The VAS forms were designed like a ruler from 0 to 100 mm, 0 indicated no symptoms and 100 mm the most severe complaints possible for the given symptom. The content validity of the VAS instruments used to assess the anatomical locations involved in the angioedema attack and their suitability for patients' assessments of the severity of acute angioedema symptoms, have been described elsewhere[13]. For each location, the data collected included the overall severity VAS score

and VAS scores designed for specific locations. In addition to the VAS scores an Investigator Symptom Score (ISS) was collected for each location. Affected locations could be abdominal, oro-facial-pharyngeal-laryngeal (OFPL), genito-urinary or peripheral. The exact peripheral location f.e. left hand or lower back, was described by the physician. Physicians scored the attack on a 0 to 5 scale from no symptoms to life-threatening symptoms. An angioedema attack was eligible for inclusion in the trial when the overall severity VAS score of at least one location of the attack had been rated by the patients as ≥50 mm on a 100-mm scale at presentation. A second or third attack location was considered significant when the ISS was ≥ 2 (mild symptoms) or the overall severity VAS score was rated by the patient ≥ 20 mm at any time point for any additional location. Further, a second location was only eligible when swelling occurred in a clearly distinct anatomical site f.e. left hand and right hand but not left foot and left knee. As a part of the clinical protocols C-reactive protein levels were measured.

Data Analysis

Data not normally distributed are given as the median and range or the interquartile range. Difference in gender and in use of prophylactic medication was evaluated with Fisher's exact test, difference in age and self reported attacks per year with the Man Whitney test. Differences in VAS scores, CRP levels and time interval after a multi-location attack were evaluated with the Man Whitney test. The analyses were done with Graph Pad Prism 5 and Microsoft Excel 2003.

RESULTS

Patients included in the study

Data from 119 HAE patients suffering from an acute HAE attack were analysed. The average age of the population included was 37 years (SD 14); 43 patients were male and 76 female. Seventy (59%) patients used prophylactic therapy for HAE. Androgens were used by 55 (46%) patients whereas 11 (9%) patients used prophylactic C1INH therapy. The median number of historical attacks per year as reported by the patient was 20 (0-150). Functional C1INH levels were below 50% of normal in all patients and were below the detection limit of the assay in the majority of the patients (Table 1). The median C4 level was 57 ug/L (range 7.25-320 ug/m normal range 100-400 ug/mL, see Table 1).

Symptoms and frequency of multi-location angioedema attacks

All 119 patients had at least one eligible angioedema attack; 49 patients had more than one attack during the study period. In total there were 219 eligible attacks.

Thirty-three of the 119 patients (28%) had a multi-location attack as their first attack included in the study, i.e. they had symptoms at more than one anatomical location (Table 2). Notably, 2 patients had an attack involving 5 anatomical sites. Twenty-nine (88%) of the 33 multi-location attacks involved at least one peripheral location, most frequently the hands and arms. In 11 (33%) patients the multi-location attack only involved peripheral locations.

Furthermore, 16 (48%) patients had abdominal symptoms as part of their multi-location attack; 10 (30%) patients had OFPL symptoms and 6 (18%) patients had genito-urinary symptoms (Table 2).

Table 1. Baseline characteristics of the HAE patients included

	Swelling at 1 location	Swelling at >1 location	All patients	p-value
Patients (n)	71	48	119	
Male (n)	16	27	43 (36%)	
Female (n)	32	44	76 (64%)	.698
Age (yrs)	37 (13-71)[a]	35 (16-67)[a]	36 (13-71)[a]	.308
Prophylactic treatment (n)	41 (58%)	30 (63%)	71 (60%)	.704
Androgens (n)	34 (48%)	21 (44%)	55 (46%)	.710
C1INH (n)	5 (7%)	6 (13%)	11 (9%)	.347
Attacks/year (n)	20 (0-150)[a]	24(3-106)[a]	20 (0-150)[a]	.435
fC1INH at screening (U/ml)	<28	<28	<28 (<28-60)[b]	.645
C4 at screening (µg/mL)	56 (7.25-320)	69 (7.25-214)	57 (7.25-320)[a]	.352

a. median (range) b. both median and minimum under detection limit of the assay

Table 2. Anatomical locations of swelling in 33 HAE patients that had a multi-location attack as their first attack during the study period

Total attacks (n)	119
Attack at single location (n)	86 (72%)
Attack at multiple locations (n)	33 (28% of all attacks)
Two locations	20 (60% of multi-location attacks)
Three locations	8 (24%)
Four locations	3 (9%)
Five locations	2 (6%)
Peripheral	30 (90% of multi-location attacks)
Hand/hands	19 (58%)/ 6 (18%)
Foot/feet	12 (40%)/ 7 (23%)
Knee(s)	3 (9%)
Back	1 (3%)
Elbow(s)	1 (3%)
Buttock	1 (3%)
Chest	1 (3%)
Breast	1 (3%)
Abdominal	16 (48%)
Oro-facial-pharyngeal-laryngeal	10 (30%)
Genito-urinary	6 (18%)

Forty-nine patients had in total 100 subsequent hospital admissions because of a repeated attack during the study period. The interval between the first and second hospital admission varied from 3 to 562 days with a median interval of 51 days (iq range = 22-138). There was no significant difference in the time interval until the second attack after a multi-location or a single location attack (p=0.382).

Thirty-four of these 100 (34%) repeated attacks were multi-location attacks (Table 3). Altogether, 48 (40%) of 119 patients had a total of 67 multi-location attacks. Combinations of abdominal with peripheral symptoms were with 22 (34%) cases most reported. There was no difference in age (p=0.308) gender (p=0.698), use of prophylactic medication (p=0.56), and number of reported attacks on a yearly basis (median:20 range 0-150, p:0.435) between patients with at least one multi-location attack during the study and patients that had a single affected location (Table 1). Laryngeal involvement was suspected when patients had difficulty with breathing (VAS score >20mm), a changing voice (VAS score >50mm) and/ or difficulty with swallowing (VAS >50mm). Patients that had a multi-location attack with one location in the OFPL region were not more likely to have laryngeal involvement as part of their OFLP location compared to patients that had OFPL swelling as their only attack location (p=.768).

The frequency of a multi-location attack in the subgroup of 49 patients that had repeated was with 34% comparable with that of the total study population. In total, the database contained data of 25 subsequent attacks occurring after a multi-location attack. Fourteen (56%) multi-location attacks were followed by another multi-location attack, suggesting that multi-location attacks are often followed by another multi-location attack.

VAS scores of multi-location symptoms

VAS scores for overall severity of symptoms were collected for each location separately except for the cases in which a patient had two or more peripheral attack locations. In the latter case only one overall severity VAS was filled in for the symptoms at various peripheral locations combined. Therefore, overall severity VAS data from two or more different locations were available for 46 attacks.

The course of the overall severity VAS scores for the different locations of multi-location attacks could be discriminated in 5 different patterns (Table 4; Figure 1). As a first pattern symptoms at different sites had a comparable course: they either increased or decreased together. This pattern was subdivided in cases in which VAS scores of the different locations at no point in time differed by more than 20 mm (n=10) and in the cases in which the difference in VAS scores at some time point was more than 20 mm (n=16). A third pattern showed an increase of VAS score in one location whereas the VAS score of the other location decreased on at least one time point (n=13), leading to a difference in VAS scores of >20 mm in the course of the attack. In a fourth category the attack initially was a single location attack with a second location becoming manifest later (n=5). Finally, in a fifth category symptoms at one location had resolved while symptoms at another location were still at least moderate, i.e. >20 mm by VAS, two time points later (n=2).

Table 3. Frequency of multiple location attacks in 119 HAE patients

attack included	Attacks (n)	Multi-location attacks n (%)
attack #1	119	33 (27%)
attack #2	49	17 (35%)
attack #3	23	12 (52%)
attack #4	10	3 (30%)
attack #5	6	0 (0%)
attack #6	4	2 (50%)
attack #7	3	0
attack #8 – 12	5[a]	0
Total	219	67 (30%)

a.1 patient had 12 attacks that were included in the study

Table 4. Overview of the frequency of various courses of VAS scores in multi-location attacks

	n (%)	Example in Figure 1:
Simultaneous increase or decrease of VAS scores at different locations with < 20 mm difference in VAS score at any time point	10 (22%)	A
Simultaneous increase or decrease of VAS scores at different locations with > 20 mm difference in VAS score at >1 time point	16 (34%)	B
Increase of VAS score of one location decrease of VAS score of the other with > 20 mm difference in VAS score	13 (28%)[a]	C
Start of location 2 >30min after location 1	5 (11%)	D
End of location 2 > 2 hour after location 1	2 (4%)	E

a. 2 patients had 3 affected areas of which 1 location in each patient showed a different clinical course

Six patients that received saline treatment experienced a multi-location attack. In 4 of the saline treated patients VAS scores showed a similar course. In one saline-treated case OFPL symptoms were slowly resolving while symptoms in the right hand were still developing (see Figure 1, Example C). In another patient abdominal symptoms started to resolve 15 minutes after start of saline infusion while symptoms in the right and left foot still worsened and did not decrease until 4 hours after start of saline treatment.

Altogether, in 26 (56%) patients the VAS scores in all locations trended together. In 18 episodes (44%) this was not the case.

Course of single versus multi-location attacks

In general, overall severity VAS scores did not differ between the most severe location of a multi-location attack and the overall severity VAS score of a single location attack. However, VAS scores of the most severe location of multi-location attacks were significantly higher

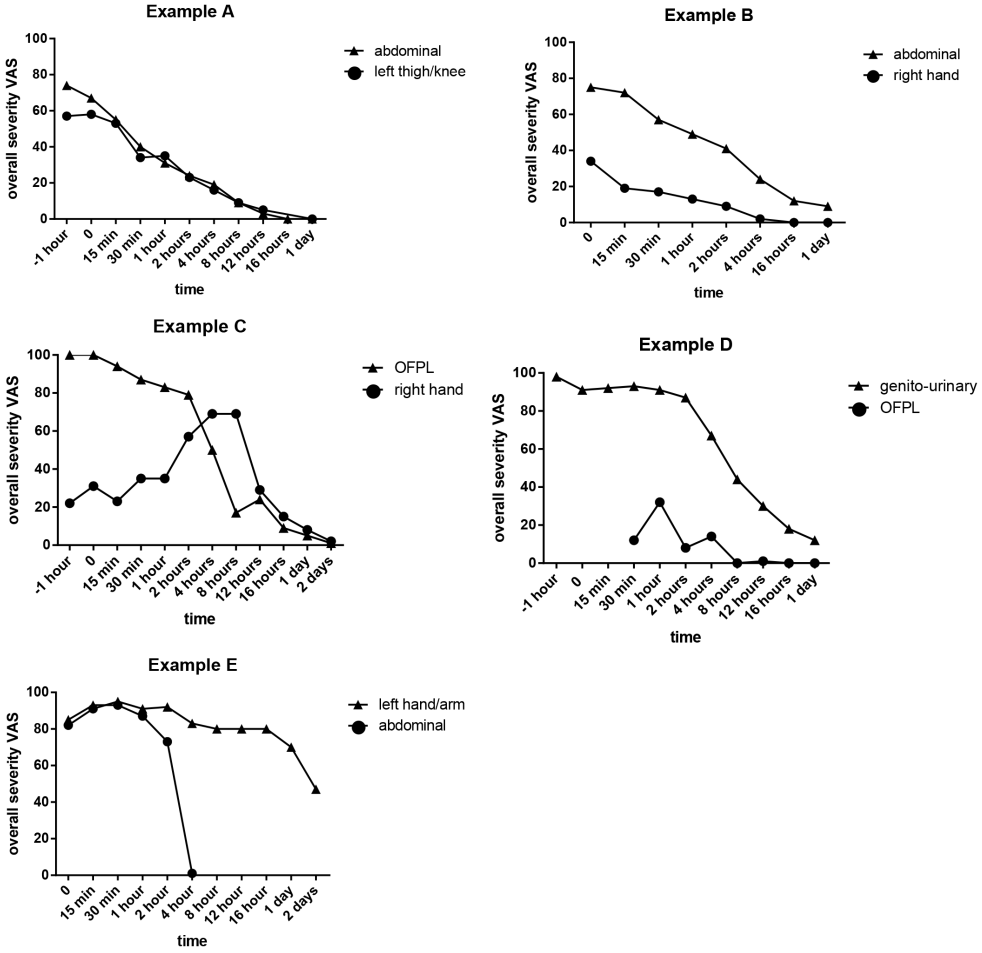

Figure 1. Examples of the course of overall severity VAS scores at two locations. Data of 5 patients are depicted, the actual anatomical locations involved are given. Example A: VAS scores in abdomen and left thigh and knee differ less than 20mm and decrease simultaneously. Example B: VAS scores in abdomen and right hand differ by more than 20mm until 16 hours after onset but the course is similar. Example C: OFPL VAS scores start at 100 mm and rapidly drop after 2 hours while VAS scores of the right hand start at 20 mm and increase with almost 50 mm until 8 hours after saline infusion. Example D: Initially there are only genito-urinary symptoms, one hour after drug infusion VAS scores show a spike in OFPL symptoms. Example E: VAS scores of abdominal symptoms fully resolve between 2 and 4 hours after drug infusion while symptoms in the right arm last for two days.

one hour before rhC1INH respectively saline infusion (p=0.019) and significantly lower 48 hour after infusion (p=.0036), compared to those of single location attacks.

The time until all symptoms had become minimal, i.e. were scored below 20 mm on VAS, was significantly longer in multi-location attacks (median:480 minutes, interquartile range:183-1107 for multi-location attacks) as compared to single location attacks (median: 241 minutes, interquartile range: 77-505 minutes; p= 0.0149).

CRP in single versus multi-location attacks

CRP levels of patients with a multi-location attack during the study period did not significantly differ from patients that did have single location attacks, except at 22 days after the attack when CRP levels in the multi-location group were higher (p=0.013). The lowest CRP levels were found in patients that only had one severe attack and only in a single location (n=48); at any time point CRP levels in this group were lower than in patients that had more than one angioedema attack during the study period (n=49) (screening: p=.002; at 45 minutes before treatment: p=.0004; at 24 hours after start of treatment: p=.013; and at 22 days after the attack p=.0002).

DISCUSSION

The aim of this study was to evaluate the frequency and clinical symptoms of multi-location attacks in patients with HAE attacks. Four main findings regarding such attacks were obtained. First, more than a third (40%) of the HAE patients included had at least one multi-location attack during the study. Secondly, symptoms could occur in up to five anatomical locations in individual patients. Thirdly, symptoms in different locations seemed to wax and wean together in most attacks. And lastly, patients with multi-location attacks have a higher chance that their subsequent attack also is a multi-location attack as compared to patients with a single location attack.

Though multi-location attacks in HAE have been mentioned in the literature[1,3-10], a detailed description of such attacks in a large cohort of patients has not been reported. Using clinical trial data does not have the recall bias of retrospective studies using patient questionnaires. Therefore, considering the number of attacks, the number of patients, as well as the methodology used, the results reported here give a reliable estimate of the occurrence of multi-location attacks in HAE. Since most attacks in this analysis were treated with rhC1INH, the course described for these attacks may not represent their natural history.

Our data support that attacks located at multiple anatomical sites are common in HAE, occurring in about 30% of severe attacks. Previous studies reported that 25% up to 55% of attacks at hospital admission were multi-location attacks[7-9]. Likewise, data from patient questionnaires showed that 24% of HAE patients had a multi-location attack as their last attack[6] and 50% of patients reported to have had a multi-location attack in the past[7]. The multi-location attacks reported in our study involved various combinations of distinct and sometimes remote anatomical sites. This observation was also made in a study with patients experiencing an abdominal attack; 51% of patients also had swellings at other sites then the intestine[9]. Furthermore, in general symptoms at different anatomical sites tended to develop and resolve more or less simultaneously rather than having an independent course such as resolving in one place while emerging in the other. In the few patients in whom a second symptomatic location developed later in the course of the attack the course of the symptoms at this new location mostly resembled that of the symptoms at the initial location. Though we observed in some attacks that one location resolved more rapidly

than the other (see example E, figure 1), this pattern was the exception. Therefore, our data support that the course of most symptoms of different anatomical locations in one patient is comparable during an HAE attack. Differences in the course of some symptoms may reflect local differences of fluid resorption rate by the involved tissues rather than basic differences in the biochemical processes leading to angioedema. The finding that symptoms can trend together in a number of symptomatic sites was noted before in a secondary analysis of RCT data by Vernon and colleagues[10]. This observation was based on patient symptom reports. However, in this secondary analysis the symptom report data about multi-location attacks was not further analysed or shown.

The finding that multi-location attacks tend to reoccur in some HAE patients has not been previously described to our knowledge. Additional investigations are necessary to confirm this since the subgroup with multi-location attacks and subsequent attacks in this study was relatively small. At the moment we can only speculate about the reasons for this observation.

Angioedema attacks in HAE can apparently be triggered by local activation of factor XII due to insufficient control by C1INH by minor trauma[1]. An intriguing question is how symptoms can occur at multiple sites in the body during an attack which is triggered by a local activation process. One may postulate in particular cases that such local activation processes occur at two different places in the body. However, here we report that the course in time of symptoms at various sites of multi-location attacks is quite comparable, which is unexpected in case angioedema at the involved sites would be triggered by independent local activation processes. Moreover, it is difficult to envision that multiple local activation processes would occur so frequently, i.e. in about 30% of the severe attacks, and would occur at more than 2 places – i.e. at up to 5 places - in the body at the same time. Therefore, we postulate that the data reported here support the hypothesis that local angioedema formation in HAE patients result from a systemic rather than a local activation process. We suggest that this systemic activation process involves fluid-phase activation of factor XII and the contact system leading to increased levels of bradykinin and breakdown products in the circulation[14-18]. The bradykinin B2 receptor (B2R) is considered to be a key receptor increasing vasopermeability in HAE attacks[19,20]. However, in case of a systemic activation of the contact system, the distribution of this receptor throughout the whole vasculature cannot explain localization of attacks. Therefore, we suggest reconsidering the role of the bradykinin B1 receptor (B1R). B1R can increase vascular leakage but in contrast to B2R, is not constitutively expressed throughout the circulation. Involvement of B1R in angioedema formation in HAE has been suggested in a study in which increased vasopermeability in mesenteric microvessels in rats by active phase HAE plasma was shown to be dependent on both B1R and B2R[21]. We suggest that local upregulation of B1R by cytokines such as interleukine-1β and tumour necrosis factor-α (TNF-α)[22,23] explains localization of angioedema triggered by a systemic activation of the contact system. Therefore, we suggest that local upregulation of B1R at various anatomical sites possibly triggered by low systemic levels of such cytokines could explain the occurrence of multi-location attacks in HAE. Notably,

two recent analyses of CRP levels and cytokines in HAE patients support low grade systemic release of cytokines in many HAE patients[24,25].

In conclusion, severe angioedema attacks often affect multiple sites in the body in HAE patients. These clinical observations support that HAE attacks are triggered by a systemic rather than a local activation process.

AUTHORS AND CONTRIBUTORS

All authors participated in the study design and protocol for data analysis. The analyses were carried out by Zonne Hofman who also prepared the first draft of the manuscript. Erik Hack and Anurag Relan contributed to the interpretation of the data and revised the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

Zonne Hofman declares that she has no conflict of interest. Erik Hack has received consultancy fees from Pharming Technologies in the past. Anurag Relan is an employee of Pharming Technologies.

REFERENCES

1. Osler, W. (1888), Hereditary angio-neurotic oedema. *Am J Med Sci*. 95, 362-367.
2. Donaldson, V.H. Evans, R.R. (1963), A biochemical abnormality in hereditary angioneurotic edema: absence of serum inhibitor of C1-esterase. *Am J Med*. 31,37-44.
3. Bork, K. Meng, G. Staubach, P. and Hardt, J. (2006), Hereditary Angioedema: New Findings Concerning Symptoms, Affected Organs, and Course. *Am J Med*. 119, 267-274.
4. Agostoni, A. Aygören-Pürsün, E. Binkley, K. *et al*. (2004), Hereditary and acquired angioedema: Problems and progress: Proceedings of the third C1 esterase inhibitor deficiency workshop and beyond. *J Allergy Clin Immunol*. 114, S51-131.
5. Nzeako, U.C. Frigas, E. Tremaine, W.J. (2001), Hereditary angioedema: a broad review for clinicians. *Arch Intern Med*. 161, 2417-2429.
6. Caballero, T. Aygören-Pürsün, E. Bygum, A. *et al*. (2013), The humanistic burden of hereditary angioedema: Results from the Burden of Illness Study in Europe. *Allergy Asthma Proc*. Advance online publication. doi: 10.2500/ aap.2013.34.3685.
7. Cicardi, M. Bergamaschini, L. Marasini, B. Boccassini, G. Tucci, A. and Agostoni, A. (1982), Hereditary Angioedema: An Appraisal of 104 Cases. *Am J Med Sci*. 284, 2-9. 8. Kusuma, A. Relan, A. Knulst, A.C. *et al*. (2012), Clinical Impact of Peripheral Attacks in Hereditary Angioedema Patients. *Am J Med*. 125, 937.e17-24.
8. Rubinstein, E. Stolz, L.E. Sheffer, A.L. Stevens, C. Bousvaros, A. (2014), Abdominal attacks and treatment in hereditary angioedema with C1-inhibitor deficiency. *BMC Gastroenterol*. 14, 71.
9. Vernon, M.K. Rentz, A.M. Wyrwich, K.W. White, M.V. Grienenberger, A. (2009), Psychometric validation of two patient-reported outcome measures to assess symptoms severity and changes in symptoms in hereditary angioedema. *Qual Life Res*. 18, 929-939.
10. Choi, G. Soeters, M.R. Farkas, H. *et al*. (2007), Recombinant human C1-inhibitor in the treatment of acute angioedema attacks. *Transfusion*. 47, 1028-1032.
11. Zuraw, B. Cicardi, M. Levy, R.J. *et al*. (2010), Recombinant human C1-inhibitorfor the treatment of acute angioedema attacks in patients with hereditary angioedema. *J Allergy Clin Immunol.* 126, 821-827.
12. McMillan, C.V. Speight, J. Rela, A. Bellizzi, L. Haase, G. Cicardi, M.(2012), Content validity of visual analog scales to assess symptom severity of acute angioedema attacks in adults with hereditary angioedema: an interview study. *Patient*. 5:113-126.
13. Nussberger, J. Cugno, M. Amstutz, C. Cicardi, M. Pellacani, A. and Agostoni, A. (1998), Plasma bradykinin in angio-oedema. *Lancet*. 351, 1693–1697.
14. Zuraw, B.J. (2008), Hereditary angioedema. *N Engl J Med*. 359,1027-1036.
15. Pappalardo, E. Caccia, S. Suffritti, C. Tordai, A. Zingale, L.C. Cicardi, M. (2008), Mutation screening of C1 inhibitor gene in 108 unrelated families with hereditary angioedema:functional and structural correlates. *Mol Immunol*. 45, 3536-3544.
16. Maurer, M. Bader, M. Bas, M. *et al*. (2011), New topics in bradykinin research. *Allergy*. 66, 1397–1406.
17. Nussberger, J. Cugno, M. Cicardi, M. and Agostoni, A. (1999), Local bradykinin generation in hereditary angioedema. *J Allergy Clin Immunol.*104,1321–1322.
18. Leeb-Lundberg, L.M. Marceau, F. Muller-Esterl, W. Pettibone, D.J. Zuraw, B.L. (2005), International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev*. 57, 27–77.
19. Han, E.D. MacFarlane, R.C. Mulligan, A.N. Scafidi, J. Davis, A.E. 3rd. (2002), Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. J Clin Invest.109, 1057–1063.

20. Bossi, F. Fischetti, F. Regoli, D. *et al*. (2009), Novel pathogenic mechanism and therapeutic approaches to angioedema associated with C1 inhibitor deficiency. *J Allergy Clin Immunol.* 124, 1303-1310.

21. Zhou. X. Polgar, P. Taylor, L. (1998) Roles for interleukin-1beta, phorbol ester and a post-transcriptional regulator in the control of bradykinin B1 receptor gene expression. *Biochem J*. 330, 361–366.

22. Phagoo, S.B. Yaqoob, M. Herrera-Martinez, E. McIntyre, P. Jones, C. Burgess, G.M. (2000), Regulation of bradykinin receptor gene expression in human lung fibroblasts. *Eur J Pharmacol*. 397, 237–246.

23. Hofman, Z.L.M. Relan, A. Hack, C.E. (2014), C-reactive protein in Hereditary Angioedema. *Clin Exp Immunol*. 177, 280-286.

24. Arcoleo, F. Salemi, M. La Porta, A. *et al*. (2014), Upregulation of cytokines and IL-17 in patients with hereditary angioedema. *Clin Chem Lab Med*. 52, e91-93.

5

Angioedema attacks of hereditary angioedema: Local manifestations of a systemic activation process

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SUMMARY

Hereditary angioedema (HAE) due to a deficiency of functional C1-inhibitor (C1INH) becomes clinically manifest as attacks of angioedema. C1INH is the main inhibitor of the contact system. Poor control of a local activation process of this system at the site of the attack is believed to lead to the formation of bradykinin (BK), which upon interaction with BK2-receptors (B2R) on the endothelium increases local vasopermeability and mediates angioedema. However, several observations in HAE patients are difficult to explain from a pathogenic model claiming a local activation process at the site of the angioedema attack. Therefore, we postulate an alternative model for angioedema attacks in HAE, which assumes a systemic, fluid-phase activation of the contact system to generate BK and its breakdown products. Interaction of these peptides with endothelial receptors that are locally expressed in the affected tissues rather than with receptors constitutively expressed by the endothelium throughout the whole body, explains that such a systemic activation process results in local manifestations of an attack. Particularly the BK-receptor 1 (B1R), which is induced on the endothelium by inflammatory stimuli such as kinins, and cytokines, meets the specifications of the involved receptor. The pathogenic model discussed here also provides an explanation why angioedema may occur at multiple sites during an attack and why HAE attacks respond well to modest increases of circulating C1INH activity levels, since inhibition of fluid-phase factor XIIa and kallikrein requires lower C1INH levels than inhibition of activator-bound factors.

INTRODUCTION

Hereditary angioedema (HAE) resulting from a deficiency of the function of the plasma protein C1-inhibitor (C1INH) becomes clinically manifest as repeated attacks of angioedema in subcutaneous or submucosal tissues at various anatomical sites[1-3]. These attacks typically worsen over 24 hours and last for 2-5 days. Attacks can be lethal when located in the submucosal tissue of the larynx[4], cause severe morbidity when located in the mucosa of the gastrointestinal tract[5], or result in disability when subcutaneous edema impairs function of the extremities[6]. The frequency and locations of attacks vary widely among HAE patients, and often occur without any obvious trigger.

C1INH is a serine-protease inhibitor (serpin) that controls factor XIIa, kallikrein and factor XIa of the contact system and activated C1s, C1r and mannan binding lectin associated proteases (MASPs) of the complement system. Therefore, deficiency of C1INH leads to uncontrolled activation of complement and contact systems and the generation of vasoactive peptides. Indeed, patients with HAE have ample evidence for increased activation of both systems[7-11]. Activation of complement does not correlate with clinical symptoms of HAE and also occurs in asymptomatic patients[7-9]. Therefore, the main vasoactive peptide mediating angioedema in HAE supposedly is derived from the contact system, i.e. bradykinin (BK)[11-13]. Indeed, a bradykinin-receptor antagonist restores abnormal vasopermeability in C1INH-deficient mice[14], and attenuates HAE attacks[15].

Angioedema during attacks of HAE results from a local increase in vasopermeability in the affected tissue. Activation of the contact system leading to the generation of BK which mediates this increase, is believed to be a local process as well[3,16,17]. However, several observations in HAE patients are difficult to understand from a local activation process as the trigger for angioedema attacks *i.e.* swellings often occur at multiple sites during one attack[18] and can be preceded by muscle aches, rash and fatigue[19,20]. These observations rather suggest systemic activation of the contact system as the precipitating event. Here we discuss how a systemic activation process of the contact system can result in localized angioedema attacks in HAE. According to this model localization of angioedema is determined by a local expression of the involved receptors for vasoactive peptides on the endothelium. This new pathogenic model may better explain some clinical and biochemical observations in HAE.

THE CONTACT SYSTEM

The contact system consists of the proteins factor XII (FXII), prekallikrein (PK), high molecular weight kininogen (HK) and factor XI (FXI)[21,22]. FXI connects the contact system to coagulation, and will not be further discussed here. The name contact system refers to that the system becomes activated upon contact of blood with negatively charged surfaces such as glass[23]. Other *in vitro* activators of the contact system include kaolin, celite and dextran sulphate[24]. *In vivo* activators include inorganic polyphosphate released from platelets[25,26], mast cell-derived heparin[27], nucleosomes exposed on neutrophil extracellular traps[28], fibrin clots[29], collagen[30] and misfolded proteins[31].

Activation of the contact system starts with activation of FXII, which subsequently converts PK into kallikrein. Kallikrein has two substrates (see *Figure 1)*: it reciprocally activates additional FXII and it cleaves HK to yield BK and cleaved HK. Notably, reciprocal activation of FXII and PK endows the contact system with an all or nothing-like behavior during activation[32,33]. Alternatively, when bound to HK, PK can auto-activate. Auto-activation can be accelerated by heat shock protein-90 that can be secreted by endothelial cells[34]. Another possible PK activator is prolylcarboxypeptidase expressed by endothelial cells[35]. Once PK is activated the reciprocal FXII activation will amplify contact system activation. In addition, activated FXII (FXIIa) and kallikrein can also activate some complement and fibrinolytic factors and mediate cross-talk between contact, complement and fibrinolytic systems[34,36-38].

A main biologically active peptide generated during contact activation is BK. BK is a nonapeptide cleaved from HK by kallikrein. The sequence of BK starts and ends with arginine[38]. In biological fluids BK is rapidly processed by several peptidases. The N-terminal Arg^{1} -Pro^{2} is cleaved by aminopeptidase P[39], whereas at the C-terminal BK is processed by enzymes that cleave either the Pro^{7} –Phe^{8} bond or the C-terminal Phe^{8} –Arg^{9} bond. These enzymes are subdivided in two groups; kininase II and kininase I. The kininase II enzymes, to which angiotensin-converting enzyme (ACE) and neprilysin belong, cleave the Pro^{7} – Phe^{8} bond. The kininase I enzymes cleave the Phe^{8} – Arg^{9} bond. Carboxypeptidase N (CPN) and carboxypeptidase M (CPM) are, amongst others, kininase I enzymes [40,41]. Removal of the C-terminal arginine by kininase I enzymes yields *des*Arg^{9}-BK. BK, its degradation products and its receptors are reviewed elsewhere[42].

In addition to BK, another kinin can be generated from HK but also from low molecular weight kininogen (LMWK), which is Lys-BK, also known as kallidin. This is a decapeptide with an additional lysine at the N-terminus as compared to BK. Also Lys-BK is processed by carboxypeptidases to yield Lys-*des*Arg^{9}-BK[42]. BK, Lys-BK and their metabolites *des*Arg^{9} BK and Lys-*des*Arg^{9} BK interact with the G-coupled protein receptors B1R and B2R on endothelial cells to regulate vasopermeability[42,43].

Notably, several other proteases that are inhibited by C1INH, can cleave BK or related peptides from HK such as factor seven activating protease and MASP[44,45]. The role of these proteases in the generation of BK *in vivo* is currently unknown, and will not be discussed further here.

INVOLVEMENT OF THE CONTACT SYSTEM IN HAE

C1INH is the major inhibitor of FXIIa and kallikrein in the circulation[46-49]. Hence, decreased levels of C1INH result in a poor control of contact activation in the circulation. Or in other words, reciprocal activation of FXII and PK at low levels of C1INH will require less FXIIa than at normal C1INH levels. Thus, C1INH deficiency will allow profound activation of the contact system upon generation of minute amounts of FXIIa. At physiological concentration C1INH will prevent such reciprocal activation by small amounts of FXIIa as it rapidly inactivates FXIIa and kallikrein (see *Figure 1*).

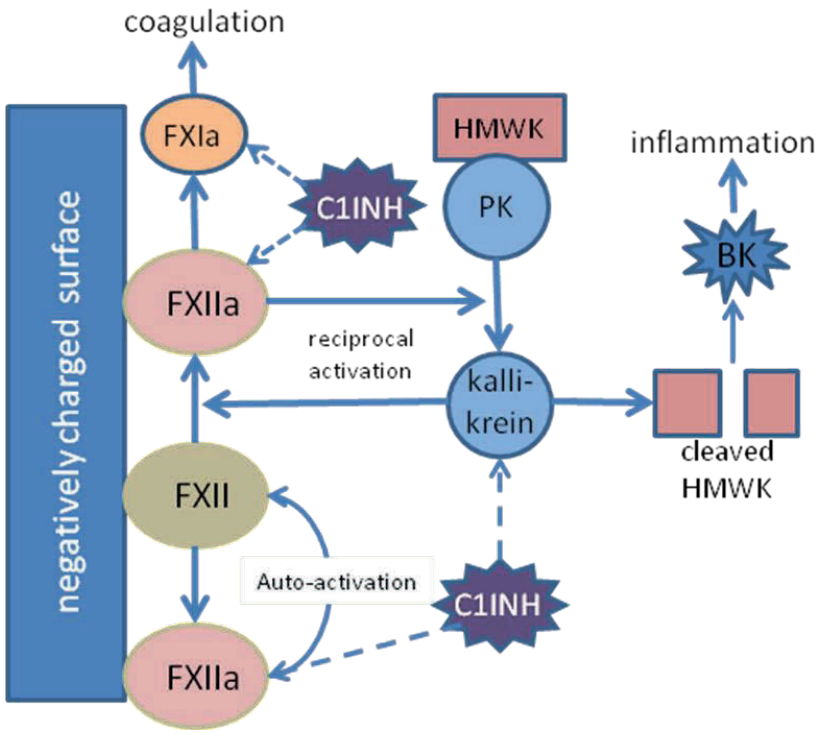

Figure 1. The contact system of coagulation. Activation of the system is triggered when FXII becomes activated upon binding to an activator. Solid arrows indicate activation, dotted line indicate inhibition. Note the reciprocal activation of FXII and prekallikrein, and the inhibition of all contact system proteases by C1INH.

There is ample evidence for involvement of the contact system in HAE attacks. First, during attacks circulating levels of cleaved HK as well as bradykinin are increased, while levels of contact system proteins such as factor XII (FXII), prekallikrein (PK) and HK are decreased[10,12,13,17,50-54] Moreover, HAE patients have increased kallikrein-dependent enzymatic activity in plasma during attacks[55,56]. Second, mutations of the FXII gene are associated with angioedema in the absence of C1INH deficiency supporting a key role of FXII in mediating angioedema attacks[57,58] Finally, drugs that inhibit kallikrein[59] or block the interaction of BK with B2R[14,15], diminish the increased vasopermeability in C1INH-deficient mice and reduce the intensity and duration of a attacks in HAE patients.

CONTACT ACTIVATION IN HAE: A LOCAL PROCESS?

The local increase of vasopermeability in HAE attacks is supposed to result from contact activation in the extravascular space locally in the affected tissues[3]. Indeed, blister fluid from the affected site contains free kallikrein activity[16]. Moreover, BK levels in the venous compartment of the affected limb are higher than in that of the unaffected limb as

demonstrated in two HAE patients with a peripheral attack[17] arguing for involvement of a local activation process mediating angioedema. Yet, several observations suggest systemic contact activation during angioedema attacks. First, locally bound plasma prekallikrein in principle will generate fluid-phase FXIIa either via activation of additional fluid-phase native FXII or via further processing of locally bound FXIIa into a small, fluid-phase fragment of FXIIa, termed β-FXIIa[60,61]. β-FXIIa contains the active site of FXIIa and can activate fluid-phase PK, which in its turn activates fluid-phase FXII. At low levels of functional C1INH fluid-phase FXIIa and kallikrein are inefficiently controlled and diffuse away from the site of activation while still being active[60,61]. Indeed, kallikrein activity in plasma of HAE patients is increased during attacks[54]. It is difficult to see how fluid phase dissemination of contact activation, even when triggered by a local activator, can be prevented at low C1-inhibitor levels.

Second, FXII bound to activators [63,64] and kallikrein bound to endothelial cells[65] are protected from inactivation by C1INH and require supra-physiological levels of C1INH for efficient inhibition. Therefore C1INH is more likely to play a role regulating systemic, fluid-phase contact activation. In other words, C1INH prevents dissemination of the contact activation process by inhibiting fluid-phase active proteases whereas it only has a modest effect on activator- or cell-bound contact system proteases. HAE patients have less than half of normal C1INH function and are therefore at risk of uncontrolled systemic fluid-phase contact activation.

The clinical importance of fluid phase contact system activation in HAE is demonstrated in placebo-controlled trials with therapeutic C1INH products[66-69]. Relatively modest increases of circulating C1INH are efficacious as a treatment for angioedema attacks[70]. An increase of C1INH activity in the circulation shuts down FXIIa and kallikrein activity in the circulation as C1INH forms complexes with these proteases that are rapidly cleared by the liver. Rapid clearance of fluid-phase active contact proteases from the circulation as a main therapeutic principle of C1INH products may explain why half-life in the circulation of C1INH products is less important for therapeutic efficacy[70].

C1INH forms complexes with FXIIa and kallikrein. Upon *in vitro* activation with ellagic acid less of such C1INH complexes are formed in plasma from HAE patients compared to plasma from healthy controls, and even less in plasma from symptomatic HAE patients[71]. This supports that the capacity of C1INH deficient plasma to inhibit fluid-phase contact system proteases is limited. Levels of FXIIa-C1INH or kallikrein-C1INH complexes in biological fluids are assessed to measure contact activation. Impaired formation of FXIIa-C1INH and kallikrein-C1INH complexes at low functional activity of C1INH explains why these complexes are not suitable to monitor contact activation in HAE[72-74]. Therefore, other parameters reflecting contact activation have been measured in HAE patients, such as free FXIIa and kallikrein activity in plasma, and circulating levels of cleaved HK[10,50-54] Levels of circulating cleaved HK during remission correlate with attack frequency[55], and in particular during attacks most of the circulating pool of HK is cleaved. It is difficult to envision how such a profound cleavage of HK could result from a local activation process. Rather, cleavage of most of the circulating pool of HK points to uncontrolled kallikrein activity widespread in the circulation. These

findings are more consistent with a systemic activation process though activation may have started locally.

Several studies report BK levels during HAE attacks. The interpretation of this data is less straightforward as it might appear, amongst others because assays to measure BK are notoriously difficult to reproduce. BK levels in HAE patients are normal to slightly elevated during remission and increase significantly during attacks[12,13,17]. These increased circulating levels of BK have been claimed to originate from the site of angioedema[17]. However, intravenous infusion of BK in healthy volunteers at such high levels that it causes a mild drop in blood pressure did not result in measurable levels of BK in venous blood due to the rapid breakdown of BK by peptidases into the more stable product BK1-5 (Arg-Pro-Pro-Gly-Phe)[75]. Indeed animal studies have shown that more than 99% of BK injected in the venous compartment is cleared during a single passage of blood through the lungs[76]. Therefore, detectable BK levels in the venous compartment of the unaffected site in HAE patients may point to a profound systemic contact activation supervening an initial local activation process and saturating clearance mechanisms of BK in the lungs.

A systemic activation process, possibly with enhanced BK formation at symptomatic sites, would explain that swellings may develop in more than one location. Over one-third of symptomatic HAE patients admitted to the hospital for medical treatment indeed report multiple affected areas, and up to 5 separate anatomical locations may be involved[18]. In most cases the onset and duration of the various affected sites developed in a comparable manner[18]. Comparable symptom onset and symptom termination at multiple sites might be explained by assuming onset of a systemic activation and depletion of systemic contact activation factors as their respective cause. Another clinical observation is that HAE patients frequently (up to 85-90%) experience prodromal symptoms such as unusual fatigue, rash, and muscle aches[19,20]. All these systemic manifestations are difficult to explain from a local activation process triggering the attack, but are easily understood when a systemic process is assumed to occur during angioedema attacks.

BK-RECEPTORS INVOLVED IN HAE ATTACKS

Contact activation ultimately leads to the formation of the biologically active peptides, BK and Lys-BK, which upon cleavage by kininase I may yield *des*Arg^9-BK and Lys-*des*Arg^9-BK. Binding of BK and Lys-BK to the bradykinin receptor B2R, and of *des*Arg^9-BK and Lys-*des*Arg^9-BK to B1R on endothelial cells results in increased vascular permeability[42,43].

B2R is considered to be an important receptor involved in HAE. This receptor is constitutively expressed on endothelial cells and has high affinity for BK and Lys-BK. Cleavage by any of the kininases decreases the affinity of either peptide for B2R[42]. Accordingly, calcium-signaling experiments show a marked B2R response to BK and Lys-BK but in much lesser extent to *des*Arg^9 BK and Lys-*des*Arg^9 BK[78]. Icatibant, a B2R antagonist, is an effective treatment for HAE attacks[17,77]. In the presence of kinins B2R is rapidly desensitized and B1R is induced and stabilized on the membrane[79-81].

B1R is exposed on endothelial cells upon stimulation with kinins[82,83], cytokines[84-86] and endotoxins[87]. These inflammatory factors induce B1R expression in a synergistic fashion: Lys-*des*Arg^9-BK stimulates IL-1β production and both Lys-*des*Arg^9-BK and IL-1β increase B1R expression[88,89]. Endothelial cells can also secrete factors that influence BK-receptor expression. For example, a soluble form of the receptor for the globular head of C1q (gC1qR) secreted by endothelial cells, can bind to these cells to enhance B2R expression and to induce B1R up-regulation[90].

B1R has high affinity for Lys-*des*Arg^9-BK and to a lesser extent for Lys-BK and *des*Arg^9-BK but not for BK[42]. However, agonist efficacy and potency do not correspond with the reported affinities[78]. Calcium-signaling experiments show that both *des*Arg peptides are the most potent agonists and not Lys-BK or BK (Table 1)[78]. Likewise, nitric oxide release after B1R binding is comparable for both *des*Arg peptides while the affinity of Lys-*des*Arg^9-BK exceeds that of *des*Arg^9 by three orders of magnitude[91]. This differential effect of BK, Lys-BK, *des*Arg^9-BK and Lys-*des*Arg^9-BK on B2R and B1R, respectively, predicts that once generated, BK will first interact with B2R, and then, after conversion by CPN or CPM, with B1R.

Little is known about levels of BK degrading enzyme in HAE. Plasma levels of APP, the main enzyme for *des*Arg^9-BK degradation, inversely correlate with disease severity in C1INH deficient HAE patients, suggesting a role for *des*Arg^9-BK in HAE attacks[92]. Interestingly, in patients with HAE due to a FXII mutation disease severity inversely correlates with ACE and CPN activity rather than with APP levels[93].

The role of B1R in HAE is open to debate. C1INH deficient mice showed no decrease in vascular leakage after injection with a B1R antagonist[14]. On the other hand blocking both B1R and B2R receptor prevents increased permeability induced by plasma of HAE patients in *in vitro* and *in vivo* models[90], demonstrating that a BK peptide can be generated in HAE plasma that interacts with B1R at physiological levels. Human *des*Arg^9-BK has been refuted as a physiologic agonist of B1R because of its low affinity for this receptor. Yet this peptide is active at relevant physiological levels in a functional assay of B1R (see Table 1). Therefore, we suggest that the BK peptide with agonistic activity towards B1R generated in HAE plasma

Table 1. Kinin affinities as determined with radioligand binding assays for human B1R[a] and the kinin-induced increase in [Ca^{2+}] for human B1R[b].

	K_i (nM)	EC_{50} (M)	Max effect (A.U.)
BK	>10,000	$2.2x\ 10^{-8}$	2.8
Lys-BK	2.54	$2.8x\ 10^{-8}$	2.8
desArg^9-BK	1930	$1.6x\ 10^{-9}$	4.2
Lys-desArg^9-BK	0.12	$8.7x\ 10^{-10}$	4.2

a) Leeb-Lundberg LMF, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International Union of Pharmacology. XLV. Classification of the Kinin Receptor Family: from Molecular Mechanisms to Pathophysiological Consequences. *Pharmacol Rev*. 2005:**57**:27–77.b) Zubakova R, Gille A, Faussner A, Hilgenfeldt U. Ca2+ signalling of kinins in cells expressing rat, mouse and human B1/B2-receptor. *Int Immunopharmacol*. 2008;**8**:276-81.

is *des*Arg^{9}-BK. Several other observations also point towards possible B1R involvement in the pathogenesis of HAE attacks. First, various cytokines are elevated during these attacks[94]. Also, plasma levels of CRP, which reflect cytokines production *in vivo*, increase after abdominal attacks and are often slightly elevated during asymptomatic periods in HAE patients[95]. These data provide evidence for the presence of agonists of B1R upregulation in HAE. Interestingly, HAE patients with high titers of Epstein Barr nuclear antigen have more frequent upper airway attacks than patients with low titers[96]. It can be speculated that local reactivation of the virus, presumably resulting in local induction of cytokines predispose to laryngeal attacks by local induction of B1R.

Second, B2R is rapidly desensitized upon interaction with BK. In contrast, B1R takes several hours to become up-regulated but once present, B1R persists longer on the cell membrane[80,81]. One may speculate therefore, that vasopermeability is initiated BK interacting with B2R, and sustained by *des*Arg^{9}-BK interacting with B1R. Indeed, HAE attacks have a slow onset of several hours and symptoms can last for days if untreated. Moreover, *des*Arg-peptides exhibit a 3 to 5-times longer half-life in plasma than Arg-peptides[97].

Another puzzling clinical observation is that symptomatic HAE patients have no hypotension in spite of elevated and detectable levels of BK in their circulation. This is in marked contrast to healthy humans who develop hypotension upon injection with small amounts of BK[74]. Further, infusion with the FXII activator dextran sulfate or BK in pigs induces a rapid but transient hypotension[98,99]. The hypotensive effect of BK is reversed with the B2R antagonist Icatibant, showing it is mediated via B2R. Therefore, the lack of hypotension in HAE patients with elevated BK levels probably reflects that B2R on the endothelial cells are inactive due to rapid internalization upon initial stimulation by BK. Even if B2R would only be desensitized for hypotensive effects and not for its effects on vasopermeability, an increase of systemic BK levels, as occurs in symptomatic HAE patients, would trigger the formation of generalized angioedema, which is not found in HAE. Hence, one must conclude that, presumably as a result of initial contact activation and possibly even before the start of an angioedema attack, systemic increases of BK leads to rapid desensitization of B2R. B1R can also mediate hypotension but only upon upregulation throughout the whole circulation, for example as occurs in endotoxic shock[98-101]. However, B1R expression in HAE likely is local and not generalized. Local B1R expression, with desensitization of B2R in the vessel bed, could yield local edema without hypotension in the presence of systemic contact activation. This also explains that swellings can develop at multiple locations simultaneously during HAE attacks in the absence of generalized edema and hypotension since local expression of B1R may occur at more than one anatomical site.

MECHANISMS OF LOCALIZATION OF ANGIOEDEMA

Localization of angioedema attacks in particular sites of the vascular bed either results from local generation of BK, or, in case of systemic generation of BK, from local expression of BK receptors. In case of local BK production, B2R and/or B1R may be involved, whereas in case

of systemic BK production, localization of angioedema implies involvement of B1R since B2R is expressed throughout the circulation.

According to the local activation model[3], a local trigger such as minor tissue trauma initiates contact activation, which subsequently is kept localized by assembly of FXII and HK on the endothelium[102,103]. As discussed above, there is abundant evidence for systemic activation of the contact system in HAE attacks. Yet, it cannot be excluded that local assembly of contact factors on the endothelium even in case of a systemic activation may generate higher local levels of BK peptides, and contributes to localization of angioedema.

In case of systemic contact activation and BK generation, localization of angioedema is determined by local receptor expression, in particular of B1R. In humans *des*Arg^9-BK, the major plasma agonist for this receptor in many animals, has a relatively low affinity for B1R arguing against involvement of this peptide as an agonist for B1R in angioedema. Yet, in HAE plasma BK-derived peptides can be generated at sufficient concentration – and with longer half-life[97] - to stimulate B1R[90]. As discussed above, the potency of *des*Arg^9-BK to stimulate B1R is not reflected by its affinity. Recent work shows that the agonistic activity of *des*Arg^9-BK for B1R is enhanced by carboxypeptidase-M (CPM)[104-107]. This glycosylphosphatidylinositol (GPI)-anchored enzyme is expressed on human endothelial cells[41], has a high affinity for BK and cleaves off its C-terminal Arg. Although some CPM is constitutively expressed by the endothelium, expression is induced by cytokines and endotoxines[108], which also induce B1R expression. CPM augments B1R signaling in three ways. First, it generates the B1R agonists *des*Arg^9-BK (and Lys-*des*Arg^9-BK which however is not involved in angioedema attacks as it is not generated in plasma during contact activation). Second, CPM can serve as a 'co-receptor' for B1R on the cell membrane and generate B1R agonists at a high concentration in close proximity of B1R, which overcomes the relatively low affinity of these agonists for the receptor. Third, CPM can directly activate B1R by conformational cross-talk upon binding of BK[105]. Due to these effects CPM lowers the effective concentration of *des*Arg^9 BK necessary to stimulate B1R from 100µM to 10nM. Therefore, in spite of its relatively low affinity for B1R in humans, *des*Arg^9-BK can interact with B1R at relatively low levels[104-106].

The B2R antagonist Icatibant has a high affinity for B2R, and a low affinity for B1R. Clinical efficacy of Icatibant points to involvement of B2R in HAE rather than B1R. However, the affinity of Icatibant for B1R (K_i 437 nM) is still higher than that of *des*Arg^9-BK for B1R (K_i 1930 nM)[109]. Moreover, Icatibant may intervene with the interaction of *des*Arg^9-BK with CPM and B1R. Finally, a scenario in which initial edema formation is mediated by B2R to be mediated by B1R in the later stages is possible. Indeed, blockade of both B1R and B2R is needed to fully prevent vascular leakage induced by HAE patient plasma in a mouse model[90].

Thus it can be concluded that mechanisms of BK-mediated vasopermability in angioedema are complex and leave room for both the local activation as well as for the systemic contact activation "model" in HAE.

SYSTEMIC CONTACT ACTIVATION LEADING TO LOCAL ANGIOEDEMA FORMATION: AN ALTERNATIVE PATHOGENIC MODEL

Local activation of the contact system and stimulation of local B2R by BK generated has been put forward to explain the formation of angioedema[3]. However, as discussed above the changes of contact system parameters during HAE attacks are more consistent with a systemic activation process. Moreover, there are compelling considerations that B1R contributes to local vasopermeability increase in HAE attacks. Putting these pieces together provides a pathogenic model for the formation of local angioedema in HAE (Figure 2). According to this model, contact activation starts at some time somewhere in the body for example due to a minor trauma. Because of the low C1INH levels the activities of FXIIa and kallikrein are insufficiently controlled leading to extensive fluid-phase activation of the contact system, which disseminates throughout the circulation. As a consequence pronounced degradation of circulating HK occurs leading to systemic increases of BK. Systemic BK generated in the beginning of this activation process desensitizes B2R on the endothelium throughout the circulation[80,81]. Angioedema develops at sites where expression of B1R and CPM by endothelial cells is increased due to local production of cytokines and other agonists. In case of little or no expression of B1R systemic contact activation may stay unnoticed and does not lead to clinical symptoms. Indeed asymptomatic HAE patients have evidence for systemic activation of the contact system[53]. An attack may resolve spontaneously because systemic activation of the contact system stops when native contact factors, in particular HK, are depleted[10]. Alternatively, active disease may stop because expression of B1R is transient.

CONCLUSIONS

We describe a new model of the pathogenesis of angioedema attacks in HAE due to C1INH deficiency. We postulate that episodes of systemic contact activation lead to local

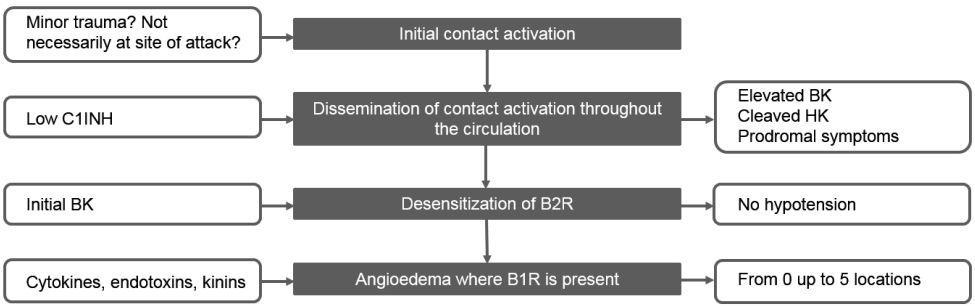

Figure 2. Systemic contact activation leads to local angioedema. Contact activation is initiated upon minor trauma or for unknown reasons. C1INH deficiency allows uncontrolled systemic activation of the contact system which leads to profound HK cleavage and continuous increased generation of BK in the circulation. Upon initial triggering by BK, B2R becomes functionally inactive due to desensitization. BK levels are preferentially degraded by carboxypeptidase N or M into *des*Arg^9-BK, which induces angioedema at sites where B1R is expressed.

angioedema through expression of B1R by local endothelial cells. A factor favoring local increase of vasopermeability besides local B1R expression is increased CPM expression in the affected site, which just as B1R can be induced by cytokines. Biochemical changes of the contact system during attacks of HAE patients support this model, which also provides an explanation for clinical symptoms such as multiple locations of attacks and prodromal symptoms. Finally, systemic contact activation also explains efficacy of C1INH products during attacks since C1INH halts ongoing contact activation in the circulation. The new model may help to guide further studies on the pathogenesis of angioedema attacks in HAE.

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REFERENCES

1. Zuraw BJ. Hereditary angioedema. *N Engl J of Med*. 2008;**359**:1027-1036.
2. Agostoni A, Aygören-Pürsün E, Binkley KE, Blanch A, Bork K, Bouillet L *et al*. Hereditary and acquired angioedema: Problems and progress: Proceedings of the third C1 esterase inhibitor deficiency workshop and beyond. *J Allergy Clin Immunol*. 2004;**114**:S51-131.
3. Longhurst H, Cicardi M. Hereditary angio-edema. *Lancet* 2012;**379**:474–481.
4. Bork K, Siedlecki K, Bosch S, Schopf RE, Kreuz W. Asphyxiation by laryngeal edema in patients with hereditary angioedema. *Mayo Clin Proc*. 2000;**75**:349-354.
5. Bork K, Meng G, Staubach P, Hardt J. Treatment with C1 inhibitor concentrate in abdominal pain attacks of patients with hereditary angioedema. *Transfusion* 2005;**45**:1774-1784.
6. Kusuma A, Relan A, Knulst AC, Moldovan D, Zuraw B, Cicardi M, Levy RJ, Nuijens JH, Hack CE. Clinical impact of peripheral attacks in hereditary angioedema patients. *Am J Med.*. 2012;**125**:937.e17-24.
7. Zuraw BL, Sugimoto S, Curd JG. The value of rocket immunoelectrophoresis for C4 activation in the evaluation of patients with angioedema or C1-inhibitor deficiency. *J Allergy Clin Immunol*. 1986;**78**:1115-1120
8. Gompels MM, Lock RJ, Morgan JE, Osborne J, Brown A, Virgo PF. A multicentre evaluation of the diagnostic efficiency of serological investigations for C1 inhibitor. *J Clin Pathol.* 2002;**55**:145-147.
9. Van Doorn MBA, Burggraaf J, van Dam T, Eerenberg A, Levi M, Hack CE, *et al*. A phase I study of recombinant human C1 inhibitor in asymptomatic patients with hereditary angioedema. *J Allergy Clin Immunol*. 2005;**116**:876-883.
10. Schapira M, Silver LD, Scott CF, Schmaier AH, Prograis LJ Jr, Curd JG, Colman RW. Prekallikrein activation and high-molecular-weight kininogen consumption in hereditary angioedema. *N Engl J Med*.1983;**308**:1050-1053.
11. Cugno M, Hack CE, de Boer JP, Eerenberg AJ, Agostoni A, Cicardi M. Generation of plasmin during acute attacks of hereditary angioedema. *J Lab Clinl Med*.1993;**121**:38-43.
12. Nussberger J, Cugno M, Cicardi M. Bradykinin-Mediated Angioedema. *N Engl J Med*. 2002;**347**:621-622.
13. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A. Plasma bradykinin in angio-oedema. *Lancet* 1998;**351**:1693-1697**.**
14. Han ED, MacFarlane RC, Mulligan AN, Scafidi J, Davis AE, *et al*. Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. *J Clin Invest*. 2002;**109**:1057-1063.
15. Cicardi M, Banerji A, Bracho F, Malbrán A, Rosenkranz B, Riedl M, *et al*. Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema. *N Engl J Med*. 2010;**363**:532-541.
16. Curd JG, Prograis LJ Jr, Cochrane CG. Detection of active kallikrein in induced blister fluids of hereditary angioedema patients. *J Exp Med*. 1980;**152**:742-747.
17. Nussberger J, Cugno M, Cicardi M, Agostoni A. Local bradykinin generation in hereditary angioedema. *J Allergy Clin Immunol*. 1999;**104**:1321-1322.
18. Hofman ZLM, Relan A, Hack CE. Hereditary Angioedema attacks: local swelling at multiple sites. *Clin Rev Allergy Immunol*. 2014 Dec 20. [Epub ahead of print]
19. Prematta MJ, Kemp JG, Gibbs JG, Mende C, Rhoads C, Craig TJ. Frequency, timing, and type of prodromal symptoms associated with hereditary angioedema attacks. *Allergy Asthma Proc*. 2009;**30**:506-511.
20. Magerl M, Doumoulakis G, Kalkounou I, Weller K, Church MK, Kreuz W, Maurer M. Characterization of prodromal symptoms in a large population of patients with hereditary angio-oedema. *Clin Exp Dermatol*. 2014;**39**:298-303.

21. Schmaier AH. Assembly, activation, and physiologic influence of the plasma kallikrein/kinin system. *Int Immunopharmacol*. 2008;**8**:161-165.

22. Colman RW, Schmaier, AE. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood* 1997;**90**:3819-3843.

23. Ratnoff OD, Rosenblum JM. Role of Hageman factor in the initiation of clotting by glass. Evidence that glass frees Hageman factor from inhibition. *Am J Med*. 1958;**25**:160-168.

24. Ogston D, Ogston CM, Ratnoff OD, Forbes CD. Studies on a complex mechanism for the activation of plasminogen by kaolin and by chloroform: the participation of Hageman factor and additional cofactors. *J Clin Invest*. 1969; **48**:1786-1801.

25. Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renné T. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*. 2009;**139**:1143-1156.

26. Faxälv L, Boknäs N, Ström JO, Tengvall P, Theodorsson E, Ramström S, Lindahl TL. Putting polyphosphates to the test: evidence against platelet-induced activation of factor XII. *Blood.* 2013;**122**:3818-3824.

27. Oschatz C, Maas C, Lecher B, Jansen T, Bjorkqvist J, Tradler T, Sedlmeier R,Burfeind P, Cichon S, Hammerschmidt S, Muller-Esterl W, Wuillemin WA, Nilsson G, Renné T. Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. *Immunity*. 2011; **34**:258–268.

28. Von Brühl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*. 2012;**209**:819-835.

29. Sanchez J, Elgue G, Larsson R, Nilsson B, Olsson P. Surface-adsorbed fibrinogen and fibrin may activate the contact activation system. *Thrombosis Research*. 2008;**122**:257-263.

30. van der Meijden PE, Munnix IC, Auger JM, Govers-Riemslag JW, Cosemans JM, Kuijpers MJ, et al. Dual role of collagen in factor XII-dependent thrombus formation. *Blood*. 2009;**114**:881-890.

31. Maas C, Govers-Riemslag JW, Bouma B, Schiks B, Hazenberg BP, Lokhorst HM, *et al*. Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. *J Clin Invest*. 2008;**118**:3208-3218.

32. Chung DW, McMullen BA, Davie EW. Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry.* 1986;**25**:2410-2417.

33. Cochrane CG, Revak SD, Wuepper KD. Activation of Hageman factor in solid and fluid phases: a critical role of kallikrein. *J Exp Med*. 1973;**138**:1564–1583.

34. Joseph K, Tholanikunnel BG, Bygum A, Ghebrehiwet B, Kaplan AP. Factor XII- independent activation of the bradykinin-forming cascade: Implications for the pathogenesis of hereditary angioedema types I and II. *J Allergy Clin Immunol*. 2013;**132**:470-475.

35. Shariat-Madar Z, Mahdi F, Schmaier AH. Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator. *J Biol Chem*. 2002;**277**:17962-17969.

36. Ghebrehiwet B, Silverberg M, Kaplan AP. Activation of the classical pathway of complement by Hageman factor fragment. *J Exp Med*. 1981;**153**:665–676.

37. Gailani D, Renné T. Intrinsic pathway of coagulation and arterial thrombosis. *Arterioscler Thromb Vasc Biol*. 2007;**27**:2507-2513.

38. Kaplan AP, Joseph K, Shibayama Y, Nakazawa Y, Ghebrehiwet B, Reddigari S, et al. Bradykinin formation—plasma and tissue pathways and cellular interactions. *Clin Rev Allergy Immunol*. 1998;**16**:403-429.

39. Vanhoof G, De Meester I, Goossens F, Hendriks D, Scharpé S, Yaron A. Kininase activity in human platelets: cleavage of the Arg1-Pro2 bond of bradykinin by aminopeptidase P. *Biochem Pharmacol.* 1992;**44**:479–487.

40. Erdös EG. Kinins, the long march-a personal view. *Cardiovasc Res*. 2002;**54**:485–491.

41. Skidgel RA, Davis RM, Tan F. Human carboxypeptidase M. Purification and characterization of a membrane-bound carboxypeptidase that cleaves peptide hormones. *J Biol Chem*. 1989;**264**:2236-2241.

42. Leeb-Lundberg LMF, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International Union of Pharmacology. XLV. Classification of the Kinin Receptor Family: from Molecular Mechanisms to Pathophysiological Consequences. *Pharmacol Rev*. 2005:**57**:27–77.

43. Marceau F, Hess JF, Bachvarov DR. The B1 Receptors for Kinins. *Pharmacol Rev*. 1998; **50**:357-386.

44. Dobó J, Major B, Kékesi KA, Szabó I, Megyeri M, Hajela K, Juhász G, Závodszky P, Gál P. Cleavage of kininogen and subsequent bradykinin release by the complement component: mannose-binding lectin-associated serine protease (MASP)-1. *PLoS One*. 2011;**6**:e20036.

45. Etscheid M, Beer N, Fink E, Seitz R, Johannes D. The hyaluronan binding serine protease from human plasma cleaves HMW and LMW kininogen and releases bradykinin. *Biol Chem*. 2002; **383**:1633–1643.

46. Pixley RA, Schapira M, Colman RW. The regulation of human factor XIIa by plasma proteinase inhibitors. *J Biol Chem*.1985;**260**:1723-1729.

47. Chan JY, Burrowes CE, Habal FM, Movat HZ. The inhibition of activated factor XII (Hageman factor) by antithrombin III: the effect of other plasma proteinase inhibitors. *Biochem Biophys Res Commun*.1977; **74**:150-158.

48. van der Graaf F, Koedam JA, Bouma BN. Inactivation of kallikrein in human plasma. *J Clin Invest*.1983;**71**:149-158.

49. Schapira M, Scott CF, Colman RW. Contribution of plasma protease inhibitors to the inactivation of kallikrein in plasma. *J Clin Invest*. 1982;**69**:462-468.

50. Cugno M, Cicardi M, Bottasso B, Coppola R, Paonessa R, Mannucci PM, Agostoni A. Activation of the coagulation cascade in C1-inhibitor deficiencies. *Blood*.1997; **89**:3213-3218.

51. Joseph K, Tholanikunnel TE, Kaplan AP. Treatment of episodes of hereditary angioedema with C1 inhibitor: serial assessment of observed abnormalities of the plasma bradykinin-forming pathway and fibrinolysis. *Ann Allergy Asthma Immunol*. 2010;**104**:50-54.

52. Cugno M, Cicardi M, Coppola R, Agostoni A. Activation of factor XII and cleavage of high molecular weight kininogen during acute attacks in hereditary and acquired C1-inhibitor deficiencies. *Immunopharmacology.* 1996;**33**:361-364.

53. Berrettini M, B Lammle, T White, MJ Heeb, HP Schwarz, B Zuraw, J Curd, JH Griffin. Detection of in vitro and in vivo cleavage of high molecular weight kininogen in human plasma by immunoblotting with monoclonal antibodies. *Blood.* 1986;**68**:455-461.

54. Lämmle B, Zuraw BL, Heeb MJ, Schwarz HP, Berrettini M, Curd JG, Griffin JH. Detection and quantitation of cleaved and uncleaved high molecular weight kininogen in plasma by ligand blotting with radiolabeled plasma prekallikrein or factor XI. Thromb Haemost. 1988;**8**;59:151-161

55. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin mediated angioedema due to hereditary C1-inhibitor deficiency. *Clin Exp Allergy*. 2014;**44**:1503-1514.

56. Defendi F, Charignon D, Ghannam A, Baroso R, Csopaki F, Allegret-Cadet M, Ponard D, Favier B, Cichon S, Nicolie B, Fain O, Martin L, Drouet C; National Reference Centre for Angioedema CREAK. Enzymatic assays for the diagnosis of bradykinin-dependent angioedema. *PLoS One.* 2013 ;8:e70140.

57. Cichon S, Martin L, Hennies HC, et al. Increased activity of coagulation factor XII (Hageman factor) causes hereditary angioedema type III. *Am J Hum Genet.* 2006;**79**:1098-1104.

58. Dewald G, Bork K. Missense mutations in the coagulation factor XII (Hageman factor) gene in hereditary angioedema with normal C1 inhibitor. *Biochem Biophys Res Commun*. 2006;**343**:1286-1289.

59. Cicardi M, Levy RJ, McNeil DL, Li HH, Sheffer AL, Campion M, Horn PT, Pullman WE. Ecallantide for the treatment of acute attacks in hereditary angioedema. *N Engl J Med*. 2010;**363**:523-531.

60. Dunn JT, Kaplan AP. Formation and structure of human Hageman factor fragments. *J Clin Invest*. 1984;**70**:627-631.

61. Pathak M, Wilmann P, Awford J, Li C, Hamad BK, Fischer PM, Dreveny I, Dekker LV, Emsley J. Coagulation factor XII protease domain crystal structure. *J Thromb Haemost.* 2015;**13**:580-591.

62. Kaplan AP, Austen KF. A prealbumin activator of prekallikrein. II. Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin. *J Exp Med*. 1971;**133**:696-712.

63. Pixley RA, Schmaier A, Colman RW. Effect of negatively charged activating compounds on inactivation of factor XIIa by CI inhibitor. *Arch Biochem Biophys*. 1987;**256**:490-498.

64. Dors DM, Nuijens JH, Huijbregts CC, Hack CE. A novel sensitive assay for functional factor XII based on the generation of kallikrein-C1-inhibitor complexes in factor XII-deficient plasma by glass-bound factor XII. *Thromb Haemost*. 1992;**67**:644-648.

65. Schousboe I. Binding of activated Factor XII to endothelial cells affects its inactivation by the C1-esterase inhibitor. *Eur J Biochem*. 2003; **270**:111-118.

66. Ravindran S, Grys TE, Welch RA, Schapira M, Patston PA. Inhibition of plasma kallikrein by C1-inhibitor: role of endothelial cells and the amino-terminal domain of C1-inhibitor. *Thromb Haemost*. 2004;**92**:1277-1283.

67. Craig TJ, Levy RJ, Wasserman RL, Bewtra AK, Hurewitz D, Obtułowicz K, Reshef A, Ritchie B, MoldovanD, Shirov T, Grivcheva-Panovska V, Kiessling PC, Keinecke H, Bernstein JA. Efficacy of human C1 esterase inhibitor concentrate compared with placebo in acute hereditary angioedema attacks. *J Allergy Clin Immunol*. 2009;**124**:801-808.

68. Zuraw BL, Busse PJ, White M Jacobs J, Lumry W, Baker J, Craig T, *et al.* Nanofiltered C1 inhibitor concentrate for treatment of hereditary angioedema. *N Engl J Med*. 2010;**363**:513-522.

69. Zuraw B, Cicardi M, Levy RJ, Nuijens JH, Relan A, Visscher S, Haase G, Kaufman L, Hack CE. Recombinant human C1-inhibitor for the treatment of acute angioedema attacks in patients with hereditary angioedema. *J Allergy Clin Immunol*. 2010;**126**:821-827.

70. Hack CE, Relan A, van Amersfoort ES, Cicardi M. Target levels of functional C1-inhibitor in hereditary angioedema. *Allergy*. 2012;**67**:123-130.

71. Konings J, Cugno M, Suffritti C, Ten Cate H, Cicardi M, Govers-Riemslag JW. Ongoing contact activation in patients with hereditary angioedema. *PLoS One.* 2013;**8**:e74043.

72. Nuijens JH, Huijbregts CC, Eerenberg-Belmer AJ, Abbink JJ, Strack van Schijndel RJ, Felt-Bersma RJ, Thijs LG, Hack CE. Quantification of plasma factor XIIa-Cl(-)-inhibitor and kallikrein-Cl(-)-inhibitor complexes in sepsis. *Blood*. 1988;**72**:1841-1848.

73. de Smet BJ, de Boer JP, Agterberg J, Rigter G, Bleeker WK, Hack CE. Clearance of human native, proteinase-complexed, and proteolytically inactivated C1-inhibitor in rats. *Blood*. 1993;**81**:56-61.

74. Wuillemin WA, Bleeker WK, Agterberg J, Rigter G, Ten Cate H, et al. Clearance of human factor XIa-inhibitor complexes in rats. *Br J Haematol*. 1996;**93**:950-954.

75. Murphey LJ, Hachey DL, Oates JA, Morrow JD, Brown NJ. Metabolism of bradykinin in vivo in humans: identification of BK1-5 as a stable plasma peptide metabolite. *J Pharmacol Exp Ther*. 2000;**294**:263-269.

76. Prechel MM, Orawski AT, Maggiora LL, Simmons WH. Effect of a new aminopeptidase P inhibitor, apstatin, on bradykinin degradation in the rat lung. *J Pharmacol Exp Ther.*1995;**275**:1136-1142.

77. Lumry WR, Li HH, Levy RJ, Potter PC, Farkas H, Moldovan D, *et al*. Randomized placebo-controlled trial of the bradykinin B2 receptor antagonist icatibant for the treatment of acute attacks of hereditary angioedema: the FAST-3 trial. *Ann Allergy Asthma Immunol.* 2011;**107**:529-537.

78. Zubakova R, Gille A, Faussner A, Hilgenfeldt U. Ca2+ signalling of kinins in cells expressing rat, mouse and human B1/B2-receptor. *Int Immunopharmacol*. 2008;**8**:276-281.

79. Marceau F, Sabourin T, Houle S, Fortin JP, Petitclerc E, Molinaro G,,*et al*. Kinin receptors: functional aspects. *Int Immunopharmacology.* 2002;**2**:1729-1739.

80. Enquist J, Skröder C, Whistler JL, Leeb-Lundberg F. Kinins promote B2 Receptor endocytosis and delay constitutive B1 receptor endocytosis. *Mol Pharmacol*. 2007;**71**:494-507.

81. Wolsing DH, Rosenbaum JS. Bradykinin-stimulated inositol phosphate production in NG108-15 cells is mediated by a small population of binding sites which rapidly desensitize. *J Pharmacol Exp Ther*. 1991;**257**:621–633.

82. Pan ZK, Zuraw BL, Lung CC, Prossnitz ER, Browning DD, Ye RD. Bradykinin stimulates NF-kappaB activation and interleukin 1beta gene expression in cultured human fibroblasts. *J Clin Invest*. 1996;**98**:2042-2049.

83. Schanstra JP, Bataillé E, Marin Castaño ME, Barascud Y, Hirtz C, Pesquero JB, Pecher C, Gauthier F, *et al*. The B1-agonist [des-Arg10]-kallidin activates transcription factor NF-kappaB and induces homologous upregulation of the bradykinin B1-receptor in cultured human lung fibroblasts. *J Clin Invest*. 1998;**101**:2080–2091.

84. Zhang Y, Brovkovych V, Brovkovych S, Tan F, Lee BS, Sharma T, Skidgel RA.

85. Dynamic receptor-dependent activation of inducible nitric-oxide synthase by ERK-mediated phosphorylation of Ser745. *J Biol Chem*. 2007;**282**:32453-32461.

86. McLean PG, Ahluwalia A, Perretti M. Association between kinin B1 receptor and leukocyte trafficking across mouse mesenteric post-capillary venules. *J Exp Med*. 2000;**192**:367–380.

87. Brovkovych V, Zhang Y, Brovkovych S, Minshall RD, Skidgel RA. A novel pathway for receptor-mediated post-translational activation of inducible nitric oxide synthase. *J Cell Mol Med*. 2011;15:258-69.

88. Regoli D, Marceau F, Lavigne J. Induction of B1 receptors for the kinins in the rabbit by bacterial lipopolysaccharide. *Eur J Pharmacol.* 1981;**71**:105-115.

89. Phagoo SB, Poole S, Leeb-Lundberg LM. Autoregulation of bradykinin receptors: agonists in the presence of interleukin-1beta shift the repertoire of receptor subtypes from B2 to B1 in human lung fibroblasts. *Mol Pharmacol*. 1999;**56**:325-333.

90. Christiansen SC, Eddleston J, Woessner KM, Chambers SS, Ye R, Pan ZK, et al. Up-regulation of functional kinin B1 receptors in allergic airway inflammation. *J Immunol.* 2002;**169**:2054-2060.

91. Bossi F, Fischetti F, Regoli D, Durigutto P, Frossi B, Gobeil F, Ghebrehiwet B, Peerschke EI, Cicardi M, Tedesco F. Novel pathogenic mechanism and therapeutic approaches to angioedema associated with C1 inhibitor deficiency. *J Allergy Clin Immunol.* 2009;**124**:1303-1310.

92. Stanisavljevic S, Ignjatovic T, Deddish PA, Brovkovych V, Zhang K, Erdös EG, Skidgel RA. Angiotensin I-converting enzyme inhibitors block protein kinase C epsilon by activating bradykinin B1 receptors in human endothelial cells. *J Pharmacol Exp Ther*. 2006;**316**:1153-1158.

93. Drouet C, Désormeaux A, Robillard J, Ponard D, Bouillet L, Martin L, *et al*. Metallopeptidase activities in hereditary angioedema: effect of androgen prophylaxis on plasma aminopeptidase P. *J Allergy Clin Immunol*. 2008;**121**:429-433.

94. Charignon D, Ghannam A, Defendi F, Ponard D, Monnier N, López Trascasa M, *et al.* Hereditary angioedema with F12 mutation: factors modifying the clinical phenotype. *Allergy*. 2014;**69**:1659-65.

95. Arcoleo F, Salemi M, La Porta A, Selvaggio V, Mandalà V, Muggeo V, *et al.* Upregulation of cytokines and IL-17 in patients with hereditary angioedema. *Clin Chem Lab Med*. 2014;**52**:e91-93.

96. Hofman ZL, Relan A, Hack CE. C-reactive protein levels in hereditary angioedema. *Clin Exp Immunol*. 2014 ;**177**:280-286.

97. Csuka D, Varga L, Farkas H, Füst G. Strong correlation of high EBNA-1-IgG levels with edematous attacks involving upper airway mucosa in hereditary angioedema due to C1-inhibitor deficiency. *Mol Immunol.* 2012;**49**:649-654.

98. Blais C, Marc-Aurèle J, Simmons WH, Loute G, Thibault P, Skidgel RA, Adam A. Des-Arg^{9}-bradykinin metabolism in patients who presented hypersensitivity reactions during hemodialysis: role of serum ACE and aminopeptidase P. *Peptides*. 1999;**20**:421–430.

99. Siebeck M, Cheronis JC, Fink E, Kohl J, Spies B, Spannagl M, Jochum M, Fritz H. Dextran sulfate activates contact system and mediates arterial hypotension via B2 receptor. *J Appl Physiol.* 1994;**77**:2675-2680.

100. Barratt-Due A, Johansen HT, Sokolov A, Thorgersen EB, Hellerud BC, Reubsaet JL, *et al*. The role of bradykinin and the effect of the bradykinin receptor antagonist icatibant in porcine sepsis. *Shock*. 2011;**36**:517-523.

101. Cayla C, Todiras M, Iliescu R, Saul VV, Gross V, Pilz B, Chai G, Merino VF, Pesquero JB, Baltatu OC, Bader M. Mice deficient for both kinin receptors are normotensive and protected from endotoxin-induced hypotension. *FASEB J*. 2007;**21**:1689-1698.

102. Merino VF, Todiras M, Campos LA, Saul V, Popova E, Baltatu OC, Pesquero JB, Bader M. Increased susceptibility to endotoxic shock in transgenic rats with endothelial overexpression of kinin B(1) receptors. *J Mol Med*. 2008;**86**:791-798.

103. Joseph K, Tholanikunnel BG, Ghebrehiwet B, Kaplan AP. Interaction of high molecular weight kininogen binding proteins on endothelial cells. *Thromb Haemost*. 2004;**91**:61–70.

104. Joseph K, Ghebrehiwet B, Peerschke EI, Reid KB, Kaplan AP. Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: identity with the receptor that binds to the globular "heads" of C1q (gC1q-R). *Proc Natl Acad Sci U S A*. 1996;**93**:8552–8557.

105. Zhang X, Tan F, Brovkovych V, Zhang Y, Skidgel RA. Cross-talk between carboxypeptidase M and the kinin B1 receptor mediates a new mode of G protein-coupled receptor signaling. *J Biol Chem*. 2011;**286**:18547–18561.

106. Zhang X, Tan F, Zhang Y, Skidgel RA. Carboxypeptidase M and kinin B1 receptors interact to facilitate efficient B1 signaling from B2 agonists. *J Biol Chem*. 2008; **283**:7994–8004.

107. Zhang X, Tan F, Skidgel RA. Carboxypeptidase M is a positive allosteric modulator of the kinin B1 receptor.*J Biol Chem*. 2013;**288**:33226-33240.

108. Sangsree S, Brovkovych V, Minshall RD, Skidgel RA. Kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells by generating bradykinin B1 receptor agonists. *Am J Physiol Heart Circ Physiol*. 2003;**284**:1959-1968.

109. Schremmer-Danninger E, Offner A, Siebeck M, Roscher AA. B1 bradykinin receptors and carboxypeptidase M are both upregulated in the aorta of pigs after LPS infusion. *Biochem Biophys Res Commun*.1998;**243**:246-252.

110. Charignon D, Späth P, Martin L, Drouet C. Icatibant, the bradykinin B2 receptor antagonist with target to the interconnected kinin systems. *Expert Opin Pharmacother*. 2012;**13**:2233-2247.

6

Cleaved kininogen as a biomarker for bradykinin release in hereditary angioedema

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CAPSULE SUMMARY

Cleaved high-molecular weight kininogen (cHK) in plasma is a biomarker for bradykinin formation. We developed an immuno-assay to detect cHK in plasma. cHK plasma levels are increased in asymptomatic C1-INH-HAE patients and increase further during angioedema attacks.

TO THE EDITOR

Bradykinin is a vasoactive inflammatory mediator that plays a key role in hereditary angioedema (HAE). It is released from its precursor high-molecular weight kininogen (HK) by plasma kallikrein, with cleaved HK (cHK) as a by-product. Direct determination of bradykinin has proven valuable, but is technically complicated due to instability and small peptide size[1,2]. Western blotting studies have shown elevated cHK production in HAE[3,4], underpinning its potential value as a clinical biomarker for bradykinin-mediated pathology. However, western blotting is a laborious and semi-quantitative method, which motivates the development of new bioassays[5]. Here we describe the development of an immuno-assay for clinical evaluation of cHK levels.

Experimental procedures are described in this article's Online Repository at www.jacionline.org.

Fig 1A shows a western blot of the purified HK and cHK preparations that were used for assay development (reduced; antibody reacts with HK light chain). As reported earlier, cHK contains two light chain fragments[6]. We selected a monoclonal variable domain of a heavy-chain-only antibody (V_HH-D1) against cHK, as well as a monoclonal V_HH (H4) against total (c)HK and used these as capture-, and detection antibody, respectively. In a microtiter plate assay setup, purified cHK is detected ~5 times better than purified HK (Fig 1B). When normal pooled plasma (NPP) is incubated with βFXIIa (1 μg/mL, synonymous to FXIIf), all HK is converted into cHK (Fig E1A). Initially, the assay setup detected cHK with low, but significant signals (Fig E1B). We next established that the anionic polymer DXS increases assay sensitivity in the presence of inhibitors that prevent contact activation (Fig 1C; enzyme inhibition controls in Fig E1D/E). Furthermore, DXS enhances assay sensitivity in the absence of plasma enzymes (Fig E1C).

Next we prepared an assay standard by mixing NPP with activated NPP (in the presence of inhibitors) at varying ratios. At a plasma sample dilution of 1:32, changes of 2.5% in cHK levels within a range of ~0-20% from baseline levels in NPP can be detected (Fig 1D; western blot Fig 1E). At sample dilutions 1:64 and 1:128, detection ranges are ~10-70% (data not shown) and ~20-100%, respectively (Fig E2A; western blot E2B). Further investigation showed that this immuno-assay setup captures complexes composed of (c)HK and DXS as a function of plasma cHK levels (Fig E3).

Next we investigated cHK levels in C1-INH-HAE patients. The index cohort consisted of 71 patients (from Hungary), diagnosed with C1-INH-HAE due to C1-INH deficiency (n=60, type I) or dysfunction (n=11, type II) and 44 healthy controls. We analyzed 78 remission samples (71 patients) and 28 samples (19 patients) collected during angioedema attacks. Median cHK levels in healthy controls were 6.4% (range 2.7-15.0%; Fig 2A), 15.2% (range 1.8-74.7%) during remission ($p<0.0001$, vs controls) and 29.6% (range 6.3-58.6%) during angioedema attacks ($p<0.0001$ and $p=0.0027$, vs controls and remission, respectively). We then measured cHK levels in a validation cohort of 62 C1INH-HAE patients (from Italy) diagnosed with type I (n=58) or type II (n=4) C1INH-HAE and 26 healthy controls. We analyzed 64 remission

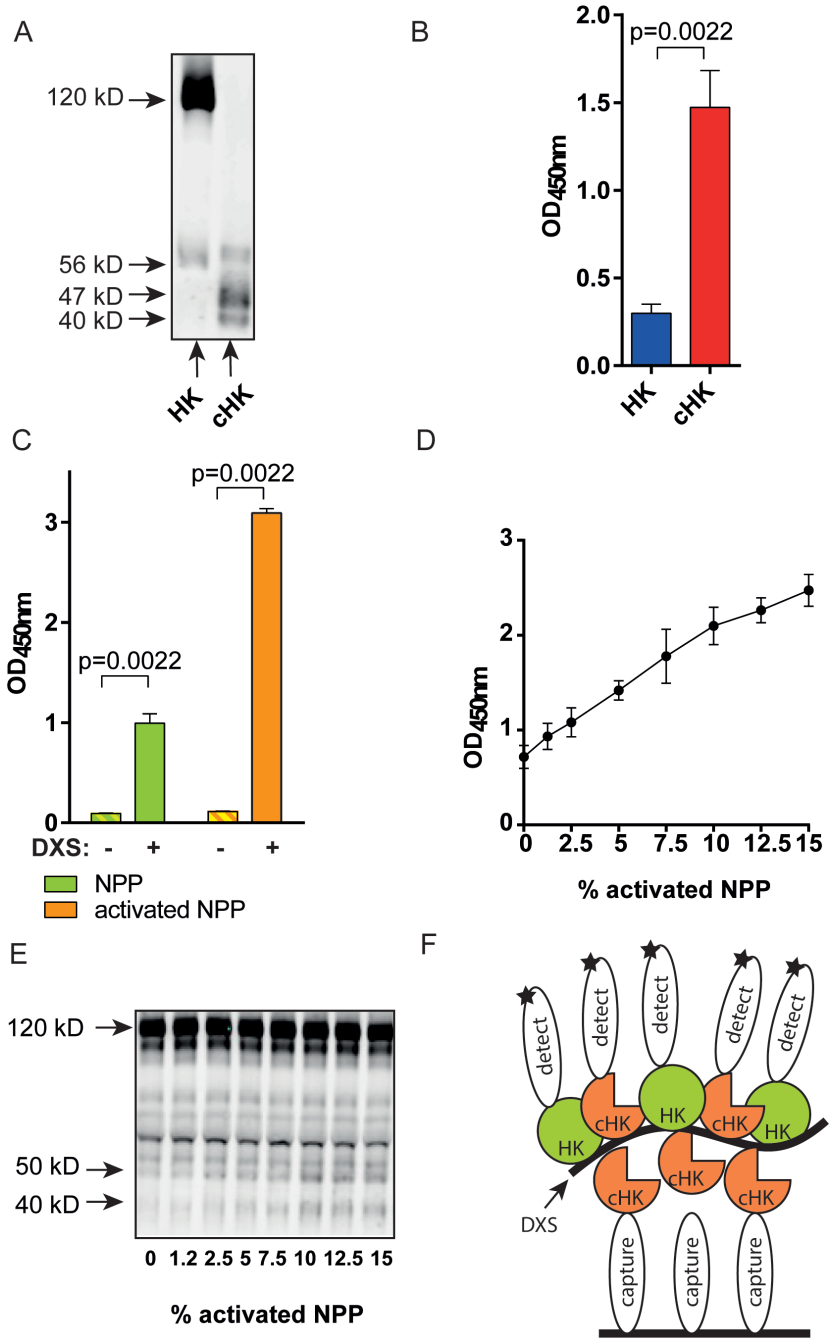

Figure 1. Assay development. A) Western blot (reduced), or B) immuno-assay of purified HK and cHK. C) Immuno-assay of (activated) NPP (1:32 dilution) with or without DXS. D, E) Immuno-assay behavior with a range of cHK in plasma (1:32 dilution) and accompanying western blot (reduced). F) Immuno-assay principle. Data represents βmean ± SD of 3 separate experiments, replicates were analyzed with Mann-Whitney t-test.

samples (62 patients) and 14 samples collected during angioedema attacks (12 patients). Median cHK levels in healthy controls were 6.4% (range 3.4-17.4%; Fig 2B), 17.7% (range 3.8-64.3%) during remission ($p<0.0001$, vs controls) and 37.2% (range 10.9-74.7%) during angioedema attacks ($p<0.0001$ and $p=0.0039$, vs controls and remission, respectively). Based on a selected cut-off of the 97.5 percentile of healthy controls (dotted lines in

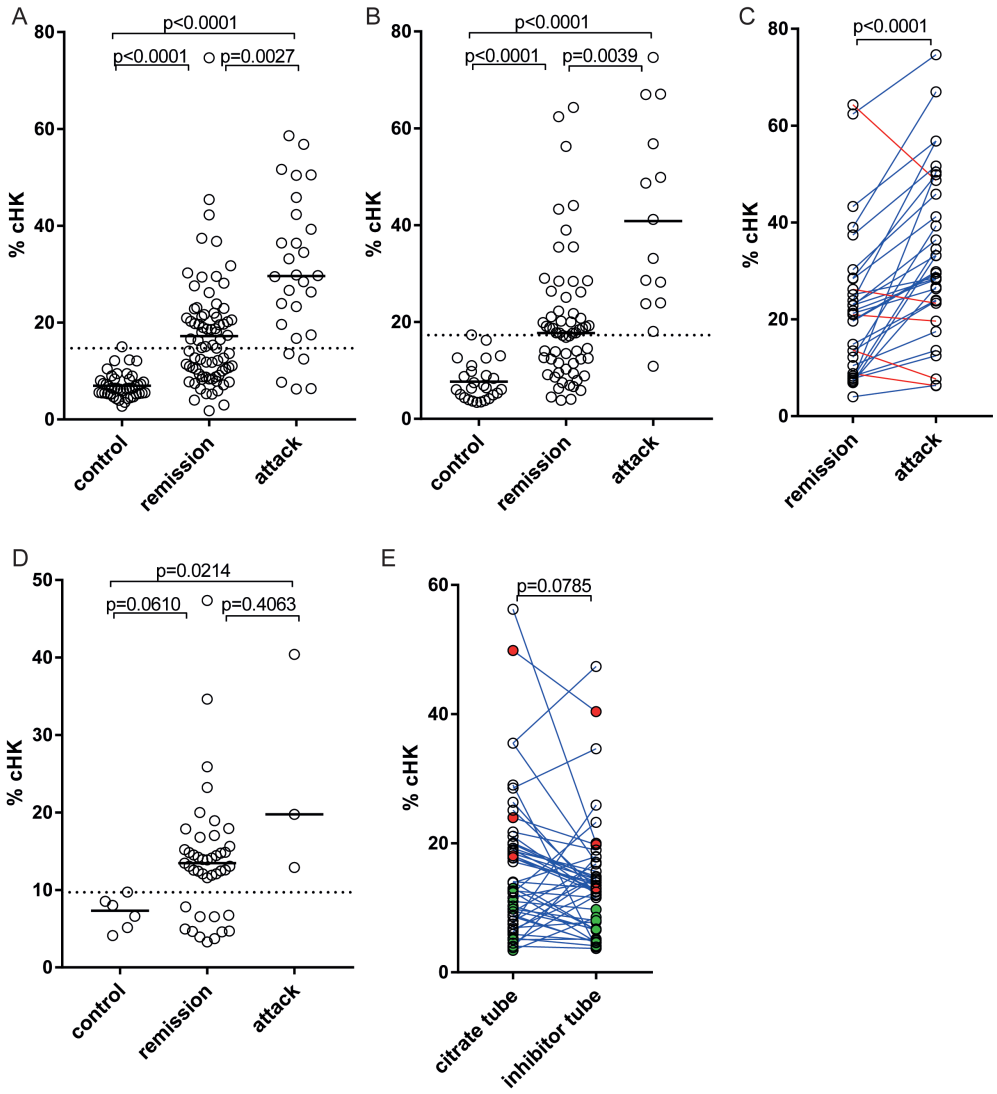

Figure 2. cHK levels in C1-INH-HAE patients. A) Hungarian cohort. B) Italian cohort. C) Paired analysis of remission and attack. Blue lines: increases (28/33), red lines: decreases (5/33). D) Samples in inhibitor tubes. E) Paired analysis of samples in sodium-citrate or inhibitor tubes. Open circles: remission, red: attack, green: controls. Sample dilutions described in Methods. Kruskal-Wallis test (A, B, D) and Wilcoxon matched-pairs signed rank test (C,E).

Fig 2A,B,D), sensitivity, specificity and positive predictive value were determined for each cohort (Table E1). Repeated determinations of cHK levels (9 samples with cHK levels ranging from 2.4 to 30%; three experiments on separate days) showed a standard deviation of 1.8% (range 0.8-3.7%). For 33 patients, plasma was collected both during remission and angioedema attacks. Paired analysis showed that cHK levels increase by 10% (average) during angioedema attacks ($p<0.0001$; Fig 2C).

We next investigated the role of pre-analytical contact activation[7]. Blood of 48 patients was simultaneously collected in sodium citrate tubes, and in collection tubes with enzyme inhibitors (p100). cHK levels in inhibitor tubes were slightly, but not significantly lower than those in citrated plasma ($p=0.0785$; median difference 3.0%; Fig 2E). A subset (5/48) of samples had high cHK levels in citrated plasma (>25%), but >10% lower in plasma with enzyme inhibitors. This suggests a contribution of *ex vivo* cHK formation. Median cHK levels in samples collected in tubes with enzyme inhibitors were 7.3% for healthy controls (n=6) (Fig 2D; range 4.1-9.7%). In samples of HAE patients, these were 13.5% during remission (n=43; range 3.3-47.4%; $p=0.0610$ vs controls) and 19.8% during angioedema attacks (n=3; range 12.9-40.4%; $p=0.0214$ and $p=0.4063$, vs controls and remission, respectively). Similar results were obtained with samples that were collected in a previously published combination of inhibitors (Fig E4)[3,4].

Data on yearly angioedema attack frequency was available for patients in the Hungarian cohort. We found that remission cHK levels did not correlate with angioedema attack frequency ($p=0.3618$, Fig E5E). In 96 samples from the Italian cohort, C1-INH antigen levels and function, C4 and C1q levels were determined. All parameters had a weak to intermediate negative correlation with cHK levels (Fig E5A-D). HK cleavage was previously analyzed by western blot for 19 samples from the Italian cohort[3]. We compared these data to our immuno-assay. There was a strong positive correlation between cHK levels detected by the two methods ($p=0.0013$, $r= 0.6836$, Fig E5F). However, baseline cHK levels are much lower when estimated by immuno-assay ($5.2\% \pm 1.1$) in comparison to estimation by western blot under these specific conditions (~ 35%)[3].

In line with previous studies in HAE (and idiopathic non-histaminergic acquired angioedema)[3,4,8,9] we found elevated cHK levels in plasma of HAE patients during remission. This is not directly attributable to *ex vivo* contact activation and indicates that the baseline rate of HK cleavage in HAE patients is increased, but also suggests the presence of additional disease modifiers.In conclusion, we developed a sensitive immuno-assay for cHK detection. This method might be implemented in C1INH-HAE patient care. Determination of cHK may be useful for investigation of bradykinin in HAE and beyond[8,9].

REFERENCES

1. Cugno M, Tedeschi A, Nussberger J. Bradykinin in idiopathic nonhistaminergic angioedema. Clin Exp Allergy 2017;47:139-140.
2. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A. Plasma bradykinin in angio-oedema. Lancet 1998;351:1693–7.
3. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency. Clin Exp Allergy 2014;44:1503–14.
4. Banerji A, Busse P, Shennak M, Lumry W, Davis-Lorton M, Wedner HJ, et al. Inhibiting Plasma Kallikrein for Hereditary Angioedema Prophylaxis. N Engl J Med. 2017;376:717–28.
5. Faucette R, Conley G, Cosic I, Kopacz K, Chikwamba K, Sexton DJ. An ELISA for the detection of cleaved high molecular weight kininogen in human plasma. Allergy Eur J Allergy Clin Immunol 2016;71 Supplem:528–91.
6. Reddigari S and Kaplan AP. Cleavage of human high-molecular weight kininogen by purified kallikreins and upon contact activation of plasma. Blood 1988;71:1334–40.
7. Kaplan AP, Joseph K. Complement , Kinins , and Hereditary Angioedema : Mechanisms of Plasma Instability when C1 Inhibitor is Absent. Clin Rev Allergy Immunol. 2016;51:207–15
8. Giard C, Nicolie B, Drouet M, Lefebvre-Lacoeuille C, Le Sellin J, Bonneau J, et al. Angio-Oedema Induced by Oestrogen Contraceptives Is Mediated by Bradykinin and Is Frequently Associated with Urticaria. Dermatology 2012;225:62–9.
9. Baroso R, Sellier P, Defendi F, Charignon D, Ghannam A, Habib M, et al. Kininogen cleavage assay: Diagnostic assistance for kinin-mediated angioedema conditions. PLoS One 2016;11.

SUPPLEMENTAL FIGURES

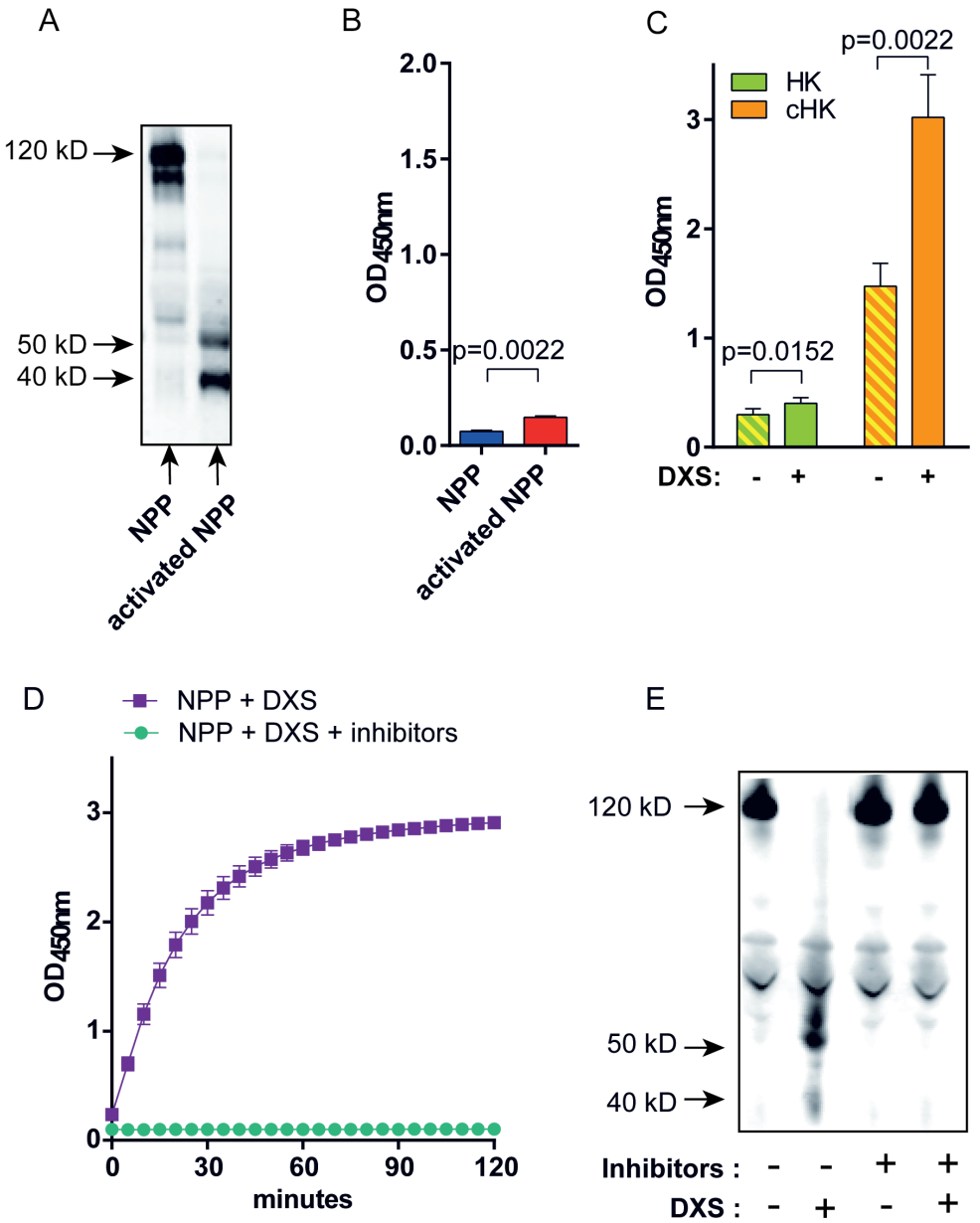

Figure E1. DXS enhances detection of purified cHK, while enzyme inhibitors in sample dilution buffer prevent contact activation. Detection of cHK in normal pooled plasma (NPP) and activated NPP by A) western blot (reduced) or B) immuno-assay (1:32). C) Immuno-assay of purified (c)HK +/- DXS. Enzyme inhibitors block DXS-triggered contact activation, determined by D) chromogenic assay or E) (c)HK western blot (reduced).Data represent mean ± SD, replicates were analyzed with Mann-Whitney t-test. Images represent 3 separate experiments

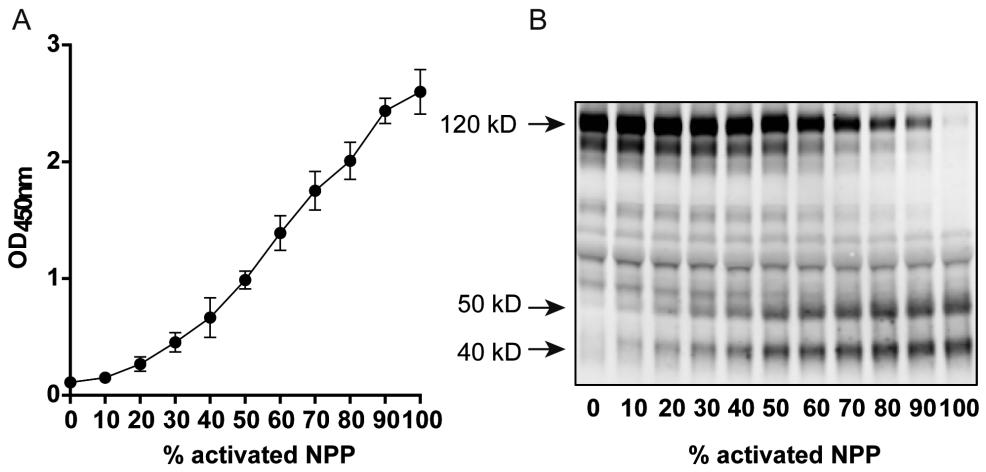

Figure E2. Cleaved high molecular weight kininogen (cHK) detection in plasma between the range of 20-100%. Plasma samples with increasing levels of cHK were prepared by mixing normal pooled plasma (NPP) with fully activated NPP in the presence of enzyme inhibitors. A) immuno-assay (1:128 dilution). B) Corresponding western blot in the same sample range (reduced). Data represent mean ± SD of 3 separate experiments.

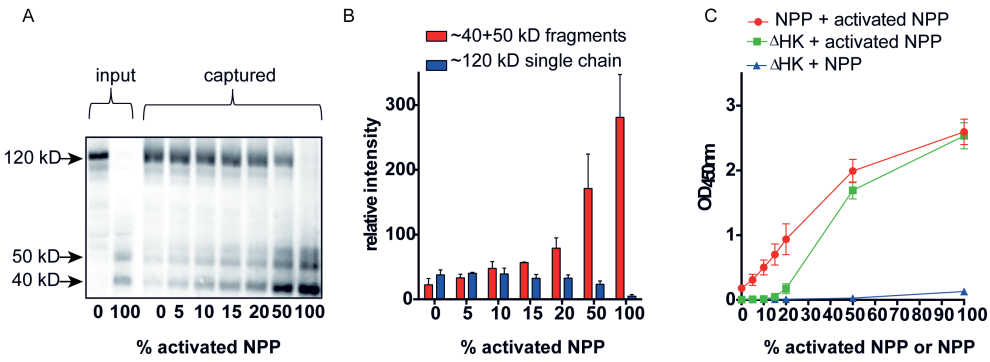

Figure E3. Assembly of (cleaved) high molecular weight kininogen on dextran sulfate improves assay sensitivity. A) Western blot (reduced) of "input" NPP and products that are captured by VhH-D1. B) Densitometric quantification of HK and light-chain fragments (normalized for "input" signals). C) Detection of cHK by immuno-assay (1:64 dilution) in NPP, or kininogen-depleted plasma (ΔHK). When cHK levels are low, HK acts as a signal amplifier. Data represent mean ± SD of 3 separate experiments.

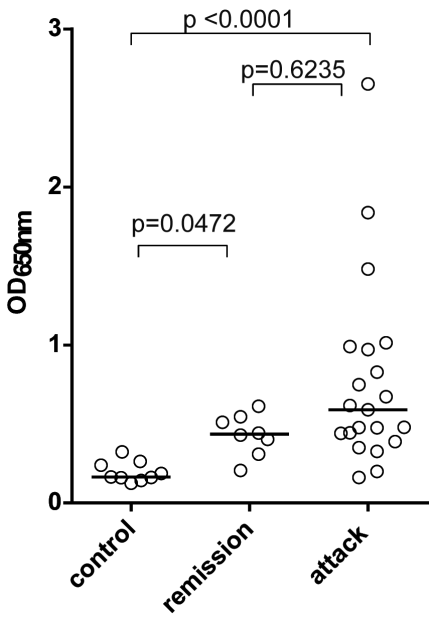

Figure E4. cHK detection in C1-INH-HAE patient plasma samples with hexadimethrine bromide. Data points represent patient or control plasma sample, lines indicate medians. Samples were analyzed at 1:32 dilution, in the presence of 8 µg/mL DXS to overcome hexadimetrine bromide. Kruskal-Wallis test was used for multiple comparison.

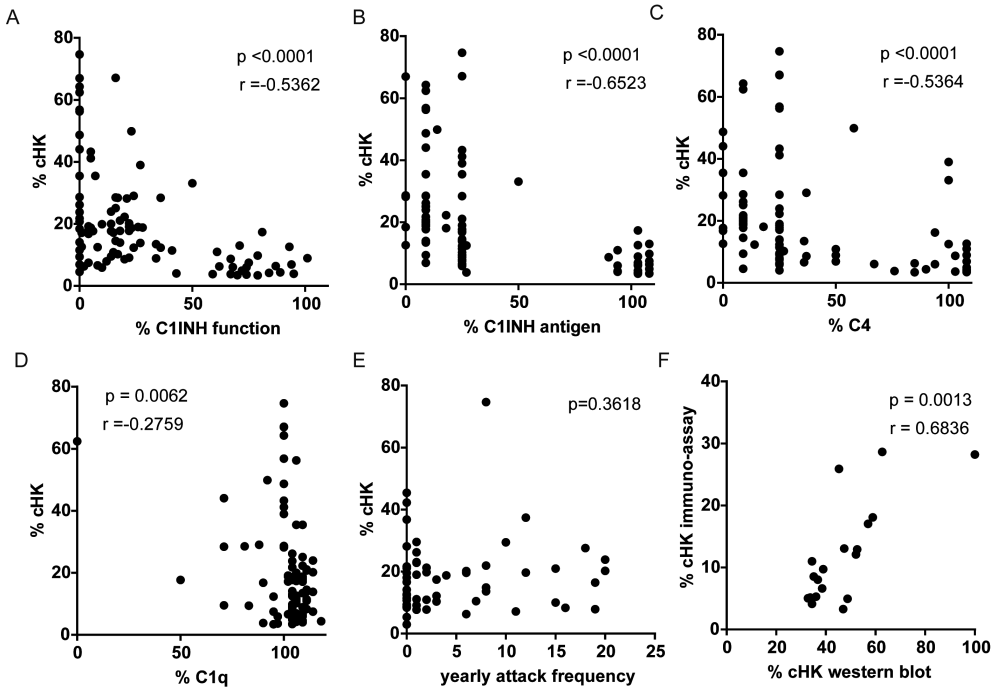

Figure E5. Correlation of cHK levels with C1-INH activity, complement parameters and yearly angioedema attack frequency. Comparison of cHK detection by immuno-assay or western blot (non-reduced). Plasma cHK levels of C1-INH-HAE patients or healthy controls are plotted against A) C1-INH function, B) C1-INH antigen level, C) C4 or D) C1q (levels expressed as a percentage of normal values), E) yearly angioedema attack frequency, and F) previously determined cHK levels previously analyzed by western blot in the Italian cohort[E1]. Correlation was calculated with Spearman's r.**Supplemental table**

Table E1. Sensitivity, specificity and positive predictive value of the cHK immuno-assay per cohort.

	Hungarian cohort		Italian cohort	
	Remission	**Attack**	**Remission**	**Attack**
Sensitivity	54%	83%	53%	93%
Specificity	98%	98%	100%	100%
Positive predictive value	98%	96%	100%	100%

Cut-off values used: 97th percentile of healthy control samples were used which were 14.7% cHK for the Hungarian cohort and 17.7 % for the Italian cohort.

SUPPLEMENTAL METHODS

Plasma samples from C1-INH-HAE patients and healthy volunteers

Blood was collected from healthy volunteers after written informed consent. According to standard procedures 9 volumes of blood were collected in 1 volume of sodium citrate (3.2% wt/vol). Samples were centrifuged twice at 2000 xg for 10 minutes shortly after collection. Plasma samples of ±50 healthy volunteers was pooled (referred to as normal pooled plasma, NPP) and stored in aliquots at -80 °C until use. This protocol was approved by University Medical Center Utrecht ethical committee.

Blood from C1-INH-HAE patients was collected as part of routine procedure during the annual control visits at the 3rd Department of Internal Medicine, Semmelweis University, Budapest, Hungary. The diagnosis HAE was confirmed by clinical and laboratory criteria (positive family history, clinical symptoms of angioedema, low functional C1-INH level, low C4, normal C1q concentrations as advised by international guidelines[E2]). Samples were collected from patients during remission; in each case, at least two weeks elapsed since the date of the last attack. Samples obtained during angioedema attacks were collected prior to acute treatment and within 6 hours after the onset of the symptoms. According to standard procedures, 9 volumes of blood to 1 volume of sodium citrate (3.2% wt/vol) was centrifuged at 3500 xg for 10 minutes. Thereafter, plasma was stored at -80°C until use. In addition, citrated plasma from healthy family members (8 samples), and from healthy donors (who had been referred for routine medical evaluation, and volunteered for the study) was collected with the same procedure and after written consent. The study protocol was approved by the institutional review board of Semmelweis University, Budapest.

To corroborate the data obtained in this cohort, we analyzed plasma samples of a second cohort of C1-INH-HAE patients. Blood was collected in the course of routine diagnostic procedures and all patients gave oral informed consent that remaining plasma could be used for research purposes. The Ethical Committee of the University of Milan approved that written informed consent is not necessary if the plasma had been obtained during routine diagnostics and that this plasma could be used for research investigating the pathophysiology of hereditary angioedema. The diagnosis of HAE was based on the presence of at least one recommended clinical and one recommended laboratory criteria. Blood samples were taken from all of the patients at least eight days from an angioedema attack (remission samples), and from selected patients within the first six hours of the beginning of an angioedema attack (attack samples). According to standard procedures, blood was collected using sodium citrate as anticoagulant (3.2% wt/vol). After centrifugation at 2000 xg for 20 min at room temperature, the plasma was divided into aliquots and stored at -80°C until tested. Citrated plasma from healthy donors was collected with the same procedure.

To evaluate the effect of blood collection on cHK levels, plasma samples were collected in collected in sodium citrate containers as well as BD bioscience EDTA-P100 tubes containing inhibitors to prevent pre-analytic contact activation[E3] for direct comparison. A subset of samples was collected in tubes containing 100 mM benzamidine, 400 µg/mL hexadimethrine bromide, 2 mg/mL soybean trypsin inhibitor, 263 µM leupeptin, and 20 mM,

aminoethylbenzene-sulphonyl fluoride dissolved in acid/citrate/dextrose (100 mM sodium citrate, 67 mM citric acid, and 2% dextrose, pH 4.5)[E1].

Reagents

Aprotinin, bromophenol blue, dextran sulfate mol. (average) wt. 500.000, DL-dithiothreitol (DTT), glycine, glycerol, Isopropyl[1]-D-1-thiogalactopyranoside (IPTG), KCl, mouse monoclonal anti-c-Myc antibody, skimmed milk powder, NaCl, Na_2HPO_4, NaH_2PO_4, triethylamine (TEA) and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA). Talon Superflowbeads for His-tag purification were from GE Healthcare (Little Chalfont, UK). Imidazole was from Merck (Darmstadt, Germany). PolySorp and MaxiSorpmicrotitre plates, NeutrAvidin Protein and PageBlue, were from Thermo Scientific (Waltham, Mass). Chromogenic assays were performed in Costar "V" Vinyl microtitre plates (Corning, NY, USA). Spectra/Por dialysis membranes (MWCO 3.5 kD) were purchased from Spectrumlabs (CA, USA). Bolt 4-12% Bis-Tris Plus Gels, MOPS buffer, Alexa Fluor 680 Donkey anti-sheep IgG, EZ-Link Sulfo-NHS-LC-Biotin and One-shot TOP10 Chemically Competent E.coli were from Life Technologies (Carlsbad, CA, USA). Immobilon-FL and ethanol were from Merck-Millipore (Amsterdam, The Netherlands). Polyclonal affinity-purified HK antibody was from Affinity Biologicals (Ancaster, Ontario, Canada). Peroxidase-conjugated polyclonal rabbit anti-sheep and anti-mouse antibodies were from Dako (Heverlee, Belgium). HK, cHK and β-FXIIa, were from Enzyme Research laboratories (South Bend, Ind). HK immunodepleted plasma was from StagoBNL (Leiden, the Netherland). Odyssey Blocking Reagent was from LI-COR (Hamburg, Germany) was from CSL-Behring (Breda, The Netherlands). 3,3',5,5'-Tetramethyl-benzidine (TMB) was from Tebu Bio (Heerhugowaard, The Netherlands). Streptavidin-poly-HRP was from Sanquin Blood Supply (Division Reagents, Amsterdam, the Netherlands). Phe-Pro-Arg-chloromethylketone (PPACK) was purchased from Haematologic Technologies (Essex Junction, Vt) Ampicillin (Amp) was obtained from Carl Roth GmbH (Karlsruhe, Germany), 2x Yeast Tryptone capsules were from MP Biomedicals (Santa Ana, CA, USA). H-D-Pro-Phe-Arg-pNA (L-2120) was from Bachem (Bubendorf, Switserland).Tris-HCl was from Roche (Woerden, the Netherlands).

Phage display and V_HH production

A phage-display library of Llama glama variable fragments of heavy-chain-only antibody fragments against cHK was obtained by immunization of two Llamas, with human cHK and generation of a phage-display library as previously described by de Maat *et al*[E4] . Animal experiments were approved by the ethical committee of the University of Utrecht, in compliance with European guidelines for animal research. Phagemid vector pUR8100 containing a myc-tag, his-tag, ampicillin resistance and a lacI repressor gene was used. TG1 E.coli were used for production of phages and Top10 E.coli for the production of V_HHs.

cHK and HK were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin according to the manufacturer's instructions. A polysorp microtiter plate was coated with 5 µg/mL NeutrAvidin protein in PBS pH 7.4 overnight at 4°C and blocked with filtered 1% skimmed

milk powder and 0.1%Tween-20 (w/v) in PBS (mPBSt) pH 7.4. Next, 500 ng/mL biotinylated cHK in mPBSt pH 7.4 was incubated on the NeutrAvidin coated plate for 1 hour at room temperature (RT). Isolated phages were blocked with mPBSt pH 7.4 for 1 hour and incubated on the plate. After washing thoroughly with PBSt pH 7.4, cHK bound phages were eluted with 0.1 M trimethylamine and added to 1 M Tris-HCl. TG1 E.coli were infected using the cHK-selected phage-pool, and single colonies were obtained to produce monoclonal- V_HHs. Culture supernatants containing monoclonal cHK V_HHs were incubated on a NeutrAvidin coated plate containing 500 ng/mL biotin-immobilized cHK or HK for 1 hour. After washing, bound V_HHs were detected via myc-tag using anti-myc-antibodies. V_HHs with the highest specificity towards cHK compared to HK were selected for production and purification. V_HHs were produced and purified as described previously[4]. In brief, selected V_HHs were produced in 2xYT medium, production was induced with 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG). Cell pellets of the production cultures were lysed by freeze-thawing. Talon beads, binding the V_HH his-tag, were added to the PBS suspension of the cell lysate. Talon bead bound V_HH was eluted by 150 mM imidazole in PBS, pH 7.4 and dialyzed overnight in PBS.

In selection studies, 9 selected VHHs were coated at a concentration of 5 µg/mL in PBS (pH 7.4) on maxisorp plates overnight (o/n) at 4°C. Plates were blocked with 200 µL PBS supplemented with 1% (w/v) skimmed milk powder and 0.1% (w/v) Tween-20 pH 7.4 for 1 hour at RT, and incubated with 250 ng/mL cHK or HK for 1 hour at RT. After washing, bound cHK was detected with polyclonal sheep anti-human HK IgG, and polyclonal rabbit anti-sheep HRP-conjugated IgG.

IMMUNO-ASSAY FOR cHK

Buffers

Phosphate buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCL, 9.2 mM Na_2HPO_4, 1.76 mM KH_2PO4, pH 7.4. A second type of PBS with lower pH consisted of 127.9 mM NaCl, 6.2 mM Na_2HPO_4, 3.7mM NaH_2PO_4, pH 7.0. PBS-tween (PBSt) was supplemented with 0.1% (w/v) Tween-20. Skimmed milk-PBSt (mPBST) was PBSt with 1%, w/v, skimmed milk powder filtered with a 0.45 µm filter prior to use. Inhibitor mix was mPBSt containing 200 µM PPACK and 100 KIU/mL aprotinin. Diluted inhibitor mix was inhibitor mix containing 20 µM PPACK and 10 KIU/mL aprotinin.

Immuno-assay procedure

VHH-D1 was coated on 96-wells maxisorp plates at 5 µg/mL in 50 µL PBS (pH 7.4) per well o/n at 4°C. During all following steps the plates were incubated on a plate shaker (600 rpm) at RT. First, plates were blocked with 200 µL mPBSt pH 7.0 for 1 hour. Samples were prepared by dilution in inhibitor mix pH 7.0, where after DXS (0.5 µg/mL final concentration; 5 µL DXS solution was added to 200 µL sample) was added to inhibitor mix to enhance (c)HK binding to the plates. After 10 minutes incubation and mixing, 50 µL sample per well was added. Plates were incubated for 1 hour at RT, and washed three times with 200 µL PBSt pH 7.0. Next,

50 µL 2 µg/mL V_HH-H4 was added in diluted inhibitor mix pH 7.0 for 1 hour, and plates were washed three times with 200 µL PBSt pH 7.0. Next 50 µL 0.5 µg/mL streptavidin poly-HRP was added in diluted inhibitor mix pH 7.0 for 1 hour, and plates were washed three times with 200 µL PBSt pH 7.0. TMB substrate conversion (100 µL) was allowed up to 10 minutes and stopped by adding 0.3M H_2SO_4 and analyzed with a micro-plate reader at 450nm (Versamax, Molecular Devices, Sunnyvale, United States).

Samples tested in the assay included 250 ng/mL purified cHK or HK in diluted inhibitor mix pH 7.0, as well as plasma samples in inhibitor mix. For some experiments, normal pooled plasma (NPP) was activated with 1 µg/mL βFXIIa. Prior to activation both NPP and activators were pre-warmed to 37°C. To stop activation, NPP was diluted 1:32, 1:64 or 1:128 in inhibitor mix pH 7.0.

Calibration standards were made by activating NPP with 1 µg/mL βFXIIa for 6 minutes, western blotting of HK confirmed HK was fully cleaved under these conditions. Activated NPP was mixed with NPP at various ratios to obtain a calibration standard.

Western blotting

NPP was activated with βFXIIa as described above and sampled 1:40 in reducing sample buffer (15.5% glycerol, 96.8 mM Tris-HCL, 3.1% SDS, 0.003% bromophenol blue and 25 mM DTT). Samples were incubated for 10 min at 100°C, and 4 µL sample per lane was run on a 4-12% Bis-Tris gradient gel in 1X MOPS buffer. For purified HK and cHK, 7 ng per lane was loaded (reduced). Proteins were transferred onto Immobilon-FL membranes. After blocking with Odyssey Blocking Reagent membranes were incubated with polyclonal sheep anti-human HK IgG (1:4000, reacts with light chain HK) in Odyssey Blocking Reagent and, after washing with tris buffered saline (50 mM Tris, 150 mM NaCl, pH=7.4, TBS) 1% Tween-20 (w/v; TBSt), with Alexa 680 donkey anti-sheep IgG (1:5000). Membranes were scanned using Infrared fluorescence detection system (Odyssey). LI-COR Odyssey V3.0 software was used to quantify signal density.

To quantify HK cleavage, the density of the 120 kD band of non-cleaved HK was compared with that of the 50 and 40 kD fragment bands of cHK. Density of the fragments in fully contact activated plasma was generally lower than density of the 120 kD zymogen band. In order to analyze the relative amount of fragment present compared to zymogen, fragment density was multiplied by (density zymogen in plasma/ density fragments in fully contact activated plasma).

The kininogen content captured by V_HH-D1 on maxisorp plates was analyzed by first incubating sample on V_HH-D1 coated plates as described above, only in this case 500 µL instead of 200 µL per condition was prepared. Eight wells per condition were incubated for 1 hour, after washing thoroughly, 40 µL sample buffer containing 25 mM DTT was added and transferred from well to well to concentrate the kininogen content in the sample buffer added, 15 µL per condition, per lane, was loaded on gel.

To analyze kininogen cleavage in NPP in PBSt pH 7.0 buffer in the presence or absence of 200 µM PPACK and 100 KIU/mL aprotinin, NPP was diluted 1:32 and incubated at 37°C for 60 minutes. Next, the samples were diluted 1/3 in sample buffer containing DTT, 15 µL per lane was loaded on gel.

Western blotting of healthy control and C1INH-HAE patients was performed under non-reducing conditions as described by Suffritti *et al*[E1].

Plasma assay of kallikrein-like activity

NPP was diluted 1:32 in mPBSt pH 7.0 or inhibitor mix pH 7.0 and DXS with a final concentration of 0.5 µg/mL was added. After 30 minutes, chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mM final concentration) was added and substrate conversion was measured with a micro-plate reader at 405nm (Versamax, Molecular Devices, Sunnyvale, United States).

Complement system activity

C1-INH antigen levels and functionalit, C4 and C1q levels were determined in plasma from C1INH-HAE patient remission samples and healthy controls of the validation cohort as described before[E1].

Statistical analysis

Graphpad Prism 7 software was used for data analysis. D'Agostino'sK2 test was used to determine sample distribution. Mann-Whitney t-test was used to compare groups of technical replicates. Kruskal-Wallis test was used for difference in cHK levels per disease state and inter cohort differences corrected for multiple testing with Dunn's multiple comparison's test. Wilcoxon-paired rank test was used for comparison of disease state within one patient and sample collection in citrated tubes and inhibitor tubes obtained at the same time. Correlations were analyzed using Spearmans r test.

Standards were included on each plate and plotted in GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif) using a sigmoidal 4PL fit model to which sample values were related.

Baseline levels of cHK in NPP were extrapolated from the OD of NPP and OD of NPP mixed with set amounts of activated NPP (0 to 15%) of three individual experiments. Prior to extrapolation, graphs of the experiments were controlled for linearity using Graphpad software (R squares: 0.95, 0.99, 0.97, respectively). Next the following calculation was used:

$$\textit{Baseline cHK} = \textit{OD NPP} / (\textit{OD NPP} - \textit{OD x\% activated NPP}/ \textit{x\%})$$

The determined baseline cHK level of 5.2% ± 1.1 represents the mean and SD of 21 samples.

REFERENCES

E1. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency. Clin Exp Allergy 2014;44:1503–14.

E2. Cicardi M, Aberer W, Banerji A, Bas M, Bernstein J a, Bork K, et al. Classification, diagnosis, and approach to treatment for angioedema: consensus report from the Hereditary Angioedema International Working Group. Allergy [Internet] 2014;69:602–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24673465

E3. Yi J, Kim C, Gelfand CA. Inhibition of intrinsic proteolytic activities moderates preanalytical variability and instability of human plasma. J Proteome Res 2007;6:1768–81.

E4. De Maat S, van Dooremalen S, de Groot PG, Maas C. A nanobody-based method for tracking factor XII activation in plasma. Thromb Haemost 2013;110:458–68.

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Triggered cleavage of high molecular weight kininogen to monitor drug activity of oral kallikrein inhibitor ATN-249 in a Phase I trial

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ABSTRACT

Background

Uncontrolled bradykinin release in Hereditary Angioedema (HAE) results in attacks of tissue swelling. Plasma kallikrein (PKa) releases bradykinin by cleaving high molecular weight kininogen (HK) into cleaved HK (cHK). The oral PKa inhibitor ATN-249 is under development as treatment for HAE. Its pharmacokinetics and safety were investigated in a single ascending dose study in healthy study subjects.

Aim

To develop an assay that functionally monitors the presence of ATN-249 in human plasma.

Methods

Plasma samples were collected predose, 2, 4, 9, 12 and 24 hours after intake of ATN-249. cHK generation was analyzed by ELISA after *ex vivo* triggering of study sample plasmas with ellagic acid. Alternatively, PKa activity in ellagic acid-triggered plasma was measured with a fluorogenic substrate.

Results

ATN-249 dose-dependently inhibits cHK generation in plasma samples from study subjects of the single ascending dose study after *ex vivo* triggering of plasma with ellagic acid. Triggered cHK generation in predose samples from healthy study subjects was 68.1%, ±17.5% (i.e. ~70% of the total plasma HK pool was cleaved). This process was significantly inhibited 2 hours after drug intake for 100 mg (after a high caloric meal); 2 and 4 hours with 150mg, 200mg and 400mg and 2, 4, 6, 9 and 12 hours after intake of 800 mg ATN-249 compared to placebo. Maximum inhibition occurred 2 hours after intake of 800mg ATN-249; at this point cHK generation was decreased with 40,5%. Inhibition was more pronounced on PKa activity analyzed in a fluorogenic substrate conversion assays. Dose dependent inhibition of triggered PKa generation was detectable up to 24 hours after intake of 150 mg or 800 mg ATN-249 and the maximum mean inhibition (99% inhibition) was observed 2 hours after intake of 800 mg ATN-249.

Conclusion

We developed a functional assay for monitoring drug activity of ATN-249 by triggering PKa activity in plasma using cHK as a read-out. With this assay we demonstrate the biological activity of the oral kallikrein inhibitor ATN-249 *ex vivo* in healthy subjects.

INTRODUCTION

When plasma kallikrein (PKa) cleaves high molecular weight kininogen (HK), the vasoactive peptide bradykinin is released[1]. Bradykinin promotes vasodilation and increased vascular permeability and is notorious for causing tissue swelling in hereditary angioedema (HAE)[2]. Most HAE patients either have a deficiency in- or a dysfunction of C1-esterase inhibitor (C1-INH), which is respectively classified as HAE-1 or HAE-2[3]. This serine protease inhibitor controls two enzymes that are involved in bradykinin production: activated factor XII (FXIIa), and PKa. Hence, C1-INH deficiency results in bouts of uncontrolled bradykinin production[3].

Currently, therapeutic options for prophylaxis of angioedema attacks consist of twice weekly C1-INH administration; either intravenous[4] or subcutaneous[5] or (twice) monthly subcutaneous injection with a monoclonal antibody targeting PKa[6]. Alternatively, attenuated androgens are used as prophylaxis but these are known for their unfavorable side effects[7]. Oral treatment with a PKa inhibitor seems an appealing alternative. To meet this need, the oral PKa inhibitor ATN-249 is under development. This therapy holds promise as a prophylactic drug for HAE-1 and -2. ATN-249 was tested in a phase I single ascending dose study in healthy study participants.

In the present study, we sought for evidence to support the biological activity of ATN-249 in plasma of these study subjects. The presence of ATN-249 in plasma should prevent bradykinin release, when exposed to a trigger for PKa activity *ex vivo*. However, bradykinin is very hard to measure directly due to its small size (9 amino acids) and half-life of seconds due to plasma kininases[1,2,8]. Cleaved HK (cHK), the byproduct of bradykinin release, is generally accepted as a surrogate marker for bradykinin production[9]. This can be determined by western blotting[9,10]. As an alternative, we recently developed a nanobody-based ELISA that detects cHK and validated this assay in two cohorts of HAE patients[11]. Contrary to the commonly used immunoblot method, cHK ELISA allows for high throughput of samples and quantification of data. The aim of this study is to evaluate whether our cHK ELISA can be used to functionally monitor for the presence of ATN-249 in human plasma, exposed to a trigger for PKa activity *ex vivo*. cHK generation was compared to PKa activity detected with an enzyme activity assay using a fluorogenic substrate.

METHODS

Plasma samples

A randomized, double-blind, placebo-controlled single ascending dose and crossover food effect study was performed with ATN-249. After written informed consent, 48 healthy males received a single oral dose of 50 mg, 100 mg, 150 mg, 200 mg, 400 mg, or 800 mg ATN-249. Subjects in the 100 mg dose cohort received their first dose under fasted condition and after a 7-day washout, a second dose 30 minutes after the start of a high fat, high caloric meal. Each dose cohort consisted of 6 subjects that were given active compound and 2 subjects that were given placebo. Serial blood draws were collected in sodium citrate blood-collection tubes (Greiner Bio-one, Kremsmünster, Austria). Collections took place pre-dose,

and at 2 hours, 4 hours, 9 hours, 12 hours and 24 hours after ATN-249 intake. Samples were centrifuged at 2000 xg for 10 minutes at 4 °C shortly after collection and stored at -80°C. Prior to analyses, plasma samples were thawed at 37 °C, vortexed for 5 seconds and 50 µL aliquots were stored in deepwell storage plates (Thermo Scientific, Waltham, Mass) at -80°C until analysis.

In addition, citrated plasma samples of ~50 healthy volunteers were collected and pooled (referred to as normal pooled plasma, NPP). Following standard procedures blood was collected in sodium citrate (3.2% wt/vol) blood-collection tubes (Becton Dickson Vacutainers, Vianen, the Netherlands). Tubes were centrifuged twice at 2000 xg for 10 minutes shortly after collection and stored in aliquots at -80 °C until use. Samples underwent 1 extra freeze-thaw cycle when being pooled. This protocol was approved by University Medical Center Utrecht ethical committee and volunteers signed written informed consent.

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MATERIALS

Bovine Serum Albumin (BSA), ellagic acid (E2250), dextran sulfate molecular (average) weight 500.000 (DXS), skimmed milk powder, NaCl, Na_2HPO_4, NaH_2PO_4, and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA). 96 well flat bottom MaxiSorp microtitre plates and FluorNunc(™) framed 16 well flat bottom black microtitre plates and 0.8 mL polyprolypene deepwell storage plates (AB-0765) and storage plate cap strips (AB0981) and were from Thermo Scientific (Waltham, Mass). 1.2 mL polypropylene tubes were from BRAND GMBH + CO KG (Wertheim, Germany). βFXIIa, was from Enzyme Research laboratories (South Bend, Ind). 3,3',5,5'-Tetramethyl-benzidine (TMB) was from Tebu Bio (Heerhugowaard, The Netherlands). Streptavidin-poly-HRP was from Sanquin Blood Supply (Division Reagents, Amsterdam, The Netherlands). Phe-Pro-Arg-chloromethylketone (PPACK) was purchased from Haematologic Technologies (Essex Junction, VT, USA). Anti-cleaved kininogen specific monoclonal VhH-D1 and biotinylated monoclonal anti-kininogen VhH-H4 were produced in our lab as published[11]. Substrate Z-FR-AMC · HCl (I-1160, Bachem, Ca, USA). Microplate reader SpectraMax M4 was used for absorbance measurements and SepctraMax iD3 for fluorescence measurements (Molecular Devices, Sunnyvale, United States).

Triggered cHK generation assay

96-wells flat bottom microtiter plates were coated with 5 µg/mL VhH D1 (50 µL per well) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 9.2 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4.) overnight at 4°C. The plates were blocked for 2 hours with 150 µL PBS per well (consisting of 127.9 mM NaCl, 6.2 mM Na_2HPO_4, 3.7 mM NaH_2PO_4, pH 7.0) supplemented with 0.1% (v/v) Tween-20 (PBST) and 1% (w/v) skimmed milk (mPBST) , while shaking at 600 rpm at room temperature. To generate an assay standard, NPP was activated with 1 µg/mL βFXIIa (final concentration), by incubating 1 volume βFXIIa in PBS (pH 7.4) with 9 volumes NPP at 37°C for 10 minutes. Enzymatic activity was stopped by diluting plasma 128-fold in mPBST, containing 50 µM PPACK. Non-activated plasma was prepared by adding

1 volume PBS to 9 volumes NPP which was diluted 128-fold in mPBST containing 50 µM PPACK. Activated (100% kininogen cleavage) and non-activated diluted plasma in mPBST containing 50 µM PPACK were mixed to generate a standard curve with a cHK range from 0 to 100% (standard curve samples contained 0%, 10%, 20%, 30%, 50%, 70%, 80% and 100% activated plasma).

To determine plasma activation time and concentration of the activator, 100 µL NPP was added to 1.2 mL polypropylene tubes, EA (40 µM) and βFXIIa (50 nM) were dissolved in HEPES-buffered saline (HBS; 10 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L $MgSO_4$ and 5 mmol/L KCl pH 7.4). Plasma and activator were prewarmed at 37°C for at least 5 min prior to activation. 100 µL activator was added to NPP and resuspended vigorously. After 1, 5 and 10 minutes, activation was stopped by dilution of the activated plasma sample 4-fold in mPBST containing 200 µM PPACK. The samples were stepwise-diluted further (17.6-fold), so that the final plasma dilution was 1:141 in mPBST, containing 50 µM PPACK. In this way, sample plasma dilutions wereequal to the plasma dilutions that were used in the assay standard. In a comparable manner, titration series of EA (40 µM -300 nM) and βFXIIa (25-0.39 nM) were used to activate NPP by adding 50 µL activator to 50 µL NPP. Activation was stopped after 3 minutes.

For experiments in plasma samples from study subjects, samples (50 µL) were thawed at 37°C, vortexed and centrifuged for 2 min at 200g. Ellagic acid (EA) was prediluted in HBS to a concentration of 5 µM. Both EA in HBS (5 µM) and plasma samples were prewarmed at 37°C for 10 minutes prior to activation. Plasma samples were activated by adding 50 µL EA (5 µM in HBS, 2,5 µM final concentration in 100 µL sample) to the 50 µL plasma sample in a 96-deep well plate, while resuspending vigorously. After 3 minutes contact activation was stopped.

For all experiments, DXS (0.5 µg/mL final concentration in samples) was added to both standard curve samples and test samples. Directly after addition of DXS and after 10 minutes incubation the samples are vigorously mixed on a plate vortex. The samples were added in duplicate (50 µL per well) to the blocked maxisop plates and incubated for 1 hour while shaking 600 rpm at room temperature. Plates were washed 3x with 150 µL PBST. Detection VhH H4-biotin (2 µg/mL in mPBST,25 µM PPACK) was added (50µL per well) and was incubated for 1 hour while shaking 600 rpm at room temperature. Plates were washed 3x with 150 µL PBST. Streptavidin-poly-HRP (0.5 µg/mL) in mPBST,25 µM PPACK) was added (50µL per well) and incubated for 30 minutes while shaking 600 rpm at room temperature. Plates were washed 3x with 150 µL PBST. 100 µL TMB was added and substrate conversion was measured at 650nm for 20 minutes measuring absorbance every minute. A calibration curve was generated in GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif) using a sigmoidal 4PL fit model on which the test samples were interpolated.

Plasma Kallikrein activity assay

96-well flat bottom black plates were blocked with 150 µL HBS containing 0.1% BSA (w/v) at 37 C for 20 min on a plate heater. Plasma (NPP or study samples) was thawed at 37 C for 5-10 minutes, vortexed, and 15 µL per well was added.

Next, 20 µL per well I-1160in HBS was added (final concentration 50 µM) followed by 15 µL ellagic acid (final concentration 2,5 µM) after which plates were immediately placed in the plate reader. Substrate conversion was monitored at 360/460 (excitation/emission) every minute for 30 minutes by an iD3 Spectramax plate reader at 37 °C, shaking samples prior to every read. Relative fluorescent units (RFU) after 15 minutes were used for data analysis.

Data analysis

Data was analyzed using GraphPad Prism 7 software. Column Statistics was used for descriptive statistics and normality testing, a sigmoidal 4PL fit model was used to interpolate samples and calculate an IC50 and Dunnett's multiple comparisons test was used for analysis of effect on cHK generation and PKa activity. Spearman's rank test was used to calculate correlation between cHK and PKa. Kruskal-Wallis test was used for differences among consecutive samples of study subjects receiving placebo. In addition to analyzing cHK values, cHK values normalized to pre-dose lcHK levels for individual study subjects were analyzed, where:

*Relative cHK level = cHK at time point-x/pre-dose cHK *100.*

Likewise, RFU of PKa substrate conversion were normalized to pre-dose RFU for individual study subjects:

*Relative PKa activity = RFU at time point-x/pre-dose RFU*100.*

RESULTS

Assay development

We set out to develop a functional assay for PKa-blocking drug activity. When plasma is incubated with FXIIa, or EA, cHK formation is triggered (Figure 1A).Typically, cHK is first generated during extensive contact activation but then further degraded to become undetectable in our ELISA setup (Figure 1A). We therefore restricted plasma activation times to 3 minutes. Next, we titrated both activators to determine a concentration at which robust cHK generation occurs within 3 minutes. Concentrations of 6.25 nM for βFXIIa and 2.5 µM EA were selected as trigger (Figure 1B,C). We went forward with EA as triggering compound as this results in consistent, repeatable cHK generation. By comparison, when PKa activity

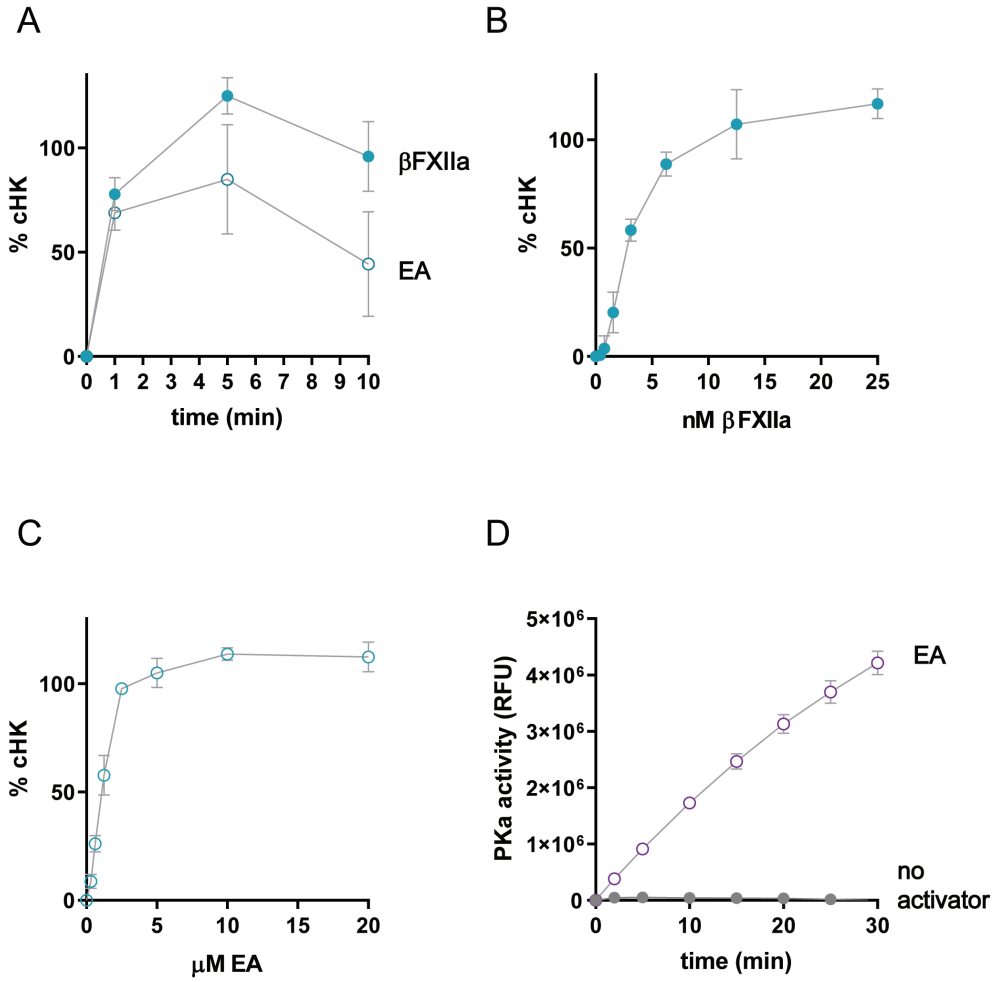

Figure 1. Assay development. A) Cleaved high molecular weight kininogen (cHK) generation over time after stimulation with 25 nM βFXIIa (closed symbols) or 20 μM ellagic acid (EA, open symbols). cHK generation after 3 minutes exposures to concentration ranges of **B)** βFXIIa or **C)** EA. The Y-axis represents cHK levels where 0% reflects cHK in normal pooled plasma (NPP), 100% fully cleaved HK in NPP. **D)** Plasma kallikrein (PKa) activity in NPP during ellagic acid stimulation (2.5 μM) or no activator (HBS). Mean and SD of three individual experiments are shown.

is triggered with 2.5 μM EA, robust amidolytic PKa activity is generated (Figure 1D), analysis time was set at 15 minutes for PKa activity throughout this work.

The plasma of healthy study subjects displays variation in ex vivo triggered cHK generation.

We then applied the cHK trigger assay on plasma collected form healthy study participants included in the ATN-249 single ascending dose study. When analyzing predose cHK

generation in all 48 study participants we observed considerable variation in cHK generation in response to EA; mean cHK 68.1%, SD ±17.5%, range:31.51-101.8% (Figure 2A). We aimed to determine if this variation existed throughout all samples or was explained by interindividual variation. cHK generation in consecutive samples over time within study subjects receiving placebo appeared stable (Figure 2B); with the lowest individual mean cHK generation of 34,6% SD5,5% and highest mean cHK generation of 87,4% SD6,1%. A comparable phenomenon was seen for PKa activity in the enzyme activity assay. There was a broad range of PKa activity in predose samples. However, when analyzing consecutive samples over time within subjects receiving placebo we observed significant differences in cHK generation ($p<0.0001$) and PKa activity ($p<0.0001$) among patients (Figure 2C and D). This interindividual variation suggest an endogenous propensity for PKa activation and cHK generation, rather than methodological issues underlies the variation in cHK generation observed among healthy volunteers.

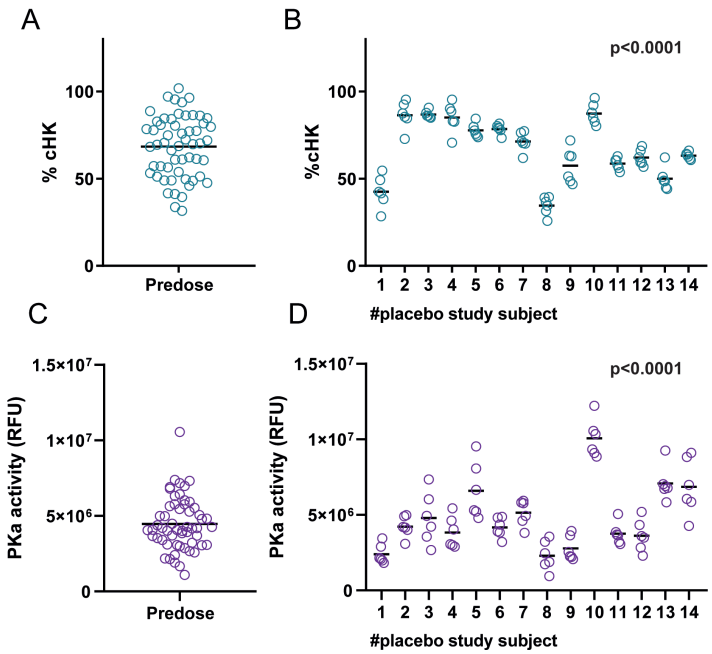

Figure 2. Healthy study subjects display a specific propensity to cHK and PKa generation and upon ellagic acid stimulation. Cleaved high molecular weight kininogen (cHK) generation after 3 minutes of ellagic acid stimulation (2,5 µM) in **A)** all 56 single ascending dose study samples prior to drug intake (predose), line represents mean **B)** all 12 study subjects receiving placebo predose, 2,4,9,12 and 24 hours after placebo intake, lines represent means. Y-axis represents cHK; 0% reflects cHK in normal pooled plasma (NPP), 100% fully cleaved HK in NPP. Plasma kallikrein (PKa) activity in **C)** all 56 single ascending dose study samples prior to drug intake (predose), line represents mean **D)** all 12 study subjects receiving placebo predose, 2,4,9,12 and 24 hours after placebo intake, lines represent means. Y-axis represents Relative Fluorescent Units (RFU) 15 minutes after start of stimulation. Differences among study subjects were tested using Kruskal-Wallis test.

cHK generation is dose dependently inhibited in study subjects receiving ATN-249

Decrease in cHK generation was seen in 2, 4, 9 and 12 hour after intake of 800 mg ATN-249 compared to subjects receiving placebo (Figure 3A), with a maximum absolute mean difference of -34.5% cHK after 4 hour (Supplementary Table 1). We next, analyzed these data after normalization to correct for interindividual differences in triggered cHK generation. Decrease in cHK generation was seen 2 and 4 hours after drug intake for subjects receiving 150 mg and 200 mg ATN-249. When normalizing data to pre-dose cHK generation, decrease was observed 2, 4, 6, 9 and 12 hours after intake of 800 mg ATN-249 compared to placebo (Figure 3B), with a maximum relative mean difference of -40.5% after 2 hour (Supplementary Table 2). In addition, decrease in generated cHK was seen after 2 and 4 hour after drug intake in subjects receiving 100 mg (fed state, only after 2 hour), 150 mg, 200 mg, and 400 mg. Mean inhibition for absolute and relative cHK values per cohort per time point are displayed in Supplementary Tables 1 and 2.

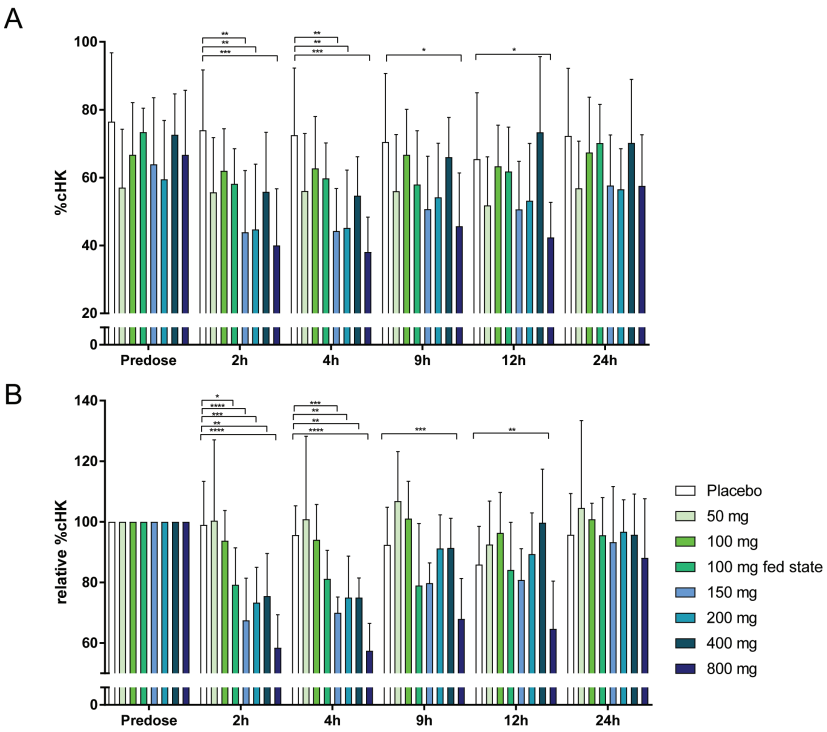

Figure 3. cHK generation is dose dependently inhibited in study subjects receiving ATN-249. A) Cleaved high molecular weight kininogen (cHK) generation after 3 minutes of ellagic acid stimulation (2500nM) in plasma from 48 healthy study subjects; 6 per active treatment, 12 placebo, 8 tested both under fasted and fed conditions. Y-axis represents cHK; 0% reflects cHK in normal pooled plasma (NPP), 100% fully cleaved HK in NPP. **B)** data normalized to individual pre-dose cHK generation per study subject. Bars represent mean and SD, differences among placebo and active cohorts were calculated with Dunnett's multiple comparison test * = p<.05 **= p<.01 ***=p<.001, ****=p<.0001.

In the PKa substrate conversion assays, the effects of ATN-249 were more pronounced and detectable at later timepoints compared to effects on cHK generation. Inhibition compared to placebo was observed for all concentrations ATN-249 after 2,4 and 9 hours and the effect lasted up to 24 hours for concentrations ATN-249 of 150 mg, 200 mg, 400 mg and 800 mg (Figure 4A). When analyzing PKa activity relative to predose activity inhibition was observed for all concentrations after 2 hours, for all but 50 mg after 4 and 9 hour and all but 50 mg and 100 mg after 12 hours. The effect lasted up to 24 hour for 150 mg and 800 mg ATN-249 (Figure 4B). Maximal inhibition was seen 2 hours after intake of 800mg ATN-249 with a mean relative difference of -99% compared to placebo (Supplementary Table 4). Mean inhibition for absolute and relative PKa activity per cohort per time point are displayed in Supplementary Tables 3 and 4.

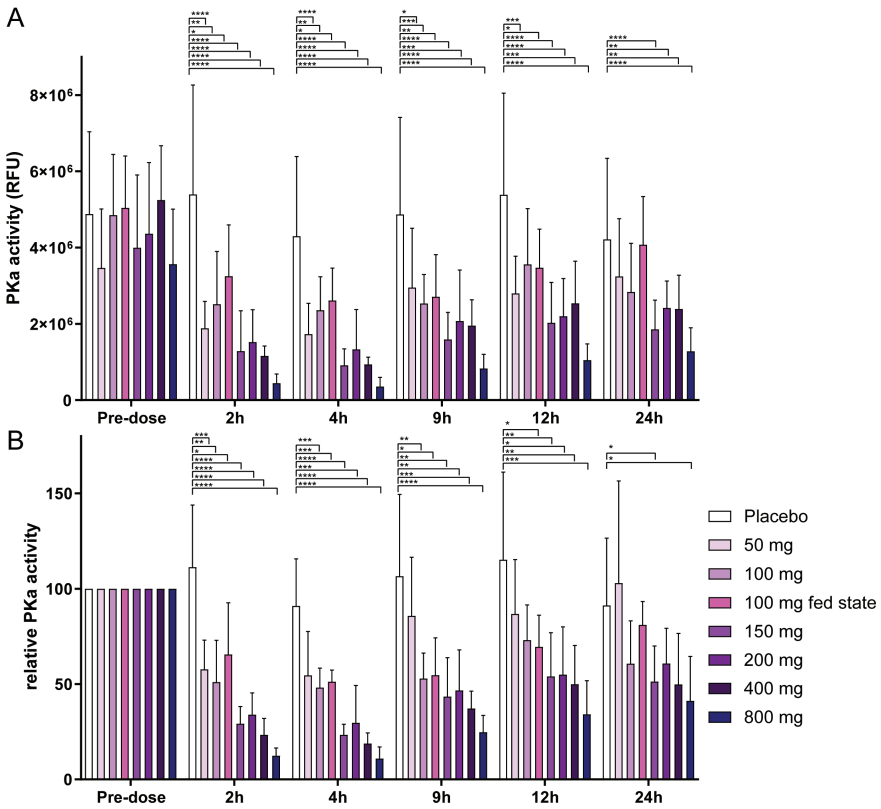

Figure 4. PKa generation is dose dependently inhibited in study subjects receiving ATN-249. A) Plasma kallikrein (PKa) activity after ellagic acid stimulation (2500nM) in plasma from 48 healthy study subjects; 6 per active treatment, 12 placebo, 8 tested both under fasted and fed conditions. Y-axis represents Relative Fluorescent Units (RFU) 15 minutes after start of stimulation. **B)** data normalized to individual pre-dose PKa activity per study subject. Bars represent mean and SD, differences among placebo and active cohorts were calculated with Dunnett's multiple comparison test * = p<.05 **= p<.01 ***=p<.001, ****=p<.0001.

DISCUSSION

We developed a cHK trigger assay using a cHK ELISA as read-out and demonstrated *ex vivo* biological activity of the oral kallikrein inhibitor ATN-249 in healthy subjects. Upon triggering of plasma samples from healthy study subjects of a single ascending dose study a dose dependent inhibitory effect on cHK generation was observed. The effect lasted up to 12 hours after ingestion of 800 mg ATN-249. These findings reflect the presence of a relevant free fraction of the drug in plasma from study subjects that prevents bradykinin release.

We compared triggered cHK generation monitored by ELISA to to triggered PKa activity in an enzyme activity assays. Since cHK is a direct marker for bradykinin release, as it is the byproduct of bradykinin generation, it is the more clinically relevant biomarker. Any PKa-targeting HAE therapy will have to disturb this biological interaction. However, enzyme activity assays are also a practical and commonly used tool to study PKa activity[12,13] (PMID). In these enzyme activity assays conversion of a chemical substrate by PKa is tested while, conversion by PKa of the natural substrate HK is measured in the cHK trigger assay. When comparing these two methods PKa activity on chemical substrate conversion was more easily inhibited by ATN-249. These differences may be explained by technical factors as the experimental set-ups are not one on one comparable. It may also be explained by biological factors as the cleavage kinetics of HK can be more favorable then the chemical substrate. Although the two parameters show moderate correlation, we stress that no direct comparison should be made between these two assay types. The results do however complement each other. The PKa assay appears to be more sensitive, demonstrating biological availability of the drug for longer periods of time after intake of lower drug concentrations. While the cHK assay adds the clinically relevant information that the compound indeed prevents bradykinin release.

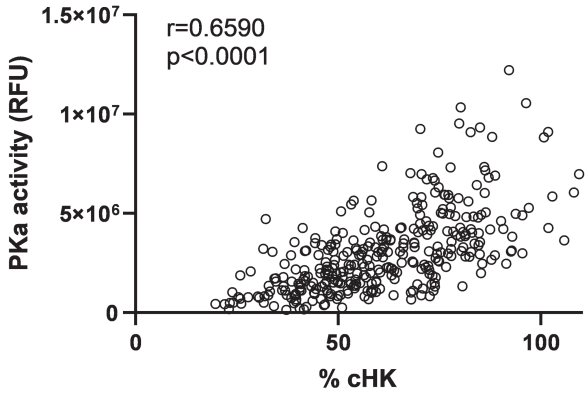

Figure 5. cHK generation correlates to PKa activity. Plasma kallikrein (PKa) and cleaved high molecular weight kininogen (cHK) measured in all 336 study samples, placebo and active treatment over time. Y-axis represents PKa activity in Relative Fluorescent Units (RFU) 15 minutes after stimulation with 2500nM ellagic acid (EA). X-axis represents cHK 3 minutes after stimulation with 2500nM EA; 0% reflects cHK in normal pooled plasma (NPP), 100% fully cleaved HK in NPP. Correlation was calculated using Spearman correlation coefficient.

Interestingly, we found that healthy subjects demonstrated considerable variability in sensitivity to the EA trigger, both on PKa enzyme activity and cHK generation. This specific propensity was consistent over time in subjects receiving placebo. This observation appears to be in line with the finding that healthy controls and HAE-1 and 2patients present with a wide range in cHK levels (2.7-17.4% for healthy controls, 1.8-74.7% for HAE patients during remission)[11]. This individual sensitivity to contact activation may give a clue to why some develop frequent angioedema while others do not. This was also proposed by Lara-Marquez and colleagues, that demonstrated increased sensitivity to the triggering compound DXS in patients with HAE-1 and 2, HAE with normal C1-INH levels and idiopathic non histaminergic angioedema[13].

Although the extent of therapeutic efficacy in HAE cannot be translated 1 on 1 from the inhibitory effect of a drug in a contact activation triggered assay, this cHK trigger assay holds promise as a practical tool to monitor drug activity in healthy study subjects. The trigger used is unlikely representative for cHK generation during an angioedema attack. In our experimental set-up the mean cHK level generated in study subjects prior to drug intake was 68.1% within 3 minutes. We previously showed that HAE-1 and 2 patients have median cHK levels of 16% during remission (compared to 6.44% in healthy subjects) and 33% during angioedema attacks[11]. Our triggered system therefore represents supraphysiological cHK generation. The maximum mean inhibition reached was 34.5% (absolute cHK inhibition), 4 hours after ingestion of 800 mg ATN-249. We expect that partial inhibition under our supraphysiological assay conditions are sufficient to abolish a milder bout of contact activation occurring in HAE patients. In addition to a cHK trigger assay, monitoring untriggered cHK in future phase II and phase III studies in HAE patients may demonstrate efficacy of ATN-249 on physiological contact activation in the context of HAE.

In conclusion, the cHK trigger assay can be used as a tool to demonstrate *ex vivo* efficacy on bradykinin release. cHK generation correlates well to PKa activity measured in an enzymatic assay but HK cleavages tends to be harder to control in our systems. With the current study we demonstrated *ex vivo* biological activity of the oral kallikrein inhibitor ATN-249 in healthy subjects from a single ascending dose study. We propose that both triggered and untriggered cHK measurement are useful companion diagnostics in HAE drug development.

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CONFLICT OF INTEREST

This work was sponsored by Attune Pharmaceuticals. All authors declare no conflict of interest.

REFERENCES

1. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A. Plasma bradykinin in angio-oedema. *Lancet*. 1998;351(9117):1693-1697. doi:10.1016/S0140-6736(97)09137-X
2. Nussberger J, Cugno M, Cicardi M, Agostoni A. Local bradykinin generation in hereditary angioedema. *J Allergy Clin Immunol*. 1999;104(6):1321-1322. doi:10.1016/S0091-6749(99)70030-8
3. Maurer M, Magerl M, Ansotegui I, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. *Allergy*. 2018;73(8):1575-1596. doi:10.1111/all.13384
4. Zuraw BL, Kalfus I. Safety and Efficacy of Prophylactic Nanofiltered C1-inhibitor in Hereditary Angioedema. *Am J Med*. 2012;125(9):938.e1-938.e7. doi:10.1016/j.amjmed.2012.02.020
5. Longhurst H, Cicardi M, Craig T, et al. Prevention of Hereditary Angioedema Attacks with a Subcutaneous C1 Inhibitor. *N Engl J Med*. 2017;376(12):1131-1140. doi:10.1056/NEJMoa1613627
6. Banerji A, Busse P, Shennak M, et al. Inhibiting Plasma Kallikrein for Hereditary Angioedema Prophylaxis. *N Engl J Med*. 2017;376(8):717-728. doi:10.1056/NEJMoa1605767
7. Zuraw BL, Davis DK, Castaldo AJ, Christiansen SC. Tolerability and Effectiveness of 17-α-Alkylated Androgen Therapy for Hereditary Angioedema: A Re-examination. *J allergy Clin Immunol Pract*. 4(5):948-955.e15. doi:10.1016/j.jaip.2016.03.024
8. Cugno M, Tedeschi A, Nussberger J. Bradykinin in idiopathic nonhistaminergic angioedema. *Clin Exp Allergy*. 2016. doi:10.1111/cea.12869
9. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency. *Clin Exp Allergy*. 2014;44(12):1503-1514. doi:10.1111/cea.12293
10. Reddigari S and Kaplan AP. Cleavage of human high-molecular weight kininogen by purified kallikreins and upon contact activation of plasma. *Blood*. 1988;71(5):1334-1340.
11. Hofman ZLM, de Maat S, Suffritti C. Cleaved kininogen as a biomarker for bradykinin release in hereditary angioedema. *J Allergy Clin Immunol*. 2017. doi:10.1016/j.jaci.2017.07.012
12. Cornpropst M, Collis P, Collier J, et al. Safety, pharmacokinetics, and pharmacodynamics of avoralstat, an oral plasma kallikrein inhibitor: phase 1 study. *Allergy*. 2016;71(12):1676-1683. doi:10.1111/all.12930
13. Lara-Marquez ML, Christiansen SC, Riedl MA, Herschbach J, Zuraw BL. Threshold-stimulated kallikrein activity distinguishes bradykinin- from histamine-mediated angioedema. *Clin Exp Allergy*. 2018;48(11):1429-1438. doi:10.1111/cea.13219

Supplementary Table 1. Dunnett's multiple comparisons test on cHK values

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
Predose				
Placebo vs. 50 mg	19,43	-1,715 to 40,57	ns	0,0858
Placebo vs. 100 mg period 1	9,776	-11,37 to 30,92	ns	0,7163
Placebo vs. 100 mg period 2	3,115	-18,03 to 24,26	ns	0,9994
Placebo vs. 150 mg	12,59	-8,555 to 33,73	ns	0,46
Placebo vs. 200 mg	17,02	-4,12 to 38,17	ns	0,1691
Placebo vs. 400 mg	3,887	-17,26 to 25,03	ns	0,996
Placebo vs. 800 mg	9,836	-11,31 to 30,98	ns	0,7108
2h				
Placebo vs. 50 mg	18,3	-2,848 to 39,44	ns	0,1195
Placebo vs. 100 mg period 1	12	-9,147 to 33,14	ns	0,512
Placebo vs. 100 mg period 2	15,79	-5,352 to 36,94	ns	0,2309
Placebo vs. 150 mg	30,07	8,926 to 51,21	**	0,0015
Placebo vs. 200 mg	29,25	8,104 to 50,39	**	0,0021
Placebo vs. 400 mg	18,14	-3,009 to 39,28	ns	0,1251
Placebo vs. 800 mg	33,96	12,81 to 55,1	***	0,0002
4h				
Placebo vs. 50 mg	16,48	-4,659 to 37,63	ns	0,1944
Placebo vs. 100 mg period 1	9,799	-11,35 to 30,94	ns	0,7142
Placebo vs. 100 mg period 2	12,72	-8,423 to 33,86	ns	0,4487
Placebo vs. 150 mg	28,27	7,121 to 49,41	**	0,0033
Placebo vs. 200 mg	27,32	6,178 to 48,47	**	0,0049
Placebo vs. 400 mg	17,86	-3,285 to 39	ns	0,1351
Placebo vs. 800 mg	34,45	13,31 to 55,59	***	0,0002
9h				
Placebo vs. 50 mg	14,47	-8,101 to 37,05	ns	0,3806
Placebo vs. 100 mg period 1	3,77	-17,37 to 24,91	ns	0,9971
Placebo vs. 100 mg period 2	12,49	-8,656 to 33,63	ns	0,4686
Placebo vs. 150 mg	19,8	-1,34 to 40,95	ns	0,0766
Placebo vs. 200 mg	16,29	-4,854 to 37,43	ns	0,2042
Placebo vs. 400 mg	4,427	-16,72 to 25,57	ns	0,9929
Placebo vs. 800 mg	24,81	3,662 to 45,95	*	0,0136
12h				
Placebo vs. 50 mg	13,62	-7,526 to 34,76	ns	0,376
Placebo vs. 100 mg period 1	2,091	-19,05 to 23,23	ns	0,9996
Placebo vs. 100 mg period 2	3,636	-17,51 to 24,78	ns	0,9973
Placebo vs. 150 mg	14,77	-6,378 to 35,91	ns	0,2935
Placebo vs. 200 mg	12,24	-8,899 to 33,39	ns	0,4899
Placebo vs. 400 mg	-7,922	-29,07 to 13,22	ns	0,8661
Placebo vs. 800 mg	23,09	1,942 to 44,23	*	0,0257

Supplementary Table 1. (continued)

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
24h				
Placebo vs. 50 mg	15,47	-5,677 to 36,61	ns	0,2496
Placebo vs. 100 mg period 1	4,916	-16,23 to 26,06	ns	0,9875
Placebo vs. 100 mg period 2	2,145	-19 to 23,29	ns	0,9996
Placebo vs. 150 mg	14,68	-6,463 to 35,82	ns	0,2992
Placebo vs. 200 mg	15,76	-5,383 to 36,9	ns	0,2326
Placebo vs. 400 mg	2,093	-19,05 to 23,24	ns	0,9996
Placebo vs. 800 mg	14,78	-6,364 to 35,92	ns	0,2925

Supplementary Table 2. Dunnett's multiple comparisons test on relative cHK values

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
Predose				
Placebo vs. 50 mg	0	-16,71 to 16,71	ns	>0,9999
Placebo vs. 100 mg period 1	0	-16,71 to 16,71	ns	>0,9999
Placebo vs. 100 mg period 2	0	-16,71 to 16,71	ns	>0,9999
Placebo vs. 150 mg	0	-16,71 to 16,71	ns	>0,9999
Placebo vs. 200 mg	0	-16,71 to 16,71	ns	>0,9999
Placebo vs. 400 mg	0	-16,71 to 16,71	ns	>0,9999
Placebo vs. 800 mg	0	-16,71 to 16,71	ns	>0,9999
2h				
Placebo vs. 50 mg	-1,416	-18,13 to 15,3	ns	0,9997
Placebo vs. 100 mg period 1	5,213	-11,5 to 21,93	ns	0,9401
Placebo vs. 100 mg period 2	19,73	3,019 to 36,44	*	0,0127
Placebo vs. 150 mg	31,5	14,79 to 48,21	****	<0,0001
Placebo vs. 200 mg	25,64	8,932 to 42,36	***	0,0005
Placebo vs. 400 mg	23,47	6,759 to 40,18	**	0,0018
Placebo vs. 800 mg	40,54	23,83 to 57,26	****	<0,0001
4h				
Placebo vs. 50 mg	-5,163	-21,88 to 11,55	ns	0,9428
Placebo vs. 100 mg period 1	1,584	-15,13 to 18,3	ns	0,9996
Placebo vs. 100 mg period 2	14,49	-2,224 to 31,2	ns	0,1184
Placebo vs. 150 mg	25,71	8,999 to 42,42	***	0,0005
Placebo vs. 200 mg	20,66	3,943 to 37,37	**	0,008
Placebo vs. 400 mg	20,67	3,953 to 37,38	**	0,008
Placebo vs. 800 mg	38,23	21,52 to 54,94	****	<0,0001

Supplementary Table 2. (continued)

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
9h				
Placebo vs. 50 mg	-14,48	-32,33 to 3,361	ns	0,1632
Placebo vs. 100 mg period 1	-8,647	-25,36 to 8,065	ns	0,6089
Placebo vs. 100 mg period 2	13,41	-3,307 to 30,12	ns	0,1719
Placebo vs. 150 mg	12,62	-4,095 to 29,33	ns	0,2215
Placebo vs. 200 mg	1,183	-15,53 to 17,9	ns	0,9997
Placebo vs. 400 mg	1,014	-15,7 to 17,73	ns	0,9998
Placebo vs. 800 mg	24,45	7,735 to 41,16	***	0,001
12h				
Placebo vs. 50 mg	-6,662	-23,37 to 10,05	ns	0,8296
Placebo vs. 100 mg period 1	-10,48	-27,19 to 6,232	ns	0,4042
Placebo vs. 100 mg period 2	1,757	-14,96 to 18,47	ns	0,9996
Placebo vs. 150 mg	5,066	-11,65 to 21,78	ns	0,9478
Placebo vs. 200 mg	-3,509	-20,22 to 13,2	ns	0,9928
Placebo vs. 400 mg	-13,83	-30,54 to 2,881	ns	0,149
Placebo vs. 800 mg	21,17	4,455 to 37,88	**	0,0062
24h				
Placebo vs. 50 mg	-8,835	-25,55 to 7,877	ns	0,5868
Placebo vs. 100 mg period 1	-5,147	-21,86 to 11,56	ns	0,9436
Placebo vs. 100 mg period 2	0,1675	-16,54 to 16,88	ns	>0,9999
Placebo vs. 150 mg	2,42	-14,29 to 19,13	ns	0,9994
Placebo vs. 200 mg	-0,9775	-17,69 to 15,73	ns	0,9998
Placebo vs. 400 mg	0,008428	-16,7 to 16,72	ns	>0,9999
Placebo vs. 800 mg	7,627	-9,085 to 24,34	ns	0,7275

Supplementary Table 3. Dunnett's multiple comparisons test on PKa activity.

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
Pre-dose				
Placebo vs. 50 mg	1414547	-316180 to 3145273	ns	0,1538
Placebo vs. 100 mg	30865	-1731696 to 1793426	ns	>0,9999
Placebo vs. 100 mg fed state	-160318	-1770668 to 1450031	ns	0,9996
Placebo vs. 150 mg	884339	-1103433 to 2872110	ns	0,7413
Placebo vs. 200 mg	517823	-1439378 to 2475025	ns	0,9723
Placebo vs. 400 mg	-364067	-2016486 to 1288351	ns	0,9914
Placebo vs. 800 mg	1318262	-345498 to 2982021	ns	0,178
2h				
Placebo vs. 50 mg	3511176	1819949 to 5202403	****	<0,0001
Placebo vs. 100 mg	2879666	945919 to 4813413	**	0,0013
Placebo vs. 100 mg fed state	2145505	230646 to 4060365	*	0,0216

Supplementary Table 3. (continued)

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
Placebo vs. 150 mg	4109387	2313757 to 5905018	****	<0,0001
Placebo vs. 200 mg	3873434	2144212 to 5602657	****	<0,0001
Placebo vs. 400 mg	4234142	2610758 to 5857527	****	<0,0001
Placebo vs. 800 mg	4949139	3327293 to 6570984	****	<0,0001
4h				
Placebo vs. 50 mg	2568889	1285196 to 3852582	****	<0,0001
Placebo vs. 100 mg	1943647	629532 to 3257762	**	0,0013
Placebo vs. 100 mg fed state	1686660	386392 to 2986929	**	0,0056
Placebo vs. 150 mg	3385941	2216629 to 4555254	****	<0,0001
Placebo vs. 200 mg	2969943	1577518 to 4362367	****	<0,0001
Placebo vs. 400 mg	3363200	2227714 to 4498686	****	<0,0001
Placebo vs. 800 mg	3942608	2802164 to 5083051	****	<0,0001
9h				
Placebo vs. 50 mg	1914654	61906 to 3767402	*	0,0399
Placebo vs. 100 mg	2334049	849058 to 3819041	***	0,0006
Placebo vs. 100 mg fed state	2156047	543099 to 3768994	**	0,0041
Placebo vs. 150 mg	3275216	1806210 to 4744222	****	<0,0001
Placebo vs. 200 mg	2794939	1065033 to 4524845	***	0,0004
Placebo vs. 400 mg	2914455	1454179 to 4374732	****	<0,0001
Placebo vs. 800 mg	4039233	2640526 to 5437939	****	<0,0001
12h				
Placebo vs. 50 mg	2586278	970627 to 4201929	***	0,0005
Placebo vs. 100 mg	1825093	-20950 to 3671136	ns	0,0539
Placebo vs. 100 mg fed state	1911265	281938 to 3540592	*	0,0145
Placebo vs. 150 mg	3356572	1706581 to 5006562	****	<0,0001
Placebo vs. 200 mg	3182556	1562257 to 4802854	****	<0,0001
Placebo vs. 400 mg	2843387	1175829 to 4510945	***	0,0002
Placebo vs. 800 mg	4337412	2866026 to 5808799	****	<0,0001
24h				
Placebo vs. 50 mg	974188	-713084 to 2661461	ns	0,4946
Placebo vs. 100 mg	1379805	-154956 to 2914565	ns	0,0959
Placebo vs. 100 mg fed state	140517	-1383846 to 1664880	ns	0,9997
Placebo vs. 150 mg	2362284	1080265 to 3644302	****	<0,0001
Placebo vs. 200 mg	1797048	537397 to 3056699	**	0,002
Placebo vs. 400 mg	1824718	495250 to 3154186	**	0,0031
Placebo vs. 800 mg	2935548	1702991 to 4168105	****	<0,0001

Supplementary Table 4. Dunnett's multiple comparisons test on relative PKa activity.

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Summary	Adjusted P Value
Pre-dose				
2h				
Placebo vs. 50 mg	53,54	21,78 to 85,30	***	0,0007
Placebo vs. 100 mg	60,27	22,23 to 98,32	**	0,0017
Placebo vs. 100 mg fed state	45,74	1,439 to 90,05	*	0,0419
Placebo vs. 150 mg	82,11	53,87 to 110,4	****	<0,0001
Placebo vs. 200 mg	77,31	48,03 to 106,6	****	<0,0001
Placebo vs. 400 mg	87,94	59,83 to 116,0	****	<0,0001
Placebo vs. 800 mg	98,91	71,81 to 126,0	****	<0,0001
4h				
Placebo vs. 50 mg	36,4	-0,4556 to 73,26	ns	0,0533
Placebo vs. 100 mg	42,81	19,76 to 65,85	***	0,0002
Placebo vs. 100 mg fed state	39,81	18,67 to 60,94	***	0,0002
Placebo vs. 150 mg	67,62	46,67 to 88,57	****	<0,0001
Placebo vs. 200 mg	61,33	28,91 to 93,75	***	0,0005
Placebo vs. 400 mg	72,21	51,24 to 93,18	****	<0,0001
Placebo vs. 800 mg	80,11	58,96 to 101,3	****	<0,0001
9h				
Placebo vs. 50 mg	20,78	-31,51 to 73,08	ns	0,7792
Placebo vs. 100 mg	53,65	15,85 to 91,44	**	0,0036
Placebo vs. 100 mg fed state	51,91	10,44 to 93,38	*	0,0104
Placebo vs. 150 mg	63,14	21,06 to 105,2	**	0,0021
Placebo vs. 200 mg	59,89	17,16 to 102,6	**	0,004
Placebo vs. 400 mg	69,32	32,97 to 105,7	***	0,0002
Placebo vs. 800 mg	81,78	45,50 to 118,1	****	<0,0001
12h				
Placebo vs. 50 mg	28,46	-22,63 to 79,54	ns	0,4817
Placebo vs. 100 mg	42,22	-0,4727 to 84,92	ns	0,0535
Placebo vs. 100 mg fed state	45,68	4,124 to 87,24	*	0,0271
Placebo vs. 150 mg	61,25	15,32 to 107,2	**	0,0062
Placebo vs. 200 mg	60,31	12,54 to 108,1	*	0,01
Placebo vs. 400 mg	65,34	21,44 to 109,3	**	0,0023
Placebo vs. 800 mg	81,13	39,00 to 123,3	***	0,0001
24h				
Placebo vs. 50 mg	-11,72	-95,84 to 72,40	ns	0,9957
Placebo vs. 100 mg	30,52	-9,324 to 70,36	ns	0,1829
Placebo vs. 100 mg fed state	10,23	-21,46 to 41,91	ns	0,9152
Placebo vs. 150 mg	39,93	3,742 to 76,13	*	0,0267
Placebo vs. 200 mg	30,44	-5,592 to 66,48	ns	0,1228
Placebo vs. 400 mg	41,42	-3,416 to 86,25	ns	0,0761
Placebo vs. 800 mg	50,04	9,222 to 90,85	*	0,0133

II

Insight in bradykinin driven disease beyond hereditary angioedema

8

High occurrence of antihistamine resistance in patients with recurrent idiopathic angioedema

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ABSTRACT

Antihistamines are the most prescribed therapy in recurrent idiopathic angioedema, yet little is known about their efficacy. Herein, we report on clinical improvement with antihistamine therapy in 120 patients evaluating angioedema attack frequency. A high incidence (36%) of antihistamine refractory cases was observed. Forty percent of patients on antihistamine prophylaxis suffered from 1 or more angioedema attacks per month. Our findings stress the need for additional treatment options for recurrent idiopathic angioedema.

INTRODUCTION

Angioedema is characterized by swelling of subcutaneous or mucosal tissue that may last up to 72 hours and often recurs. Swellings can be disfiguring and lead to impaired functioning and quality of life[1]. Upper airway swellings require immediate medical care[2,3]. Angioedema may be driven by bradykinin and/or mast-cell mediators including histamine. A disease classification is made based upon the underlying disease mechanism[4]. Bradykinin induced angioedema includes all types of hereditary angioedema, acquired C1-esterase inhibitor deficiency and angiotensin converting enzyme (ACE) inhibitor induced angioedema. Mast-cell mediator induced angioedema includes IgE mediated allergic angioedema and non-IgE mediated angioedema with urticaria. When no underlying cause can be identified, idiopathic angioedema is diagnosed[4]. The disease mediator of idiopathic angioedema is unknown but the disease is considered as part of the spectrum of chronic spontaneous urticaria and suspected to be mast-cell mediator induced[4,5].

Antihistamines are first line therapy for idiopathic angioedema although their efficacy was never established in randomized control trials. Only two observational studies in a cohort of Italian angioedema patients report that 84% of patients are diagnosed as idiopathic histaminergic angioedema, i.e. respond to antihistamines[6,7]. To what extent patients respond in terms of reduction in angioedema attack frequency and severity is not reported, leaving clinicians with limited information on the most commonly prescribed therapy for angioedema.

We undertook a retrospective evaluation of therapeutic management of recurrent idiopathic angioedema with antihistamines in a tertiary treatment center. Attack frequency and need for acute treatment during follow-up are reported, as well as usage of add-on therapy.

METHODS

We performed a retrospective analysis of medical records in all patients diagnosed with recurrent angioedema who visited our Dermatology/Allergology out-patient clinic between January 2008 and April 2017. The study was approved by the medical ethical committee at the University Medical Center Utrecht and the need for individual patient consent was waived.

Patients were included when they experienced: recurrent spontaneous angioedema, duration of disease of >6 weeks and had at least one follow-up visit to evaluate therapeutic management. Main exclusion criteria were other disease subtypes than idiopathic angioedema (e.g. angioedema with urticaria, hereditary angioedema, allergic angioedema, ACE-inhibitor induced angioedema and acquired C1-esterase inhibitor deficiency angioedema). Based on the treating physicians' evaluation, antihistamine prophylaxis was started or increased in dose. After approximately 4 weeks, symptoms were evaluated and where necessary adjusted and followed-up. Various types of mainly second generation H1-receptor antagonists were prescribed. Clinical characteristics, including historical attack

frequency, were collected at first visit and at evaluation of maximum prescribed therapy. Attack frequency was categorized as ≥1 per week, ≥1 per month but <1 per week, ≥1 per year but <1 per month and no further attacks. Improvement was defined as shift into a lower attack frequency category. Patients reporting no improvement or receiving add-on therapy such as omalizumab or cyclosporine, were defined as antihistamine-refractory or 'non-responder'. To assess attack severity, necessity for acute treatment was scored based on treatment intensity ranging from: (additional) antihistamines and/or corticosteroids, adrenaline auto-injector, emergency medical care to hospital or intensive care admission. GraphpadPrism7.04 was used for statistical analysis. Groups were compared with Mann-Whitney test or Wilcoxon test in case of paired samples.

RESULTS

511 medical records were screened, and 120 patients included. Patients' characteristics are described in Table 1. Most common site of angioedema was the face (n= 105; 88%). Alarmingly, 28 patients (23%) reported to have experienced suspected laryngeal angioedema described as having dyspnea, difficulty swallowing or sudden changing voice.

At first visit or during follow-up, 99 patients started on antihistamine prophylaxis or had a dose increase. 21 patients did not receive prophylaxis, or their dose prescribed prior to first visit was not increased. The majority of patients (54%) was followed-up for >6 months. Details on therapy and attack frequency in all patients are listed in Table 2.

We evaluated change in attack frequency during follow-up. In 95 of the 120 patients attack frequency at first visit and during maximal prescribed antihistamine therapy was reported (missing data at first visit n=15, missing data during follow-up n=17, Table 2). During follow-up, attack frequency decreased significantly ($p<0.0001$, Figure 1A) in patients receiving intervention. A non-significant reduction in attack frequency was observed in patients not receiving prophylactic antihistamine therapy or a dose escalation ($p=.09$). Of these 17 patients that did not receive intervention and had data on attack frequency reported, 59% improved (Figure 1B). This demonstrates that perceived effectiveness of antihistamine therapy may in part reflect natural disease remission. However, it should be noted that these patients in general had a lower attack frequency at first visit ($p=0.01$, Figure 1A) and often reported an emergency care visit or hospitalization in their history (55%) therefore, comparison with the intervention group should be made with caution.

Of the 78 patients that received prophylactic antihistamine therapy, 50 patients (64%) improved, in 28 patients (36%) attack frequency did not improve and even got worse in 4 patients (5%). It should be noted that 9 patients who did not improved only received a 1 or 2-fold daily dose and may have benefitted from higher doses. However, a significant proportion of patients experienced frequent attacks even at high doses of antihistamines (Figure 1C). Among all patients receiving antihistamine prophylaxis (n=99, including those with missing data on attack frequency at first evaluation) 40% suffered from at least 1 attack per month (Table 2), stressing that antihistamine therapy often fails to fully suppress symptoms.

Table 1. Clinical characteristics

	Total group (n=120)
Female (%)	78 (65%)
Male (%)	42 (35%)
Mean age at first consult (range)	
All patients	51 (13-86)
Duration of disease (%)	
<1 year	44 (37%)
1 - <5 years	50 (42%)
5 - <10 years	14 (12%)
10 years or >	12 (10%)
Family history with AE; first grade family member* (%)	
Unknown	51 (43%)
No	59 (50%)
Yes	10 (8%)
Referral (%)	
General practitioner	63 (53%)
Secondary or tertiary treatment center	57 (48%)
Locations of AE attacks (%)	
Facial	105 (88%)
Oropharyngeal	93 (78%)
Laryngeal	28 (23%)
Abdominal	8 (7%)
Peripheral	37 (31%)

*Hereditary angioedema based on C1 esterase deficiency was excluded in patients with a positive family history by means of C4 screening

Fifty (42%) patients reported that prior to the first visit they had sought immediate medical attention for at least one attack (Table 2). 7 patients reported admission to the hospital or intensive care because of a severe attack and one patient required intubation. During follow-up only 5 (4%) patients sought immediate medical care; 2 did not receive antihistamine prophylaxis and 3 were on ≥4-fold daily dose. No patients were admitted to the hospital. This decreased incidence of need for acute treatment suggests relatively well controlled disease in patients on antihistamine prophylaxis.

Eleven antihistamine-refractory patients received add-on therapy and had their attack frequency reported. Nine patients received omalizumab: 7 of 9 patients improved. 5 went into full remission, 1 improved to <1 attack per month. In contrast, 3 patients had ≥1 attack per month despite omalizumab therapy, indicating moderate responsiveness in one patient that went from weekly to monthly attacks and no response in the other 2 cases

Table 2. Prophylactic treatment, angioedema attack frequency and necessity for acute attack treatment at baseline and follow-up.

	Baseline (n=120)	**Follow-up (n=120)**
Prophylactic treatment (%)		
Unknown	3 (3%)	0 (0%)
None	62 (52%)	18 (15%)
Antihistamine mono-therapy	45 (38%)	87 (73%)
	27 (23 %)	17 (14%)
One daily dose	11 (9%)	30 (25%)
Twofold daily dose	2 (2%)	7 (6%)
Threefold daily dose	5 (4%)	21 (18%)
Fourfold daily dose	0 (0%)	12 (10%)
> Fourfold daily dose	7 (6%)*	15 (13%)***
Antihistamines + add-on	3 (3%)**	0 (0%)
Other treatment		

	Baseline (n=120)	**All n=120**	**No intervention n=21†**	**Antihistamines only n=99**	**+ Add-on n=99**
Attack frequency (%)					
Unknown	17 (14%)	15 (13%)	3 (14%)	11 (11%)	12 (12%)
≥ 1 per week	42 (35%)	15 (13%)	2 (10%)	17 (17%)	13 (13%)
≥ 1 per month	44 (37%)	24 (20%)	4 (19%)	23 (23%)	20 (20%)
≥ 1 per year	17 (14%)	30 (25%)	6 (29%)	23 (23%)	24 (24%)
No attack	0 (0%)	36 (30%)	6 (29%)	25 (25%)	30 (30%)
Acute attack treatment (%)††					
Unknown	15 (13%)	18 (15%)	3 (14%)	15 (15%)	
None	11 (9%)	51 (43%)	6 (29%)	45 (45%)	
1. Antihistamines	25 (21%)	26 (22%)	8 (38%)	18 (18%)	
2. Antihistamines and/or corticosteroids	14 (12%)	17 (14%)	2 (10%)	16 (16%)	
3. Epipen	5 (4%)	3 (3%)	1 (5%)	2 (2%)	
4. Urgent care center	43 (36%)	5 (4%)	2 (10%)	3 (3%)	
5. Hospitalization or Intensive care	7 (6%)	0 (0%)	0 (0%)	0 (0%)	

*Antihistamines combined with leukotriene antagonist (n=2), corticosteroids (n=3), H2 antagonist (n=1), tranexamic acid (n=1).

**Monotherapy with tranexamic acid (n=2) or H2 antagonist (n=1).

*** Antihistamines combined with omalizumab (n=8), omalizumab and tranexamic acid (n=1), leukotriene antagonist (n=1), cyclosporine (n=1), tranexamic acid (n=2), sulfalazine (n=1), H2 antagonist (n=1).

†no prophylaxis (n=18) no antihistamine dose increase (n=3)

†† overview of the most invasive reported symptomatic treatment used by patients during an acute attack including the reported need for medical care where invasiveness was reported from 1 to 5.

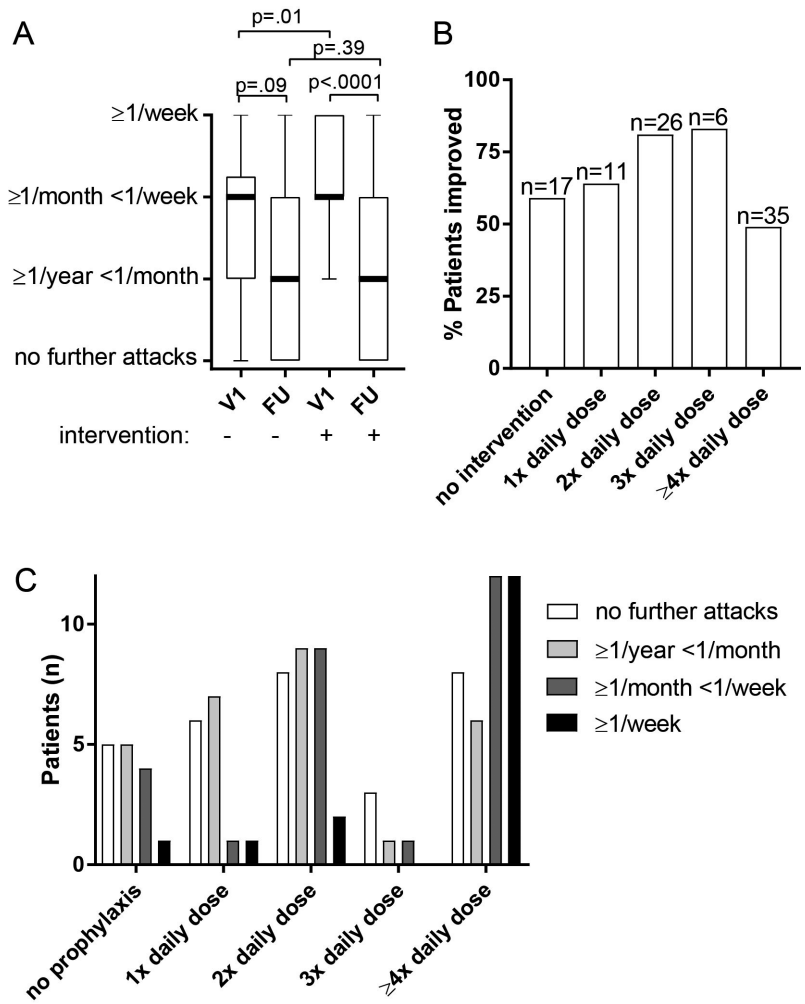

Figure 1. Attack frequency in relation to antihistamine prophylaxis. Attack frequencies were evaluated at maximum antihistamine dose prescribed (prior to add-on therapy) A) Box-and-whiskers-plot of attack frequency at first visit (V1) and follow-up(FU) with (+),or without intervention (-), bold line=median, Mann-Whitney and Wilcoxon test were used comparing groups respectively paired samples. B) Percentage of patients reporting improvement per maximum dose antihistamines prescribed, improvement was defined as shift into a lower attack frequency group, n=total patients per group, patients with missing data on attack frequency could not be evaluated for improvement (n=25). C) Attack frequency per maximum dose prescribed.

DISCUSSION

Overall, the majority of our cohort (64%) improved under prophylactic therapy with antihistamines. However, 40 (40%) patients still experienced ≥1 attack per month despite treatment. Previous studies reported that 15-16% of idiopathic angioedema patients were 'non-histaminergic' i.e. were antihistamine-refractory, compared to 36% within the current

study. Notably, in these two previous studies, lower doses of antihistamines were prescribed (up to two-fold daily dose)[6,7]. It was previously observed that chronic spontaneous urticaria patients may benefit from a four-fold maximum daily dose or even higher[8]. Differences in prevalence of antihistamine-refractory patients may be explained by different definitions of improvement. Clinical evaluation of angioedema is complicated by variability in natural course of disease. In addition, antihistamine treatment was often (46%) already started in primary or secondary care. Moreover, patients that only came for an initial visit but were then loss to follow-up, possibly due to good response, were excluded. This could potentially contribute to an over-representation of antihistamine-refractory patients in our cohort.

Our findings underline the urgent need for additional treatment options in idiopathic angioedema as monotherapy with antihistamines is often insufficient. As the underlying mechanism of idiopathic angioedema is unknown add-on therapy is based on a trial-and-error. In this study, add-on therapy with omalizumab, a monoclonal antibody targeting IgE, resulted in complete disease remission in 5 out of 9 patients. So far, case reports describe a total of 23 patients with antihistamine refractory idiopathic angioedema on omalizumab that all showed complete remission[9]. Alternatively, other case reports describe successful use of therapy commonly prescribed in patients with hereditary angioedema; such as C1-esterase inhibitor concentrate, bradykinin receptor antagonist and kallikrein inhibition[10]. Hence, these therapies constitute potential treatment options for antihistamine-refractory patients, but their efficacy should be further investigated in clinical trials.

In conclusion, in this retrospective study we observed a high incidence of anti-histamine refractory patients. This stresses further efforts in exploring novel treatment options for idiopathic angioedema.

DECLARATIONS

Ethics approval

The study was approved by the local medical ethical committee at the University Medical Center Utrecht and the need for individual patient consent was waived.

Consent for publication

Not applicable since no details on individual person's data were used.

Availability of data and materials

Contact the corresponding author H Röckmann for an anonymized database of this study.

Competing interest

CM is inventor and has a financial interest in SERPINx BV.. CEH has a financial interest in Prothix BV. AK received research funding from Novartis and is a member of the national and international advisory board from Novartis for CSU. HR is a member of the national advisory board from Novartis for CSU. ZH and NW have no conflict of interest to declare.

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AUTHORS CONTRIBUTIONS

ZH prepared the first draft of this manuscript, NW reviewed all medical records. All authors read and approved the final manuscript.

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REFERENCES

1. Sussman G, Abuzakouk M, Bérard F, et al. Angioedema in chronic spontaneous urticaria is underdiagnosed and has a substantial impact: Analyses from ASSURE-CSU. *Allergy*. 2018;73(8):1724-1734. doi:10.1111/all.13430
2. Bork K, Barnstedt S-E. Laryngeal edema and death from asphyxiation after tooth extraction in four patients with hereditary angioedema. *J Am Dent Assoc*. 2003;134(8):1088-1094. http://www.ncbi.nlm.nih.gov/pubmed/12956349.
3. Crochet J, Lepelley M, Yahiaoui N, et al. Bradykinin mechanism is the main responsible for death by isolated asphyxiating angioedema in France. *Clin Exp Allergy*. 2019;49(2):252-254. doi:10.1111/cea.13297
4. Maurer M, Magerl M, Ansotegui I, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. *Allergy*. 2018;73(8):1575-1596. doi:10.1111/all.13384
5. Zuberbier T, Aberer W, Asero R, et al. The EAACI/GA2LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: The 2013 revision and update. *Allergy Eur J Allergy Clin Immunol*. 2014;69(7):868-887. doi:10.1111/all.12313
6. Zingale LC, Beltrami L, Zanichelli A, et al. Angioedema without urticaria: a large clinical survey. *CMAJ*. 2006;175(9):1065-1070. doi:10.1503/cmaj.060535
7. Mansi M, Zanichelli A, Coerezza A, et al. Presentation, diagnosis and treatment of angioedema without wheals: a retrospective analysis of a cohort of 1058 patients. *J Intern Med*. 2015;277(5):585-593. doi:10.1111/joim.12304
8. van den Elzen MT, van Os-Medendorp H, van den Brink I, et al. Effectiveness and safety of antihistamines up to fourfold or higher in treatment of chronic spontaneous urticaria. *Clin Transl Allergy*. 2017;7:4. doi:10.1186/s13601-017-0141-3
9. Bucher MC, Petkovic T, Helbling A, Steiner UC. Idiopathic non-histaminergic acquired angioedema: a case series and discussion of published clinical trials. *Clin Transl Allergy*. 2017;7:27. doi:10.1186/s13601-017-0164-9
10. van den Elzen M, Go MFCL, Knulst AC, Blankestijn MA, van Os-Medendorp H, Otten HG. Efficacy of Treatment of Non-hereditary Angioedema. *Clin Rev Allergy Immunol*. 2018;54(3):412-431. doi:10.1007/s12016-016-8585-0

9

Recombinant human C1 esterase inhibitor for prophylaxis of idiopathic non-histaminergic angioedema: a case series

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ABSTRACT

Background

Idiopathic non-histaminergic angioedema is a disorder with recurrent swelling episodes that do not respond to antihistamine therapy. The underlying cause of this disease is unknown. Bradykinin, a vasoactive peptide that causes swelling in hereditary angioedema, is suspected to be involved. We evaluated the effects of recombinant human C1 inhibitor (rhC1-INH) to prevent attacks of idiopathic non-histaminergic angioedema.

Methods

We conducted a single center, consecutive, cross-over, case-series study. Adult patients were included who had no apparent cause for angioedema and who had at least 2 attacks per month in the 6 months prior to inclusion that were unresponsive to prophylactic treatment with 4 times the standard daily dose of antihistamines. Eligible patients were observed for 4 months. During months 2 and 3 (treatment period) they received intravenous rhC1-INH (50 IU/kg; maximum 4200 IU) twice weekly, while month 1 and 4 served as control periods. The primary endpoint was the number of angioedema attacks during the treatment period. Attacks and severity of attacks were recorded daily using the angioedema activity score. The angioedema-related quality of life score was recorded monthly. In addition, blood samples were collected at 5 time points during treatment and control periods in search for disease-related biomarkers. Markers of contact activation, inflammation and fibrinolysis were measured. This study is registered with EudraCT number 2016-005083-34.

Results

Three patients were enrolled between 12-03-2018 and 9-7-2018. One patient experienced 2 attacks during the treatment period versus 14 attacks during the control period, of which 1 attack during treatment occurred following a missed dose 6 days after receiving the previous dose. A second patient had 4 attacks during the treatment period versus 7 during the control period. The third patient had 7 attacks during the treatment period versus 3 attacks during the control period. Therapy was well-tolerated. No biomarkers for disease or disease severity were identified, except for possibly D-dimer levels, which were elevated in 2 patients.

Conclusion

Clinical responses of the 3 patients with idiopathic non-histaminergic angioedema described in this report to twice weekly rhC1-INH treatment was variable. Further studies are needed to establish the place of rhC1-INH in the treatment of idiopathic non-histaminergic angioedema.

INTRODUCTION

Angioedema is characterized by episodes with submucosal and subcutaneous swelling. Swelling attacks can occur everywhere in the body, but most often affects lips, eyelids and tongue[1,2]. Besides pain and disfigurement, angioedema can lead to functional impairment such as dysphagia and dyspnea and is potentially lethal when the airway is compromised. An episode with angioedema can last for hours up to days, and have a great impact on everyday life[3,4].

The pathophysiological mechanism explaining the occurrence of angioedema is not fully understood. Two vasoactive peptides are considered as key players in acute swelling; histamine and bradykinin.

Histamine released by mast cells and basophils is the supposed vasoactive compound in allergic angioedema and angioedema associated with chronic spontaneous urticaria(CSU)[3]. Bradykinin generated upon activation of the contact system is the supposed vasoactive compound in other forms of angioedema. The contact system comprises factor XII (FXII), plasma prekallikrein (PK) and high molecular weight kininogen (HK). Active FXII (FXIIa) activates PK into plasma kallikrein (PKa) that liberates bradykinin from HK[5]. Bradykinin is associated with hereditary angioedema (HAE) and angiotensin converting enzyme (ACE) induced angioedema[4]. The mediator of idiopathic angioedema is unknown, histamine involvement is likely as 64-85% of patients improve on antihistamine therapy[6,7]. Patients that do not respond to antihistamine therapy are diagnosed as idiopathic non-histaminergic angioedema[6,8]. Bradykinin is suspected as mediator of disease. Elevated levels of bradykinin were detected in four patients with idiopathic non-histaminergic angioedema during acute attacks. Moreover, case studies report that patients with idiopathic non-histaminergic angioedema may benefit from therapies interfering with bradykinin formation or activity such as C1-INH infusions and a bradykinin receptor antagonist[9,10]. These therapies have been developed for hereditary angioedema (HAE) as most patients with this disease lack or have dysfunctional C1-INH (called HAE-1 respectively 2) resulting in uncontrolled bradykinin release[4,11].

Recombinant human C1-INH (rhC1-INH) is an effective treatment for acute attacks and prophylaxis of angioedema in HAE type 1 and 2[12–15]. A randomized controlled crossover trial demonstrated that twice weekly infusion with 50 IU/kg (max 4200 IU) resulted in a mean difference of -4.4 attacks per month[15]. Moreover, therapy is safe and well tolerated. We aimed to find evidence for effectivity of rhC1-INH prophylaxis in idiopathic non-histaminergic angioedema in a consecutive case series of patients.

In addition, we evaluated potential biomarkers for idiopathic non-histaminergic angioedema to identify biomarkers for disease, disease severity or therapy response. Markers evaluated included cleaved HK (cHK), a byproduct of bradykinin release[16]; FXIIa- and PKa-C1-INH complexes, which reflect activation of these enzymes, and their subsequent inhibition by C1-INH[17]; C4 levels and C1-INH function, both markers of C1-INH dysfunction or deficiency in HAE[4]; the fibrin breakdown product D-dimer, which is commonly associated with thrombotic

events, but is also elevated in HAE[18] and CSU[19]; and total immunoglobulin E (IgE), C-reactive protein (CRP) and leukocyte count as general markers of (allergic) inflammation.

METHODS

Study design and participants

This single center, consecutive, cross-over case-series study was performed at the dermatology and allergology department of the University Medical Center Utrecht in the Netherlands. All patients enrolled were observed for 4 months. Month 1 served as a control period during which patients were observed without receiving rhC1-INH. Then followed a two month-treatment period (months 2 and 3; treatment period) during which they received rhC1-INH twice weekly. Finally, this was followed by one month-period (months 1 and 4; control period) during which they did not receive rhC1INH (Figure 1A).

Patients were eligible when they 1) were 18 years or older; 2) had angioedema attacks despite treatment with 4 times the standard daily dose of antihistamines; 3) had no identifiable allergic or other cause of their angioedema; 4) had at least 2 attacks per month during the past six months, with at least one attack in the month prior to treatment; and

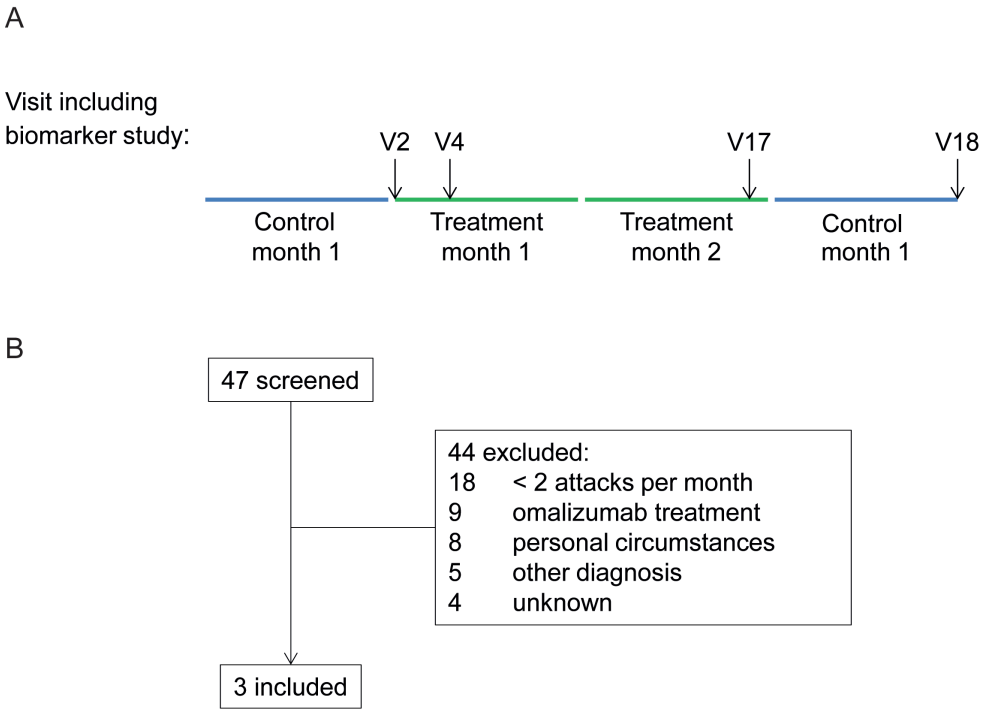

Figure 1. Study design and flow-chart of inclusions. A) Treatment (blue lines) and control (green lines) sequence, each line represents a 4-week time frame. During treatment patients received rhC1-INH 50 IU/mL twice weekly, arrows indicate visit were blood was drawn for biomarkers studies. B) Flow-chart of screening, inclusion, exclusion and (main) reason for exclusion.

5) had signed written informed consent. Patients were excluded when they 1) had wheals accompanying angioedema; 2) had used ACE-inhibitors in the past 6 months; 3) received therapy with omalizumab, cyclosporine, methotrexate, azathioprine or mycophenolic acid; 4) had rabbit allergy; 5) were pregnant or gave breastfeeding; 6) had a condition that could compromise the safety of the patient such as renal or hepatic insufficiency or malignancies; 7) likely suffered from another condition (eg allergic angioedema, drug-hypersensitivity, mastocytosis or HAE). Standard HAE screening at our center comprised patient history and C4 levels; in addition, C1-INH function was determined as one of the study markers.

Patients could continue prophylactic treatment with antihistamine therapy or antifibrinolytic therapy. They also continued to use rescue medication during acute angioedema attacks as prescribed by their treating physician which mostly consisted of increasing doses of antihistamines, oral steroids and intramuscular adrenaline.

Approval for this study by the local medical ethical committee at the UMC Utrecht and the central committee for research involving human subjects (Centrale Commissie Mensgebonden Onderzoek, CCMO) was obtained.

Procedures

Two vials of lyophilized rhC1-INH powder were reconstituted with 14 mL sterile water for injection each and mixed gently. The calculated amount of solution (50 IU/kg; max 4200 IU) was aspirated into two 20 mL syringes 30 minutes prior to drug administration and kept at room temperature until use. The drug was administered intravenously via a winged infusion set over a time course of 5 minutes. In case a patient was unable to attend a treatment visit, the drug was scheduled to be administered the next day or rescheduled after the last treatment for a maximum of two missed administrations, prolonging the treatment period by a maximum of one week.

Blood was drawn at inclusion (visit 1), at the final visit (visit 18) and at the first (visit 2), third (visit 4), and last treatment visit (visit 17) prior to drug infusion. Blood (9 volumes) was collected in standard sodium citrate tubes (1 volume sodium citrate, 3.2% w/v), EDTA tubes (1.8 mg EDTA per mL blood) and specific sample collection/anticoagulant tubes (SCAT) containing 25 μM PPACK (Phe-Pro-Arg-chloromethylketone), 11 mM sodium citrate, 0.1 % Mannitol (w/v). Within one to two hours after collection blood tests were performed, or plasma was collected, aliquoted and stored at -80 until use. A urine sample was collected at first visit in women under the age of 50 to exclude pregnancy via urine βHCG

The attack frequency and severity were recorded in the angioedema activity score (AAS) form on a daily basis. The AAS measures severity of angioedema with a daily survey questioning if angioedema was present followed by an assessment on duration, physical symptoms, daily activities, influence on appearance and overall severity[20]. Weekly AAS results (AAS7; range 0-105) were used to determine AAS scores per 4 weeks (AAS28; range 0-420). In addition, the research physician recorded attack date, location, rescue treatment used and possible triggers for the attack in the patient's medical record. An attack was separated from a subsequent attack when there had been an angioedema-free period between reported

swellings on subsequent days as scored on the AAS form. The angioedema-related quality of life (AE-QoL) questionnaire was scored each month. The AE-QoL assesses the influence on the overall quality of life and separate domains comprising of functioning, fatigue/mood, fear/shame and nutrition. The AE-QoL questionnaire score ranges from 0-100% where 0% indicates angioedema has no influence on (this domain of) quality of life[21].

A detailed medical history and physical examination were recorded at first visit and adverse events and concomitant drug use were registered at each following visit. As part of thrombogenic risk assessment the Wells evaluation for deep vein thrombosis (score of 1 or more required further testing) and pulmonary embolism (score of 2 or more required further testing) were performed. The occurrence of angioedema and potential medical intervention for angioedema including hospitalization were not considered (serious) adverse events but were recorded separately.

Laboratory assessments

C4 levels and total IgE were determined at visit 1. Women <50 years of age were tested for urine βHCG by dipstick test to exclude pregnancy. At visits 2,4,17 and 18, CRP, D-dimer levels, and leukocyte counts were determined. C1-INH function was determined at visits 2,4,17 and 18 (Sanquin, the Netherlands) with a chromogenic C1s assay. In this assay, samples are incubated with a fixed amount of active C1s and residual C1s activity was measured. Further analyses were performed in our research laboratory with plasma collected at visits 2,4,17 and 18. C1-INH, FXII, PK and HK antigen were visualized with immunoblotting. For this, SCAT collected plasma was diluted 40 times in reducing sample buffer (15.5% glycerol, 96.8 mM Tris-HCL, 3.1% SDS, and 0.003% bromophenol blue, 25 mM DTT), boiled for 10 minutes, and 5 to 10 µL per samples was loaded and ran on a 4-12% Bis-Tris gel at 165V for 60 minutes and transferred onto Immobilon-FL membranes at 125V for 55 minutes. For detection polyclonal goat anti human IgG antibodies (anti-human FXII CI20055AP, anti-human PK CI20090A, anti-human HK CI20027AP, anti-human C1-INH CL200323AP, Cedarlane, Burlington, Canada) and Alexa Fluor 680 donkey anti-sheep IgG (lot#1878516, Dako, Glostrup, Denmark) were used.

cHK[16], a marker for bradykinin release and C1-INH in complex with FXIIa or PKa, markers for FXII and PK activation[22], were determined in SCAT plasma via ELISA partly using in-house produced nanobodies as described before, upper normal limit was based upon previous measurements in ~50 healthy individuals for cHK and ~20 healthy controls for C1-INH complexes. In addition, a C1-INH-PKa trigger assay was set-up to evaluate the capacity of C1-INH in plasma to form C1-INH-PKa complexes. For this assay, 50 µL citrated plasma was added into 1.2 mL polypropylene tubes (BRAND, Wertheim, Germany) and placed at 37C for 5 minutes. Next, 25 µL PKa (15 µg/mL) dissolved in HEPES-buffered saline (HBS; 10 mM HEPES, 150 NaCl, pH=7.4) was added to plasma. Reaction was stopped after 1 minute by further diluting the sample in mHBSt (HBS containing 1% skimmed milk powder (w/v), 0.1% Tween-20 w/v) containing 200 µM PPACK ((Phe-Pro-Arg-chloromethylketone, Haematologic Technologies, Essex Junction, VT, USA) to a final plasma dilution of 1:32. For reference, pooled plasma for ~30 healthy volunteers was activated with 30 µg/mL dextran sulphate,

MW 500.000, at 37C or HBS, reaction was stopped after 30 minutes in mHBSt containing 200 µM PPACK (Haematologic Technologies) to a final plasma concentration of 1:32. Dextran sulphate-activated plasma was defined as 100% activated plasma and HBS activated plasma as 0% activated plasma. Diluted 100% and 0% activated plasma samples were mixed to obtain samples with varying amounts of PKa-C1-INH complexes. PKa-C1-INH complexes were determined with ELISA as described by de Maat and colleagues[22].

Outcomes

The primary outcome was the number of angioedema attacks observed in the 2-month treatment period compared with the 2-month control period. Clinical response was defined as a reduction in attack frequency of 50% in the treatment period compared to the control period. Secondary outcomes were the AAS and AE-QoL scores during the treatment period compared to control period. Outcomes were assessed per case.

Data analysis

As this is a case-series study no statistical test were performed other than descriptive statistics. Descriptive statistics and graphical demonstration of clinical data and laboratory outcomes were performed with GraphPad Prism 8 software.

Role of the funding source

The study was design and performed by the study team of the UMC Utrecht at the Dermatology and Allergology clinic. Pharming Technologies, the subsidizing partner was informed on the study protocol and notified regarding inclusions and progress in order to organize drug delivery. In addition, Pharming Technologies, donated 6 months of treatment after the last study visit for one patient (referred to as case 1 or patient 1).

RESULTS

Patients with idiopathic angioedema suffering from frequent attacks visiting the dermatology and allergology outpatient clinic at the UMC Utrecht or patients responding through a newsletter of the Dutch patient society for angioedema, were screened for eligibility. Between March 2018 and November 2018 47 patients were screened; in 18 patients attack frequency was <2 per month; 9 patients were using omalizumab; 8 patients could not visit the clinical center twice weekly because of personal reasons; 5 patients did not meet a diagnosis of idiopathic non-histaminergic angioedema, but suffered from CSU with angioedema and wheals, idiopathic generalized edema or ACE-induced angioedema; finally, 4 patients refused to participate without further explanation. Three patients were found eligible during initial screening and visited the hospital to be enrolled in the study after finalizing the screening visit (Figure 1B).

Case 1

A 31-year-old woman presented with recurrent angioedema for 4 years. Her medical history reported an oral allergy syndrome (including allergies to apple, various nuts and carrot), allergic rhinitis, asthma, eczema, latex and penicillin allergy. Swellings evolved spontaneously, were non-pruritic, occurred in the face and upper airways and lasted for several hours up to 5 days. Initially, the patient experienced one or less attacks per month but over the last 6 months prior to study inclusion attack frequency increased to 3 swellings per month. Angioedema was not accompanied by wheals or signs of anaphylaxis. Fatigue was reported as a prodromal symptom. Prophylactic therapy with 4 times daily dose of a second-generation antihistamine did not improve her angioedema attacks. Symptoms from allergic rhinitis included itchy, puffy eyelids, that improved with antihistamine therapy were recognized by the patients as a distinct symptom from angioedema. Acute treatment consisted of oral steroids and intramuscular adrenaline injections which usually resulted in improvement of symptoms within hours up to 4 to 5 days. Swellings were not influenced by estrogen containing anti-contraceptives that were used in the past. There was no family history of angioedema and C4 levels were 0.30 g/L (normal range 0.1-0.47 g/L).

Despite angioedema and accompanying fatigue, the patient strived to minimize absence from work or other activities. However, frequent visits to the emergency care department for upper airway swellings strongly interfered with daily activities.

During the control period of 2 months the patient experienced 14 angioedema attacks (Figure 2) both in the first- and second-month upper airway swellings needed to be treated and observed at the emergency care department. The AAS28 during the control months was 58 for the first and 175 for the second month of the control period (Figure 3A). During the treatment period the patient experienced two attacks, of which one occurred after a treatment interval of 6 days due to a missed dose. The AAS28 was 8 respectively 21, and when corrected for the swelling following the missed dose 8 and 0. The AE-QoL questionnaire scores are represented in Figure 4A, most improvement was reported on functioning during the two treatments months. Therapy was tolerated well; two episodes of headache were mentioned of which one was during the treatment period that can possibly be related to rhC1-INH treatment.

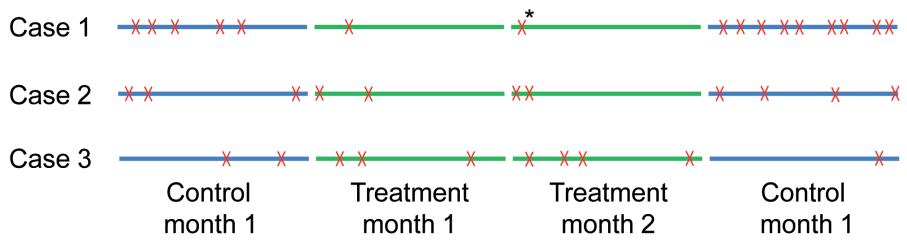

Figure 2. Attack frequency per study case. Treatment (blue lines) and control (green lines) sequence, each line represents a 4-week time frame, red cross indicates an angioedema attack. *) this attack occurred after a missed gift and 5 days after the previous received gift.

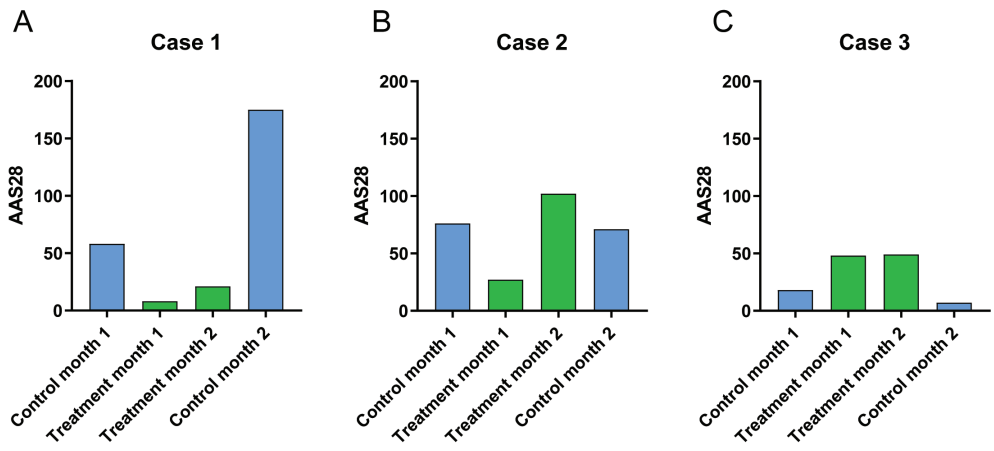

Figure 3. Angioedema activity score per case. AAS28 scores for A) case 1 B) case 2 C) case 3, blue bars indicate AAS28 during an control month, green bars AAS28 during a treatment month. * this bar indicates the severity of a single attack that occurred after a missed gift and 5 days after the previous received gift.

After the final study visit the patient continued treatment with rhC1-INH for six months during which she remained symptom-free. Cessation of treatment resulted in an immediate relapse of symptoms with an average attack frequency of 3 per month. The patient was admitted to the intensive care once after cessation of rhC1-INH, where symptoms improved after administration of C1-INH infusion. Monthly treatment with 300 mg omalizumab for three months did not improve symptoms and was accompanied by side effects including malaise and sore muscles. Prophylactic treatment with C1-INH was continued.

Case 2

A 37-year-old woman presented with recurrent angioedema for 7 months. Her medical history reported oral allergy syndrome (including allergies to various nuts and apple), allergic rhinitis and unexplained arthralgia. Start of angioedema initially appeared to relate to an episode with joint pain and increased intake of non-steroidal anti-inflammatory drugs (NSAIDs). The patient was screened for autoimmune disorders, vasculitis and possible underlying malignancies but no explanation for her symptoms were found. After spontaneous resolution of joint pain and cessation of NSAIDs, recurrent idiopathic angioedema remained. Angioedema evolved spontaneously and was often already present at awakening. Swellings were non-pruritic and sometimes accompanied by a tingling sensation, they occurred in the face and upper airways and hands and were not accompanied by wheals or symptoms of anaphylaxis. Attack frequency had increased from one to up to 8 attacks per month with an average of 4 attack per month in the previous 6 months. No prodromal symptoms were reported but angioedema was accompanied by malaise. Prophylactic therapy with 4 times daily dose of a non-sedating antihistamine did not improve angioedema. Acute treatment

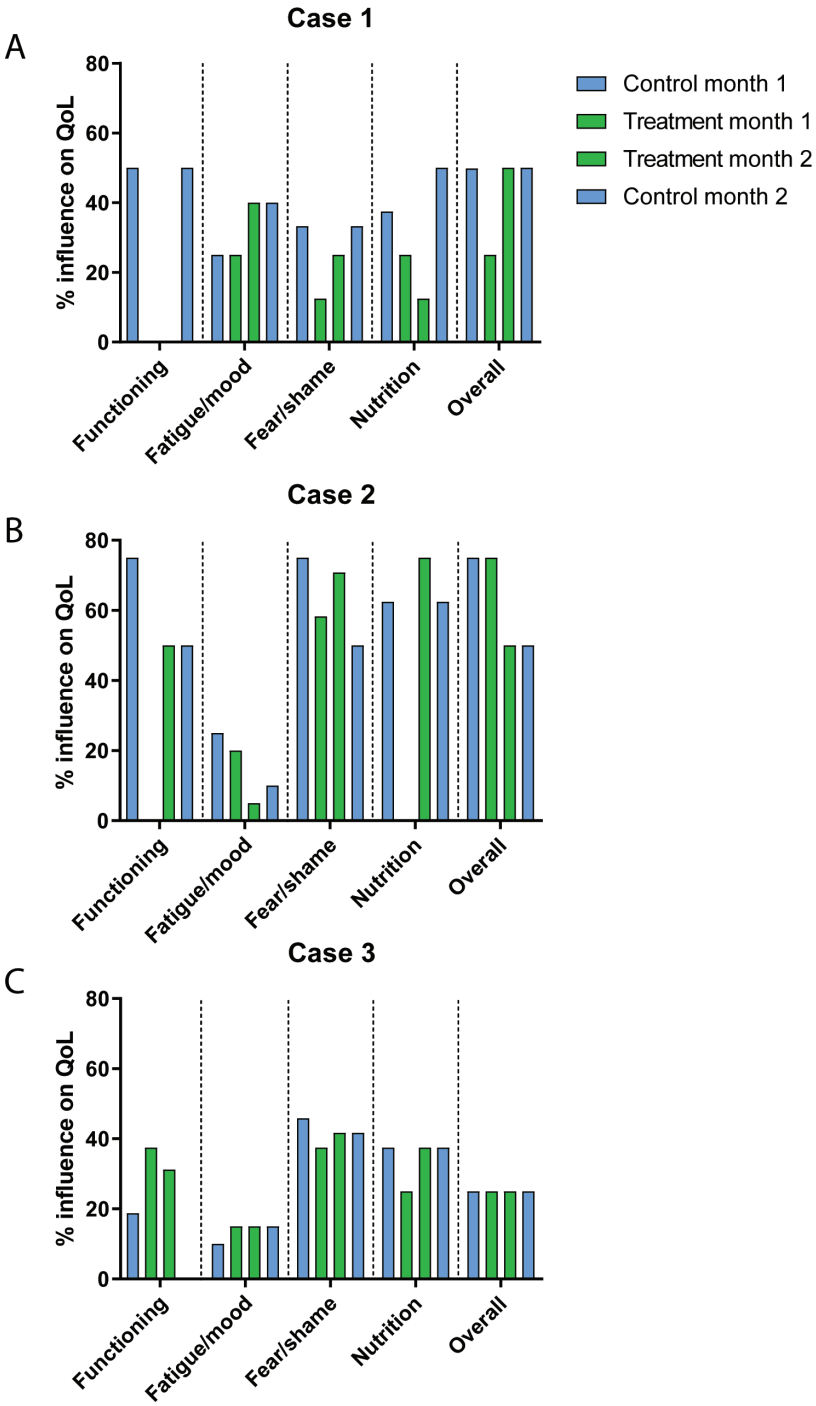

Figure 4. Angioedema related quality of life score per case. AE-QoL questionnaire scores for A) Case 1 B) Case 2 C) Case 3. AE-QoL ranges from 0-100% were 0 indicates no negative influence on life, blue bars indicate AE-QoL during an control month, green during a treatment month.

consisted of oral steroids and a sedating antihistamine; this treatment had variable result but in general failed to improve symptoms. Swellings did not relate to intake of an estrogen containing anti contraceptives or previous pregnancies. There was no family history of angioedema and C4 levels were 0.15 g/L (normal range 0.10-0.47 g/L). Angioedema strongly influenced the patient's life and contributed to a temporary disability leave from work.

During the 2 months of the control period the patient experienced 7 angioedema attacks (Figure 2). The AAS28 was 76 during the first and 71 during the second control month (Figure 3B) During the treatment months the patient experienced 4 angioedema attacks. One mild swelling evolved in the lips after the first dose of rhC1-INH, a second swelling was accompanied by an episode of allergic rhinitis. A third mild swelling of the face occurred that resolved within a few hours. However, a fourth swelling of the face that was again mild continued into ongoing evolving and decreasing mild swelling of the face lasting for eleven days. The AAS28 was 27 during the first and 138 during the second treatment month. The AE-QoL questionnaire scores are presented in Figure 4B, most improvement was reported on functioning and nutrition during the first treatment month, but scores worsened during the second treatment month.

Therapy with rhC1-INH was tolerated well. The patient reported swelling of the lip and a tingling sensation five hours after the first administration of rhC1-INH. Therefore, she was observed for 6 hours after receiving the second dose at the allergology ward. No signs of an allergic response to therapy were observed and subsequent doses were tolerated well. During the treatment period the patient experienced an episode with dyspnea upon exertion and thoracic pain. She was evaluated at the emergency care department where a pulmonary embolism, pneumonia and cardiac pathology were excluded. Symptoms resolved spontaneously and it was deemed unlike that they were related to rhC1-INH.

After the final study visit monthly treatment with 300 mg Omalizumab was started. After six months of treatment the patient appeared to improve but experienced a relapse while still on treatment two months later.

Case 3

A 59-year-old man presented with recurrent angioedema for 10 years. His medical history reported a spinal disc herniation. Swellings evolved spontaneously, were non-pruritic, often already present at awakening and lasted for up to 2 days. Most swelling episodes were located in the face, tongue and oral cavity, but swelling had also occurred in the groin and hands. Over the years, attack frequency ranged from less than once a month to multiple swellings per week. During the 6 months prior to the study the patient reported 3 swellings per month. Angioedema was not accompanied by wheals or signs of anaphylaxis. Attacks were often preceded by a tingling sensation. Prophylactic therapy with 4 times the daily dose of a non-sedating antihistamine did not improve angioedema. Acute treatment consisted of oral steroids and intramuscular adrenaline injections. The patient reported improvement of symptoms within 4 hours after intake of oral steroids. There was no family history of angioedema and C4 levels were 0.27 g/L (normal range 0.1-0.47 g/L). Angioedema

did not interfere with work, however swellings hindered social activities and food intake, especially at breakfast.

During the control period of 2 months, the patient experienced 3 angioedema attacks (Figure 2). The AAS28 was 18 during the first and 7 during the second control month (Figure 3C). During the treatment months the patient experienced 7 angioedema attacks, one of which was treated with 20 mg prednisolone that resulted in resolution of swelling within 2 hours. The AAS28 was 48 and 49, respectively. The AE-QoL questionnaire scores are presented in Figure 4C, most improvement was reported on functioning during the second control month.

Therapy was tolerated well, one episode with headache was mentioned during the treatment period that possibly was related to rhC1-INH treatment. The patient experienced a herpes labialis during the treatment period that resolved spontaneously and reported to have had this at least one time before prior to the study. An increase in angioedema frequency was observed during the treatment months. However, attacks did not tend to occur immediately following drug infusion and were not considered to be an allergic or hypersensitivity response to therapy.

After the final study visit the patient did not continue treatment with rhC1-INH. As the patient felt symptoms were tolerable at the moment, no additional treatment with Omalizumab was started.

Biomarker evaluation

As treatment with rhC1-INH inhibits FXIIa and PKa and thereby prevents HK cleavage, ergo bradykinin release, we investigated biomarkers of the contact system. Figure 5 shows the proteins of the contact system including C1-INH on immunoblot under reducing conditions. No variation of protein bands in subsequent samples of the same patient were observed indicating no gross variations of C1-INH or contact system protein levels during the control period. Moreover, levels were comparable to those in healthy pooled plasma apart from case 3 that appears to have decreased PK levels. For all 3 cases, cHK levels were within the normal range (upper normal limit cHK <20%; case 1 7.8±0.6 (mean±SD); case 2 13.5±0.6; case 3 11.1±0.7). Furthermore, levels in subsequent samples varied only little (Figure 6A). C1-INH complexes with FXIIa or PKa, respectively, were undetectable in all plasma samples obtained during this study (Figure 6B/C).

For all 3 cases, levels of functional C1-INH were within the normal range (0.63-1.82 U/mL; mean case 1.37±0.2; case 2 0.87±0.1; case 3 1.14±0.0; Figure 6D). Remarkably, case 1 had an elevated C1-INH function at visit 18 which was during the control period following weeks with multiple severe angioedema attacks. In addition to C1-INH function on C1s activity we assessed the capacity of plasma to form PKa-C1-INH complexes upon incubation of plasma with 5 µg/mL PKa. Three healthy donors formed 9.2, 8.9 and 8.7% PKa-C1-INH complexes and pooled healthy plasma 8.9% PKa-C1-INH (Figure 6E). Tested in plasma from subsequent measurements the patients formed 11.0±0.9, 9.1±0.7 respectively 8.9±3.4% PKa-C1INH complexes (Figure 6F). There were no differences in the capacity to generate PKa-C1-INH

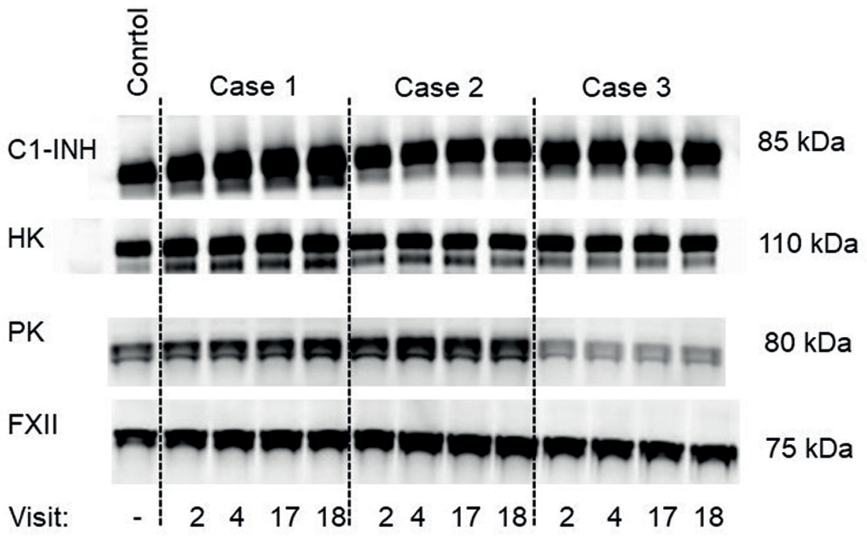

Figure 5. Proteins of the contact system. Immunoblot of the proteins of the contact system C1-INH (C1-esterase inhibitor), HK (high molecular weight kininogen), PK (plasma kallikrein) and FXII (factor XII). Control represents a healthy pooled plasma sample. Visit 4 and 17 samples were collected during treatment prior to the next gift, visit 2 and 18 samples were collected at the end of the two control months.

complexes between control and treatment periods, neither was this capacity different between patients and healthy controls.

D-dimer levels were borderline elevated in case 1, with a mean D-dimer level of 0.54±0.06 mg/L (normal limit <0.5 mg/L). Case 2 had a mean D-dimer level of 0.29±0.04 mg/L. Both case 1 and 2 showed little variation is subsequent measurements, no relation between D-dimer level disease severity and or (response to) treatment could be made. Case 3 had markedly elevated D-dimer levels (mean 1.42±0.13 mg/L), the highest level(1.64 mg/L) was measured one day after an angioedema attack. Overall, D-dimer was elevated in 2 out of 3 cases, there was no clear relation with disease severity or (response to) treatment (Figure 7) though an elevation of D-dimer was seen after one angioedema attack in one patient.

CRP levels in all samples tested, were within the normal range (Figure 8A). Leukocyte count was borderline elevated at visit 18 with 10,8*10^8 cells/L (normal range 4-10*10^8 cells/L) for case 1 following weeks with multiple severe angioedema attacks and within the normal limits for case 2 and 3 (Figure 8B). Total IgE was only measured at the first study visit and slightly above the cut of range of 100 kU/L in all three cases (Table 1).

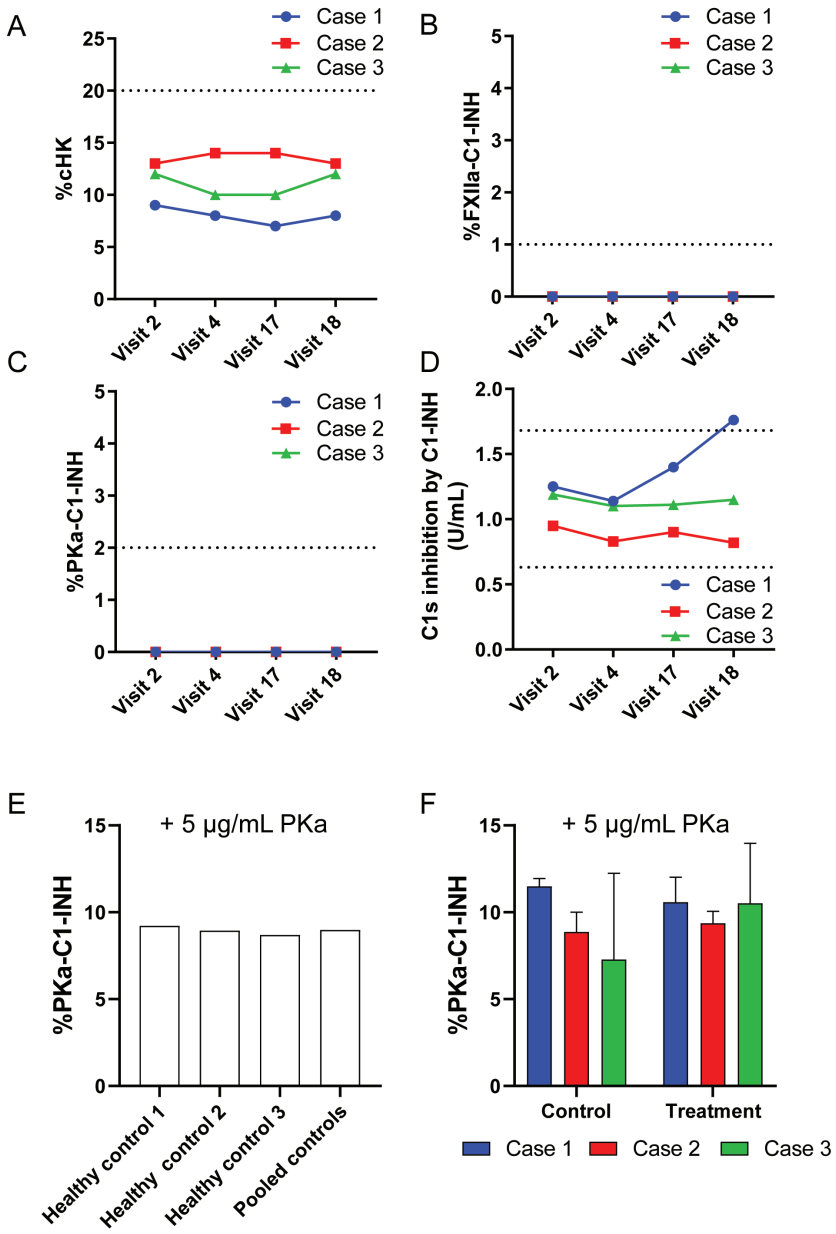

Figure 6. Biomarkers of contact activation. Visit 2 sample was collected prior to the first recombinant human C1-esterase inhibitor (rhC1-INH), visit 4 and 17 prior to third and sixteenth gift, visit 18 sample at final visit. Dotted lines represents upper range or normal ranges. **A)** % Cleaved kininogen (cHK), 100% indicates cleavage of the total HK pool. **B)** % Factor XIIa-C1-INH and **C)** Kallikrein (PKa)-C1-INH complex, 100% indicates FXIIa/PKa-C1INH generation after 30 min dextran sulphate induced contact activation in pooled plasma. **D)** C1-INH inhibition of C1s activity. PKa-C1-INH complex formed, 1 minute after incubation with 5 µg/mL PKa in **E)** 3 healthy controls and pooled plasma from ~30 controls **F)** in patients during control (Visit 2&18) or treatment (visit 4&17) 100% indicates FXIIa/PKa-C1INH generation after 30 min dextran sulphate induced contact activation in pooled plasma.

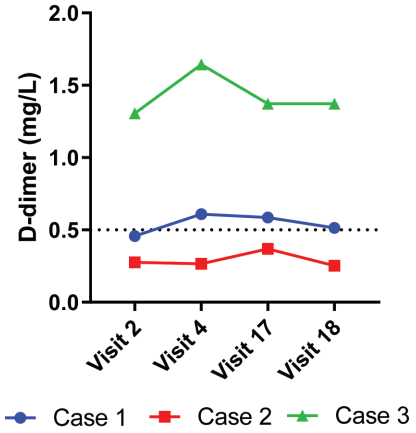

Figure 7. D-dimer levels. Visit 2 sample was collected prior to the first recombinant human C1-esterase inhibitor (rhC1-INH), visit 4 and 17 prior to third and sixteenth gift, visit 18 sample at final visit. Dotted lines represent upper range.

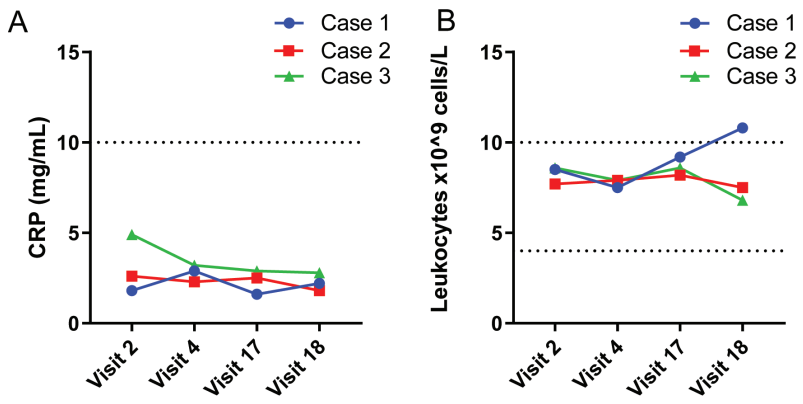

Figure 8. C-reactive protein and leukocytes. Visit 2 sample was collected prior to the first recombinant human C1-esterase inhibitor (rhC1-INH), visit 4 and 17 prior to third and sixteenth gift, visit 18 sample at final visit. Dotted lines represents upper range or normal ranges. A) C-reactive protein B) leukocyte count x10^9 cells/L.

Table 1. C4 and Total IgE levels at first visit

	C4 (g/L) Reference 0,1-0,47	**Total IgE(kU/L) Reference 0-100**
Case 1	0,3	275
Case 2	0,15	163
Case 3	0,27	254

DISCUSSION

In this consecutive, cross-over case-series study of 3 patients with idiopathic non-histaminergic angioedema, less attacks were observed in one patient during treatment with twice weekly rhC1-INH. However, 2 patients did not improve during treatment with rhC1-INH. D-dimer levels were elevated in 2 patients. Our variable outcomes suggest that idiopathic non-histaminergic angioedema is a heterogeneous disease. A biomarker that could identify therapy responders is essential, unfortunately, the here tested set of biomarkers was unable to do so. This study motivates that rhC1-INH supplementation may be used in some patients with idiopathic non-histaminergic angioedema.

Although not uncommon in low-incidence diseases, case reports and case-series are deemed as low-value in terms of demonstrating drug efficacy. In order to increase the value of our findings we choose a cross-over design in which each case provided its own control data and included two control periods to minimize the effect of natural variation in disease severity that is expected over time. As we report three consecutive cases, we avoid publication bias of only reporting on positive outcomes, giving a more balanced and realistic view on therapeutic outcomes. In order to investigate therapy response in a relative short period of time, preventing unnecessary lengthy treatment for study participants, we only included patients with a high attack frequency. This introduced a selection bias for patients with severe disease. In addition, study participation was time demanding and more likely to be acceptable for patients with a high disease burden.

In two patients no clear improvement in attack frequency was seen. The dose and treatment interval of rhC1-INH were based upon efficacy in HAE-1 and 2 and may have been insufficient for idiopathic non-histaminergic angioedema. Alternatively, symptoms may not have been bradykinin driven in these two cases. Case 3 even experienced an increase in symptoms during the treatment period. This might be explained by 1) a herpetic infection during the second treatment month triggering facial swelling and 2) natural variation, as this patient experience one attack right before the first and right after the second control period. In case 2 we suspect a possible misdiagnosis, although no other diagnosis could be made the medical history of unexplained arthralgia, considerable malaise during attacks and the observation of mild swelling that tended to linger for over a week instead of clearly evolve and resolve are atypical for idiopathic angioedema.

To our knowledge, this is the first case report describing successful use of rhC1-INH prophylaxis in idiopathic non-histaminergic angioedema and the first consecutive, cross-over, case-series study to C1-INH prophylaxis in this disease. Three previous case reports describe successful use of plasma derived C1-INH prophylaxis in 4 patients with idiopathic non-histaminergic angioedema[23–25]. We deem it highly likely that the improvement observed in our study is caused by rhC1-INH therapy as there was a clear difference in attack frequency during treatment and control period. Moreover, continuation for 6 months after the final study visit again resulted in improvement followed by a relapse after discontinuation. It should be noted that the steep rise in attack frequency during the second control month

may be in part explained by an unreported viral infection. Leukocyte count and C1-INH function, both biomarkers of an acute phase response, were elevated.

Bradykinin involvement in idiopathic non-histaminergic angioedema is suspected based on drug effectiveness in case reports[10] and laboratory assesments[9]. However, a growing number of case reports demonstrate efficacy of the anti IgE monoclonal antibody omalizumab in idiopathic non-histaminergic angioedema[26]. The mechanism of action of omalizumab in angioedema are not well understood, but it suggests that mast cells contribute to the symptoms in therapy responders. Although bradykinin could still be a contributing factor in a mast-cell induced disease its relevance is uncertain. These seemingly contrasting findings in angioedema literature are in line with our observation that only 1 out of 3 patients responded to rhC1-INH. Moreover, therapy with omalizumab failed to improve symptoms in the here described rhC1-INH responder (case 1) and non-responder (case 2). Likely, idiopathic non-histaminergic angioedema represents a pluriform population covering a multitude of diseases of which some are bradykinin-driven.

A challenge arising is how to identify patients with idiopathic non-histaminergic angioedema that could benefit from rhC1-INH. None of the here-reported biomarkers could distinguish our responder from the non-responders. Interestingly, D-dimer was clearly elevated in one of the two non-responders. D-dimer is associated with antihistamine resistance in CSU and possibly linked to omalizumab resistance[19,27]. As our here presented case 3 patient failed to respond to rhC1-INH, high D-dimer levels do not appear to be a predictor of bradykinin mediated disease.

A report by Lara-Marquez and colleagues demonstrated that dextran sulphate stimulated kallikrein activity distinguished idiopathic histaminergic from non-histaminergic angioedema[28]. This novel test holds promise for diagnosing bradykinin mediated angioedema, but we so far failed to set-up a comparable test in our lab. We therefore feel that a second challenge ahead besides the development of diagnostic assay lies in validation, availability and standardization of tests used in treatment centers worldwide.

In summary, twice-weekly rhC1-INH administration effectively prevented angioedema attacks in one patient. Two patients showed no benefit of treatment, but therapy was well tolerated in all three cases. rhC1-INH may be a valid treatment option for some patients with idiopathic non-histaminergic angioedema but biomarkers to identify bradykinin-mediated angioedema are urgently needed.

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CONFLICT OF INTEREST

TS is an employee at Pharming, CM was a consultant of Pharming. The other authors declare no conflict of interest.

REFERENCES

1. Longhurst H, Cicardi M. Hereditary angio-oedema. *Lancet (London, England)*. 2012;379(9814):474-481. doi:10.1016/S0140-6736(11)60935-5
2. Schulkes KJG, Van den Elzen MT, Hack EC, Otten HG, Bruijnzeel-Koomen CAFM, Knulst AC. Clinical similarities among bradykinin-mediated and mast cell-mediated subtypes of non-hereditary angioedema: a retrospective study. *Clin Transl Allergy*. 2015;5(1):5. doi:10.1186/s13601-015-0049-8
3. Zuberbier T, Aberer W, Asero R, et al. The EAACI/GA2LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: The 2013 revision and update. *Allergy Eur J Allergy Clin Immunol*. 2014;69(7):868-887. doi:10.1111/all.12313
4. Maurer M, Magerl M, Ansotegui I, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. *Allergy*. 2018;73(8):1575-1596. doi:10.1111/all.13384
5. de Maat S, Maas C. Factor XII : form determines function. *J Thromb Heamostasis*. 2016;14:1498-1506. doi:10.1111/jth.13383
6. Mansi M, Zanichelli A, Coerezza A, et al. Presentation, diagnosis and treatment of angioedema without wheals: a retrospective analysis of a cohort of 1058 patients. *J Intern Med*. 2015;277(5):585-593. doi:10.1111/joim.12304
7. Hofman ZLM, van West N, Hack CE, Knulst AC, Maas C, Röckmann H. High occurrence of antihistamine resistance in patients with recurrent idiopathic angioedema. *Clin Transl Allergy*. 2019;9:35. doi:10.1186/s13601-019-0274-7
8. Cicardi M, Aberer W, Banerji A, et al. Classification, diagnosis, and approach to treatment for angioedema: consensus report from the Hereditary Angioedema International Working Group. *Allergy*. 2014;69(5):602-616. doi:10.1111/all.12380
9. Cugno M, Tedeschi A, Nussberger J. Bradykinin in idiopathic nonhistaminergic angioedema. *Clin Exp Allergy*. 2016. doi:10.1111/cea.12869
10. van den Elzen M, Go MFCL, Knulst AC, Blankestijn MA, van Os-Medendorp H, Otten HG. Efficacy of Treatment of Non-hereditary Angioedema. *Clin Rev Allergy Immunol*. 2018;54(3):412-431. doi:10.1007/s12016-016-8585-0
11. Gösswein T, Kocot A, Emmert G, et al. Mutational spectrum of the C1INH (SERPING1) gene in patients with hereditary angioedema. *Cytogenet Genome Res*. 2008;121(3-4):181-188. doi:10.1159/000138883
12. Zuraw B, Cicardi M, Levy RJ, et al. Recombinant human C1-inhibitor for the treatment of acute angioedema attacks in patients with hereditary angioedema. *J Allergy Clin Immunol*. 2010;126(4):821-827.e14. doi:10.1016/j.jaci.2010.07.021
13. Moldovan D, Reshef A, Fabiani J, et al. Efficacy and safety of recombinant human C1-inhibitor for the treatment of attacks of hereditary angioedema: European open-label extension study. *Clin Exp Allergy*. 2012;42(6):929-935. doi:10.1111/j.1365-2222.2012.03984.x
14. Reshef A, Moldovan D, Obtulowicz K, et al. Recombinant human C1 inhibitor for the prophylaxis of hereditary angioedema attacks: a pilot study. *Allergy*. 2013;68(1):118-124. doi:10.1111/all.12060
15. Riedl MA, Grivcheva-Panovska V, Moldovan D, et al. Recombinant human C1 esterase inhibitor for prophylaxis of hereditary angio-oedema: a phase 2, multicentre, randomised, double-blind, placebo-controlled crossover trial. *Lancet (London, England)*. 2017;390(10102):1595-1602. doi:10.1016/S0140-6736(17)31963-3
16. Hofman ZLM, de Maat S, Suffritti C. Cleaved kininogen as a biomarker for bradykinin release in hereditary angioedema. *J Allergy Clin Immunol*. 2017. doi:10.1016/j.jaci.2017.07.012

17. Cugno M, Nuijens J, Hack E, et al. Plasma levels of C1- inhibitor complexes and cleaved C1- inhibitor in patients with hereditary angioneurotic edema. *J Clin Invest*. 1990;85(4):1215-1220. doi:10.1172/JCI114555

18. Reshef A, Zanichelli A, Longhurst H, Relan A, Hack CE. Elevated D-dimers in attacks of hereditary angioedema are not associated with increased thrombotic risk. *Allergy*. 2015;70(5):506-513. doi:10.1111/all.12587

19. Asero R. D-dimer: a biomarker for antihistamine-resistant chronic urticaria. *J Allergy Clin Immunol*. 2013;132(4):983-986. doi:10.1016/j.jaci.2013.04.037

20. Weller K, Groffik A, Magerl M, et al. Development, validation, and initial results of the Angioedema Activity Score. *Allergy*. 2013;68(9):1185-1192. doi:10.1111/all.12209

21. Weller K, Groffik A, Magerl M, et al. Development and construct validation of the angioedema quality of life questionnaire. *Allergy*. 2012;67(10):1289-1298. doi:10.1111/all.12007

22. de Maat S, Björkqvist J, Suffritti C, et al. Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations. *J Allergy Clin Immunol*. 2016;138(5):1414-1423.e9. doi:10.1016/j.jaci.2016.02.021

23. Gravante C, Carucci L, Bova M, Petraroli A, Genovese A, Marone G. Prophylactic treatment with plasma-derived C1 inhibitor in idiopathic non-histaminergic angioedema. *Pediatr Allergy Immunol*. 2016;27(6):658-659. doi:10.1111/pai.12592

24. Stahl MC, Harris CK, Matto S, Bernstein JA. Idiopathic nonhistaminergic angioedema successfully treated with ecallantide, icatibant, and C1 esterase inhibitor replacement. *J Allergy Clin Immunol Pract*. 2014;2(6):818-819. doi:10.1016/j.jaip.2014.05.006

25. Shroba J, Hanson J, Portnoy J. Current treatment options for idiopathic angioedema. *Ann Allergy Asthma Immunol*. 2015;115(5):429-433. doi:10.1016/j.anai.2015.07.023

26. Bucher MC, Petkovic T, Helbling A, Steiner UC. Idiopathic non-histaminergic acquired angioedema: a case series and discussion of published clinical trials. *Clin Transl Allergy*. 2017;7:27. doi:10.1186/s13601-017-0164-9

27. Asero R, Marzano A V, Ferrucci S, Cugno M. D-Dimer Plasma Levels Parallel the Clinical Response to Omalizumab in Patients with Severe Chronic Spontaneous Urticaria. *Int Arch Allergy Immunol*. 2017;172(1):40-44. doi:10.1159/000453453

28. Lara-Marquez ML, Christiansen SC, Riedl MA, Herschbach J, Zuraw BL. Threshold-stimulated kallikrein activity distinguishes bradykinin- from histamine-mediated angioedema. *Clin Exp Allergy*. 2018;48(11):1429-1438. doi:10.1111/cea.13219

10

Evidence for bradykinin release in chronic spontaneous urticaria

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ABSTRACTS

Background

Chronic spontaneous urticaria (CSU) is characterized by recurrent itchy wheals and/or angioedema and is believed to be driven by mast cell activation. It was shown that excessive mast-cell activation during anaphylaxis initiates contact activation resulting in bradykinin release. Evidence for bradykinin release was never demonstrated in CSU.

Objective

To study biomarkers of bradykinin release in CSU.

Methods

Plasma samples of CSU patients were collected during routine visits at the outpatient clinic. Cleaved kininogen, factor XIIa-C1-inhibitor (FXIIa-C1-INH), kallikrein-C1-INH, plasmin-antiplasmin (PAP) complexes and soluble urokinase receptor (suPAR) levels were determined by ELISA. Clinical data was collected from medical records and associated with lab parameters.

Results

One-hundred seventeen samples from 88 CSU patients and 28 samples from healthy controls were analyzed. Thirty-nine (44%) patients had wheals only, 35 (40%) patients had wheals and angioedema, 14 (16%) patients had idiopathic angioedema. Median cHK level in patients was 9.1% (range: 1.4-21.5%) and significantly increased compared to healthy controls (median 6.0% range: 0-19.9%; p=0.0005). cHK levels normalized in patients during disease remission (median 6.5% range 1.5-20.8%) and were associated with disease severity but not dependent on the presence of angioedema or presence of an acute angioedema attack. C1-INH-complexes and suPAR levels were not elevated in patients compared to healthy controls. PAP-levels in patients were elevated compared to healthy controls but there was no correlation between PAP and cHK.

Conclusions

cHK levels were elevated in symptomatic CSU patients compared to healthy controls, indicating increased bradykinin production. cHK increase was not limited to patients with angioedema. Whether this elevated bradykinin generation has clinical implications in the pathology of CSU is open to debate.

INTRODUCTION

Chronic spontaneous urticaria (CSU) is hallmarked by recurrent itchy wheals and/or angioedema. The pathophysiology of CSU is not fully understood[1]. Mast cell and histamine involvement are strongly implicated and patients demonstrate clinical response to the anti-IgE monoclonal antibody omalizumab and antihistamine therapy[2,3].

Angioedema is a common symptom of CSU. When patients experience angioedema without wheals this CSU subtype can be referred to as idiopathic angioedema[4]. Angioedema results from increased vascular permeability followed by swelling of subcutaneous or submucosal tissues. Angioedema in CSU is believed to be histamine induced, while angioedema related to angiotensine converting enzyme (ACE)-inhibitor use and hereditary angioedema (HAE) are driven by the vasoactive peptide bradykinin[4]. ACE-inhibitors prevent the breakdown of bradykinin thereby increasing bradykinin half-life. In HAE types 1 and 2, bradykinin levels are increased because patients lack the main inhibitor of bradykinin production: C1-inhibitor (C1-INH)[5]. C1-INH inhibits actived factor XII (FXIIa) and plasma kallikrein (PKa). FXIIa converts plasma prekallikrein (PK) into PKa that will cleave bradykinin from high molecular weight kininogen (HK)[6]. Therefore, C1-INH deficiency results in uncontrolled bradykinin production.

The various subtypes of angioedema are classified as bradykinin induced or mast cell induced, especially when wheals and angioedema coincide swellings are considered mast cell and not bradykinin induced[4,7]. However, research in anaphylaxis suggests that mast cell activation and bradykinin release co-exist. A study performed in wasp-venom allergic individuals demonstrated increased markers of bradykinin generation after a wasp sting in those with severe anaphylaxis *e.g.* angioedema and or shock[8]. While markers were low prior to the sting and in individuals with a mild response. In addition, activation of the fibrinolytic system was also observed during anaphylaxis as plasmin-antiplasmin (PAP) complexes increased[9]. A later study found increased levels of cleaved HK (cHK), a marker for bradykinin release, in patients visiting the emergency medicine department with a (food or drug) allergic response[10]. These observations in humans were explained in mice-models of IgE mediated anaphylaxis where drop in blood pressure and increased vasopermeability during excessive mast cell activation were reversed by bradykinin receptor antagonism and FXII deficiency[10,11].

Little is known about bradykinin formation in CSU as it is generally considered a mast cell and histamine mediated disease. We recently developed a sensitive ELISA-based method to asses cHK generation[12]. Using this method we revisited cHK generation in CSU. In addition, C1-INH in complex with FXIIa or PKa were determined as well as markers of the fibrinolytic system as these were previously associated with mast cell activation in anaphylaxis[9].

METHODS

Study participants and blood collection

All patients with a diagnosis of CSU defined as having spontaneous, recurrent wheals and/or angioedema for at least 6 weeks visiting the dermatology and allergology outpatient department were asked to provide blood samples at first and selected follow-up visits. This study was approved by the local ethical committee (protocol#13-272). Of these, all patients with written informed consent and at least one collected sample at first visit between January 2014 and February 2016 were included and data was analyzed for this study. Patient characteristics (age, sex, CSU subtype, disease severity, treatment) were retrieved from medical records by the physician of the research team. Disease severity was categorized into 1) symptom free; complete disease control for at least one month, 2) mild; recent symptoms but no need for additional treatment 3) moderate to severe; recent symptoms, need for additional treatment and/or frequent use of rescue medication. When available the urticaria control test (UCT) score was collected[13]. During the timeframe of this study the UCT was not yet collected routinely at every visit.

Venipuncture was performed at inclusion during a regular control visit at the outpatient clinic. One additional blood draw was performed in 20 patients during random control visits, 2 additional blood draws in 2 patients and 3 in one patient.

Blood was collected in standard blood collection tubes containing 1.8mg EDTA per mL blood and specific sample collection/anticoagulant tubes (SCAT) containing 25 μM PPACK (Phe-Pro-Arg-chloromethylketone), 11 mM sodium citrate, 0.1 % Mannitol (w/v) to prevent post-blood draw enzymatic activity in samples. Blood was centrifuged at 2000 x g for 10 minutes shortly after blood draw and stored at -80 until use.

In addition, plasma from healthy donors was collected (with written informed consent; approval by the local ethical committee of the University Medical Center Utrecht; protocol#07-125). Blood was collected in standard sodium citrate tubes (10% sodium citrate, 3.2% wt/vol). Blood was centrifuged twice at 2000 x g for 10 minutes shortly after blood draw and stored at -80 until use. Plasma from ~30 healthy donors was pooled for control pooled plasma and plasma from 28 patients was stored individually for control plasma.

Biomarker assays

cHK[12], FXIIa-C1-INH complexes[14], PKa-C1-INH complexes[14], plasmin-anti-plasmin (PAP) complexes[15] and soluble urokinase receptor (suPAR)[16] levels were determined by ELISA, as previously published. cHK, FXIIa-C1-INH, PKa-C1-INH and PAP complexes in patients were determined in citrated plasma containing PPACK to prevent post-blood draw contact activation, suPAR levels were determined in EDTA plasma. cHK and C1-INH complex measurements are expressed as percentages. One-hundred % cHK reflects total HK cleavage in control pooled plasma generated by incubating plasma with 1 μg/mL bèta-FXIIa (Hematologic Technology, Burlington, United Kingdom) at 37°C for 10 minutes after which reaction was stopped by diluting plasma 64x in a phosphate buffered saline buffer

(mPBSt: 127.9 mM NaCl, 6.2 mM Na_2HPO_4, 3.7 mM NaH_2PO_4, pH 7.0 supplemented with 0.1% Tween-20 wt/vol and 1% skimmed milk powder wt/vol, containing 50 µM PPACK (Sigma-Aldrich, Zweindrecht the Netherlands)). One-hundred % C1-INH complex indicates control pooled plasma incubated with dextran sulphate Mr ~500,000 (Sigma-Aldrich) at 37ºC for 30 minutes after which reaction was stopped by diluting 32x in mPBSt containing 50 µM PPACK. Calibration curves were created by mixing 100% activated plasma with unactivated control pooled plasma both diluted in mPBSt containing PPACK. Plasma dilution in calibration curves was equal to plasma dilution of patient samples[12,14].

Data analysis

Graphpad Prism 8 was used for data analysis. cHK and C1-INH complex levels were interpolated from a calibration curve using a sigmoidal 4PL fit model. The first collected sample per patient was included for analysis unless indicated otherwise. Groups were compared using Mann-Whitney t-test or Wilcoxon test for paired samples, multiple groups with Kruskal-Wallis test using Dunn's correction for multiple testing. Correlation was tested with Spearman's rank test.

RESULTS

Eighty-eight patients were included (70% female; mean age 44 years, range 18-78), clinical data and blood samples from 117 visits were collected (Table 1). Twenty-eight samples from healthy donors were included as controls (74% female; mean age 39 years, range 23-65). Thirty-nine patients had wheals (44%), 35 (40%) patients had both wheals and angioedema, 14 (16%) patients had idiopathic angioedema. At first visit in this study, the majority of patients received prophylactic therapy with antihistamines (n=65, 74%). Further prophylactic treatement at first visit was omalizumab (n=11, 13%), steroids (n=7, 8%) and leukotriene antagonists (n=5, 6%). 18 patients (20%) did not receive any prophylaxis but all had on-demand treatment available also mainly consisting of antihistamines (Table 1). Most patients (n=59, 67%) described moderate to severe symptoms at first visit, needing additional therapy. Four (5%) patients were symptom free at first visit. When analyzing all visits including follow-up visits 16 (14%) of patients reported to be symptom free (Table 1).

Cleaved kininogen levels are increased in CSU patients

cHK and C1-INH in complex with FXIIa or PKa were determined to assess contact activation in CSU. Median cHK level in patients was 9.1% (range:1.4-21.5%) and significantly increased compared to healthy controls (median 6.0% range: 0-19.9% p=0.0006). When classifying by disease severity cHK in healthy controls was significantly lower than in CSU patients with mild symptoms (median cHK 10.6% range 1.4-17.2%, p<0.0001) and moderate to severe symptoms (median cHK 9.0% range 2.7-18.71%, p=0.0043) but not compared to symptom free patients (median cHK 6.5% range 1.5-20.8%, p>0.9999, Figure 1A). cHK levels in symptom free patients were significantly lower than in patients with mild symptoms (p=0.0296) and

Table 1. Patients characteristics, therapy and disease severity per visit

Number of patients	88	
Male (%)	30%	
Female (%)	70%	
Mean age (range)	44 (18-78)	
CSU subtype n (%)		
Wheals	39 (44%)	
Wheals and angioedema	35 (40%)	
Angioedema	14 (16%)	
Prophylactic therapy at visit n (%)*:	first visit (n=88)	All visits (n=117†)
None	18 (20%)	19 (16%)
Antihistamines	65 (74%)	91 (78%)
Omalizumab	11 (13%)	30 (26%)
Steroids	7 (8%)	9 (8%)
Leukotriene antagonist	5 (6%)	7 (6%)
Tranexamic acid	2 (2%)	3 (3%)
Cyclosporine	2 (2%)	2 (2%)
Methotrexate	1 (1%)	1 (1%)
Mycophenolic acid	1 (1%)	1 (1%)
Chloroquine	1 (1%)	1 (1%)
Disease severity at visit n (%):**	first visit (n=88)	All visits (n=117†)
Symptom free	4 (5%)	16 (14%)
Mild	25 (29%)	35 (30%)
Moderate to severe	59 (67%)	66 (56%)

CSU= chronic spontaneous urticaria.
*includes combinations of therapies.
† includes 29 follow-up visits from 23 patients
**Disease severity defined as: symptom free = complete disease control, mild = symptoms partly controlled no need for additional therapy, moderate to severe = uncontrolled symptoms, need for additional therapy.

lower compared to patients with moderate to severe symptoms, however this difference was not statistically significant (p=0.3018, Figure 1A). An urticaria control test (UCT) score was reported at 33 visits, the average score was 11 (SD5.6) and trended towards negative correlation with cHK (p=0.06, r=-0.28, Supplemental figure 1) There were no differences in cHK levels among patients with wheals, wheals and angioedema or idiopathic angioedema (Figure 1B, p=0.91).

In three patients with idiopathic angioedema, one or more samples were collected during an angioedema attack. All of these patients experienced a high attack frequency with 2 to 10 attacks monthly while using antihistamine prophylaxis of 1 to 4 times the daily recommended dose. In addition, one of these patients received tranexamic acid and one tranexamic acid and omalizumab which only led to partial improvement of symptoms.

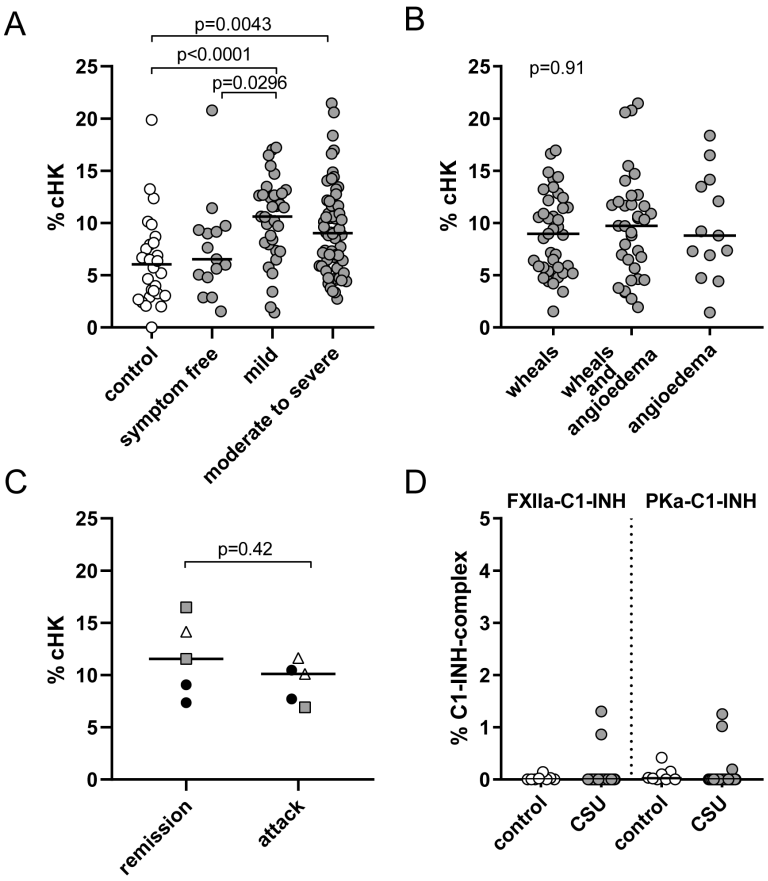

Figure 1. Biomarkers of the contact system in chronic spontaneous urticaria. Cleaved kininogen (cHK) levels were determined by ELISA, 100% represents total cleavage of the HK pool. **A)** cHK levels in healthy controls (n=28) and chronic spontaneous urticaria (CSU) patients (114 measurements in 86 patients) per disease severity; symptom free (n=15), mild (n=35), moderate to severe (n=64) **B)** cHK per disease phenotype, dots reflect measurements in 86 patients **C)** cHK during remission and angioedema attack in 3 patients with angioedema without urticaria, each symbol represents 1 patient. **C)** FXIIa-C1-INH and PKa-C1-INH complexes were determined by ELISA, 100% represents complex formation after 30 minutes of dextran sulphate induced contact activation in control pooled plasma. Healthy controls (n=9) compared to CSU (n=86). Lines indicate median, Kruskal-Wallis test and Mann-Whitney t-test were used for comparison.

No increase in cHK could be observed in samples collected during an angioedema attack compared to samples in between attacks (Figure 1C, p=0.42).

FXIIa-C1-INH and PKa-C1-INH complex levels were mainly below detection limits in patients and healthy controls (Figure 1D). Distribution of cHK levels among age and sex were analyzed but no relation with age or sex was found (Supplemental figure 2).

We analyzed changes in cHK levels detected in subsequent samples over time per patient. In only 11 of the 23 cases change in disease severity corresponded to change in cHK

levels, where change was defined as >4% cHK difference. Disease severity and cHK levels per patient, including additional information on immunosuppressive therapy and omalizumab used per time point are shown in Supplemental figure 3 (red lines indicate disease severity, blue lines cHK).

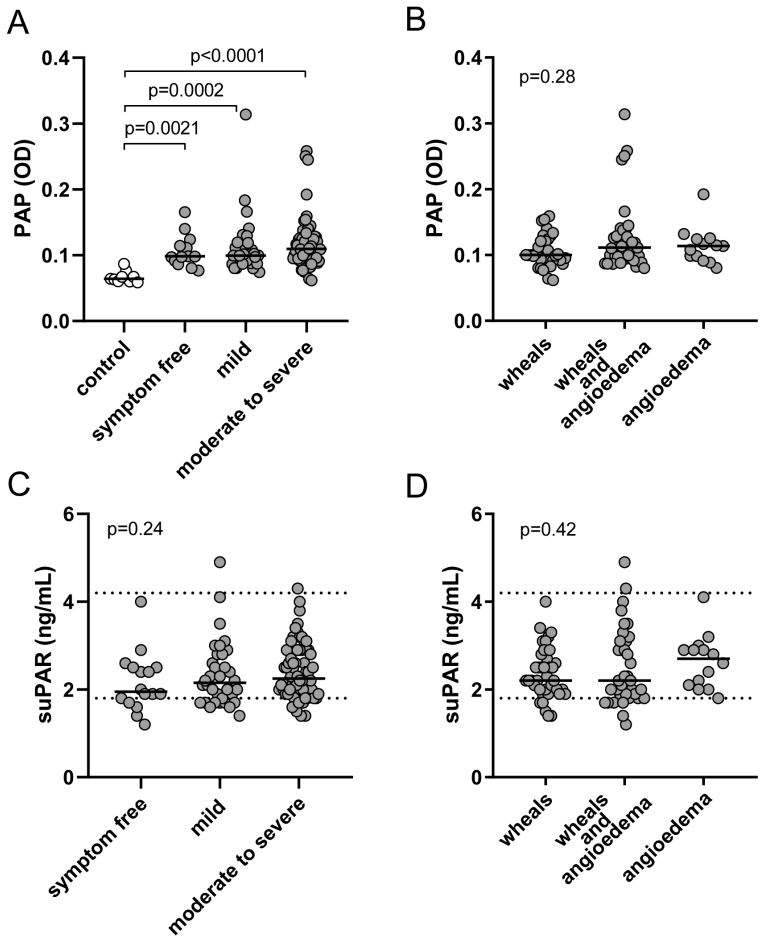

Figure 2. Biomarkers of the fibrinolytic system in chronic spontaneous urticaria. Plasmin-anti-plasmin (PAP) levels were determined by ELISA data represented in optical density (OD) **A)** PAP levels in healthy controls (n=9) and chronic spontaneous urticaria (CSU) patients (111 measurements in 85 patients) per disease severity; symptom free (n=15), mild (n=34), moderate to severe (n=62) **B)** PAP levels per disease phenotype, dots reflect measurements in 85 patients. Soluble urokinase receptor (suPAR) levels were determined by ELISA (116 measurement in 88 patients), dotted lines indicate upper and lower reference range in healthy controls **C)** suPAR levels per disease severity (symptom free n=16, mild n=34, moderate to severe n=66), **D)** suPAR levels per disease phenotype, dots reflect measurements in 88 patients. Lines indicate median, Kruskal-Wallis test was used for comparison.

Plasmin-antiplasmin levels were increased in CSU patients

Activation of the fibrinolytic system was analyzed by measurement of PAP complexes and suPAR levels. We observed increased PAP levels in CSU patients compared to healthy controls (p=<0.0001). This differences was there for symptom free patients (p=0.0021), patients with mild symptoms (p=0.0002) and moderate to severe symptoms (p<0.0001, Figure 2A). There was no association with disease phenotype (p=0.28, Figure 2B). Soluble uPAR levels were only determined in patient samples as reference ranges from healthy control populations were available. Measurements were done in 116 samples, in only two patients levels were slightly increased, 20 measurements in 17 (19%) patients were below the lower cut-off value. Four patients with lowered levels were symptom free, 8 had mild symptoms and 7 moderate to severe symptoms. There was no difference in suPAR levels among the disease severity groups (p=0.24, Figure 2C) or disease phenotypes (p=0.42, Figure 2D). Furthermore, PAP and suPAR did not correlate to cHK levels or each other (Figure 3 A-C) .

DISCUSSION

We evaluated if there is evidence for bradykinin release in CSU and found that cHK, a marker for bradykinin production, was elevated in CSU patients compared to healthy controls. cHK levels normalized in patients that were in disease remission. Increased cHK was not limited to CSU patients with angioedema. In addition, PAP levels were also increased reflecting fibrinolytic activety in CSU.

The increase in cHK levels observed in this CSU cohort was within the healthy control range; roughly not surpassing 20% cHK. We previously showed that even in HAE types 1 and 2 half of the HAE patients had cHK levels within the normal range during remission and that a slight rise within this range could indicate an angioedema attack[12]. This was in line with results from a study assessing cHK levels in HAE via western blot[17]. There appears to be no

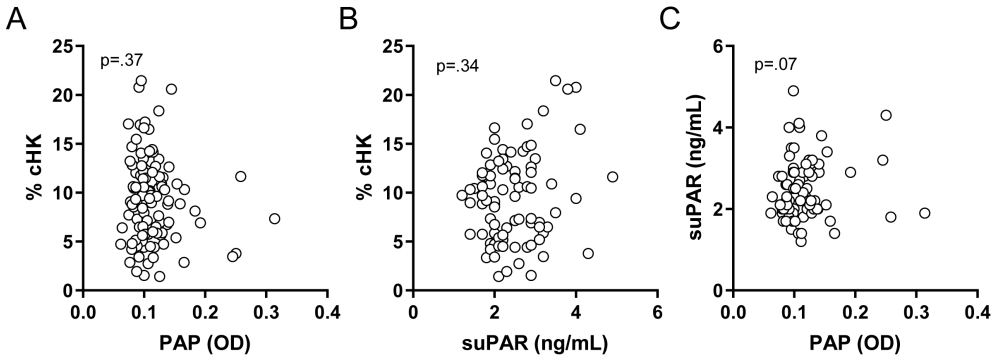

Figure 3. Cleaved kininogen levels and biomarkers of the fibrinolytic system do not correlate. Cleaved kininogen (cHK), plasmin-anti-plasmin (PAP) and soluble urokinase receptor (suPAR) were determined by ELISA. cHK plotted against **A)** PAP levels **B)** suPAR levels. **C)** suPAR levels plotted against PAP levels. Spearman's rank test was used.

lower threshold for an angioedema attack[12,17]. It is therefore possible that the overall slightly increased cHK levels in CSU contribute to a state of increased vascular permeability.

Bradykinin levels or HK degradation in CSU were only studied in very small numbers of patients and limited to cases with angioedema. One study reported no decrease in HK antigen on western blot in 7 cases with idiopathic angioedema[10]. This different outcome can be explained by the sensitivity of cHK detection versus loss of HK antigen on western blot. Another study observed decreased HK levels in 10 patients with angioedema provoked by oral contraceptive intake, notably 8 of them also reported wheals, symptoms improved and HK normalized after cessation of oral contraceptives[18]. Evidence is surfacing that bradykinin contributes to idiopathic angioedema that is unresponsive to antihistamine therapy (idiopathic non-histaminergic angioedema). Increased bradykinin levels were detected in 4 cases of idiopathic non-histaminergic angioedema during angioedema attacks[19]. Moreover, *ex vivo* plasma stimulation with a FXII activator in patients with idiopathic non-histaminergic angioedema resulted in increased PKa activity[20], suggesting increased sensitivity to bradykinin production. Both studies did not found increased bradykinin levels or sensitivity to bradykinin production in idiopathic angioedema patients that did responded to antihistamine therapy (idiopathic histaminergic angioedema)[20,21].We determined cHK in three patients with idiopathic angioedema during an acute attack but did not observe a further increase in cHK. All three showed no clear benefit from antihistamine therapy but two continued to have a partial and complete response to omalizumab suggesting mast cell involvement in their disease pathology. In our study cHK levels in patients with idiopathic angioedema did not differ from those with wheals. Our findings appear to be in line with previous observations in idiopathic histaminergic angioedema as the cHK increase detected is very subtile and within the range of healthy controls. However, using our sensitive method of cHK detection we observed possible clinical relevant difference and are the first to report elevated cHK levels in CSU, unrelated to the occurrence of angioedema.

Involvement of the fibrinolytic system in CSU is topic of debate. The fibrin degradation product D-dimer is associated with CSU and it correlates with disease severity and antihistamine resistant urticaria[22,23]. However, urokinase-type plasminogen activator (uPA), soluble uPA receptor (suPAR) and plasminogen activator inhibitor-1 were not elevated in one study on CSU patients[24]. We add to these previous observations our finding of increased PAP levels in CSU compared to healthy controls. There is a large bout of evidence demonstrating that activation of the fibrinolytic system and contact system go hand in hand[9,14,25–28]. It is strongly suggested that plasmin initiates bradykinin release in HAE with normal C1-INH function[14,28]. On the other hand, mast cells are pointed out as a source of fibrinolysis via excretion of tissue plasminogen activator, fibrinogenolytic properties of bèta-tryptase[29] and expression of uPAR[30]. In addition, we found decreased suPAR levels in 19% of this CSU cohort. While increased suPAR levels are associated with inflammatory disease[31–33], cardiovascular disease[34], cancer[35] and overall mortality, decreased levels are not connected to pathology. Other than a possible compensatory mechanism it is hard to envision a contribution of suPAR to CSU. With this study, we could not establish a correlation between PAP levels and

cHK. This may be explained by a mismatch in half-life of circulating cHK and circulating PAP levels. It is uncertain if cHK generation and plasmin activity in CSU are intertwined.

Whether our observation of increased cHK generation is of clinical relevance in CSU or if cHK is just an innocent bystander requires investigations. We can only speculate that increased bradykinin turn-over in CSU, as reflected by our biomarker study, may contribute to increased vasopermeability, possibly priming the vasculature for events leading to wheals and/or angioedema. A recent case series including two CSU patients described that relapse in symptoms after initial successful omalizumab treatment drastically improved after cessation of ACE-inhibitors. It was suggested that ACE-inhibitors, which increase bradykinin levels, contributed to disease severity in these two CSU cases[36].

We here report on increased cHK levels in CSU reflecting increased bradykinin release. CSU is considered a multifactorial, mast-cell mediated disease and we cautiously introduce the idea of including bradykinin in this multifactorial model. If, and to what extent bradykinin indeed contributes to CSU remains to be answered.

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DATA SHARING STATEMENT

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

CONFLICT OF INTREST

CM and SM are inventors and have a financial interest in SERPINx BV. CEH has a financial interest in Prothix BV. AK received research funding from Novartis and is a member of the national and international advisory board from Novartis for CSU. HR is a member of the national advisory board from Novartis for CSU. ZH, MvdE and JK have no conflict of interest to declare.

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AUTHORS CONTRIBUTIONS

ZH prepared the first draft of this manuscript, collected clinical data and performed analysis on the data. MvdE wrote the protocol for the biobank study, included the majority of patients and reviewed the manuscript. ZH and JK carried out experimental work. CM, CEH, SM, ACK and RH supervised the work, and revised the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Puxeddu I, Pratesi F, Ribatti D, Migliorini P. Mediators of Inflammation and Angiogenesis in Chronic Spontaneous Urticaria: Are They Potential Biomarkers of the Disease? *Mediators Inflamm*. 2017;2017:4123694. doi:10.1155/2017/4123694
2. Zuberbier T, Aberer W, Asero R, et al. The EAACI/GA2LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: The 2013 revision and update. *Allergy Eur J Allergy Clin Immunol*. 2014;69(7):868-887. doi:10.1111/all.12313
3. Maurer M, Rosén K, Hsieh H-J, et al. Omalizumab for the treatment of chronic idiopathic or spontaneous urticaria. *N Engl J Med*. 2013;368(10):924-935. doi:10.1056/NEJMoa1215372
4. Maurer M, Magerl M, Ansotegui I, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. *Allergy*. 2018;73(8):1575-1596. doi:10.1111/all.13384
5. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A. Plasma bradykinin in angio-oedema. *Lancet*. 1998;351(9117):1693-1697. doi:10.1016/S0140-6736(97)09137-X
6. de Maat S, Maas C. Factor XII: form determines function. *J Thromb Haemost*. 2016;14(8):1498-1506. doi:10.1111/jth.13383
7. Cicardi M, Aberer W, Banerji A, et al. Classification, diagnosis, and approach to treatment for angioedema: consensus report from the Hereditary Angioedema International Working Group. *Allergy*. 2014;69(5):602-616. doi:10.1111/all.12380
8. van der Linden PW, Hack CE, Eerenberg a J, Struyvenberg A, van der Zwan JK. Activation of the contact system in insect-sting anaphylaxis: association with the development of angioedema and shock. *Blood*. 1993;82(6):1732-1739. http://www.ncbi.nlm.nih.gov/pubmed/8400230.
9. van der Linden PW, Hack CE, Struyvenberg A, et al. Controlled insect-sting challenge in 55 patients: correlation between activation of plasminogen and the development of anaphylactic shock. *Blood*. 1993;82(6):1740-1748.
10. Sala-Cunill A, Björkqvist J, Senter R, et al. Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions. *J Allergy Clin Immunol*. 2015;135(4):1031-1043.e6. doi:10.1016/j.jaci.2014.07.057
11. Oschatz C, Maas C, Lecher B, et al. Mast Cells Increase Vascular Permeability by Heparin-Initiated Bradykinin Formation In Vivo. 2011. doi:10.1016/j.immuni.2011.02.008
12. Hofman ZLM, de Maat S, Suffritti C. Cleaved kininogen as a biomarker for bradykinin release in hereditary angioedema. *J Allergy Clin Immunol*. 2017. doi:10.1016/j.jaci.2017.07.012
13. Weller K, Groffik A, Church MK, et al. Development and validation of the Urticaria Control Test: a patient-reported outcome instrument for assessing urticaria control. *J Allergy Clin Immunol*. 2014;133(5):1365-1372, 1372-6. doi:10.1016/j.jaci.2013.12.1076
14. de Maat S, Björkqvist J, Suffritti C, et al. Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations. *J Allergy Clin Immunol*. 2016;138(5):1414-1423.e9. doi:10.1016/j.jaci.2016.02.021
15. Bouma B, Maas C, Hazenberg B, Lokhorst H, GEBBINK M. Increased plasmin- a 2-antiplasmin levels indicate activation of the fibrinolytic system in systemic amyloidoses. *J Thromb Heamostasis*. 2007;5:1139-1142.
16. Haupt TH, Petersen J, Ellekilde G, et al. Plasma suPAR levels are associated with mortality , admission time , and Charlson Comorbidity Index in the acutely admitted medical patient : a prospective observational study. *Crit Care*. 2012;16(4):R130. doi:10.1186/cc11434

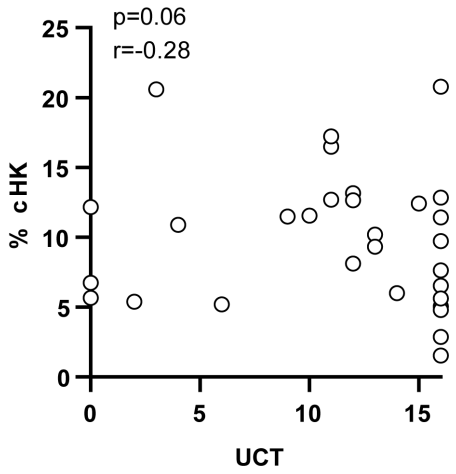
17. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency. *Clin Exp Allergy*. 2014;44(12):1503-1514. doi:10.1111/cea.12293
18. Giard C, Nicolie B, Drouet M, et al. Angio-Oedema Induced by Oestrogen Contraceptives Is Mediated by Bradykinin and Is Frequently Associated with Urticaria. *Dermatology*. 2012;225(1):62-69. doi:10.1159/000340029
19. Cugno M, Tedeschi A, Nussberger J. Bradykinin in idiopathic nonhistaminergic angioedema. *Clin Exp Allergy*. 2016. doi:10.1111/cea.12869
20. Lara-Marquez ML, Christiansen SC, Riedl MA, Herschbach J, Zuraw BL. Threshold-stimulated kallikrein activity distinguishes bradykinin- from histamine-mediated angioedema. *Clin Exp Allergy*. 2018;48(11):1429-1438. doi:10.1111/cea.13219
21. Nussberger J, Cugno M, Cicardi M. Bradykinin-mediated angioedema. *N Engl J Med*. 2002;347(8):621-622. doi:10.1056/NEJM200208223470820
22. Asero R, Marzano A V, Ferrucci S, Cugno M. D-Dimer Plasma Levels Parallel the Clinical Response to Omalizumab in Patients with Severe Chronic Spontaneous Urticaria. *Int Arch Allergy Immunol*. 2017;172(1):40-44. doi:10.1159/000453453
23. Asero R. D-dimer: a biomarker for antihistamine-resistant chronic urticaria. *J Allergy Clin Immunol*. 2013;132(4):983-986. doi:10.1016/j.jaci.2013.04.037
24. Kasperska-Zajac A, Brzoza Z, Rogala B. Blood urokinase plasminogen activator system in chronic urticaria. *Arch Dermatol Res*. 2007;298(8):409-411. doi:10.1007/s00403-006-0715-8
25. Hofman Z, de Maat S, Hack CE, Maas C. Bradykinin: Inflammatory Product of the Coagulation System. *Clin Rev Allergy Immunol*. 2016;51(2):152-161. doi:10.1007/s12016-016-8540-0
26. Cugno M, Hack CE, de Boer JP, Eerenberg AJ, Agostoni A, Cicardi M. Generation of plasmin during acute attacks of hereditary angioedema. *J Lab Clin Med*. 1993;121(1):38-43. http://www.ncbi.nlm.nih.gov/pubmed/8426080.
27. Van Geffen M, Cugno M, Lap P, Loof A, Cicardi M, Van Heerde W. Alterations of coagulation and fibrinolysis in patients with angioedema due to C1-inhibitor deficiency. *Clin Exp Immunol*. 2012;167(3):472-478. doi:10.1111/j.1365-2249.2011.04541.x
28. Bork K, Wulff K, Steinmüller-Magin L, et al. Hereditary angioedema with a mutation in the plasminogen gene. *Allergy*. 2018;73(2):442-450. doi:10.1111/all.13270
29. Valent P, Baghestanian M, Bankl HC, et al. New aspects in thrombosis research: possible role of mast cells as profibrinolytic and antithrombotic cells. *Thromb Haemost*. 2002;87(5):786-790. http://www.ncbi.nlm.nih.gov/pubmed/12038777.
30. Rossi FW, Prevete N, Rivellese F, et al. The Urokinase/Urokinase Receptor System in Mast Cells: Effects of its Functional Interaction with fMLF Receptors. *Transl Med @ UniSa*. 2016;15:34-41. http://www.ncbi.nlm.nih.gov/pubmed/27896225.
31. Tsai P-K, Tsao S-M, Yang W-E, Yeh C-B, Wang H-L, Yang S-F. Plasma Soluble Urokinase-Type Plasminogen Activator Receptor Level as a Predictor of the Severity of Community-Acquired Pneumonia. *Int J Environ Res Public Health*. 2019;16(6). doi:10.3390/ijerph16061035
32. Slot O, Brünner N, Locht H, Oxholm P, Stephens RW. Soluble urokinase plasminogen activator receptor in plasma of patients with inflammatory rheumatic disorders: increased concentrations in rheumatoid arthritis. *Ann Rheum Dis*. 1999;58(8):488-492. doi:10.1136/ard.58.8.488
33. Legány N, Toldi G, Distler JHW, et al. Increased plasma soluble urokinase plasminogen activator receptor levels in systemic sclerosis: possible association with microvascular abnormalities and extent of fibrosis. *Clin Chem Lab Med*. 2015;53(11):1799-1805. doi:10.1515/cclm-2015-0079

34. Sörensen NA, Nikorowitsch J, Neumann JT, et al. Predictive value of soluble urokinase-type plasminogen activator receptor for mortality in patients with suspected myocardial infarction. *Clin Res Cardiol*. April 2019. doi:10.1007/s00392-019-01475-1

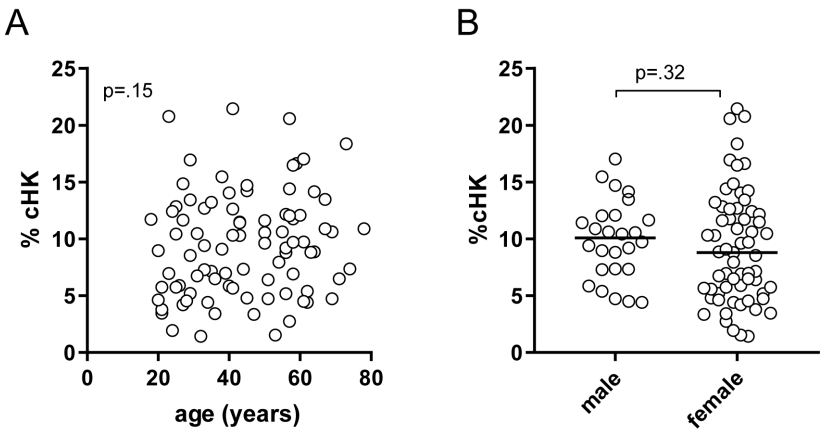
35. Loosen SH, Tacke F, Püthe N, et al. High baseline soluble urokinase plasminogen activator receptor (suPAR) serum levels indicate adverse outcome after resection of pancreatic adenocarcinoma. *Carcinogenesis*. February 2019. doi:10.1093/carcin/bgz033

36. Asero R. ACE inhibitors may interfere with omalizumab in chronic spontaneous urticaria. *J Eur Acad Dermatol Venereol*. 2017;31(8):e358-e359. doi:10.1111/jdv.14155

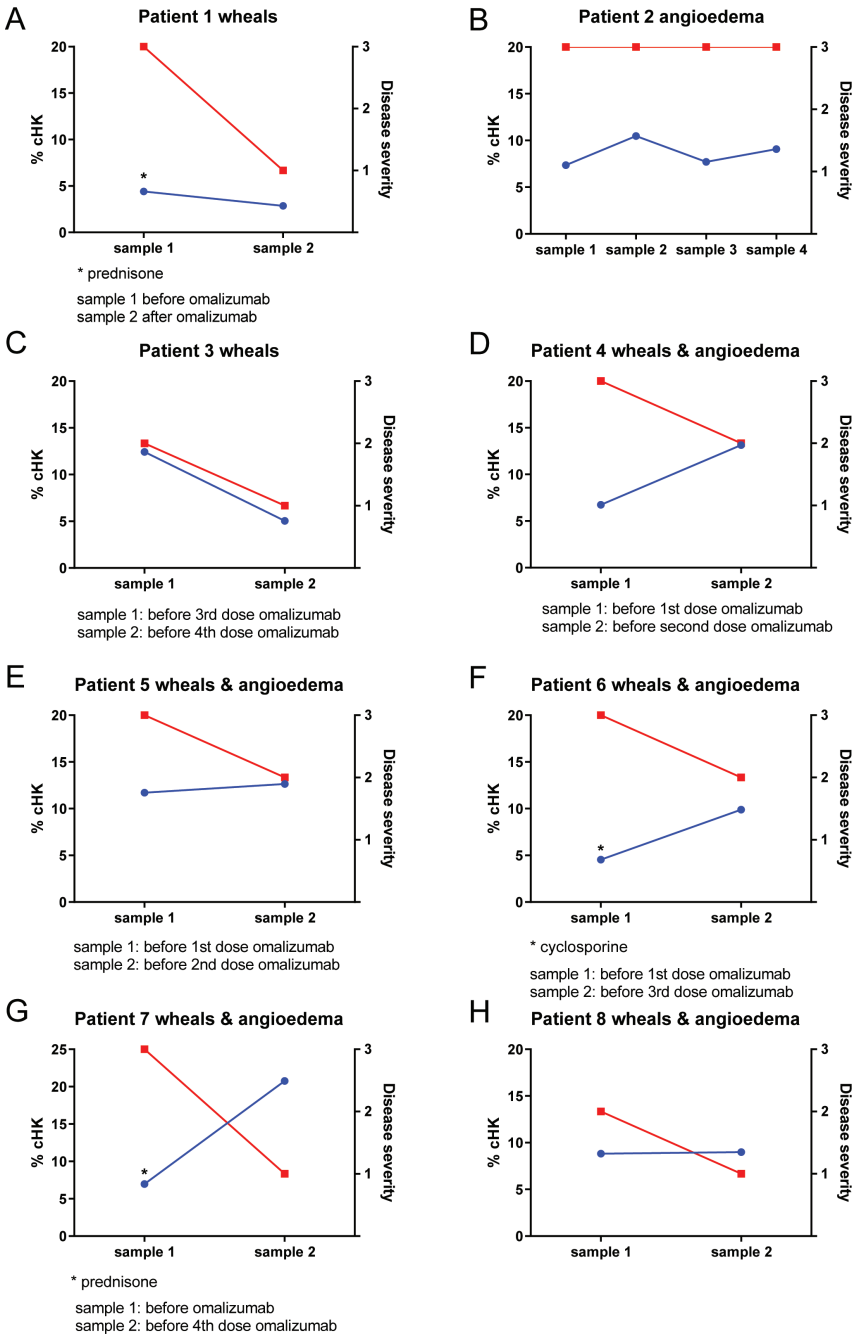
SUPPLEMENTAL DATA



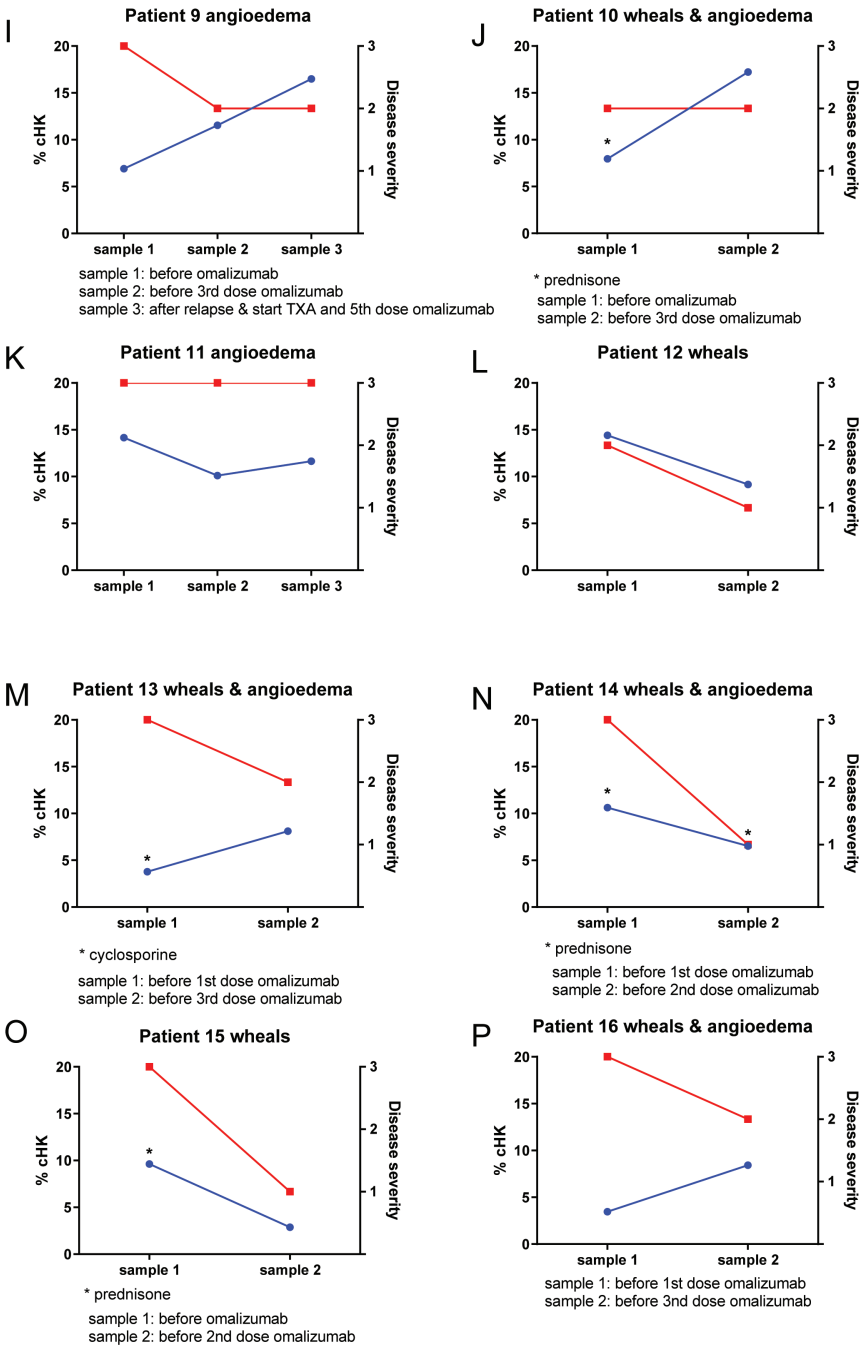
Supplemental figure 1. Cleaved kininogen levels per urticaria control test score. Cleaved kininogen (cHK) levels were determined by ELISA (Y-axis) the urticaria control test (UCT) consist of a 16 point score where 16 means complete disease control and 0 uncontrolled disease (X-axis, for 31 visits the UCT was reported and cHK was determined). Spearman's rank test was used.



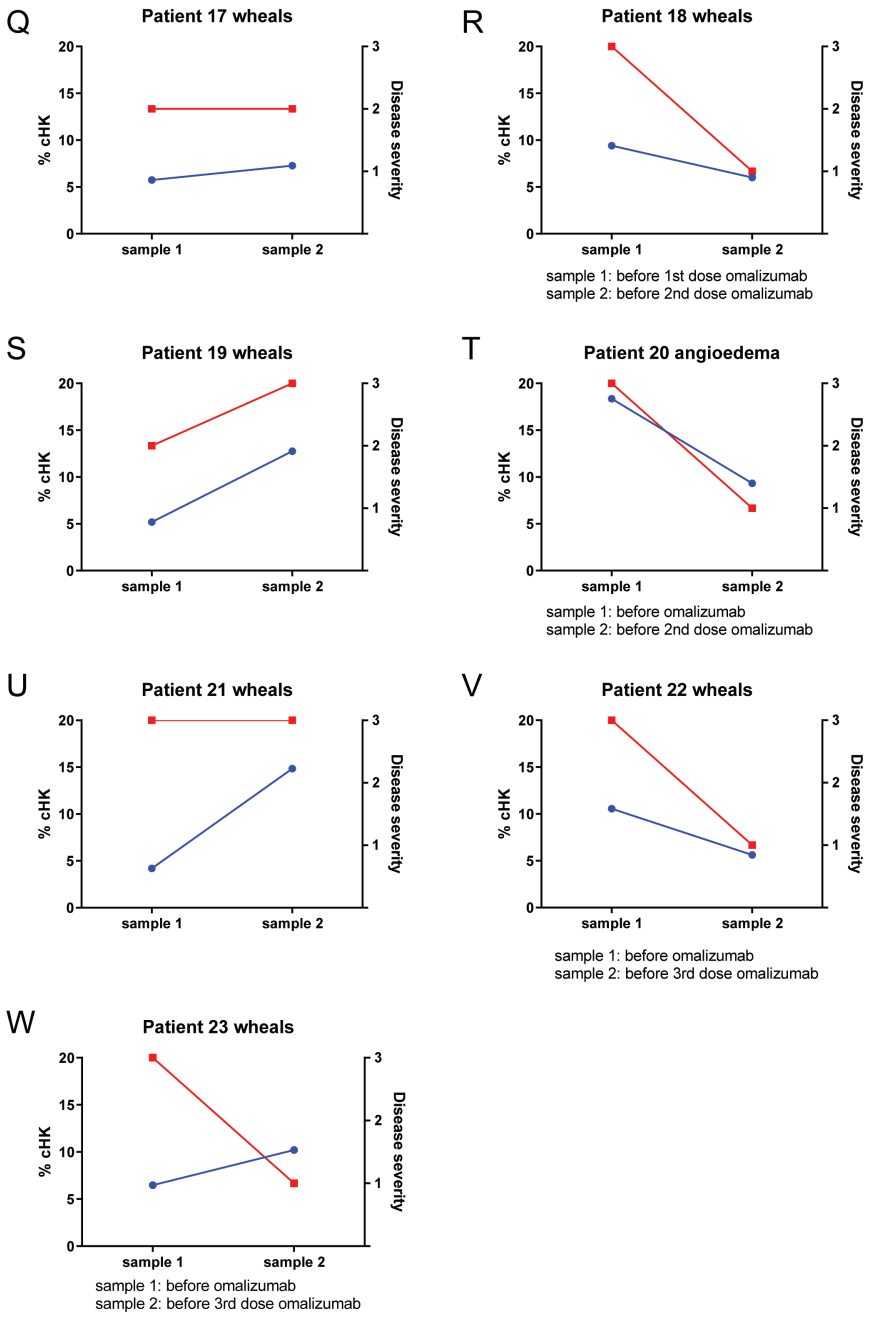
Supplemental figure 2. Cleaved kininogen levels per age and sex. Cleaved kininogen (cHK) levels were determined by ELISA. **A)** cHK did not correlate with age and **B)** did not differ between male (n=26) and female patients (n=60). Dots reflect measurements in 86 patients, lines indicate median, Spearman's rank test and Mann-Whitney t-test were used.



Supplemental figure 3. Cleaved kininogen levels related to disease severity in 23 patients. Cleaved kininogen (cHK) levels determined by ELISA and disease severity in 23 patients of which blood was collected at two or more time points. Blue lines indicates cHK red indicates disease severity where 1 = symptom free, 2= mild symptoms, 3 = moderate to severe symptoms. Where indicated patients received add-on therapy next to prophylactic antihistamine treatment.



Supplemental figure 3. (continued)



Supplemental figure 3. (continued)

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A mutation in the kringle domain of human Factor XII that causes autoinflammation, disturbs zymogen quiescence and accelerates activation

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ABSTRACT

Coagulation factor XII (FXII) drives production of the inflammatory peptide bradykinin. Pathological mutations in the F12 gene, which encodes FXII, causes acute tissue swelling in hereditary angioedema (HAE). Interestingly, a recently identified point mutation in FXII (W268R) is not associated with HAE. Instead, FXII-W268R carriers experienced cold-inducible urticarial-rash, arthralgia, fever and fatigue. We aimed to investigate the molecular characteristics of FXII-W268R and how these differ from HAE-associated FXII mutations. We expressed wild type FXII (FXII-WT), mutants FXII-W268R, FXII-T309R (causes HAE) and other FXII variants in HEK293 freestyle cells. We analyzed expression medium, cell lysates and purified proteins for FXII activation by chromogenic substrate assays, western blotting and ELISA. Recombinant FXII-W268R forms increased amounts of intracellular cleavage products that are also present in expression medium. These fragments display enzymatic activity. An active-site incapacitated mutant FXII-W268R-S544A demonstrates that intracellular fragmentation is largely dependent on FXII autoactivation. Purified FXII-W268R is highly sensitive to activation by plasma kallikrein and plasmin compared to FXII-WT or FXII-T309R. Furthermore, binding studies demonstrate that FXII-W268R exposes a plasminogen binding site that is cryptic in FXII-WT. In plasma, recombinant FXII-W268R spontaneously triggers high molecular weight kininogen (HK) cleavage. Our findings suggest that the FXII-W268R mutation influences protein conformation. This leads to exposure of the activation loop, which is normally concealed in FXII-WT. This results in intracellular autoactivation and constitutive low-grade secretion of activated FXII. Combined, these findings help to explain the chronically increased contact activation in carriers of the FXII-W268R mutation.

INTRODUCTION

Factor XII (FXII) is a 78 kDa liver-expressed serine protease zymogen that circulates in blood plasma (30 µg/mL; 375 nM). It consists of 5 N-terminal domains, which are connected to the C-terminal protease domain by a proline-rich region. The protease domain contains an activation loop with a cleavage motif for plasma kallikrein (PKa) and plasmin. Cleavage after arginine at position 353 of this sequence generates activated FXII (αFXIIa). This full-length two-chain disulfide-linked enzyme can be truncated to generate βFXIIa (~28 kDa)[1]. Both forms of FXIIa can activate plasma prekallikrein, as well as FXII zymogen (autoactivation). As a result of this reciprocal feedback, initial FXII activation is rapidly amplified. FXII is part of the plasma contact system. This system is activated by anionic surface materials, which can be artificial or of natural origin. When FXII binds to these surfaces, it changes conformation[2,3]. This promotes FXII activation through activation loop exposure. The interplay of FXII(a) with PK(a) is dependent on high molecular weight kininogen (HK); this protein is bound to PK in plasma and has surface-binding properties. The contact system is linked to the blood coagulation system but is also responsible for production of bradykinin. This peptide is liberated from its precursor HK by PKa and is a mediator of pain and vascular leakage[4,5].

The connection between FXII activation, bradykinin release and human pathology was first identified in hereditary angioedema (HAE). HAE is a vascular leakage disorder hallmarked by acute attacks of bradykinin-mediated tissue swelling. HAE often results from deficiency in C1-esterase inhibitor (C1-INH), the main plasma inhibitor of FXIIa and PKa. In addition, bradykinin mediated HAE can also be caused by mutations in the F12 gene (encodes FXII). So far, five different mutations in FXII are associated with HAE[6–9]. All of these are located in the proline-rich region, a 53-amino acid sequence that is unique to FXII. Three of these mutations alter the enzymatic processing of FXII during activation through introduction of cleavage sites that are sensitive to plasmin[10] and thrombin[11]. The resulting truncated FXII products display an enhanced sensitivity for activation[10–12].

Recently, a new FXII mutation (FXII-W268R) was reported in the kringle domain of FXII[13,14]. Interestingly, FXII-W268R carriers did not present with acute symptoms of HAE. All four identified FXII-W268R carriers are first-degree family members. They suffer from an autoinflammatory syndrome characterized by cold-inducible urticaria, arthralgia, fever and fatigue. Plasma from these subjects contains markedly increased biomarkers of contact activation. Furthermore, clinical symptoms improve upon administration of a bradykinin receptor antagonist. Strikingly, this pathogenic FXII mutation (W268R) is located only 9 amino acids before the proline-rich region begins, which harbors mutations that cause HAE. This raises the question how FXII-W268R influences protein function and how this differs from mutations that cause HAE. We here report the investigation on the molecular characteristics of FXII with the mutation W268R.

RESULTS

FXII-W268R is spontaneously active

Western blot analyses of expression medium containing FXII-W268R showed that it is partly fragmented (Fig. 1*A*). This fragmentation is predominantly seen under reducing conditions (non-reduced western blot in Fig. S1) and is much less pronounced during expression of FXII-WT or FXII-T309R (that causes HAE). The smallest fragment that FXII-W268R forms is ~30 kDa, resembling the light chain of FXIIa (which contains the protease domain). Chromogenic substrate assays showed that expression medium containing FXII-W268R exhibits spontaneous enzyme activity that is equivalent to 21 ±2.4 nM αFXIIa (progress curves in Fig. 1*B*, quantitation of FXIIa activity in Fig. 1*C*). Furthermore, incubation of FXII-W268R expression medium with plasma prekallikrein results in an accelerated development of enzyme activity, compared to expression medium containing FXII-WT or FXII-T309R (Fig. 1*D*). In a similar manner, prekallikrein activator activity is seen in expression medium containing FXII-W268R that was cloned without the purification tag (tagless FXII-W268R), but not in expression medium containing tagless FXII-WT (Fig. S2*A*). We subsequently found that the spontaneous enzymatic activity in FXII-W268R expression medium is sensitive to inhibition by the chemical serine protease inhibitor PPACK, C1-INH, and the FXIIa-inhibiting monoclonal antibody OT2 (Fig. 1*E*;[15]). Furthermore, enzymatic activity that develops in FXII-W268R expression medium in the presence of plasma prekallikrein is fully inhibited by PPACK and partially inhibited by C1-INH and OT2 (Fig. S3). When C1-INH is added to expression medium of the FXII-W268R mutant, FXIIa-C1-INH complexes form (Fig. 1*F*). These combined experiments show that FXII-W268R is already active upon production.

FXII-W268R is prone to intracellular autoactivation

The spontaneous enzymatic activity in expression medium that is displayed by FXII-W268R led us to investigate the possibility of intracellular FXII-W268R activation. Hereto, we analyzed lysates of cells that express FXII-W268R by western blotting. Increased levels of ~30 kDa FXII fragments were found in lysates of cells that express FXII-W268R compared to those expressing FXII-WT or FXII-T309R (Fig. 2*A*). This suggests that FXII-W268R has an increased propensity towards intracellular cleavage and activation. To evaluate the contribution of autoactivation of FXII to the intracellular activation of FXII-W268R, we expressed an active site mutant, FXII-W268R-S544A. In this 'silent' mutant, the active site serine is replaced by alanine. FXII-W268R-S544A is unable to autoactivate (i.e. cleave and activate other FXII molecules) and cannot activate plasma prekallikrein (Fig. S4). This FXII variant showed markedly less intracellular fragmentation (Fig. 2*A),* indicating that intracellular FXII-W268R cleavage predominantly reflects autoactivation.

Alternatively, we investigated if other intracellular enzymes are involved in the intracellular misprocessing of FXII-W268R. Proprotein convertases are abundant in most cell types, 9 subtypes are important for intracellular processing of secretory proteins, with furin as the archetypical example[16]. *In silico* predictions suggest that the W268R mutation introduces

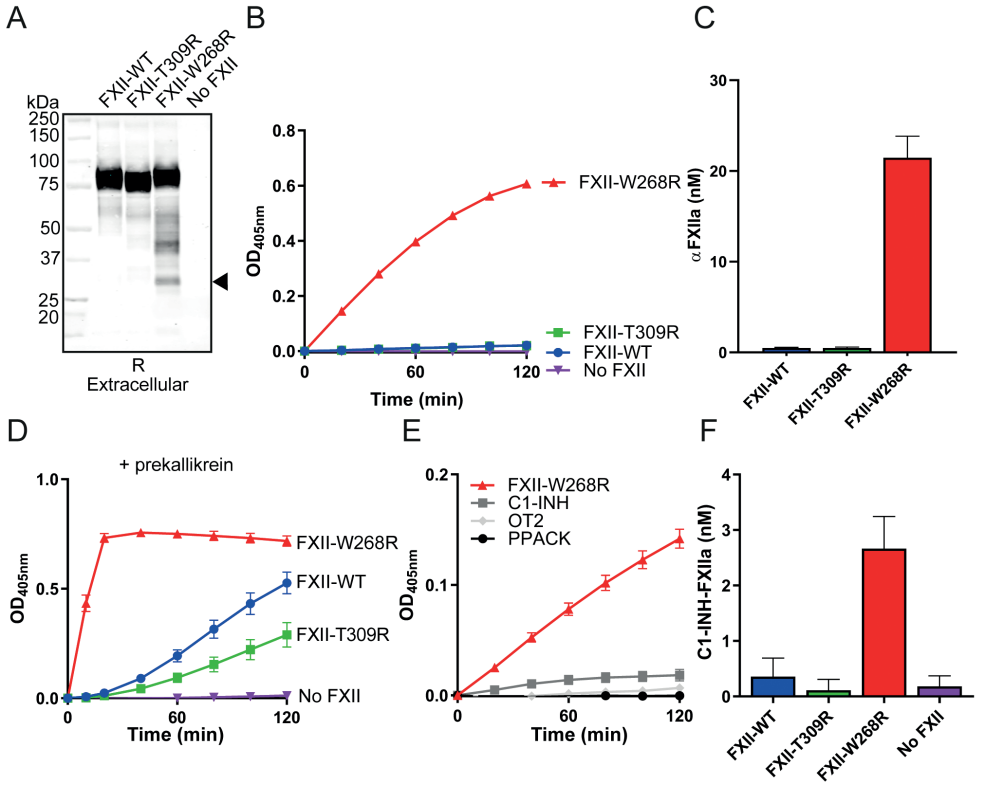

Figure 1. Factor XII-W268R displays spontaneous activity during protein expression. *A*, western blot of expression medium under reducing (R) conditions. The closed arrow indicates the FXIIa light chain. Image is representative for three separate experiments. *B*, chromogenic substrate assay for FXIIa activity in expression medium. *C*, quantification of FXIIa activity in expression medium. Substrate conversion (linear) was monitored in the first 20 minutes of the assays and fitted to a standard curve of αFXIIa in expression medium. *D*, chromogenic substrate assay for plasma prekallikrein activator activity in expression medium. *E*, Effects of PPACK, C1-INH or antibody OT2 on FXIIa activity in expression medium. *F*, FXIIa-C1-INH complex formation in expression medium after incubation with C1-INH, a sigmoidal 4PL fit model was used to interpolate concentrations from a standard curve. Figure panels represent mean and SD of three repeated experiments.

an RXRXXR consensus motif for proprotein convertases in FXII (residues 263-270; Table S1). To question its functionality, we developed four FXII-W268R variants (S267R-W268R and R265A-W268R), respectively improving or disrupting this consensus motif and their active site-incapacitated counterparts (S267R-W268R-S544A and R265A-W268R-S544A; Table S2). Western blot analyses of expression medium containing these FXII-W268R variants showed that FXII fragmentation occurs irrespective of disruption or improvement of the consensus motif for proprotein convertases (Fig. 2*B*). Disabling FXII-autoactivation (S267R-W268R-S544A and R265A-W268R-S544A) prevents this FXII fragmentation to an extent that is comparable to that of FXII-WT and FXII-W268R-S544A (Fig. 2*B*). Furthermore, disruption

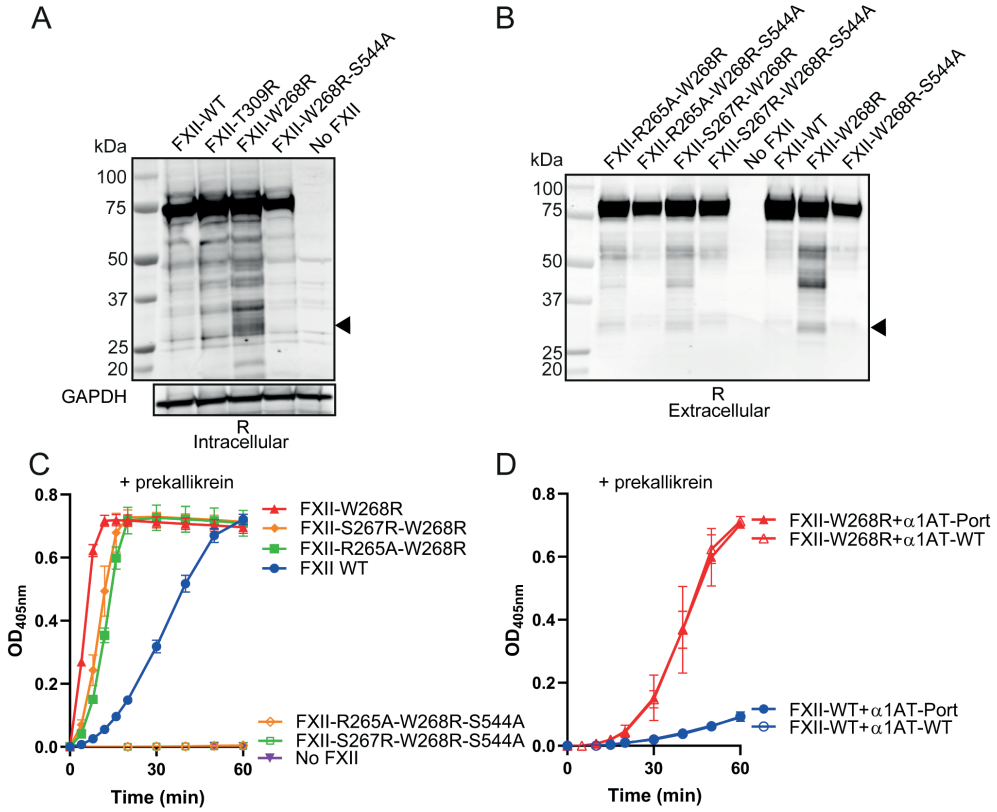

Figure 2. FXII-W268R is prone to intracellular autoactivation. *A*, western blot of transfected cell lysates under reducing (R) conditions. Cell lysates of mock-transfected cells were included as a control (No FXII). The closed arrow indicates FXIIa light chain. Where indicated, the FXII active site is incapacitated (S544A). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a lane loading control. *B*, western blot (reduced; R) of expression medium containing FXII variants with altered putative proprotein convertase motifs. Where indicated, the FXII active site is incapacitated (S544A). Images are representative for three separate experiments. *C*, chromogenic substrate assay for plasma prekallikrein activator activity in expression medium of FXII variants with altered putative proprotein convertase motifs (closed symbols). Where indicated, the FXII active site is incapacitated (S544A; open symbols). *D*, chromogenic substrate assay for plasma prekallikrein activator activity of FXII-WT and FXII-W268R after co-expression with wild-type α1AT (α1AT-WT) or α1AT-Portland. Data represent means and SD of three separate experiments.

or improvement of this motif did not influence plasma prekallikrein-activator activity (Fig. 2*C*), whereas plasma prekallikrein-activator potential was completely lost upon additional disabling of FXII-autoactivation. It is therefore unlikely that the W268R mutation introduces a new functional proprotein convertase cleavage motif.

In addition, co-expression of FXII-W268R with α1-antitrypsin-Portland (α1AT-Portland; inhibits furin[17]) does not reduce plasma prekallikrein-activator activity (Fig. 2*D*), ruling out a role for furin. Together, these findings show that where FXII-WT is resistant to intracellular

autoactivation during protein expression, FXII-W268R is not. We hypothesized that this is the result of inappropriate activation loop exposure by this pathogenic mutant.

Mutation W268R accelerates FXII activation

We next examined the sensitivity of FXII-W268R for activation by either PKa or plasmin. We first purified FXII-W268R via its N-terminal strep-tag. This removes active, truncated βFXIIa, lacking the purification tag (SDS-PAGE with coomassie stain of purified FXII variants in Fig. S5). As a result, the spontaneous activity of purified FXII-W268R is substantially reduced (Fig. 3). Transient exposure to PKa activates both FXII-WT (6.4±0.4 nM αFXIIa), and FXII-T309R (3.0±0.6 nM αFXIIa) to a limited extent (progress curves in Fig. 3*A,* quantification of FXIIa activity in 3*B*). By comparison, FXII-W268R displays a strongly increased activity after exposure to PKa (52 nM ± 3.6 nM) (progress curves in Fig. 3*A,* quantification of FXIIa activity in 3*B*). Tagless versions of these FXII variants behaved highly similarly (Fig. S2*B,C*). In a similar manner, transient exposure of FXII-W268R to plasmin generates 2.5 nM ± 0.6 nM αFXIIa activity, approximately 5-fold more than FXII-WT (0.2 nM ± 0.07 nM) and FXII-T309R (0.5 nM ± 0.2 nM) (progress curves in Fig. 3*C,* quantification of FXIIa activity in 3*D*). We next investigated the influence of surface binding on FXII-W268R activation. Anionic surfaces, like kaolin, enhance FXII (auto)activation. In contrast to activation in solution, FXII-WT and FXII-W268R develop similar enzymatic activity in the presence of kaolin (35 nM ± 3.0 nM and 30 nM ± 3.9 nM αFXIIa, respectively; progress curves in Fig. 3*E,* quantification of FXIIa activity in 3*F*).

We next performed a similar experiment with short dextran sulfate polymers (DXS-15k; Mr ~15000). Due to their short chain length[3], FXII autoactivation is limited (progress curves in Fig. 3*G,* quantification of FXIIa activity in Fig. 3*H*). However, the presence of DXS-15k does enhance activation of FXII-WT by PKa approximately 4-fold (3.9 nM ± 0.74 nM αFXIIa without DXS; 16.9 ± 3.9 nM αFXIIa with DXS). Under the same conditions, FXII-W268R already becomes activated by PKa in the absence of DXS-15k, (98.6 nM ± 8.1 nM αFXIIa; Fig. 3*G*,*H*). Taken together, FXII-W268R behaves as if it is already in a surface-bound, unfolded conformation exposing its activation loop where FXII-WT does not.

Mutation W268R enhances activating cleavage of FXII

We next investigated FXII-W268R with an ELISA that recognizes two-chain FXIIa, which forms when FXII is cleaved after R353. This ELISA does not require an operational active site for target recognition [18]. In good agreement with our chromogenic substrate activity assays, FXII-W268R generates much more FXIIa in response to PKa than FXII-WT or FXII-T309R (Fig. 4*A*). Similarly, more FXIIa forms when this mutant is exposed to plasmin (Fig. 4*B*). We next performed these experiments with active-site incapacitated mutants (S544A). As before, the W268R mutation accelerates formation of a two-chain form of FXII by PKa or plasmin (Fig. 4*C,D*). These combined experiments suggest that cleavage site R353 is cryptic in FXII-WT, but uncovered by the W268R mutation, making it sensitive to activation in solution.

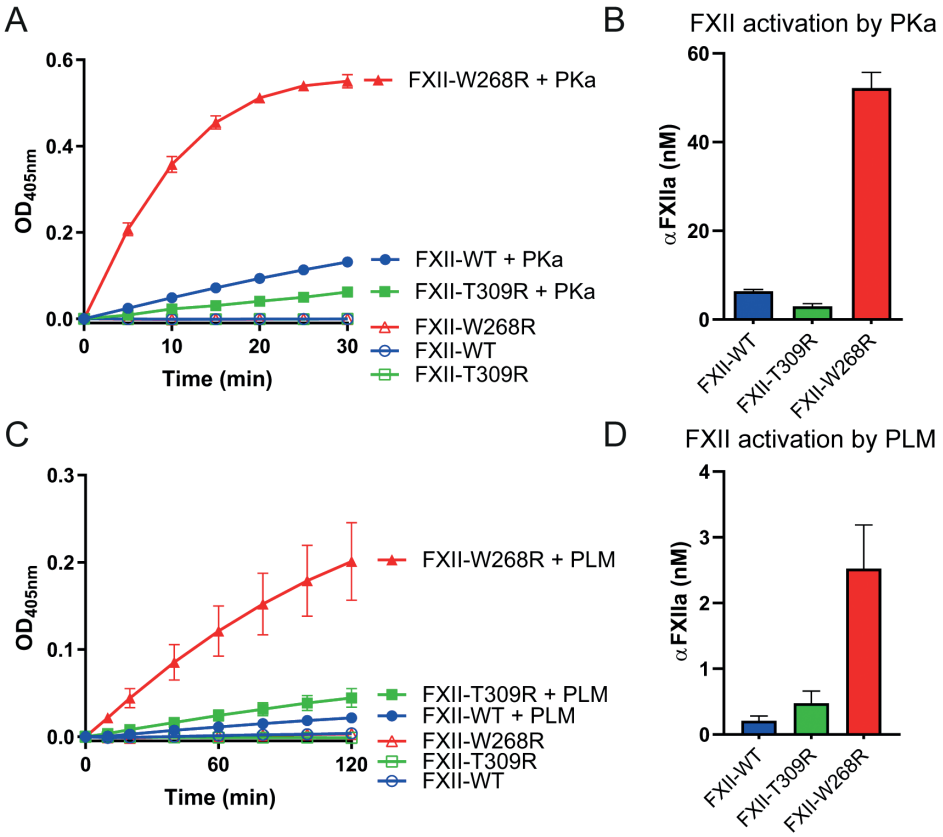

Figure 3. Mutation W268R accelerates FXII activation. *A*, chromogenic substrate assay for FXIIa activity of purified FXII variants after exposure to plasma kallikrein (PKa; 1 µg/mL, closed symbols) or buffer (open symbols). *B*, quantification of FXIIa activity. Substrate conversion (linear) was monitored in the first 5 minutes of the assays and fitted to a standard curve of αFXIIa (0-125 nM). *C*, chromogenic substrate assay for FXIIa activity of purified FXII variants after exposure to plasmin (PLM; 25 µg/mL, closed symbols) or buffer (open symbols). *D*, quantification of FXIIa activity. Substrate conversion (linear) was monitored in the first 10 minutes of the assays and fitted to a standard curve of αFXIIa (0-25 nM). Figures represent means and SD of three repeated experiments.

In further experiments, we examined whether the W268R mutation influences the interaction of FXII with its natural binding partners. We were unable to detect a stable binding interaction between plasma prekallikrein and immobilized FXII-WT or FXII-W268R in direct binding studies (by ELISA; data not shown). In contrast, plasminogen binds to immobilized FXII-WT, but only when it is immobilized in the presence of DXS (Fig. 4*E*). This suggests that FXII contains a cryptic binding site for plasminogen, which is exposed by surface binding. Remarkably, plasminogen binds robustly to immobilized FXII-W268R without DXS (Fig. 4*E*), suggesting an open conformation. This behavior is specific for this mutation, as it was not the case for several FXII mutations that cause HAE (Fig. S6). Together,

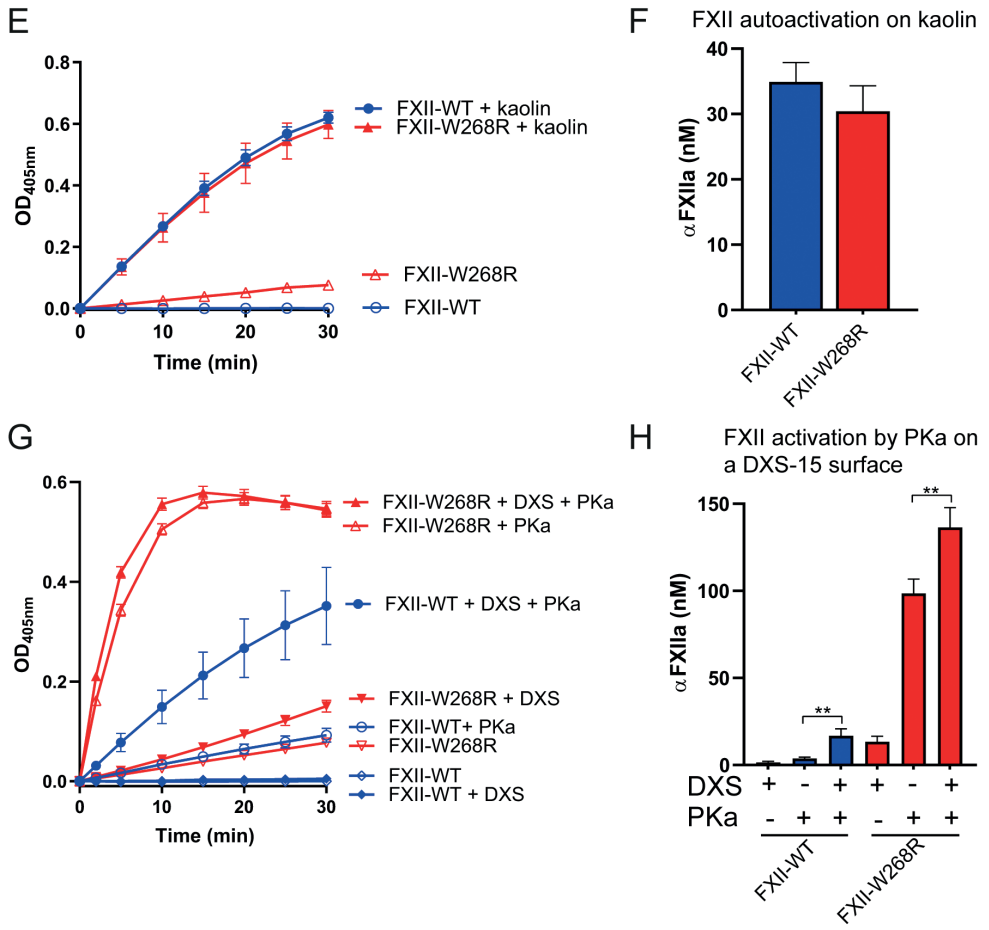

Figure 3 (continued) Mutation W268R accelerates FXII activation. ***E*, chromogenic substrate assay for FXIIa activity triggered by kaolin (closed symbols) or buffer (open symbols).** *F*, quantification of kaolin-triggered FXIIa activity. Spontaneous background activity of FXII variants was subtracted before analyses. Substrate conversion (linear) was monitored in the first 5 minutes of the assays and fitted to a standard curve of αFXIIa (0-125 nM). *G*, chromogenic substrate assay for FXIIa activity after pre-incubation with dextran sulfate (DXS-15k) and subsequent exposure to PKa. FXII-WT and FXII-W268R are indicated by closed circles and closed triangles, respectively. Controls without DXS are indicated by open symbols. Activation of FXII-WT and FXII-W268R in the absence of PKa (autoactivation) is indicated by diamonds and inverted triangles, respectively. *H*, quantification of PKa-triggered FXIIa activity in the absence or presence of DXS-15k. Substrate conversion (linear) was monitored in the first 5 minutes of the assays and fitted to a standard curve of αFXIIa (0-150 nM). Spontaneous background activity of FXII variants was subtracted before analyses. Figures represent means and SD of three repeated experiments.

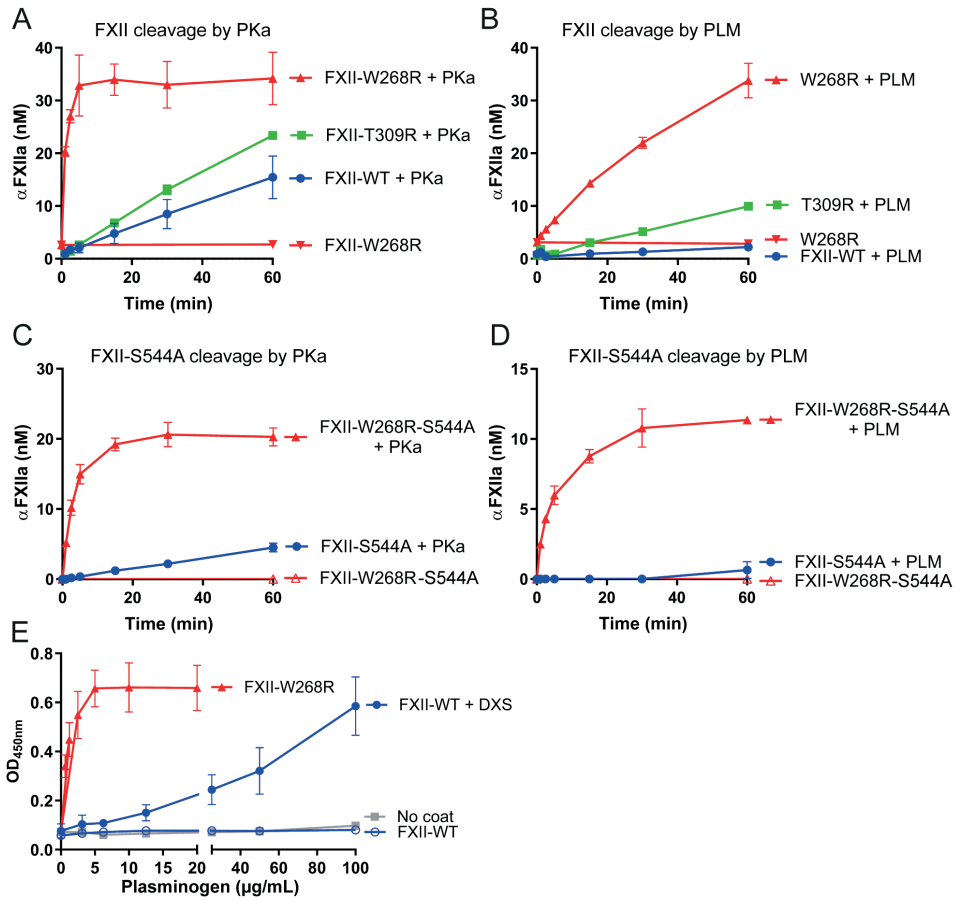

Figure 4. Mutation W268R enhances activating cleavage of FXII. Formation of two-chain FXIIa, monitored by ELISA during exposure to *A*, PKa, or *B*, plasmin (PLM). Formation of two-chain FXII by active-site incapacitated FXII variants (S544A), monitored by ELISA during exposure to *C*, PKa, or *D*, PLM. A sigmoidal 4PL fit model was used to interpolate FXIIa concentrations from a standard curve. *E*, plasminogen binding to immobilized FXII variants in the absence or presence of dextran sulfate (DXS). All data represent means and SD of three separate experiments.

our results suggest that the conformation of FXII determines its activation rate, as well as the interaction with binding partners.

FXII-W268R activity in plasma overrides C1-INH function

In physiology, FXIIa and PKa are inhibited by C1-INH. We next investigated how FXII-W268R interacts with the plasma contact system by western blotting. We first examined FXII fragmentation in buffer in the absence or presence of PKa. Over a 5-minute timespan, 15 µg/mL FXII-WT, FXII-W268R or a 1:1 combination of both (7.5 µg/mL each, to mimic heterozygous FXII-W268R carriers) did not display fragmentation (Fig. 5*A,C*). In contrast, progressive FXII fragmentation was observed in the presence of PKa where FXII-W268R was present

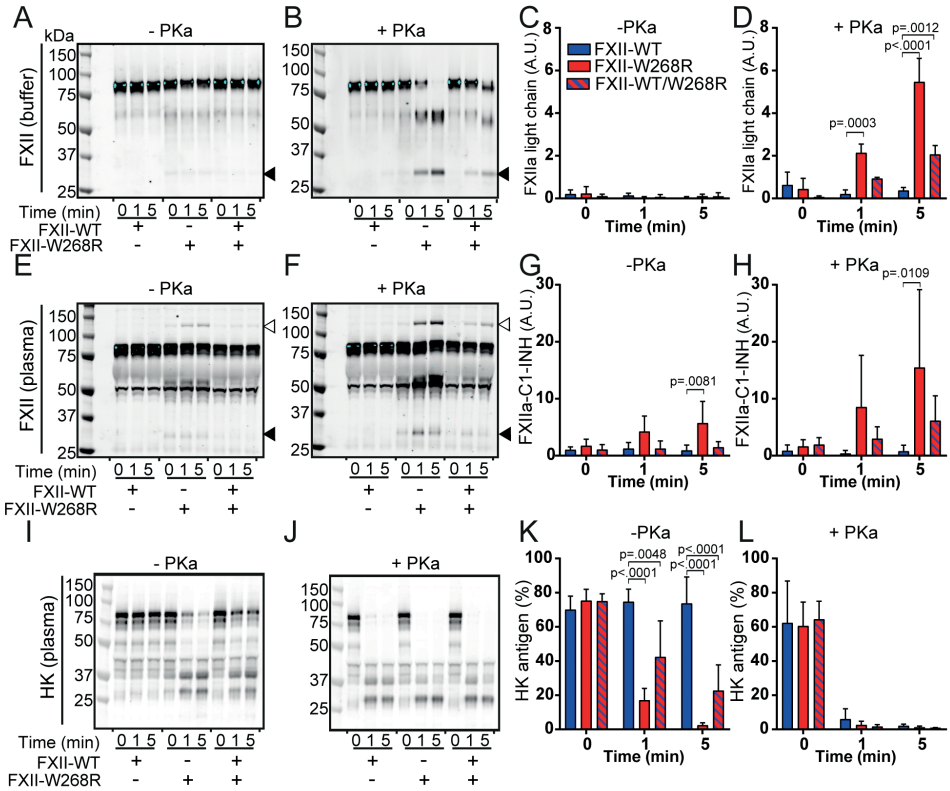

Figure 5. FXII-W268R activity in plasma overrides C1-INH activity. FXII and HK fragmentation were analyzed by western blot (reduced) in a buffered system or in FXII-immunodepleted plasma in the absence or presence of PKa. *A*, Spontaneous FXII fragmentation in buffer. *B*, FXII cleavage by PKa in buffer. Black arrows indicate FXIIa light chain. C,D, densitometric quantification of FXIIa light chain, Y-axis reflects band intensity of panels *A* and *B*, respectively. *E*, Spontaneous FXII fragmentation in plasma. *F*, FXII cleavage by PKa in plasma. Open arrows indicates FXIIa-C1INH complex. *G*,*H*, densitometric quantification of FXIIa-C1INH complex bands, Y-axis reflects band intensity. *I*, Spontaneous HK cleavage in plasma in the presence of FXII variants. *J*, PKa-triggered HK cleavage in the presence of FXII variants. *K*,*L* densitometric quantification of HK antigen, Y-axis reflect band intensity. Conditions with FXII-WT were compared to FXII-W268R and FXII-WT/W268R using 2-way ANOVA, *= p<.05, ** = p<.01, ***=p<.001, ****=p<.0001.

(Fig. 5*B,D*). Similarly, when we resupplemented FXII-immunodepleted plasma with FXII-W268R, we observed spontaneous formation of ~130 kDa bands that correspond to FXIIa-C1-INH complexes (Fig. 5*E*; indicated by open arrows; densitometric quantification in Fig. 5*G*), as well as FXIIa light chain (Fig. 5*E*; indicated by closed arrows). This was more apparent in the presence of PKa (Fig. 5*F,H*). Finally, we analyzed HK cleavage as a reflection of bradykinin production. Where resupplementation of FXII-immunodepleted plasma with FXII-WT did not provoke any HK cleavage, substantial HK cleavage was seen in the presence of FXII-W268R (Fig. 6*I,K*). As expected, the direct addition of PKa to plasma leads to HK cleavage (Fig. 6*J,L*).

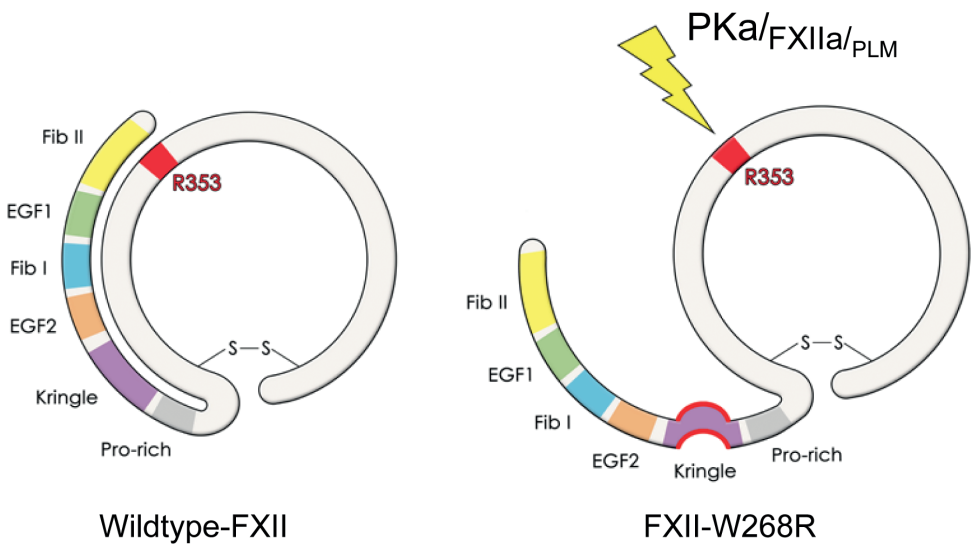

Figure 6. Graphical representation of hypothesized molecular mechanism behind FXII-W268R. Domain abbreviations: Fib II; fibronectin type II domain, EGF1; epidermal growth factor-like domain 1, Fib I; Fibronectin type I domain, EGF2; epidermal growth factor-like domain 2, Kringle; kringle domain, pro-rich; proline-rich region.

These combined observations indicate that the presence of FXII-W268R overrides the inhibitory capacity of C1-INH, triggering HK cleavage in plasma and are consistent with observations in FXII-W268R carriers[13,14].

DISCUSSION

In the present study, we investigated the biochemical features of the FXII mutant FXII-W268R that was recently discovered in a family with autoinflammation[13,14]. We found that this pathogenic variant has a generally increased susceptibility for enzyme activation, unlike mutant FXII-T309R, a biochemically comparable mutation that causes HAE. This remarkable behavior of FXII-W268R is not restricted to the extracellular environment; activation already begins intracellularly. The finding that a fraction of FXII-W268R is secreted as an active enzyme and has a lowered threshold for activation, enables it to override physiological C1-INH activity in plasma. This matches the clinical observations of excessive contact activation in plasma of FXII-W268R carriers.

Based on our findings, we hypothesize that the increased susceptibility for activation of FXII-W268R is related to its conformation (graphically summarized in Fig. 6). However, this might not be the only explanation for the observed clinical phenotype. It is becoming increasingly clear that leukocytes contain a distinct pool of FXII[19]. The presence of intracellular FXIIa in these cells might trigger inflammasome activation, in turn leading

to uncontrolled IL-1β production. Alternatively, this mutation might influence the non-proteolytic contributions of FXII to inflammation[20].

At the same time, there is a growing body of evidence that the conformation of FXII is paramount to its function. Firstly, the binding of FXII to a negatively charged surface changes its conformation. This mediates (auto)activation and causes FXII to expose its activation loop[2,3]. In the present study, we show that the W268R mutation influences FXII in a strikingly similar way. We previously proposed that this is the result of a 'closed' protein conformation, in which the N-terminus of FXII shields its activation loop[1]. This concept is not completely novel, it is also described for the serine proteases plasminogen[21], Factor IX[22], prothrombin[23] and the metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13)[24,25]. Each of these enzymes displays increased functional properties in an open conformation, which mostly is the result of binding to phospholipid surfaces, protein cofactors or polymeric target proteins.

Although our data is consistent with the hypothesis that FXII adopts an open conformation as a result of the W268R mutation, we did not directly demonstrate this. Currently available data on FXII structure is limited to the protease domain[26], βFXIIa[27,28], and the FnI-EGF2 tandem domain[29].

The W268R mutation is located in the kringle domain of FXII. The functional role of this domain in FXII has not been thoroughly investigated. Kringle domains are amongst others conserved in members of the plasminogen activation system, hepatocyte growth factors and prothrombin[30]. However, the kringle domain peptide sequence of FXII has low similarity to those of plasminogen, which are important for lysine-dependent binding to fibrin. Although FXII has fibrin-binding properties and is implicated in fibrinolysis, it remains unclear whether this is dependent on lysine-dependent binding of its kringle domain[31,32].

Interestingly, the two kringle domains of prothrombin are not only important for interactions with Factor Va and Factor Xa. Kringle 1 is involved in ensuring a closed prothrombin conformation through intramolecular binding to its serine protease domain[23]. Disruption or reinforcement of this interaction directly influences the 'open/closed' conformational equilibrium, which impacts prothrombin activation.

We, as well as others, previously proposed that an 'open/closed' mechanism controls the FXII activation[1,23]. Based on our current findings, one can speculate that the kringle domain of FXII has a role in regulation of the 'open/closed' equilibrium of this enzyme.

Although our findings point towards the kringle domain as a regulator of zymogen quiescence, based on the identification of a human pathogenic mutation in this domain, it cannot be ruled out that other domains are involved. Indeed, although a monoclonal antibody against the kringle domain enhances FXII activation by PKa[33], the same holds true for monoclonal antibodies against the adjacent EGF2 domain[11] and the N-terminally positioned FnII domain[34].

This current work reveals the biomolecular differences between FXII-W268R and previously studied FXII-HAE mutations. Throughout our studies, we learned that FXII-

W268R was extremely sensitive to activating cleavage, but not as the result of inappropriate truncation. Moreover, FXII-W268R already activates intracellularly where FXII-T309R does not. Our binding studies show that FXII-W268R exposes a binding site for plasminogen, that is cryptic in FXII-WT or FXII with HAE mutations.

Furthermore, studies with active-site incapacitated FXII mutants and ELISAs, show us that this mutant exposes its activation loop whereas incapacitated FXII-WT does not. Together, these findings strongly suggest that mutation W268R forces FXII into an 'open' conformation. This contrasts the FXII-HAE mutants that we studied earlier: these are in a quiescent state until they are truncated, which requires a specific enzymatic environment. We hypothesize that the continuously increased potential for activation matches the chronic, diffuse clinical picture of autoinflammatory urticaria, while the accelerated activation of the FXII-HAE mutations after truncation matches the acute clinical picture of HAE.

Our work presented here on the FXII-W268R mutation supports a pivotal role for the contact system and bradykinin in pathology beyond HAE. To our knowledge, this is the first study to suggest that a properly controlled conformation of FXII is decisive for the regulation of the contact system in human physiology.

EXPERIMENTAL PROCEDURES

Reagents

Aprotinin, benzamidine hydrochloride hydrate, bromophenol blue, D-desthiobiotin, dextran sulfate (DXS) mol. (average) wt. 500.000 and DXS mol. (average) wt. 15000 (DXS-15k), DL-dithiothreitol (DTT), DNA oligonucleotides, EDTA, glycine, glycerol, hexadimethrine bromide (Polybrene), KCL, Dulbecco's Phosphate Buffered Saline (PBS), skimmed milk powder, NaCl, Na_2HPO_4, NaH_2PO_4, Soy Bean Trypsin Inhibitor (SBTI), bovine serum albumin (BSA), and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA). Restriction enzymes BsmBI, HindIII, XmaI, BsaI, EcoRI, and NotI and T4 DNA polymerase, CutSmart Buffer were from New England Biolabs (Ipswich, Mass, USA). 293fectin transfection reagent, CloneJET PCR cloning kit, pJET1.2/blunt vector, Gibco FreeStyle 293 Expression Medium, Nalgene Single-Use PETG Erlenmeyer Flasks, PolySorp- and MaxiSorp microtitre plates and PageBlue were from Thermo Scientific (Waltham, Mass., USA). Costar "V" Vinyl microtitre plates were from (Corning, NY, USA). Spectra/Por dialysis membranes (MWCO 3.5 kDa) were purchased from Spectrumlabs (CA, USA). Bolt 4-12% Bis-Tris Plus Gels, MOPS buffer, Alexa Fluor 680 Donkey anti-sheep IgG, One-shot TOP10 Chemically Competent E.coli were from Life Technologies (Carlsbad, CA, USA). Immobilon-FL and ethanol were from Merck-Millipore (Amsterdam, the Netherlands). Sodium acetate and $CaCl_2$ were from Merck (Darmstadt, Germany). HEK293F cells were from ATCC (LGC Standards GmbH, Wesel, Germany), Tris-HCl was from Roche (Woerden, the Netherlands), and HEPES was from VWR International (Amsterdam, the Netherlands). Kaolin (light) was purchased from BDH (Poole, UK). DNA gel clean up and plasmid purification kits were from QIAGEN (Hilden, Germany). Polyclonal affinity-purified FXII antibody CL20055 was from Cederlane (Burlington, Canada). Anti-human Factor XII antibody

clone OT2 and streptavidin-poly-HRP were from Sanquin Blood Supply (Division Reagents, Amsterdam, the Netherlands). Anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Clone 4G5; sc-51906) was from Santa Cruz Biotechnology (Dallas, TX, USA). Polyclonal affinity-purified HK antibody 1518BR1 was from Affinity Biologicals (Ancaster, Ontario, Canada) Peroxidase-conjugated polyclonal rabbit anti-sheep antibodies were from Dako (Heverlee, Belgium). PKa, αFXIIa and βFXIIa, were from Enzyme Research laboratories (South Bend, Ind., USA). Odyssey Blocking Reagent and donkey anti-mouse IRdye 800 antibody were from LI-COR (Hamburg, Germany). Streptokinase (Streptase) was purchased from CSL-Behring (Breda, the Netherlands), 3,3',5,5'-tetramethyl-benzidine (TMB) was from Tebu Bio (Heerhugowaard, the Netherlands). Phe-Pro-Arg-chloromethylketone (PPACK) was purchased from Haematologic Technologies (Essex Junction, Vt, UK) Ampicillin (Amp) was obtained from Carl Roth GmbH (Karlsruhe, Germany), H-D-Pro-Phe-Arg-pNA (L2120) was from Bachem (Bubendorf, Switzerland). Plasma purified C1-INH was purchased from Alpha Diagnostics (San Antonio,TX, USA). Blasticidin-S hydrochloride were from Enzo Life Sciences (Raamsdonksveer, the Netherlands). 2x Yeast Tryptone broth and agar capsules were from MP Biomedicals (Santa Ana, CA, USA). Strep-Tactin Sepharose beads were from IBA-Life Sciences (Goettingen, Germany). Gene fragments were ordered from integrated DNA technologies (IDT, Coralville, USA). FXII immunodepleted plasma was from Invitrogen (Carlsbad, CA, USA). Plasminogen was purified from plasma as described [10] and converted to plasmin (2.17 mg/mL) by a 30 minute incubation with 2000 IU/mL streptokinase in 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH=7.4 (HBS) at 37°C.

Factor XII cloning, protein expression and purification

FXII-WT and HAE mutants T309K, T309R and Del&Ins (c971_1018+24del72) were developed as described previously[10]. For FXII-Dup (Pro279-Pro284dup), the two segments at both sides of the reported pathogenic duplication[7] were amplified by PCR (Table S3; Fragment 1: N-Frag_For & Pro279-Pro284dup_P1_Rev. Fragment 2: Pro279-Pro284dup_P2_For & C-Frag_Rev). Subsequently, the two PCR products were fused and amplified by PCR via overhanging primer sequences.

Mutants FXII-W268R and FXII-S544A were developed through two-step assembly. First, the insert encoding the FXII-WT cDNA sequence was amplified by PCR in three segments (annotated as N, M and C, respectively. Primer sequences in Table S3). Segments with the desired mutations were ordered as double stranded DNA fragments. First, the N and M segments were digested with XmaI. Subsequently, the fragments were fused by ligation. The fused product (NM) was amplified via PCR. Hereafter, the NM and C segments were digested with BsaI and fused together by ligation. For FXII triple mutants R265A-W268R, R265A-W268R-S544A, S267R-W268R, S267R-W268R-S544A and W268R-S544A the complete cDNA sequences were ordered as double stranded DNA fragments that were codon-optimized for expression in human cell lines. Fragments were first ligated into the pJET1.2/blunt vector according to manufacturer instructions. Inserts were next digested with EcoRI,

and NotI and ligated into the mammalian expression vector pSM2 encoding an N-terminal IgK secretion signal and 2xstrep-tags for affinity purification (based on pcDNA6/V5-His A [10]). For FXII-WT and W268R expressed without the N-terminal 2xstrep-tags (tagless FXII) cDNA sequences were amplified by PCR from the FXII-WT or W268R cDNA sequence using the primers tagless_FXII_For and FXII_Rev Primer sequences in Table S3). The resulting cDNA fragment contains the N-terminal IgK secretion signal but lacks the 2xstrep-tags. The cDNA product was ligated into the pJET1.2/blunt vector according to manufacturer instructions. Inserts were digested with HindIII and NotI and ligated into the HindIII and NotI restricted mammalian expression vector pcDNA6/V5-His A. Sequences were verified by sanger sequencing prior to transfection.

HEK293F cells, at a concentration of 1,1*10^6 cells/mL, were transfected with 293fectin and cultured in Gibco FreeStyle 293 Expression Medium with 2 mL cell suspensions in 6-well culture plates. After 4 days, cell suspensions were collected and centrifuged at 500xg for analysis of expression medium and cell pellets. Stable cell lines were selected for resistance to 5 µg/mL blasticidin in the presence of penicillin and streptomycin. Resistant cells were expanded and grown in 2L Erlenmeyer flasks for cell culture, cells were centrifuged at 500xg and supernatant medium was harvested twice weekly. FXII inhibitor mix, consisting of SBTI (0.024 g/L), benzamidine (0.174 g/L) and Polybrene (0.056 g/L), was added to the supernatant prior to storage at -20 °C. Supernatants were thawed, concentrated and the buffer was exchanged (100 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 8.0, containing FXII inhibitor mix) on a 10-kDa dialysis membrane in a Quixstand benchtop system (GE Life Sciences, Fairfield, Conn, USA). Strep-Tactin Sepharose beads were used for purification of recombinant FXII via strep-tag, 12.5 µM PPACK was added to washing and elution buffers. Purified recombinant FXII was dialyzed against 4 mM sodium acetate–HCL and 150 mM NaCl (pH 5.5). Protein concentrations were routinely determined by absorption spectroscopy at 280 nm, coomassie protein staining or western blotting (accompanied by densitometric analyses of bands) to ensure equal protein amounts in experiments. Table S2 displays on overview of constructs used.

α1-antitrypsin expression

α1AT-WT and α1AT-Portland[17] in pSM2 were co-transfected with FXII constructs where indicated. A SERPINA1 gene fragment was designed with two BsmBI restriction sites between reactive center loop (P4-P4') allowing for later insertion of the desired reactive center loop DNA template, this SERPINA1-BsmBI fragment was ligated into pSM2 and digested via the BsmBI sites. DNA oligonucleotides encoding for amino acid sequences AIPMSIPP (P4-P4' reactive center loop of α1AT-WT), or RIPRSIPP (P4-P4' reactive center loop of α1AT-Portland) were ligated into the predigested SERPINA1-BsmBI template.

Western blotting

FXII in expression medium

Samples from expression medium were diluted in 3x sample buffer (30% glycerol, 190 mM Tris-HCL, 6% SDS, and 0.006% (m/v) bromophenol blue) with or without 25 mM DTT. HEK293F cell pellets of 2 mL expressions, seeded with $2{,}2*10^6$ cells were washed, resuspended in Dulbecco's PBS and lysed by adding 3x sample buffer with 25mM DTT, heated for 10 minutes at 95°C.

Purified FXII in plasma and buffer

For activation experiments in plasma, 10 µL recombinant FXII-WT, FXII-W268R or a 1:1 combination of both (final FXII concentration 15 µg/mL) were added to 15 µL FXII immunodepleted plasma or 15 µL HBS containing 0,05% BSA (w/v). Directly after mixing, samples were placed at 37°C for 2 minutes and activation was started with 5 µL PKa (final concentration 12.5 µg/mL) or 5 µL HBS as a control. Samples were taken after 1 and 5 minutes and diluted 20x in 1x sample buffer containing 8.3mM DTT. Prior to activation, t=0 samples were created.

All samples were heated for 10 minutes at 95°C and centrifuged for 5 minutes at 2000xg. Samples were separated on 4-12% Bis-Tris gels at 165V for 60 minutes in MOPS buffer and proteins were transferred onto Immobilon-FL membranes at 125V for 55 minutes in blotting buffer (14.4 g/L glycine, 3.03 g/L Tris-HCL, 20% ethanol). Membranes were blocked for 1-2 hours at room temperature using Odyssey blocking reagent diluted 1:1 with Tris-buffered saline (TBS; 50 mM Tris-HCL and 150 mM NaCl, pH 7.4) 0.1% Tween-20. Factor XII or HK were detected with affinity-purified polyclonal antibodies (1:2000) by overnight incubation at 4°C. Next, primary antibodies were detected with Alexa Fluor 680-conjugated donkey anti-sheep IgG (1:5000). GAPDH in lane loading controls was detected with a monoclonal antibody (1:4000) and a IRdye 800-conjugated donkey anti-mouse antibody (1:10000). Membranes were washed with TBSt and distilled water and analyzed on a near-infrared Odyssey scanner (LI-COR).

FXIIa-C1-INH complex ELISA

Methods on VhH selection and production for this assay are described before[18]. VhH 1B12 (5 µg/mL), a monoclonal VhH directed against complexed C1-INH was coated o/n at 4°C on 96-wells MaxiSorp plates. Plates were tapped dry and blocked with HBS containing 0.05% (v/v) Tween-20 (HBSt) and 1% (w/v) skimmed milk powder (mHBSt). Plasma purified C1-INH was added to expression medium at a final concentration of 5 µg/mL. As a reference standard, βFXIIa was added to expression medium from untransfected HEK293F cells and incubated with 5 µg/mL C1-INH. After 10 minutes incubation with purified C1-INH, 100 µL per sample was pipetted on the plate and incubated for 1 hour at room temperature while shaking. Plates were washed with HBSt. The captured FXIIa-C1-INH complexes were detected by 5 µg/mL biotinylated polyclonal VhH targeted against βFXIIa (5 µg/mL) in mHBSt and after rinsing

by streptavidin-poly-HRP (1:2000) in mHBSt. Finally, plates were rinsed and 50 µL/well TMB substrate was added. Substrate conversion was stopped by adding 25 uL/well H_2SO_4 (0.3 M) and absorbance was measured at 450 nm. GraphPad Prism 7.02 software was used to interpolate FXIIa-C1-INH concentrations, using a sigmoidal 4PL fit model to the βFXIIa-C1-INH reference standard.

FXIIa ELISA

VhH B7, a monoclonal VhH directed against FXIIa[18] was coated o/n at 4°C on a 96-wells MaxiSorp plates. Plates were tapped dry and blocked with mHBSt. Ten µg/mL recombinant FXII in HBSt was incubated with 1 µg/mL PKa or 25 µg/mL plasmin. At indicated time points, reactions were stopped by diluting samples five times in mHBSt, supplemented with 250 µM PPACK. Fifty µL per sample was pipetted on the blocked plates and incubated for 1 hour at room temperature while shaking. A reference range of purified αFXIIa in mHBSt containing 200 µM PPACK was included as a standard. Captured FXIIa was detected by the biotinylated monoclonal VhH B2 targeted against βFXIIa (5 µg/mL). Further detection steps were performed as described above (FXIIa-C1-INH complex ELISA).

Protein binding assays

To study binding interactions between FXII and plasminogen, 5 µg/mL recombinant FXII in HBS was coated on 96-well PolySorp plates (o/n at 4°C) in the presence of 100 µM PPACK and in the presence of 1 µg/mL DXS, where indicated. Plates were tapped dry and blocked with 2% BSA in HBS (w/v). A concentration series of plasminogen was diluted in HBS containing 1% (w/v) BSA in the presence of 10 µM PPACK and incubated on plates for 1 hour. After rinsing, bound plasminogen was detected using a polyclonal goat anti-human plasminogen antibody (1:2000) followed by a rabbit anti-sheep IgG-HRP antibody (1:4000) in the presence of 10 µM PPACK. Finally, plates were rinsed and 50 µL/well TMB substrate was added. Substrate conversion was stopped by adding 25 µL H_2SO_4 (0.3 M) and absorbance was measured at 450 nm.

Chromogenic substrate assays

Chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mM) was used to detect FXIIa and/or PKa activity. 96-wells costar plates were blocked with HBS containing 1% BSA (w/v; filtered on 0.20 micron filters) for at least 20 minutes. All sample incubations were performed 37°C. Substrate conversion was read out at 405 nm at 37°C.

Spontaneous activity in medium was determined in 40 µL expression medium containing FXII to which 10 µL chromogenic substrate H-D-Pro-Phe-Arg-pNA was added (0.5 mM final concentration). Amidolytic FXIIa activity was quantified by determining the initial (linear) slopes of substrate conversion for 20 minutes, which were fitted to a standard curve of αFXIIa in expression medium (0-25 nM). Where indicated, inhibitors PPACK (200 µM), C1-INH (25 µg/mL) or monoclonal antibody OT2 (5 µg/mL) for 15 minutes prior to addition of chromogenic substrate H-D-Pro-Phe-Arg-pNA.

Plasma prekallikrein activator activity in 40 µL expression medium containing FXII was measured by addition of 10 µL chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mM final concentration) and plasma prekallikrein (5 µg/mL final concentration), after which PKa-like activity was measured. The effect of inhibitors PPACK (200 µM), C1-INH (25 µg/mL) and mouse monoclonal antibody OT2 (5 µg/mL) was investigated by pre-incubation for 15 minutes, after which 10 µL chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mM final concentration) and plasma prekallikrein (5 µg/mL) were added and PKa-like activity was measured.

FXII activation of purified recombinant FXII variants by PKa or plasmin was investigated by pre-incubating FXII (10 µg/mL in HBS, 0.2% m/v BSA) with PKa (1 µg/mL, in HBS 0.2% BSA), plasmin (25 µg/mL in HBS 0.2% BSA), or buffer (HBS, 0.2% BSA) for 15 minutes at 37°C, followed by a 5-minute incubation with aprotinin for all conditions (100 KIU/mL in HBS 0.2% BSA) to inhibit PKa or plasmin. FXIIa activity was detected by conversion chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mM) and quantified by determining the initial (linear) slopes of substrate conversion for 5 minutes, which were fitted to a standard curve of αFXIIa (0-125 nM) in HBS, 0.2% BSA. Residual PKa activity (monitored as a control without FXII) was routinely subtracted where FXII activation by PKa was investigated.

FXII activation of purified recombinant FXII variants by kaolin was measured by pre-incubating FXII (10 µg/mL in HBS, 0.2% m/v BSA) with kaolin (50 µg/mL) or buffer (HBS) for 15 minutes at 37°C after which chromogenic substrate H-D-Pro-Phe-Arg-pNA (10 µL, 0.5 mM) was added and FXIIa activity detected. FXIIa activity was quantified by determining the initial (linear) slopes of substrate conversion for 5 minutes, which were fitted to a standard curve of αFXIIa (0-125 nM) in HBS, 0.2% BSA.

FXII activation of purified recombinant FXII variants by PKa in the presence of DXS-15 was measured by pre-incubating FXII (10 µg/mL in HBS, 0.2% m/v BSA) with DXS-15 (10 µg/mL in HBS) or buffer (HBS) for 15 minutes at 37°C after which PKa (1 µg/mL, in HBS 0.2% BSA) or buffer (HBS, 0.2% BSA) was added and incubated for 15 min at 37°C after which aprotinin (100 KIU/mL in HBS 0.2% BSA) was added and incubated for 5 minutes at 37°C. chromogenic substrate H-D-Pro-Phe-Arg-pNA (10 µL, 0.5 mM final concentration) was added and FXIIa activity was quantified by determining the initial (linear) slopes of substrate conversion for 5 minutes, which were fitted to a standard curve of αFXIIa (0-156 nM) in HBS, 0.2% BSA. Residual PKa activity (monitored as a control without FXII) was routinely subtracted where FXII activation by PKa was investigated.

STATISTICAL ANALYSIS

Graphpad Prism 7.02 software was used for statistical analysis were indicated. Two-way ANOVA with Dunn's correction of multiple testing was used for comparison of repeated experiments with biological samples.

DISCLOSURE OF CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

ACKNOWLEDGEMENTS

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REFERENCES

1. de Maat S, Maas C. Factor XII : form determines function. *J Thromb Heamostasis*. 2016;14:1498-1506. doi:10.1111/jth.13383
2. Samuel M, Pixley RA, Villanueva MA, Colman RW, Villanueva GB. Human factor XII (Hageman factor) autoactivation by dextran sulfate. Circular dichroism, fluorescence, and ultraviolet difference spectroscopic studies. *J Biol Chem*. 1992;267(27):19691-19697.
3. Citarella F, Wuillemin WA, Lubbers YT, Hack CE. Initiation of contact system activation in plasma is dependent on factor XII autoactivation and not on enhanced susceptibility of factor XII for kallikrein cleavage. *Br J Haematol*. 1997;99(1):197-205. http://www.ncbi.nlm.nih.gov/pubmed/9359524.
4. Hall SM, LeBaron L, Ramos-Colon C, et al. Discovery of Stable Non-opioid Dynorphin A Analogues Interacting at the Bradykinin Receptors for the Treatment of Neuropathic Pain. *ACS Chem Neurosci*. 2016;7(12):1746-1752. doi:10.1021/acschemneuro.6b00258
5. De Maat S, Hofman ZLM, Maas C. Hereditary angioedema: the plasma contact system out of control. *J Thromb Haemost*. 2018;16(9):1674-1685. doi:10.1111/jth.14209
6. Dewald G, Bork K. Missense mutations in the coagulation factor XII (Hageman factor) gene in hereditary angioedema with normal C1 inhibitor. *Biochem Biophys Res Commun*. 2006;343(4):1286-1289. doi:10.1016/j.bbrc.2006.03.092
7. Kiss N, Barabás E, Várnai K, et al. Novel duplication in the F12 gene in a patient with recurrent angioedema. *Clin Immunol*. 2013;149(1):142-145. doi:10.1016/j.clim.2013.08.001
8. Bork K, Wulff K, Meinke P, Wagner N, Hardt J, Witzke G. A novel mutation in the coagulation factor 12 gene in subjects with hereditary angioedema and normal C1-inhibitor. *Clin Immunol*. 2011;141(1):31-35. doi:10.1016/j.clim.2011.07.002
9. Gelincik A, Demir S, Olgaç M, et al. Idiopathic angioedema with F12 mutation: is it a new entity? *Ann Allergy Asthma Immunol*. 2015;114(2):154-156. doi:10.1016/j.anai.2014.11.018
10. de Maat S, Björkqvist J, Suffritti C, et al. Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations. *J Allergy Clin Immunol*. 2016;138(5):1414-1423.e9. doi:10.1016/j.jaci.2016.02.021
11. Ivanov I, Matafonov A, Sun M-F, et al. A mechanism for hereditary angioedema with normal C1 inhibitor: an inhibitory regulatory role for the factor XII heavy chain. *Blood*. 2019;133(10):1152-1163. doi:10.1182/blood-2018-06-860270
12. de Maat S, Clark CC, Boertien M, et al. Factor XII truncation accelerates activation in solution. *J Thromb Haemost*. 2019;17(1):183-194. doi:10.1111/jth.14325
13. Scheffel J, Mahnke N, Hofman Z, et al. 10th Congress of International Society of Systemic Auto-Inflammatory Diseases (ISSAID). *Pediatr Rheumatol*. 2019;17(S1):18. doi:10.1186/s12969-019-0313-x
14. Mahnke N.A., Scheffel J., Wu J., de Maat S., Maas C., Hofman Z.L, Ennis S., Holloway J.W., Pengelly R.J., Maurer M. KK. 45th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF) Zurich, Switzerland, March 7-10, 2018. *Exp Dermatol*. 2018;27(3):e2-e106. doi:10.1111/exd.13486
15. Dors DM, Nuijens JH, Huijbregts CC, Hack CE. A novel sensitive assay for functional factor XII based on the generation of kallikrein-C1-inhibitor complexes in factor XII-deficient plasma by glass-bound factor XII. *Thromb Haemost*. 1992;67(6):644-648. http://www.ncbi.nlm.nih.gov/pubmed/1509403.
16. Seidah NG, Sadr MS, Chrétien M, Mbikay M. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. *J Biol Chem*. 2013;288(30):21473-21481. doi:10.1074/jbc.R113.481549

17. Jean F, Stella K, Thomas L, et al. α1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: Application as an antipathogenic agent. *Proc Natl Acad Sci U S A*. 1998;95(13):7293-7298. doi:10.1073/pnas.95.13.7293

18. de Maat S, van Dooremalen S, de Groot PG, Maas C. A nanobody-based method for tracking factor XII activation in plasma. *Thromb Haemost*. 2013;110(3):458-468. doi:10.1160/TH12-11-0792

19. Stavrou EX, Fang C, Bane KL, et al. Factor XII and uPAR upregulate neutrophil functions to influence wound healing. *J Clin Invest*. 2018;128(3):944-959. doi:10.1172/JCI92880

20. Renné T, Stavrou EX. Roles of Factor XII in Innate Immunity. *Front Immunol*. 2019;10:2011. doi:10.3389/fimmu.2019.02011

21. Xue Y, Bodin C, Olsson K. Crystal structure of the native plasminogen reveals an activation-resistant compact conformation. *J Thromb Haemost*. 2012;10(7):1385-1396. doi:10.1111/j.1538-7836.2012.04765.x

22. Johnson DJD, Langdown J, Huntington JA. Molecular basis of factor IXa recognition by heparin-activated antithrombin revealed by a 1.7-A structure of the ternary complex. *Proc Natl Acad Sci U S A*. 2010;107(2):645-650. doi:10.1073/pnas.0910144107

23. Chinnaraj M, Planer W, Pozzi N. Structure of Coagulation Factor II: Molecular Mechanism of Thrombin Generation and Development of Next-Generation Anticoagulants. *Front Med*. 2018;5:281. doi:10.3389/fmed.2018.00281

24. South K, Luken BM, Crawley JTB, et al. Conformational activation of ADAMTS13. *Proc Natl Acad Sci U S A*. 2014;111(52):18578-18583. doi:10.1073/pnas.1411979112

25. Clark CC, Mebius MM, De Maat S, et al. Truncation of ADAMTS13 by Plasmin Enhances Its Activity in Plasma. *Thromb Haemost*. 2018;118(3). doi:10.1055/s-0038-1627460

26. Pathak M, Wilmann P, Awford J, et al. Coagulation factor XII protease domain crystal structure. *J Thromb Haemost*. 2015;13(4):580-591. doi:10.1111/jth.12849

27. Dementiev A, Silva A, Yee C, et al. Structures of human plasma β-factor XIIa cocrystallized with potent inhibitors. *Blood Adv*. 2018;2(5):549-558. doi:10.1182/bloodadvances.2018016337

28. Pathak M, Manna R, Li C, et al. Crystal structures of the recombinant β-factor XIIa protease with bound Thr-Arg and Pro-Arg substrate mimetics. *Acta Crystallogr Sect D, Struct Biol*. 2019;75(Pt 6):578-591. doi:10.1107/S2059798319006910

29. Beringer DX, Kroon-Batenburg LMJ. The structure of the FnI-EGF-like tandem domain of coagulation factor XII solved using SIRAS. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2013;69(Pt 2):94-102. doi:10.1107/S1744309113000286

30. Castellino FJ, Beals JM. The genetic relationships between the kringle domains of human plasminogen, prothrombin, tissue plasminogen activator, urokinase, and coagulation factor XII. *J Mol Evol*. 1987;26(4):358-369. doi:10.1007/BF02101155

31. Konings J, Govers-Riemslag JWP, Philippou H, et al. Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin. *Blood*. 2011;118(14):3942-3951. doi:10.1182/blood-2011-03-339572

32. Mitchell JL, Lionikiene AS, Georgiev G, et al. Polyphosphate colocalizes with factor XII on platelet-bound fibrin and augments its plasminogen activator activity. *Blood*. 2016;128(24):2834-2845. doi:10.1182/blood-2015-10-673285

33. Ravon DM, Citarella F, Lubbers YT, Pascucci B, Hack CE. Monoclonal antibody F1 binds to the kringle domain of factor XII and induces enhanced susceptibility for cleavage by kallikrein. *Blood*. 1995;86(11):4134-4143. http://www.ncbi.nlm.nih.gov/pubmed/7492770.

34. Citarella F, te Velthuis H, Helmer-Citterich M, Hack CE. Identification of a putative binding site for negatively charged surfaces in the fibronectin type II domain of human factor XII--an immunochemical and homology modeling approach. *Thromb Haemost*. 2000;84(6):1057-1065. http://www.ncbi.nlm.nih.gov/pubmed/11154114.

SUPPLEMENTAL DATA

Supplementary table 1. General proprotein convertase cleavage site predictions[I]

protein	position	context	score	probability
FXII-WT	268	RDRLSW/EY	-	-
FXII-W268R	268	RDRLSR/EY	0.114	Low
FXII-S267R-W268R	268	RDRL**R**R/EY	.594	High
FXII-R265A-W268R	268	RD**A**LSR/EY	.05	Low
FXII-WT	326	ALPAKR/EQ	.832	High
FXII-WT	353	LSSMTR/VV	.857	High

/ indicates cleavage site
Red-bold indicates introduced point mutation changing the likelihood of cleavage at the 268 arginine.
I)ProP 1.0 Server: http://www.cbs.dtu.dk

Supplementary table 2. Factor XII sequence variations (FASTA sequence: http://www.uniprot.org/uniprot/P00748)

IPPWEAPKEHKYKAEEHTVVLTVTGEPCHFPFQYHRQLYHKCTHKGRPGPQPWCATTPNFDQDQRWGYCLEPKK
VKDHCSKHSPCQKGGTCVNMPSGPHCLCPQHLTGNHCQKEKCFEPQLLRFFHKNEIWYRTEQAAVARCQCKGP
DAHCQRLASQACRTNPCLHGGRCLEVEGHRLCHCPVGYTGAFCDVDTKASCYDGRGLSYRGLARTTLSGAPCQ
PWASEATYRNVTAEQARNWGLGGHAFCRNPDNDIRPWCFVLNRDRLSWEYCDLAQCQTPTQAAPPTPVSPRL
HVPLMPAQPAPPKPQPTTRTPPQSQTPGALPAKREQPPSLTRNGPLSCGQRLRKSLSSMTRVVGGLVALRGAH
PYIAALYWGHSFCAGSLIAPCWVLTAAHCLQDRPAPEDLTVVLGQERRNHSCEPCQTLAVRSYRLHEAFSPVSYQ
HDLALLRLQEDADGSCALLSPYVQPVCLPSGAARPSETTLCQVAGWGHQFEGAEEYASFLQEAQVPFLSLERCSA
PDVHGSSILPGMLCAGFLEGGTDACQGDSGGPLVCEDQAAERRLTLQGIISWGSGCGDRNKPGVYTDVAYYLAW
IREHTVS

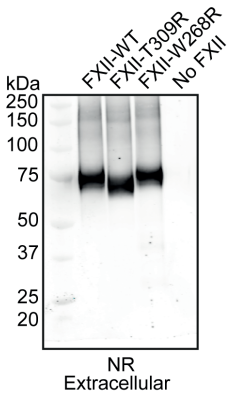
Color	Mutant	Properties
Yellow	FXII-W268R	Identified in one family with autoinflammatory symptoms
	FXII-R265A-W268R	Incapacitated proprotein convertase motif
	FXII-S267R-W268R	Enhanced proprotein convertase motif
Green	FXII-S554A	Incapacitated active site
Yellow/green	FXII-W268R-S554A	FXII-W268R with incapacitated active site
Yellow/green	FXII-R265A-W268R-S544A	FXII- Incapacitated proprotein convertase motif -W268R-with incapacitated active site
Yellow/green	FXII-S267R-W268R-S544A	FXII- Enhanced proprotein convertase motif -W268R-with incapacitated active site
Blue	FXII-T309R, FXII-T309K	Identified in HAE-FXII*
Pink	FXII c.892_909dup (FXII-dup)	Sequence duplicated in HAE-FXII*
Underlined	FXIIc.971_1018+4del48 (FXII-del&ins)	Changed to RSREGAGRARFRPAGRGLSVLSPCSST in HAE-FXII*

* de Maat et al. *J Thromb Haemost*. 2018 Sep;16(9):1674-1685. doi: 10.1111/jth.14209.

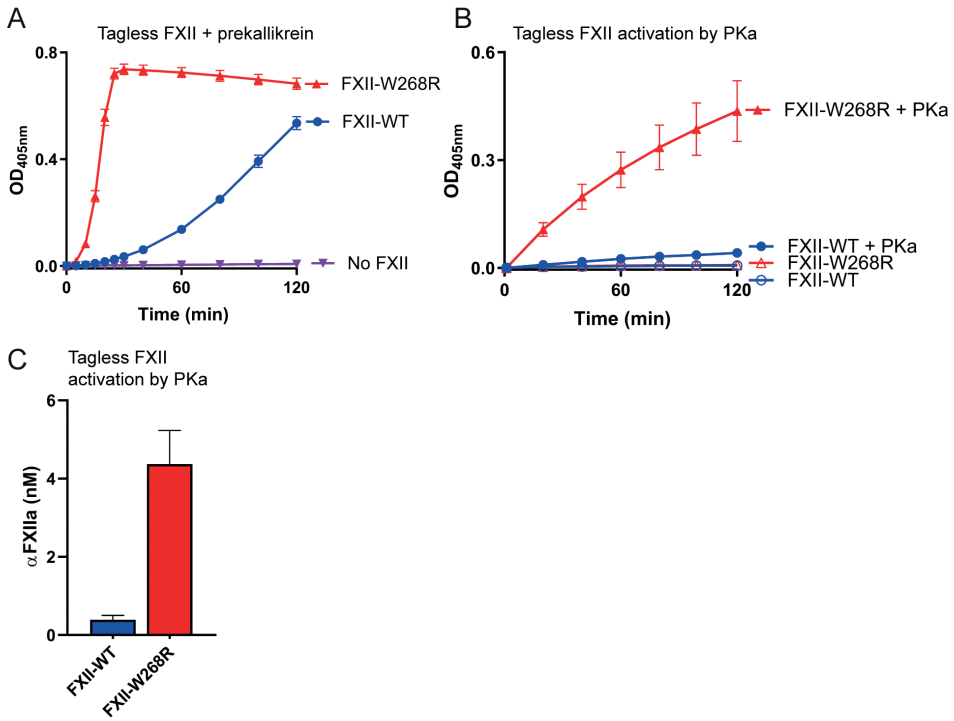
Supplementary table 3. Primer sequences.

Pro279-Pro284dup_P1_Rev: GTCGGAGGCGCCGCCTGGGTTGGAGGCGCCGCCTGGGTTGGGGTCTGGCACTGTGCCAGGTCG
Pro279-Pro284dup_P2_For: CCAACCCAGGCGGCGCCTCCAACCCAGGCGGCGCCTCCGACCCCGGTGTCCCCTAGGCTTC
N-Frag_For: GGAGAATTCGAAAACCTGTATTTTCAGTCTATTCCACC
N-Frag_Rev: GCTCCCGGGGTCTGGGACTGAGGCGGGG
M-Frag_For: GACCCCGGGAGCCTTGCCGGCGAAGCG
M-Frag_Rev: CGTGGTCTCGGAGGGTCGCGCGGCGCCGCTTG
C-Frag_For: GACCCTCCGAGACCACGCTCTGCCAGG
C-Frag_Rev: CGAGCGGCCGCTCATCAGGAAACGGTGTGCTCCCG
FXII_tagless_For: GAAAAGCTTATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTCCACCTTGGGAAGCCC

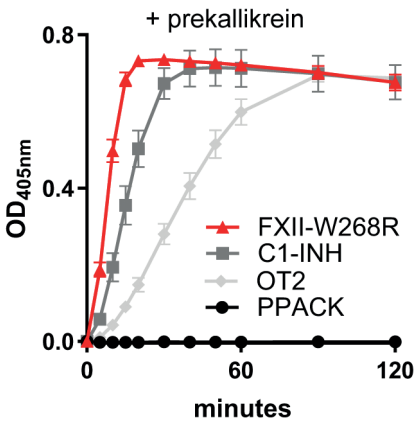
FXII_Rev (for tagless constructs): CGAGCGGCCGCTCATCAGGAAA



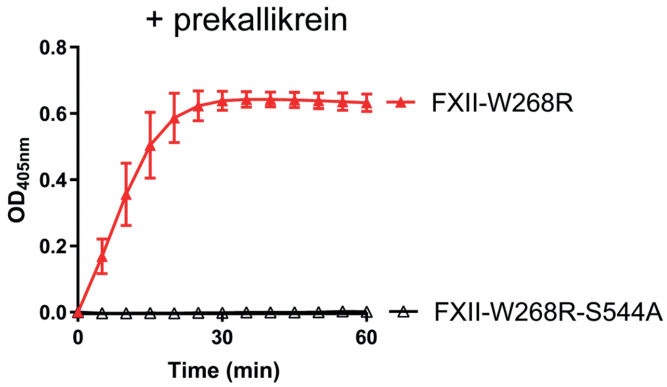
Supplemental figure 1. Western blot of expression medium containing factor XII variants under non-reducing (NR) conditions. The image is representative of three separate experiments.



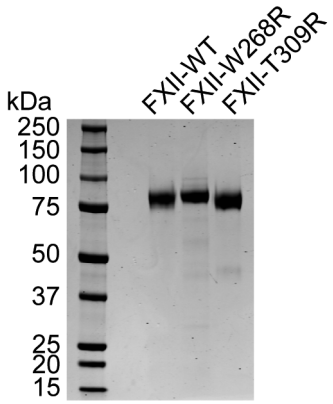
Supplemental figure 2. Tagless factor XII variants. *A*, chromogenic substrate assay for plasma prekallikrein activator activity in expression medium. *B*, chromogenic substrate assay for FXIIa activity of FXII variants in expression medium after exposure to plasma kallikrein (PKa; closed symbols) or buffer (open symbols). Forty µL cell culture supernatant was incubated for 30 minutes at 37°C with 20 µL PKa (prediluted in cell culture medium; final concentration 2 µg/mL). Subsequently, 20 µL aprotinin (200 KIU/mL final concentration was added. After 15 minutes incubation, 20 µL H-D-Pro-Phe-Arg-pNA was added and substrate conversion monitored. *C*, quantification of FXIIa activity. Substrate conversion (linear) was monitored in the first 20 minutes of the assays and fitted to a standard curve of αFXIIa (0-12.5 nM). Figure panels represent mean and SD of three repeated experiments.



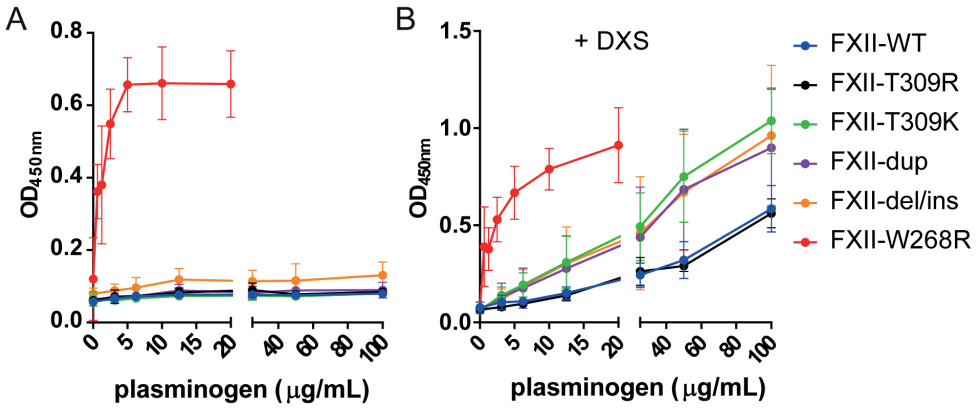
Supplemental figure 3. The effect of PPACK, C1-INH or monoclonal antibody OT2 on prekallikrein activator activity in expression medium. Data represents mean and SD of three repeated experiments.



Supplemental figure 4. Plasma prekallikrein activator activity of FXII-W268R and active-site incapacitated FXII-W268R-S544A in expression medium. Data represents mean and SD of three repeated experiments.



Supplemental figure 5. Purity analysis of factor XII variants after affinity purification. Protein (1 µg/lane) was separated by SDS-PAGE under reducing conditions on 4-12% gels and stained with Coomassie blue.



Supplemental figure 6. Unlike FXII-W268R, FXII-HAE variants do not expose a plasminogen binding site. Plasminogen binding to immobilized FXII variants, including FXII-HAE mutations FXII-T309R, FXII-T309K, FXII-del/ins or FXII-dup in the absence (A) or presence (B) of dextran sulfate (DXS). Data represent means and SD of three separate experiments.

12

The Fibronectin Type II Domain of Factor XII Ensures Zymogen Quiescence

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ABSTRACT

Factor XII (FXII) zymogen activation requires cleavage after arginine 353 located in the activation loop. This cleavage can be executed by activated FXII (FXIIa; autoactivation) or plasma kallikrein. Previous studies proposed that the activation loop of FXII is shielded to regulate FXII activation and subsequent contact activation. In this study, we aimed to elucidate this mechanism by expressing and characterizing 7 consecutive N-terminally truncated FXII variants as well as full-length wild-type (WT) FXII. As soon as the fibronectin type II domain is lacking (FXII Δ1-71), FXII cleavage products are seen on western blot. These fragments display spontaneous amidolytic activity, indicating that FXII without the fibronectin type II domain is susceptible to autoactivation. Additionally, FXII Δ1-71 is more easily activated by plasma kallikrein than full-length WT FXII. To exclude a contribution of autoactivation, we expressed active-site incapacitated FXII variants (S544A). FXII Δ1-71 S544A is highly susceptible to cleavage by plasma kallikrein indicating that the activation loop is exposed. In surface binding experiments we found that the fibronectin type II domain is non-essential for binding to polyphosphate or kaolin, whereas the following epidermal growth factor-like (EGF-1) domain is indispensable. Binding of full-length FXII S544A to polyphosphate or kaolin increases its susceptibility to cleavage by plasma kallikrein. Moreover, the activation of full-length WT FXII by plasma kallikrein increased approximately 6-fold in the presence of kaolin. Combined, these findings suggest that the fibronectin type II domain shields the activation loop of FXII, ensuring zymogen quiescence.

INTRODUCTION

Factor XII (FXII) is the initiating enzyme of the plasma contact system. It can independently activate when it binds to anionic surfaces and polymers. This process is heavily dependent on a cleavage event after arginine 353 (R353) located in the activation loop (spanning residues 340–467, mature protein numbering) that can be executed by activated FXII (FXIIa; autoactivation) and plasma kallikrein (PKa).[1,2] As a result, PKa is an important catalyst for FXII activation and subsequent contact activation.[3]

Contact activation has two functions: 1) coagulation via the intrinsic pathway and 2) bradykinin liberation from high molecular weight kininogen (HK). This latter molecule is a cofactor for PKa in contact activation.

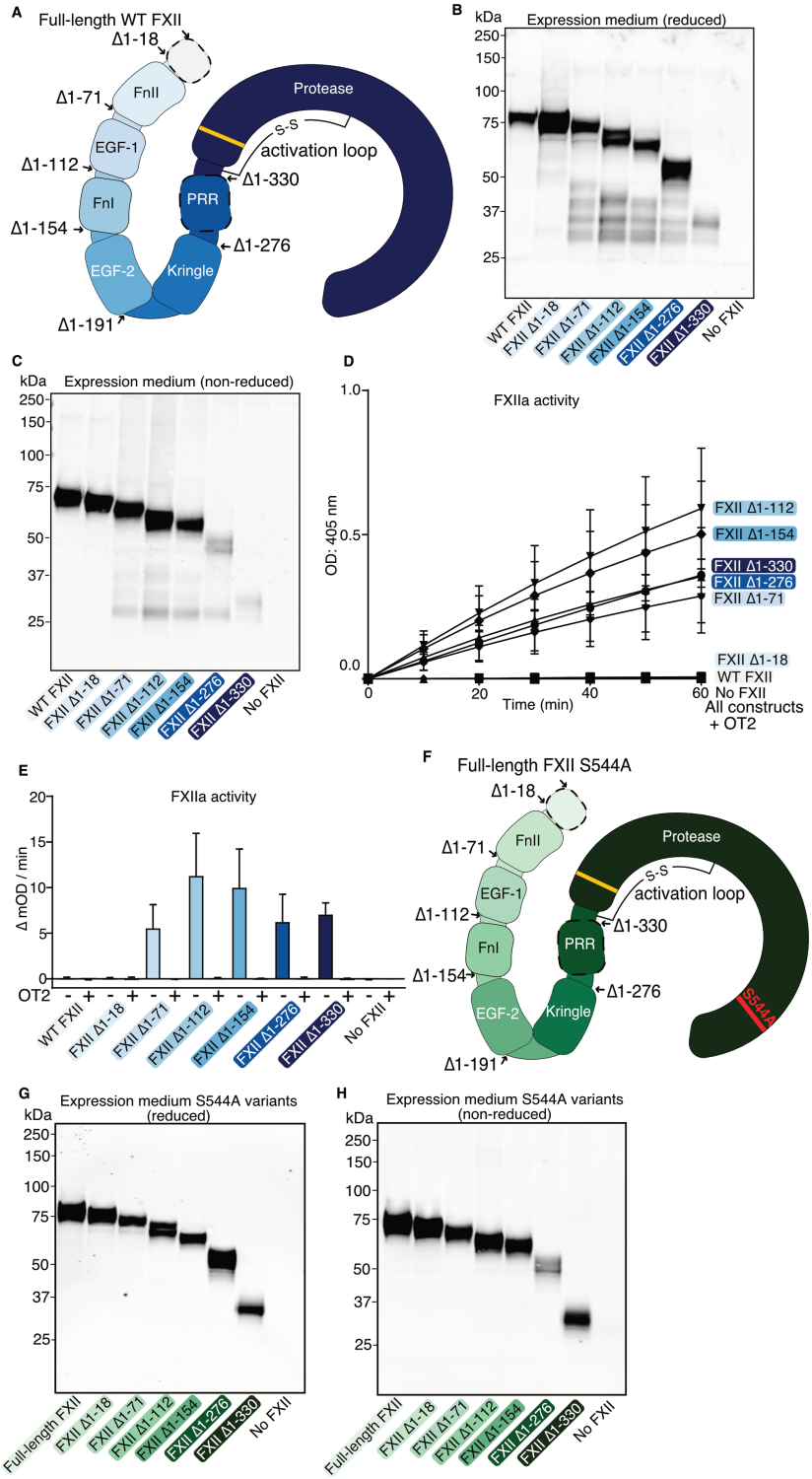
FXII consists of six domains. Its peptide sequence starts with an unstructured peptide sequence that is implicated in the interaction with factor XI[4], which is followed by a fibronectin type II domain (FnII), an epidermal growth factor-like domain (EGF-1), and a fibronectin type I domain (FnI) that are implicated in surface- and receptor binding. These domains are followed by a second EGF-like domain (EGF-2), a kringle domain (kringle) and a serine protease domain (protease) (graphical representation in ►**Fig. 1A**). A proline-rich region (PRR; 54 amino acids) connects the kringle and protease domain. The PRR contains cleavage sites for leukocyte proteases[5] and is a hotspot for mutations that cause hereditary angioedema (HAE).[6] Three out of five of these mutations introduce additional cleavage sites for plasmin or thrombin.[7,8] Truncation of FXII through cleavage in the PRR accelerates activation by PKa.[5] Similarly, FXII binding to anionic polymers increases its rate of activation by PKa.[9] This can be recapitulated by the binding of monoclonal antibodies to the N-terminal domains of FXII.[10] Together, these observations have led us and others to believe that the surface binding domains of FXII have a regulatory role in its activation mechanism.[8,11,12]

We hypothesize that the activation loop is usually shielded as a result of intramolecular self-association between N-terminal domain(s) and the protease domain. This phenomenon is described for several other plasma proteases (thrombin, plasminogen and ADAMTS-13).[13-15] Currently, there are no crystal structures available for full-length FXII to support this concept.[2,16] In the present study, we investigated the molecular mechanisms that control the sensitivity of FXII for activating cleavage.

Methods

Reagents

Aprotinin (from bovine lung), Bovine serum albumin (BSA), Bromophenol blue, Dithiothreitol (DTT), Dulbecco's Phosphate Buffered Saline (PBS), EDTA, Glycerol, Glycine, NaCl, Na_2HPO_4, NaH_2PO_4, and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA). DNA oligonucleotides were from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 680 Donkey anti-sheep IgG, Alexa Fluor 680 Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary antibody, Bolt 4-12% Bis-Tris plus gels, S.O.C. medium, One Shot TOP10 Chemically Competent E. coli and 10x TBE (Tris-Borate EDTA) buffer were from Invitrogen (Carlsbad, CA, U.S.A.). Restriction


A
Full-length WT FXII
Δ1-18
Δ1-71
FnII
EGF-1
Δ1-112
FnI
Δ1-154
EGF-2
Kringle
Δ1-191
PRR
Δ1-276
Δ1-330
S-S
activation loop
Protease
B
kDa
Expression medium (reduced)
250
150
100
75
50
37
25
WT FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
FXII Δ1-154
FXII Δ1-276
FXII Δ1-330
No FXII
C
kDa
Expression medium (non-reduced)
250
150
100
75
50
37
25
WT FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
FXII Δ1-154
FXII Δ1-276
FXII Δ1-330
No FXII
D
FXIIa activity
OD: 405 nm
1.0
0.5
0.0
0
20
40
60
Time (min)
FXII Δ1-112
FXII Δ1-154
FXII Δ1-330
FXII Δ1-276
FXII Δ1-71
FXII Δ1-18
WT FXII
No FXII
All constructs
+ OT2
E
FXIIa activity
Δ mOD / min
20
15
10
5
0
OT2
- + - + - + - + - + - + - + - +
WT FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
FXII Δ1-154
FXII Δ1-276
FXII Δ1-330
No FXII
F
Full-length FXII S544A
Δ1-18
Δ1-71
FnII
EGF-1
Δ1-112
FnI
Δ1-154
EGF-2
Kringle
Δ1-191
PRR
Δ1-276
Δ1-330
S-S
activation loop
Protease
S544A
G
Expression medium S544A variants
(reduced)
kDa
250
150
100
75
50
37
25
Full-length FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
FXII Δ1-154
FXII Δ1-276
FXII Δ1-330
No FXII
H
Expression medium S544A variants
(non-reduced)
kDa
250
150
100
75
50
37
25
Full-length FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
FXII Δ1-154
FXII Δ1-276
FXII Δ1-330
No FXII

enzymes *EcoRI*, *NotI*, Cutsmart buffer, T4 ligase buffer and T4 ligase were from New England Biolabs (Ipswich, MA, U.S.A.). Polyethylenimine MW 25.000 (PEI) was from Polysciences (Warrington, PA, U.S.A.). CloneJET PCR cloning Kit, HF phusion polymerase, Gibco FreeStyle 293 Expression Medium, HEK293F cells, and Gibco Opti-MEM + GlutaMAX reduced serum medium were from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Anti-FXII affinity-purified polyclonal antibody (Sheep IgG; CL20055AP) was from Cedarlane Ltd. (Burlington, Canada). Anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Clone 4G5; sc-51906) was from Santa Cruz Biotechnology (Dallas, TX, U.S.A). Immobilon-FL, and Ethanol were from Merck-Millipore (Amsterdam, the Netherlands). Peptide substrate H-D-Pro-Phe-Arg-pNA (L2120) was from Bachem (Bubendorf, Switzerland). Plasma kallikrein was purchased from Enzyme Research laboratories (ERL; South Bend, U.S.A.). Costar vinyl 96 well plate was purchased from Corning (Kennebunk, ME, U.S.A.). Odyssey blocking reagent was purchased from LI-COR (Lincoln, NE, U.S.A.). Tris-HCl was from Roche (Woerden, the Netherlands) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from VWR International (Amsterdam, the Netherlands). Kaolin (Light) was from BDH ltd. (Poole, UK). Nucleospin Plasmid-, NucleoSpin Gel and PCR Clean-up kits were from Macherey-Nagel (Düren, Germany). Calcium pre-adsorbed platelet size polyphosphate (polyP) (average polymer size of 70 phosphate residues) was a kind gift from prof. dr. Thomas Renné (Universitätsklinikum Hamburg-Eppendorf, Germany).

Molecular cloning and protein expression

cDNA sequences of FXII truncation variants with an N-terminal *EcoRI* and C-terminal *NotI* restriction site were generated by PCR using HF phusion polymerase (primer sequences listed in Table S1), based on the F12 cDNA (reference sequence (NM_000505.3) containing the silent mutation 1140T>C (PMID 27130860). Alternatively, PCR was performed on F12 cDNA with the additional mutation 1387T>G, that leads to incapacitation of the active-site by changing the active-site serine into an alanine residue (S544A). These sequences were ligated into pSM2, a pCDNA6/V5-His A expression vector with a N-terminal murine

Figure 1. The fibronectin type II domain of factor XII regulates zymogen quiescence. (A) Factor XII (FXII) truncation strategy. Arrows indicate the starting residues for each FXII variant. The first region spanning residues 1-18, and the proline-rich region (PRR), are indicated with dashed lines. EGF-like domain 1 (EGF-1), fibronectin type 1 domain (FnI), EGF-like domain 2 (EGF-2), kringle domain (kringle), serine protease domain (protease) (B,C) Western blots of FXII variants in expression medium (under reducing and non-reducing conditions, respectively). (D) Spontaneous FXIIa activity in expression medium and the effect of the inhibiting anti-FXII serine protease domain antibody OT2. Conditioned expression medium of mock-transfected cells (no FXII) was included as a control. (E) Initial rates of substrate conversion (first 30 minutes). (F) Truncation strategy of active-site incapacitated FXII S544A variants. Arrows indicate the starting residues for each FXII variant. (G,H) Western blots of active-site incapacitated FXII S544A variants in expression medium (under reducing and non-reducing conditions, respectively). Data represent mean ± SD of three separate experiments, all performed in duplicate. Western blots are representative for three independent experiments.

Igκ secretion signal and two N-terminal strep-tags inserted through the *HindIII* and *EcoRI* restriction sites (PMID 27130860). DNA sequences were verified by sequence PCR. HEK 293F cells (1.1 x 10^6/mL) in Gibco FreeStyle 293 expression medium were (transiently) transfected with 1 µg/mL of plasmid DNA in a DNA:PEI ratio of 1:3, dissolved in Opti-MEM. After 24 hours, expression medium was replaced in order to remove excess PEI. Five days later, the cells were centrifuged at 500x*g* for 10 minutes after which the supernatant expression medium was centrifuged at 5.000x*g* for 10 minutes in order to remove cell debris and stored at -20°C until use. Prior to comparative functional experiments, FXII antigen levels were equalized by western blotting. Throughout experiments, conditioned expression medium of non-transfected cells (no FXII) was included as a control.

Chromogenic substrate enzyme activity assays

The chromogenic substrate H-D-Pro-Phe-Arg-pNA was used to detect FXIIa and/or plasma kallikrein-like activity. C costar vinyl 96 well plates were blocked with 200 µL filtered HBS-BSA (10 mM HEPES, 150 NaCl, pH 7.4 and 1.0% BSA (w/v)) for >20 minutes at 37°C. All incubations of conditioned expression medium (with equal FXII concentrations) with indicated reagents (final in-assay concentrations are stated) were performed at 37°C. Substrate conversion was measured on a SpectraMax ID3 (Molecular devices) at 405 nm for 120 minutes with 1 minute intervals at 37°C.

Spontaneous FXIIa activity

Spontaneous FXIIa activity of FXII variants was determined by incubating FXII variants in expression medium (70 µL) with vehicle control conditioned expression medium (10 µL) or with OT2 antibody (10 µL; 10 µg/mL) for 20 minutes after which the substrate was added (20 µL; 0.5 mM).

Activation of FXII variants by plasma kallikrein

The activation of FXII variants by plasma kallikrein (PKa) was determined by incubating FXII variants in expression medium (80 µL) with PKa (10 µL pre-diluted in HBS-BSA 0.2% (w/v); 2 µg/mL) for 45 minutes after which PKa was inhibited with aprotinin (10 µL pre-diluted in HBS-BSA (0.2% (w/v)); 200 KIU/mL) for 15 minutes prior to substrate addition (20 µL; 0.42 mM).

Activation of FXII variants by plasma kalllikrein in the presence of a surface

The activation of FXII variants by plasma kallikrein (PKa) in the presence of a surface (kaolin) was determined by incubating FXII variants in expression medium (80 µL; equal FXII concentrations) with kaolin (10 µL pre-diluted in TBS (50 mM TRIS, 150 mM NaCl, pH 7.4); 0.46 mg/mL) or with buffer control (10 µL TBS) for 15 minutes. Subsequently, PKa (10 µL pre-diluted in HBS-BSA 0.2% (w/v); 1.85 µg/mL) or buffer control (10 µL HBS-BSA 0.2% (w/v)) was added and incubated for 45 minutes. Next, PKa was inhibited with aprotinin (10 µL pre-

diluted in HBS-BSA 0.2% (w/v); 185 KIU/mL) during 15 minutes after which the substrate was added (20 µL; 0.38 mM).

SDS-PAGE and western blotting

SDS-PAGE and western blotting samples (specific conditions outlined below, all incubations were performed at 37°C) are prepared by 3-fold dilution of the sample into (non-)reducing sample buffer (1x sample buffer: 10% glycerol, 0.06 M Tris-HCl, 2% SDS, Bromophenol blue, with or without 8.3 mM DTT) after which the samples are heated for 10 minutes at 95°C. Samples (60 µL) were separated on 4-12% Bis-Tris gels at 165V for 70 minutes in MOPS buffer. Proteins were transferred to Immobilon-FL membranes at 125V for 60 minutes in blotting buffer (25 mM Tris, 192 mM Glycine, 20% (v/v) Ethanol). Membranes were blocked for 2 hours at room temperature (RT) with blocking buffer (Odyssey blocking reagent diluted 1:1 with TBS 0.1% Tween (v/v) (TBS-T)). FXII was detected with polyclonal affinity-purified goat anti-human factor XII and GAPDH with monoclonal mouse anti-human GAPDH diluted 1:4000 in blocking buffer overnight at 4°C. Membranes were washed with TBS-T, and primary antibodies were detected with donkey anti-sheep IgG Alexa Fluor 680 or Alexa Fluor 680 goat anti-mouse IgG (H+L) diluted 1:10:000 in blocking buffer for 1 hour at RT. Membranes were washed with TBS-T and analyzed on a near infrared Odyssey scanner (LI-COR). Band densitometry was performed with Odyssey software (LI-COR).

FXII- and FXII S544A variants in expression medium and HEK293F cell lysates

FXII variants in expression medium were diluted 3-fold in (non-)reducing sample buffer. Transfected cells (1.1 x 10^6/mL) were washed, resuspended in Dulbecco's phosphate buffered saline and lysed by 3-fold dilution in reducing sample buffer.

Cleavage of FXII S544A variants by plasma kallikrein

Active-site incapacitated FXII (S544A) variants in expression medium (80 µL) were incubated with plasma kallikrein (PKa) (20 µL pre-diluted in TBS; 5 µg/mL) or with buffer control (20 µL TBS) for 30 minutes after which the samples were diluted 3-fold in sample buffer.

Polyphosphate and kaolin binding of FXII S544A variants

FXII S544A variants in expression medium (150 µL) were incubated with polyphosphate (polyP) (16.5 µL pre-diluted in HBS; 10 mg/mL) or kaolin (16.5 µL pre-diluted in TBS; 500 µg/mL) for 15 minutes. Subsequently, the polyP and kaolin were pelleted by centrifugation at 5000x*g* for 5 minutes. Western blot samples were taken prior to addition of polyP or kaolin (input sample; with TBS or HBS buffer control) and taken from the supernatant after pelleting (supernatant sample). Finally, polyP or kaolin pellets were taken up in 60 µL 1x reducing sample buffer.

Cleavage of FXII S544A variants by plasma kallikrein in the presence of a surface.

FXII S544A variants in expression medium (100 µL) were incubated with polyP (5.55 µL pre-diluted in HBS; 10 mg/mL), kaolin (5.55 µL pre-diluted in TBS; 500 µg/mL), or buffer control (5.55 µL TBS or HBS) for 15 minutes. Subsequently, PKa (5.55 µL pre-diluted in HBS; 5 µg/mL) or buffer control (5.55 µL HBS) is added and incubated for 30 minutes after which samples are diluted 3-fold in sample buffer.

Data analysis

Statistics were performed by One-Way ANOVA. $P<0.05$ was considered significant. All data analyses were performed with Prism Graphpad 7.0 (Graphpad Software).

RESULTS

Removal of the fibronectin type II domain disturbs FXII zymogen quiescence

We developed DNA constructs encoding 7 N-terminally truncated FXII variants through consecutive N-terminal domain deletion (also including the PRR) while retaining the subsequent inter-domain linker sequence, as well as a full-length control construct (WT FXII) (►**Fig. 1A**). We used UNIPROT (www.uniprot.org/uniprot/P00748) as a source for domain annotation, except that we included the N-terminal VVLT amino acids (residues 19-22 of the mature FXII sequence) as part of the FnII domain, based on FnII domain solution structure studies.[17] Six out of the 7 truncated FXII variants were effectively secreted (►**Supplementary Fig. 1A**). FXII starting with the kringle domain (FXII Δ1-191) was not secreted, but was present in the lysates of transfected cells, indicating intracellular retention (►**Supplementary Fig. 1B**). Therefore, this FXII variant was omitted from further experiments. Western blot analysis of conditioned expression medium shows that full-length WT FXII and FXII Δ1-18 are expressed as single-chain proteins (►**Fig. 1B**). In contrast, FXII is partially fragmented when the FnII domain is lacking (FXII Δ1-71, and further truncations) (►**Fig. 1B**). These cleavage products are predominantly visible under reducing conditions (►**Fig. 1B,C**), suggesting that they result from a cleavage event within the activation loop. Next, we investigated enzyme activity with a chromogenic substrate assay. No spontaneous enzymatic activity was found for full-length WT FXII and FXII Δ1-18 (►**Fig. 1D**). However once the FnII domain is lacking (FXII Δ1-71, and further truncation) spontaneous enzymatic activity that is sensitive to inhibition by the FXIIa inhibiting antibody OT2 is observed (►**Fig 1D,E**).[7] This suggests that FXII without the FnII domain is susceptible to autoactivation. Indeed, expression of active-site incapacitated forms of FXII (S544A), which are unable to autoactivate, eliminates FXII fragmentation (►**Fig. 1F-H** and ►**Supplementary Fig. 1C,D** for cell lysates).These findings show that the absence of the FnII domain disturbs zymogen quiescence.

The fibronectin type II domain shields the activation loop of FXII

We next investigated the susceptibility of FXII to activation by PKa for the first four FXII variants; full-length WT FXII, FXIIΔ1-18, FXII lacking the FnII domain (FXII Δ1-71), and FXII

without the following EGF-1 domain (FXII Δ1-112). Transient exposure to PKa activates full-length WT FXII and FXII Δ1-18 to a limited extent (►**Fig. 2A**). As before, FXII Δ1-71 and FXII Δ1-112 already display FXIIa activity prior to exposure to PKa. After exposure to PKa, FXII Δ1-71 and FXII Δ1-112 display a strongly increased activity (►**Fig. 2A,B**; corrected for autoactivation). This indicates that the R353 site in the activation loop of FXII is more easily cleaved by PKa in the absence of the FnII domain.

In western blotting experiments we next investigated PKa cleavage of the activation loop in active-site incapacitated FXII S544A variants (to exclude a contribution of FXII

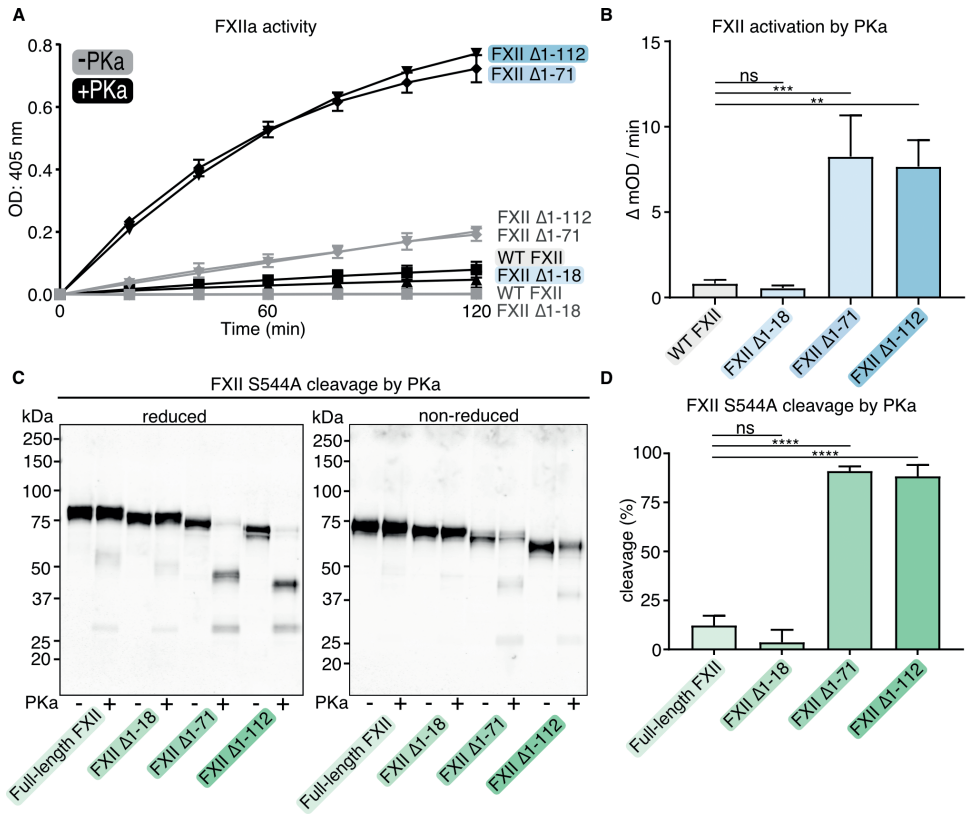


Figure 2. The fibronectin type II domain shields the activation loop of factor XII. (A) FXIIa activity of FXII variants in expression medium after activation by plasma kallikrein (PKa; black lines) or buffer control (grey lines). Data are corrected for residual PKa activity in conditioned expression medium of non-transfected cells. Raw data in ►**Supplementary Fig 2.** (B) Initial rate of substrate conversion (first 30 minutes) by FXIIa after exposure to PKa, corrected for spontaneous FXIIa activity of each construct and residual PKa activity. Data represent mean ± SD of three separate experiments, all performed in duplicate. (C) Western blots of active-site incapacitated FXII S544A variants in expression medium after cleavage by PKa (under reducing and non-reducing conditions). (D) Densitometric quantification of FXII S544A cleavage by PKa of three separate experiments. Western blots are representative for three independent experiments. Data represent mean ± SD and were analyzed with one-way ANOVA. Non-significant (ns), P<0.01=*** P<0.001=*** and p<0.0001=****.

autoactivation). Full-length FXII S544A and FXII Δ1-18 S544A are largely resistant against cleavage by PKa, whereas FXII Δ1-71 S544A and FXII Δ1-112 S544A are efficiently cleaved to form a two-chain disulfide-linked product (►**Fig. 2C,D**). Together, these data show that the activation loop is exposed in FXII variants that lack the FnII domain.

The EGF-1 domain is the predominant surface binding site of FXII.

Multiple domains of FXII have been reported to act as binding sites for activating surfaces but their relative contribution remains unclear.[18] The FnII domain is described to mediate binding to kaolin and dextran sulfate.[19,20] Remarkably, our findings indicate that the FnII domain also shields the activation loop of FXII. We hypothesized that when the FnII domain binds to the surface, it is displaced, thereby unshielding the activation loop. Hereto, we investigated the binding of active-site incapacitated FXII S544A variants to polyphosphate (polyP) and kaolin in pull-down experiments. As expected, both full-length FXII S544A and FXII Δ1-18 S544A bind to polyP and kaolin, concentrating it on these surfaces and removing it from solution after centrifugation (►**Fig. 3A-D**). FXII lacking the FnII domain (FXII Δ1-71 S544A) can surprisingly still efficiently bind to both polyP and kaolin. In contrast, FXII lacking the EGF-1 domain (FXII Δ1-112 S544A, and further truncations) have lost this capacity. This shows the critical importance of the EGF-1 domain for binding to polyP and kaolin.

Surface binding exposes the activation loop of FXII by displacing the fibronectin type II domain

Earlier studies reported that the binding of FXII to dextran sulfate enhances the rate of activation by PKa by exposing the activation loop.[9] We hypothesized that a similar mechanism is in place when FXII binds to polyP or kaolin. Indeed, in the presence of either of these two surfaces, full-length FXII S544A and FXII Δ1-18 S544A become highly susceptible to cleavage by PKa (►**Fig. 4A-D**). As before, FXII Δ1-71 S544A and FXII Δ1-112 S544A that lack the FnII domain are highly susceptible to cleavage by PKa in the absence of a surface. Taken together, these findings suggest that FXII surface binding (in which the EGF-1 domain plays an important role) displaces the neighboring FnII domain to unshield the FXII activation loop.

Finally, we investigated the susceptibility of FXII to activation by PKa in the presence of kaolin. Hereto, we incubated FXII with high a concentration of kaolin (this limits FXII autoactivation).[4,21] Kaolin binding of full-length WT FXII and FXII Δ1-18 dramatically increases their susceptibility for PKa-mediated activation by 5.7±1.0 and 9.4±2.7-fold respectively (►**Fig. 4E,F,I**). As before (►**Fig. 2A,B**), PKa-mediated activation of FXII Δ1-71 and FXII Δ1-112 takes place irrespective the presence of kaolin (►**Fig. 4G-I**). These findings show that kaolin binding displaces the FnII domain to unshield the activation loop of FXII.

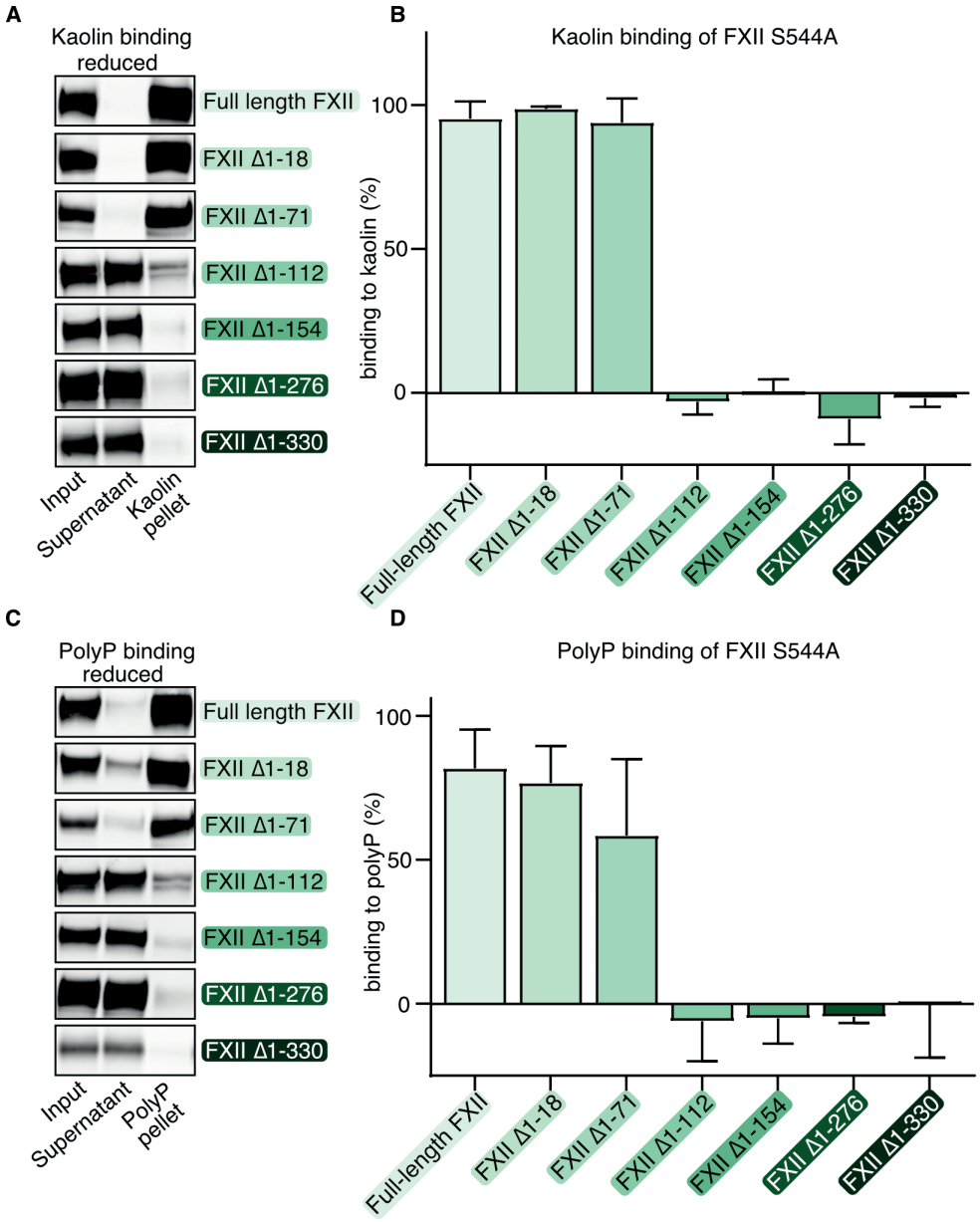
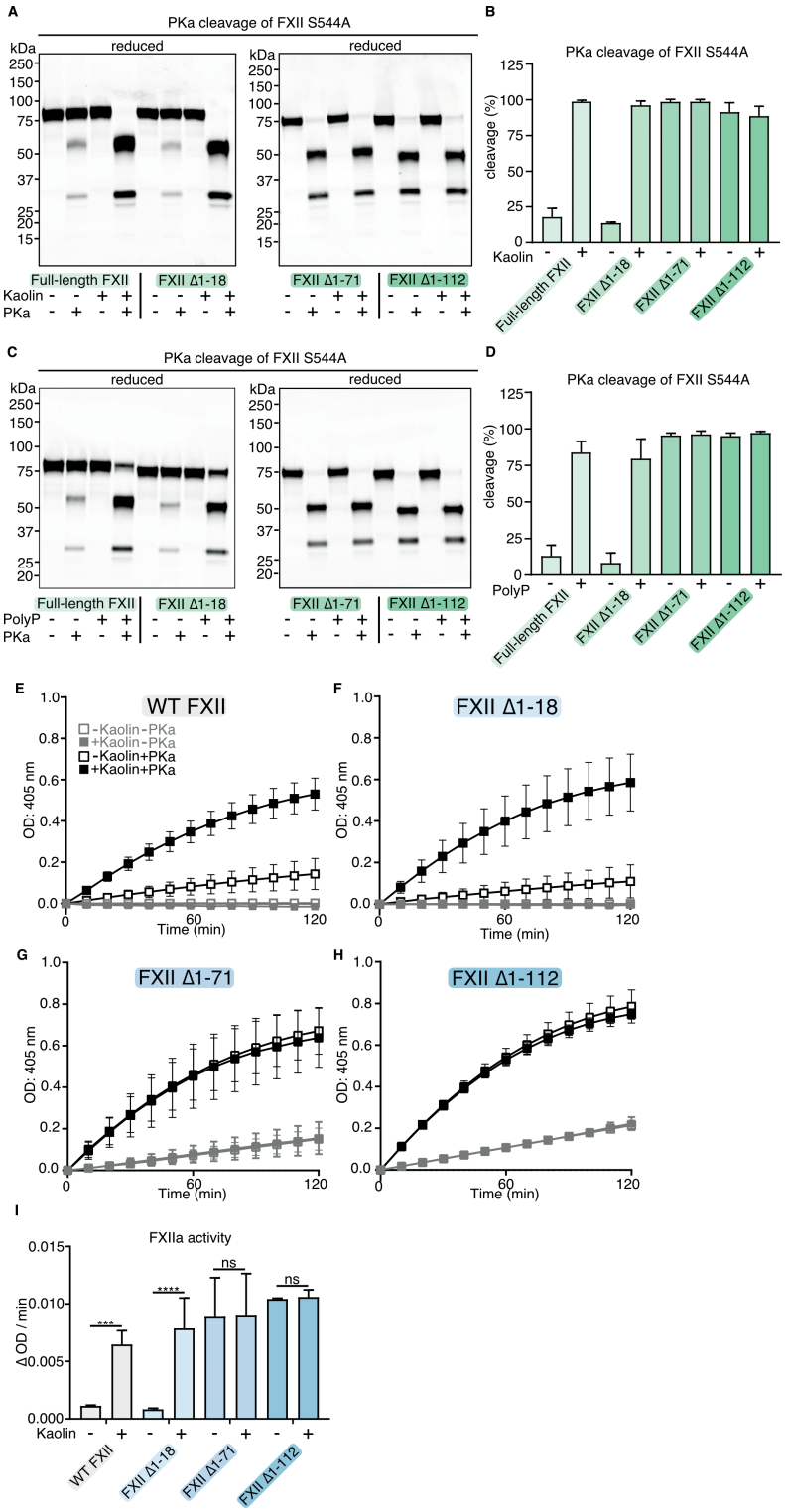


Figure 3. The EGF-like 1 domain is the predominant binding site of factor XII for polyphosphate and kaolin. (A) Reduced western blot of active-site incapacitated FXII S544A variants in expression medium that were incubated with polyphosphate (polyP) (10 mg/mL) or kaolin (500 µg/mL) and were pelleted by centrifugation. Input, supernatant, and pellets were analyzed for the presence of active-site incapacitated FXII S544A. Western blots are representative for three independent experiments. Full-size image of these western blots are shown in ▶ **Supplementary Fig 3A,B**). (B) Densitometric quantification of polyP and kaolin binding of active-site incapacitated FXII S544A variants. Data represent the relative loss of FXII antigen in supernatant after pulldown with kaolin or polyP for three separate experiments.


A
PKa cleavage of FXII S544A
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Full-length FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
Kaolin
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PKa cleavage of FXII S544A
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Kaolin
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FXII Δ1-18
FXII Δ1-71
FXII Δ1-112
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PKa cleavage of FXII S544A
reduced
PolyP
PKa
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PKa cleavage of FXII S544A
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+Kaolin-PKa
-Kaolin+PKa
+Kaolin+PKa
OD: 405 nm
Time (min)
F
FXII Δ1-18
G
FXII Δ1-71
H
FXII Δ1-112
I
FXIIa activity
ΔOD / min
0.015
0.010
0.005
0.000
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WT FXII
FXII Δ1-18
FXII Δ1-71
FXII Δ1-112

DISCUSSION

The implication of FXII in both human thrombotic and inflammatory disease has sparked a new interest in structure-function research of FXII and its activation mechanism. In this study, we show that the N-terminally positioned FnII domain negatively regulates FXII activation. Truncated FXII variants that lack this domain are prone to autoactivation and are highly susceptible towards activation by PKa in solution, indicating that the activation loop of FXII is exposed when the FnII domain is missing. Based on similarities with other plasma proteases (thrombin, plasminogen and ADAMTS-13)[13-15], we hypothesize that the FnII domain is involved in an intramolecular association with another site that is located on the protease domain. This would help to explain how this domain shields the distant activation loop. Future studies to determine the protein structure of full-length FXII are needed to further explore this concept.

In line with previous studies, we found that surface binding accelerates FXII activation by PKa.[9] In earlier FXII truncation studies, the contributions of the FnII, EGF-1 and FnI domains to surface binding were not independently investigated.[20] Surprisingly, we found that the EGF-1 domain is critical for both polyP and kaolin binding. To our knowledge, this has not been previously reported.[18] Our current study provides additional insight by delineating the separate functions of the FnII and EGF-1 domain: the former shields the activation loop, while the latter mediates surface binding. Thus, FXII is resistant against activation by PKa in solution, unless its FnII domain is displaced during surface binding or removed through enzymatic truncation.

The N-terminally consecutively truncated FXII variants of our study are obviously not physiological. Several human mutations in FXII introduce cleavage sites that are targetable by plasmin and thrombin, resulting in HAE.[7,8] Their cleavage leads to undesired FXII truncation which exposes its activation loop and accelerates its activation in solution. The phenomenon of truncation, leading to accelerated FXII activation, may not be restricted to pathogenic mutations: natural truncation by PKa at position R334, as well as by other leukocyte-associated enzymes shows strikingly similar effects.[5] However, the physiological relevance of enzymatic truncation is presently unknown.

Figure 4. Surface binding exposes the activation loop of factor XII by displacing the fibronectin type II domain. (A,C) Western blots (reduced) of active-site incapacitated FXII S544A variants in expression medium after pre-incubation with polyphosphate (polyP) (10 mg/mL) or kaolin (500 µg/mL) and subsequent exposure to plasma kallikrein (PKa; 5 µg/mL). Western blots are representative for three independent experiments. (B,D) Densitometric quantification of PKa-mediated cleavage of active-site incapacitated FXII S544A variants in the absence or presence of polyP or kaolin for three separate experiments. (E-H) FXIIa activity of FXII variants after exposure to kaolin, PKa, or both. (E) Full-length WT FXII (F) FXII Δ1-18 (G) FXII Δ1-71 (H) FXII Δ1-112. Data are corrected for residual PKa activity. Raw data in ►**Supplementary Fig 4.** (I) Initial rates of substrate conversion (first 30 minutes). Data represent mean ± SD of three separate experiments, all performed in duplicate. Data were analyzed by one-way ANOVA Non-significant (ns), P<0.001=***,p<0.0001=****.

In conclusion, we have found that the FnII domain is a critical negative regulator of FXII activation. Based on our findings we expect that displacement of this domain during surface binding will ultimately stimulate surface-dependent coagulation. Alternatively, removal of this domain through enzymatic truncation will accelerate FXII activation in solution and subsequent surface-independent activation of the kallikrein-kinin system.

ADDENDUM

C.C. Clark, Z.L.M. Hofman, W. Sanrattana, S. de Maat, and C. Maas performed experiments, were involved in the development of the concept, design and interpretation of data and wrote the manuscript.

ACKNOWLEDGEMENTS

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DISCLOSURE OF CONFLICT OF INTERESTS

The authors state that they have no conflict of interest.

REFERENCES

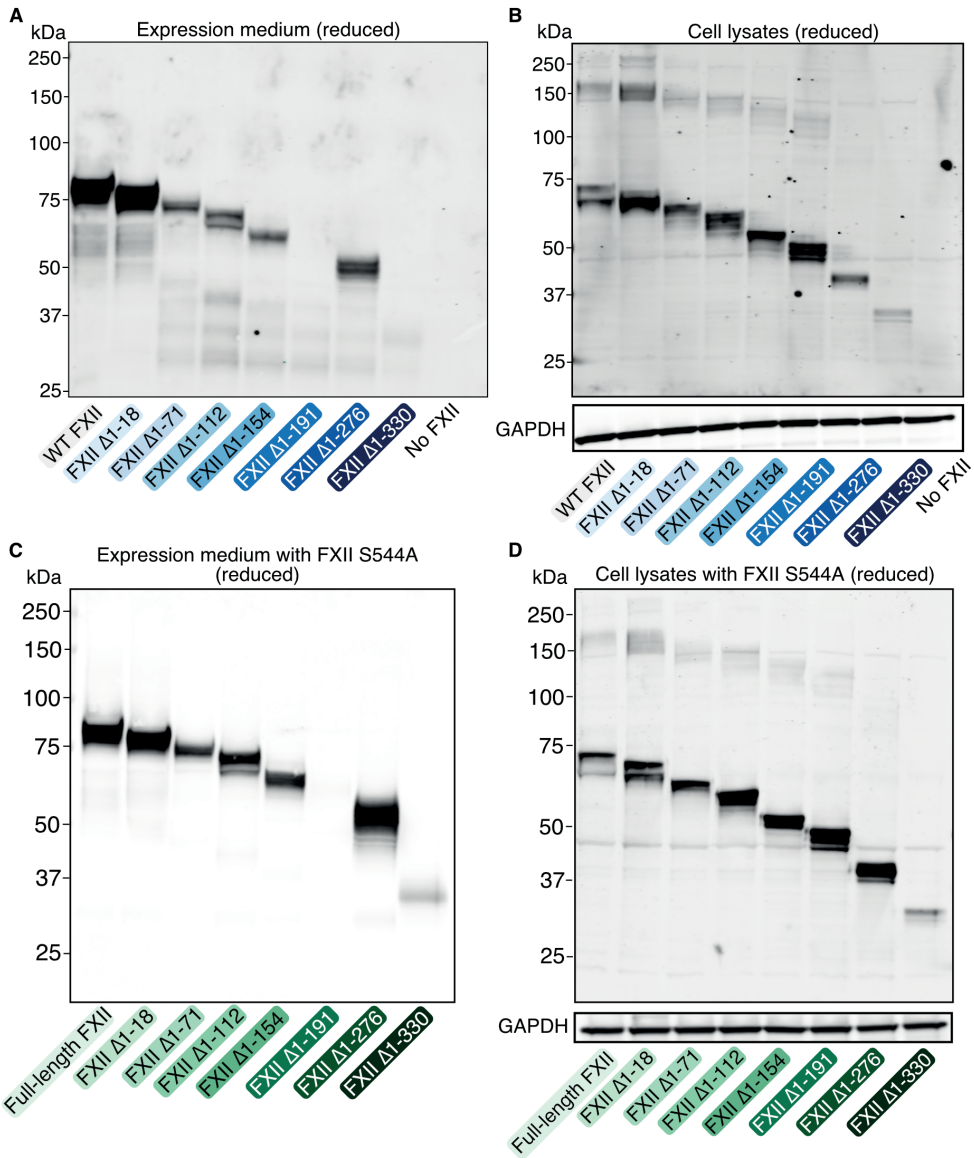
1. Hovinga JK, Schaller J, Stricker H, Wuillemin WA, Furlan M, Lämmle B. Coagulation factor XII Locarno: the functional defect is caused by the amino acid substitution Arg 353-->Pro leading to loss of a kallikrein cleavage site. Blood 1994;84:1173–81
2. Dementiev A, Silva A, Yee C, et al. Structures of human plasma β-factor XIIa cocrystallized with potent inhibitors. Blood Adv 2018;2:549–58
3. Asmis LM, Sulzer I, Furlan M, Lämmle B. Prekallikrein deficiency: The characteristic normalization of the severely prolonged aPTT following increased preincubation time is due to autoactivation of factor XII. Thromb Res 2002;105:463-70
4. Citarella F, Fedele G, Roem D, Fantoni A, Hack CE. The second exon-encoded factor XII region is involved in the interaction of factor XII with factor XI and does not contribute to the binding site for negatively charged surfaces. Blood 1998;92:4198–206
5. de Maat S, Clark CC, Boertien M, et al. Factor XII truncation accelerates activation in solution. J Thromb Haemost 2019;17:183–94
6. de Maat S, Hofman ZLM, Maas C. Hereditary angioedema: the plasma contact system out of control. J Thromb Haemost 2018;16:1674–85
7. de Maat S, Björkqvist J, Suffritti C, et al. Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations. J Allergy Clin Immunol 2016;138:1414-23.e9
8. Ivanov I, Matafonov A, Sun MF, et al. A mechanism for hereditary angioedema with normal C1 inhibitor: an inhibitory regulatory role for the factor XII heavy chain. Blood 2019;133:1152–63
9. Citarella F, Wuillemin WA, Lubbers YTP, Hack CE. Initiation of contact system activation in plasma is dependent on factor XII autoactivation and not on enhanced susceptibility of factor XII for kallikrein cleavage. Br J Haematol 1997;99:197–205
10. Nuijens JH, Hijbregts CCM, Eerenberg-Belmer AJM, Meijers JCM, Bouma BM, Hack CE. Activation of the contact system of coagulation by a monoclonal antibody directed against a neodeterminant in the heavy chain region of human coagulation factor XII (Hageman factor). J Biol Chem 1989;264:12941–9
11. Jukema BN, de Maat S, Maas C. Processing of Factor XII during Inflammatory Reactions. Front Med 2016;3:1–7
12. Philippou H. Heavy chain of FXII: not an innocent bystander! Blood 2019;133:1008–9
13. Chinnaraj M, Planer W, Pozzi N. Structure of coagulation factor II: Molecular mechanism of thrombin generation and development of next-generation anticoagulants. Front Med 2018;5:1–6
14. Law RHP, Caradoc-Davies T, Cowieson N, et al. The X-ray crystal structure of full-length human plasminogen. Cell Rep 2012;1:185–90
15. South K, Luken BM, Crawley JTB, et al. Conformational activation of ADAMTS13. Proc Natl Acad Sci 2014;111:18578–83
16. Pathak M, Wilmann P, Awford J, et al. Coagulation factor XII protease domain crystal structure. J Thromb Haemost 2015;13:580–91
17. Pickford AR, Potts JR, Bright JR, Phan I, Campbell ID. Solution structure of a type 2 module from fibronectin: Implications for the structure and function of the gelatin-binding domain. Structure 1997;5:359–70
18. de Maat S, Maas C. Factor XII: form determines function. J Thromb Haemost 2016;14:1498–506

19. Citarella F, te Velthuis H, Helmer-Citterich M, Hack CE. Identification of a putative binding site for negatively charged surfaces in the fibronectin type II domain of human Factor XII. Thromb Haemost 2018;84:1057–65

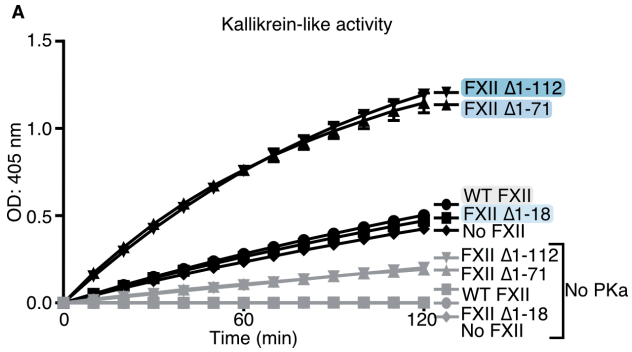
20. Citarella F, Ravon DM, Pascucci B, Felici A, Fantoni A, Hack CE. Structure/function analysis of human factor XII using recombinant deletion mutants: Evidence for an additional region involved in the binding to negatively charged surfaces. Eur J Biochem 1996;238:240–9

21. Wiggins BYRC, Cochrane CC. The autoactivation of rabbit Hageman factor. J Exp Med 1979;150:1122–33

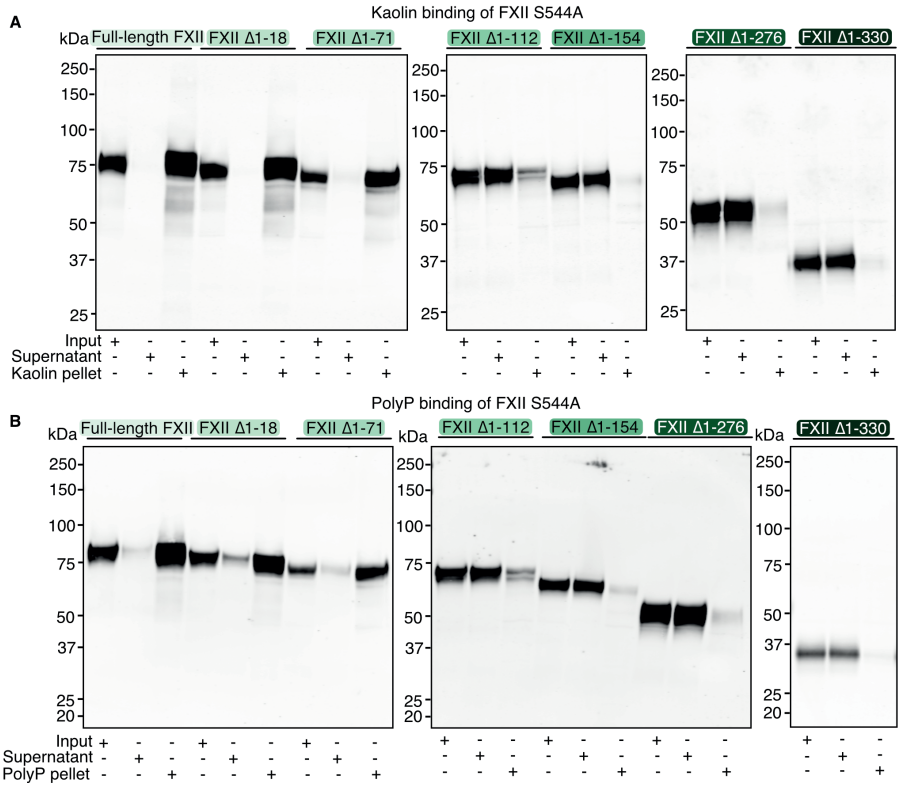
SUPPLEMENTAL DATA



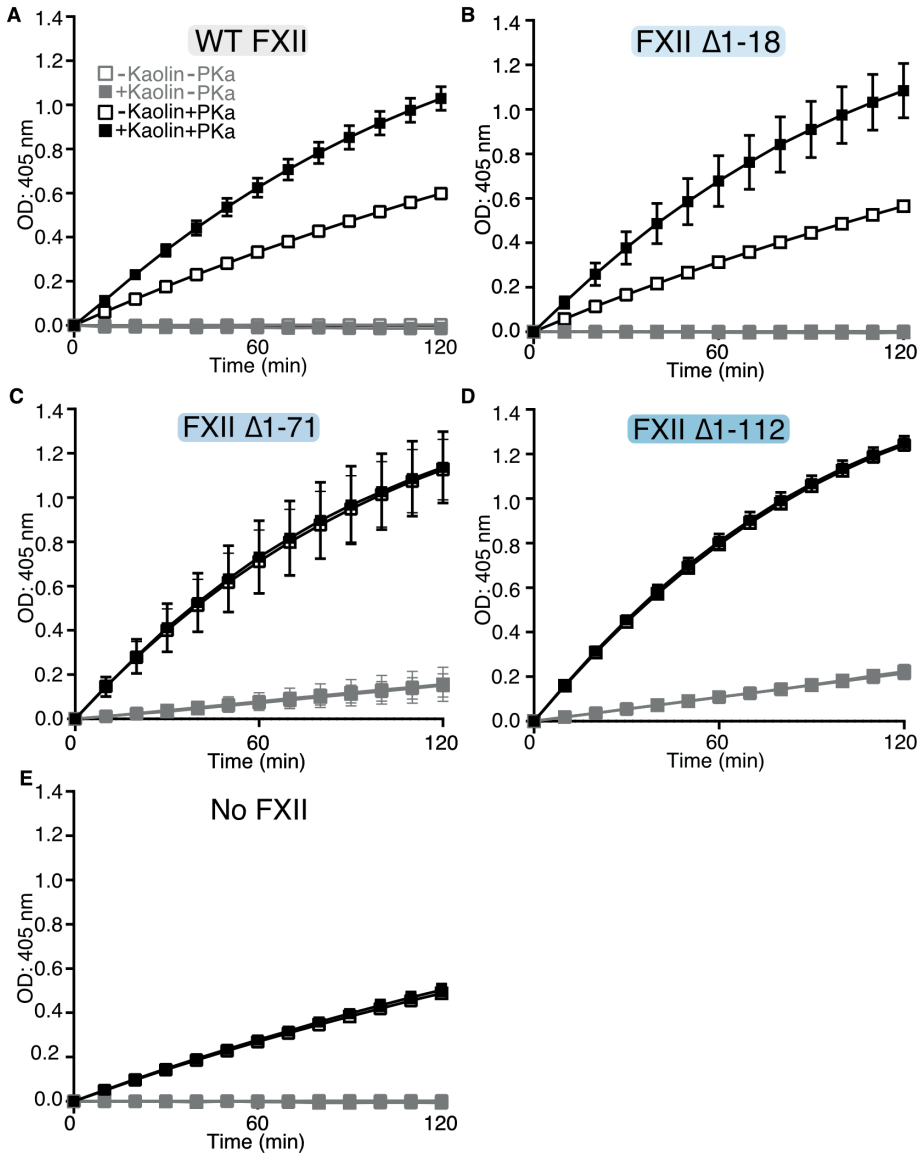
Supplementary Figure S1. Expression of factor XII variants. (A) Reduced western blot of FXII variants in expression medium or (B) lysates of transfected HEK293F cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a lane loading control. (C) Reduced western blot of active-site incapacitated FXII S544A variants in expression medium or (D) lysates of transfected HEK293F cells.



Supplementary Figure S2. The fibronectin type II domain shields the activation loop of factor XII. (A) Kallikrein-like activity in the presence of FXII variants in expression medium after exposure to plasma kallikrein (PKa; black lines) or buffer control (grey lines). Data represent mean ± SD of three separate experiments, all performed in duplicate.



Supplementary Figure S3. The EGF-like 1 domain is the predominant binding site of factor XII for polyphosphate (polyP) and kaolin. (A) Reduced western blot of active-site incapacitated FXII S544A variants in expression medium that were incubated with kaolin (500 µg/mL) or polyP (10 mg/mL). PolyP and kaolin were pelleted by centrifugation. Input, supernatant, and pellets were analyzed for the presence of FXII S544A. Western blots are representative for three independent experiments.



Supplementary Figure S4. Surface binding exposes the activation loop of factor XII by displacing the fibronectin type II domain. (A-D) kallikrein-like activity in the presence of FXII variants after exposure to kaolin, plasma kallikrein (PKa), or both. (A) Full-length WT FXII (B) FXII Δ1-18 (C) FXII Δ1-71 (D) FXII Δ1-112 (E) No FXII.

Supplementary Table S1. Molecular cloning (primers)

Primer	Sequence 5'- 3'
Full-length_For	TTTGAATTCATTCCACCTTGGGAAGCCC
Δ1-18_For	GCGGAATTCGTCGTTCTCACTGTCACCGGGGAGCCCTG
Δ1-71_For	TGTGAATTCCCCAAGAAAGTGAAAGACCACTGC
Δ1-112_For	CACGAATTCAAAGAGAAGTGCTTTGAGCCTCAGC
Δ1-154_For	CAGGAATTCGCCAGCCAGGCCTGCCGCACC
Δ1-191_For	TTCGAATTCGTGGACACCAAGGCAAGCTGCTATGATG
Δ1-276_For	GCAGAATTCCAGACCCCAACCCAGGCGGC
Δ1-330_For	CAGGAATTCTCCCTGACCAGGAACGGCCCA
FXII_Rev	CGAGCGGCCGCTCATCAGGAAA

13

General discussion

Since the discovery of bradykinin, the medical field got familiarized with bradykinin driven inflammation within the scope of hereditary angioedema (HAE). We learned that C1-esterase inhibitor (C1-INH) deficiency or dysfunction (HAE-1 and -2) result in overproduction of bradykinin due to insufficient control of the contact system. With this knowledge, therapies treating and preventing swelling were developed. However, there is still a lot to be discovered. Observations in HAE incite new questions on how bradykinin production and local vascular permeability are regulated.

With this thesis I aimed to gain more insight in the mechanisms of bradykinin production in order to increase our understanding of HAE and extend this knowledge to potential bradykinin driven diseases beyond HAE.

PART I: INSIGHT IN HEREDITARY ANGIOEDEMA

There is a wide variety in disease severity among patients with HAE type 1 and 2, even among family members that share the same mutation. Some patients experience multiple angioedema attacks per week while others can go years or decades without swelling. This variation in disease is not fully explained by the severity of C1-INH deficiency/dysfunction as the majority of genotype-phenotype studies could not relate mutations or measured C1-INH function to clinical severity[1–4]. It is generally recognized that other factors besides C1-INH function influence clinical outcome[5]. Understanding these factors would provide insight into regulation of bradykinin driven inflammation.

In **chapter 6** we developed a nanobody based ELISA to detect cleaved high molecular weight kininogen (cHK). As cHK is the byproduct of bradykinin release, it is considered to reflect bradykinin production. We analyzed cHK in patients with HAE-1 and -2 and found that cHK levels were elevated in patients compared to healthy controls and increased further during angioedema attacks. This corresponded to findings in previous studies were cHK was measured with immunoblotting[6]. We observed that remission cHK levels did not correlate with yearly angioedema attack frequency. In contrast, one previous study demonstrated normalization of cHK levels in patients with less than 3 attacks per year, but no correlation was found between cHK levels and attack frequencies of more than 3 per year[6]. Combined, these data implicate that the amount cHK generated, ergo the amount of bradykinin produced during remission, does not fully explain the number of angioedema attacks occurring. Moreover, we found that an angioedema attack could be accompanied by only a small increase in cHK that was within the normal range of healthy controls. In contrast, cHK levels could be over 50%, meaning that halve of the total HK pool is cleaved, yet these patients did not have an episode with angioedema. Although overall cHK levels increased during an angioedema attack, there was no threshold cHK level after which an event occurred. It appears that other factors contribute to the occurrence of an angioedema attack after HK is cleaved and bradykinin is released in circulation.

Notably, there was a considerable spread in cHK levels detected in HAE patients during remission ranging from 1.8-74.9% cHK (100% indicates cleavage of all HK in pooled healthy control plasma). A correlation was found between C1-INH function and cHK levels. When

taking healthy controls out of the equation (data not shown), a weak correlation remained. Although less pronounced, cHK levels in healthy controls also demonstrated considerable variation ranging from 2.7-17.4%. Indicating that variation in the amount of bradykinin produced may not be limited to impaired C1-INH activity. In **chapter 7**, we developed a cHK generation assay. With this assay we stimulated contact activation in plasma using elagic acid and measured cHK generation with our ELISA. Using this assay showed efficacy of a novel kallikrein inhibiting compound in healthy study subjects. In line with our *in vivo* findings of variation in cHK levels in healthy controls and HAE patients (**chapter 6**), we observed considerable but consistent variation in *ex vivo* triggered cHK generation between healthy study subjects receiving placebo. These data reflect that low C1-INH function is indeed associated with increased HK cleavage; HAE-1 and -2 patients had increased levels., However, other factors appear to contribute to the amount of bradykinin generated as well as we detected a wide range of cHK levels within our cohorts. In addition, variation in the amount cHK produced *in vivo*, or after *ex vivo* stimulation of the contact system also seems to occur in healthy individuals.

Bradykinin B1 receptor expression as a possible regulator of angioedema attacks

Based upon our analysis of cHK levels in HAE, we can dissect our possible factors contributing to the HAE disease phenotype into 1) those regulating the effects of bradykinin after it is release and 2) those contributing to increased bradykinin production. In **chapter 5** we review on bradykinin B1 receptor expression as a possible candidate for regulating the effects of bradykinin after its release. We postulate that the occurrence and location of angioedema is determined by bradykinin B1 receptor. The bradykinin B1 receptor is expressed on the endothelium in response to inflammatory stimuli such as cytokines or kinins[7,8]. After binding its ligand des-Arg9-bradykinin (a bradykinin metabolite), it generates a prolonged vasodilatory effect. In contrast, the bradykinin B2 receptor is constitutively expressed but is rapidly internalized after binding its ligand bradykinin, resulting in a short-lasting vasodilation[7,8]. Transient bradykinin B1 receptor expression may help explain why even in the presence of high cHK levels patients may not present with angioedema as we observed in **chapter 6.** In addition, bradykinin B1 receptor expression gives a possible explanation for our observation in **chapter 4.** Here we described the occurrence of multiple swelling sites during angioedema attacks, the local presence of the bradykinin B1 receptor may determine the location of the swelling in the presence of systemic contact activation. In **chapter 3** we observed that HAE patients often have increased c-reactive protein (CRP) levels, although the elevation is very subtle it reflects the presence of cytokines that can upregulate bradykinin B1 receptor expression acting together with bradykinin that upon binding the bradykinin B2 receptor initiates bradykinin B1 receptor expression.

Mutations in hereditary angioedema with normal C1-INH function reveal key factors in bradykinin driven disease

We postulated the hypothesis of bradykinin receptor expression as determining for the occurrence of angioedema. However, more factors ranging from the chain-reaction of FXII activation to intracellular pathways following interaction of bradykinin with its receptors may influence the clinical outcome in HAE. Discoveries in HAE genetics help indicate key factors for bradykinin regulation.

Though very rare, some families present with HAE but the C1-INH function is normal and no mutation in the SERPING1 gene coding for C1-INH can be found. Genetic studies in families with HAE and normal C1-INH function revealed mutations in genes encoding for Factor XII (FXII)[9,10], plasminogen (PLG)[11], angiopoietin-1 (ANGPT1)[12], HK and the kininogen splice variant low molecular weight kininogen (LK, both encoded by the KNG1 gene)[13]. Of these, ANGPT1 points towards another potential post-bradykinin production regulatory mechanism complementary to bradykinin receptor expression. ANGPT1 prevents vascular leakage upon binding to the tyrosine kinase receptor TIE2. Binding attenuates the vascular permeability enhancing effects of bradykinin[14,15] as well as vascular endothelial growth factor (VEGF)[16]. The ANGPT1 A119S variant, associated with HAE, impairs multimerization of the protein which is key to efficient TIE2 binding[14]. This identifies ANGPT1 as an important factor in preventing angioedema. Even though it is uncertain if swelling in ANGPT1-HAE is caused by bradykinin, it is very likely that ANGPT1 regulates the effects of bradykinin after its production.

FXII, plasminogen and HK, all associated with HAE with normal C1-INH likely represent key players regulating the amount of bradykinin produced. As the discovery of the HAE-PLG mutation K330E and HAE-KNG1 mutation M379K are very recent it is still unknown how these mutant proteins exactly result in increased bradykinin production. However, 5 symptomatic HAE-PLG patients were successfully treated with either bradykinin receptor antagonists or plasma derived C1-INH therapy[11]. In addition, there are several lines of evidence linking plasmin to contact system activation which we reviewed in **chapter 2**. Biomarkers of fibrinolysis increase during HAE attacks and plasmin was described to enzymatically activate FXII *in vitro* connecting plasmin to bradykinin production[17].

Better understood are the pathophysiological mechanisms of FXII mutations in HAE. So far 5 mutations in the f12 gene encoding for FXII have been found in HAE[10,18,19]. All mutations are located in the proline-rich region of FXII. For three mutations it has been shown that they introduce a novel cleavage site for plasmin[20] and, as later work showed, for thrombin[21]. Cleavage at the mutated site results in truncation of FXII followed by accelerated activation of FXII, plasma prekallikrein (PK) activation and bradykinin release in solution. As FXII initiates the contact activation system, knowledge on its regulation is key for understanding the pathophysiological conditions in which bradykinin is produced. In **part II** of this thesis we further explored FXII function and its role in bradykinin mediated disease beyond HAE.

PART II: INSIGHT IN BRADYKININ DRIVEN DISEASE BEYOND HEREDITARY ANGIOEDEMA

While HAE is a rare disease, angioedema is common, with a self-reported prevalence of 7%[22]. Angiotensin converting enzyme (ACE)-induced angioedema and acquired C1-INH deficiency are two examples of bradykinin mediated disease besides HAE[23]. For other forms of angioedema, the contribution of bradykinin is not established. Mast-cell activity and histamine release are suspected to cause allergic angioedema and angioedema in chronic spontaneous urticaria (CSU) a disease characterized by recurrent itchy wheals, and/or angioedema that is not triggered by an allergen[23]. In part II of this thesis, we explore bradykinin driven inflammation beyond HAE.

Resistance to antihistamine therapy is common in idiopathic angioedema

When no underlying cause for angioedema can be found and there is no family history suggestive of HAE, patients are diagnosed with idiopathic angioedema. Antihistamines are prescribed on a trial-and-error basis as randomized controlled trials proving efficacy are lacking. In **chapter 8,** we performed a retrospective study on antihistamine prophylaxis in a cohort of 120 idiopathic angioedema patients. We found that 36% of patients did not improve on antihistamine prophylaxis. This underlines and emphasizes the observation in two previous retrospective studies in an Italian cohort describing inefficacy of antihistamine treatment in 16% of idiopathic angioedema patients. Our study stresses that antihistamine prophylaxis is often insufficient to prevent swelling in idiopathic angioedema.

When idiopathic angioedema patients do not respond to antihistamine treatment they are classified as idiopathic non-histaminergic angioedema. Evidence for the involvement of bradykinin in idiopathic non-histaminergic angioedema is surfacing. A previous study demonstrated increased bradykinin levels in 4 out of 4 patients with idiopathic non-histaminergic angioedema during an angioedema attack[24]. As direct bradykinin measurement is complicated by its short plasma half-life and small molecule size this finding was never repeated in larger cohorts. Interestingly, it was observed that plasma samples from patients with idiopathic non-histaminergic angioedema generated more plasma kallikrein (PKa) activity after stimulation with the FXII-activating compound dextran-sulphate compared to healthy controls. And also more than patients with idiopathic angioedema that responded to antihistamine therapy[25]. This would suggest that idiopathic non-histaminergic angioedema patients are prone to increased bradykinin production upon stimulation of contact activation. Lastly, case reports describe successful use of bradykinin receptor antagonists or C1-INH concentrate infusion therapy in patients with idiopathic non-histaminergic angioedema[26]. Altogether, multiple lines of evidence suggest that targeting bradykinin could be a treatment strategy for idiopathic non-histaminergic angioedema.

Recombinant human C1-INH, a potential treatment for idiopathic non-histaminergic angioedema

In **chapter 9**, we describe the study design and preliminary data of a prospective, consecutive, cross-over, case-series study using recombinant human C1-INH as prophylaxis for idiopathic non-histaminergic angioedema. All patients included in this study received twice weekly rhC1-INH for 2 months while recording their angioedema symptoms. One month prior to treatment and the month after treatment patients recorded their symptoms for comparison. We described findings of the 3 first included patients. One patient benefitted from rhC1-INH treatment. We can only speculate on the reasons why rhC1-INH did not improve symptoms in the 2 other cases. The dosage or treatment interval might have been insufficient. Or rhC1-INH might not have been able to prevent bradykinin release because contact system activation took place locally on the endothelial cell surface or in the perivascular space as C1-INH is less capable of inhibiting surface bound enzymes and rhC1-INH does not cross the endothelial barrier[27,28]. Most importantly, swellings observed in the two non-responders may not have been bradykinin mediated.

We were unable to identify a biomarker that could predict therapy response. Notably increased D-dimer levels were observed in one non-responder which may point towards an active role of the fibrinolytic system in his pathology. Interestingly, antihistamine resistance in patients with chronic spontaneous urticaria (CSU) is associated with increased d-dimer levels[29]. Despite that fact that there are multiple lines of evidence connecting activation of the fibrinolytic system[30] with bradykinin release we failed to demonstrate that the patient with angioedema and increased d-dimer levels benefitted from rhC1-INH treatment.

This study for the first time demonstrated efficacy of rhC1-INH in a patient with idiopathic non-histaminergic angioedema. However, we observed inefficacy in two consecutive cases. Idiopathic non-histaminergic angioedema may be an umbrella term for diseases with different pathophysiological mechanisms. Better understanding of swellings in idiopathic non-histaminergic angioedema is necessary to select the right therapeutic strategy and continue to further clinical trials for this disease.

Evidence for bradykinin release in chronic spontaneous urticaria

In **chapter 8** and **9** we differentiate idiopathic angioedema in 'histaminergic' and 'non-histaminergic' based upon clinical efficacy of antihistamine therapy. It is questionable if such a black-and-white distinction between histaminergic and supposedly bradykininergic angioedema should be made. As reviewed in **chapter 2**, mast-cell activation can also indirectly lead to bradykinin production. It was demonstrated that heparin released by mast-cells can activate FXII, thereby activating the contact system[31]. Mice studies and biomarker research in human patients with an anaphylactic response point towards involvement of FXII and bradykinin[31–33].

In **chapter 10** we performed a biomarker study in patients with CSU. The underlying mechanism of CSU is not fully understood but it is considered a non-IgE driven mast-cell

mediated disease and patients are treated with antihistamine therapy and the anti-IgE monoclonal antibody omalizumab. We found that cHK levels were increased in CSU patients compared to healthy controls and normalized in patients that were in disease remission. These data reflect the hypothesis that increased bradykinin production can be observed in mast-cell related pathologies. We hypothesized that cHK levels would be higher in CSU patients with angioedema compared to patients with wheals, but this was not the case. By design, this biomarker study does not demonstrate a causal relation between bradykinin release and symptoms in CSU. cHK may be an innocent bystander in CSU not contributing to symptoms. However, we speculate that mast-cell activity induces bradykinin production in CSU and contribute to angioedema when additional risk factors are present to enhance the effects of bradykinin. Besides a possible contribution of bradykinin to the development of angioedema in CSU, bradykinin may also contribute to a general state of increased vascular permeability, enhancing wheal formation. It is known that intradermal bradykinin injection does not result in local angioedema but a painful, mildly itchy, wheal and flare reaction[34,35]. Moreover, histamine and bradykinin can work synergistically as is demonstrated in allergic rhinitis. Interfering with either mast-cells, histamine or bradykinin improves symptoms of allergic rhinitis[36–38], and sensitivity to intranasal histamine improves allergic rhinitis after pretreatment with bradykinin receptor antagonist[36]. Our study generates the first evidence that bradykinin might be involved in CSU but more evidence is needed to confirm an active role for bradykinin.

FXII-W268R mutation causes a novel bradykinin mediated disease that does not present with angioedema

A novel FXII mutation is broadeningour understanding of the phenotype of bradykinin driven inflammation. A point mutation located in the kringle domain of FXII (W268R) was identified in a four-generation family suffering from an autoinflammatory syndrome. Disease co-segregated with mutation carriers. Symptoms consisted of cold-induced generalized urticarial rash, fatigue, fever, headache and arthralgia[39–41]. These combined symptoms are common in patients with the hereditary autoinflammatory disease cryopyrin associated periodic syndrome (CAPS). CAPS is an interleukin-1β (Il-1β) driven disease[42]. Most patients carry mutations in the NLRP3 gene encoding for cryopyrin, resulting in inappropriate inflammasome activation followed by IL-1β production. In 15% of patients with CAPS-like symptoms, no NLRP3 mutation can be identified[43]. The FXII-W268R carriers presented with normal to mildly elevated levels of the inflammatory biomarker CRP and a trial with IL-1β receptor antagonist in two FXII-W268R carriers resulted in limited, respectively moderate improvement but needed to be discontinued due to severe local side reactions[39,40]. Treatment with a bradykinin receptor antagonist in one patient immediately improved symptoms, followed by a relapse after cessation of therapy. In collaboration with the discoverers of this mutations we demonstrated massive HK cleavage and markedly increased levels of FXIIa-C1-INH and PKa-C1-INH complexes in the blood plasma of affected subjects[41]. The combined observation of clinical efficacy of a bradykinin receptor antagonist and increased biomarkers

of contact activation conclude that this novel autoinflammatory syndrome is a bradykinin mediated disease. The term FXII associated cold autoinflammatory syndrome (FACAS) was proposed to denote this disease.

The question arises how this FXII-W268R mutation results in increased bradykinin release and in what aspect it differs from the various mutations that cause FXII-HAE. In **chapter 11** we investigated the biochemical behavior of FXII-W268R and compared it to wildtype FXII (FXII-WT) and the HAE related FXII mutation FXII-T309R. We found that FXII-W268R but not FXII-WT or FXII-T309R was partly fragmented and active upon production by HEK293freestyle cells. Fragmentation was already observed intracellularly and was reversed by mutagenesis of the FXII active site creating the inactive variant FXII-W268R-S544A. This demonstrates that FXII-W268R activates more easily and, unlike wildtype FXII, even intracellularly. In addition, activation of purified FXII-W268R by PKa and plasmin was accelerated compared to FXII-WT and FXII-T309R. Interestingly, FXII-W268R exposes a plasminogen binding site that is only exposed in FXII-WT and FXII-T309R when bound to a negatively charged surface. The combined observations that FXII-W268R is partly active upon excretion and that activation of the protein is accelerated explain the profound presence of systemic biomarkers of contact activation seen in FXII-W268R.

Impaired FXII quiescence causing bradykinin mediated disease

For the first time, we identified a clear link between bradykinin and a disease beyond HAE with the discovery of FXII-W268R in FACAS. As FXII initiates bradykinin release and its dysregulation is now connected to two diseases, it can be stated that regulation of FXII is key for bradykinin production *in vivo*.

FXII normally becomes active upon cleavage after R353[44], with a second cleavage event at R334 the surface binding domains of FXII(a) are removed creating a truncated form of FXIIa; βFXIIa. Both cleavage events are mediated by FXIIa and PKa activity. FXII activation is accelerated upon binding to a negatively charged surface, it is suggested that surface binding spatially removes the surface binding domains away from the activation loop were R353 is located, exposing it for activation[45,46]. Studies on FXII-HAE mutations demonstrated that FXII activation accelerates after cleavage of the mutated site in the proline-rich region by plasmin or thrombin, creating truncated FXIIa[20,21]. Following up on this discovery it was demonstrated that FXII-WT has naturally occurring cleavage sites in the proline-rich region for cathepsin K and neutrophil elastase[47]. Likewise, truncation at these sites accelerates FXII activation, implicating that conditions were these enzymes are released, such as during neutrophil activation, accelerate FXII activation[47]. The FXII-W268R mutation does not result in truncation of FXII. In stead, it appears that the activation loop is always exposed. FXII-W268R is already prone to intracellular autoactivation, it has an accelerated capacity for autoactivation and activation by PKa and plasmin and exposes a plasminogen binding site that is normally cryptic. Based on our biochemical studies, we hypothesize that the W268R mutation leads to an altered conformation exposing the activation loop.

To deepen our understanding of FXII regulation we carried out a functional study on FXII in **chapter 12**. By step wise N-terminally deletion of FXII domains, 7 FXII truncation variants were created. Spontaneous FXII fragmentation and FXIIa activity upon production in HEK293freestyle cells was observed after deletion of the fibronectin type II (FnII) domain and onwards. Cleavage by PKa was accelerated after truncation of the FnII domain. Surface binding accelerated the activation rate of FXII-WT by PKa but not of the FnII domain truncated variant. Altogether, these functional data point towards a regulatory mechanism were FXII is protected against activation by intramolecular shielding of the activation loop. Surface binding or truncation displaces the FnII domain exposing R353.

THE CLINICAL PHENOTYPE OF BRADYKININ MEDIATED DISEASE IN HAE AND BEYOND

The most well-known and distinct clinical phenotype of bradykinin mediated disease is angioedema. Although there are clinical differences among the subtypes of bradykinin mediated angioedema, their swelling attacks are very similar. Bradykinin involvement in FACAS sheds a new light on the clinical phenotype of bradykinin driven inflammation. The conditions leading to different clinical outcomes are not well understood.

In **chapter 5** we review on the possibility of local upregulation of the bradykinin B1 receptor as a determinant of the occurrence of an angioedema attacks in HAE-1 and 2. This disease is associated with an overall increased bradykinin production throughout circulation and high cHK levels can be detected both during remission and angioedema attacks. Patients often present with angioedema at multiple sites and mild systemic features like fatigue, malaise, muscle ache and the skin rash erythema marginatum prior to swellings[48,49]. Poor systemic control of contact activation can be explained by lack of C1-INH activity in circulation.

Angioedema HAE with normal C1-INH and idiopathic non-histaminergic angioedema appears to arise more locally. Erythema marginatum is not described, patients less often present with multiple swelling sites[50] and although only determined in limited numbers of patients, HK consumption is very subtle or undetectable[20]. Swelling occurs more often in the face and upper airways compared to HAE type 1 and 2, pointing towards a different underlying mechanism. Increased fibrinolytic activity localized to the endothelial surface was put forward as a local mechanism for FXII activation and increased bradykinin production[30]. Treatment with antifibrinolytic agents appears more successful in HAE with normal C1-INH and idiopathic non-histaminergic angioedema[51–53] compared to HAE-1 and 2. Notably, a side effect of thrombolytic therapy in stroke patients, especially seen in those also receiving ACE-inhibitor therapy, is angioedema of the tongue and lips[54,55]. In addition, severe tongue swelling is common in HAE with a plasminogen mutation. Combined, these data suggest that the face and upper airways are sites with increased fibrinolytic activity potentially explaining locally increased bradykinin release.

The question remains why FACAS, a disease state with a seemingly systemic clinical character, does not present with angioedema. The underlying pathophysiological

mechanism is currently being researched. Immunohistochemistry revealed macrophage and neutrophil infiltration in FACAS lesional skin that co-localized with IL-1β. FXII expression was observed in intra- and perivascular neutrophils[39]. Moreover, incubation of donor peripheral blood mononuclear cells with FXII-W268R but not FXII-WT elicited IL-1β production[39]. The researchers propose a combined model of bradykinin and IL-1β induced disease where neutrophils are a local source of FXIIa in the skin triggering local bradykinin generation. Together, FXIIa and bradykinin incite IL-1β production upon interaction with monocytes and macrophages[56,57].

In **chapter 11**, we propose that increased (auto)activation in FXII-W268R leads to continuous excretion of FXIIa. In contrast to FACAS, HAE is not associated with excessive FXII activation, only a minute amount of FXIIa is necessary to start the cascade leading to HK cleavage[20]. In HAE with FXII mutations or other conditions with normal FXII, FXII is secreted as an inactive molecule, and as proposed in **chapter 12**, protected for activation until FXII is enzymatically truncated or bound to a surface. This is reflected by the amount of FXIIa-C1-INH complex detected in various diseases. These are ranging from 0 to 2% in healthy individuals and CSU patients (**chapter 10**), from 0-4% in two patients with HAE-FXII during an angioedema attack[20] and from 22-67% in three FACAS patients (100% reflects maximal FXIIa-C1-INH complex in dextran sulphate triggered plasma)[41]. Independent of bradykinin, FXIIa may work as a proinflammatory protein inducing cytokine production in macrophages and neutrophils creating circumstances different from HAE. It was previously demonstrated that incubation of FXIIa with bone marrow-derived macrophages stimulates Il-1β, Il-6, Il-12, Cxcl1, Ccl2, and TNF-α release[56]. Incubation with FXII resulted in significantly less cytokine production compared to FXIIa. This macrophage activation of FXIIa was independent of FXIIa enzymatic activity but depended on cell-signaling via binding to the urokinase-type plasminogen activator receptor (uPAR)[56]. In **chapter 11** we suggest that FXII-W268R has an altered conformation, we show that FXII-W268R exposes a plasminogen binding site that is normally cryptic. Possibly, the binding site of FXII for uPAR is also cryptic but exposed in FXIIa and FXII-W268R which would enable FXII-W268R to activate macrophages even in its inactive form. Still, bradykinin is a major culprit in FACAS as rash, fatigue and headache fully disappeared upon treatment with a bradykinin receptor antagonist.

As discussed, we introduced the concept that the combined presence of bradykinin release in circulation and IL-1β results in bradykinin B1 receptor upregulation followed by local angioedema (**chapter 5**). Our recent findings in FACAS are inconsistent with this hypothesis as widespread contact activation and the presence of IL-1β present with a different clinical phenotype. How bradykinin B1 receptor expression fits in the disease model of HAE and FACAS awaits to be established.

From FACAS we learned that the combined model of bradykinin, cytokines and FXIIa can present with urticarial-like rash. This creates a new perspective on our findings in **chapter 10** where we observed increased cHK levels in CSU. Mast-cell activation may locally activate FXII via excretion of heparin, leading to FXIIa driven cytokine induction and bradykinin release. Under these circumstances, bradykinin may contribute to the rise of both angioedema and

urticaria. Various Th-1 and Th-2-cell associated cytokines were detected in CSU, but contrary to CAPS their contribution to disease is unclear[58].

Altogether, our research indicates that bradykinin driven inflammation is not limited to HAE nor to the clinical symptom angioedema. Figure 1 gives an overview of factors that were highlighted in this thesis as potentially important for bradykinin driven inflammation.

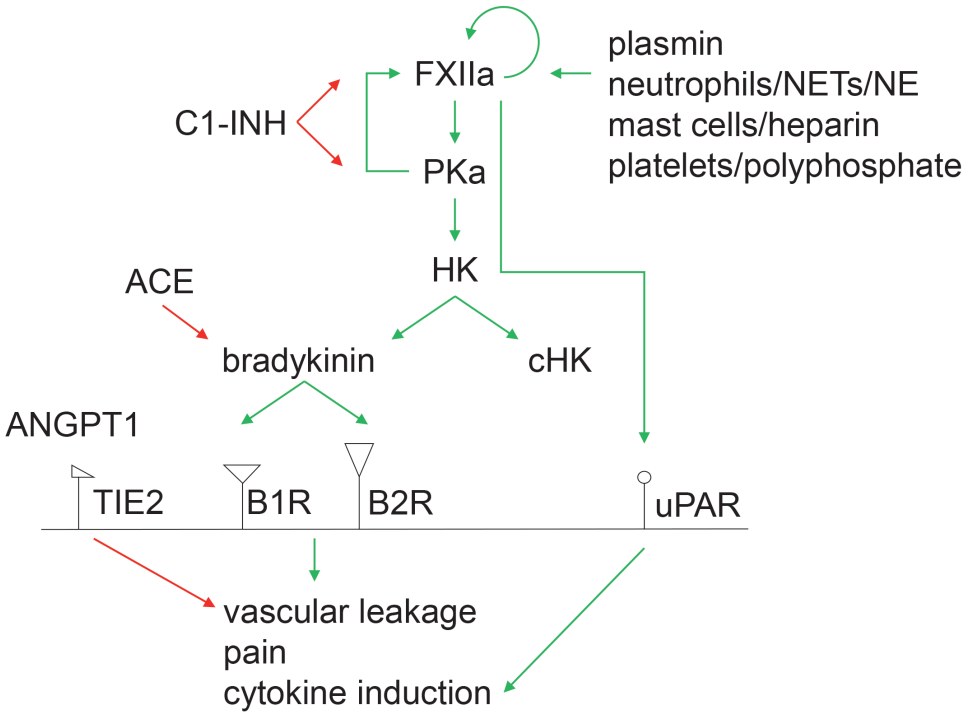


Figure 1. The contact system and factors influencing bradykinin driven inflammation. FXIIa: active Factor XII, PKa: plasma kallikrein, HK: high molecular weight kininogen, cHK: cleaved HK, C1-INH: C1-esterase inhibitor, ACE: angiotensin-converting enzyme, ANGPT1: angiopoeitin-1, TIE2: ANGPT1 receptor, B1R: bradykinin B1 receptor, B2R: bradykinin B2 receptor, uPAR: urkinase-type plasminogen activator receptor, NETs: neutrophil extracellular traps, NE: neutrophil elastase.

CLINICAL IMPLICATIONS

- We developed a cHK ELISA (**chapter 6**) allowing for high throughput and quantitative measurement of cHK, a biomarker for bradykinin release. This assay can support HAE patient care and drug development by monitoring bradykinin release. In **chapter 7** we demonstrate its practical use in a phase I clinical trial testing the efficacy of a kallikrein inhibitor after triggering *ex vivo* contact activation.
- The cHK ELISA is of limited use in diagnosing a bradykinin mediated disease in individual patients, sensitivity in HAE type 1 and 2 patients is only 53-54%. In addition, cHK levels did not predict response to rhC1-INH in idiopathic non-histaminergic angioedema (**chapter 9**). The cHK ELISA can be used to detect mean differences in cHK on a population base (**chapter 10**) thereby supporting further research to bradykinin mediated disease.
- We observed improvement in one patient with idiopathic non-histaminergic angioedema treated with rhC1-INH. Hereby strengthening the evidence that this can be a bradykinin mediated disease and rhC1-INH can be used to treat patients with idiopathic non-histaminergic angioedema. However, two patients with idiopathic non-histaminergic disease did not respond to rhC1-INH treatment suggesting heterogeneity in the underlying mechanisms of disease in this population.
- In collaboration with Scheffel and colleagues[39–41] we identified a novel bradykinin mediated disease and demonstrated in **chapter 11** that the FXII-W268R mutation leads to continuous excretion of FXIIa and accelerated activation. This led to the insight that bradykinin can contribute to symptoms like urticarial rash, fatigue, arthralgia and headache in human pathology. Future research to CAPS and CSU should include bradykinin and FXII as a possible disease mediators and potential therapeutic targets.

CONCLUSIONS

Bradykinin driven inflammation is not limited to HAE or to the clinical presentation of angioedema. We demonstrated FXII dysregulation in FACAS. This novel bradykinin mediated autoinflammatory disease does not present with angioedema. We propose that FXII regulation is key in bradykinin driven disease. Conditions that trigger FXII activation determine when and where bradykinin will be produced. Therapies developed for HAE-1 and 2 are potential treatment options for other bradykinin driven diseases. Currently we do not have a biomarker available that can predict clinical efficacy of targeting bradykinin. Better identification of bradykinin mediated diseases is necessary in order to continue with clinical trials to make these therapies available for diseases beyond HAE. Bradykinin is a potential therapeutic target in idiopathic angioedema, chronic spontaneous urticaria and unexplained autoinflammatory diseases that do not respond to current therapies.

REFERENCES

1. Bygum A, Fagerberg CR, Ponard D, Monnier N, Lunardi J, Drouet C. Mutational spectrum and phenotypes in Danish families with hereditary angioedema because of C1 inhibitor deficiency. *Allergy*. 2011;66(1):76-84. doi:10.1111/j.1398-9995.2010.02456.x
2. Bafunno V, Bova M, Loffredo S, et al. Mutational spectrum of the c1 inhibitor gene in a cohort of Italian patients with hereditary angioedema: description of nine novel mutations. *Ann Hum Genet*. 2014;78(2):73-82. doi:10.1111/ahg.12052
3. Kelemen Z, Moldovan D, Mihály E, et al. Baseline level of functional C1-inhibitor correlates with disease severity scores in hereditary angioedema. *Clin Immunol*. 2010;134(3):354-358. doi:10.1016/j.clim.2009.11.002
4. Mete Gökmen N, Gülbahar O, Onay H, et al. Deletions in SERPING1 Lead to Lower C1 Inhibitor Function: Lower C1 Inhibitor Function Can Predict Disease Severity. *Int Arch Allergy Immunol*. 178(1):50-59. doi:10.1159/000492583
5. Zuraw BL, Christiansen SC. HAE Pathophysiology and Underlying Mechanisms. *Clin Rev Allergy Immunol*. 2016;51(2):216-229. doi:10.1007/s12016-016-8561-8
6. Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency. *Clin Exp Allergy*. 2014;44(12):1503-1514. doi:10.1111/cea.12293
7. Mesquita TRR, Campos-Mota GP, Lemos VS, et al. Vascular Kinin B1 and B2 Receptors Determine Endothelial Dysfunction through Neuronal Nitric Oxide Synthase. *Front Physiol*. 2017;8:228. doi:10.3389/fphys.2017.00228
8. Leeb-Lundberg LMF, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev*. 2005;57(1):27-77. doi:10.1124/pr.57.1.2
9. Hardt J, Meinke P, Witzke G, Wulff K, Wagner N, Bork K. A novel mutation in the coagulation factor 12 gene in subjects with hereditary angioedema and normal C1-inhibitor. *Clin Immunol*. 2011;141(1):31-35. doi:10.1016/j.clim.2011.07.002
10. Dewald G, Bork K. Missense mutations in the coagulation factor XII (Hageman factor) gene in hereditary angioedema with normal C1 inhibitor. *Biochem Biophys Res Commun*. 2006;343(4):1286-1289. doi:10.1016/j.bbrc.2006.03.092
11. Bork K, Wulff K, Steinmüller-Magin L, et al. Hereditary angioedema with a mutation in the plasminogen gene. *Allergy*. 2018;73(2):442-450. doi:10.1111/all.13270
12. Bafunno V, Firinu D, D'Apolito M, et al. Mutation of the angiopoietin-1 gene (ANGPT1) associates with a new type of hereditary angioedema. *J Allergy Clin Immunol*. 2018;141(3):1009-1017. doi:10.1016/j.jaci.2017.05.020
13. Bork K, Wulff K, Rossmann H, et al. Hereditary angioedema cosegregating with a novel kininogen 1 gene mutation changing the N-terminal cleavage site of bradykinin. *Allergy*. May 2019. doi:10.1111/all.13869
14. d'Apolito M, Santacroce R, Colia AL, Cordisco G, Maffione AB, Margaglione M. Angiopoietin-1 haploinsufficiency affects the endothelial barrier and causes hereditary angioedema. *Clin Exp Allergy*. 2019;49(5):626-635. doi:10.1111/cea.13349
15. Bouillet L, Mannic T, Arboleas M, et al. Hereditary angioedema: key role for kallikrein and bradykinin in vascular endothelial-cadherin cleavage and edema formation. *J Allergy Clin Immunol*. 2011;128(1):232-234. doi:10.1016/j.jaci.2011.02.017
16. Gavard J, Patel V, Gutkind JS. Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. *Dev Cell*. 2008;14(1):25-36. doi:10.1016/j.devcel.2007.10.019

17. Rosen FS, Alper CA, Pensky J, Klemperer MR, Donaldson VH. Genetically determined heterogeneity of the C1 esterase inhibitor in patients with hereditary angioneurotic edema. *J Clin Invest*. 1971;50(10):2143-2149. doi:10.1172/JCI106708

18. Bork K, Wulff K, Meinke P, Wagner N, Hardt J, Witzke G. A novel mutation in the coagulation factor 12 gene in subjects with hereditary angioedema and normal C1-inhibitor. *Clin Immunol*. 2011;141(1):31-35. doi:10.1016/j.clim.2011.07.002

19. Kiss N, Barabás E, Várnai K, et al. Novel duplication in the F12 gene in a patient with recurrent angioedema. *Clin Immunol*. 2013;149(1):142-145. doi:10.1016/j.clim.2013.08.001

20. de Maat S, Björkqvist J, Suffritti C, et al. Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations. *J Allergy Clin Immunol*. 2016;138(5):1414-1423.e9. doi:10.1016/j.jaci.2016.02.021

21. Ivanov I, Matafonov A, Sun M-F, et al. A mechanism for hereditary angioedema with normal C1 inhibitor: an inhibitory regulatory role for the factor XII heavy chain. *Blood*. 2019;133(10):1152-1163. doi:10.1182/blood-2018-06-860270

22. Madsen F, Attermann J, Linneberg A. Epidemiology of Non-hereditary Angioedema. *Acta Derm Venereol*. 2012;92(5):475-479. doi:10.2340/00015555-1389

23. Maurer M, Magerl M, Ansotegui I, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2017 revision and update. *Allergy*. 2018;73(8):1575-1596. doi:10.1111/all.13384

24. Cugno M, Tedeschi A, Nussberger J. Bradykinin in idiopathic nonhistaminergic angioedema. *Clin Exp Allergy*. 2016. doi:10.1111/cea.12869

25. Lara-Marquez ML, Christiansen SC, Riedl MA, Herschbach J, Zuraw BL. Threshold-stimulated kallikrein activity distinguishes bradykinin- from histamine-mediated angioedema. *Clin Exp Allergy*. 2018;48(11):1429-1438. doi:10.1111/cea.13219

26. van den Elzen M, Go MFCL, Knulst AC, Blankestijn MA, van Os-Medendorp H, Otten HG. Efficacy of Treatment of Non-hereditary Angioedema. *Clin Rev Allergy Immunol*. 2018;54(3):412-431. doi:10.1007/s12016-016-8585-0

27. Dors DM, Nuijens JH, Huijbregts CC, Hack CE. A novel sensitive assay for functional factor XII based on the generation of kallikrein-C1-inhibitor complexes in factor XII-deficient plasma by glass-bound factor XII. *Thromb Haemost*. 1992;67(6):644-648. http://www.ncbi.nlm.nih.gov/pubmed/1509403.

28. Farrell C, Hayes S, Relan A, van Amersfoort ES, Pijpstra R, Hack CE. Population pharmacokinetics of recombinant human C1 inhibitor in patients with hereditary angioedema. *Br J Clin Pharmacol*. 2013;76(6):897-907. doi:10.1111/bcp.12132

29. Asero R. D-dimer: a biomarker for antihistamine-resistant chronic urticaria. *J Allergy Clin Immunol*. 2013;132(4):983-986. doi:10.1016/j.jaci.2013.04.037

30. de Maat S, de Groot PG, Maas C. Contact system activation on endothelial cells. *Semin Thromb Hemost*. 2014;40(8):887-894. doi:10.1055/s-0034-1395159

31. Oschatz C, Maas C, Lecher B, et al. Mast Cells Increase Vascular Permeability by Heparin-Initiated Bradykinin Formation In Vivo. 2011. doi:10.1016/j.immuni.2011.02.008

32. van der Linden PW, Hack CE, Eerenberg a J, Struyvenberg A, van der Zwan JK. Activation of the contact system in insect-sting anaphylaxis: association with the development of angioedema and shock. *Blood*. 1993;82(6):1732-1739. http://www.ncbi.nlm.nih.gov/pubmed/8400230.

33. Sala-Cunill A, Björkqvist J, Senter R, et al. Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions. *J Allergy Clin Immunol*. 2015;135(4):1031-1043.e6. doi:10.1016/j.jaci.2014.07.057

34. Fadel R, Ramboer I, Chatterjee N, Rihoux JP, Derde MP. Cetirizine inhibits bradykinin-induced cutaneous wheal and flare in atopic and healthy subjects. *Allergy*. 2000;55(9):888-891. http://www.ncbi.nlm.nih.gov/pubmed/11003455.

35. Hosogi M, Schmelz M, Miyachi Y, Ikoma A. Bradykinin is a potent pruritogen in atopic dermatitis: a switch from pain to itch. *Pain*. 2006;126(1-3):16-23. doi:10.1016/j.pain.2006.06.003

36. Turner P, Dear J, Scadding G, Foreman JC. Role of kinins in seasonal allergic rhinitis: icatibant, a bradykinin B2 receptor antagonist, abolishes the hyperresponsiveness and nasal eosinophilia induced by antigen. *J Allergy Clin Immunol*. 2001;107(1):105-113. doi:10.1067/mai.2001.111145

37. Rajakulasingam K, Polosa R, Lau LC, Church MK, Holgate ST, Howarth PH. Comparative nasal effects of bradykinin and histamine: influence on nasal airways resistance and plasma protein exudation. *Thorax*. 1993;48(4):324-329. doi:10.1136/thx.48.4.324

38. Gawlik R, Grzanka A, Jawor B, Czecior E. Specific immunotherapy with mugwort pollen allergoid reduce bradykinin release into the nasal fluid. *Postep dermatologii i Alergol*. 2016;33(4):269-275. doi:10.5114/ada.2016.61602

39. Scheffel J, Mahnke NA, Hofman ZLM, et al. Cold-induced urticarial autoinflammatory syndrome related to factor XII activation. *Pediatr Rheumatol*. 2019;17(S1):18. doi:10.1186/s12969-019-0313-x

40. Mahnke NA, Scheffel J, Wu J, et al. A novel urticarial and Factor XII mutation-related autoinflammatory syndrome. *Exp Dermatol*. 2018;27(3):e2-e106. doi:10.1111/exd.13486

41. Hofman ZLM, Krause K, Mahnke NA, et al. Human Factor XII Mutation W268R Causes Urticarial Autoinflammatory Syndrome through Intracellular Misprocessing. *Res Pract Thromb Haemost*. 2018;2:1-368. doi:10.1002/rth2.12125

42. Levy R, Gérard L, Kuemmerle-Deschner J, et al. Phenotypic and genotypic characteristics of cryopyrin-associated periodic syndrome: a series of 136 patients from the Eurofever Registry. *Ann Rheum Dis*. 2015;74(11):2043-2049. doi:10.1136/annrheumdis-2013-204991

43. Toplak N, Frenkel J, Ozen S, et al. An international registry on autoinflammatory diseases: the Eurofever experience. *Ann Rheum Dis*. 2012;71(7):1177-1182. doi:10.1136/annrheumdis-2011-200549

44. Ivanov I, Matafonov A, Sun M-F, et al. Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. *Blood*. 2017;129(11):1527-1537. doi:10.1182/blood-2016-10-744110

45. de Maat S, Maas C. Factor XII : form determines function. *J Thromb Heamostasis*. 2016;14:1498-1506. doi:10.1111/jth.13383

46. Citarella F, te Velthuis H, Helmer-Citterich M, Hack CE. Identification of a putative binding site for negatively charged surfaces in the fibronectin type II domain of human factor XII--an immunochemical and homology modeling approach. *Thromb Haemost*. 2000;84(6):1057-1065. http://www.ncbi.nlm.nih.gov/pubmed/11154114.

47. de Maat S, Clark CC, Boertien M, et al. Factor XII truncation accelerates activation in solution. *J Thromb Haemost*. 2019;17(1):183-194. doi:10.1111/jth.14325

48. Magerl M, Doumoulakis G, Kalkounou I, et al. Characterization of prodromal symptoms in a large population of patients with hereditary angio-oedema. *Clin Exp Dermatol*. 2014;39(3):298-303. doi:10.1111/ced.12285

49. Prematta MJ, Kemp JG, Gibbs JG, Mende C, Rhoads C, Craig TJ. Frequency, timing, and type of prodromal symptoms associated with hereditary angioedema attacks. *Allergy asthma Proc*. 30(5):506-511. doi:10.2500/aap.2009.30.3279

50. Bork K, Gül D, Hardt J, Dewald G. Hereditary angioedema with normal C1 inhibitor: clinical symptoms and course. *Am J Med*. 2007;120(11):987-992. doi:10.1016/j.amjmed.2007.08.021

51. Wintenberger C, Boccon-Gibod I, Launay D, et al. Tranexamic acid as maintenance treatment for non-histaminergic angioedema: analysis of efficacy and safety in 37 patients. *Clin Exp Immunol*. 2014;178(1):112-117. doi:10.1111/cei.12379

52. Mansi M, Zanichelli A, Coerezza A, et al. Presentation, diagnosis and treatment of angioedema without wheals: a retrospective analysis of a cohort of 1058 patients. *J Intern Med*. 2015;277(5):585-593. doi:10.1111/joim.12304

53. Bucher MC, Petkovic T, Helbling A, Steiner UC. Idiopathic non-histaminergic acquired angioedema: a case series and discussion of published clinical trials. *Clin Transl Allergy*. 2017;7:27. doi:10.1186/s13601-017-0164-9

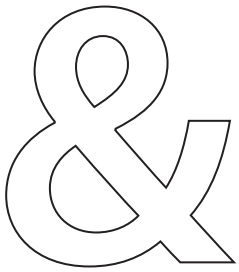
54. Vigneron C, Lécluse A, Ronzière T, et al. Angioedema associated with thrombolysis for ischemic stroke: analysis of a case-control study. *J Intern Med*. July 2019. doi:10.1111/joim.12962

55. Lekoubou A, Philippeau F, Derex L, et al. Audit report and systematic review of orolingual angioedema in post-acute stroke thrombolysis. *Neurol Res*. 2014;36:687-694. doi:10.1179/1743132813Y.0000000302

56. Vorlova S, Koch M, Manthey HD, et al. Coagulation factor XII induces pro-inflammatory cytokine responses in macrophages and promotes atherosclerosis in mice. *Thromb Haemost*. 2017;117(1):176-187. doi:10.1160/TH16-06-0466

57. Toossi Z, Sedor JR, Mettler MA, Everson B, Young T, Ratnoff OD. Induction of expression of monocyte interleukin 1 by Hageman factor (factor XII). *Proc Natl Acad Sci U S A*. 1992;89(24):11969-11972. doi:10.1073/pnas.89.24.11969

58. Radonjic-Hoesli S, Hofmeier KS, Micaletto S, Schmid-Grendelmeier P, Bircher A, Simon D. Urticaria and Angioedema: an Update on Classification and Pathogenesis. *Clin Rev Allergy Immunol*. 2018;54(1):88-101. doi:10.1007/s12016-017-8628-1



Appendix

NEDERLANDSE SAMENVATTING

Bradykinine is een ontstekingspeptide dat episodes met zwellingen veroorzaakt in mensen met de ziekte erfelijk angio-oedeem. Deze zwellingen kunnen voorkomen in de huid en slijmvliezen. Angio-oedeem van de handen of voeten kan belemmerend zijn in het dagelijks functioneren, evenals ontsierende zwellingen in het gelaat. Ernstige complicaties treden op wanneer er oedeem in de darmen of de keel ontstaat. Oedeem in de darmen is zeer pijnlijk en een zwelling in de keel kan tot verstikking leiden.

De meeste patiënten met erfelijk angio-oedeem hebben een tekort aan C1-esterase remmer. Deze remmer circuleert in de bloedbaan en voorkomt activatie van het contact systeem. Het contact systeem is een enzymatische cascade, welke begint met activatie van Factor XII (FXII). FXII activeert het enzym plasma kallikreïne. Dit enzym knipt bradykinine uit zijn precursor eiwit hoog molecuulgewicht kininogeen. Een tekort aan C1-esterase remmer zorgt dus voor een te veel aan bradykinine. Wanneer bradykinine bindt aan zijn receptoren op de vaatwand verwijden de vaten en verhoogt de doorlaatbaarheid van het endotheel. Een te hoge lokale doorlaatbaarheid van de bloedvaten resulteert in angio-oedeem.

De ontdekking dat C1-esterase remmer tekort de oorzaak was van erfelijk angio-oedeem heeft ons veel geleerd over de gevolgen van bradykinine productie. Er zijn verschillende therapieën ontwikkeld die ingrijpen op bradykinine en bradykinine vorming voor de behandeling van erfelijk angio-oedeem. Deze therapieën zouden ook werkzaam kunnen zijn in andere ziekteprocessen waar bradykinine bij betrokken is. Er is echter nog weinig bekend over de rol van bradykinine in andere ziekten dan erfelijk angio-oedeem.

Het doel van dit proefschrift is inzicht te krijgen in de mechanismes achter bradykinine productie om het begrip van de ziekte erfelijk angio-oedeem te vergroten en om deze kennis toe te passen bij onderzoek naar andere ziekten die mogelijk door bradykinine worden veroorzaakt.

Deel I: Inzichten in erfelijk angio-oedeem

Om onderzoek te kunnen doen naar bradykinine vorming hebben wij een methode ontwikkeld om bradykinine productie te kunnen meten in bloedplasma. Bradykinine is maar enkele seconde aanwezig in de circulatie; het wordt snel afgebroken. Dit maakt bradykinine lastig meetbaar. Daarom hebben wij een ELISA ontwikkeld die het bijproduct van bradykinine vorming meet; gekliefd (hoog molecuulgewicht) kininogeen. In dit proefschrift demonstreerde wij de werkzaamheid van deze ELISA door metingen te doen in bloedplasma van patiënten met erfelijk angio-oedeem. Wij vonden verhoogde waarden gekliefd kininogeen in patiënten. De waarden waren het hoogst tijdens een episode met angio-oedeem maar zelfs in afwezigheid van zwellingen hadden patiënten verhoogde gekliefd kininogeen waarden ten opzichte van gezonde personen. Opvallend genoeg relateerde de hoeveelheid gekliefd kininogeen niet aan de angio-oedeem aanvalsfrequentie. Met andere woorden, een patiënt met erfelijk angio-oedeem kan veel meer bradykinine aanmaken dan iemand die de ziekte niet heeft, toch leidt dit niet altijd tot een episode met angio-oedeem. Wij gaan ervan uit dat er meer factoren zijn dan alleen C1-esterase remmer deficiëntie of de hoeveelheid bradykinine in circulatie die bijdragen aan het ontstaan van angio-oedeem.

In dit proefschrift postuleren wij de hypothese dat de bradykinine B1 receptor bij zou kunnen dragen aan het ontstaan van een zwelling. Deze receptor komt alleen tot expressie op de vaatwand in aanwezigheid van interleukines of bradykinine. Wij toonden aan dat patiënten met erfelijk angio-oedeem vaker dan gezonde personen verhoogde ontstekingswaarden in het bloed hebben wat wijst op de aanwezigheid van interleukines. Daarnaast beschreven wij dat patiënten met erfelijk angio-oedeem vaak zwellingen op meerde plekken tegelijk in hun lichaam hebben tijdens een ziekte episode. Als mogelijke verklaring voor dit fenomeen veronderstellen wij dat er alleen een zwelling ontstaat wanneer er lokaal op de vaatwand bradykinine B1 receptor tot expressie is gekomen.

Deel II: Inzichten in bradykinine gedreven ziekten voorbij erfelijk angio-oedeem

In het tweede deel van dit proefschrift hebben wij onderzoek gedaan naar een mogelijke rol voor bradykinine in andere ziektes dan erfelijk angio-oedeem. Verreweg de meeste patiënten met klachten van angio-oedeem hebben géén erfelijk angio-oedeem. Soms is een allergie de oorzaak van de zwellingen maar vaak kan er geen oorzaak gevonden worden. De diagnose "idiopathisch angio-oedeem" wordt dan gesteld. Men gaat er vanuit dat idiopathisch angio-oedeem wordt veroorzaakt door histamine. Dit ontstekingspeptide komt vrij bij mestcel activatie. De mestcel is onder andere betrokken bij allergie maar kan ook in afwezigheid van een allergische prikkel ziekte veroorzaken. Idiopathisch angio-oedeem wordt dus behandeld met antihistaminica, maar wij weten dat deze therapie niet altijd afdoende is. In dit proefschrift beschrijven wij dat 36% van de patiënten met idiopathisch angio-oedeem, die dagelijks antihistamine tabletten gebruikt, hier niet door verbeterd. Het vermoeden bestaat dat in antihistamine therapieresistente patiënten het angio-oedeem wordt veroorzaakt door bradykinine. Wij hebben dit verder onderzocht door drie patiënten met antihistamine resistent idiopathisch angio-oedeem te behandelen met recombinant C1-esterase remmer infusie. Tijdens behandeling met tweewekelijks recombinant C1-esterase remmer, had 1 patiënt aanzienlijk minder angio-oedeem aanvallen. Bij het stoppen van therapie kwamen de klachten direct terug en klachten verbeterde weer bij herstarten van de behandeling. Echter, in twee patiënten had de therapie geen effect. Wij vermoeden dat er verschillende mechanismes aan de ziekte idiopathisch angio-oedeem ten grondslag liggen. Alleen in sommige patiënten wordt een zwelling veroorzaakt door bradykinine en is het geven van recombinant C1-esterase remmer zinvol. Helaas konden wij met het meten van verschillende factoren in het bloed, waaronder gekliefd kininogeen, niet voorspellen welke patiënt ging reageren.

Angio-oedeem komt ook voor in patiënten met chronisch spontaan urticaria. Patiënten met deze ziekte hebben last van netelroos met hierbij soms ook angio-oedeem. Wij detecteerden verhoogde waarden van gekliefd kininogeen in het bloedplasma van patiënten met chronisch spontaan urticaria ten opzichte van gezonde personen. Opvallend genoeg waren de waarden ook verhoogd in patiënten die alleen last hadden van netelroos en géén angio-oedeem hadden. Over het algemeen wordt aangenomen dat mestcellen chronisch spontaan urticaria veroorzaken. Wij kunnen met ons onderzoek niet aantonen

dat bradykinine ook een rol speelt in deze ziekte. Wel vermoeden wij dat bradykinine bij kan dragen aan de toegenomen doorlaatbaarheid van de vaten welke optreedt bij angio-oedeem en netelroos in patiënten met chronisch spontaan urticaria.

Recent werd een nieuwe mutatie gevonden in FXII. In heel zeldzame gevallen zijn Factor XII mutaties de oorzaak van erfelijk angio-oedeem. Echter, de familie waarin deze nieuwe mutatie werd aangetoond had géén klachten van angio-oedeem. Zij hadden last van koude geïnduceerd netelroos, hoofdpijn, artralgie, koorts en algehele malaise. Dit zijn symptomen passend bij auto-inflammatoire ziekten. In samenwerking met de ontdekkers van deze mutatie lieten wij zien dat dragers van de mutatie extreem verhoogde waarden van gekliefd kininogeen hadden, ook waren er aanwijzingen voor verhoogde Factor XII activiteit en plasma kallikreïne activiteit. Behandeling met een bradykinine receptor antagonist, een therapie ontwikkelt voor erfelijk angio-oedeem, in een draagster van deze Factor XII mutatie zorgde direct voor verbetering van de klachten. Deze nieuwe aandoening wordt FACAS genoemd; Factor XII associated cold autoinflammatory syndrome.

In dit proefschrift onderzochten wij de functionele veranderingen die optreden in de FACAS-mutatie. Het betreft een puntmutatie resulterend in een aminozuur substitutie in het kringle domein van Factor XII (FXII-W268R). Wij brachten FXII-W268R recombinant tot expressie en zagen dat FXII-W268R gelijk na productie al sporen van activiteit vertoonde. Deze activiteit was al in de cel aanwezig. Met ons onderzoek lieten wij zien dat FXII-W268R gemakkelijk auto-activeert en gevoeliger is voor activatie door plasma kallikreïne en plasmine. Dragers van de FXII-W268R mutatie hebben waarschijnlijk een continue verhoogde FXII activiteit. Dit verklaart waarom dragers van de mutatie extreem veel bradykinine produceren en baat hebben bij behandeling ontwikkeld voor erfelijk angio-oedeem. Met de ontdekking van de FXII-W268R mutatie is voor het eerst een duidelijk verband gelegd tussen bradykinine en een ziekte buiten angio-oedeem.

Ontregeling van FXII in FACAS, maar ook FXII mutaties die leiden tot erfelijk angio-oedeem, veroorzaakt ziekte. Wij denken daarom dat FXII een belangrijke rol speelt in bradykinine gedreven pathologie. Door onderzoek te doen naar FXII regulatie proberen wij meer inzicht te krijgen in pathologische omstandigheden die resulteren in bradykinine vorming. Onze onderzoeksgroep toonde eerder aan dat in erfelijk angio-oedeem truncatie van het FXII molecuul door plasmine zorgt voor versnelde activatie van FXII. FXII-W268R laat continue een versnelde activatie zien.

Activatie van FXII treedt op door enzymatische klieving na de arginine op positie 353 (R353). Wij stellen ons voor dat R353 cryptisch in het FXII-eiwit ligt waardoor FXII onder normale omstandigheden beschermd is voor activatie. In dit proefschrift hebben wij deze hypothese met functionele proeven onderzocht. Door het maken van verschillende truncatie-varianten van FXII kwamen wij tot de bevinding dat truncatie na het fibronectine type II domein leidt tot spontane activiteit van FXII. Wij vermoeden dat dit domein nodig is voor het afschermen van R353. Binding aan negatief geladen oppervlakte en enzymatische truncatie van FXII zorgen er voor dat R353 wordt blootgesteld door het fibronectine type II domein te verplaatsen of te verwijderen. Activatie van mestcellen, neutrofiele granulocyten

of bloedplaatjes zorgt voor uitscheiding van negatief geladen deeltjes en enzymen die FXII kunnen trunceren waardoor R353 beschikbaar komt voor klieving. Mogelijk zorgt activiteit van deze celtypen ervoor dat in het lichaam omstandigheden ontstaan waarin FXII activeert met bradykinine productie als gevolg.

Klinische implicaties

- De gekliefd kininogeen assay is een praktische, kwantificeerbare methode om onderzoek te doen naar bradykinine productie. Deze test biedt ondersteuning aan klinisch geneesmiddelenonderzoek naar middelen voor erfelijk angio-oedeem.
- De gekliefd kininogeen assay kan niet in een individu aantonen of ziekte bradykinine gedreven is. Wel kan deze test gebruikt worden om groepen patiënten te vergelijken, om zo meer inzicht te krijgen mogelijk bradykinine gedreven processen.
- Sommige patiënten met idiopathisch angio-oedeem die niet reageren op antihistamine therapie, kunnen baat hebben bij recombinant C1-esterase remmer therapie. Op dit moment kunnen wij nog niet voorspellen bij welke patiënten de therapie effectief zal zijn.
- Bradykinine levert een belangrijke bijdrage aan het ziektebeeld FACAS. Bij patiënten met onbegrepen auto-inflammatoire ziekten en mogelijk ook bij therapieresistent chronisch spontaan urticaria zou bradykinine een rol kunnen spelen. In toekomstig onderzoek moeten bradykinine en FXII worden meegenomen als mogelijke targets voor de behandeling van deze ziektebeelden.

Conclusies

Bradykinine gedreven inflammatie is niet alleen beperkt tot erfelijk angio-oedeem. In het ziektebeeld FACAS, waarbij geen angio-oedeem optreedt maar wel symptomen van autoinflammatie voorkomen speelt bradykinine een belangrijke rol. Condities waarin overmatige FXII-activatie optreedt spelen waarschijnlijk een belangrijke rol in het ontstaan van bradykinine gedreven inflammatie. Therapieën ontwikkeld voor erfelijk angio-oedeem kunnen werkzaam zijn in andere bradykinine gedreven ziektebeelden. Een voorbeeld hiervan is idiopathisch angio-oedeem. Op dit moment is er nog geen bloedonderzoek dat kan voorspellen of een patiënt baat zal hebben bij therapie gericht op bradykinine. Betere identificatie van bradykinine gedreven ziekte is nodig om werkzaamheid van deze therapieën te kunnen onderzoeken. Bradykinine is een potentieel target in de behandeling van idiopathisch angio-oedeem, netelroos en auto-inflammatoire syndromen waarbij andere behandelingen onsuccesvol zijn.

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&

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CURRICULUM VITAE

Zonne Hofman was born on 16 September 1989 in Zeist, the Netherlands. In 2009 she started studying Medicine at the University of Utrecht. During her bachelor and master she was active in research at the laboratory for translational immunology as part of the honours programme. Zonne completed her master's degree *cum laude* and was awarded the Alexandre Suerman MD/PhD stipend from the UMC Utrecht. Her research was under the supervision of Professor Hack, Professor Pasterkamp and Dr. Maas. Her work focusses on angioedema and the contact system. During her PhD she was awarded several travel grants and awards and presented her work at international congresses in the field of allergy and immunology as well as thrombosis and hemostasis. Zonne was invited as chairperson for the international meeting on primary angioedema with normal C1-inhibitor in Italy. During her PhD project she also worked as a physician at the van Creveld Clinic working with hereditary angioedema patients. In collaboration with the patient organization she organized an angioedema patient meeting in Utrecht. After obtaining her PhD her ambition is to continue translational research in immunology. But first she will pursue her career as rheumatologist, starting at the St. Antonius hospital in Nieuwegein.