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Impaired control of the contact system in hereditary angioedema with normal C1-inhibitor

Running title: Contact system in normal C1-inhibitor hereditary angioedema

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Abstract

Background: Hereditary angioedema (HAE) comprises HAE with C1-inhibitor deficiency (C1-INH-HAE) and HAE with normal C1-INH activity (nl-C1-INH-HAE), due to mutations in factor XII (FXII-HAE), plasminogen (PLG-HAE), angiopoietin 1 (ANGPT1-HAE), kininogen 1 genes (KNG1-HAE) or angioedema of unknown origin (U-HAE). The Italian network for C1-INH-HAE (ITACA) created a registry including different forms of angioedema without wheals.

Objective: We analyzed clinical and laboratory features of a cohort of Italian subjects with nl-C1-INH-HAE followed by ITACA to identify specific biomarkers.

Methods: 105 nl-C1-INH-HAE patients were studied. Plasma concentrations of cleaved high molecular weight kininogen (cHK), Vascular Endothelial Growth Factors (VEGFs), angiopoietins (Angs) and secreted phospholipase A2 enzymes (sPLA2) were evaluated.

Results: We identified 43 FXII-HAE patients, 58 U-HAE and 4 ANGPT1-HAE. We assessed a prevalence of 1:1.4 x 10^6 for FXII-HAE and 1:1.0 x 10^6 for U-HAE. cHK levels in U-HAE patients were similar to controls in plasma collected using protease inhibitors cocktail (PIC), but they significantly increased in absence of PIC. In FXII-HAE patients cHK levels, in absence of PIC, were significantly higher than in controls. We found a significant increase of VEGF-A, VEGF-C, Ang1 levels in U-HAE patients compared to controls. In FXII-HAE only VEGF-C levels were increased. Ang2 concentrations and sPLA2 activity were not modified. The levels of these mediators in ANGPT1-HAE patients were not altered.

Conclusions: Our results suggest that pathogenesis of FXII-, ANGPT1- and U-HAE moves through an unbalanced control of kallikrein activity, with bradykinin as most likely mediator. VEGFs and Ang1 participate in the pathophysiology of U-HAE increasing the basal vascular permeability.

INTRODUCTION

Angioedema is a local, self-limiting edema due to periodic increase in vascular permeability. Affected individuals suffer from chronically recurrent swellings localized to the skin and/or to the mucous membranes of the upper respiratory and gastrointestinal tracts [1]. Angioedema can occur with or without hives and with different pathophysiologic mechanisms. Angioedema occurring independently of hives is referred to as primary angioedema and can be due either to mast-cell derived mediators or to the release of bradykinin, although other mechanisms are also envisaged [2, 3]. Recurrent angioedema can be hereditary or acquired as reported in the HAWK (Hereditary Angioedema International Working Group) classification [4]. The most common form of hereditary angioedema (HAE) is caused by deficiency of C1 esterase inhibitor (C1-INH-HAE), but HAE can also occur with normal plasma levels of C1-INH (nl-C1-INH-HAE). This form of HAE can be due to mutations in genes coding for coagulation Factor XII (*F12*, FXII-HAE), angiopoietin 1 (*ANGPT1*, ANGPT1-HAE), plasminogen (*PLG*, PLG-HAE) and kininogen 1 gene (KNG1-HAE) [5]. In a relevant number of patients, in whom angioedema is clearly hereditary, genetic cause is not identified: these patients are classified as having angioedema of unknown origin (U-HAE) [4-7]. All HAE share similar clinical phenotypes, with absence of wheals and are non-responsive to H1-antihistamine therapy.

Angioedema with deficiency of C1-INH is due to mutations in *SERPING1* gene (LRG_105; ENSG00000149131; OMIM #606860) and it was first identified in 1963 (C1-INH-HAE, OMIM #106100) [8]. C1-INH deficiency causes an uncontrolled activation of the contact/kallikrein-kinin systems resulting in local release of the vasoactive peptide bradykinin (BK) as reported by Fields *in vitro* [9] and by Nussberger *in vivo* [10]. The clinical expression of C1-INH-HAE is heterogeneous among patients [11, 12], with a clinical spectrum varying from a minority of asymptomatic cases to patients suffering from weekly disabling and life-threatening attacks.

Mutations in *F12* gene (LRG_145, ENSG00000131187, OMIM #610619) encoding human coagulation FXII were the first identified gene variants leading to HAE with normal levels of C1-INH in plasma (FXII-HAE, OMIM # 610618) [13, 14]. FXII-HAE phenotype is almost exclusively expressed by females [15, 16]. de Maat *et al*. showed that mutations in *F12* gene introduce a cleavage site for plasmin. This facilitates conversion of FXII protein into its active form FXIIa, which can in turn generate active kallikrein and bradykinin leading to angioedema [17]. Ivanov *et al.* have recently demonstrated that Factor XII with Lys/Arg substitutions for Thr309 can be cleaved by thrombin and factor XIa generating the truncated species δFXII, which

in turn activates kallikrein [18]. In ANGPT1-HAE the mechanism of angioedema implies that this mutation could impair the interaction of angiopoietin-1 with its endothelial membrane receptor TIE2, leading to a vascular leakage and angioedema [19]. We have recently found the c.807G>T, p.(Ala119Ser) *ANGPT1* mutation in a female patient with apparently non-hereditary recurrent angioedema [20]. No pathogenetic mechanism has been envisaged for HAE related to mutation in plasminogen and kininogen 1 genes.

In 2012 an Italian network for C1-INH-HAE (ITACA) was established and provided a database of patients with C1-INH-HAE [21, 22]. Starting from the ITACA database, a web based multi-centre global registry was created with the support of the Italian HAE association. Moreover, a separate registry was built to include different forms of angioedema not associated to wheals. In this paper we report the first large survey on genetic characteristics, laboratory measurements and clinical features of Italian subjects diagnosed with HAE with normal C1-INH followed by the ITACA network.

We previously reported that C1-INH-HAE patients showed increased plasma levels of cleaved high molecular weight kininogen (cHK) [23], and vascular permeability factors such as Vascular Endothelial Growth Factors (VEGFs), Angiopoietins (Angs) and secreted phospholipase A2 enzymes (sPLA2) when compared to healthy controls [24, 25]. In order to identify specific biomarkers in different forms of HAE, we measured cHK (as indirect evidence of bradykinin generation), VEGFs and Angs concentrations and sPLA2 activity in patients with FXII- and U-HAE.

MATERIALS AND METHODS

Patients

The study includes patients with recurrences of angioedema without hives resistant to second-generation antihistamine, administered at a dosage up to 4 times the one used for allergic disorders, and at least one family member, within the second degree, with history of recurrent angioedema. Patients with history of urticaria were excluded. A written informed consent for genetic and clinical studies was obtained from subjects enrolled in the study. The ITACA registry was approved by the local Institutional Review Boards of participating centres. The study was conducted in accordance with the Principles of the Declaration of Helsinki. For each patient a detailed clinical history was obtained. Data regarding age, gender, ethnicity, age at first symptoms and age at diagnosis, delay in diagnosis, location and frequency of angioedema attacks, estrogens exposure, complement parameters and therapy were recorded. As control group, data on demographic characteristics of the Italian general population were collected from the Italian Institute for Statistic (January 2018). (https://www.istat.it/it/archivio/208951).

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes according to standard protocols. Mutational screening of *SERPING1*, *F12*, *ANGPT1* and *PLG* coding region and exon/intron boundaries was performed by direct DNA sequencing, as described elsewhere [19, 26, 27]. We have standardized the PCR conditions using primers designed with Primer3 software (www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) and chosen on the basis of known sequences of *SERPING1*, *F12*, *ANGPT1* and *PLG* as reported in ENSEMBL database (Wellcome Trust Sanger Institute, Cambridge, United Kingdom): *SERPING1* ENSG00000149131, *F12* ENSG00000131187, *ANGPT1* ENSG00000154188 and *PLG* ENSG00000122194. Briefly, polymerase chain reactions were carried out in 50 µl samples in a Bio-Rad thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each sample contained 0.15 µg of genomic DNA, 0.3 µM of each primer, 200 µM of dNTP, 1X PCR buffer (with 1.5 mM $MgCl_2$), and 1.5 U of AmpliTaqR Gold Polymerase (Applied Biosystems Inc., Foster City, CA, USA). PCR products were purified and subjected to direct-cycle sequence analysis using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

The Data Collection instrument software provided the raw intensity data into a file called *.ab1 file. The primary analysis tool Sequencing Analysis Software used a base-caller algorithm that performs base calling for pure and mixed base calls, analyses the background signal noise and gives a quality score to that base. In order to view bases, assembly multiple samples and compare to a reference sequence (alignment), the Sequencher v.4.7 tool (Gene Codes, Corp.) was used. Variants causing HAE were described according to the Human Genome Variation Society recommendations (http://varnomen.hgvs.org/; v.19.01).

Complement parameters

Blood samples were diluted with sodium citrate solution (0.11 mol/l) and then centrifuged (20 min, 2000 g, 22°C). The plasma samples collected were immediately frozen and stored at -80°C until tested. C1-INH activity was measured using a colorimetric assay (Technochrome C1-INH, Technoclone GmbH, Vienna, Austria). Normal values of activity of C1-INH are greater than 0.7 Unit C1 INH/ml (>70%). All patients enrolled in this study showed a C1-INH functional activity higher than 50%, as previously reported [28]. C1-INH and C4 antigen levels were measured by means of radial immunodiffusion (RID) (NOR-Partigen, Siemens Healthcare Diagnostics, Munich, Germany).

Cleavage of high-molecular weight kininogen

Measurements were conducted collecting blood in tubes containing sodium citrate, tubes containing the protease inhibitors cocktail (PIC) previously described [29] and commercial tubes (BD EDTA-P100, code 366448) with PIC added by the manufacturer. PIC prevents *in vitro* activation of contact system that occurs during blood collection and handling. Blood samples from all patients were obtained at least 8 days apart from an angioedema attack. The cleavage of HK was assessed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis (a modification of the method described by Berrettini *et al*.) [30, 23]. The amount of cHK was expressed as a percentage of total HK [31].

Determination of VEGFs and Angs

Plasma levels of angiogenic and lymphangiogenic mediators were measured using commercially available ELISA kits for VEGF-A, VEGF-C, Ang1 and Ang2 (R&D System) according to the manufacturer's instructions. The ELISA analytical ranges are 31.1–2,000 pg/ml for VEGF-A, 62–4,000 pg/ml for VEGF-C, 156.25–10,000 pg/ml for Ang1 and 31.1–4,000 pg/ml for Ang2 [24].

201 Phospholipase A_2 activity assay

202 Activity of PLA_2 in plasma of patients and healthy controls was measured by Life Technologies
203 EnzChek®phospholipase A_2 assay.

204 Statistical analysis

205 Data were analyzed using the GraphPad Prism 5 software package. Data were tested for normal
206 distribution using the D'Agostino-Pearson normality test. If normality was not rejected at 0.05
207 significance level, we used parametric tests, in particular Kruskal-Wallis test. Otherwise, for not-
208 normally distributed data we used nonparametric tests. Statistical analysis was performed by one-
209 way analysis of variance (ANOVA), followed by Dunnett's test (when comparison was made
210 against a control) or Bonferroni's test (when comparison was made between each pair of groups).
211 Correlations between two variables were assessed by Spearman rank correlation analysis and
212 reported as coefficient of correlation (r). A p value ≤ 0.05 was considered statistically significant.

213

RESULTS

Genetic diagnosis

We identified 105 Italian subjects with nl-C1-INH-HAE. Genotyping showed that none of them had mutations in *SERPING1,* 43 were FXII-HAE and 4 ANGPT1-HAE as reported previously [19]. The remaining 58 subjects had no mutations in *F12, ANGPT1* and *PLG* and were classified as U-HAE (Tab. S1). On the basis of demographic data of the Italian population in 2018 (60,494,000 inhabitants), we can derive a minimum prevalence equal to 1:1.4 x 10^6 for FXII-HAE, 1:1.0 x 10^6 for U-HAE and 1:5.8 x 10^5 for nl-C1-INH-HAE.

FXII-HAE

The 43 FXII-HAE patients (11 males and 32 females, ratio 1:2.9; median age 39 years, range 5-88) belong to 9 unrelated families, 5 of them already described [6, 27]. Pedigrees of the four newly reported families are given in Fig. S1. All bear the most frequent missense mutation c.1032C>A p.(Thr309Lys) in heterozygous state. As previously described, we observed a variable penetrance of the missense mutation: 44.4% of females with the mutation were symptomatic.

Genetic analysis of the entire gene revealed the presence, in homozygous and heterozygous state, of single nucleotide polymorphisms (SNPs), described previously, and not correlated with clinical phenotype (Tab. S2).

Including the FXII-HAE families already described, the pathogenic mutation was present in 32 females (53.5% with history of recurrent angioedema) and in 11 men all asymptomatic for angioedema (Tab 1).

Median age of symptoms onset was 21 years (range 5-76) with a median delay in diagnosis of 13 years (range 0-42). The most frequent angioedema locations were face (91% of patients), abdomen (74%) and peripheral (trunk, limbs, genitals) (65%) (Tab. 2). Patients reporting attacks involving laryngeal mucosa were 39% and tongue 26%. In 20 symptomatic subjects reliably recording attack, the median frequency of angioedema was 4 (range 1-13) per year and median attack duration 42 hours (range 12-90). Factors triggering attacks in most patients were hormone replacement therapy (1/1), oral contraceptive (OC) (19/19) and pregnancy (11/16). One subject reported attacks (1.5/month) only during pregnancy. One subject experienced a single attack that occurred during therapy with estroprogestins. Physical or psychological stress were reported as triggering factors by a minority (4/23) of symptomatic patients. One patient became symptomatic after exposure to angiotensin converting enzyme inhibitor (ACEI). The patient stopped having

attacks two months after ACEI withdrawal. Three patients were started on ACEI prescribed by their general practitioner. They did not experience attack recurrences and remained on the same medication.

Treatment of attacks

Icatibant was used in 9 patients for 26 attacks. Seven patients responded with disappearance of angioedema within 12 hours from treatment. Two patients were considered non-responsive because the attacks remission initiated >24 hours from treatment. Five patients were treated with plasma derived C1-inhibitor and one with fresh frozen plasma. All attacks became negligible within 12-hours from treatment. Two patients reported tranexamic acid to be effective in reducing severity and duration of attacks; one patient found this treatment inefficacious. Data regarding attacks were analyzed retrospectively.

Prevention of attacks

Due to the frequency of recurrences (≥1 attack/month after removal of potential triggering factors), eight patients started long term prophylaxis. Six patients used tranexamic acid (duration of treatment 17-46 months; dose: 1.5-2 g/day) with significant reduction of recurrences (≤3 attack/year). Due to an unprovoked portal vein thrombosis, one patient was started on progestin instead of tranexamic acid. Upon this treatment, ongoing for 4 years, attacks were reduced from 2/month to 1/year. One patient suffering from cutaneous and abdominal symptoms started on tranexamic acid that failed in controlling cutaneous attacks. Plasma derived C1 inhibitor twice a week was added to the prophylactic regimen and was able to control cutaneous, but not abdominal symptoms. Combination therapy (plasma derived C1-INH and tranexamic acid) is still ongoing.

Plasma derived C1 inhibitor was used for short-term prophylaxis before esophago-gastro-duodenoscopy (EGDS) (3 patients), bronchoscopy (1 patient) and dental procedures (3 patients). Upon short term prophylaxis all medical interventions were uneventful. Previous dental extraction without prophylaxis in 2 patients repeatedly resulted in angioedema of the face and oral mucosa.

Recently a prophylaxis with plasma derived C1-INH (1000 U every 4 days) has been administered during pregnancy to two sisters due to symptoms worsening (severity and increase in the number of attacks), with an almost complete control on cutaneous and abdominal symptoms.

U-HAE

Fifty-eight patients, in 38 independent families spanning 2-4 generations, were diagnosed with U-HAE (median age 44 years, range 12-82, Tab.1). Pedigrees of some U-HAE families are reported in Fig. S2. Twenty-four patients were males (41.4%) and 34 females (58.6%), with a ratio of 1:1.4. Angioedema symptoms presented no gender related differences. Median age of symptom onset was 23 years (range 1-69) with median delay in diagnosis similar to that observed in FXII-HAE (10 years, range 1-55) (Tab.1). The most frequent angioedema locations were face (87%) and skin (63%) with attacks involving laryngeal or upper airways and tongue in 40% and 27% of cases, respectively (Tab. 2). Interestingly, attacks involving abdomen were found significantly lower in U-HAE (42%) than in FXII-HAE patients. The mean number of acute attacks was 6 per year with mean attack duration of 2 days (range 3 hours to 5 days).

In 28 patients angioedema recurrences worsened under specific circumstances: oral contraceptives (5 patients), menstrual cycle (2 patients), pregnancy (2 patients), exposure to high temperatures (5 patients), recurring infections (5 patients), physical trauma (4 patients), ACEI therapy (3 patients), and emotional distress (2 patients).

Treatment of attacks

Six patients treated their acute attacks with tranexamic acid, three with plasma derived C1 inhibitor and one patient with Icatibant plus tranexamic acid with resolution in 12 hours. Icatibant alone was used by 2 patients and seemed efficacious in one of them. Data regarding attacks were analyzed retrospectively.

Twenty-one patients with one or more attacks per month were on prophylactic treatment with tranexamic acid. Eleven had consistent (<3 attacks/year) and persistent (ongoing treatment for 4-5 years) attack reduction. Ten patients stopped the treatment due to absence of efficacy. No side effects were reported.

Laboratory studies

Contact system activation

Cleaved HK (cHK) is an indirect measure of the bradykinin released upon activation of the contact system. Levels of cHK are higher in plasma from patients deficient in C1-INH and further increase when plasma is collected without protease inhibitors. We measured plasma levels of cHK in samples from 72 healthy subjects (11 in sodium citrate and 61 with PIC), 19 patients with FXII-HAE (sodium citrate only) and 58 patients with U-HAE (35 samples collected in sodium citrate and 23 with PIC) (Fig 1). Mean levels of cHK in samples from healthy subjects collected with and

without PIC were not significantly different [36% (32-38) vs 33% (31-36), median values (interquartile ranges)]. In U-HAE patients during remission, cHK levels were similar to those in healthy subjects in samples with PIC [33% (30-41) vs 36% (32-38), respectively] and significantly higher in absence of PIC [50% (46-55) vs 33% (31-36); $p<0.01$, respectively]. Moreover, in FXII-HAE patients cHK levels, measured in absence of PIC, were not significantly different from U-HAE, but significantly higher than in normal subjects [50% (47-56) vs 33% (31-36); $p<0.01$] (Fig. 2).

Vasoactive mediators

We evaluated the concentrations of different angiogenic and lymphangiogenic factors in 34 healthy controls, in 15 FXII-HAE, in 31 U-HAE and 4 ANGPT1 patients in remission. Figure 3 shows that VEGF-A (panel A) plasma levels of U-HAE patients were higher than in healthy controls [VEGF-A: 3.5 (0-17.5) vs 0 (0-0.7) pg/ml, median values (interquartile ranges)]. VEGF-C concentrations were also elevated in U-HAE patients compared to controls (Fig. 3B) [VEGF-C: 674 (492-843) vs 154 (97-211) pg/ml; $p<0.01$]. Plasma levels of VEGF-A were not increased in FXII-HAE patients compared to controls (panel A) [0 (0-0) vs 0 (0-0.7) pg/ml], while VEGF-C concentration (panel B) was significantly higher [350 (192-442) vs 154 (97-211) pg/ml; $p<0.01$]. Interestingly, Ang1 was increased only in U-HAE but not in FXII-HAE patients compared to controls [U-HAE: 3.7 (2.6-5.6); FXII-HAE 2.7 (0.8-3) vs controls 2.1 (1.6-2.6) ng/ml); $p<0.01$] (Fig. 3C). In contrast, Ang2 levels, did not differ in the groups [U-HAE 120.2 (65.6-175), FXII-HAE 27.2 (0-153) vs controls 77 (0.1-244) pg/ml; $p=0.273$] (Fig. 3D). Moreover Fig. 3 showed that the concentrations of VEGF-A (panel A), VEGF-C (panel B), Ang1 (panel C) and Ang2 (panel D) were not altered in ANGPT1-HAE patients compared to healthy controls. In FXII-HAE and U-HAE patients in remission, plasma levels of cHK did not correlate with VEGF-A and Ang2 concentrations. (Fig. 4).

$sPLA_2$ activities, elevated in patients with C1-INH-HAE [25], showed no differences when measured in FXII-HAE, U-HAE and ANGPT1-HAE patients. Interestingly, the concentrations of these mediators did not differ between symptomatic and asymptomatic FXII-HAE patients (data not shown).

Discussion

Here we reported the cohort of 105 patients with nl-C1-INH-HAE present in the database from ITACA, the network of Italian angioedema centers. Forty-three patients (9 families) had FXII-HAE, 4 (1 family) ANGPT-HAE and 58 (38 families) U-HAE. In 2015, the ITACA database of patients with C1-INH-HAE listed 920 living subjects belonging to 367 families [21]. The numbers suggest that frequency of nl-C1-INH-HAE is about 1/10 compared to that of HAE due to C1-INH deficiency. Bork et al. reported a cohort of 265 German patients with nl-C1-INH-HAE from 88 unrelated families: 23 had FXII-HAE and 65 U-HAE [7]. Neither ANGPT-HAE, PLG-HAE nor KNG1-HAE had been described at the time of the publication. Assuming that the two cohorts represent the majority of diagnosed patients in both countries, since population in Germany is 1.3 times larger than in Italy, prevalence of nl-C1-INH-HAE is nearly double in Germany than in Italy. Separating FXII-HAE and U-HAE, prevalence in Germany vs Italy is 1.2 and 2.5 folds respectively. Thus, we can conclude that nl-C1-INH-HAE and particularly FXII-HAE have different distribution in Europe. Four different *F12* variants can lead to FXII-HAE [32-35], but a single one, c.1032C>A p.(Thr309Lys), accounts for the large majority of all cases worldwide. This variant originates from a common founder [36] and acts as a gain of function mutation [18]. In contrast, C1-INH-HAE has an identical prevalence worldwide and is caused by loss of function *SERPING1* variants rarely shared by independent families and frequently identified as *de novo* mutations [37]. All these findings make it likely that FXII-HAE may have different distribution in Europe. Geographical distribution of U-HAE seems intermediate between C1-INH-HAE and FXII-HAE, but this setting likely assembles different genotypes that have just started being identified.

In 2017 Bork performed exome analysis by NGS in families with U-HAE [38]. Four of seven had the mutation c.9886A>G p.(Lys330Glu) located in the gene coding for plasminogen (*PLG*). Segregation studies within these families demonstrated that this mutation was associated with the presence of angioedema symptoms. The missense mutation ANGPT1 c.807G>T p.(Ala119Ser), was detected in all symptomatic members of an Italian family with U-HAE but not in asymptomatic family members [19]. Today PLG-HAE and ANGPT-HAE have been separated from U-HAE and we expect the same to happen with the discovery of novel involved genes. Recently, Bork found that a new variant in the KNG1 gene leads to a novel type of HAE, HAE with normal C1-INH and a specific variant in the KNG1 gene or HAE-KNG1 [5].

In terms of clinical phenotype, our results on FXII-HAE and U-HAE are consistent with the existing literature and confirm that disease expression in U-HAE is similar to C1-INH-HAE, while FXII mutations cause angioedema when present in women: symptomatic men are rare exceptions. In addition to gender restriction, severity of FXII-HAE is very sensitive to estrogens levels: frequency of angioedema increases during pregnancy and estrogen-based treatments [13, 14, 39]. The only exception is the FXII-HAE population described in Brazil, where 53% of males have symptoms of angioedema [40]. Brazilian and German patients also differ greatly in levels of plasminogen activation inhibitor-2 [41]. The reason for-these differences is still unexplained.

In terms of clinical presentation, our cohorts of nl-C1-INH-HAE confirm similarities with other reports [40, 42, 7]. Median age of symptoms onset was 21 years for FXII-HAE and 23 for U-HAE, most frequent angioedema location was face [38]. Patients experienced also attacks involving laryngeal mucosa and tongue. We did not record deaths for laryngeal edema, which were reported in both FXII-HAE and U-HAE German patients [7]. Compared to C1-INH-HAE where symptoms onset is within the second decade of life [43], angioedema in nl-C1-INH-HAE tends to start during the third decade.

Genotyping allows precise diagnosis in nl-C1-INH-HAE with defined genetic defect, while the definition of U-HAE relies on the clinical characteristics of the angioedema and on its presence in two or more members in the same family [4]. No biochemical test for diagnosing nl-C1-INH-HAE has yet been developed, due to the poor knowledge that we have of the mechanisms leading to angioedema.

Unclear disease prevalence, blurred diagnosis and lack of specific target for therapy prevented so far pivotal trials in nl-C1-INH-HAE, which remains without therapy. This is strikingly in contrast with C1-INH-HAE where 8 different drugs are on the market and 5 are in different phases of clinical development. All these treatments target bradykinin or its release [44]. Since it is widely accepted that nl-C1-INH-HAE is bradykinin mediated, drugs for C1-INH-HAE may be effective even in HAE where functional C1-INH levels, measured using a commercial chromogenic assay, are above 50% of normal. In addition, when functional C1-INH levels were measured in nl-C1-INH-HAE based on inhibition of factor XIIa or kallikrein, a range of 60-75% of normal was reported [45]. Data from off label experience tend to confirm the role of bradykinin in nl-C1-INH-HAE and data that we presented here move in the same direction [46-49]. However, lack of uniform diagnostic criteria for patients' recruitment and a significant interindividual variability in

response to treatment, leaves without convincing treatment strategy to approach nl-C1-INH-HAE even within an off-label area.

Attempts have been made at unravelling mechanisms leading to angioedema in patients with normal C1-INH. Kaplan and Austen in 1971 demonstrated that plasmin is able to cleave factor XII to release activators of prekallikrein [50]. de Maat *et al*. showed that mutations in *F12* gene that lead to FXII-HAE create a novel cleavage site for plasmin in mutant proteins and mutated factor XII has a facilitated plasmin cleavage [17]. Extrapolating from this evidence and from the favourable therapeutic effect of the plasmin inhibitor tranexamic acid on non-histaminergic angioedema with normal C1-INH [6, 51-52], a role for plasmin in kinin mediated angioedema can be envisaged.

Previous studies have found important changes in the components of the systems regulated by C1-inhibitor that depend on its deficiency. Increased formation of bradykinin in citrated plasma collected from C1-INH-HAE patients was demonstrated by Fields *et al*. [9]. Plasma kallikrein and cleavage of its substrate HK are higher in patients with C1-INH-HAE in resting conditions than in normal subjects and increase further during attacks [53-55]. Hofman et al. using an ELISA method showed that cleaved kininogen is biomarker of bradykinin release in hereditary angioedema [56]. We used a western blot based assay to quantify cHK in plasma. With this method as with other methods aimed at detecting activation of the contact system, pre-analytic variability should be carefully considered. At blood drawing, contact system activates and when plasma kallikrein is poorly controlled, as in C1-INH deficiency [57], massive cleavage of HK occurs unless blood is protected by direct collection in an anti-protease cocktail (Fig 1). Evidence that blocking plasma kallikrein prevents cleavage of HK, comes from studies with lanadelumab [58]. Therapeutic doses of lanadelumab block plasma kallikrein for several weeks and prevent cleavage of HK even if blood is drawn in absence of anti-protease cocktails [59]. We previously reported that under anti-protease protection, plasma levels of cHK not only differentiate C1-INH-HAE patients from normal subjects, but also differentiate C1-INH-HAE patients outside and during attacks and those with different degrees of disease severity [23, 55]. Baroso *et al*. [60] found levels of cleaved HK significantly higher in angioedema patients with normal C1-INH compared to healthy donors. Here we found that under analogous anti-protease protected conditions, plasma levels of cHK in patients with nl-C1-INH-HAE were not significantly different from healthy controls. When blood was drawn without anti-protease cocktail, the levels of cHK in both FXII-HAE and U-HAE were significantly higher than in healthy control plasma collected in identical conditions. These data

suggest that generation of active kallikrein is facilitated in plasma from patients with nl-C1-INH-HAE, compared to healthy controls, but to a lesser extent than in patients with genetic deficiency of C1-INH. Accordingly, Lara-Marquez *et al*. [61] measuring plasma kallikrein activity in samples stimulated ex-vivo with sub-maximal doses of dextran sulphate, found that all patients with HAE generate significantly more kallikrein than normal subjects or patients with histaminergic angioedema. Across HAE patients, kallikrein generation was higher in C1-INH deficient plasma than in plasma with normal C1-INH. These data lead to conclude that all HAE, are characterized by reduced control of kallikrein generation. Nevertheless, further studies are needed to confirm whether the differences between forms with and without C1-INH deficiency can be used as biomarkers for distinguishing among pathogenetic mechanisms that impact on treatment strategies.

Plasma levels of vasopermeability factors VEGFs and ANGPT1 [24] and $sPLA_2$ [25] are increased in patients with C1-INH-HAE, and they further increase in patients with elevated cHK concentrations and experiencing a higher frequency of angioedema attacks [24]. Measuring these factors in nl-C1-INH-HAE, we found that U-HAE patients in remission have higher plasma levels of VEGF-A compared to healthy controls. Moreover, the concentrations of this mediator in U-HAE patients are similar to those of C1-INH-HAE patients (data reported in our previous paper [24]). The plasma levels of VEGF-C and ANGPT1 were increased in U-HAE patients compared to controls but not in comparison with C1-INH-HAE patients [24]. In FXII-HAE and ANGPT1 patients the relevance of these differences remains unclear.

In conclusion, our nationwide-based study shows that HAE with normal C1-INH is rarer in Italy than in Germany where it was originally identified. The term nl-C1-INH-HAE collects analogous clinical picture caused by different and in part yet unknown genetic defects. Reduced control of kallikrein activity characterizes all forms of nl-C1-INH-HAE. Based on this finding we can assume that bradykinin is the main mediator of symptoms in all nl-C1-INH-HAE, but with different pathogenetic mechanisms for its release.

Tab. 1 Demographic and clinical features of the patients

Demographic and clinical features	FXII-HAE (n=43)	U-HAE (n=58)	P value
Age-years	**39 (29-57)**	**44 (34-51)**	0.64
Females (n)	**32 (74.4%)**	**34 (58.6%)**	0.095
Families (n)	**9**	**38**	
Caucasian patients	**100%**	**100%**	
Symptomatic males	**0**	**26 (41.3%)**	
Age at onset (years)	**21 (18-24)**	**23 (12-32)**	0.97
Diagnosis delay (years)	**13 (5-24)**	**10 (4.5-21)**	0.84
Attack duration (hours)	**42 (24-48)**	**48 (24-72)**	0.51
Attack frequency (n/year)	**4 (3-6)**	**6 (3-12)**	0.005

Data reported in the table were expressed as median values (interquartile ranges) and analysed by using t test. $p<0.05$

Tab. 2 Distribution of angioedema attacks in FXII-HAE and U-HAE patients.

	Larinx	Abdomen	Face	Tongue	Peripheral sites
FXII-HAE (%)	39	74	91	26	65
U-HAE (%)	40	42	87	27	63

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FIGURE LEGEND

Figure 1. lmmunoblotting of cleaved HK in plasma collected from U-HAE patients using NaCit (NA, lane 1) or protease inhibitors (PI, lane 2), from normal subjects (N, lane 3) and from FXII-HAE patients using NaCit (NA, lane 4). The normal pattern is a major band with a Mr of 130,000 and a band with a Mr of 107,000. Samples from U-HAE patients in NaCit show the appearance of a third band with a Mr of 98,000. Cleaved HK levels (% of total) are indicated.

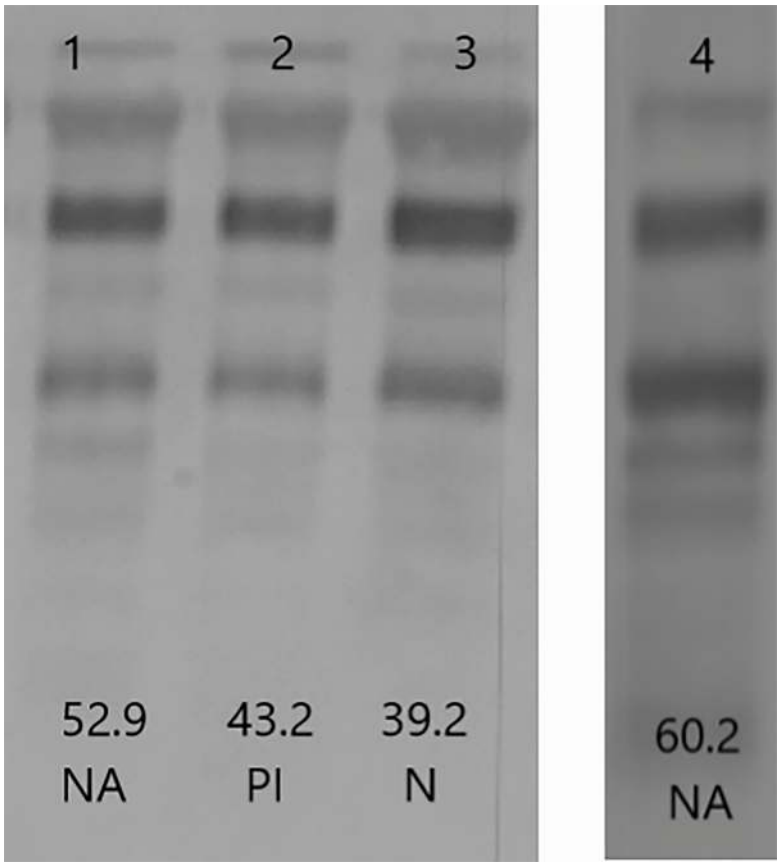
Figure 2. Levels of cleaved HK (expressed as the percentage of total HK) in plasma collected from healthy subjects, FXII-HAE patients, and U-HAE patients using sodium citrate (NaCit) or a mixture of inhibitors. Levels of cleaved HK are shown as the median (horizontal black line), the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 72 healthy subjects (11 samples collected in sodium citrate and 61 with inhibitors), 19 FXII-HAE (samples collected in sodium citrate), 58 U-HAE (35 samples collected in sodium citrate and 23 with inhibitors). * $p < 0.01$

Figure 3. Plasma concentrations of VEGF-A, VEGF-C, Ang1 and Ang2 in FXII-HAE, U-HAE and ANGPT1 patients. Plasma VEGF-A (A), VEGF-C (B), Ang1 (C) and Ang2 (D) in controls (Healthy) and in patients with FXII-HAE, U-HAE and ANGPT1 in remission. Data are shown as the median (horizontal black line), the 25thand 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 34 controls, 15 FXII-HAE, 31 U-HAE and 4 ANGPT1 patients. * $p < 0.01$

Figure 4. Correlations between two variables: cleaved high-molecular-weight kininogen (cHK) and VEGF-A (A, E); cHK and VEGF-C (B, F); cHK and Ang1 (C, G); cHK and Ang2 (D, H) were assessed in FXII-HAE (A-D) and U-HAE (E-H) by Spearman rank correlation analysis. A p value ≤ 0.05 was considered statistically significant. NS: non-significant

Figure S1. Pedigree structure of the families with FXII-HAE and genotype data of F12 locus

Figure S2. Pedigree structure of some families with U-HAE



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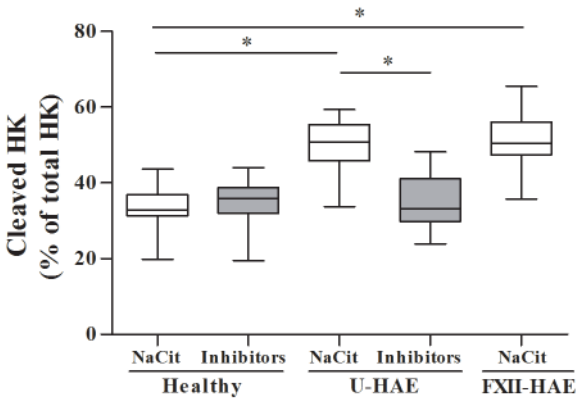

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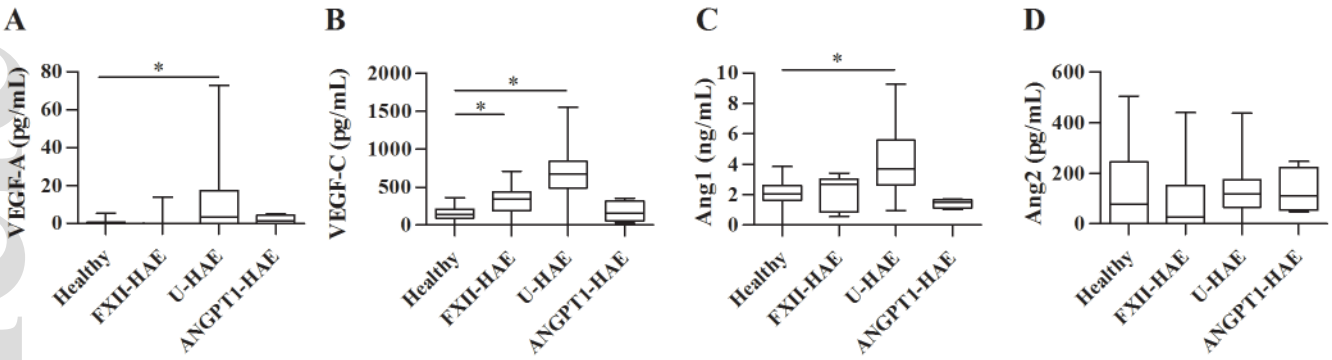

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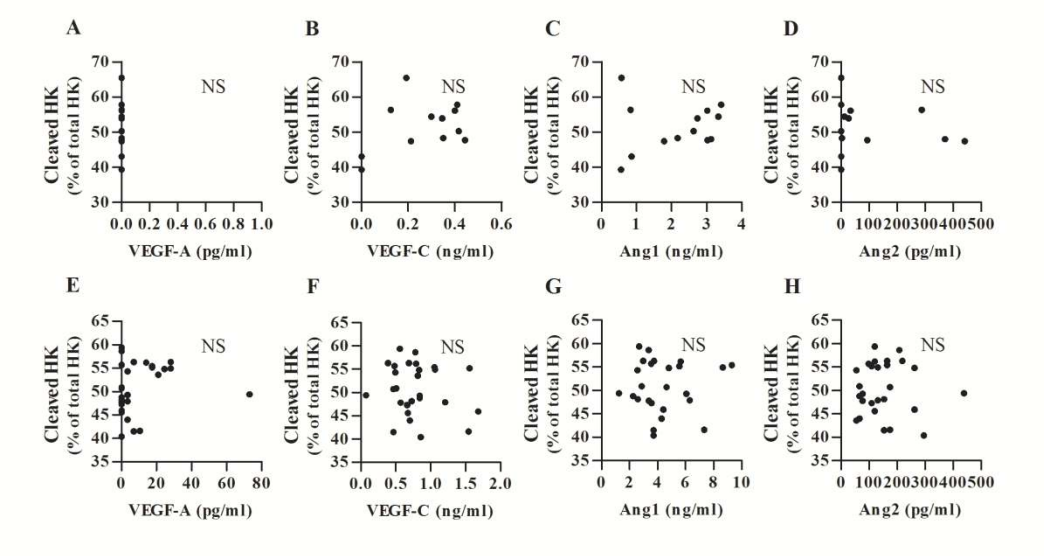

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