

C1-Inhibitor function using contact phase proteases as target: evaluation of an innovative assay

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Abbreviations: AAE, acquired angioedema; α 2M, alpha2-Macroglobulin; AU, arbitrary unit; AUC, area under curve; BK, bradykinin; C1Inh, C1-Inhibitor; C1Inh-HAE, hereditary angioedema with C1Inh deficiency; CV, coefficient of variation; FXII, Factor XII; FXIIa, activated Factor XII; HAE, hereditary angioedema; HK, high-molecular-weight kininogen; NPV, negative predictive values; OC, estrogen-containing oral contraception; PPV positive predictive values; pKK prekallikrein; ROC, receiver operating characteristics

Abstract

Background. Controlling prekallikrein activation by C1-Inhibitor (C1Inh) represents the most essential mechanism for angioedema patient protection. C1Inh function in the plasma is usually measured based on the residual activity of the C1s protease not involved in the pathological process. We have hereby proposed an alternative enzymatic measurement of C1Inh function based on contact phase activation and correlation with angioedema diagnostic requirements.

Methods. The contact phase was reconstituted using the purified components, with C1Inh standard or plasma sample. The kinetics of the amidase activity were monitored using Pro-Phe-Arg-pNA, independently of alpha2-Macroglobulin (α 2M). We prevented any interference from a possible high plasma kininogenase activity by pre-incubating the samples with protease inhibitor. Receiver operating characteristics (ROC) were used to calculate the assay's diagnostic performance.

Results. The calibration curve was built using C1Inh standard (threshold limit $0.10 \times 10^{-3}\text{U}$, *i.e.* 0.2pmol) and C1Inh function was quantified in the sample, with a reference interval established based on healthy individuals (n=281; men: 0.61-1.10U/mL, median: 0.85U/mL; women: 0.42-1.08U/mL, median: 0.74U/mL). The median values of female donors were lower than those of the others due to estrogen, yet C1Inh function remained within the reference interval. The ROC curve calculation provided the following optimum diagnostic cut-off values: women 0.36U/mL (area under curve [AUC]: 0.99; sensitivity: 93.48%; specificity: 99.37%); men 0.61U/mL (AUC: 1; sensitivity: 100.0%; specificity: 100.0%).

Conclusion: The performance outcome provided features suitable for angioedema diagnostic or follow-up. Established by means of the kinin formation process, this assay should be preferred over the method based on a C1s protease target.

Introduction

C1-Inhibitor (C1Inh), whose gene name is *SERPING1*, is a plasma glycoprotein that controls the activation of the contact phase (1,2), complement (3–5), Factor XI of the intrinsic coagulation (6) and fibrinolysis. It is an acute-phase protein that can increase by up to 2.5-fold during inflammation (7) or after androgen treatment (8). C1Inh belongs to the Serpin family, which consists of Serine protease inhibitors that all share a unique structural architecture despite their modest sequence identity (9).

Hereditary angioedema (HAE) results from contact phase activation, which is associated with kinin production and potentially caused by C1Inh deficiency. This C1Inh-deficiency HAE form (C1Inh-HAE) is characterized by episodic local subcutaneous and submucosal swelling, which may prove life-threatening when attacks involve the upper airways due to the risk of asphyxiation. Kinins, namely bradykinin (BK) and *desArg*⁹-BK, are the primary mediators of this disease and are generated by contact phase activation. Angioedema could be precipitated by estrogens, *i.e.* estrogen-dependent HAE with exacerbations after puberty and/or with high estrogen states. Angioedema could also be associated with anti-C1Inh autoantibodies or lymphoproliferation in the contexts of acquired angioedema (AAE) (10). Most of these conditions are associated with transient or long-lasting decreased C1Inh function. In cases of estrogen-dependent HAE, this occurs during pregnancy or some oral contraception conditions.

The contact phase consists of the zymogens Factor XII (FXII), prekallikrein (pKK), and high-molecular-weight kininogen (HK). Their contact with negatively charged surfaces induces a conformational change in FXII, resulting in its activation (FXIIa) (10,11), which in turn activates pKK to generate kallikrein (KK) leading to the subsequent cleavage of HK and BK production. KK can develop a positive feedback loop and reciprocally activates FXII.

The contact phase is efficiently controlled by C1Inh, which represents the primary plasma control for FXIIa and KK (12,13). Plasminogen-activator inhibitor-1 also possesses a low FXIIa-blocking activity (14) while α 2-Macroglobulin (α 2M) non-specifically targets KK proteolytic function.

When KK is generated *in situ*, C1Inh contributes to 86% of its control (15). α 2M, a large-spectrum protease inhibitor, does not significantly inhibit FXIIa and has proven only moderately efficient in inhibiting KK (reviewed by 10). Compared to pKK, the plasma concentration of C1Inh is in excess, approximately 4- to 5-times higher in moles.

The K_{on} thermodynamic constraints of C1Inh favor plasma KK control ($1.7 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$) compared to those of FXIIa ($3.7 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$) (2) or C1s protease ($43 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$) (16), suggesting that plasma KK control by C1Inh is more effective than that of FXIIa or C1s protease.

The optimal management of HAE depends on early diagnosis especially considering that the mortality risk of untreated angioedema is approximately 40% due to asphyxiation (17,18). Investigating C1Inh is essential for angioedema diagnosis (19,20), providing a clear distinction between different HAE conditions, *i.e.*, C1Inh-HAE vs. HAE with normal C1Inh function (nC1Inh-HAE). In some cases, nC1Inh-HAE is found, for example when a C1Inh mutant affects FXII or KK control but not C1s, an assay that is specially designed for the contact phase is needed, and the common C1s protease is not suitable as target.

Two methods are currently available on the market for measuring C1Inh function: chromogenic and immunoenzyme assays (21). Both have been developed using a C1 protease as the analytical target, primarily the C1s protease. However, the primary mediator of swelling in angioedema patients is BK, whose production is controlled by plasma KK. A recent approach used biotinylated active enzymes (FXIIa or KK) and took advantage of the abundance of enzyme-

C1Inh complex as a readout of the C1Inh function in plasma (22). The authors demonstrated high sensitivity using FXIIa, while C1Inh targeted only 60% of the protease when using KK, presumably due to it being inhibited by α 2M. C1Inh was nevertheless established as more efficient in the inactivation of KK (Figure 1). This prompted us to consider a functional laboratory assay conducted without the interference of α 2M or plasma protease, specially designed to decipher pathological angioedema conditions using contact phase proteins, the natural target.

Material & Methods

Samples

Citrate plasma samples from adult patients and donors were prepared by centrifugation of freshly collected blood at $2,000 \times g$ for 10 min to harvest the platelet-free plasma. The samples were immediately aliquoted and stored at -80°C until use. Patients had confirmed angioedema diagnosis: C1Inh-HAE; AAE; estrogen-dependent angioedema) or were healthy individuals (controls).

Reagents and purified proteins

Substrate L-2120 (Bachem[®], Bubendorf, Switzerland), α 2M (BioMac[®], Leipzig, Germany), contact phase proteins FXIIa, pKK, HK (Enzyme Research[®], Swansea, United Kingdom), and purified C1Inh (Berinert[®], CSL Behring GmbH, Paris, France) were all obtained as indicated above.

Assay procedure

The contact phase was reconstituted *in vitro* in a 96-well plate using purified proteins FXIIa, pKK and HK. These were contained in either C1Inh standard or patient plasma supplemented by a Serine protease inhibitor as a source of C1Inh, according to patent application FR1160851

(INPI No BR071761/HL/ADA) (23). Briefly, equimolar amounts of purified HK and pKK were mixed with FXIIa, then C1Inh standard or protease inhibitor pre-treated plasma was added and incubated at 37°C for 10 min. The kinetics of the contact phase activation was monitored at 405 nm using the substrate L-2120 at pH 7.8 and a spectrophotometer (Multiskan Go_ThermoFisher®, Saint Herblain, France). The C1Inh antigenic levels in the plasma samples were measured using a laser nephelometer BNII (Dade Behring®, Marburg GmbH, Germany).

Receiving operator characteristics (ROC) and statistics

The assays diagnostic performance was evaluated by ROC curve analysis (Graphpad® software). This was supplemented by calculations of cut-off values, sensitivity and specificity values, positive and negative predictive values (PPV and NPV), and the corresponding area under the curve (AUC) data, with 95% confidence intervals (CI). Statistical analyses were performed using the Mann-Whitney test. Two distinct data sets have been presented, one for women, one for men. *P*-values <0.05 were considered statistically significant.

Ethics

All procedures were performed in accordance with the principles expressed in the Helsinki declaration and French ethical policies governing the use of the biological sample collection (Ministry of Health authorization). The Institutional Review Board of Grenoble University Hospital (South-East committee V) stated that all sample collection and processing methods fulfilled these requirements.

Results

Titration curve of C1Inh

C1Inh was evaluated for its KK control capacity in *in vitro* contact phase activation by FXIIa, using increasing doses of C1Inh standard (24) and different incubation times. One C1Inh unit (U) was defined as the required C1Inh quantity able to reconstitute the Serpin function present in 1 mL of freshly citrated human plasma, equivalent to 270 mg of antigenic C1Inh. Figure 2A illustrates the kinetics of pKK activation (V_{max}) in the presence of C1Inh. The V_{max} linearly decreased from 100% for the control without C1Inh and ranging from 92% to 15% with increased doses of C1Inh in line with observations made by Joseph et al (25). As decided based on the titration curve, the limit of detection was fixed at 0.10×10^{-3} U C1Inh, a lower value than that found using the residual C1s activity (26), making this assay nearly 25-times more sensitive (0.2pmol vs 5pmol).

Impact of $\alpha 2M$ on the C1Inh titration curve

Overall $\alpha 2M$ was found to contribute to 35% of the pKK activation control. Due to its high plasma concentration, its impact on the system was tested using double the concentration used for C1Inh, corresponding to human plasma concentrations. As shown in Figure 2B, increased $\alpha 2M$ concentrations did not decrease the V_{max} value. The C1Inh titration curves were all found to be similar in the presence or absence of increasing $\alpha 2M$ concentrations. The $\alpha 2M$ impact on the contact phase control was found to be negligible in the assay conditions (10- min incubation time). This is consistent with its low value of K_{on} interaction for KK and its inability to control the hydrolysis of small peptide substrates. In this context, while V_{max} decreased linearly with increasing C1Inh doses, increasing $\alpha 2M$ concentrations had no effect. These experimental

conditions, along with the negligible impact of α 2M in the assay, were all recorded for the next part of the study.

Normal references

Normal values were established based on 281 healthy individuals (158 women and 123 men). As shown in Figure 3, we recorded distinct data for women and men (0.61-1.10U/mL, median: 0.85 U/mL for men; 0.42-1.08U/mL, median: 0.74U/mL for women). The titration curve linearity enabled us to calculate function in relation to the antigenic level, *i.e.*, the Serpin specific activity (in U/mg or U/pmol, normal values: 3.2U/mg or 0.34U/nmol), as well as to estimate the abundance of functional Serpin (% of the whole C1Inh protein).

ROC analysis and assay performance

The assay's performance was evaluated using ROC analysis on C1Inh-deficient patients (n=93; 69 with Type I HAE, 11 with Type II HAE, and 13 with AAE). By means of ROC analysis, we defined the optimum diagnostic cut-off value for C1Inh function assay for women as 0.36U/mL (AUC: 0.99, sensitivity: 93.48%, specificity: 99.37%), and that for men as 0.61U/mL (AUC: 1, sensitivity: 100%, specificity: 100%) (Figure 4).

Reproducibility, repeatability, and limits of detection

By testing the reproducibility on pathological and control samples, we obtained average of 0.41U/mL and 1.10U/mL (coefficient of variation [CV]:4% and 17%), respectively. We next tested repeatability, applying the average values of 0.33U/mL and 0.96U/mL (CV:4% and 18%), respectively. The detection limit was 0.11U/mL, corresponding to the value obtained from the

titration curve and the 1 μ L testing volume. Predictive values for the evaluation of C1Inh function and HAE diagnosis are presented in Figure 4B. Both indicators of performance for HAE diagnosis are consistent with high excellence in terms of AUC, sensitivity, and specificity.

Estrogen intake impact on C1Inh function

C1Inh function was investigated in healthy women taking estrogen-containing oral contraception (+OC, n=59). The data was compared with that taken from healthy women not taking oral contraception (-OC, n=51). We found a significant difference between the two group medians ($p<0.01$; Figure 5A). However, the reference intervals remained nearly identical. The difference between the median values of the OC group (n=59) and the healthy females during the genital period (-OC and + OC groups, n=110) was not significant (Figure 5A). This suggests that even if the median value was low compared to that of the -OC group, these women's estrogen intake did not modify the biological indication of C1Inh function value in the healthy group. C1Inh protein was revealed to be cleaved during the active period of estrogen-dependent angioedema conditions (+OC), with associated low function (27). Samples from +OC patients identified as nC1Inh-HAE were investigated for C1Inh function and anti-C1Inh immunoblotting (n=39), including +OC patients with active disease (n=23) and +OC patients after withdrawal of the OC pill (n=16) (Figure 5B). We examined the relationship between a cleaved C1Inh species (inactive C1Inh) in the sample and a loss of function revealed by the two protease targets, namely contact phase and C1s. Figure 5C presents three illustrative examples of the association of a decreased C1Inh function with C1Inh cleavage. The data demonstrates that C1Inh function when using contact phase as the target and when it is detected to be within the normal range, was associated with a native species. In some instances the C1Inh function assay using C1s protease as target

was unable to really discriminate between different forms. An additional advantage of this assay is its ability to distinguish between the OC-dependent angioedema and the non-induced condition, which it does much better than the former method using C1s protease as target.

Discussion

This study sought to evaluate a new C1Inh function assay and its performance on patient plasma samples for diagnosis. C1Inh function was the primary test conducted for the biological diagnosis of angioedema (20). pKK activation and BK generation were the reported causes for the angioedema attacks observed in C1Inh-deficient cases. One definite advantage of our assay was the use of C1Inh natural target, namely the natively-produced KK protease, the major enzyme involved in the physiopathology of angioedema. We implemented specific experimental conditions rendering the method independent of α 2M control (Figure 2B), in line with the slower inhibition of KK by α 2M than by C1Inh, which could explain why the presence of this inhibitor is not sufficient to prevent attacks in HAE patients (28). The assay's efficient performance was demonstrated by ROC curves, including patients with C1Inh-HAE and AAE, revealing excellent markers and signifying this test's suitability for establishing a biological diagnosis of angioedema. This feature was valuable in its ability to distinguish between the two principal angioedema conditions, with or without C1Inh function decrease.

The units used to define C1Inh function were based on the international standard (24), the same as that used for C1Inh concentrates. In this way we could work with unambiguous information on the actual Serpin function, contributing also to applications in patient follow-up during replacement therapy using plasma or recombinant C1Inh concentrates (29). Alternatively, the function could be expressed with reference to C1Inh antigenic level, *i.e.*, Serpin specific activity

(U/mg or U/pmol). This calculation provides the biologist with a useful indication of the circulating species (native and functional, cleaved and non-functional), along with an estimate of the abundance of functional Serpin (%). Anti-C1Inh immunoblotting confirmed this estimate for us, with a stronger correlation achieved when using contact phase activation as the target rather than C1s protease (Figure 5D).

Moreover, the detection limit was lower than that obtained using the former assays based on residual C1s esterase or amidase activity. The advantage of a low detection limit offers a unique opportunity to develop a high-throughput analysis of patient-specific applications of C1Inhconcentrates in some contexts of severe kinin-dependent diseases.

This method is able to detect the angioedema conditions presenting with a *SERPING1* mutation, which differentially controls the protease C1s as opposed to KK, the latter corresponding to the pathological situation. Mutation positions were reported to be of great strategic importance for C1Inh control of kinin formation (30), which commonly-used testing methods with C1s target are unable to effectively evaluate , as shown in Table I. C1Inh function has often been considered as a marker of estrogen-dependent angioedema during the active period of disease, *e.g.*, conditions with FXII gain-of-function (27,31). Owing to the high analytical sensitivity of this assay, our results emphasized this concept (Figure 5B), demonstrating good discrimination between conditions within the active period and after inducer withdrawal, in line with circulating C1Inh species. In contrast, the assay using C1s as the target exhibited less discriminatory power in patients after inducer withdrawal. This emphasizes the hypothesis that C1Inh function could be proposed as a metabolic criterion for estrogen-dependent angioedema, including FXII gain-of-

function (27). With its high analytical sensitivity, the assay conducted in our study achieved this goal.

Methods using contact phase proteins had already been introduced in the literature. Recently, an assay took advantage of an ELISA method using biotinylated active enzymes (FXIIa or KK) (22). Its rationale was founded on the formation of FXIIa-C1Inh and KK-C1Inh complexes after incubation with plasma and detection of bound C1Inh. Prior reports have also demonstrated the possibility of analyzing C1Inh function using C1Inh-protease complexes (32,33). The levels of FXIIa-C1Inh and KK-C1Inh complexes may not, however, be proportionate to HK cleavage by KK and concomitantly to the extent of BK release () *in vivo* (32). Furthermore, the impaired formation of FXIIa-C1Inh and KK-C1Inh complexes following contact phase activation due to low C1Inh function, as well as the rapid clearance of protease-C1Inh complexes, could explain why these complexes cannot monitor contact activation in HAE (32–35). This is the reason why our described method, based on the residual enzyme activity after contact phase activation, is a promising tool for biological diagnosis due to its independence from the protease-C1Inh complexes.

We hereby present a new C1Inh function assay providing an enzymatic readout, applicable to patient samples tested for the purposes of angioedema diagnosis and follow-up, offering the advantage of targeting all contact phase proteases responsible for HK cleavage and BK production. After considering all the benefits of this new method, as outlined above, we are convinced of its value and thus to invite biologists to adopt a technical concept using the contact phase protease as C1Inh target and help physicians set up an improved diagnostic process.

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Author Contributions: AG and CD were responsible for the planning and performance of experiments, data analysis, and manuscript preparation; PS developed the experimental work, PS and DC conducted the statistical analyses; FD, DP and ALL carried out the patient sample selection; BF reviewed the manuscript. All authors read and approved the final manuscript.

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References

1. Schapira M, Scott CF, Colman RW. Contribution of plasma protease inhibitors to the inactivation of kallikrein in plasma. *J Clin Invest* 1982;**69**:462–468.
2. Pixley RA, Schapira M, Colman RW. The regulation of human factor XIIa by plasma proteinase inhibitors. *J Biol Chem* 1985;**260**:1723-1729.
3. Sim RB, Reboul A, Arlaud GJ, Villiers CL, Colomb MG. Interaction of 125I-labelled complement subcomponents C-1r and C-1s with protease inhibitors in plasma. *FEBS Lett* 1979;**97**:111-115.
4. Rossi V, Cseh S, Bally I, Thielens NM, Jensenius JC, Arlaud GJ. Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and -2. *J Biol Chem* 2001;**276**:40880-40887.

5. Davis AE, Mejia P, Lu F. Biological activities of C1 inhibitor. *Mol Immunol* 2008;**45**:4057-4063.
6. Willemin WA, Minnema M, Meijers JC, Roem D, Eerenberg AJ, Nuijens JH, et al. Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role for C1-inhibitor. *Blood* 1995;**85**:1517-1526.
7. Liu D, Lu F, Qin G, Fernandes SM, Li J, Davis AE. C1 inhibitor-mediated protection from sepsis. *J Immunol* 2007;**179**:3966-3972.
8. Drouet C, Désormeaux A, Robillard J, Ponard D, Bouillet L, Martin L, et al. Metallopeptidase activities in hereditary angioedema: effect of androgen prophylaxis on plasma aminopeptidase P. *J Allergy Clin Immunol* 2008;**121**:429-433.
9. Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem* 2001;**276**:33293-33296.
10. Kaplan AP, Joseph K. Pathogenic mechanisms of bradykinin mediated diseases: dysregulation of an innate inflammatory pathway. *Adv Immunol* 2014;**121**:41-89.
11. Renné T, Schmaier AH, Nickel KF, Blombäck M, Maas C. In vivo roles of factor XII. *Blood* 2012;**120**:4296-4303.
12. Björkqvist J, Sala-Cunill A, Renné T. Hereditary angioedema: a bradykinin-mediated swelling disorder. *Thromb Haemost* 2013;**109**:368-374.
13. Revak SD, Cochrane CG. The relationship of structure and function in human Hageman factor. The association of enzymatic and binding activities with separate regions of the molecule. *J Clin Invest* 1976;**57**:852-860.
14. Schapira M. Major inhibitors of the contact phase coagulation factors. *Semin Thromb Hemost* 1987;**13**:69-78.
15. Harpel PC, Lewin MF, Kaplan AP. Distribution of plasma kallikrein between C-1 inactivator and alpha 2-macroglobulin in plasma utilizing a new assay for alpha 2-macroglobulin-kallikrein complexes. *J Biol Chem* 1985;**260**:4257-4263.
16. Kerr FK, Thomas AR, Wijeyewickrema LC, Whisstock JC, Boyd SE, Kaiserman D, et al. Elucidation of the substrate specificity of the MASP-2 protease of the lectin complement pathway and identification of the enzyme as a major physiological target of the serpin, C1-inhibitor. *Mol Immunol* 2008;**45**:670-677.

17. Bork K, Siedlecki K, Bosch S, Schopf RE, Kreuz W. Asphyxiation by laryngeal edema in patients with hereditary angioedema. *Mayo Clin Proc* 2000;**75**:349-354.
18. Bork K, Hardt J, Witzke G. Fatal laryngeal attacks and mortality in hereditary angioedema due to C1-INH deficiency. *J Allergy Clin Immunol* 2012;**130**:692-697.
19. Maurer M, Magerl M, Metz M, Siebenhaar F, Weller K, Krause K. Practical algorithm for diagnosing patients with recurrent wheals or angioedema. *Allergy* 2013;**68**:816-819.
20. Cicardi M, Aberer W, Banerji A, Bas M, Bernstein JA, Bork K, et al. Classification, diagnosis, and approach to treatment for angioedema: consensus report from the Hereditary Angioedema International Working Group. *Allergy* 2014;**69**:602-616.
21. Wagenaar-Bos IGA, Drouet C, Aygören-Pursun E, Bork K, Bucher C, Bygum A, et al. Functional C1-inhibitor diagnostics in hereditary angioedema: assay evaluation and recommendations. *J Immunol Methods* 2008;**338**:14-20.
22. Joseph K, Bains S, Tholanikunnel BG, Bygum A, Aabom A, Koch C, et al. A novel assay to diagnose hereditary angioedema utilizing inhibition of bradykinin-forming enzymes. *Allergy* 2015;**70**:115-119.
23. Ghannam A, Defendi F, Favier B, Drouet C. Method for dosing the control capacity of C1Inh. Patent Application (Application #20140315232 issued October 23, 2014) - Justia Patents Database. <http://patents.justia.com/patent/20140315232> (accessed 12 Jan 2015).
24. Thelwel C, Rigsby P, Longstaff C, ISTH-SSC Subcommittee on Factor XI and Contact System. An international collaborative study to establish the WHO 1st international standards for C1-inhibitor, plasma and concentrate. *J Thromb Haemost* 2011;**9**:2097-2099.
25. Joseph K, Tuscano TB, Kaplan AP. Studies of the mechanisms of bradykinin generation in hereditary angioedema plasma. *Ann Allergy Asthma Immunol* 2008;**101**:279-286.
26. Drouet C, Alibeu C, Ponard D, Arlaud GJ, Colomb MG. A sensitive method to assay blood complement C1- inhibitor activity. *Clin Chim Acta* 1988;**174**:121-130.
27. Martin L, Raison-Peyron N, Nöthen MM, Cichon S, Drouet C. Hereditary angioedema with normal C1 inhibitor gene in a family with affected women and men is associated with the p.Thr328Lys mutation in the F12 gene. *J Allergy Clin Immunol* 2007;**120**:975-977.
28. Kaufman N, Page JD, Pixley RA, Schein R, Schmaier AH, Colman RW. Alpha 2-macroglobulin-kallikrein complexes detect contact system activation in hereditary angioedema and human sepsis. *Blood* 1991;**77**:2660-2667.

29. Reshef A, Moldovan D, Obtulowicz K, Leibovich I, Mihaly E, Visscher S, et al. Recombinant human C1 inhibitor for the prophylaxis of hereditary angioedema attacks: a pilot study. *Allergy* 2013;**68**:118-124.
30. López-Lera A, Favier B, de la Cruz RM, Garrido S, Drouet C, López-Trascasa M. A new case of homozygous C1-inhibitor deficiency suggests a role for Arg378 in the control of kinin pathway activation. *J Allergy Clin Immunol* 2010;**126**:1307-1310.e3.
31. Charignon D, Ghannam A, Defendi F, Ponard D, Monnier N, López Trascasa M, et al. Hereditary angioedema with F12 mutation: factors modifying the clinical phenotype. *Allergy* 2014;**69**:1659-1665.
32. Nuijens JH, Huijbregts CC, Eerenberg-Belmer AJ, Abbink JJ, Strack van Schijndel RJ, Felt-Bersma RJ et al. Quantification of plasma factor XIIa-C1(-)-inhibitor and kallikrein-C1(-)-inhibitor complexes in sepsis. *Blood* 1988;**72**:1841-1848.
33. De Smet BJ, de Boer JP, Agterberg J, Rigter G, Bleeker WK, Hack CE. Clearance of human native, proteinase-complexed, and proteolytically inactivated C1-inhibitor in rats. *Blood* 1993;**81**:56-61.
34. Cugno M, Nuijens J, Hack E, Eerenberg A, Frangi D, Agostoni A et al. Plasma levels of C1- inhibitor complexes and cleaved C1- inhibitor in patients with hereditary angioneurotic edema. *J Clin Invest* 1990;**85**:1215-1220.
35. Konings J, Cugno M, Suffritti C, Ten Cate H, Cicardi M, Govers-Riemslog JWP. Ongoing contact activation in patients with hereditary angioedema. *PloS One* 2013;**8**:e74043.

Figure legends

Figure 1. Schematic representation of contact phase and its control. Contact phase consists of 3 plasma proteins that assemble when blood comes into contact with triggers, for example, negatively charged surfaces: FXII, plasma prekallikrein (pKK), and the nonenzymatic cofactor high-molecular-weight kininogen (HK); 80% of blood HK-pKK association is bound onto the endothelium. FXII and HK directly bind to polyanions; surface binding induces a conformational change in FXII, leading to limited proteolytic activity. Consecutively, activated FXII (FXIIa) cleaves pKK, generating plasma kallikrein (KK). In an amplified reaction, KK efficiently activates further FXII proenzyme molecules and cleaves its cofactor HK to yield bradykinin.

Proenzyme activation and protease activities are under C1-Inhibitor (C1Inh) and alpha-2 Macroglobulin (α 2M) control; the percentage of the participant to the control is indicated in the context of *in situ* contact phase generation.

Figure 2. C1-Inhibitor titration curve. (A) Kinetics (% of V_{max} without C1-Inhibitor, C1Inh) of prekallikrein (pKK) activation with increased doses of C1Inh (n=4). Two scales are shown for C1Inh: pmol, (solid line and squares) and $U \times 10^{-3}$, (dashed line and circles). (B) Impact of alpha2-Macroglobulin (α 2M) on C1Inh titration curve: kinetics of pKK activation with increased doses of C1Inh, α 2M or C1Inh and α 2M together (n=2). Results $\pm$ standard error of the mean (SEM).

Figure 3. Distribution of normal values. C1-Inhibitor (C1Inh) function in 280 healthy subjects (123 men and 158 women): solid line: median; dashed line: interquartile range; **: $P < 0.01$; ***: $P < 0.001$

Figure 4. Diagnostic outcomes of C1-Inhibitor (C1Inh) function assay for the diagnosis of C1Inh deficiency. (A) Receiving operator characteristics (ROC) curves for male (left panel) and female (right panel) individuals carrying a *SERPING1* mutation or presenting with acquired angioedema (AAE) *versus* blood donors. (B) Diagnostic values for C1-Inhibitor (C1Inh) function. The data was collected from women (healthy: n=158; C1Inh deficient: n=46, HAE Type I: n=39; HAE Type II: n=2; and AAE: n=5) and men (healthy, n=123, C1Inh deficient: n=47; HAE Type I: n=30; HAE Type II: n=9; and AAE: n=8). (C) Healthy/patient subject distribution for males shown in the left panel (healthy, n=123, patients, n=47), and for females in the right panel (healthy, n=158, patients, n=46), *****: $P < 0.0001$.

Figure 5. Distribution of C1-Inhibitor function (U/mL) in different situations. (A) Healthy females: C1-Inhibitor (C1Inh) function in female blood donors (n=110), not taking estrogen-

containing contraception (healthy -OC: n=51) or taking estrogen contraception (healthy +OC: n=59), the horizontal bars show the median values. (B) Estrogen-dependent angioedema patients: distribution of C1Inh function using our test, between patients with active disease (n=23) and patients after OC pill withdrawal (n=16). (C) Estrogen-dependent angioedema patients: distribution of C1Inh function using C1s as target, between patients with active disease (n=23) and patients after OC pill withdrawal (n=16). (D) Anti-C1Inh immunoblotting of plasma and corresponding C1Inh function: illustration of three examples: one control, one patient during active disease under estrogen intake (+OC), and one patient undergoing withdrawal from OC pill (-OC); the Table shows C1Inh function using C1s or kallikrein (KK) as target. **: $P<0.01$; ****: $P<0.0001$.

Table I. Distinct C1Inh control function: testing using contact phase and C1s protease as targets. Samples from patients carrying three *SERPING1* mutations (14 heterozygous, 1 homozygous) and belonging to 6 families are tested for C1Inh function using the presently described and the conventional testing (% of median in brackets). Normal references: contact phase proteases (0.61-1.10 U/mL, median: 0.85U/mL for men; 0.42-1.08U/mL, median 0.74U/mL for women) and C1s protease (17.2-27.4 AU/mL; median 22.1 AU/mL). Mutation positions are according to hae.enzim.hu. Bold figures correspond to nearly normal C1Inh function of an individual with an exceptionally normal amidase activity as biological phenotype.

Patient ID	Gender	C1-Inhibitor function		<i>SERPING1</i> mutation
		Contact phase proteases (U/mL)	C1s protease (AU/mL)	
59K	male	<0.11 (<12.9%)	5.4 (24.3%)	I271T
59B1	male	<0.11 (<12.9%)	5.4 (24.3%)	I271T
59B2	female	<0.11 (<12.9%)	8.4 (37.8%)	I271T
59S	female	<0.11 (<12.9%)	9.6 (43.2%)	I271T
59C	female	<0.11 (<12.9%)	3.4 (15.3%)	I271T
179D1	male	<0.11 (<12.9%)	8.5 (38.25%)	R378C
179D2	female	0.20 (27.0%)	13.5 (60.7%)	R378C
193J	female	0.45 (60.8%)	5.5 (24.7%)	L107R
202C	female	0.11 (12.9%)	13.2 (59.4%)	R378C
120J	female	0.14 (18.9%)	11.4 (51.3%)	R378C
120N	male	0.23 (27.0%)	15.6 (70.2%)	R378C
120R	male	0.11 (12.9%)	12.8 (57.6%)	R378C
203GZ	male	<0.11 (<12.9%)	4 (18.2%)	R378C/R378C
203GZ5	female	<0.11 (<12.9%)	10.9 (49%)	R378C
203GZ6	male	0.11 (12.9%)	12.4 (56%)	R378C

Figure 1

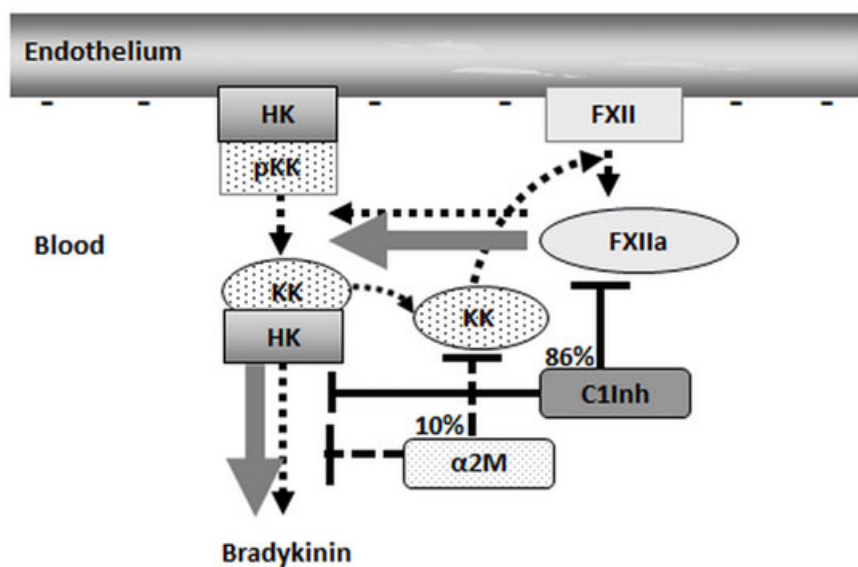


Figure 2

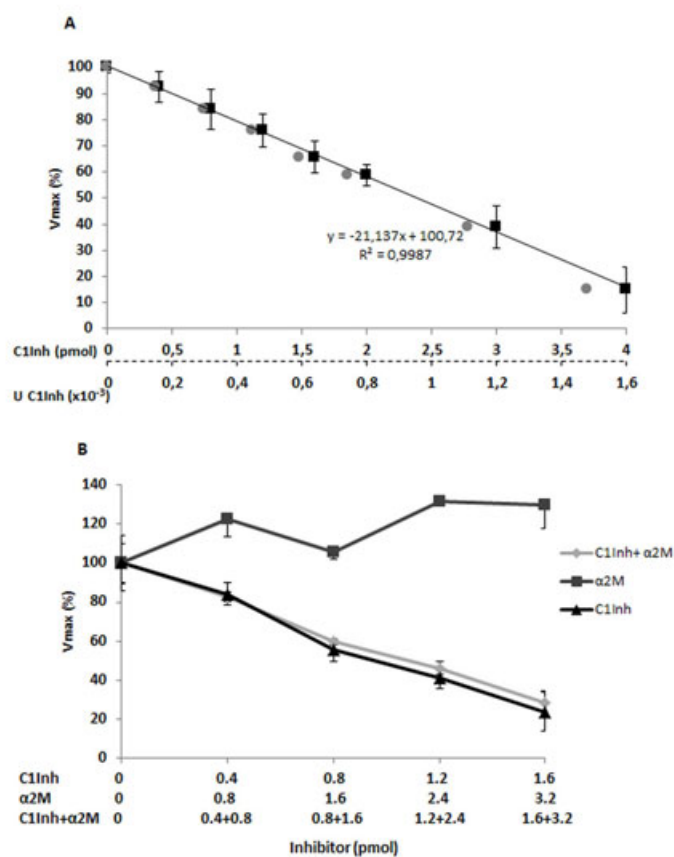


Figure 3

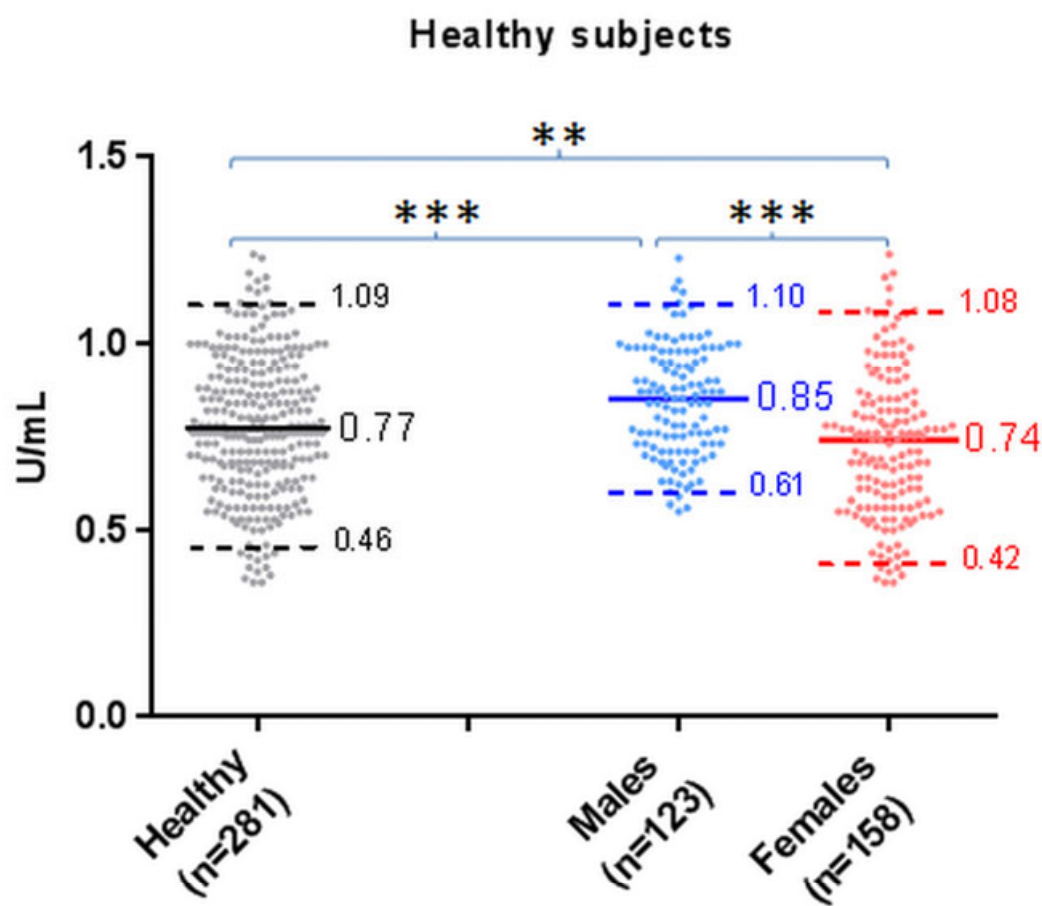


Figure 4

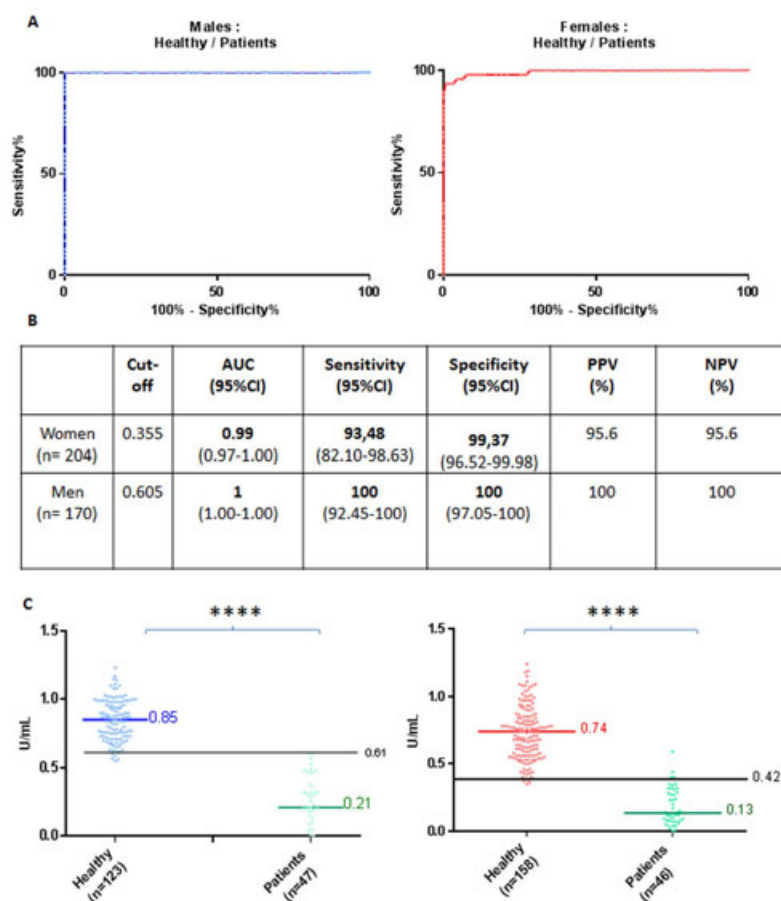


Figure 5

