

ORIGINAL ARTICLE

Clinical Mechanisms in Allergic Disease

Threshold-stimulated kallikrein activity distinguishes bradykinin- from histamine-mediated angioedema

Maria L. Lara-Marquez¹  | Sandra C. Christiansen¹ | Marc A. Riedl¹  |
Jack Herschbach² | Bruce L. Zuraw^{1,2} 

¹Division of Rheumatology, Allergy and Immunology, Department of Medicine, UC San Diego, La Jolla, California

²Medicine Service, San Diego VA Healthcare, San Diego, California

Correspondence:

Bruce L. Zuraw, UC San Diego, 9500 Gilman Dr., Mail Code # 0732, La Jolla, CA 92037-0732.

Email: bzuraw@ucsd.edu

Funding information

US HAE Association

Summary

Background: The lack of specific biomarkers makes the diagnosis of hereditary angioedema (HAE) with normal levels of C1-inhibitor (C1INH) protein (HAE-nl-C1INH) and idiopathic non-histaminergic angioedema (INHA) difficult. Confirming or excluding these diagnoses is a significant challenge for clinicians evaluating patients with angioedema.

Objective: To develop a reliable biomarker that would aid the diagnosis of HAE-nl-C1INH and INHA.

Methods: A total of 154 consecutive patients referred for angioedema at a single centre were enrolled and evaluated. Subjects were clinically phenotyped based on clinical history and response to treatment by clinicians blinded to laboratory assay results. Plasma kallikrein activity was measured by the cleavage of the fluorometric substrate Z-Phe-Arg-AMC-HCL in plasma samples stimulated ex vivo with submaximal doses of dextran sulphate.

Results: Stimulated plasma kallikrein activity (mean relative fluorescence units/min $\pm$ SD) was significantly increased in both HAE-nl-C1INH (1804 $\pm$ 600) and INHA (1579 $\pm$ 371) subjects compared to non-swelling controls (171 $\pm$ 46) and histaminergic angioedema (133 $\pm$ 30) subjects. Using a threshold cut-off based on the normal controls, HAE-nl-C1INH and INHA subjects could be differentiated from histaminergic angioedema subjects with high sensitivity (negative predictive value 86%-89%) and specificity (positive predictive value 80%-100%).

Conclusion and Clinical Relevance: The stimulated kallikrein activity assay allows differentiation of bradykinin- from histamine-mediated angioedema. The assay could feasibly be considered as a potential clinical tool for the diagnosis of bradykinin-mediated angioedema.

KEYWORDS

hereditary angioedema, hereditary angioedema with normal C1 inhibitor, idiopathic non-histaminergic angioedema, bradykinin, histamine, kallikrein, biomarker, diagnosis, high-molecular weight kininogen, angioedema

1 | INTRODUCTION

Recurrent angioedema without urticaria can be mediated by different mediators, predominantly histamine or bradykinin. Compared to

histamine-mediated angioedema, bradykinin-mediated angioedema is distinguished by its increased severity, longer duration of swelling and lack of response to treatment with antihistamines, corticosteroids or epinephrine.¹ Differentiating bradykinin- from histamine-

mediated angioedema is of critical importance to prevent morbidity and mortality.² The ability to diagnose bradykinin-mediated angioedema with normal C1 inhibitor (C1INH), however, has been severely limited by the lack of any available diagnostic tests.

Hereditary angioedema (HAE) due to C1INH deficiency (HAE-C1INH) and acquired C1INH deficiency are the best characterized forms of bradykinin-mediated angioedema.^{3–6} A novel form of HAE with normal C1INH (HAE-nl-C1INH) was initially described in 2000.^{7,8} Subsequently, a minority of HAE-nl-C1INH patients were found to have mutations in F12 (the gene encoding FXII; HAE-FXII) that segregated with disease activity.^{9–12} HAE-FXII is autosomal dominant with low penetrance.¹³ Most recently, mutations in angiopoietin-1 and plasminogen have also been linked to HAE-nl-C1INH.^{14,15} Most HAE-nl-C1INH patients, however, do not have a known mutation and are called HAE-unknown (HAE-U). Distinguishing HAE-U from idiopathic histaminergic angioedema (IHA) is very challenging due to the lack of any definitive biomarker for either condition. Currently, the diagnosis of HAE-U is based on consensus clinical criteria, although the sensitivity and specificity of this approach are unknown.¹⁶ Another group of angioedema patients is clinically similar to HAE-U but lack a compelling family history of angioedema. This group has been classified as idiopathic non-histaminergic angioedema (INHA) and also lacks any biomarker to establish the diagnosis.¹⁷

Recent evidence strongly supports the hypothesis that HAE-FXII is mediated by bradykinin.^{18,19} Circumstantial evidence suggests that HAE-U and INHA are also bradykinin-mediated^{20,21} and may respond to bradykinin-targeted medications.^{13,22–24} The lack of a validated clinical biomarker to differentiate bradykinin-mediated angioedema from histamine-mediated angioedema has caused considerable difficulty for patients and physicians. Many patients are denied access to medicines that appear to be helpful because of the lack of a clear-cut diagnosis. Alternatively, some patients are labelled as having HAE-nl-C1INH and prescribed bradykinin-targeted medications despite very weak evidence that their angioedema is bradykinin-mediated. Development of a robust clinical assay to help identify patients with bradykinin-mediated angioedema is an urgent unmet need with major clinical and economic implications.

In this study, we describe a simple assay that discriminates histamine- from bradykinin-mediated angioedema. This assay is based on activation of plasma kallikrein following *ex vivo* stimulation of the contact system in plasma. Utilizing a threshold dose of the stimulator, we could differentiate subjects with bradykinin-mediated from IHA without urticaria. We propose that this assay can help identify patients with possible HAE-U or INHA who could benefit from a trial of a bradykinin-targeted medication. By segregating these patients from IHA, this assay may also facilitate efforts to identify the underlying mechanisms of bradykinin-mediated angioedema with normal C1INH.

2 | METHODS

2.1 | Human subjects

This study was approved by the Institutional Review Board and Human Research Protections Program of the University of California

San Diego (#150613). Consecutive patients presenting with angioedema without urticaria to the US Hereditary Angioedema Association (HAEA) Angioedema Center at University of California San Diego were informed of the study and given the opportunity to participate. Subjects were required to be age 14 or above. All study subjects (and parents for minors) signed informed consent/assent forms prior to any study procedures. Control subjects included normal healthy volunteers as well as patients without angioedema or urticaria.

2.2 | Clinical phenotyping

All subjects were clinically phenotyped based on clinical history, standard laboratory values (including C1INH function chromogenic assay and the FXII SNP analysis that detects T309K and T309R [National Jewish Health Advanced Diagnostic Laboratories, Denver, CO, USA]) and response to treatment by blinded investigators (SC, MR, BZ). Using the consensus guidelines,^{16,25} subjects were assigned to seven different groups: (a) HAE-C1INH; (b) acquired C1INH deficiency; (c) HAE-XII; (d) HAE-U; (e) INHA; (f) IHA; and (g) non-swelling control (NSC). Subjects classified as potentially having INHA met the same criteria as HAE-U but did not have a positive family history of angioedema.

In the absence of definitive criteria for the diagnosis of HAE-U or INHA, the clinical diagnosis of HAE-U or INHA cannot be unequivocally established but rather represents a probability that the clinical diagnosis may be correct. We sought to increase the probability of a correct diagnosis of HAE-U or INHA by making the clinical criteria more stringent. Subjects with a diagnosis of HAE-U or INHA were further classified based on probability using two pre-defined criteria not in the consensus guidelines: (a) history of rapid and durable responses to a bradykinin-targeted medication; and (b) either predominantly documented visible angioedema; or in patients with predominant abdominal symptoms, evidence of bowel wall edema documented by CT or MRI. To be classified as high probability, subjects needed to meet both additional criteria. A negative response to either of these reduced the probability to moderate, and negative responses to both reduced the probability to low.

2.3 | Blood drawing and processing

At the time of blood sampling, history of recent angioedema attacks and medication use was recorded. Blood was collected into plastic vacutainer tubes containing EDTA or citrate (Becton Dickinson, Franklin Lakes, NJ, USA) after removing the tourniquet and discarding at least the first 5 mL of blood. Tubes were kept at room temperature until processed within 15 minutes of the draw then centrifuged at 2000 × *g* for 10 minutes at room temperature. Plasma was aliquoted into Corning 1.2 mL internal threaded polypropylene cryogenic vials, immediately snap frozen in liquid nitrogen, and stored at –80°C until analysed. Plasma aliquots were thawed a maximum of two times. All assays were performed by ML-M or JH.

2.4 | Reagents and chemicals

The following reagents and chemicals were purchased: dextran sulfate (DXS) and dimethyl sulfoxide from Sigma-Aldrich, St. Louis, MO, USA; Z-Phe-Arg-AMC-HCl from Bachem, Torrance, CA, USA; recombinant human plasma kallikrein (rKLKB1, Lot NVH0215121) from R&D Systems, Minneapolis, MN, USA; human pre-kallikrein deficient citrated plasma from Aniera, West Chester, OH, USA; 12% Mini-Protean TGX Precast Protein Gels and 2X Laemmli premixed buffer from Bio-Rad, Hercules, CA, USA.

Fresh DXS stock and 4X assay buffer were prepared every 4 months. DXS was dissolved in distilled water (20 mg/mL), sterilized by filtration and stored at 4°C until used. The DXS was diluted in 4x assay buffer within 2 hours of the assay. Z-Phe-Arg-AMC-HCl was dissolved in dimethyl sulfoxide to 90 mmol/L stock solution and stored at −20°C. Ten minutes prior to the assay, the stock Z-Phe-Arg-AMC-HCl was diluted to the final concentration with filtered deionized H₂O and then kept at room temperature in the dark until used.

2.5 | Plasma kallikrein fluorometric activity assay

Plasma aliquots were thawed in a 37°C water bath for 4 minutes immediately prior to performing the assay. The assay was performed in 96-well plates (NUNC Black Maxisorp) with a total volume of 100 µL per well with 2-3 replicates per sample. Each aliquot included a negative control (no DXS) to calculate spontaneous kallikrein activity. The plates were prepared by adding the reagents within 6 minutes in the following order: 25 µL of 4x assay buffer (320 mmol/L NaCl, 200 mmol/L Tris-HCl, pH 7.8) with or without DXS (0, 1.25, 2.5 or 5 µg/mL final concentration); 25 µL of EDTA plasma or rKLKB1 (varying concentrations); and 50 µL of substrate (100 µmol/L of Z-Phe-Arg-AMC-HCl final concentration). The plates were immediately loaded into the spectrophotometer (Synergy H1 Reader, BioTek, Winooski, VT, USA), stabilized for 30 minutes at 37°C prior to the assay. Assay parameters were shaking the plate for 5 seconds followed by measurement of fluorescence (excitation 360 nm, emission 460 nm) every 60 seconds for 30 minutes. Results were calculated as the slope of relative fluorescence units (RFU) over the 30-minute assay, subtracting spontaneous kallikrein activity at each point.

2.6 | Plasma high molecular weight kininogen (HMWK) immunoblotting

Citrated plasma samples (10 µL) were incubated with varying concentrations of DXS (10 µL) for 30 minutes at 37°C, and the stimulation stopped by dilution with an equal volume of 2X Laemmli loading buffer. Immunoblotting for HMWK was performed under both reducing and non-reducing conditions as previously described.²⁶

2.7 | Data and statistical analysis

GraphPad PRISM 7.0a software for MAC was used to perform linear regression analysis and slope calculations (La Jolla, CA, USA). JMP

Statistical Analysis Software (SAS) was used for data analysis (Cary, NC, USA). Group comparisons used the Wilcoxon signed-rank test. Differences with a *P* value <0.05 were considered significant. Receiver operator curve (ROC) analysis was performed using a web-based calculator.²⁷

3 | RESULTS

3.1 | Subject demographics and phenotyping

A total of 154 unrelated research subjects were enrolled. The final clinical phenotype for each subject reflected the initial consultative evaluation as well as information collected on clinical follow-up. Every subject was independently phenotyped by at least two expert angioedema clinicians who were blinded to the experimental results. In the few cases where there was a difference in initial phenotype decision, subjects' clinical data were reviewed by all 3 experts until a unanimous consensus was reached. Random codes were broken, and experimental assay results linked to the subjects only after all the clinical phenotyping was finalized.

Of 154 subjects, 37 were excluded because they were found to have concomitant urticaria. Subjects considered to have low probability of HAE-nl-C1INH or INHA (*n* = 7) were also excluded. Eight subjects were felt to have features of both bradykinin-mediated and histamine-mediated angioedema. These subjects were classified in a separate group, identified as intermediate and excluded from the analysis. Additional excluded subjects were as follows: 3 subjects diagnosed with ACE inhibitor associated angioedema; 2 subjects with complex seizure disorders requiring use of multiple drugs; 2 subjects whose plasma was not processed in a timely fashion; and 5 subjects adjudicated not to have angioedema. This left 90 subjects in the analysed cohort. Subject demographics of the analysed cohort are shown in Table 1. One of the 20 subjects identified as HAE-nl-C1INH was found to have HAE-XII (T309K).

3.2 | Spontaneous kallikrein activity does not distinguish HAE-nl-C1INH or INHA from IHA

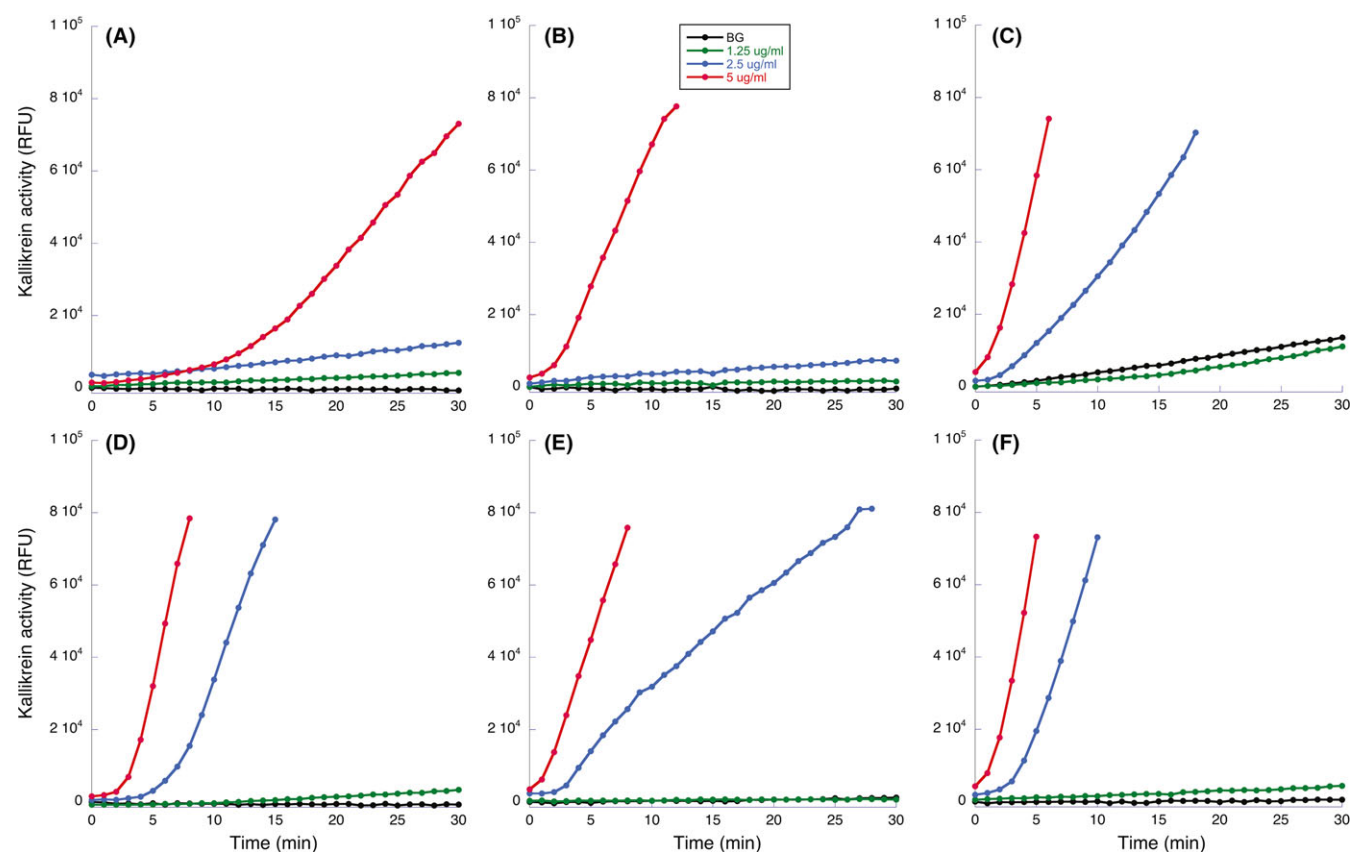
Spontaneous plasma kallikrein amidolytic activity has been reported to identify patients with HAE-nl-C1INH.²¹ As shown in Table 1, we observed a modest increase in spontaneous kallikrein activity only in the C1INH-deficient subjects. We also failed to detect any increase in spontaneous kallikrein activity in HAE-nl-C1INH or INHA subjects who were actively swelling or had experienced an angioedema attack within 1 day of the blood draw (data not shown).

3.3 | Key features of the threshold-stimulated kallikrein assay

We investigated whether plasma kallikrein activity stimulated by a minimal threshold dose of DXS could identify bradykinin-mediated angioedema subjects. Ten µg/mL DXS stimulated maximal kallikrein activity in all subjects and failed to discriminate between the groups

TABLE 1 Spontaneous and DXS-stimulated kallikrein activity

Group	N	F (%)	Age (y $\pm$ SD)	RFU/min (mean $\pm$ SEM)			
				Spontaneous	DXS stimulation		
					1.25 μ g/mL	2.5 μ g/mL	5 μ g/mL
Non-swelling	14	64.3	43.4 $\pm$ 15.5	1.8 $\pm$ 1.8	34.4 $\pm$ 8.8	170.8 $\pm$ 45.8	2910.2 $\pm$ 566.8
Histaminergic AE	24	37.5	52.5 $\pm$ 15.9	7.5 $\pm$ 4.1	21.6 $\pm$ 4.0	133.0 $\pm$ 29.8	3132.6 $\pm$ 558.9
C1INH deficiency	9	66.7	45.4 $\pm$ 16.7	160.7 $\pm$ 77.9**	348.3 $\pm$ 151.4**	2479.9 $\pm$ 437.2**	8656.8 $\pm$ 1479.1**
HAE-nl-C1INH ^a	20	100.0	46.8 $\pm$ 13.4	9.3 $\pm$ 4.4	123.6 $\pm$ 72.4*	1804.3 $\pm$ 597.8**	7097.2 $\pm$ 742.3**
INHA	23	91.3	38.6 $\pm$ 14.5	7.1 $\pm$ 4.7	212.8 $\pm$ 167.1	1578.9 $\pm$ 371.2**	6136.6 $\pm$ 627.0**

^aIncludes the HAE-FXII subject.**P* < 0.05 compared to histaminergic AE.***P* < 0.01 compared to histaminergic AE.**FIGURE 1** Dextran sulfate stimulates kallikrein activity in a dose-dependent manner. Plasma was stimulated ex vivo with various doses of dextran sulfate (DXS) as indicated, and plasma kallikrein activity measured as relative fluorescence units (RFU) over 30 min. BG refers to background or spontaneous kallikrein activity. Panels are as follows: A, non-swelling control; B, histaminergic angioedema; C, HAE-C1INH; D, HAE-FXII; E, HAE-U; and F, INHA

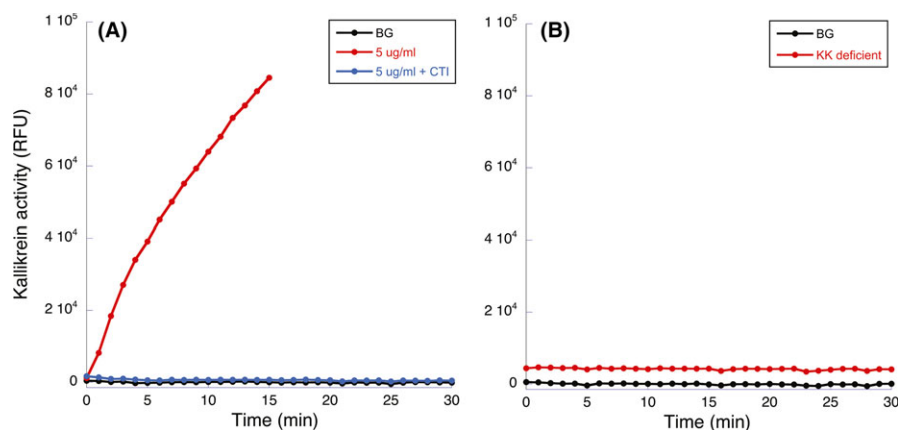
(data not shown). Figure 1 shows kallikrein activity in 6 representative subjects (A, non-swelling control; B, histaminergic angioedema; C, HAE-C1INH; D, HAE-FXII; E, HAE-U; F, INHA) stimulated with 0, 1.25, 2.5 or 5 μ g/mL DXS.

Five μ g/mL DXS gave a near maximal response but failed to clearly discriminate between the subjects. After 2.5 μ g/mL DXS, kallikrein activity was clearly increased in the HAE-C1INH, INHA and HAE-nl-C1INH subjects compared to the IHA and NSC subjects. At

1.25 μ g/mL DXS and below, only the HAE-C1INH subject showed significant kallikrein activity.

We investigated the specificity and reproducibility of the kallikrein assay. Because the fluorometric substrate used in our assay could potentially be cleaved by other proteases, we assessed the dependence of the cleavage on activation of the plasma contact system. DXS stimulated cleavage of the substrate was completely abrogated in normal plasma by pre-incubation with corn trypsin inhibitor,

FIGURE 2 Stimulated plasma kallikrein activity assay is specific for contact system activation. Panel A. Normal plasma was stimulated with dextran sulfate (DXS) 5 $\mu\text{g}/\text{mL}$ in the absence (red line) or presence (blue line) of CTI. Spontaneous kallikrein activity is shown by the black line. Panel B. Spontaneous kallikrein activity in normal plasma is shown by the black line. Kallikrein activity in plasma kallikrein-deficient plasma stimulated with DXS 5 $\mu\text{g}/\text{mL}$ is shown by the red line



a potent and highly specific FXII inhibitor (Figure 2A). Similarly, we observed no RFU over the 30-minute assay when prekallikrein deficient plasma was stimulated with 5 $\mu\text{g}/\text{mL}$ DXS (Figure 2B). These results confirm that the DXS-stimulated activity measured in our assay was FXII- and plasma kallikrein-dependent.

We assessed the reproducibility of the assay both in multiple replicates performed in a single assay as well as in multiple aliquots from the same subject analysed on different days. The average variance of multiple replicates of the same sample run on the same day averaged 7.0%. Variance of the plasma sample from seven different subjects run on separate days was 14.5%.

3.4 | The threshold-stimulated kallikrein assay distinguishes subjects with HAE-nl-C1INH or INHA from IHA

We analysed kallikrein activity in the entire cohort of subjects using plasma samples that were stimulated with DXS at 0, 1.25, 2.5 and 5.0 $\mu\text{g}/\text{mL}$. Table 1 shows that 2.5 and 5.0 $\mu\text{g}/\text{mL}$ DXS provided significant separation in kallikrein activity between the presumed bradykinin-mediated angioedema subjects (HAE-C1INH, acquired C1INH deficiency, HAE-nl-C1INH and INHA) and non-bradykinin-mediated angioedema subjects (IHA and NSC).

Sensitivity and specificity of the stimulated kallikrein activity using 2.5 or 5.0 $\mu\text{g}/\text{mL}$ DXS for the diagnosis of HAE-nl-C1INH or INHA were assessed based on our clinical phenotyping utilizing the kallikrein activity measured in the NSC group (mean - 170.8; SD - 171.4). We performed a sensitivity analysis, using the mean NSC kallikrein activity plus either 1, 2 or 3 standard deviations (75-99.75 confidence interval) as the upper limit for kallikrein activity. The sensitivity analysis was performed using four subject populations: (a) entire cohort; (b) entire cohort minus HAE-U or INHA subjects who had received a bradykinin-targeted medication anytime from just before to 6 days prior to the blood draw; (c) all subjects minus the C1INH deficient subjects; and (d) all subjects minus the C1INH deficient subjects as well as subjects who had received a bradykinin-targeted medication within the prior 6 days.

Table 2 shows the predictive value for the kallikrein assay (Table 2a after stimulating with 2.5 $\mu\text{g}/\text{mL}$ and Table 2b after

stimulating with 5.0 $\mu\text{g}/\text{mL}$ DXS). The positive predictive value (PPV), negative predictive value (NPV) and odds ratio (OR) are all considerably higher after stimulation with 2.5 $\mu\text{g}/\text{mL}$ DXS compared to 5.0 $\mu\text{g}/\text{mL}$ DXS. Therefore, we concentrated on the 2.5 $\mu\text{g}/\text{mL}$ DXS results. As expected, the sensitivity decreases and the specificity increases as the stringency of the upper limit of normal is increased. The PPV ranged from (80%-100%) while the NPV was more variable (63.33%-89.47%). Excluding subjects who had recently received a bradykinin-targeted medication improved the NPV (85.71%-89.47%), suggesting that the medication may have interfered with the assay.

Individual 2.5 $\mu\text{g}/\text{mL}$ DXS-stimulated kallikrein activity is shown in Figure 3A while Figure 3B shows the activity in subjects who had not recently received a bradykinin-targeted medication. In every case, HAE-C1INH, HAE-nl-C1INH and INHA groups had significantly elevated kallikrein activity compared to IHA and NSC groups. No significant differences were seen between the HAE-U-, INHA- and C1INH-deficient groups. As our HAE-nl-C1INH and INHA groups are predominantly female, we asked whether there was a gender difference in the assay. We found no difference in the 2.5 $\mu\text{g}/\text{mL}$ DXS-stimulated kallikrein activity between females vs males in our IHA and NSC groups (data not shown).

Receiver operator curve (ROC) analysis is a standard method to evaluate the value of a diagnostic test. We evaluated the threshold-stimulated kallikrein assay for the diagnosis of recurrent angioedema without urticaria in subjects who had not recently received bradykinin-targeted treatment as this represents the clinical scenario for which the test would be most useful. As shown in Figure 4, the kallikrein assay showed excellent characteristics as a diagnostic test based on area-under-the curve >0.91 in the ROC analysis.

3.5 | DXS-stimulated kallikrein activity can be modelled using rKLKB1

We constructed standard curves of kallikrein activity using rKLKB1, performed in an identical manner to the threshold-stimulated kallikrein assay except that varying amounts of rKLKB1 were added in place of plasma. Figure 5 shows the linear characteristics of the rKLKB1 activity in this assay. By interpolating the mean kallikrein activity, we could estimate that the mean amount of kallikrein

TABLE 2 Sensitivity analysis of predictive value of the threshold-stimulated kallikrein assay for the diagnosis of HAE and INHA

Population	Threshold SD ^a	True “+”	False “+”	True “-”	False “-”	Sensitivity (%)	Specificity (%)	PLR	NLR	OR	PPV	NPV
a) Dextran sulfate 2.5 µg/mL												
All	1	35	4	34	17	67.31	89.47	6.39	0.37	17.50	89.74%	66.67%
	2	33	2	36	19	63.46	94.74	12.06	0.39	31.26	91.67%	65.45%
	3	30	0	38	22	57.69	100.00	>100 ^b	0.42	>100 ^b	100.00%	63.33%
All subjects minus recently treated	1	25	4	34	4	86.21	89.47	8.19	0.15	53.13	86.21%	89.47%
	2	24	2	36	6	79.31	94.74	15.07	0.22	69.00	92.00%	85.71%
	3	23	0	38	6	79.31	100.00	>100 ^b	0.21	>100 ^b	100.00%	86.36%
All minus C1INH deficient	1	26	4	34	17	60.47	89.47	5.74	0.44	13.00	86.67%	66.67%
	2	24	2	36	19	55.81	94.74	10.60	0.47	22.74	92.31%	65.45%
	3	21	0	38	22	48.84	100.00	>100 ^b	0.51	>100 ^b	100.00%	63.33%
All minus C1INH deficient and recently treated	1	16	4	34	4	80.00	89.47	7.60	0.22	34.00	80.00%	89.47%
	2	14	2	36	6	70.00	94.74	13.30	0.32	42.00	87.50%	85.71%
	3	14	0	38	6	70.00	100.00	>100 ^b	0.30	>100 ^b	100.00%	86.36%
b) Dextran sulfate 5.0 µg/mL												
All	1	38	11	27	14	73.08	71.05	2.52	0.38	6.66	77.55%	65.85%
	2	25	3	35	27	48.08	92.11	6.09	0.56	10.80	89.29%	56.45%
	3	10	0	38	42	19.23	100.00	>100 ^b	0.81	>100 ^b	100.00%	47.50%
All subjects minus recently treated	1	23	11	27	6	79.31	71.05	2.74	0.29	9.41	67.65%	81.82%
	2	17	3	35	12	58.62	92.11	7.43	0.45	16.53	85.00%	74.47%
	3	8	0	38	21	27.59	100.00	>100 ^b	0.72	>100 ^b	100.00%	64.41%
All minus C1INH deficient	1	31	11	27	12	72.09	71.05	2.49	0.39	6.34	73.81	69.23
	2	21	3	35	22	48.84	92.11	6.19	0.56	11.14	87.50%	61.40%
	3	6	0	38	37	13.95	100.00	>100 ^b	0.86	>100 ^b	100.00%	50.67%
All minus C1INH deficient and recently treated	1	16	11	27	4	80.00	71.05	2.76	0.28	9.82	59.26%	87.10%
	2	13	3	35	7	65.00	92.11	8.23	0.38	21.67	83.33%	21.67%
	3	4	0	38	16	20.00	100.00	>100 ^b	0.80	>100 ^b	100.00%	70.37%

PLR, positive likelihood ratio; NLR, negative likelihood ratio; OR, odds ratio; PPV, positive predictive value; NPV, negative predictive value.

^aUpper limit of normal range calculated as NSC group mean value plus the indicated number of standard deviations.

^bCannot calculate value due to infinite result.

activity stimulated by 2.5 µg/mL of DXS in the studied groups were as follows: HAE-nl-C1INH, 0.37 µg/mL; INHA, 0.29 µg/mL; IHA and NSC, 0.09 µg/mL; and C1INH deficiency, 0.50 µg/mL.

3.6 | Increased threshold-stimulated kallikrein activity correlates with increased susceptibility to ex vivo HMWK cleavage

In a limited number of subjects, we assessed whether threshold DXS-stimulated kallikrein activity also tracked with enhanced susceptibility to DXS-induced HMWK cleavage. Figure 6 shows reduced HMWK immunoblots of plasmas from six representative subjects (A, NSC; B, IHA; C, HAE-C1INH; D, HAE-XII; E, HAE-U; and F, INHA) that were stimulated with various doses of DXS. The kallikrein activity at 2.5 µg/mL DXS is also shown for each sample. Plasmas that displayed an increased kallikrein activity demonstrated corresponding cleavage of HMWK at lower doses of DXS, supporting the

physiologic relevance of the kallikrein activity. Non-reduced HMWK immunoblots gave similar results (data not shown).

4 | DISCUSSION

Current consensus guidelines for the diagnosis of HAE-U and INHA ultimately depend on excluding alternative diagnoses. With the exception of the relatively small percentage of HAE-nl-C1INH patients with identified mutations, there are no diagnostic tests that can confirm the diagnosis of HAE-nl-C1INH or INHA. The absence of a confirmative assay causes substantial clinical problems, involving both under- and over-diagnosis of these conditions. Under-diagnosis carries an attendant risk of morbidity and mortality due to the failure to treat bradykinin-mediated angioedema attacks with effective drugs. Over-diagnosis leads to inappropriate use of costly medications and the failure to pursue other

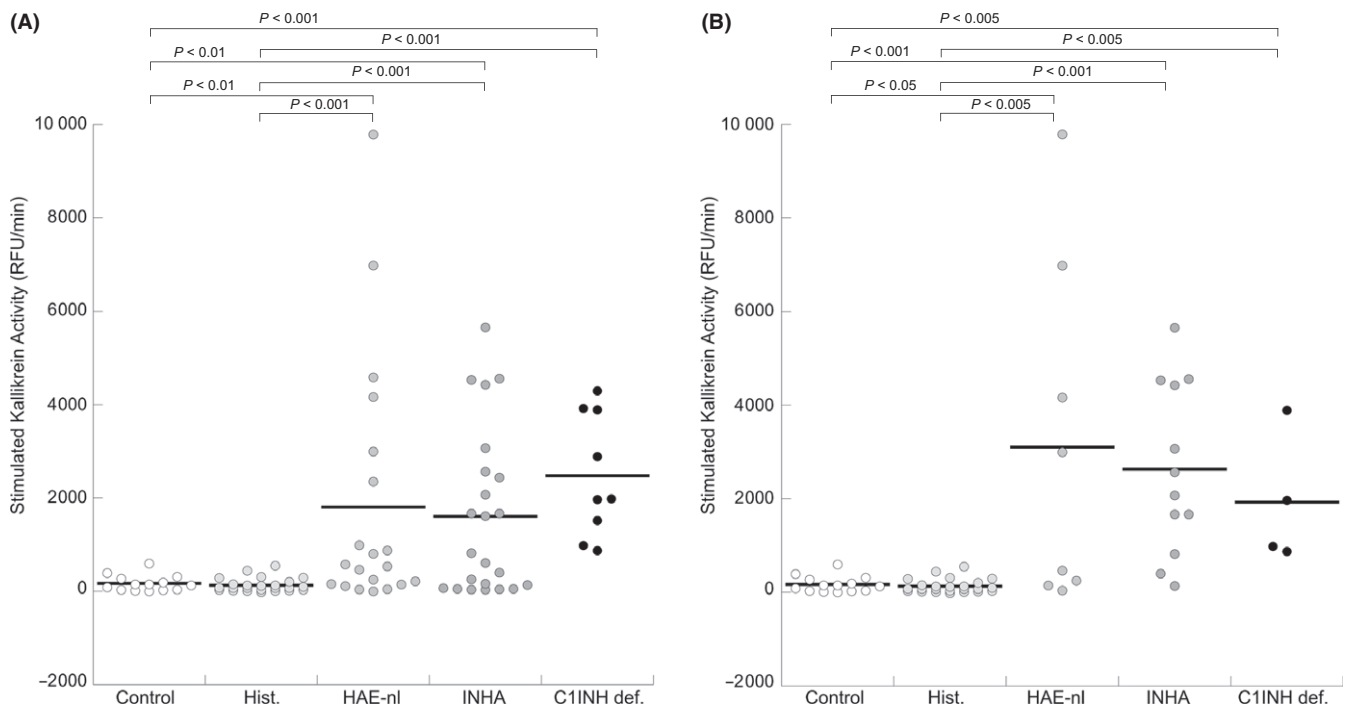


FIGURE 3 Kallikrein activity is increased in subjects in the HAE-nl-C1INH, INHA and C1INH deficiency groups. Individual results of plasma kallikrein activity after ex vivo stimulation with dextran sulfate (DXS) 2.5 μ g/mL are shown. Mean kallikrein activity is represented by the horizontal line for each group. Differences that were statistically significant by Wilcoxon test are shown. Panel A: all subjects. Panel B: subjects receiving a bradykinin-targeted medication within 6 d of the sample collection were excluded

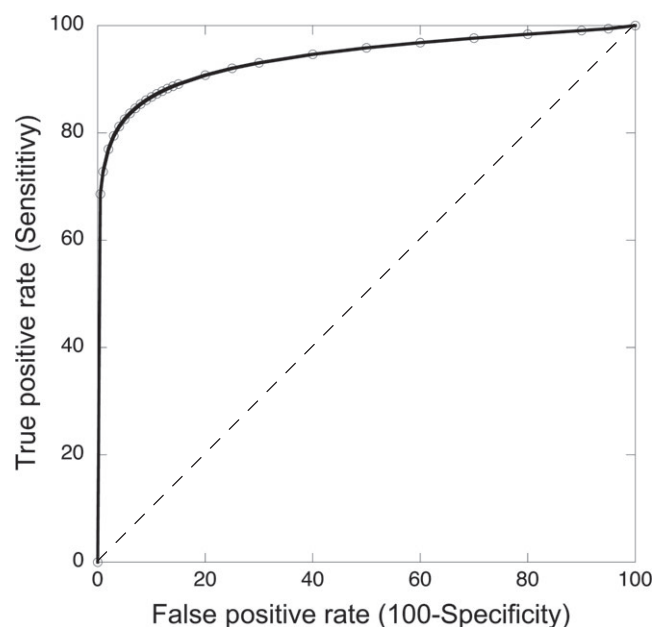


FIGURE 4 Receiver operating curve for the stimulated plasma kallikrein activity assay. The receiver operator curve (ROC) was generated using all subjects with recurrent angioedema without urticaria who had not received a bradykinin-targeted medication within 6 d of the sample collection. The line of no-discrimination is shown as a dashed line

treatments that could ameliorate symptoms. Both under- and over-diagnosis cause considerable confusion for patients and physicians. In this report, we describe a threshold-stimulated kallikrein assay

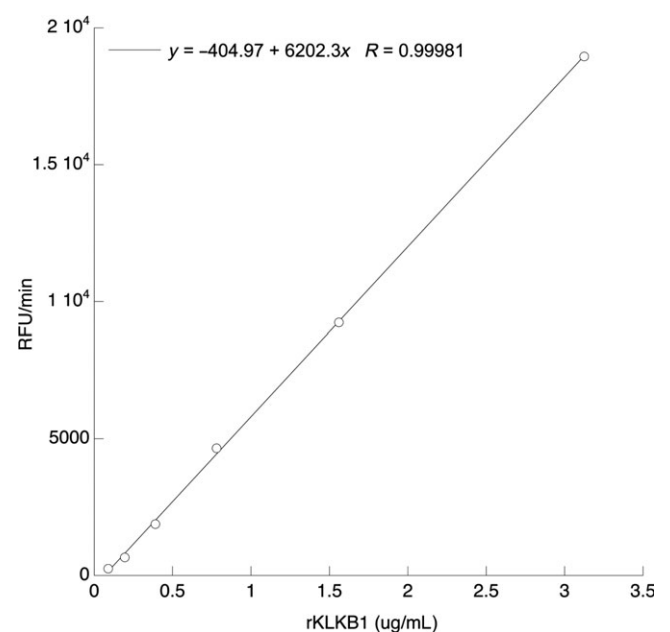


FIGURE 5 Recombinant human plasma kallikrein yields a linear curve that can be used to standardize the assay. Various doses of rKLKB1 were added in place of subject plasma and the activity measured as described

that provides a new tool to positively identify patients with HAE-U and INHA.

Clinical phenotyping was critical to the analysis and utilized stringent criteria. First, we utilized current consensus guidelines. Second, phenotype was determined by comprehensive clinical evaluation and

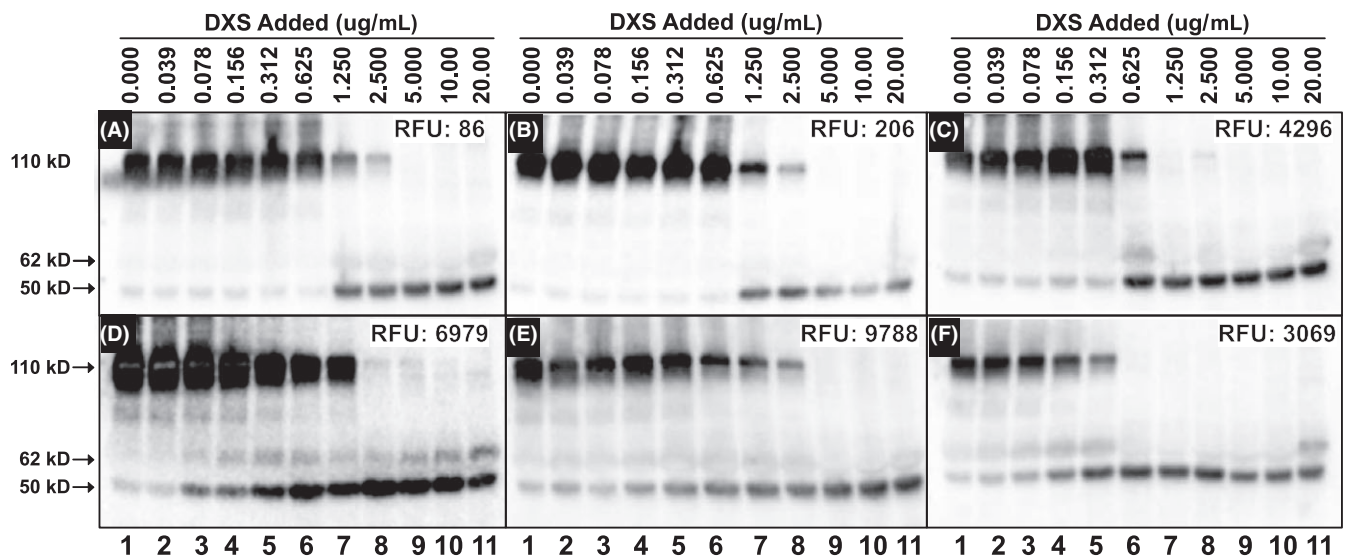


FIGURE 6 Subjects with bradykinin-mediated angioedema show cleavage of high molecular weight kininogen at a lower dose of dextran sulfate (DXS) than histaminergic angioedema or non-swelling control subjects. Plasma was incubated with various doses of DXS then immunoblotted for high molecular weight kininogen under reducing conditions. The intact one-chain kininogen migrates at an apparent molecular weight of 110 kD while the cleaved light chain appears as 62 and 50 kD bands. The relative fluorescence units (RFU) values from the same plasma samples stimulated with DXS 2.5 µg/mL are shown. The panels are as follows: A, non-swelling control; B, histaminergic angioedema; C, HAE-C1INH; D, HAE-FXII; E, HAE-U; and F, INHA

chart review by at least two physicians with expertise in angioedema. Third, subjects were subdivided into high, moderate or low probability categories based on additional strict criteria. Fourth, in the rare cases of disagreement about diagnosis or probability, all three expert clinicians reviewed the data together until a consensus was reached. Clinicians were blinded to the assay results until phenotyping was completed.

Two groups were excluded from further analysis. The first consisted of seven subjects classified as HAE-U low probability. These subjects reported predominantly episodic abdominal pain without documented imaging showing bowel wall edema and did not report clear benefit from treatment with a bradykinin pathway acting drug. While these subjects appeared to meet the consensus criteria for HAE-U, our clinical impression was that they did not have true bradykinin-mediated angioedema and likely did not have angioedema. When the kallikrein data were unblinded, we found that each of these subjects had normal kallikrein activity—corroborating the clinical impression. The second group consisted of eight subjects who could not be unequivocally phenotyped by our clinical criteria. These subjects reported a partial response to treatment with high-dose antihistamines but also described significant improvement when treated with a bradykinin-targeted medication. Interestingly, kallikrein activity in this group was elevated in five of the eight subjects, suggesting a possible mixed pathophysiology in at least some of these individuals.

Increased threshold-stimulated kallikrein activity was highly correlated with clinical phenotype. Most new patients with recurrent angioedema without urticaria will not have been exposed to a bradykinin pathway acting drug. In these patients, we found a PPV ranging from 85.71% to 89.47% and a NPV ranging from 85.71% to 89.47%

depending on the threshold cut-off. The high specificity of this assay suggests that patients with an elevated kallikrein activity should be considered eligible for a therapeutic trial with a bradykinin-targeted medication. The converse situation where a patient suspected of having HAE-U or INHA has a normal kallikrein activity should alert the physician to the possibility that this patient might not have a bradykinin-mediated angioedema. Trials of alternative mast cell-targeted medications such as leukotriene modifiers or omalizumab should be considered prior to any trial of a bradykinin-targeted medication.

The threshold-stimulated kallikrein assay should be adaptable to clinical practice. Samples can be stored at -80°C for at least a month prior to assay, the assay itself is relatively simple, fast, and rKLKB1 can be used to standardize the assay allowing comparisons between results obtained at different times or locations. The assay is entirely dependent on activation of the plasma contact system and is highly reproducible. The receiver operating characteristic curve of the assay appears excellent, supporting the potential clinical utility of the assay. Limitations of the assay include the need to collect the blood sample using good technique with rapid processing of the sample. It is important to note that recently administered bradykinin-targeted medications may interfere with the assay, although this has not been specifically tested.

Several other assays have been reported to provide useful information in diagnosing HAE-U or INHA. Spontaneous kallikrein chromogenic activity was reported to identify HAE-nl-C1INH patients.²¹ We failed to confirm any increase in spontaneous kallikrein activity in our HAE-nl-C1INH subjects. Decreased levels of PAI-2 in patients with HAE-XII and HAE-U were reported²⁸; however, subsequent reports failed to confirm the initial observation.^{29,30}

In conclusion, we report a novel biomarker for the diagnosis of HAE-U and INHA. The threshold-stimulated kallikrein assay shows high specificity and positive likelihood ratio for presumed bradykinin-mediated angioedema. The lack of an existing diagnostic test or biomarker for HAE-U or INHA has caused substantial difficulties for patients and physicians resulting in both the failure to treat patients with severe life-threatening angioedema with appropriate medications as well as inappropriate use of costly medications. The inability to establish an accurate diagnosis also severely hampers the ability to conduct genetic studies or therapeutic trials due to the non-homogeneity of the population. We propose that the threshold-stimulated kallikrein assay can be adapted for clinical use and may improve patient care while aiding future studies aimed at identifying specific causes of bradykinin-mediated angioedema.

ACKNOWLEDGEMENTS

This research was funded through a gift from the US Hereditary Angioedema Association (US HAEA). The authors wish to thank the US HAEA and our patients for their support and help, and Ramon Serrano PhD for helpful discussions.

CONFLICT OF INTEREST

Dr. Lara-Marquez has served as a paid consultant to CSL Behring, unrelated to this work. Dr. Christiansen has served as a paid consultant to CSL Behring and Shire, unrelated to this work. Dr. Riedl has received funding for research unrelated to this work from BioCryst, CSL Behring, Pharming, Shire; and has served as paid consultant unrelated to this work from Adverum, Alnylam, BioCryst, CSL Behring, Kalvista, Ionis, Shire, Pharming; and has participated as a speaker unrelated to this work for CSL Behring, Pharming and Shire. Mr. Herschbach reports no conflicts. Dr. Zuraw has served as a paid consultant to Adverum, Alnylam, BioCryst, CSL Behring, Novartis, Sanofi and Shire; and has or may receive research funding from Shire and Ionis, all unrelated to this work.

ORCID

Maria L. Lara-Marquez  <http://orcid.org/0000-0003-1014-6524>

Marc A. Riedl  <http://orcid.org/0000-0003-3460-1544>

Bruce L. Zuraw  <http://orcid.org/0000-0003-0640-6768>

REFERENCES

- Zuraw BL. Clinical practice. Hereditary angioedema. *N Engl J Med*. 2008;359:1027-1036.
- Zuraw BL, Bernstein JA, Lang DM, et al. A focused parameter update: hereditary angioedema, acquired C1 inhibitor deficiency, and angiotensin-converting enzyme inhibitor-associated angioedema. *J Allergy Clin Immunol*. 2013;131:1491-93.e25.
- Davis AE 3rd, Lu F, Mejia P. C1 inhibitor, a multi-functional serine protease inhibitor. *Thromb Haemost*. 2010;104:886-893.
- Walford HH, Zuraw BL. Current update on cellular and molecular mechanisms of hereditary angioedema. *Ann Allergy Asthma Immunol*. 2014;112:413-418.
- Christiansen SC, Zuraw BL. Laboratory approaches for assessing contact system activation. *Immunol Allergy Clin North Am*. 2017;37:527-539.
- Kaplan AP, Joseph K. Pathogenic mechanisms of bradykinin mediated diseases: dysregulation of an innate inflammatory pathway. *Adv Immunol*. 2014;121:41-89.
- Bork K, Barnstedt SE, Koch P, Traupe H. Hereditary angioedema with normal C1-inhibitor activity in women. *Lancet*. 2000;356:213-217.
- Binkley KE, Davis A 3rd. Clinical, biochemical, and genetic characterization of a novel estrogen- dependent inherited form of angioedema. *J Allergy Clin Immunol*. 2000;106:546-550.
- 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.
- 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;141:31-35.
- Kiss N, Barabas E, Varnai K, et al. Novel duplication in the F12 gene in a patient with recurrent angioedema. *Clin Immunol*. 2013;149:142-145.
- Bork K, Wulff K, Hardt J, Witzke G, Lohse P. Characterization of a partial exon 9/intron 9 deletion in the coagulation factor XII gene (F12) detected in two Turkish families with hereditary angioedema and normal C1 inhibitor. *Haemophilia*. 2014;20:e372-e375.
- Bork K, Wulff K, Hardt J, Witzke G, Staubach P. Hereditary angioedema caused by missense mutations in the factor XII gene: clinical features, trigger factors, and therapy. *J Allergy Clin Immunol*. 2009;124:129-134.
- Bafunno V, Firinu D, D'Apolito M, et al. Mutation of the angiotensin-converting enzyme 1 gene (ANGPT1) associates with a new type of hereditary angioedema. *J Allergy Clin Immunol*. 2018;141:1009-1017. in press.
- Bork K, Wulff K, Steinmuller-Magin L, et al. Hereditary angioedema with a mutation in the plasminogen gene. *Allergy*. 2018;73:442-450. in press.
- Zuraw BL, Bork K, Binkley KE, et al. Hereditary angioedema with normal C1 inhibitor function: consensus of an international expert panel. *Allergy Asthma Proc*. 2012;33(Suppl 1):S145-S156.
- Bork K, Wulff K, Witzke G, Stanger C, Lohse P, Hardt J. Antihistamine-resistant angioedema in women with negative family history: estrogens and F12 gene mutations. *Am J Med*. 2013;126(1142):e9-e14.
- Bjorkqvist J, de Maat S, Lewandrowski U, et al. Defective glycosylation of coagulation factor XII underlies hereditary angioedema type III. *J Clin Invest*. 2015;125:3132-3146.
- de Maat S, Bjorkqvist 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;138:1414-1423.
- Ghannam A, Defendi F, Charignon D, et al. Contact system activation in patients with HAE and normal C1 inhibitor function. *Immunol Allergy Clin North Am*. 2013;33:513-533.
- Defendi F, Charignon D, Ghannam A, et al. Enzymatic assays for the diagnosis of bradykinin-dependent angioedema. *PLoS ONE*. 2013;8:e70140.
- Boccon-Gibod I, Bouillet L. Safety and efficacy of icatibant self-administration for acute hereditary angioedema. *Clin Exp Immunol*. 2012;168:303-307.
- Marcos C, Lopez Lera A, Varela S, Linares T, Alvarez-Eire MG, Lopez-Trascasa M. Clinical, biochemical, and genetic characterization of type III hereditary angioedema in 13 Northwest Spanish families. *Ann Allergy Asthma Immunol*. 2012;109(195-200):e2.
- Cronin JA, Maples KM. Treatment of an acute attack of type III hereditary angioedema with ecallantide. *Ann Allergy Asthma Immunol*. 2012;108:61-62.

25. Cicardi M, Aberer W, Banerji A, et al. EAACI Huppo, classification, diagnosis, and approach to treatment for angioedema: consensus report from the hereditary Angioedema International working group. *Allergy*. 2014;69:602-616.
26. Berrettini M, Lammle B, White T, et al. Detection of in vitro and in vivo cleavage of high molecular weight kininogen in human plasma by immunoblotting with monoclonal antibodies. *Blood*. 1986;68:455-462.
27. Eng J. ROC analysis: web-based calculator for ROC curves. 2014. Baltimore: Johns Hopkins University. <http://www.jrocf.it.org>. Accessed Aug 10, 2017.
28. Joseph K, Tholanikunnel BG, Wolf B, Bork K, Kaplan AP. Deficiency of plasminogen activator inhibitor 2 in plasma of patients with hereditary angioedema with normal C1 inhibitor levels. *J Allergy Clin Immunol*. 2015;137:1822-1829.
29. Marlu R, Deroux A, Du-Thanh A, Boccon-Gibod I, Launay D, Bouillet L. Normal PAI-2 level in French FXII-HAE patients. *J Allergy Clin Immunol*. 2017;139:1719-1720.
30. Joseph K, Constantino-Silva RN, Grumach AS, et al. Reply. *J Allergy Clin Immunol*. 2017;139:1720-1721.

How to cite this article: 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;48:1429–1438. <https://doi.org/10.1111/cea.13219>