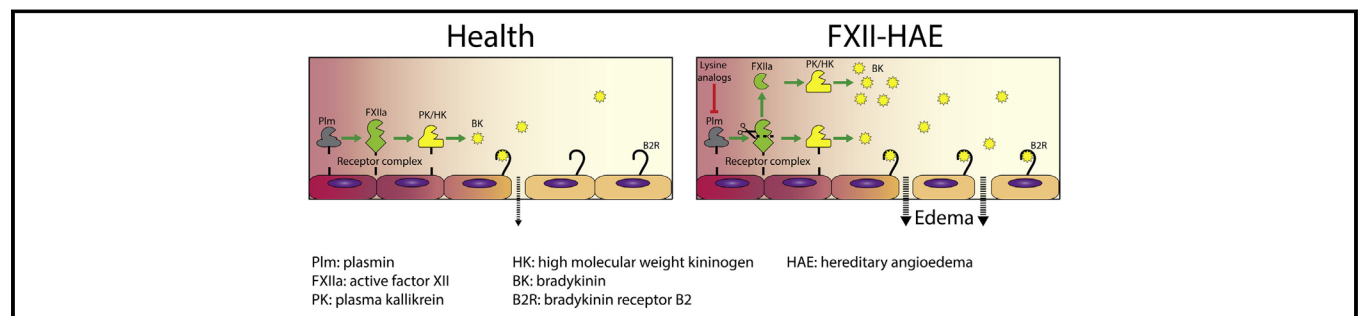


Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations

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GRAPHICAL ABSTRACT



Background: Patients with angioedema experience unpredictable attacks of tissue swelling in which bradykinin is implicated. Several distinct mutations in Factor XII (FXII) are associated with hereditary angioedema (HAE) in the presence of

normal C1 esterase inhibitor activity (FXII-HAE). The underlying disease mechanisms are unclear, which complicates diagnosis and treatment.

Objective: We sought to identify the natural trigger for FXII activation, which causes uncontrolled bradykinin production in patients with FXII-HAE.

Methods: We generated recombinant variants of FXII, representing health and disease, and studied their behavior in functional studies. We investigated bradykinin-forming pathways in blood plasma with newly developed nanobody-based analytic methods.

Results: We here report that FXII-HAE mutations collectively introduce new sites that are sensitive to enzymatic cleavage by plasmin. These FXII mutants rapidly activate after cleavage by plasmin, escape from inhibition through C1 esterase inhibitor, and elicit excessive bradykinin formation. Furthermore, our findings indicate that plasmin modulates disease activity in patients with FXII-HAE. Finally, we show that soluble lysine analogs attenuate this mechanism, explaining their therapeutic value in patients with HAE.

Conclusion: Our findings indicate a new pathway for bradykinin formation in patients with HAE, in which FXII is cleaved and activated by plasmin. This should lead to the identification of new markers for diagnosis and targets for treatment. (*J Allergy Clin Immunol* 2016;■■■■:■■■■-■■■■.)

Key words: Angioedema, bradykinin, Factor XII, plasma kallikrein, kininogen, C1 esterase inhibitor, plasmin

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Local swelling attacks of the dermis and (sub)mucosa are key features of angioedema. These unpredictable attacks can affect various areas of the body. They can be disfiguring, painful, or life-threatening and have a large effect on the patient's quality of life.¹ There are different types of angioedema that are categorized by

Abbreviations used

εACA:	ε-Aminocaproic acid
C1inh:	C1 esterase inhibitor
FXII:	Factor XII
FXIIa:	Active Factor XII
HAE:	Hereditary angioedema
HBS:	HEPES-buffered saline
HK:	High-molecular-weight kininogen
mHBS:	0.5% Skimmed milk in HBS
PAP:	Plasmin-α ₂ -antiplasmin
PBST:	PBS-Tween
PK:	Plasma kallikrein
PPACK:	Phe-Pro-Arg-chloromethylketone
PPK:	Plasma prekallikrein
TBS:	Tris-buffered saline
TEV:	Tobacco etch virus
TMB:	3,3',5,5'-Tetramethyl-benzidine
WT:	Wild-type

possible genetic inheritance and known aggravating factors.² However, it is generally unknown how swelling attacks are triggered.

Bradykinin is an important mediator of vascular leakage and strongly implicated in angioedema.³ This peptide is liberated from its nonenzymatic precursor high-molecular-weight kininogen (HK) by plasma kallikrein (PK), which in turn is activated by active Factor XII (FXIIa). Together, these factors form the contact activation system. C1 esterase inhibitor (C1inh) is the major inhibitor of FXIIa and PK and is required to keep bradykinin production under control.⁴ As a result, congenital C1inh deficiencies can lead to a hereditary form of angioedema (C1-INH-HAE). In other forms of hereditary angioedema (HAE), C1inh levels are normal.⁵ Mutations in the gene that encodes FXII are reported to segregate with disease in these patients. This strongly suggests that swelling attacks in patients with this type of angioedema are also the result of contact system activation and bradykinin release.⁶⁻⁸

The natural trigger for contact system activation in patients with HAE is unknown. However, several lines of evidence implicate the enzyme plasmin as an important player:

- (1) Clinical studies reported increased levels of plasmin-α₂-antiplasmin (PAP) complexes and D-dimer during HAE attacks.⁹⁻¹¹ During attacks, levels of plasmin activator inhibitor 1 and 2 are decreased.^{12,13} Together, these changes favor plasminogen activation and activity.
- (2) Thrombolytic therapy (which generates plasmin) can provoke angioedema in patients with adverse reactions.¹⁴⁻¹⁶
- (3) Thrombolytic therapy leads to direct activation of FXII through cleavage by plasmin.¹⁷
- (4) Antifibrinolytic agents (that target plasmin) have therapeutic value as prophylactic treatment for various types of (hereditary) angioedema.¹⁸⁻²⁰

These reports suggest that plasmin is more than a passive bystander in pathological vascular leakage.

We here report that 3 distinct familial FXII-HAE mutations lead to an increased sensitivity of FXII for activation by plasmin. The resulting accelerated activation of FXII exceeds its inactivation by C1inh. This leads to free FXIIa in plasma and ultimately causes uncontrolled bradykinin formation. Soluble

lysine analogs attenuate this mode of FXII activation and temper bradykinin release, which offers an explanation for their therapeutic value in patients with HAE. Finally, we report that variation in plasminogen levels and activity modulates contact system activation and bradykinin generation in patients with FXII-HAE. Based on these findings, we conclude that plasmin is a natural trigger for swelling attacks in patients with FXII-HAE and potentially other forms of angioedema.

METHODS

Summarized experimental approach

We generated recombinant FXII variants that represent health and disease for the functional studies on FXII-HAE. These mutants vary in protein glycosylation and the presence of (predicted) cleavage sites (schematically represented in Fig 1, A). We investigated these FXII variants in purified systems and plasma for their responsiveness to surfaces and activating enzymes. Our findings implicate plasmin as a powerful trigger of FXII-HAE. We investigated this mechanism further in the plasma of 2 patients with FXII-HAE. Finally, we investigated the capacities of lysine analogs (ε-aminocaproic acid [εACA]) and an mAb against FXII to interfere with plasmin-triggered bradykinin production in plasma.

Detailed methods can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

FXII protein expression and purification

The eukaryotic expression vector pcDNA6/V5-His A was modified by insertion of a murine Igκ secretion signal and 2 strep-tags through the *HindIII* and *EcoRI* digestion sites (called pSM2 in [Table E1](#) in this article's Online Repository at www.jacionline.org). cDNA of the F12 gene was kindly donated by Dr F. Citarella and matches the National Center for Biotechnology Information reference sequence (NM_000505.3),²¹ except for the silent mutation c.nt.1140T>C (P460P, mature protein numbering). A sequence coding for a tobacco etch virus (TEV) protease cleavage site was inserted 5' from the F12 sequence by using PCR with the Phusion Polymerase (Thermo Scientific, Waltham, Mass; see [Table E1](#), primers FXII_NTerm-TEV_For and FXII_C-Term_Short). Mutations T309K, T309R, and T309A were generated by means of site-directed mutagenesis with Pfu turbo (see [Table E1](#)). The Deletion & Insertion (Del&Ins; c.971_1018+24del48) and Deletion (Del; c.971_del48) mutants were prepared in 2 steps: N-terminal (see [Table E1](#), primers FXII-TEV_N-term_For with Del&Ins_P1_Rev or Del_P1_Rev, respectively) and C-terminal (see [Table E1](#), primers FXII_C-term_Short with Del&Ins_P3_For or Del_P2_For) fragments were generated by means of PCR that were subsequently fused and amplified by using PCR through overhanging primer sequences. In the case of the Del&Ins mutant, the primers introduced an additional sequence that encodes 27 amino acids (see [Table E1](#), primers Del&Ins_P2_For and Rev). All FXII constructs were ligated into pSM2 through *EcoRI* and *NotI*. HEK293T cells were transfected with Lipofectamine 2000. Transfected cells were selected by resistance to 5 μg/mL blasticidin, expanded, and grown in 1L cell factories. Production medium (Dulbecco modified Eagle medium, 5% FBS, 0.5% Ultrosor G, and penicillin/streptomycin) was harvested twice a week, supplemented with benzamidine (0.174 g/L), Soy Bean Trypsin Inhibitor (0.024 g/L), and Polybrene (0.056 g/L; all from Sigma-Aldrich, St Louis, Mo); centrifuged to remove cell debris; and stored at -20°C. Harvested media were thawed at 37°C, pooled, and concentrated, and buffer was exchanged (100 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1 mmol/L EDTA, pH 8.0) on a 10-kDa dialysis membrane in a Quixstand benchtop system (GE Life Sciences, Fairfield, Conn). Recombinant FXII protein was purified by using Strep-Tactin Sepharose beads (IBA-Life Sciences, Goettingen, Germany). Purified recombinant FXII was dialyzed against 4 mmol/L sodium acetate-HCl and 150 mmol/L NaCl (pH 5.4) and stored at -80°C. Protein concentrations were determined by means of absorption spectroscopy at OD_{280nm}. Absorption coefficients of the FXII mutants were determined based on their

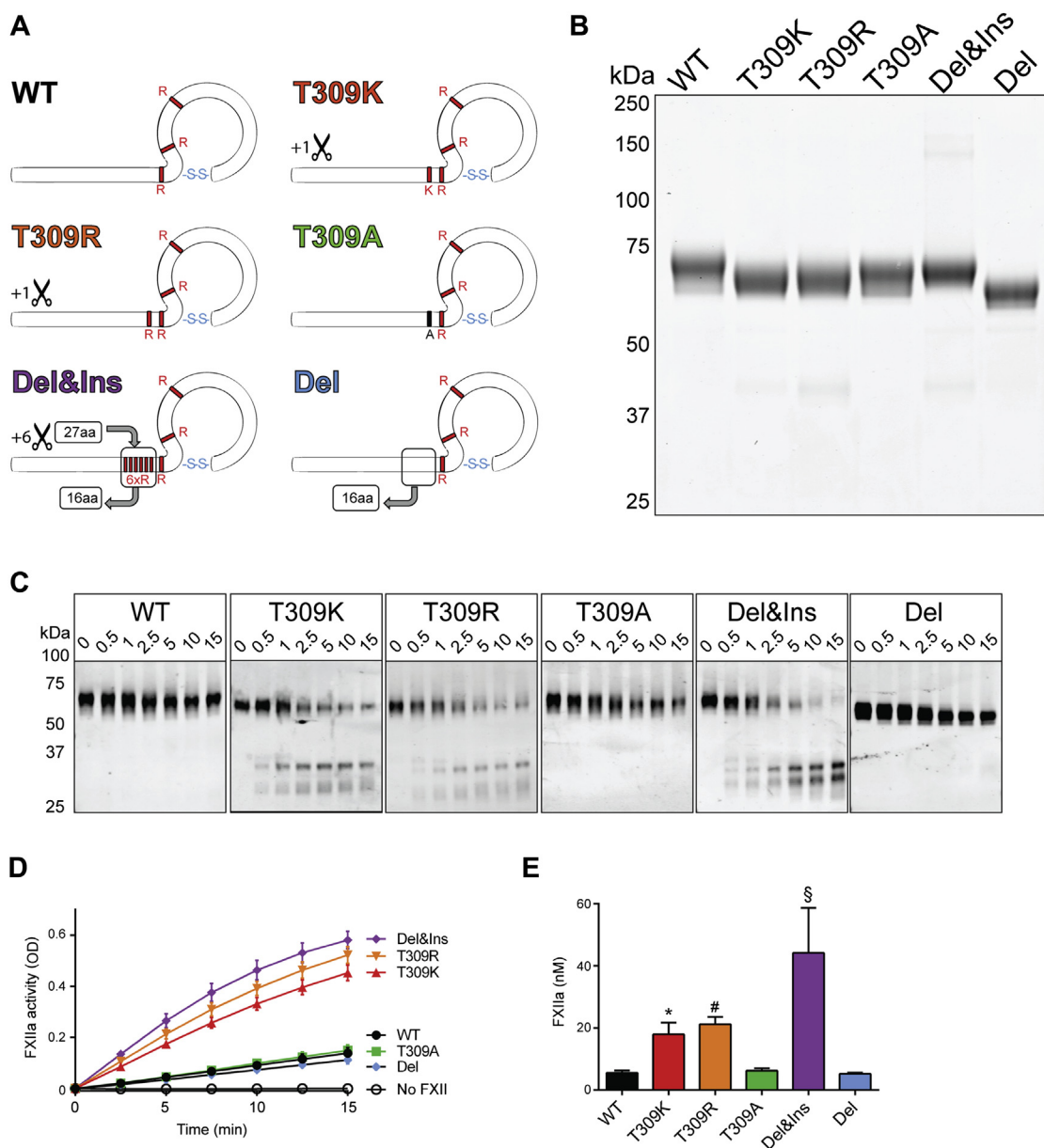


FIG 1. Mutations that cause FXII-HAE introduce cleavage sites that accelerate FXII activation by plasmin. **A**, Schematic comparison of FXII constructs. **B**, Recombinant FXII variants. **C**, FXII cleavage by plasmin (time in minutes). **D**, Enzymatic activity of FXII variants. **E**, FXIIa levels after plasmin cleavage. Values are presented as means $\pm$ SDs of 3 experiments. * $P < .05$, # $P < .005$, and § $P < .0001$ (compared with WT), 1-way ANOVA.

mature amino acid sequences using ProtParam.²² Purity and degradation were assessed through 4-12% Bis Tris-PAGE gel and Coomassie blue staining.

Contact system activation assays

Purified factor assays. Recombinant FXII (375 nmol/L) in HEPES-buffered saline (HBS) with 0.1% BSA (and antibody OT2, where indicated) was incubated with PK (0.5 μ g/mL), plasmin (50 μ g/mL and ϵ ACA where indicated), or vehicle for 15 minutes at 37°C, after which kallikrein and plasmin activity was neutralized with 100 KIU/mL aprotinin. FXIIa activity was determined through conversion of the chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mmol/L) at 405 nm (37°C). Alternatively, samples were collected in assay-specific buffers for analysis by using Western blotting or ELISA.

Plasma assays. Citrated FXII-depleted plasma was reconstituted with 375 nmol/L recombinant FXII and activated by kaolin (150 μ g/mL). Alternatively, plasma from healthy donors or patients with FXII-HAE was used. Streptokinase (400 U/mL) was added and incubated at 37°C to induce plasmin activity in plasma. Where indicated, ϵ ACA (200 nmol/L) or OT2 (225 nmol/L) was added to plasma 10 minutes before activation. Total kallikrein-like enzymatic activity was monitored at 37°C by means of cleavage of the chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mmol/L final concentration) at 405 nm. Alternatively, samples were collected in assay-specific buffers for analysis by using Western blotting or ELISA.

Plasma collection

Blood was collected from healthy volunteers who had not been receiving anticoagulant or antiplatelet medication for at least 10 days before blood

withdrawal with informed consent. Blood was anticoagulated with 10% sodium citrate (3.2% wt/vol). Platelet-poor plasma was prepared by means of double centrifugation (for 10 minutes at 2000g). The University Medical Center Utrecht Ethics Committee approved these procedures. Patients with FXII-HAE provided written informed consent, after which blood was collected during routine diagnostic procedures and labeled with a code, according to the guidelines of the Ethical Committee of the University of Milan. From patient 1, 2 remission plasma samples were obtained, as well as 3 samples that were taken at onset and 24 and 72 hours after onset of an HAE attack. Patient 2 donated plasma during remission. Plasma was isolated by means of centrifugation (for 20 minutes at 2000g), after which the plasma was divided into aliquots and stored at -80°C until tested. Experiments were performed blind to the patient's history.

RESULTS

FXII-HAE mutations change protein glycosylation and introduce new cleavage sites

All reported FXII-HAE mutations are located in the proline-rich region of the F12 gene (schematically presented in Fig 1, A). This central part of the protein is heavily charged and unique to FXII. Two types of mutations both lead to the substitution of threonine 309 by either a lysine or an arginine residue (T309K or T309R, respectively⁷). A third mutation (c.971_1018+24del72⁸) removes 16 amino acids but simultaneously introduces 27 new amino acids through intron retention.²³ We reasoned that these FXII mutants could provide new insights that might lead to the identification of the trigger for FXII activation in patients with HAE. *In silico* prediction models confirmed our earlier findings that FXII-HAE mutations alter FXII glycosylation (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org).²⁴ However, this is not the only feature of FXII that changes.

During contact system activation, PK (a trypsin-like enzyme) activates FXII by means of cleavage after an arginine at position 353 (R353).²⁵ Two more cleavages after arginines (at R334 and R343) alter the target specificity of FXIIa (schematically shown in Fig 1, A, wild-type [WT]).²⁶ Remarkably, all FXII-HAE mutations introduce 1 or more positively charged amino acids, leading to additional sites for potential cleavage by using trypsin-like enzymes (Fig 1, A, and see Fig E1, A and B). We developed recombinant variants of FXII, representing health (WT) and disease (T309K, T309R, and Del&Ins; each mutant has its own color). To dissect the contribution of defective glycosylation from that of newly introduced enzymatic cleavage sites, we designed 2 additional mutants (T309A and Del). Mutant T309A resembles T309K and T309R: 1 O-linked glycosylation at position 309 is lost, but the number of putative cleavage sites remains unchanged (Fig 1, A, and see Fig E1). In analogy to mutant Del&Ins, we developed the mutant Del. In this mutant the target sequence of 16 amino acids is deleted, without replacement by a 27-amino-acid sequence. This enables us to distinguish the role of the inserted sequence from that of the deleted sequence. The migratory behavior of these recombinant FXII variants corresponds well with the predicted variations in glycosylation and amino acid sequence length (Fig 1, B). These FXII variants were not spontaneously active in buffer (see Fig E2, A, in this article's Online Repository at www.jacionline.org) or in plasma (see Fig E2, B). We found that kaolin-triggered clotting activity and enzymatic (kallikrein-like) activity in plasma was comparable between FXII variants (see Fig E2, C and D). This suggests that mutations in FXII that cause HAE accelerate

contact system activation in a manner that is mechanistically dissimilar from surface-triggered coagulation *in vitro*.

FXII-HAE mutations collectively accelerate FXII activation by plasmin

We next investigated whether FXII-HAE mutations insert new sites for enzymatic cleavage of FXII. We considered PK to be the primary candidate for executing these cleavages.^{27,28} Unexpectedly, we found no evidence for cleavage by PK (see Fig E2, E, unreduced blots), and all FXII variants had equal activity as a result (see Fig E2, F). We next explored the possibility that another enzyme could fulfill this role. It was previously reported that plasmin, the principal enzyme of the fibrinolytic system, can cleave and activate FXII.^{17,29} When the mutants T309K and T309R were exposed to plasmin, they rapidly fragmented through cleavage of a site that was not present in WT FXII (Fig 1, C, unreduced samples). Furthermore, these mutants had higher enzymatic activity (Fig 1, D; colors correspond to Fig 1, A). The control mutant T309A was not fragmented by plasmin and displayed normal activity (Fig 1, C and D). Quantitative analyses indicate that both FXII-HAE mutants generate higher amounts of free FXIIa in response to plasmin than WT and the control mutant T309A (Fig 1, E, and see Fig E2, G). In an identical manner we found that plasmin provokes fragmentation (Fig 1, C) and hyperactivity of FXII-HAE mutant Del&Ins but not of the control mutant Del (Fig 1, D). Also, in this case FXIIa levels in solution were strongly increased (Fig 1, E, and see Fig E2, G). Together, these experiments with purified proteins revealed that FXII-HAE mutations insert new cleavage sites that accelerate FXII activation by plasmin. Next, we went on to investigate this mechanism in a plasma environment.

FXII-HAE mutants escape from inactivation by C1inh during activation by plasmin

C1inh keeps the plasma contact system under control to prevent continuous bradykinin formation in solution. This leads to the question of how bradykinin-mediated angioedema can take place when C1inh activity is normal. In the next experiments we reconstituted FXII-deficient plasma with FXII mutants, triggered plasmin activity, and investigated contact system activation. Small amounts of FXII were consumed in all cases (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Activation of FXII-HAE mutants (but not WT FXII) by plasmin leads to strongly increased levels of free FXIIa in plasma, despite the presence of C1inh (Fig 2, A). Subsequently, plasma levels of FXIIa-C1inh complexes increased (Fig 2, B, and see Fig E3, B), plasma prekallikrein (PPK) was consumed (see Fig E3, C), and PK-C1inh complexes formed (Fig 2, C, and see Fig E3, D). We found no evidence for FXI consumption under these conditions (data not shown). Ultimately, the activation of FXII-HAE mutants by plasmin accelerated HK consumption (Fig 2, D, and see Fig E3, E) and exacerbated bradykinin formation (Fig 2, D). These experiments indicate that FXII-HAE mutations collectively accelerate FXII activation by plasmin, breaking the balance between activation and inhibition of the contact system in solution.

Variation in the plasminogen activation system modulates FXII-HAE disease activity

Patients with FXII-HAE mutations are not continuously ill. This suggests that factors beyond these mutations contribute to

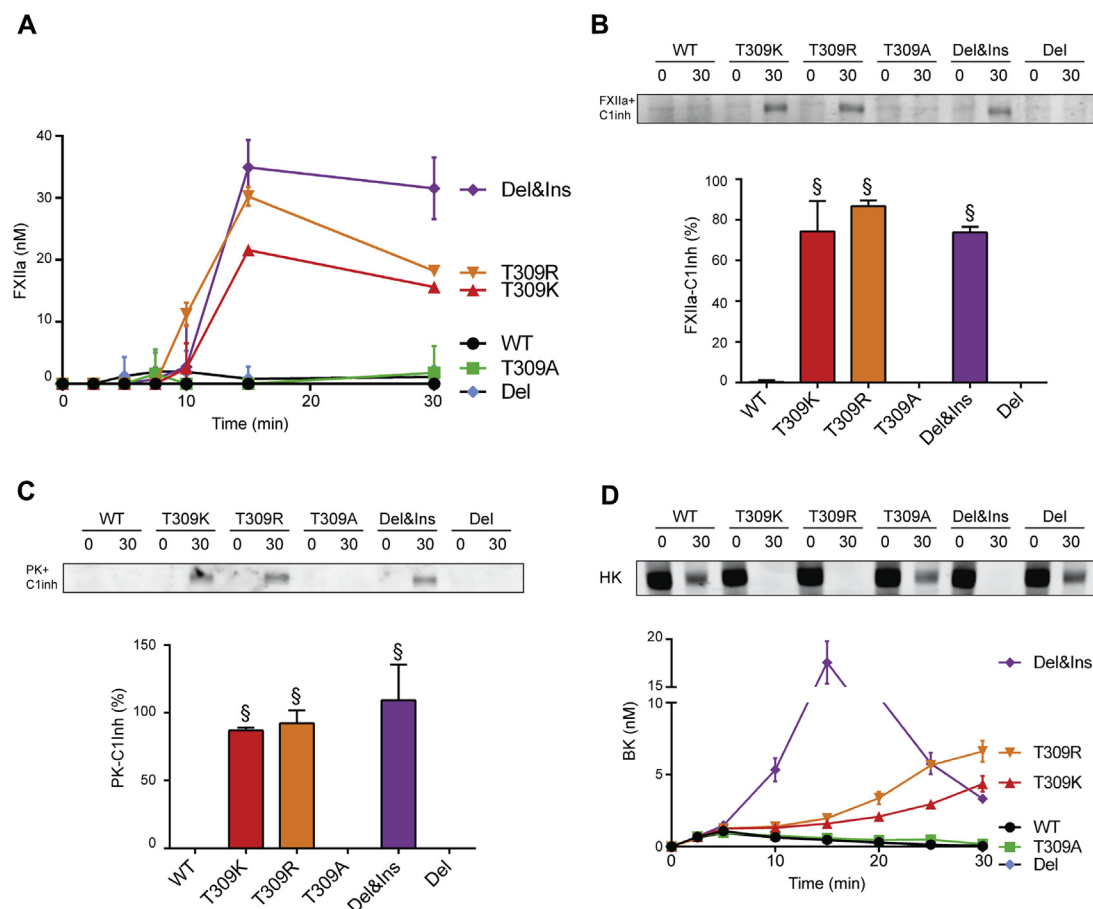


FIG 2. FXII-HAE mutants escape inactivation by C1inh during activation by plasmin in plasma. **A**, Free FXIIa production. **B** and **C**, FXIIa-C1inh and PK-C1inh complex formation after 30 minutes of plasminogen activation. **D**, HK consumption (30 minutes) and bradykinin production. *Top panels*, Western blots; *bottom panels*, ELISA. Values are presented as means $\pm$ SDs of 3 experiments. * $P < .05$, # $P < .005$, † $P < .0005$, and § $P < .0001$ (compared with WT), 1-way ANOVA.

swelling attacks. We next investigated a relationship between plasmin and FXII in 2 female patients with FXII-HAE (both heterozygous for FXII-T309K). Plasma samples were collected in remission (indicated as “basal”). In patient 1 additional samples were collected over the course of an attack. C1inh levels and activity were within the reference range in all samples (see Fig E4, A, in this article’s Online Repository at www.jacionline.org). Western blot analyses confirmed the heterozygous FXII mutation in both patients (Fig 3, A; WT = upper band, T309K = lower band), whereas other contact system factor antigen levels were normal. Furthermore, activated partial thromboplastin time clotting times in both patients were normal (data not shown).

In search of a functional link between plasmin and FXII in patients with FXII-HAE, we triggered plasmin activity *ex vivo* in these patients’ plasma samples and investigated contact system activation. Surprisingly, although mutant FXII protein is present in all FXII-HAE plasma samples (Fig 3, A), large amounts of free FXIIa were only generated in a subset of samples (Fig 3, B). When free FXIIa was generated, FXIIa-C1inh (Fig 3, C, and see Fig E4, B) and PK-C1inh (Fig 3, D, and see Fig E4, C) complexes strongly increased, and excessive bradykinin production followed (Fig 3, E). It is noteworthy that FXIIa-C1inh and PK-C1inh complexes formed less aggressively after

plasmin was triggered in the plasma of patient 1, which was collected at the end of a swelling attack (72 hours after onset; Fig 3, C and D). This indicates that patient 1 at the time of blood sampling was less sensitive to a plasmin trigger.

Patient 2 was pregnant at the time of blood sampling (a known aggravating factor for swelling attacks³⁰) and had been going through a series of attacks. When plasmin activity was triggered, her plasma responded violently: it generated free FXIIa (Fig 3, B), FXIIa-C1inh and PK-C1inh complexes (Fig 3, C and D), and bradykinin production (Fig 3, E). The presence of PAP complexes in plasma indicates that plasminogen activation has recently occurred *in vivo*.^{31,32} These PAP complexes were present in our (unprovoked) patients’ plasma and appeared to be associated with disease activity (Fig 3, F). We next went on to determine FXIIa-C1inh and PK-C1inh complexes (reflecting recently occurring contact system activation *in vivo*^{33,34}) and found that these complexes were increased in plasma from patients in a similar manner (Fig 3, G). These combined observations suggest that plasminogen activation and contact system activation coincide in patients with FXII-HAE.

Our findings raise the question of how the responsiveness of the contact system for activation by plasmin is modulated. Therefore we categorized patients’ samples by their sensitivity to a plasmin trigger (ie, samples that generate free FXIIa [$n = 3$] vs samples

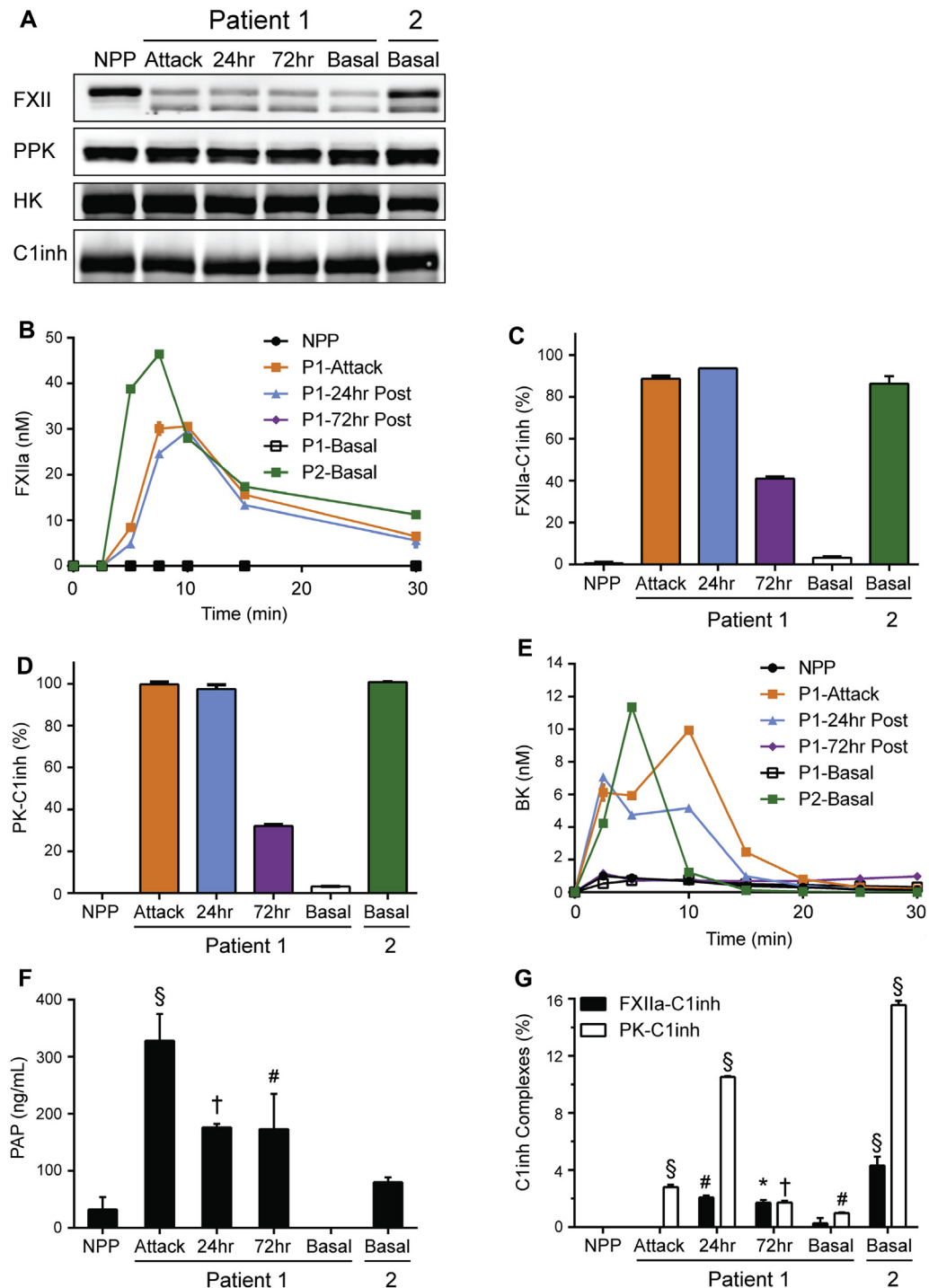


FIG 3. Plasmin triggers bradykinin production in plasma from patients with FXII-HAE. Plasma analyses of 2 patients with FXII-HAE: *basal*, remission; *NPP*, normal pooled plasma; *attack/24hr/72hr*, attack. **A**, Contact system factors. **B**, Free FXIIa production. **C** and **D**, FXIIa-C1inh and PK-C1inh complexes after 30 minutes. **E**, Bradykinin production. **F** and **G**, PAP and enzyme-C1inh complexes. Values are presented as means $\pm$ SDs of triplicate analyses. * $P < .05$, # $P < .005$, † $P < .0005$, and § $P < .0001$ (vs NPP), 1-way ANOVA.

that do not [$n = 3$]). We found that mutant FXII-T309K was specifically consumed in sensitive samples after plasmin was triggered (Fig 4, A, mutant FXII is indicated by the arrow; categorized quantifications are shown in Fig 4, B). This in turn led to a sequence of events that included formation of

FXIIa-C1inh complexes (see Fig E4, B), consumption of PPK (see Fig E4, D and E), formation of PK-C1inh complexes (see Fig E4, C), and increased consumption of HK (see Fig E4, D and E). Because FXII, PPK, HK, and C1inh antigen levels were not significantly different between sensitive and insensitive

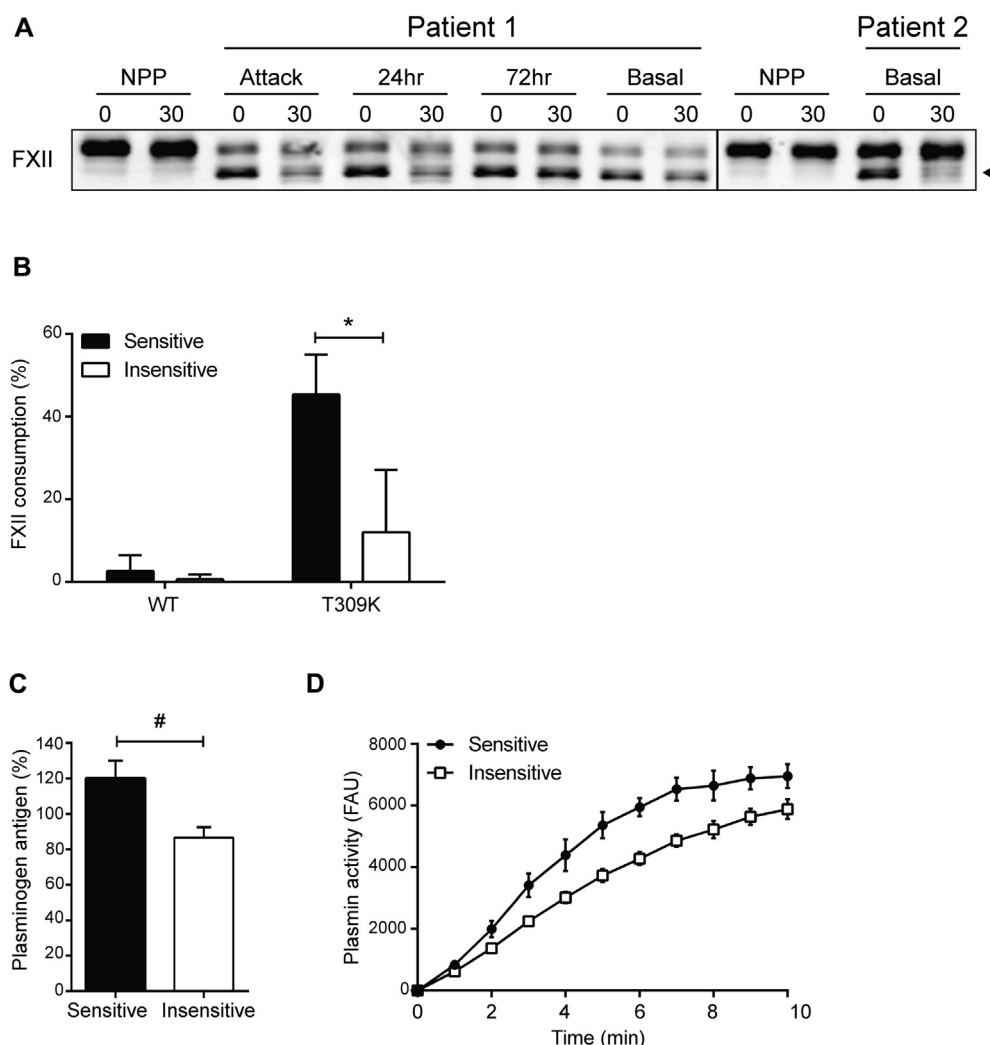


FIG 4. HAE-FXII disease activity corresponds to plasmin-forming potential. **A**, Consumption of FXII (upper bands) and FXII-T309K (lower bands) 30 minutes after plasminogen activation. **B**, FXII-WT and FXII-T309K consumption in grouped samples categorized by their capacity to generate FXIIa in response to plasmin ($n = 3$ per group). **C** and **D**, Plasminogen levels and plasmin activity in grouped samples. * $P < .05$ and # $P < .005$, unpaired Student t test.

samples (see Fig E4, F), we shifted our attention to factors of the plasminogen activation system.

The potential for plasmin-induced FXII activation was not related to variation in α_2 -antiplasmin values because its activity was normal in all samples (see Fig E4, G). In contrast, plasminogen levels fluctuated between samples and were significantly higher in sensitive samples than they were in samples that were insensitive to an identical trigger (Fig 4, C, and see Fig E4, H). Accordingly, the total amount of plasmin activity was higher in sensitive plasma samples after an identical stimulus (Fig 4, D). These experiments indicate that the plasminogen activation system and contact activation system are functionally linked and suggest that increased potential of the plasminogen activation system can prime plasma for excessive bradykinin production.

Soluble lysine analogs block FXII activation by plasmin

Studies in patients with HAE indicate that prophylactic treatment with lysine analogs (eg, ϵ ACA) or tranexamic

acid^{20,35} has therapeutic benefit, but their mechanism of action is unknown. We next investigated whether lysine analogs interfere with activation of FXII by plasmin. We exposed FXII to plasmin in the presence of ϵ ACA, neutralized plasmin activity (with aprotinin), and found that FXIIa activity was dose-dependently decreased (Fig 5, A). In contrast, FXIIa activity was normal when the same concentrations of ϵ ACA were added after activation by plasmin (see Fig E5, A, in this article's Online Repository at www.jacionline.org). Furthermore, ϵ ACA was unable to inhibit activation of FXII by PK (see Fig E5, B). These experiments show that ϵ ACA selectively interferes with the activation of FXII by plasmin but does not directly inhibit FXIIa. We next established that the mAb OT2³⁶ fully inhibits WT FXIIa after activation by plasmin (Fig 5, B; concentrations represent FXII:OT2 ratios of 2:1, 1:1, and 1:2). Furthermore, we found that both ϵ ACA and OT2 are able to control the activation and activity of all FXII-HAE mutants (Fig 5, C-E, and see Fig E5, C-E). Plasmin-triggered consumption of PPK and HK in plasma was modestly affected by both ϵ ACA and OT2 in the presence of FXII-HAE mutants (see Fig E6, A, in this article's Online

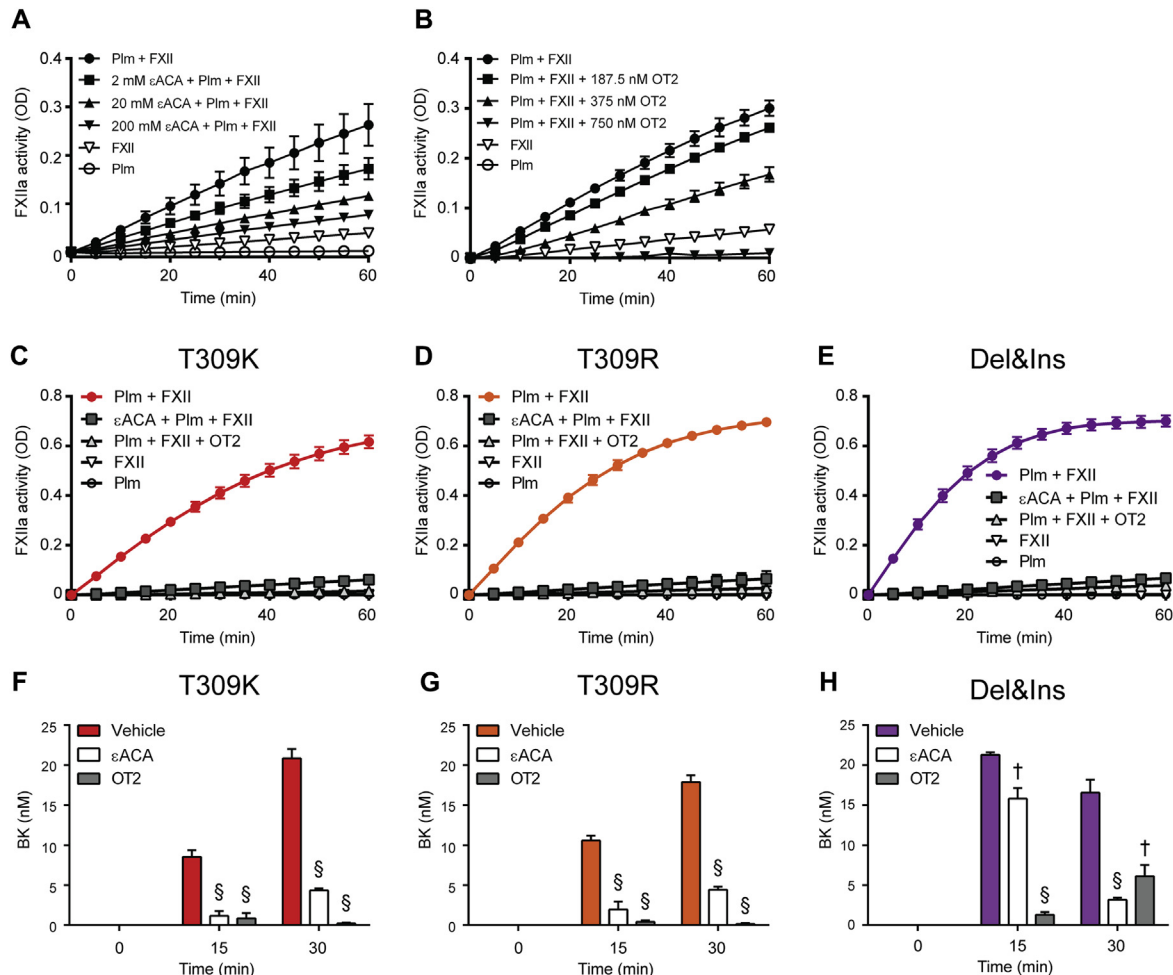


FIG 5. Strategies to control FXII activation by plasmin. **A** and **B**, Effects of ϵ ACA and OT2 on activation of FXII by plasmin (Plm). **C-E**, Effects of ϵ ACA (200 mmol/L) and OT2 (750 nmol/L) on plasmin-triggered activation of purified FXII-HAE mutants. **F-H**, Effects of ϵ ACA and OT2 on plasmin-triggered bradykinin production. Values are presented as means $\pm$ SDs of 3 values. $\dagger P < .0005$ and $\$ P < .0001$ (compared with vehicle), 1-way ANOVA.

Repository at www.jacionline.org; quantifications are shown in Fig E5, B-E). However, both these compounds significantly attenuated excessive bradykinin production in plasma that contains FXII-HAE mutants (Fig 5, F-H, and see Fig E6, F-H). Together, these results show that soluble lysine analogs can inhibit excessive contact system activation by plasmin and support their therapeutic application in patients with FXII-HAE.

DISCUSSION

HAE is tightly linked to uncontrolled bradykinin production and surrounded by several intriguing questions: How is the contact system naturally activated? How can excessive contact activation take place when C1inh activity is normal? Why do HAE attacks and bradykinin production take place locally while the contact system circulates systemically?

It is commonly assumed that the mechanism of contact system activation *in vivo* resembles its activation by negatively charged surfaces or polymers *in vitro*. We here report that 3 FXII-HAE mutations introduce cleavage sites that enhance FXII activation. Our results suggest that initial activation of the contact system

in patients with FXII-HAE is not mediated by a surface or polymer but through enzymatic cleavage. This aggressive activation mechanism temporarily renders C1inh ineffective, increasing bradykinin production.

FXII-HAE mutants are abnormally sensitive to plasmin, and changes in plasminogen levels modulate the potential for excessive contact system activation in the patient's plasma (Figs 1-4). This suggests that plasmin, rather than PK, delivers the initiating spark for contact activation in patients with FXII-HAE attacks. Only after initial activation of FXII does PK amplify the reaction. It is possible that a similar scenario exists in patients with C1-INH-HAE. Here the sensitivity of FXII for plasmin is normal, but its inactivation is compromised. It is a limitation of our study that our confirmatory experiments were performed in the plasma of only 2 patients with FXII-HAE. It will be of great interest to investigate the role of plasmin in patients with (FXII-)HAE in further studies with larger patient groups.

The identification of plasmin as a natural activator of the contact system is helpful to understand how bradykinin can be locally produced during HAE attacks.³⁷ Contact system

components can bind to and activate on the endothelial cell surface, as reviewed by de Maat et al.³⁸ The responsible receptor complex includes receptors that also mediate plasminogen activation. We propose a 3-step mechanism. First, endothelial cells become activated by subendothelial stimuli (eg, damage, infection, hypersensitivity, or hypoxia). Second, the contact activation system and plasminogen activation system assemble on the endothelial cell surface. Third, plasmin activates FXII on the cell surface. The charged glycocalyx protects these enzymes from inactivation, creating a niche for effective enzymatic crosstalk,³⁸ and facilitates localized bradykinin production.

Recently,^{39,40} several new FXII-HAE mutations were described. None of these mutations directly introduce new positively charged amino acids. The mature WT-FXII sequence contains 58 putative cleavage sites (see Fig E1). Of these 58 sites, 3 are actually cleaved by PK. It is attractive to speculate that these newly described mutations reposition pre-existing putative cleavage sites, making them susceptible to actual cleavage by a trypsin-like enzyme. This could be PK or plasmin, but it is also possible that other enzymes facilitate this cleavage of FXII.

There are several potential clinical implications coupled to our findings. Soluble lysine analogs inhibit activation of FXII by plasmin. This helps to explain the effectiveness of lysine analogs in HAE and nonhistaminergic angioedema.²⁰ Lysine analogs can interfere with the role of plasmin in (H)AE at multiple levels: in addition to inhibiting FXII activation, they might also interfere with the sequestration and activation of plasminogen, reducing potential for FXII activation. Furthermore, they can interfere with the direct cleavage of HK by plasmin (this primes it for kallikrein-mediated bradykinin release).⁴¹ Lysine analogs are rapidly absorbed after oral intake but also rapidly eliminated. It might be of interest to develop a new and more powerful therapeutic strategy that specifically prevents interaction between plasmin and the contact activation system.

The implications of our findings might extend to states of pathological vascular leakage beyond angioedema. Simultaneous plasminogen activation and contact system activation has been demonstrated in patients with anaphylaxis,^{42,43} rheumatoid arthritis,⁴⁴⁻⁴⁶ and systemic amyloidosis.^{32,33} It is attractive to speculate that the interaction between plasmin and the plasma contact system extends to states of (pathological) vascular leakage beyond HAE.

In conclusion, we propose that plasmin is a natural activator of FXII. This mechanism is meant for physiologic production of bradykinin and controlled regulation of vascular permeability. However, excessive activation of the contact system by plasmin leads to pathological vascular leakage. Our findings warrant further exploration of the plasminogen activation system as a source of biomarkers for diagnosis and therapeutic targets for treatment of FXII-HAE and possibly other forms of pathological vascular leakage.

Key messages

- Mutations in FXII that are associated with HAE introduce new cleavage sites.
- Plasmin is a natural endogenous trigger for bradykinin formation in patients with HAE.
- Therapeutic lysine analogs block plasmin-triggered bradykinin production.

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METHODS

Reagents

εACA, aprotinin, benzamidine hydrochloride hydrate, BSA, bromophenol blue, DL-dithiothreitol (DTT), DNA oligonucleotides, EDTA (IDRANAL III), glycerol, glycine, KCl, MgSO₄, hexadimethrine bromide (Polybrene), skimmed milk powder, NaCl, Na₂HPO₄, NaH₂PO₄, Soy Bean Trypsin Inhibitor, triethylamine, and Tween-20 were from Sigma-Aldrich. MaxiSorp microtiter plates, Nunc Cell Factory Systems, PageBlue, Phusion Polymerase, RNase H, and restriction enzymes *Bst*II, *Eco*RI, *Hind*III, *Not*I, and *Sfi*I were from Thermo Scientific (Waltham, Mass). Bolt 4-12% Bis-Tris Plus Gels, MOPS buffer, Gibco Dulbecco modified Eagle medium (high glucose, GlutaMAX, and HEPES), Alexa Fluor 680 Donkey Anti-Sheep IgG, pcDNA6/V5-His A, Gibco penicillin-streptomycin, EZ-Link Sulfo-NHS-LC-Biotin, Lipofectamine 2000, and One-shot B121 pLysS *E coli* were from Life Technologies (Carlsbad, Calif). Polyclonal antibodies (total IgG) to FXII, PPK, and C1inh (affinity purified) were from Cedarlane (Burlington, Ontario, Canada). Polyclonal affinity-purified antibodies to plasminogen and HK were from Affinity Biologicals (Ancaster, Ontario, Canada). Peroxidase-conjugated polyclonal rabbit anti-sheep antibody was from Dako (Heverlee, Belgium). Blasticidin-S hydrochloride and bradykinin ELISA were from Enzo Life Sciences (Raamsdonksveer, The Netherlands). Sodium acetate and CaCl₂ were from Merck (Darmstadt, Germany). Immobilon-FL, Ficoll 400, and ethanol were from Merck-Millipore (Amsterdam, The Netherlands). Chromogenic substrates H-D-Val-Leu-Lys-AMC (I1390) and H-D-Pro-Phe-Arg-pNA (L2120) were from Bachem (Bubendorf, Switzerland). FXII immune-depleted plasma and Phe-Pro-Arg-chloromethylketone (PPACK) were purchased from Haematologic Technologies (Essex Junction, Vt). α-FXIIa, β-FXIIa, and active PK were purchased from Enzyme Research laboratories (South Bend, Ind). Pfu Turbo was from Agilent Technologies (Santa Clara, Calif), and T4 ligase was from New England Biolabs (Whitby, Ontario, Canada). HEK293T cells were from ATCC (LGC Standards GmbH, Wesel, Germany), and FBS was from Lonza (Breda, The Netherlands). Strep-Tactin Superflow Sepharose was from IBA-Life Sciences (Goettingen, Germany). Lysine-Sepharose and HiLoad 26/600 Superdex 200-pg columns were from GE Healthcare (Hoevelaken, the Netherlands). Tris-HCl was from Roche (Woerden, the Netherlands), and HEPES was from VWR International (Amsterdam, The Netherlands). Kaolin (light) was purchased from BDH (Poole, United Kingdom) and routinely suspended in Tris-buffered saline (TBS; 50 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.4). Odyssey blocking reagent was purchased from LI-COR (Homburg, Germany), and streptokinase (Streptase) was purchased from CSL-Behring (Breda, The Netherlands). 3,3',5,5'-Tetramethyl-benzidine (TMB) was obtained from Tebu Bio (Heerhugowaard, The Netherlands). The kinase inhibitors apstatin, ramipril, lisinopril, and enalapril were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). C1inh was from Alpha diagnostics (San Antonio, Tex), and Streptavidin-poly-HRP was from Sanquin Blood Supply (Division Reagents, Amsterdam, The Netherlands). The Technozym PAP Complex ELISA Kit was obtained from Technoclone (Vienna, Austria). The STACHROM Antiplasmin Activity Assay Kit was purchased from Diagnostica Stago S.A.S (Asnières sur Seine, France).

In silico predictions

Mature amino acid sequences (UniProt: P00748)^{E1} were analyzed with Peptide Cutter software for the presence of putative cleavage sites that are sensitive to trypsin-like enzymes.^{E2} Changes in protein charge were analyzed with Protparam,^{E2} and changes in O-linked glycosylation with NetOGlyc server 4.0.^{E3}

Plasminogen purification

Plasminogen was purified from citrated human plasma by using lysine-sepharose affinity chromatography. In brief, 600 mL of plasma was supplemented with 3 mmol/L EDTA, filtered through paper filters to remove precipitate, and diluted to 900 mL with distilled water. A 30-mL lysine sepharose column was equilibrated with greater than 10 bed volumes of

30 mmol/L NaPO₄ and 3 mmol/L EDTA (pH 7.4). Columns were loaded with plasma at 3 mL/min and washed with greater than 30 bed volumes of 30 mmol/L NaPO₄, 3 mmol/L EDTA, and 500 mmol/L NaCl (pH 7.4) until the flow-through was free of protein. The column was eluted with 30 mmol/L NaPO₄, 3 mmol/L EDTA, 500 mmol/L NaCl, and 200 mmol/L εACA (pH 7.4). Protein-containing fractions were pooled and dialyzed against 25 mmol/L Tris-HCl and 150 mmol/L NaCl (pH 7.4). Plasminogen concentrations were determined by using OD_{280nm}. Purity was assessed on 10% SDS-PAGE gels with Coomassie blue staining. Plasmin activity was monitored at 37°C by means of cleavage of the chromogenic substrate H-D-Val-Leu-Lys-AMC (0.5 mmol/L final concentration) at an extinction of 380 nm and emission of 460 nm. Plasmin was freshly prepared for functional experiments by means of preactivation of plasminogen with 10 U/mL streptokinase in 10 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L MgSO₄, and 5 mmol/L KCl (pH 7.4 [HBS]) with 0.1% BSA for 15 minutes at 37°C, after which it was kept on ice until use.

Coagulation assays

Plasma coagulation times were recorded on an mc10 plus coagulometer (Merlin medical, Lemgo, Germany) by mixing 50 µL of prewarmed reconstituted FXII-depleted plasma with a 50-µL prewarmed mixture of kaolin (150 µg/mL) and 10 µmol/L phospholipid vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine in a 40%/40%/20% ratio). Coagulation was initiated after 2 minutes by means of recalcification with 50 µL of prewarmed CaCl₂ (8.3 mmol/L final concentration). Coagulation times were recorded up to 1000 seconds.

Western blotting

Samples from contact system activation assays were diluted 20 times in (non-)reducing sample buffer (15.5% glycerol, 96.8 mM Tris-HCl, 3.1% SDS, and 0.003% bromophenol blue ± 25 mmol/L DTT) and boiled for 10 minutes at 95°C. Samples were separated on a 4-12% Bis-Tris gels at 165 V for 65 minutes in MOPS buffer. Proteins were transferred onto Immobilon-FL membranes at 125 V for 60 minutes in blotting buffer (14.4 g/L glycine, 3.03 g/L Tris-HCl, and 20% ethanol) and blocked with blocking buffer (0.5× Odyssey blocking reagent in TBS) for 1 hour at room temperature. FXII, PPK, C1inh, and HK were detected by using overnight incubation at 4°C with polyclonal antibodies in blocking buffer (1:4000 for HK and 1:2000 for other proteins). Blots were washed with TBS-0.05% Tween 20, and primary antibodies were detected with Alexa Fluor 680 donkey anti-sheep IgG (1:7500 in blocking buffer). Blots were extensively washed with TBS-0.05% Tween 20, followed by distilled water and analyzed on a near-infrared Odyssey scanner (LI-COR).

FXIIa nanobody ELISA

An FXIIa ELISA was performed, as described previously, with minor modifications.^{E4} Briefly, 5 µg/mL nanobody B7 against FXIIa was immobilized on MaxiSorp plates in PBS (21 mmol/L Na₂HPO₄, 2.8 mmol/L NaH₂HPO₄, and 140 mmol/L NaCl [pH 7.4]) overnight at 4°C. Samples from contact system activation assays were collected by 8× dilution in 0.5% skimmed milk in HBS (mHBS) containing 200 µmol/L PPACK and 100 KIU/mL aprotinin. Wells were rinsed with PBS, blocked with 4% mHBS, and incubated with samples for 2 hours at room temperature while shaking. Next, the wells were rinsed with 0.05% PBS-Tween (PBST) and incubated with goat polyclonal anti-FXII (1:2,000 in 0.5% mHBS) for 1 hour at room temperature. After washing with PBST, wells were incubated with a peroxidase-conjugated anti-sheep polyclonal (1:8000 in 0.5% mHBS) for 1 hour at room temperature. Finally, wells were rinsed with PBST and developed with 100 µL of TMB substrate. Substrate conversion was stopped by adding 50 µL of H₂SO₄ (0.3 mol/L), and absorbance was read at 450 nm. A β-FXIIa standard curve was included on each plate, which was plotted in GraphPad Prism 6.0 software (GraphPad Software, La Jolla, Calif) by using a sigmoidal 4PL fit model to which sample concentrations of FXIIa were related.

Bacteriophage library preparation

A pair of *Llama glama* were immunized with α FXIIa-C1Inh every week for 4 weeks in total, after which venous blood was collected. Blood lymphocytes were isolated by using Ficoll gradient centrifugation, after which total RNA was isolated. RNA was transcribed into cDNA, and subsequently, residual RNA was degraded by using RNase H. IgG heavy-chain sequences were amplified from cDNA by means of PCR with an oligo-DT framework1-specific primer containing a 5' *SfiI* restriction site. The 1.3-kb PCR product was digested with *SfiI* and *Bst*II, resulting in a 300- to 400-bp fragment (Nanobody sequence) that was purified and ligated into the phagemid vector pUR8100. pUR8100 provides a c-terminal Myc- and His⁶ tag for the detection and purification of nanobodies, as well as ampicillin resistance for selection. The ligated vectors from both libraries were transformed into the *E coli* TG1 strain,^{E5} reaching a transformation efficiency of greater than 10⁷.

Nanobody development against C1Inh with cleaved reactive center loop

Two milligrams of PK was incubated with 3 mg of C1Inh in HBS and incubated at 37°C for 90 minutes. The PK-C1Inh complex was purified by means of gel filtration over a Superdex column. Purity was confirmed by using SDS-PAGE with Coomassie blue staining. The PK-C1Inh complex (5 μ g/mL in PBS) was immobilized overnight at 4°C on a 96-well MaxiSorp plate. Phages from both libraries were produced overnight and isolated through use of polyethylene glycol (Mr = 6000) precipitation (20% polyethylene glycol in 2.5 mol/L NaCl). The coated microtiter plate and phages were blocked with 2% skimmed milk powder (mass/volume) in PBS for 1 hour at room temperature. Subsequently, the phages were added to the immobilized PK-C1Inh and incubated for 3 hours at room temperature. Bound phages were extensively washed with PBST, after which bound phages were eluted by addition of triethylamine (0.1 mol/L). Eluted phages were pH neutralized by addition of Tris (1 mol/L, pH 7.5). Isolated phages were allowed to infect, exponentially growing *E coli* TG1, after which the infected bacteria were plated on yeast tryptone-agar plates containing glucose (2% m/v) and ampicillin (100 μ g/mL) and incubated overnight at 37°C.

Nanobody production

Nanobodies 1B12 and B7 were codon optimized for *E coli* (<http://eu.idtdna.com/CodonOpt>) and ligated into the pMEK219 vector through *SfiI* and *Bst*II. pMEK219 provides a LacZ promoter and c-terminal Myc and His⁶ tag for the detection and purification of nanobodies, as well as ampicillin resistance for selection. *E coli* (BL21 pLysS strain) was transformed and grown overnight at 37°C in 2xYT containing glucose (2% m/v) with ampicillin (100 μ g/mL). The overnight culture was used to inoculate an 5L fermenter (BioFlo 115, Eppendorf, Germany) containing ZYP-5052 autoinduction media (without trace metals).^{E6} Bacteria were grown for 3 hours at 37°C, followed by 20 hours at 24°C. The nanobody was isolated by immobilized metal affinity chromatography, as previously published.^{E7} Nanobodies were dialyzed against HBS and purified by means of gel filtration over a Superdex column. Protein concentrations were spectrophotometrically determined by using OD_{280nm}. Absorption coefficients of the nanobodies were determined based on amino acid sequences with ProtParam.^{E2} Purified nanobodies were assessed for impurity and degradation by using Coomassie blue staining after SDS-PAGE.

Nanobody capture ELISA for C1Inh-enzyme complexes

The C1Inh complex capture ELISA was performed similarly to the FXIIa capture ELISA. 1B12 (5 μ g/mL; a nanobody against complexed C1Inh) in HBS was immobilized on MaxiSorp plates overnight at 4°C. Samples from contact system activation assays were diluted 32× in mHBS, containing 200 μ mol/L PPACK. Wells were rinsed with PBS and blocked with 2% mHBS for 1 hour, after which the samples were incubated for 2 hours at room

temperature while shaking. Next, the wells were rinsed with PBS and 0.1% Tween 20 and incubated with polyclonal anti-PK (1:2000 in mHBS) or by 5 μ g/mL biotinylated polyclonal VHH mix that was selected against β -FXIIa^{E4} for 1 hour at room temperature. After washing with PBS and 0.5% Tween 20, wells were incubated with a peroxidase-conjugated anti-sheep polyclonal (1:8000 in 0.5% mHBS) or streptavidin-poly-HRP (1:5000 in 0.5% mHBS) for 1 hour at room temperature. Finally, wells were rinsed with PBS and 0.1% Tween 20 and developed with 50 μ L of TMB substrate. Substrate conversion was stopped with the addition of 25 μ L per well H₂SO₄ (0.3 mol/L), and absorbance was measured at 450 nm. An activated plasma standard curve (activated with 30 μ g/mL DXS-500k for 30 minutes) was included on each plate, which was plotted in GraphPad Prism 6.0 software by using a sigmoidal 4PL fit model to which sample concentrations were related.

Bradykinin ELISA

Kinase inhibitors (1.19 μ g/mL apstatin, 104 ng/mL ramipril, 21 ng/mL lisinopril, and 162 ng/mL enalapril) were added to plasma before experiments to reduce bradykinin degradation. Samples were collected and diluted 5× in ice-cold ethanol. Protein precipitate was removed by means of centrifugation (15 minutes at 1000g), after which supernatants were dried in a centrifugal evaporator. Pellets were reconstituted in buffer to the original sample volume after which they were frozen at -20°C until analysis. Samples were diluted 36 times in assay buffer and analyzed with a competitive ELISA for bradykinin, according to the manufacturer's instructions.

α_2 -Antiplasmin activity

α_2 -Antiplasmin activity was measured with a commercial chromogenic assay. Ten microliters of plasmin (13 nanokatal per milliliter) was incubated for 30 seconds with 10 μ L of 5× diluted citrated (patient) plasma in HBS. Subsequently, 40 μ L of plasmin substrate (2 mmol/L; MM-L-Tyr-Arg-pNA) was added, and absorption was measured at 405 nm. Slopes were determined and expressed as a percentage of the inhibitory capacity of normal pooled plasma (defined as 100%).

PAP complex ELISA

PAP levels were determined in 10× diluted samples, according to the manufacturer's instructions.

Statistical analysis

Statistics were performed by using 1-way ANOVA or the unpaired Student *t* test, as indicated in the figure legends. A *P* value of less than .05 was considered significant. All data analyses were performed with GraphPad Prism 6.0 software.

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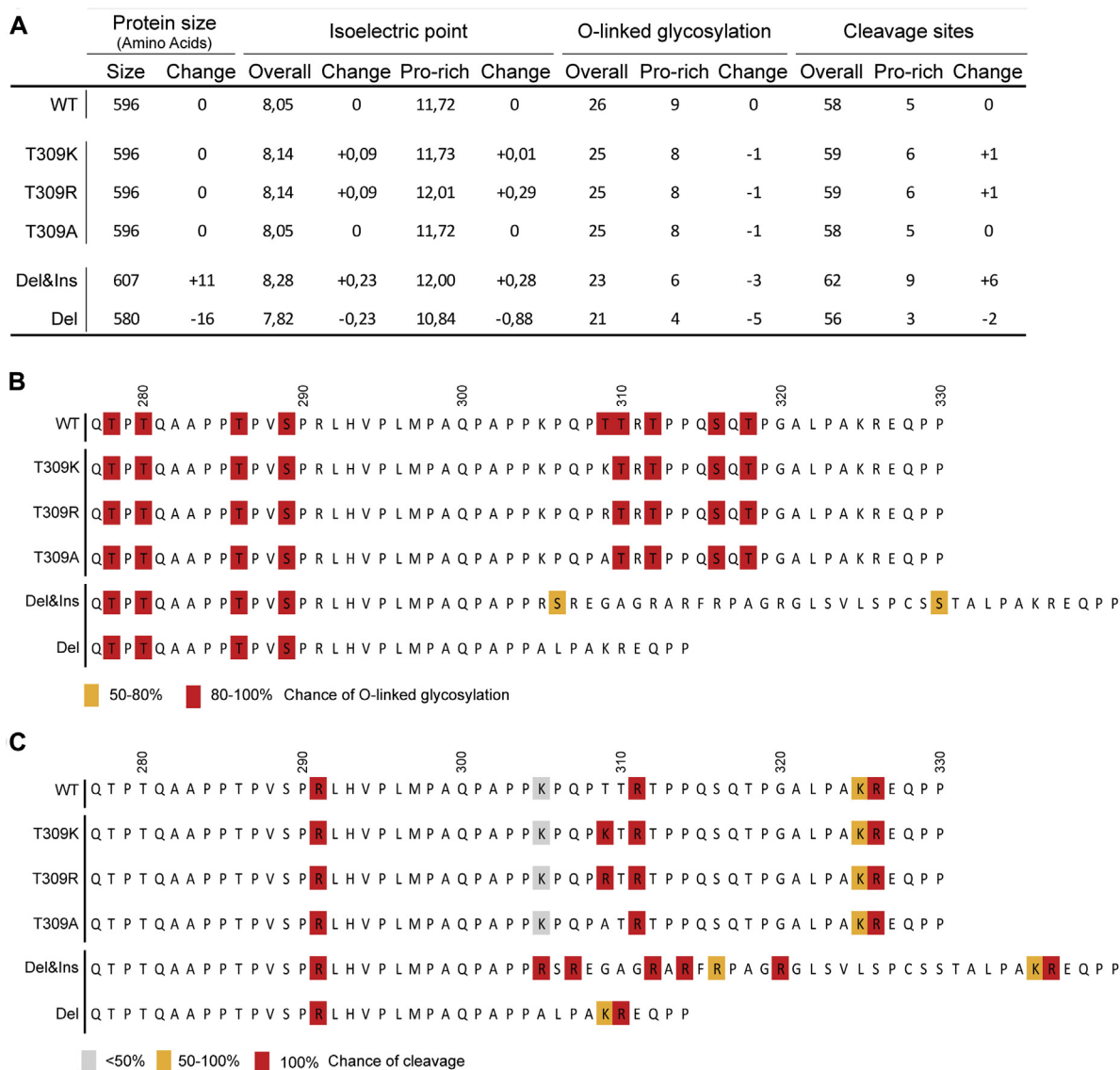


FIG E1. Mutations that cause FXII-HAE introduce novel cleavage sites in FXII. **A**, Predicted changes in protein size, isoelectric point values, and O-linked glycosylation and cleavage sites for trypsin-like enzymes in full-length FXII variants (*Overall*) or its proline-rich domain (*Pro-rich*). **B**, Map of predicted O-linked glycosylation sites in the proline-rich domain of FXII. Putative glycosylation sites are marked in red for high probability and yellow for medium probability. **C**, Map of predicted cleavage sites for trypsin-like enzymes in the proline-rich domain of FXII. Putative cleavage sites are marked in red for high probability of cleavage, yellow for medium probability, or gray for low probability.

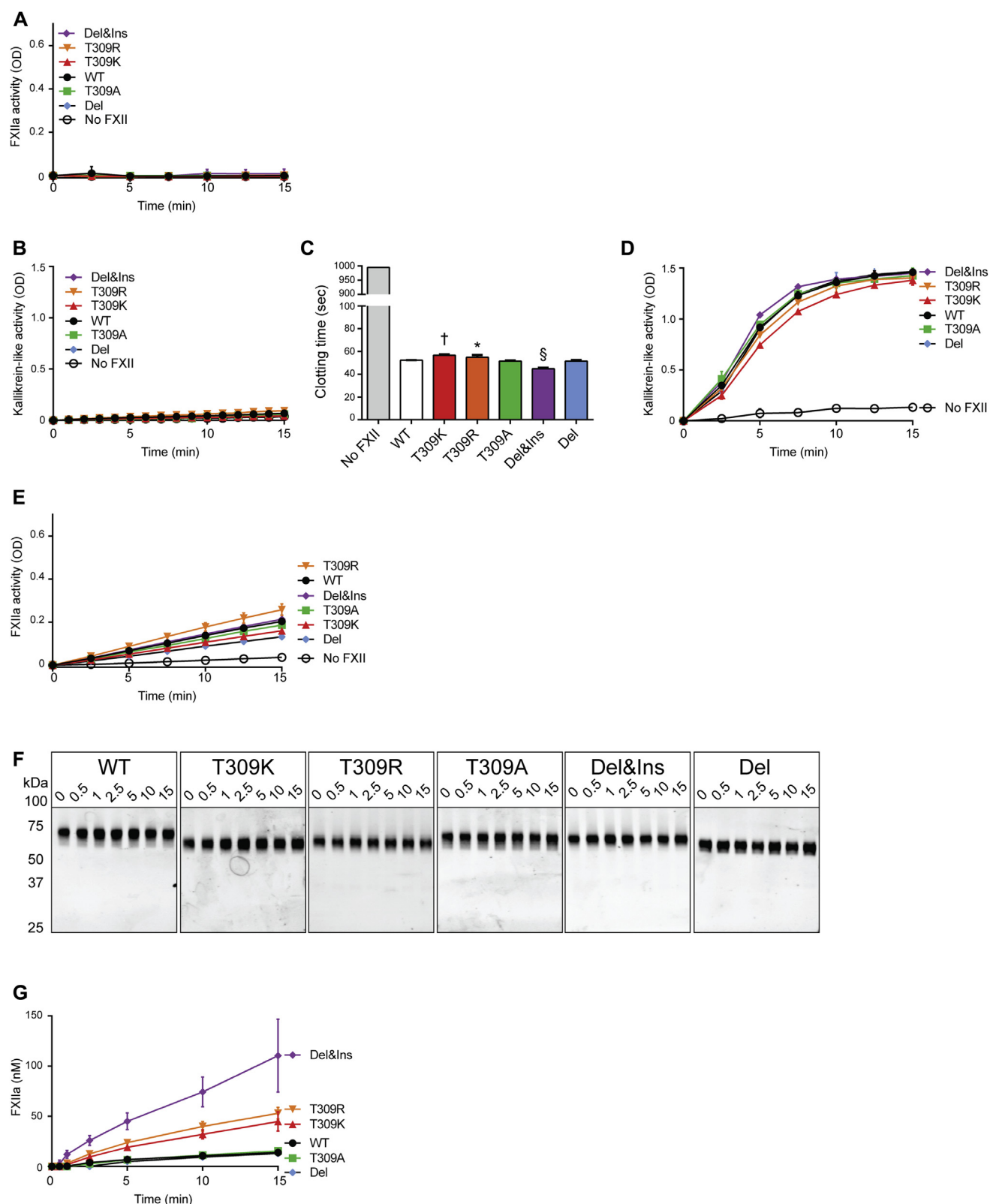


FIG E2. FXII-HAE mutations do not provoke spontaneous activity or enhance clotting potential. **A**, Spontaneous enzymatic activity of purified FXII variants. **B**, Spontaneous enzymatic activity in plasma in the presence of recombinant FXII variants. **C**, Kaolin-triggered coagulation times (dilute activated partial thromboplastin time) of FXII-deficient plasma in the presence of recombinant FXII variants. **D**, Kaolin-triggered kallikrein-like activity in reconstituted FXII-deficient plasma. **E**, Enzymatic activity of FXII variants after exposure to PK (0.5 μg/mL). **F**, FXII cleavage by PK (unreduced; time series indicated above in minutes). **G**, Time course of FXIIa production by FXII variants during exposure to plasmin. Data represent means $\pm$ SDs of 3 separate experiments, each performed in duplicate. * $P < .05$, # $P < .005$, † $P < .0005$, and § $P < .0001$ compared with WT analyzed by 1-way ANOVA.

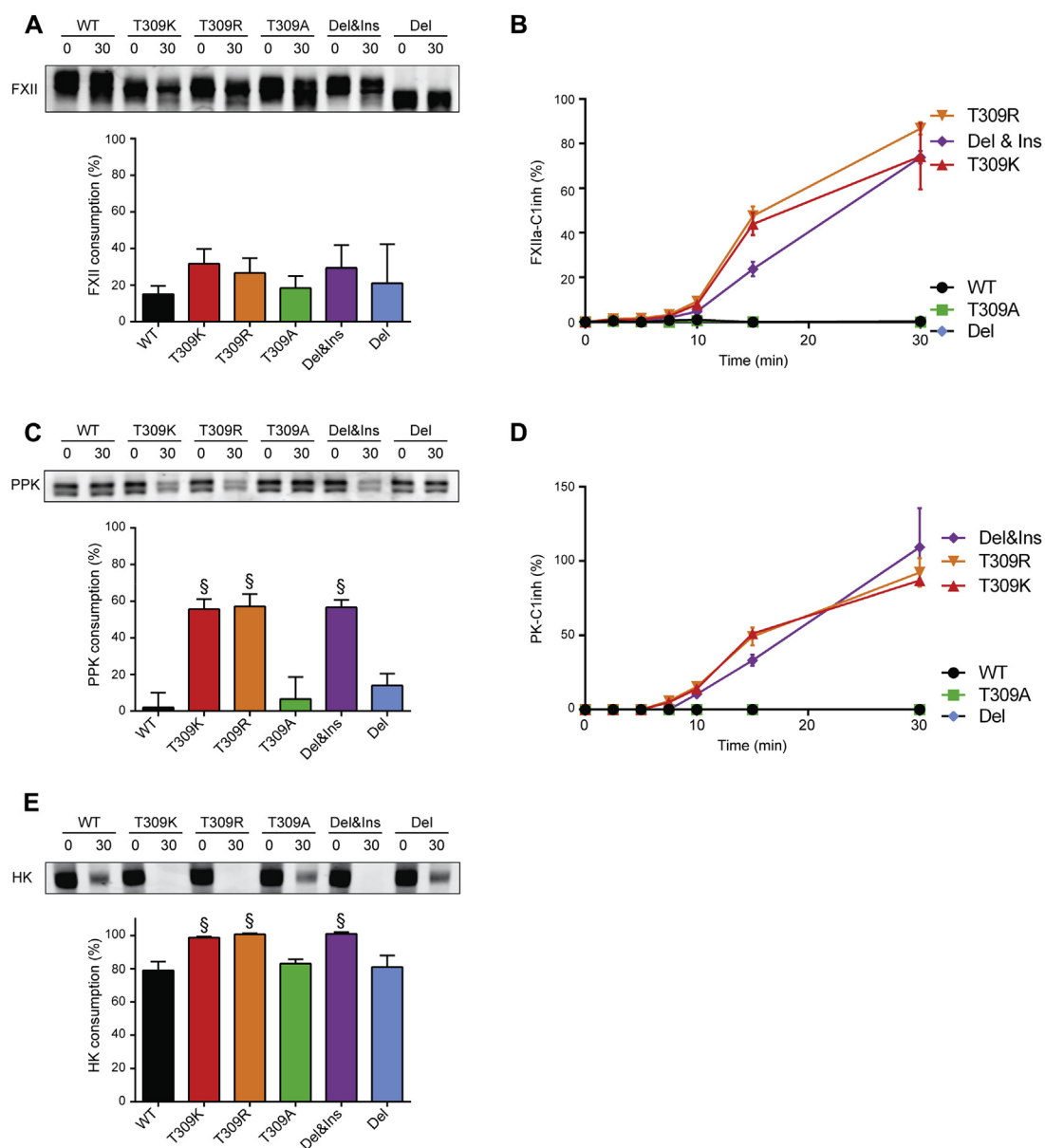


FIG E3. FXII-HAE mutants escape inactivation by C1inh during activation by plasmin in plasma. **A**, FXII consumption in plasma 30 minutes after induction of plasmin activity: *top*, Western blotting; *bottom*, quantification of repeated experiments. **B**, Time course of FXIIa-C1inh complex formation by FXII variants during exposure to plasmin. **C**, PPK consumption in plasma 30 minutes after induction of plasmin activity: *top*, Western blotting; *bottom*, quantification of repeated experiments. **D**, Time course of PK-C1inh complex formation in the presence of FXII variants during exposure to plasmin. **E**, HK consumption in plasma 30 minutes after induction of plasmin activity: *top*, Western blotting; *bottom*, quantification of repeated experiments. Data represent means $\pm$ SDs of 3 separate experiments, each performed in duplicate. * $P < .05$, # $P < .005$, † $P < .0005$, and § $P < .0001$ compared with WT analyzed by using 1-way ANOVA.

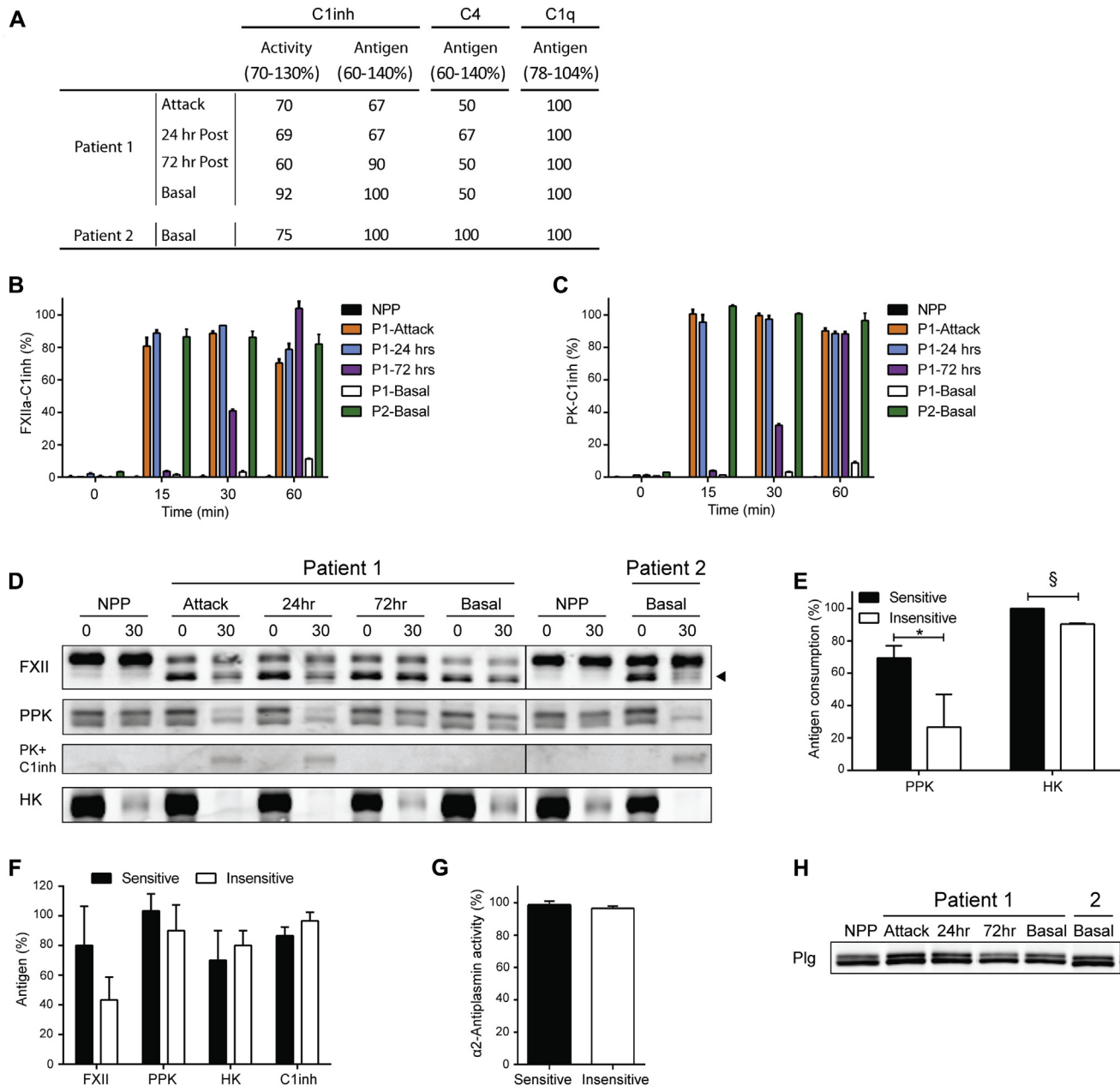


FIG E4. Plasmin triggers bradykinin production in plasma from patients with FXII-HAE and modulates disease activity. Samples were collected from 2 patients with FXII-HAE (T309K mutation) in remission (*Basal*). From patient 1, samples were repeatedly collected during a swelling attack. Normal pooled plasma (*NPP*) serves as a control. **A**, Activity and antigen levels of C1inh, C4, and C1q. **B**, Formation of FXIIa-C1inh complexes in plasma from patients with FXII-HAE after induction of plasmin activity. **C**, Formation of PK-C1inh complexes in plasma from patients with FXII-HAE after induction of plasmin activity. **D**, Plasma FXII, PPK, and HK consumption after 30 minutes of plasmin activity. **E**, Quantification of PPK and HK consumption 30 minutes after induction of plasmin activity in grouped patient samples categorized by their capacity to generate FXIIa in response to plasmin ($n = 3$ per group). **F**, FXII, PK, HK, and C1inh antigen levels in grouped patient samples ($n = 3$ per group). **G**, α_2 -Antiplasmin activity in grouped patient samples ($n = 3$ per group). **H**, Western blot of plasminogen levels. Fig E4, E-G, were analyzed by using the unpaired Student t test: * $P < .05$ and § $P < .0001$.

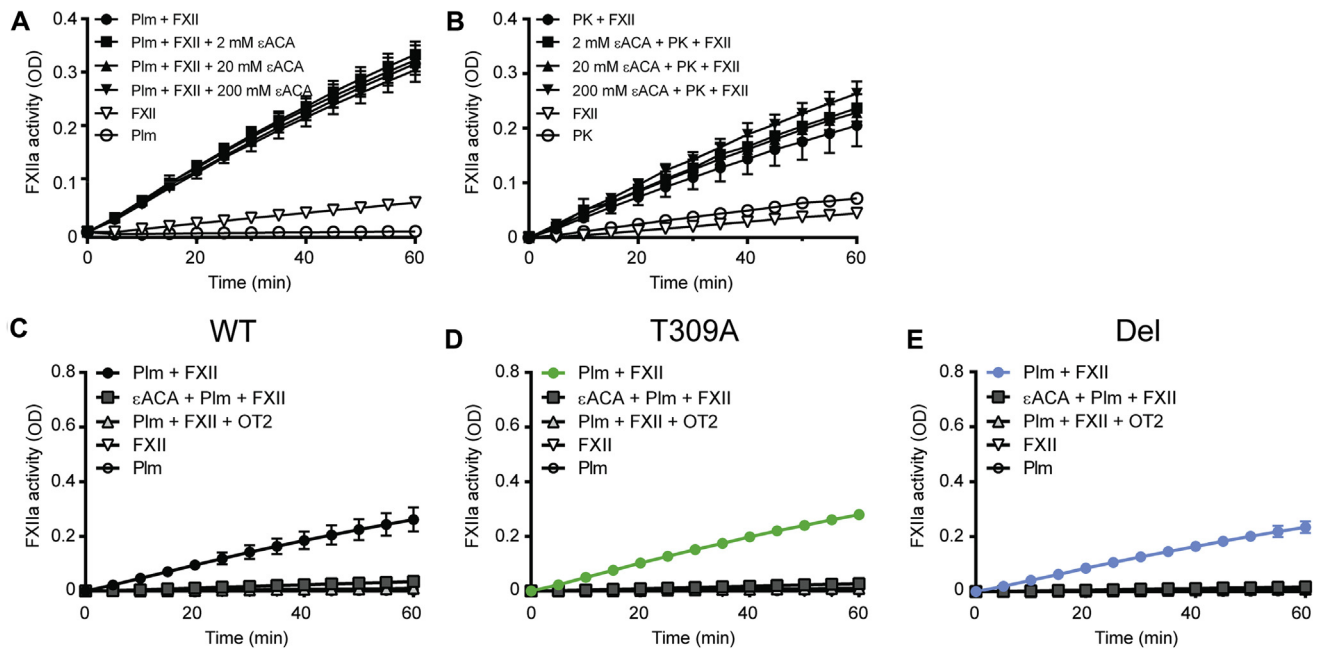


FIG E5. The soluble lysine analog ϵ ACA selectively inhibits activation of FXII by plasmin, whereas the mAb OT2 terminates FXIIa activity. **A**, ϵ ACA does not inhibit the activity of FXII after it has been activated by plasmin (*Plm*; ϵ ACA was added after activation). **B**, ϵ ACA does not inhibit activation of FXII by PK (ϵ ACA was added before activation). **C-E**, Inhibition of plasmin-triggered activation and activity of purified FXII variants (FXII-WT, FXII-T309A, and FXII-Del) by ϵ ACA or OT2 (ϵ ACA [200 mmol/L] was added before activation, and OT2 [750 nmol/L] was added after activation).

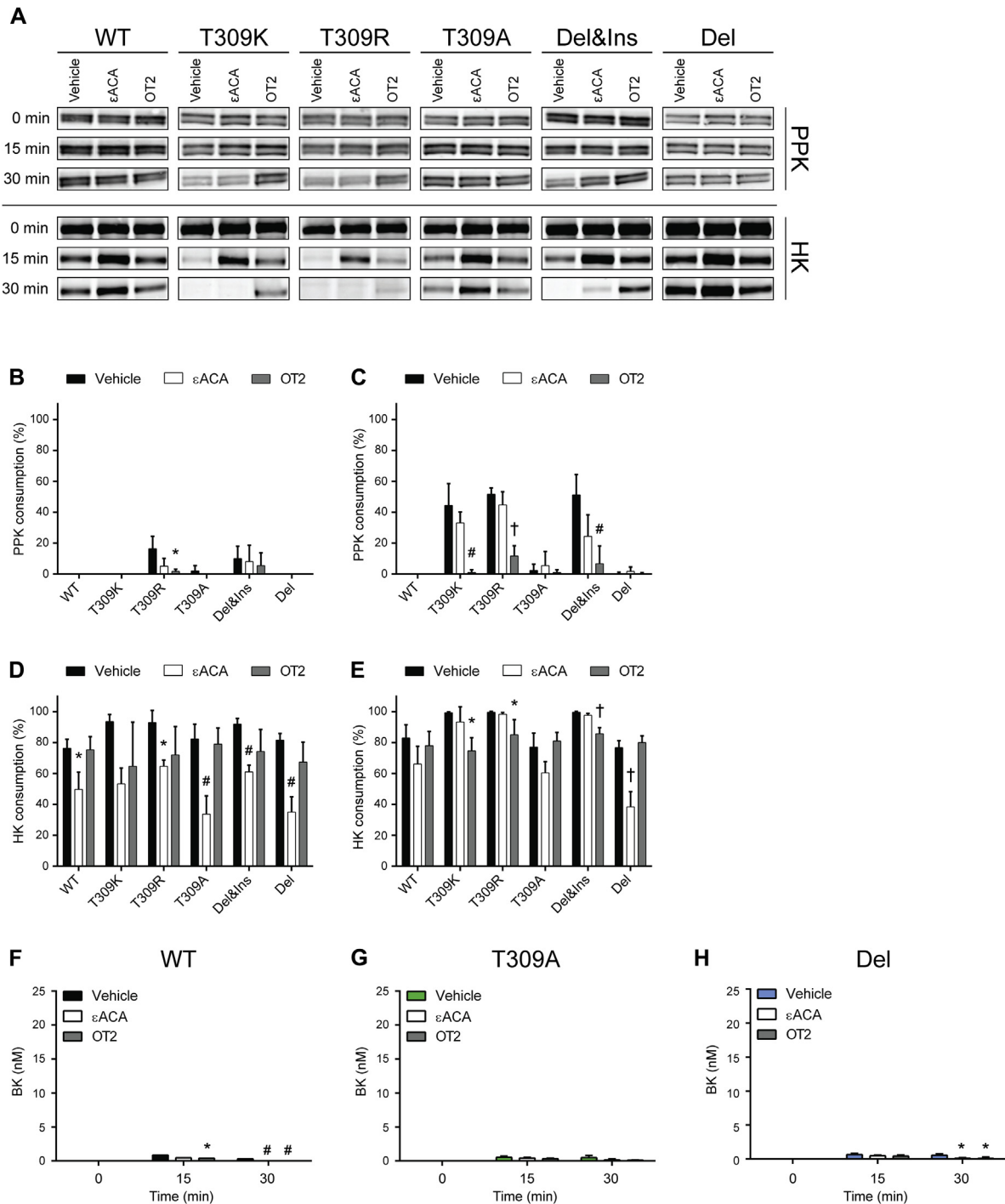


FIG E6. The soluble lysine analog ϵ ACA selectively inhibits activation of FXII by plasmin in plasma, whereas the mAb OT2 terminates FXIIa activity. **A**, Effects of ϵ ACA (200 mmol/L) or the mAb OT2 (225 nmol/L) on plasmin-triggered PPK (upper panels) and HK (lower panels) consumption in plasma. **B** and **C**, Quantification of PPK consumption after 15 and 30 minutes of plasmin activity, respectively. **D** and **E**, Quantification of HK consumption after 15 and 30 minutes of plasmin activity, respectively. **F-H**, Effects of ϵ ACA or OT2 on plasmin-triggered bradykinin production in the presence of FXII variants (FXII-WT, FXII-T309A, and FXII-Del; ϵ ACA [200 mmol/L] was added before activation, and OT2 [225 nmol/L] was added after activation). Data are means $\pm$ SDs of 3 separate experiments, each performed in duplicate. * $P < .05$, # $P < .005$, † $P < .0005$, and § $P < .0001$ compared with vehicle by using 1-way ANOVA.

TABLE E1. Molecular cloning and primer design

Primer	Sequence 5' → 3'
FXII_N-termTEV_For	GCAGAATTCGAAAACCTGTATTTTCAGTCTATTCCACCTTGGAAGCCCCCAAGGAGCA
FXII_C-Term_Short	CGAGCGGCCGCTCATCAGGAAACG
T309K_For	CCGAAGCCTCAGCCCAAGACCCGGACCCCGCCT
T309K_Rev	AGGCGGGGTCCGGGTCTTGGGCTGAGGCTTCGG
T309R_For	CCGAAGCCTCAGCCCGGACCCGGACCCCGGAC
T309R_Rev	AGGCGGGGTCCGGGTCCGGGGCTGAGGCTTCGG
T309A_For	CCGAAGCCTCAGCCCGCGACCCGGACCCCGCCT
T309A_Rev	AGGCGGGGTCCGGGTCCGGGGCTGAGGCTTCGG
FXII-TEV_N-Term_For	GGGAATTCGAAAACCTGTATTTTCAG
Del&Ins_P1_Rev	CTCGGCTCCTCGGCGGTGCCGGCTGCGGGGCATG
Del&Ins_P2_For	GGCACCGCCGAGGAGCCGAGAGGGCGCCGGGCGAGCTAGATTCCGGCCAGCCGGCCGCGGGCTCTCCGTC
Del&Ins_P2_Rev	CCGGAAGGCTGTGGAGGAGCAGGGGCTGAGGACGGAGAGCCCGCGCCGGCTGGCCGGAATCTAGCTCG
Del&Ins_P3_For	CTCTCCACAGCCTTGCCGGCGAAGCGGGAGCAG
Del_P1_Rev	TCCCGCTTCGCCGGCAAGGCCGGCGGTGCCGGCTGCGCGGGCAT
Del_P2_For	CCGCGCAGCCGGCACCGCCGGCCTTGCCGGCGAAGCGGGAGCAG
pSM2_Insert	AAGCTTACCATGGAGACAGACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGACGGCAG CAGCGCTTGAGCCACCCGCAGTTCGAAAAAGGCAGCAGCGCTTGAGCCACCCGCAGTTCGAAAAAGAATTC