

Laboratory Approaches for Assessing Contact System Activation



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KEYWORDS

- Contact system • Hereditary angioedema • C1 inhibitor • Bradykinin • C4
- C1 inhibitor complexes • Vascular permeability

KEY POINTS

- Activation of the plasma contact system generates bradykinin and causes swelling in hereditary angioedema (HAE) due to C1 inhibitor (C1INH) deficiency (HAE-C1INH), HAE associated with factor XII (FXII) mutations, and acquired C1INH deficiency (AC1D).
- Bradykinin is suspected to be the mediator of swelling in several other forms of angioedema, such as HAE of unknown type, idiopathic nonhistaminergic angioedema, and angioedema associated with inhibitors of kininases.
- Laboratory tests to detect activation of the contact system and generation of bradykinin remain limited to research settings at the current time.

INTRODUCTION

Angioedema is a common clinical finding that can reflect multiple underlying pathophysiologic mechanisms. It is the physical manifestation of fluid movement from the blood vessel into the interstitial fluid that occurs as a consequence of transiently increased vascular permeability. Unlike hydrostatic or oncotic causes of edema, angioedema results from the action of mediators on endothelial cells that disrupt the adherens junction, resulting in vascular leak.¹ Histamine, cysteinyl leukotrienes, and bradykinin are recognized as the principal biologic mediators of swelling.² A majority of angioedema cases are thought to involve mast cell activation, triggering the release of histamine and other mediators, such as leukotrienes.³ Less frequently, angioedema is linked to the generation of bradykinin. Because the prognosis and treatment of bradykinin-mediated angioedema are markedly different from those of histamine-mediated or leukotriene-mediated angioedema, it is of considerable clinical

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importance to identify whether angioedema is mast cell mediated (often called histaminergic) or bradykinin mediated. This article reviews the forms of angioedema that are likely bradykinin mediated and discusses the laboratory approaches for establishing a diagnosis.

BRADYKININ-MEDIATED ANGIOEDEMA

The kallikrein-kinin system consists of proteases (kallikreins) that cleave their substrate (kininogens) to release bioactive kinin. There are 2 primary kallikreins, tissue kallikrein and plasma kallikrein (PK), which are immunologically and functionally distinct. Tissue kallikrein is widely distributed in mammalian tissues and releases Lys-bradykinin (BK) from low-molecular-weight kininogen. To date, there is no evidence that tissue kallikrein participates in angioedema. On the other hand, there is substantial evidence showing that PK is involved in bradykinin-mediated angioedema (See Allen P. Kaplan and Kusumam Joseph's article, "[Pathogenesis of Hereditary Angioedema: the Role of the Bradykinin Forming Cascade](#)," in this issue).

Activation of the contact system results in the generation of bradykinin, a biologic mediator of enhanced endothelial permeability. Bradykinin-mediated vascular leakage in susceptible individuals can trigger attacks of angioedema typified by nonpitting asymmetric tissue swelling. There is strong evidence that implicates activation of the contact system and generation of bradykinin in the pathogenesis of swelling in HAE-C1INH. The evidence for a role of contact system activation and bradykinin in HAE with normal C1INH (HAE-nl-C1INH) is highly suggestive but currently less clearly delineated. The evidence for both forms of HAE is summarized.

Role of Bradykinin in Hereditary Angioedema due to C1 Inhibitor Deficiency

Landerman and colleagues⁴ reported in 1962 that plasma from patients with HAE failed to inhibit PK and plasma dilution factor—now known to be FXII. One year later, Donaldson and Evans⁵ established that C1INH activity was absent in patients with HAE, thus providing the definitive biochemical explanation for the cause of type I HAE. Soon thereafter, Rosen and colleagues⁶ described a group of HAE patients who had normal plasma C1INH protein levels but lacked C1INH functional activity, thereby defining type II HAE.

C1INH is the major inhibitor of both active PK and active FXII of the contact system cascade.⁷ The genesis of swelling in HAE types I and II has been established to evolve from the generation of bradykinin, engagement with the constitutively expressed B2-receptor on endothelial cells, and resultant vascular leak. Acceptance of the central role of the contact system in HAE was founded on series of observations recently reviewed.⁸ In 1980 Curd and colleagues⁹ detected active PK in the interstitial fluid from patients with HAE followed in 1982 by sequencing bradykinin as the permeability inducing factor.¹⁰ This observation was extended by Fields and colleagues,¹¹ demonstrating that bradykinin, not C2 kinin, was responsible for vascular permeability. Further proof of the central role of contact system activation in HAE included the consumption of high-molecular-weight kininogen (HMWK) and cleavage of PK during attacks¹² as well as evidence that HMWK was cleaved during HAE attacks.^{13–15}

Subsequently, plasma BK levels in HAE-C1INH subjects were shown significantly increased during attacks and normal or slightly increased during remission.^{16,17} Most recently, well-controlled clinical trials have shown that the PK inhibitor ecallantide as well as the bradykinin B2-receptor antagonist icatibant are effective in the treatment of HAE-C1INH.^{18,19} The increased circulating BK levels during attacks have been claimed to originate from the site of angioedema.¹⁷ Based on the

catabolism of bradykinin in both animal and human models, this has been interpreted as a systemic activation process of the contact system with local manifestation of disease.²⁰

Although many of the attacks in HAE are unpredictable, stress, infection, and trauma have all been implicated,²¹ which can be linked to the candidate physiologic activators (discussed previously). Enzymatic activation of PK on endothelial cells has also been associated with heat shock protein 90,²² which is secreted during cellular stress. Heat shock protein 90, which lacks enzymatic activity, can interact with plasma prekallikrein (PPK) in complex with HMWK in the presence of Zinc (Zn²⁺) to convert PPK to PK.²³ The stoichiometric generation of PK from PPK could thus play an initiating role in contact system activation and attacks of angioedema.

Role of Bradykinin in Hereditary Angioedema with Normal C1 Inhibitor

In 2000, Bork and colleagues²⁴ and Binkley and colleagues²⁵ independently described a form of HAE characterized by normal C1INH antigenic and functional levels. Thus, HAE is now classified as either HAE-C1INH or HAE-nl-C1INH. A minority (approximately 25% in Europe but much less in the United States) of patients with HAE-nl-C1INH were subsequently shown to have mutations in exon 9 of the F12 gene.²⁶ Based on this, HAE-nl-C1INH has been subdivided into HAE-XII for patients with an F12 mutation and HAE-nl-C1INH unknown type (HAE-U) for patients without an F12 mutation.²⁷ The mutant FXII has been shown to confer an increased ease of activation of the contact cascade.²⁸ FXII HAE mutations seem to create new sites susceptible to enzymatic cleavage by plasmin, thereby escaping inhibition by C1INH and providing a pathway for bradykinin formation.²⁹ Polymorphisms associated with lower levels of both aminopeptidase P and angiotensin-converting enzyme (ACE), enzymes involved in bradykinin degradation, have been reported in subjects with the FXII mutation.³⁰ As is the case for HAE types I and II, there is substantial evidence for HAE-nl-C1INH that the swelling is mediated by kinin generation.^{31,32} Successful treatment with a B2-antagonist has also provided putative evidence that bradykinin is the mediator of swelling.³³

Role of Bradykinin in Other Forms of Angioedema

Bradykinin is also thought to be the primary mediator of swelling in several other types of angioedema. AC1D is clinically nearly indistinguishable from HAE-C1INH. The pathophysiologic consequences of C1INH deficiency would be expected to be similar whether the deficiency is hereditary or acquired. Not surprisingly, angioedema attacks in patients with AC1D has been shown accompanied by elevated plasma bradykinin levels.¹⁶

Another group of patients is defined by angioedema unresponsive to antihistamines who have normal C1INH, normal FXII, and no family history of angioedema. This group has been called, idiopathic angioedema unresponsive to antihistamines.³⁴ Many of these patients do respond to bradykinin-targeted therapy, and the angioedema in these patients may be bradykinin mediated. Because of the low penetrance seen in HAE-U, and the lack of a genetic marker, it is possible that these patients are actually part of the HAE-U group.

Angioedema has additionally been reported to occur in patients treated with a variety of drugs that act on the renin-angiotensin system, especially ACE inhibitors (ACEIs) (See Cosby Stone and Nancy J. Brown's article, "[ACE-Inhibitor and Other Drug-Associated Angioedema](#)," in this issue). Plasma levels of bradykinin were shown to be very high at the time of swelling in ACE angioedema (ACEI-AE) but returned to normal after withdrawal of the drug and resolution of the angioedema.¹⁶ In 3 other patients with a history of ACEI-AE, bradykinin levels were high during ACEI treatment.³⁵

Although anecdotal clinical experience has suggested that icatibant and ecallantide have beneficial effects in the treatment of ACEI-AE, randomized clinical studies have yielded mixed but not encouraging results—possibly due to the logistical challenges involved in treating this form of angioedema. One randomized double-blind clinical trial show that icatibant was effective in treating ACEI-AE if given early in the attack.³⁶ Two subsequent studies failed to show any benefit for icatibant.³⁷

LABORATORY DIAGNOSIS OF ANGIOEDEMA

Early recognition and proper diagnosis of bradykinin-mediated angioedema are essential to avoid excess morbidity and potentially fatal outcomes from the disease. Clinically, a high index of suspicion should be maintained in cases of recurrent angioedema in absence of urticaria or unexplained spontaneously reversible abdominal pain. Ultimately, clinical suspicion must be confirmed by appropriate laboratory testing (Table 1).

Angioedema Associated with C1 Inhibitor Deficiency

Laboratory measurements are targeted for both activation of the contact system and importantly the complement cascade for which C1 inhibitor is a primary regulatory

Table 1 Clinical situations where laboratory testing for contact system activation may be most useful		
Test	Major Advantages	Major Disadvantages
C1INH level	Easy to perform; critical for diagnosis of HAE-C1INH and AC1D	Antigenic level may give deceiving result; can be false positive or negative; can be influenced by treatment
C4 level	Important for diagnosis of HAE-C1INH and AC1D	Substantial variability in the general population; can be influenced by treatment
Protease-inhibitor complexes	Easy to measure as biomarker of contact system activation; may be useful as novel assay for C1INH function	Complexes are short-lived in vivo; subject to ex vivo artifact; may be artificially low in C1INH deficiency
Cleaved protease	Directly reflects contact system activation	Difficult to measure, requires immunoblotting or antibody to neoepitope; subject to ex vivo artifact
Kallikrein activity	Simple and straightforward assay; able to be standardized	Spontaneous activity subject to ex vivo artifact; activity not completely specific
Cleaved HMWK	Stable biomarker of contact system activation; moderately sensitive	Difficult to measure, requires immunoblotting or antibody to neoepitope; subject to ex vivo artifact
Bradykinin level	Theoretically the best biomarker of contact system activation	Very difficult to measure; extremely short half-life of peptide; subject to ex vivo artifact
Fibrinolysis	Easy to measure; may reflect primary or secondary events in contact system activation	Very nonspecific and subject to variability

protein.⁷ For HAE-C1INH, commercially available laboratory evaluation is straightforward with type I HAE characterized by low C4 and C1INH antigenic levels whereas type II HAE is characterized by low C4 levels and low C1INH functional levels.²¹ There can be some variability in the measurement of C4 levels, which in particular can confound the diagnosis between attacks or during treatment with attenuated androgen therapy resulting in normalization of the C4.³⁸ Although not commercially available, the most sensitive test is a C4d/C4 ratio reflecting complement system activation in the setting of deficient C1INH functional levels. A normal C4d/C4 ratio excludes C1INH deficiency, even if the patient is not actively swelling or is receiving attenuated androgen treatment.³⁹ AC1D typically presents with low C4 and C1INH levels but can be distinguished from HAE-C1INH by the later age of onset, lack of a family history of angioedema, and often a low C1q level.⁴⁰

Bradykinin-Mediated Angioedema with Normal C1 Inhibitor

The diagnosis of HAE-nl-C1INH presently rests on a collection of criteria, including clinical expression of disease, hereditary pattern, and response to therapy.²⁷ Unlike for HAE-C1INH, there is no available laboratory confirmation or clear-cut biomarker. HAE-nl-C1INH is characterized not only by normal C4 and C1INH levels but also by a lack of SERPING1 mutations. A minority of HAE-nl-C1INH patients have a mutation in exon 9 of the F12 gene.²⁶ A diagnosis of ACEI-associated angioedema or ARB-associated angioedema is typically a diagnosis of exclusion, which is confirmed if the angioedema remits after stopping the drug.

BIOMARKERS OF CONTACT SYSTEM ACTIVATION

Biomarkers can be defined as objective measures that reflect a biologic process. Validated biomarkers of contact system activation would infer whether or not bradykinin had been generated and could thus be extremely helpful in characterizing angioedema as either bradykinin mediated or not. Beyond their use for accurate diagnosis, biomarkers of contact system activation may be valuable in patients with a known diagnosis of bradykinin-mediated angioedema to understand whether specific symptoms are related to an angioedema attack. A variety of different potential biomarkers of contact system activation are summarized, describing the assay, its interpretation, and its limitations.

Measurement of C1 Inhibitor Levels

C1INH is the major inhibitor of the early complement proteases C1r, C1s, and the mannose-binding lectin-associated serine protease (MASP) 1 and 2 as well as the contact-system proteases, PK and activated FXII.⁷ C1INH is also a minor inhibitor of plasmin and factor XIa. In the absence of sufficient C1INH levels, activation of target proteases is enhanced. Because of its key role in inhibiting the contact system proteases, low C1INH levels are a reasonable surrogate marker for contact system activation. Although it is accepted that the fundamental cause of HAE-C1INH is a deficiency of functional C1INH, it has proved difficult to show that C1INH levels directly correlate with disease severity although 1 article reported that C1INH functional levels correlated with overall HAE severity.⁴¹

C1INH levels can be measured by assessing total antigenic protein levels or by assessing C1INH functional levels. In type I HAE and AC1D, C1INH antigenic levels are reduced, and this has been a key component in the diagnosing these forms of angioedema. C1INH antigenic levels are normal in type II HAE and, therefore, this diagnosis depends on the measurement of C1INH function.

At least 3 different ways to assess C1INH function have been described. The ability of C1INH to inhibit active C1s in a solid-phase ELISA assay is the most commonly used laboratory test for C1INH function. This test, however, can show false normal values,⁴² and caution should be used in interpreting a low normal result. The chromogenic C1INH functional assay measures the ability of C1INH to inhibit C1s activity in a solution phase assay. This test is more sensitive than the ELISA assay but can show falsely low C1INH functional levels if the plasma was not prepared and handled carefully. The third assay is the measurement of C1INH function determined by its binding to immobilized activated FXII or kallikrein.⁴³ This assay has the advantage of directly measuring the ability of C1INH to complex with the relevant contact system proteases; however, this test is not yet commercially available.

C4 Levels

Measurement of C4 levels is a routine component of the standard diagnostic panel for HAE-C1INH and AC1D. Although not 100% sensitive when measured at a time a patient is not swelling, the C4 level is invariably decreased during attacks of angioedema in patients with HAE-C1INH. When FXIIa is generated, it can be further cleaved by kallikrein to generate B-FXIIa (also known as FXII_f), which can efficiently activate C4.⁴⁴ Because of this, measurement of C4 during an attack of angioedema might have some usefulness even in the situation where C1INH is normal.

Measurement of Protease-Inhibitor Complexes

A more direct biomarker for contact system activation is the measurement of in vivo formed protease-inhibitor complexes. C1INH is a member of the serpin superfamily of inhibitors.⁴⁵ Like other serine protease inhibitors, it has been described as a molecular mousetrap, with a reactive mobile loop critical for their functional activity.⁴⁶ The reactive center is fully exposed in the aqueous environment. Target proteases attack and cleave the Arg⁴⁴⁴-Thr⁴⁴⁵ bond within the C1INH reactive mobile loop leading to a thermodynamically favorable conformational rearrangement that irreversibly traps the protease within the C1INH molecule.⁴⁷ C1INH thus functions as a suicide inactivator of PK of the contact system, and detection of these complexes could serve as a biomarker of contact system activation.

C1INH forms SDS-stable complexes with FXIIa and kallikrein.^{48,49} Sandwich-type ELISA assays developed to detect these complexes were able to sensitively measure ex vivo activation of the contact system.^{50,51} Attempts to use these assays to measure in vivo activation of the contact system, however, showed modest increases in protease-inhibitor complexes despite substantial in vivo activation,^{52,53} including during HAE attacks (Zuraw, unpublished data). Presumably, these complexes are cleared rapidly in vivo, preventing a significant increase to be measured.^{54–56} A specific serpin-enzyme complex receptor was identified on human hepatoma cells and monocytes.⁵⁷ When samples are drawn rapidly (5–15 minutes) after a discrete activation of the contact system, increased kallikrein-C1INH and FXIIa-C1INH levels can be measured.⁵⁸ C1INH also forms complexes with both MASP-1 and MASP-2.^{59,60} The relevance of this stems from MASPs reported as capable of generating bradykinin from HMWK.⁶¹ An additional problem with measuring protease-C1INH levels in HAE-C1INH is that the low levels of functional C1INH limit the sensitivity of these assays⁶²; however, this would not be a problem in bradykinin-mediated angioedema not associated with C1INH deficiency.

Cleavage of Zymogen Proteases

Activation of the contact system is associated with cleavage of the zymogen proteases prekallikrein and FXII into their active proteolytic forms, kallikrein and FXII. This cleavage can be monitored by immunoblotting based on changes in the migration of the specific bands. A monoclonal antibody that recognizes only FXIIa or bFXIIa has been reported⁶³ and could be used to detect activated FXII without immunoblotting.⁶⁴ It is likely, however, that these proteases do not circulate for long before they are complexed to inhibitors or cleared.

High-Molecular-Weight Kininogen Cleavage

Coagulant levels of HMWK were shown to fall during severe attacks of angioedema in patients with HAE-C1INH.¹² Detection of HMWK cleavage by immunoblotting was first reported in 1986 and was found a highly sensitive marker for HAE-C1INH attacks.^{13–15} During HAE-C1INH attacks, HMWK was almost completely cleaved.⁶⁵ Measurement of HMWK cleavage in remission samples has also been shown to correlate with HAE-C1INH disease severity.⁶⁶ HMWK cleavage was also reported to be increased in women with HAE-nl-C1INH.⁶⁷ Although promising for use as a biomarker of contact system activation, measurement of HMWK cleavage remains laborious and subject to significant potential artifact from ex vivo contact system activation during blood drawing, processing, and storage. Although attacks of HAE are localized to 1 or more sites of swelling the degree of HMWK cleavage measured seems to point toward a systemic activation process.²⁰

Bradykinin Levels

Direct measurement of bradykinin could be an ideal test to confirm the involvement of bradykinin in angioedema; however, the short half-life of bradykinin in plasma plus the technical difficulties in measuring bradykinin have limited the applicability of this technique. Using scrupulous attention to the process of preparing the samples, elevated levels of bradykinin were detected during attacks of angioedema in patients with HAE-C1INH, AC1D, and ACEI-associated angioedema.^{16,17,68} Measurement of bradykinin metabolites that have a longer circulating half-life than bradykinin may be more feasible, although at present these assays are highly specialized.^{69,70}

Measurement of Kallikrein Activity

Kallikrein amidolytic activity can be readily measured using synthetic substrates. Several reports have described increased kallikrein activity in the plasma of patients with HAE-C1INH⁶⁶ and HAE-nl-C1INH.³² Although promising, these assays need to be validated and confirmed.

Other Assays

Although it has yet to be confirmed as a reliable biomarker, Joseph and colleagues⁷¹ showed that HAE-XII and HAE-U patients have markedly reduced plasminogen activator inhibitor type 2 levels compared with normal controls or patients with HAE-C1INH. Activation of the FXII-kallikrein pathway results in secondary activation of the fibrinolytic system. Elevations of D-dimers and plasmin- α_2 -antiplasmain complexes have been consistently observed in HAE-C1INH.^{65,72,73}

SUMMARY

The role of contact system activation and generation of bradykinin have been clearly established for HAE-C1INH (Figs. 1 and 2). Insights continue to emerge regarding the

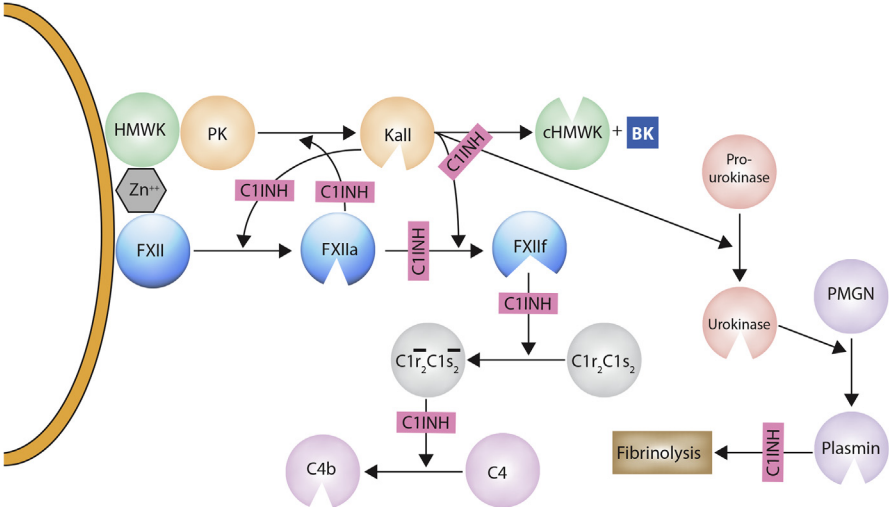


Fig. 1. Schematic of contact system activation. Figure shows the reciprocal activation of PPK (PK) and FXII to kallikrein (Kall) and FXIIa with cleavage of HMWK to cleaved HMWK (cHMWK) and release of bradykinin (BK). Kallikrein also activates prourokinase to urokinase, which activates plasminogen (PMGN) to plasmin. B-FXIIa (FXIIb) can activate the classical complement pathway. Steps inhibited by C1INH as shown.

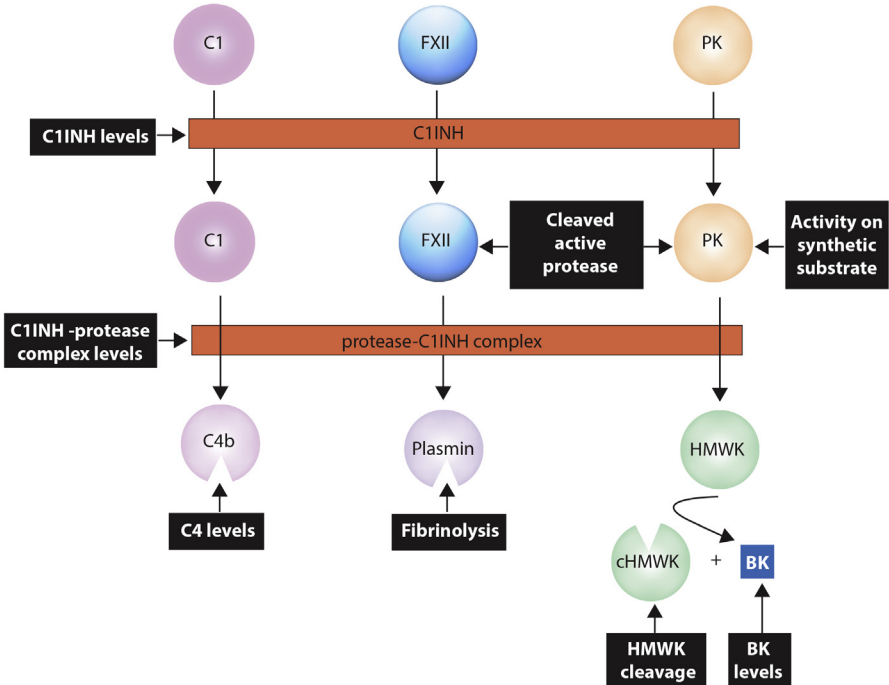


Fig. 2. Schematic of the major assays available to assess contact system activation. The contact system is shown in an abstracted manner that shows the different levels of activation and cleavage. The events measured by each of the laboratory assays are shown by black boxes with white text and arrows.

factors governing local expression of what is fundamentally a systemic disease. In experimental studies, C1INH-protease complexes, levels of functional C1INH, PK activation, and cleavage of HMWK have all been linked with disease activity. For HAE-nl-C1INH, there is compelling evidence as well for contact system activation and bradykinin generation underlying enhanced vascular permeability and angioedema attacks. Recent work has provided key insights as to the role of FXII genetic mutations and plasmin as an activator of the contact system, which eludes C1INH. To build on these findings and establish a reliable biomarker for HAE-nl-C1INH and other forms of bradykinin-mediated angioedema with normal C1INH will be the focus of ongoing research in years to come. The ability to confirm contact system activation would have important implications for the diagnosis and effective management of angioedema.

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