

High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency

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Summary

Background The inherited deficiency of C1-inhibitor (C1-INH), which can be quantitative (type I) or qualitative (type II), is characterized by recurrent attacks of oedema, and it is known as hereditary angioedema due to C1-INH deficiency (HAE-C1-INH). The frequency of symptoms varies widely among patients and in the same patient during life.

Objective To identify laboratory markers of disease severity in HAE-C1-INH patients.

Methods We studied 162 patients with differently severe HAE-C1-INH during remission, 31 HAE-C1-INH patients during attacks, and 81 normal controls, evaluating complement parameters, spontaneous plasma kallikrein activity, the capacity of plasma to inhibit exogenous kallikrein activity, and cleavage of high-molecular-weight kininogen (HK). Sixty-five HAE-C1-INH patients were screened for mutations in the C1-INH gene.

Results As expected, plasma C1-INH levels and activity and C4 levels were low in the HAE-C1-INH patients. Spontaneous plasma kallikrein activity in patients in remission was higher than in controls ($P = 0.001$) and increased during acute attacks ($P = 0.01$), whereas the capacity of inhibiting kallikrein activity was lower in patients in remission than in controls ($P = 0.001$) and further reduced during attacks ($P = 0.001$). HAE-C1-INH patients in remission had higher levels of cleaved HK than controls ($P = 0.001$), and these further increased during acute attacks ($P = 0.001$). Cleaved HK levels were higher in highly symptomatic HAE-C1-INH patients than in those with less frequent attacks ($P = 0.001$). Thirty-five different mutations in the C1-INH gene were equally distributed in patients with different attack frequencies.

Conclusions Measuring plasma levels of cleaved HK may be a sensitive mean of assessing disease severity in HAE-C1-INH patients.

Keywords angioedema, biomarkers, C1-inhibitor deficiency, disease severity, high-molecular-weight kininogen, kallikrein

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Introduction

Hereditary angioedema with C1-inhibitor (C1-INH) deficiency (HAE-C1-INH) is a rare, autosomal dominant disease caused by mutations in one of the two alleles of the C1-INH gene (SERPING1) [1, 2]. Systematic analysis of the SERPING1 mutations collected in a locus-specific database has shown that more than 200 are responsible for HAE-C1-INH [3]: 25% are large deletions/insertions, whereas the rest are small mutations (splicing defects, nonsense, missense, and frameshift mutations) [4–8]. As

a consequence, the mutated allele does not provide a plasma protein in 85% of patients (low plasma levels of antigenic and functional C1-INH, HAE-C1-INH type I) and produces a dysfunctional protein in the remaining 15% (normal plasma levels of antigenic C1-INH and low levels of functional C1-INH, HAE-C1-INH type II) [4]. C1-INH is a serine protease inhibitor that blocks the activity of C1r, C1s and mannose-binding lectin-associated serine protease (MASP) 1 and 2 in the complement system, activated factor XII (FXIIa) and plasma kallikrein (pKK) in the contact system, activated factor XI

(FXIa) and thrombin in the coagulation system, and tissue plasminogen activator (tPA) and plasmin in the fibrinolytic system [9–11].

C1-INH-deficient patients are exposed to recurrent localized oedema (angioedema) because episodic activation of the contact system leads to the massive generation of plasma kallikrein (pKK) that cleaves high-molecular-weight kininogen (HK) to release bradykinin, a potent vasoactive peptide mediating oedema formation [12–16]. It is also likely that the concomitant activation of the fibrinolytic and coagulation systems, which are also devoid of a control protein, contributes to oedema by generating plasmin and thrombin [17–19]. HAE-C1-INH is clinically characterized by episodes of subcutaneous and submucosal oedema lasting 2–5 days. The most frequently involved organs are the skin, gastrointestinal tract, oropharynx, and larynx. The disease is disabling and possibly lethal and causes disfigurement, severe abdominal pain, dysphagia, and asphyxia [20, 21].

HAE-C1-INH is a periodic disease in which patients suddenly change from complete wellness to conditions with important limitations and even life-threatening. Duration of symptomatic phases scores disease-related disability. Anxiety, attending the constant presence of impending angioedema and the ensuing impaired quality of life, complete the burden of the disease [22, 23]. Therapy aims at restoring quality of life in these patients. Several drugs can revert attacks, and their early on demand administration is recommended to reduce disease-related disability [24]. Drugs preventing attacks can be used for long-term prophylaxis [25]. Other than reducing disability, they also positively impact on patients' anxiety by decreasing angioedema recurrences [26]. The due concerns of lifelong treatments force careful identification of severe patients to weight expected benefit against potential risk. Variability of disease severity among patients and throughout time, tangles deciding on the proper therapeutic regimen. Currently, disease severity is merely foreseen on the clinical evaluation. To ground on objective parameter(s) would be a major advance for decision-making.

The aim of this study was to identify laboratory markers of disease severity by analysing the systems impaired by C1-INH deficiency in a large cohort of HAE-C1-INH patients clustered on the basis of the number of angioedema recurrences per year. The complement system was studied by measuring the plasma levels of C1-INH, C1q, and C4 and the capacity of plasma C1-INH to inhibit the esterase activity of exogenous C1s, and the contact system was studied by detecting spontaneous plasma pKK activity and measuring the capacity of plasma to inhibit the amidolytic activity of exogenous pKK. The relative amount of the cleaved band of HK generated in plasma upon cleavage by endogenous pKK was used as indirect

evidence of bradykinin generation. Finally, we determined whether the presence of a specific mutation predicted disease severity (genotype/phenotype correlation), which was assessed on the basis of the number of angioedema attacks per year.

Methods

Study population

Blood was collected in the course of routine diagnostic procedures, and all patients gave oral informed consent that remaining plasma could be used for research purposes. If consent was given by the patient, the remaining plasma sample was labelled with a code which was documented into a data sheet. The Ethical Committee of the University of Milan approved that written informed consent is not necessary if the plasma had been obtained during routine diagnostics and that this plasma could be used for research investigating the pathophysiology of hereditary angioedema. Technicians who performed the assays were blinded to the patients' history.

We studied 162 patients with HAE-C1-INH. The diagnosis was based on the presence of at least one recommended clinical and one recommended laboratory criteria [27]. Blood samples were taken from all of the patients at least 8 days from an angioedema attack (remission samples) and from selected patients within the first 6 h of the beginning of an angioedema attack (attack samples). The patients were grouped on the basis of their prophylactic treatment and the number of angioedema attacks recorded during the previous 12 months: < 3 attacks (low frequency), 3–12 attacks (intermediate frequency), and > 12 attacks (high frequency). Table 1 shows the distribution of prophylactic treatments and attack frequencies.

Complement parameters were measured in all 162 HAE-C1-INH patients (63 males and 99 females; 139 type I and 23 type II; median age 40 years, range 6–77); pKK was measured in 67 patients (24 males and 43 females; 56 type I and 11 type II; median age 44 years, range 10–76); and cleaved HK was studied in 122 patients (47 males and 75 females; 107 type I and 15 type II; median age 40 years, range 6–77). Complement, pKK, and cleaved HK were measured during attacks in, respectively, 31, 22, and 16 patients.

Sixty-five patients (25 males and 40 females; 58 type I and 7 type II; median age 40 years, range 6–77) were screened for *SERPING1* mutations.

The complement, pKK, and cleaved HK controls were, respectively, 81 (23 males and 58 females; median age 26 years, range 19–54), 73 (21 males and 52 females; median age 24 years, range 19–52), and 61 normal subjects (15 males and 46 females; median age 25 years, range 19–54).

Table 1. Frequency of angioedema attacks in patients receiving or not receiving prophylactic treatment

Prophylactic treatment	Dose range	Number of patients	< 3 attacks/year	3–12 attacks/year	> 12 attacks/year
Complement study (<i>n</i> = 162)					
No prophylaxis		82	33	17	32
Danazol	50–200 mg/day	58	34	9	15
Stanozolol	2–4 mg/day	15	2	7	6
Tranexamic acid	1.5–3 g/day	7	1	2	4
Kallikrein study (<i>n</i> = 67)					
No prophylaxis		26	16	0	10
Danazol	50–200 mg/day	31	21	2	8
Stanozolol	2–4 mg/day	8	1	2	5
Tranexamic acid	1.5–3 g/day	2	0	0	2
Cleaved HK study (<i>n</i> = 122)					
No prophylaxis		65	24	17	24
Danazol	50–200 mg/day	39	21	9	9
Stanozolol	2–4 mg/day	13	1	7	5
Tranexamic acid	1.5–3 g/day	5	1	2	2
Mutation screening (<i>n</i> = 65)					
No prophylaxis		31	14	7	10
Danazol	50–200 mg/day	24	1	0	23
Stanozolol	2–4 mg/day	7	0	3	4
Tranexamic acid	1.5–3 g/day	3	1	1	1

HK, high-molecular-weight kininogen.

Blood sampling

The samples were collected using sodium citrate 3.2% as anticoagulant, and after centrifugation at 2000 g for 20 min at room temperature, the plasma was divided into aliquots and stored at -80°C until tested.

In the case of cleaved HK, blood obtained by means of a clean venipuncture and minimal stasis was drawn into vacutainer tubes containing 100 mM benzamidine, 400 $\mu\text{g/mL}$ hexadimethrine bromide, 2 mg/mL soybean trypsin inhibitor, 263 μM leupeptin, and 20 mM aminoethylbenzene-sulphonyl fluoride dissolved in acid/citrate/dextrose (100 mM trisodium citrate, 67 mM citric acid, and 2% dextrose, pH 4.5). Nine parts of blood were mixed with one part of an anticoagulant/inhibitor cocktail mixture [28], and the samples were centrifuged within 1 h for 20 min at 2000 g and 20°C . The plasma was removed, divided into aliquots, and stored at -80°C until tested. The samples were thawed quickly to 37°C immediately before being assayed.

Laboratory methods

Complement system. C1-INH, C1q, and C4 antigen levels were measured by means of radial immunodiffusion (RID; NOR-Partigen, Siemens Healthcare Diagnostics, Munich, Germany).

C1-INH function was assessed as the capacity of plasma to inhibit the esterase activity of exogenous C1s as measured on a specific chromogenic substrate by

means of a commercially available kit (Technoclone GmbH, Vienna, Austria).

Contact system. The amidolytic activity of kallikrein in plasma and the capacity of plasma to inhibit the amidolytic activity of exogenous kallikrein were both determined using a method based on the chromogenic peptide kallikrein substrate S-2302 (Chromogenix, Instrumentation Laboratory, Bedford, MA, USA) [29]. To detect the presence of kallikrein amidolytic activity, plasma diluted 1 : 20 in 0.05 M Tris, 0.36 M NaCl, 20 mg/L polybrene, pH 7.8, was incubated with the substrate for 30 min at 37°C , and the rate of release of paranitroaniline (pNA) was photometrically measured at 405 nm. The absorbance was converted to total kallikrein-like activity using kinetic constants and expressed as mU/mL. To determine the capacity of plasma to inhibit the amidolytic activity of exogenous kallikrein, a fixed amount of purified human plasma kallikrein (0.06 U/mL) was added to serial plasma dilutions. The release of pNA was recorded after incubation with the substrate for 5 min at 37°C . The capacity of plasma to inhibit the amidolytic activity of kallikrein was expressed as a percentage of normal using a standard curve of normal human pooled plasma.

The cleavage of HK was assessed by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis (a modification of the method described by Berrettini et al.) [30]. After electrophoretic separation and the transfer of

proteins from the gel to a polyvinylidene difluoride membrane (Immobilon; EMD Millipore Corp, Billerica, MA, USA), HK was identified using goat polyclonal anti-HK light chain (Nordic, Tilburg, The Netherlands) and visualized using a biotinylated rabbit anti-goat antibody (Sigma-Aldrich Co, St. Louis, MO, USA). The apparent molecular masses of the proteins were estimated by comparing them with the high-molecular-weight protein markers (Bio-Rad Laboratories, Hercules, CA, USA). Using this method, native HK appears as a single band with an M_r of 130 000, but upon maximal cleavage, it is progressively replaced by two bands with M_r values of 107 000 and 98 000. The density of the bands was measured using a Bio-Rad GS800 densitometer. Kaolin-incubated plasma was run on each electrophoresis as a control sample. The amount of cleaved HK (with M_r bands of 107 000 and 98 000) was expressed as a percentage of total HK (the sum of the three bands) [31].

Sequencing analysis. All of the *SERPING1* exons and intron/exon boundaries were completely sequenced. DNA was extracted from peripheral white blood cells using the 'salting out' method, and its concentration was measured by means of spectrophotometric analysis at 260 and 280 nm. The C1-INH gene was divided into seven regions (A–F), each of which was amplified using previously published specific sets of primers [32], except for region A. This region was divided into two subregions: the primers for A1 were as follows: forward 5'-AAAGAAGACCAAGCGGTCAG-3', reverse 5'-CGGGTCTCTGTCCATCTGTT-3'; the primers for A2 were as follows: forward 5'-CTCTGGCCTCATTTGTTGGT-3', reverse 5'-CGTGTGCAAGGATCACACTC-3'. The reactions were carried out in 1× PCR buffer (with 1.5 mM $MgCl_2$), 0.1 mM dNTP, 0.3 μ M of each primer, 5% DMSO, 2.5 U Taq DNA polymerase, and 500 ng of DNA in a final volume of 50 μ L. For regions A1, A2, B, and D, a hot start at 95°C for 5 min was followed by 32 cycles at 94°C for 1 min and 10 s, 58°C for 1 min and 10 s, and

72°C for 1 min and 10 s. The final extension was performed for 10 min at 72°C. For regions C, E, and F, the annealing temperature was 60°C. The PCR products were size-separated on 2% agarose gels, visualized by a UV transilluminator, and purified using Millipore Montage micro PCR96 plates. The sequencing reaction was carried out using the BigDye Terminator kit V1.1 (Applied Biosystems, Carlsbad, CA, USA), and the sequencing reaction products were purified using the Millipore Montage SEQ96 Sequencing Reaction Cleanup Kit. The sequences were analysed using an ABI 310 Genetic Analyser, and the data were analysed using DNA SEQUENCING 3.7 software (Applied Biosystems).

Patients negative for mutations following the above procedure were further analysed by means of long-range PCR using Expand 20 Kb PCR Systems (Roche, Basel, Switzerland) to check for the presence of large deletions or insertions.

Statistics

As the data were positively skewed, they were log-transformed before analysis. The results are expressed as geometric mean values and 95% confidence intervals (CIs). Student's *t*-test for unpaired values was used to assess the statistical significance of the differences between groups, and correlations were assessed by means of least-squares linear regression. The association between C1-INH gene mutations and clinical manifestations was evaluated using the chi-squared test. The data were analysed using the SPSS statistical package, version 20.00 (SPSS Inc., Chicago, IL, USA), and a *P*-value of < 0.05 was considered statistically significant.

Results

Table 2 shows the results of the complement studies. In line with the diagnosis of HAE-C1-INH, all of the patients had < 50% C1-INH function and normal levels of C1q. All of the complement parameters were

Table 2. Complement parameters (expressed as % of normal pooled plasma) in healthy controls and patients with hereditary angioedema (HAE-C1-INH) during remission and during acute attacks

	C1-INH function %	C1-INH Ag %	C4 Ag %	C1q Ag %
Controls	83.8 (80.6–87.0) (<i>n</i> = 81)	99.7 (97.5–101.8) (<i>n</i> = 81)	96.7 (91.7–101.6) (<i>n</i> = 81)	98.6 (97.6–99.6) (<i>n</i> = 81)
HAE-C1-INH during remission	13.7 (11.8–15.5)* (<i>n</i> = 162)	15.4 (12.8–18.0) type I* (<i>n</i> = 139) 90.8 (69.8–111.9) type II (<i>n</i> = 23)	20.4 (16.6–24.1)* (<i>n</i> = 162)	96.2 (94.5–97.8) (<i>n</i> = 162)
HAE-C1-INH during attacks	4.9 (1.5–8.3)*** (<i>n</i> = 31)	7.1 (3.2–11.0) type I*** (<i>n</i> = 31)	9.0 (1.9–16.1)** (<i>n</i> = 31)	86.5 (78.9–94.1) [†] (<i>n</i> = 31)

Geometric mean values and 95% confidence intervals.

HAE-C1-INH, hereditary angioedema due to C1-INH deficiency.

**P* < 0.001 between controls and HAE-C1-INH patients during remission.

[†]*P* = 0.049, ***P* = 0.003, ****P* < 0.001 between HAE-C1-INH patients during remission and during an acute attack.

significantly lower during angioedema attacks than during remission. Spontaneous pKK activity in the patients in remission was higher (2.1 mU/mL, CI 1.7–2.5 mU/mL) than in the controls (1.0 mU/mL, CI 0.6–1.4 mU/mL; $P = 0.001$; Fig. 1) and increased during acute attacks (6.7 mU/mL, CI 0.7–12.8 mU/mL; $P = 0.01$). In line with this, plasma from the patients in remission inhibited kallikrein activity less than plasma from the controls (43.1%, CI 39.1–47.2% vs. 111.4%, CI 108.6–114.3%; $P < 0.001$), and this capacity was further reduced during angioedema attacks (6.5%, CI 0–16.3%; $P < 0.001$; Fig. 2). C1-INH function in the HAE-C1-INH patients during remission correlated positively with the capacity to inhibit kallikrein activity ($r = 0.656$; $P < 0.001$) and negatively with spontaneous plasma kallikrein activity ($r = -0.308$; $P = 0.011$).

The electrophoretic pattern of HK was different in the patients and controls (Fig. 3). The patients in remission had higher levels of cleaved HK than the normal controls (42.9%, CI 41.4–44.4% vs. 34.8%, CI 33.5–36.1%; $P < 0.001$), and these increased further during acute attacks (58.1%, CI 54.4–61.8%; $P < 0.001$; Fig. 4). In the 16 patients observed during attacks, plasma levels of cleaved HK were significantly higher than those measured during remission (Fig. 5; $P = 0.0001$). C1-INH function correlated negatively with cleaved HK ($r = -0.300$; $P = 0.001$).

The differences within the groups of patients receiving and not receiving prophylactic treatment were analysed by disease severity. C4 antigen levels were significantly reduced in the highly symptomatic patients not receiving prophylaxis ($P = 0.001$; Table 3),

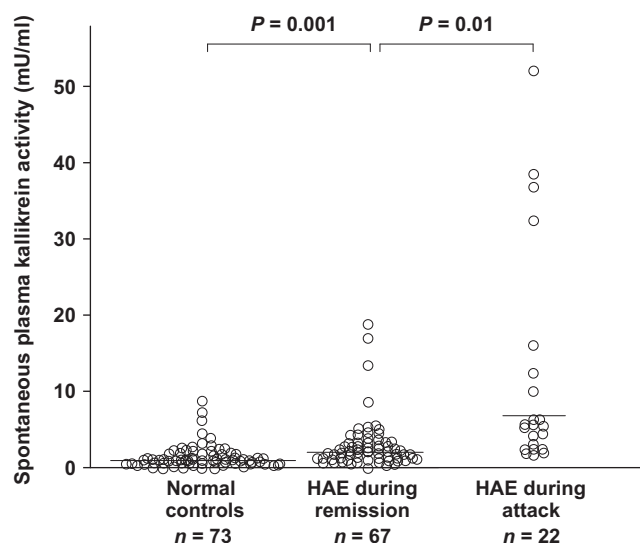


Fig. 1. Spontaneous plasma kallikrein activity (mU/mL) in normal controls, patients with hereditary angioedema due to C1-INH deficiency (HAE-C1-INH) during remission, and HAE-C1-INH patients during acute attacks.

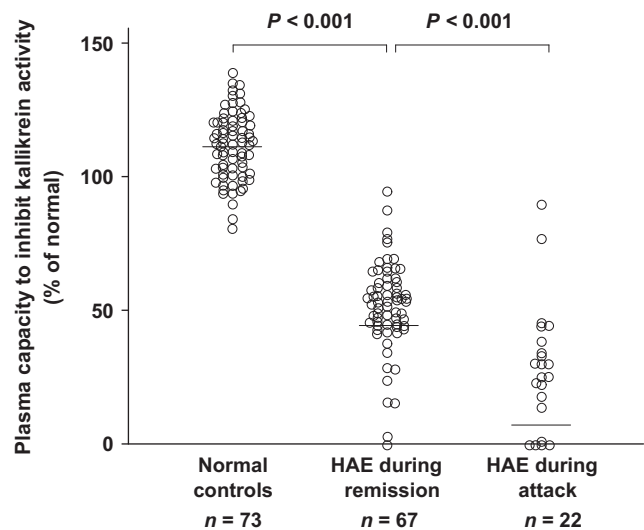


Fig. 2. Capacity of plasma to inhibit kallikrein activity (expressed as the percentage of normal capacity) in normal controls, patients with hereditary angioedema due to C1-INH deficiency (HAE-C1-INH) during remission, and HAE-C1-INH patients during acute attacks.

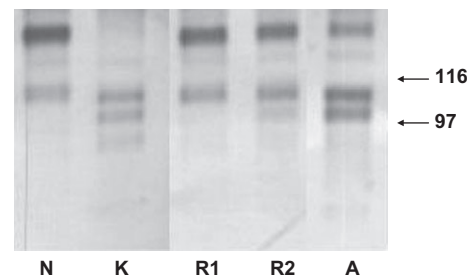


Fig. 3. Immunoblotting of cleaved high-molecular-weight kininogen (HK) in plasma taken from normal subjects (N), patients with hereditary angioedema due to C1-INH deficiency (HAE-C1-INH) during remission (R1, R2), and HAE-C1-INH patients during an angioedema attack (A). The normal pattern is a major band with an M_r of 130 000 and a faint band with an M_r of 107 000; patients show a reduction in the band with an M_r of 130 000, an increase in the band with an M_r of 107 000, and the appearance of a band with an M_r of 98 000. The pattern typical of cleaved HK can be obtained by activating normal human plasma with kaolin (K) *in vitro*.

whereas during prophylaxis with attenuated androgens, C1-INH function and C4 antigen levels were significantly increased ($P = 0.01$ and $P < 0.001$; Table 4). Spontaneous pKK activity and the capacity of plasma to inhibit kallikrein were not significantly different in the three groups (Table 3), and neither were significantly modified by prophylactic treatment with attenuated androgens (Table 4).

The mean levels of cleaved HK in the patients experiencing < 3 attacks/year were not significantly different from those observed in the healthy controls, but significantly increased with disease severity (Fig. 6). The levels were not changed by prophylactic treatment *per se*

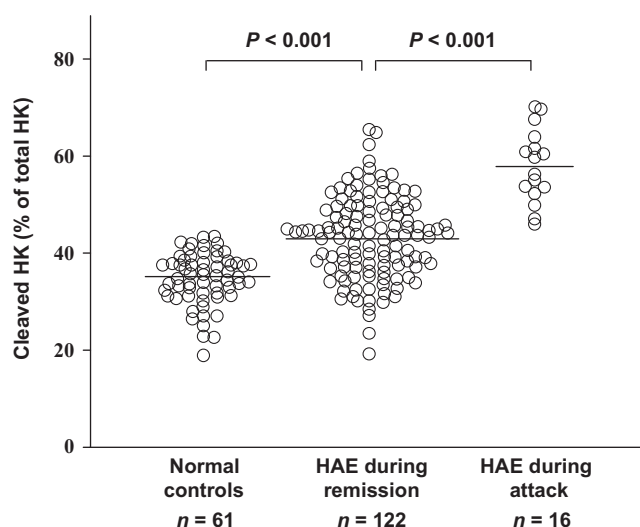


Fig. 4. Levels of cleaved high-molecular-weight kininogen (HK) (expressed as the percentage of total HK) in plasma taken from normal controls, patients with hereditary angioedema due to C1-INH deficiency (HAE-C1-INH) during remission, and HAE-C1-INH patients during acute attacks.

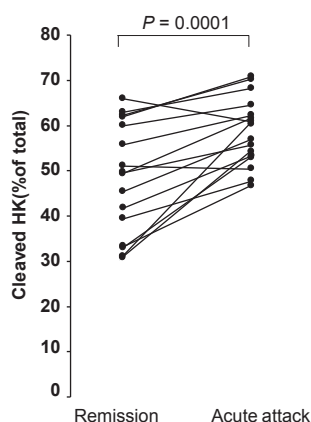


Fig. 5. Levels of cleaved high-molecular-weight kininogen (HK) (expressed as the percentage of total HK) in plasma collected from patients with hereditary angioedema due to C1-INH deficiency (HAE-C1-INH) during remission and during acute attack.

(Table 4), and the differences between the disease severity groups were identical to those observed in the patients not receiving prophylaxis. During androgen therapy, cleaved HK levels were significantly higher in the patients experiencing > 12 attacks/year than in those experiencing < 3 attacks/year (48.6%, CI 44.5–52.7 vs. 39.9%, CI 37.4–42.4; $P = 0.002$).

No differences were evident in levels of cleaved HK, spontaneous pKK activity, and plasma capacity to inhibit kallikrein activity regarding sex and age in controls and patients; moreover, no differences in the same parameters were evident in type I and type II HAE-C1-INH patients.

Mutation screening revealed 35 different mutations in *SERPING1*, six of which have never been previously described. The same mutation was present in patients with different degrees of disease severity (Table 5), but although not statistically significant, missense mutations tended to be more frequent in the patients experiencing < 3 attacks/year (10/16; 62.5%) than in those experiencing > 12 attacks/year (17/38; 44.7%). No differences regarding mean plasma levels of cleaved HK were evident among the groups of mutations.

Discussion

Angioedema symptoms in patients with hereditary C1-INH deficiency are mediated by bradykinin, which is released when HK is cleaved by plasma kallikrein. A laboratory diagnosis of HAE-C1-INH is straightforward, but there are no clearly identifiable parameters that consistently reflect different disease states. Despite the persistent C1-INH deficiency, the severity of HAE-C1-INH changes over time and event-free phases alternate with clustered attacks. We investigated whether biochemical changes in the bradykinin release system correlate with disease severity and could be suitable as predictors when making therapeutic decisions.

We concentrated on the complement and contact systems because, of the four biological systems regulated by C1-INH, they are the most affected by C1-INH deficiency. A number of previous studies (also by our group) have found important changes in the components of the two systems that depend on C1-INH deficiency and are further increased by the presence of angioedema symptoms [12–14, 16, 19, 33–46]. In this study, we not only confirmed that the levels of the complement proteins C1-INH and C4 are significantly reduced during attacks, but also discovered that the same is true of C1q which, to the best of our knowledge, has not been previously reported.

C1-INH inactivates C1 stoichiometrically by binding to its enzymatic subcomponents C1r and C1s. Activation of C1 in HAE-C1-INH was first described by Donaldson [47], and the existence of factor XII-dependent complement activation has been demonstrated by Ghebrehiwet et al. [48, 49], who showed that the Hageman factor fragments (factor XII_f) can directly activate the classic complement cascade by activating C1r and (to a lesser extent) C1s. We have previously found that the formation of C1s-C1-INH complexes in plasma is increased during angioedema attacks, which suggests further activation of the classical complement pathway [50]. The reduction in the levels of C1q, the recognition unit of the C1 complex that does not bind to C1-INH, indicates that all three of the proteins forming C1 complex are involved in activating the classical complement pathway, which accompanies angioedema

Table 3. Complement parameters (expressed as % of normal pooled plasma), spontaneous plasma kallikrein activity (mU/mL), and the capacity of plasma to inhibit kallikrein activity (% of normal pooled plasma) in healthy controls and HAE-C1-INH patients not receiving prophylactic treatment who experienced < 3 attacks/year, 3–12 attacks/year, or > 12 attacks/year

	C1-INH function %	C1-INH Ag %	C4 Ag %	C1q Ag %	Spontaneous plasma kallikrein activity (mU/mL)	Capacity to inhibit kallikrein activity %
Controls	83.8 (80.6–87.0) (n = 81)	99.7 (97.5–101.8) (n = 81)	96.7 (91.7–101.6) (n = 81)	98.6 (97.6–99.6) (n = 81)	1.0 (0.6–1.4) (n = 73)	111.4 (108.6–114.3) (n = 73)
HAE-C1-INH < 3 attacks/year	12.7 (8.2–17.1) (n = 33)	11.8 (6.1–17.5) type I (n = 31)	22.9 (16.0–29.9) (n = 33)	98.3 (96.7–99.8) (n = 33)	2.5 (0.3–4.8) (n = 16)	30.3 (19.9–40.7) (n = 16)
HAE-C1-INH 3–12 attacks/year	13.5 (9.9–17.0) (n = 17)	16.9 (10.8–23.1) type I (n = 14)	16.4 (12.6–20.1)* (n = 17)	84.1 (73.1–95.1) (n = 17)	–	–
HAE-C1-INH > 12 attacks/year	11.1 (8.4–13.8) (n = 32)	12.8 (9.5–16.1) type I (n = 28)	9.8 (5.9–13.7) [†] (n = 32)	95.4 (90.4–100.4) (n = 32)	3.1 (2.2–3.9) (n = 10)	46.6 (42.0–50.6) (n = 10)

Geometric mean values and 95% confidence intervals. All the other values are not significantly different.

HAE-C1-INH, hereditary angioedema due to C1-INH deficiency

**P* = 0.011 between HAE-C1-INH patients characterized by < 3 attacks/year and those experiencing 3–12 attacks/year.

[†]*P* = 0.001 between HAE-C1-INH patients characterized by < 3 attacks/year and those experiencing > 12 attacks/year.

attacks in patients with hereditary C1-INH deficiency. The evidence that C1q may be reduced in hereditary C1-INH deficiency further lessen its value as parameter to discriminate between hereditary and acquired C1-INH deficiency [51]. The decline in C1-INH function during angioedema positively correlated with the capacity of plasma from HAE-C1-INH patients to inhibit kallikrein amyolytic activity, which suggests that the inhibition mainly depends on the activity of C1-INH. These findings parallel those provided by Joseph et al., who showed that the increase in plasma-activated factor XII and plasma kallikrein can be reverted by replacement therapy with plasma-derived C1-INH [46].

We also confirmed previous evidence that there is increased cleavage of HK during angioedema symptoms due to bradykinin release [41]. In brief, our measurements support the view that angioedema attacks are characterized by the generation of active kallikrein, which cleaves HK to release bradykinin, and concomitant activation of the classical complement pathway. The central role of bradykinin in angioedema is demonstrated by the efficacy of drugs inhibiting the generation and the effect of bradykinin in reverting HAE-C1-INH symptoms [52–54], but the consequence of complement activation during attacks has not yet been defined [55].

When we considered the same parameters in the absence of an angioedema attack by dividing the patients into three groups on the basis of the frequency of their symptoms, we found that only the levels of C4 (among the complement proteins) and cleaved HK (in the contact system) were significantly higher in frequently symptomatic patients than in those who were only slightly symptomatic. Moreover, cleaved HK was significantly higher in patients with an intermediate

number of attacks (i.e. 3–12 per year) than in patients with 0–2 attacks per year. It was not unexpected to find that highly symptomatic patients reproduced some of the changes characterizing angioedema attacks. Symptoms of angioedema emerge when the contact system, which is unstable because of the C1-INH deficiency, is exposed to a trigger eliciting the local generation of bradykinin at a specific site, which increases vasopermeability and thus causes oedema [42, 44]. The parameters that we measure in plasma coming from the systemic circulation therefore represent the diluted effect of local events, and it can be expected that the contact system is more unstable in patients with frequent attacks and allows an ongoing low degree of bradykinin generation at various sites.

We do not yet know the factor(s) that may be responsible for changing contact system instability in patients with C1-INH deficiency. However, as the angioedema occurs at various sites, it is likely that they act systemically and not just locally. Of the two parameters that change in highly symptomatic patients, C4 is influenced by treatment with attenuated androgens. This effect was long ago described by Laurell et al. [56], who showed that treatment with danazol (the androgen derivative used for HAE-C1-INH prophylaxis) significantly changes the levels of a number of plasma proteins and increases those of C4. This explains why some HAE-C1-INH patients receiving androgen prophylaxis have normal C4 levels but still develop angioedema (M. Cicardi personal observation), which makes C4 an unreliable marker of susceptibility to symptoms.

The parameter that best correlated with the presence of angioedema or susceptibility to it was the amount of cleaved HK. This is consistent with the fact that the cleaved form of HK is directly related to the release of

Table 4. Complement parameters (expressed as % of normal pooled plasma), spontaneous plasma kallikrein activity (mU/mL), the capacity of plasma to inhibit kallikrein activity (% of normal pooled plasma), and cleaved HK levels (% of total) in HAE-C1-INH patients (during remission) not receiving prophylactic treatment and those receiving prophylactic treatment

	C1-INH function %	C1-INH Ag %	C4 Ag %	C1q Ag %	Spontaneous plasma kallikrein activity (mU/mL)	Capacity to inhibit kallikrein activity %	Cleaved HK %
HAE-C1-INH no therapy	12.2 (10.0–14.5) (n = 82)	13.1 (10.1–16.0) type I (n = 73) 65.9 (45.3–86.4) type II (n = 9)	15.5 (11.9–19.1) (n = 82)	94.0 (90.9–97.1) (n = 82)	2.7 (1.3–4.1) (n = 26)	35.8 (29.2–42.3) (n = 26)	42.9 (40.7–45.1) (n = 65)
HAE-C1-INH androgen therapy	16.1 (12.8–19.3)** (n = 73)	18.4 (13.9–22.9) type I (n = 60) 104.0 (76.0–132.1) type II* (n = 13)	29.1 (22.6–35.6)*** (n = 73)	98.4 (97.3–99.4) (n = 73)	1.7 (1.1–2.4) (n = 39)	52.2 (47.5–56.9) (n = 39)	42.2 (40.1–44.3) (n = 52)
HAE-C1-INH tranexamic acid therapy	8.2 (4.1–12.2) (n = 7)	20.0 (7.9–32.0) type I (n = 6) 45.0 type II (n = 1)	10.2 (2.8–17.6) (n = 7)	99.5 (94.8–104.2) (n = 7)	9.2 (n = 2)	24.1 (n = 2)	50.8 (42.1–59.6) (n = 5)

Geometric mean values and 95% confidence intervals.

HAE-C1-INH, hereditary angioedema due to C1-INH deficiency; HK, high-molecular-weight kininogen

* $P = 0.02$, ** $P = 0.01$, *** $P < 0.001$ between HAE-C1-INH patients not receiving prophylactic treatment and those receiving prophylactic treatment with attenuated androgens.

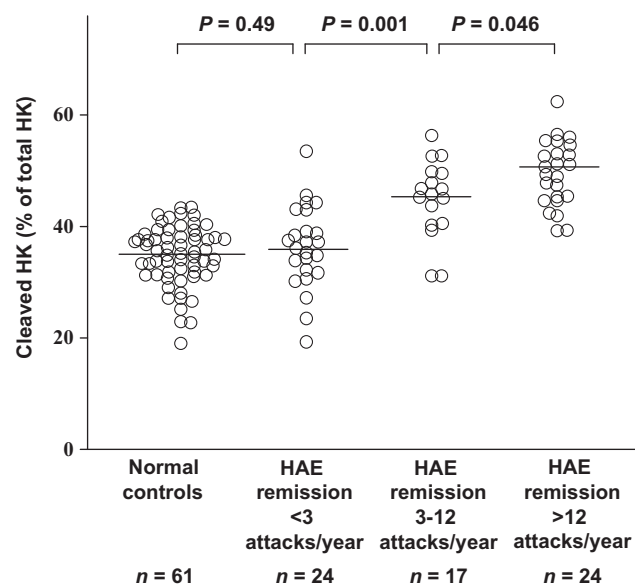


Fig. 6. Levels of cleaved high-molecular-weight kininogen (HK) (expressed as the percentage of total HK) in plasma taken from normal controls and patients with hereditary angioedema due to C1-INH deficiency (HAE-C1-INH) during remission, divided on the basis of the frequency of acute attacks.

bradykinin, the mediator of symptoms. Unfortunately, the cleaved HK levels in the three subgroups of patients based on disease severity partially overlap and prevent them from being used as an absolute parameter. This limitation is probably due to a combination of pre-analytical and analytical problems. Cleaved HK can be generated by *in vitro* activation of the contact system at the time blood is drawn, and although the extent of the activation can be limited by using antiprotease cocktails (such as that used in our samples) [19, 28, 30, 57], the residual cleaved HK reduces the sensitivity of the assay. Furthermore, quantitative immunoblotting is intrinsically insensitive. Nevertheless, despite these technical problems, our data support the value of cleaved HK as a target analyte for identifying disease severity in patients with HAE-C1-INH. Thus, our results on cleaved HK suggest that its measurement may have a prognostic value and may be useful to the decision whether or not prophylactic treatment is needed. The behaviour of spontaneous pKK activity was similar to that of cleaved HK; however, the overlapping between controls and HAE patients in remission and during attacks was more evident (Fig. 1). Plasma capacity to inhibit kallikrein activity discriminates fairly well between controls and HAE patients but less between HAE patients in remission and during attacks (Fig. 2). In line with this, recently Defendi et al. [58] measured spontaneous amidase activity plasma levels in HAE-C1-INH patients significantly higher than in healthy controls.

Table 5. Mutations in 65 HAE-C1-INH patients. Cleaved HK plasma levels are reported for each patient as single value and for each group of mutation as mean $\pm$ SD

Type of mutation	Traditional numbering	< 3 attacks/year	3–12 attacks/year	> 12 attacks/year	Cleaved HK (% of total HK)
Nonsense	2409C>T (Q67X)	0	1	0	46.3
	8729G>A (W277X)	0	0	1	36.8
	14043C>T (Q326X)	0	0	1	37.6
	16661insA (Y401X) [†]	0	1	1	48.3; 51.2
	16872C>T (R472X) [†]	2	0	3	54.0; 35.8; 50.3; 30.4; 54.5
	Total	2	2	6	44.5 $\pm$ 8.6
Large Ins/Del		0	1	3	62.9; 50.2; 34.0; 38.4 46.4 $\pm$ 12.9
Splicing	4486G>T	0	0	2	51.6; 45.2
	4516G>A*	1	0	0	61.5
	8946G>A	0	0	1	55.8
	Total	1	0	3	53.5 $\pm$ 6.9
Frameshift	2262_2263delAG (R18fsX35)	0	0	1	46.3
	8507 insT (L268fsX276)	0	0	1	59.5
	8778 delC (F293fsX298)* [†]	0	3	3	55.9; 50.3; 39.8; 54.0; 65.4; 40.4
	8779 insA (K294fsX305) [†]	1	0	1	31.1; 38.9
	16671delC (L405fsX408) [†]	1	1	2	37.7; 52.2; 41.1; 40.4
	16673 delG (L405fsX408)*	0	1	0	47.3
	16742 delA (T428fsX553) [†]	1	0	1	33.1; 38.1
	Total	3	5	9	45.4 $\pm$ 9.6
Missense	589G>A (M-22I) [†]	2	0	1	45.8; 27.7; 32.2
	2273G>T (G21G)*	0	0	1	57.0
	2647C>A (A146D)*	0	0	1	39.8
	4366C>A (T167N)	0	0	1	47.0
	8361G>C (R219P)*	0	1	0	45.7
	8821 T>A (Y308N) [†]	1	0	1	48.1; 35.8
	14224A>T (D386V)	1	0	0	38.9
	16738T>G (L427R)	0	0	1	49.5
	16764G>A (A436T) [†]	1	1	4	31.6; 46.1; 48.4; 38.4; 34.7; 39.2
	16789G>T (R444L)	0	0	1	53.1
	16789G>A (R444H)	0	0	1	44.7
	16810T>G (V451G) [†]	2	0	2	38.0; 36.6; 33.0; 35.2
	16810T>A (V451E)	0	1	0	44.2
	16867T>A (M470K)	0	0	1	66.0
	16873G>T (R472L) [†]	1	0	2	39.3; 49.4; 44.4
	16873G>A (R472Q)	2	0	0	34.4; 19.8
	Total	10	3	17	41.5 $\pm$ 9.2

HAE-C1-INH, hereditary angioedema due to C1-INH deficiency; HK, high-molecular-weight kininogen

*Novel mutations.

[†]Mutations associated with different groups of attacks/year.

We also used a genetic parameter to identify the propensity of patients to develop symptoms. It is known that patients with genetic C1-INH deficiency who share the same mutation do not necessarily present the same clinical phenotype [59, 60], but, despite the absence of any clear genotype/phenotype correlates, Bors et al. [61] have recently found that patients with missense mutations have a relatively milder phenotype. Our data tend to confirm this finding as missense mutations were 18% more frequent in asymptomatic patients than in highly symptomatic

patients; however, this difference did not reach the level of significance possibly because of the small number of cases. Moreover, a source of bias may be the fact that in our case list tested for genetic studies, the majority of HAE patients had a severe disease.

In conclusion, we identified biochemical markers that correlate with disease severity, although their sensitivity needs to be increased by improving measuring technique before they can be considered totally suitable for guiding clinical decision-making.

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Conflict of interest

Marco Cicardi received the following supports from pharmaceutical companies with interests in hereditary angioedema: Reimbursement for attending a symposium: Shire, SOBI, CSL Behring, Pharming, Viropharma. Speaking fees: Shire, SOBI, CSL Behring, Viropharma, Pharming. Fees for educational events: Shire, CSL

Behring. Research funds: Shire. Consulting agreement, advisory boards: Sigma Tau, SOBI, Viropharma, CSL Behring, BioCryst. Andrea Zanichelli received the following supports from pharmaceutical companies with interests in hereditary angioedema: Reimbursement for attending a symposium: Shire, SOBI, CSL Behring. Speaking fees: Shire, SOBI, CSL Behring, Viropharma. Fees for educational events: Shire, CSL Behring. Consulting agreement, advisory boards: Shire, CSL Behring. Chiara Suffritti, Massimo Cugno, Lorena Maggioni, Erika Bonanni, declare that they have no conflict of interest.

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