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Elevated plasma levels of vascular permeability factors in C1 inhibitor-deficient hereditary angioedema

Short Title: Increased of VEGF and Ang1/2 in hereditary angioedema

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ABSTRACT

Background: Hereditary Angioedema with C1 inhibitor deficiency (C1-INH-HAE) is a rare inherited genetic disease characterized by recurrent swelling episodes of the skin, gastrointestinal tract and upper airways. Angioedema attacks result from increased vascular permeability due to the release of bradykinin from high molecular weight kininogen. Currently, there are no biomarkers predicting the frequency of angioedema attacks. Vascular permeability is modulated by several factors, including vascular endothelial growth factors (VEGFs) and angiopoietins (Angs).

Since increased circulating levels of VEGFs and Angs have been observed in diseases associated with higher vascular permeability (e.g. systemic capillary leak syndrome and sepsis), we sought to analyze plasma concentrations of VEGFs and Angs in C1-INH-HAE patients.

Methods: 68 healthy controls and 128 C1-INH-HAE patients were studied. Concentrations of angiogenic (VEGF-A, Ang1, Ang2), anti-angiogenic (VEGF-A_{165b}) and lymphangiogenic (VEGF-C) factors were evaluated by ELISA. C1-INH functional activity was assessed by EIA.

Results: Plasma concentrations of VEGF-A, VEGF-C, Ang1 and Ang2 were higher in C1-INH-HAE patients in remission than in healthy controls. Concentration of VEGF-A was further increased in patients with lower C1-INH functional activity. C1-INH-HAE patients experiencing more than 12 angioedema attacks per year were characterized by higher plasma levels VEGF-A, VEGF-C and Ang2 compared to the other patients.

Conclusions: We hypothesize that VEGFs and Angs induce a state of “vascular pre-conditioning” that may predispose to angioedema attacks. In addition, the identification of increased plasma levels of VEGFs and Angs in C1-INH-HAE patients may prompt the investigation of VEGFs and Angs as biomarkers of C1-INH-HAE severity.

KEY WORDS]

Angiopoietins; bradykinin; kininogen; endothelial nitric oxide synthase; hereditary angioedema with C1 inhibitor deficiency; VEGF; vascular permeability;

INTRODUCTION

Hereditary Angioedema with C1 Inhibitor Deficiency (C1-INH-HAE)(1) is a rare autosomal genetic disorder presenting with recurrent episodes of swelling of the skin, gastrointestinal tract and upper airways that resolve within 48 to 96 hours(2). Two different subtypes of HAE due to C1-INH deficiency have been described(3). Type I, which accounts for about 85% of cases, is characterized by functional and antigenic C1-INH deficiency. Type II, which accounts for 15% of cases, is characterized by normal or upper normal C1-INH antigenic levels, but low functional activity. The worldwide prevalence is estimated between 1:10,000 and 1:50,000. Data recently collected by the Italian network for HAE provided a prevalence of 1:64,935 with no gender differences(4). The burden of the disease is mainly dependent on the frequency and severity of symptoms, which are extremely variable from patient to patient and even in the same patient from time to time. The mechanisms governing such variability are not known and biomarkers of disease severity are not available.

C1-INH belongs to the family of serine protease inhibitors and inhibits several proteases in the complement, contact, fibrinolytic and coagulation systems(5, 6). In humans, C1-INH deficiency leads to the activation of factor XII that produces plasma kallikrein (pKK). The latter cleaves high molecular weight kininogen (HK) and releases bradykinin (BK), which in turn promotes vascular permeability and mediates edema formation(7, 8). Compared to healthy controls, patients with C1-INH-HAE have a trend toward higher BK plasma levels, which were further increased during angioedema attacks(7). Accordingly, plasma levels of cleaved HK are increased in C1-INH-HAE patients and correlate with the number of attacks(9). BK increases endothelial permeability through the engagement of BK2R, which

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leads to the activation of the endothelial nitric oxide synthase (eNOS) and the production of nitric oxide (NO)(10). Patients with C1-INH-HAE in inter-attack periods have elevated serum levels of eNOS(11).

The activation of the eNOS/NO pathway is also mediated by the vascular endothelial growth factor (VEGF)-A. VEGF-A belongs to a family of growth factors that in humans consists of five separate gene products: VEGF-A, -B and PlGF are key regulators of blood vessel growth, -C and -D modulate lymphangiogenesis(12-14). VEGFs bind three receptor tyrosine kinases (VEGFR-1, -2, -3) expressed on blood and lymphatic endothelial cells(12) and modulate endothelial barrier structure and function(15). In particular, VEGF-A regulates physiological and pathological blood vessel growth(16). It is now known as the most potent proangiogenic factor, but it was first identified by Dvorak and colleagues for its permeabilizing activity and named vascular permeability factor (VPF). VPF/VEGF-A vasodilating activity is at least 50 times higher than the activity of histamine and is mediated through the release of NO(17-19).

A complementary endothelial pathway regulating vascular barrier function is the angiopoietin (Ang) system(20, 21). In humans, there are two main angiopoietins: Ang1 and Ang2. Ang1 is an agonist of Tie2 receptors present on endothelial cells and inhibits vascular permeability(22). Ang2 functions as partial Tie2 agonist and induces vasopermeability(23, 24). It is noteworthy that upregulation of VEGF-A and increase in the Ang2/Ang1 ratio lead to increased permeability of blood brain barrier(24, 25). In addition, increased concentrations of VEGF and/or Angs have been found in different human disorders characterized by

increased vascular permeability, namely systemic capillary leak syndrome(26) and sepsis(27, 28). The role of VEGFs and Angs in the pathophysiology of angioedema has not been investigated so far.

METHODS

Patients

We studied 128 patients followed at Milan center for HAE due to C1-INH deficiency and 68 normal control subjects. Diagnosis was based on presence of at least one recommended clinical and laboratory criteria(1). Table I summarizes the clinical characteristic of patients with C1-INH-HAE and healthy controls. Patients (117 type I and 11 type II C1-INH-HAE) belong to 78 unrelated families and their median age at symptoms onset was 6 years (interquartile range 3-14). There were no differences between the study groups in terms of age, sex and race. Because the frequency of attacks is an index of angioedema severity, patients were grouped according to the number of angioedema attacks registered during the last 12 months: 87 of them had less than 12 attacks (low frequency), while 41 complained more than 12 attacks (high frequency). Blood samples from all patients were obtained at least 8 days apart from an angioedema attack (remission sample). 34 patients were taking prophylactic therapy at the time of blood collection (5 were on tranexamic acid and 29 on attenuated androgens).

Blood sampling

Blood was collected during 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 labeled with a code which was documented into a data sheet. The controls had been referred for routine medical check-up and volunteered for the study by giving informed consent.

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. The samples were collected by means of a clean venipuncture and minimal stasis using two types of anticoagulants: sodium citrate 3.2% and, for the measurement of cleaved HK, an inhibitor cocktail containing 100 mM benzamidine, 400 µg/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) After centrifugation at 2000 g for 20 min at room temperature, the plasma was divided into aliquots and stored at -80°C until tested.

Complement system

C1-INH 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). Reference ranges were: 0.70 to 1.30 Unit C1-INH/ml (1 Unit C1-INH corresponds to the average C1-INH activity present in 1 ml of fresh citrated normal plasma). The functional activity of C1-INH was also expressed as a percentage of activity of C1-INH present in samples. 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 lower than 50%, as previously reported(29).

Determination of VEGFs and Angs

Plasma levels of angiogenic and lymphangiogenic mediators were measured using commercially available ELISA kits for VEGF-A, VEGF-A165b, VEGF-C, Ang1 and Ang2 (R&D System) according to the manufacturer's instructions.

Contact system

The cleavage of HK was assessed by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis (a modification of the method described by Berrettini *et al.*)(30). Samples were loaded on a 9% SDS-polyacrylamide gel. After electrophoretic separation, proteins were transferred from the gel to a polyvinylidene difluoride membrane using Bio-rad Trans-Blot® Turbo™ Transfer System(Bio-Rad Laboratories, Hercules, USA). HK was identified using goat polyclonal anti-HK light chain (Nordic, Tilburg, The Netherlands) and visualised using a biotinylated rabbit anti-goat antibody (Sigma Aldrich Co, St Louis, USA). The density of the bands obtained was measured

using a Bio-Rad GS-800 densitometer. The amount of cleaved HK was expressed as a percentage of total HK.

Statistical analysis

Data were analyzed with the GraphPad Prism 5 software package. Data were tested for normality using the D'Agostino-Pearson normality test. If normality was not rejected at 0.05 significance level, we used parametric tests. Otherwise, for not-normally distributed data we used nonparametric tests. Statistical analysis was performed by unpaired two-tailed t-test or two-tailed Mann-Whitney test as indicated in figure legends. Correlations between two variables were assessed by Pearson 's correlation analysis and reported as coefficient of correlation (r). A p value ≤ 0.05 was considered statistically significant. Plasma levels of VEGFs and Angs are shown as the median (horizontal black line), the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 68 controls and 128 patients.

RESULTS

Increased plasma concentrations of VEGF-A, VEGF-C, Ang1 and Ang2 in C1-INH-HAE patients

Table I summarizes the clinical characteristic of 128 patients with C1-INH-HAE and 68 healthy controls. We have evaluated the concentrations of different angiogenic and lymphangiogenic factors in C1-INH-HAE patients in remission. Figure 1A shows that VEGF-A and VEGF-C plasma levels of C1-INH-HAE patients were higher than healthy controls (VEGF-A: 0 (0-0) vs 3 (0-29) pg/ml median values (interquartile ranges); VEGF-C:

0.2 (0.09-0.6) vs 0.7 (0.4-1.2) ng/ml). The VEGF-A gene encodes for several splicing variants, such as VEGF-A_{165a} and VEGF-A_{165b}(31, 32). VEGF-A_{165b} prevents the VEGF-A_{165a} effects on increased vascular permeability, vasodilatation, and angiogenesis(32, 33). The plasma level of the VEGF-A_{165b} was not increased in C1-INH-HAE patients compared to controls (see supplementary Fig. 1).

Interestingly, both Ang2 which increases vascular permeability and its inhibitor Ang1 were increased in C1-INH-HAE patients compared to controls (Ang1: 2.2 (1.5-2.9) vs 4.5 (3.5-5.7); Ang2: 0.3 (0.1-0.4) vs 0.45 (0.3-0.7) ng/ml) (Fig. 1C and D).

There was no difference in VEGFs and /or Angs concentration between male and female values in both controls and patients (Table II). Moreover, the age of patients and the concentration of the different plasma mediators examined did not correlate (see supplementary Fig. 2).

Plasma level of VEGF-A is inversely associated with functional activity of C1-INH

Since C1-INH-HAE patients may exhibit a spectrum of residual functional activity of C1-INH, we divided the patients according to their C1-INH activity. 98 patients had less than 30% of normal functional activity of C1-INH; the remaining 30 patients had a functional activity between 30% and 50% of C1-INH. Figure 2A shows that patients with the lowest functional activity of C1-INH had higher plasma concentration of VEGF-A than patients with higher functional activity of C1-INH (3 (0-27) vs 0 (0-0) pg/ml). By contrast, the concentrations of VEGF-C, Ang1 and Ang2 did not differ between the two groups of patients (Fig. 2B-D) (VEGF-C: 0.7 (0.4-1.1) vs 0.6 (0.3-0.8) ng/ml; Ang1: 4.5 (3.7-5.7) vs 4.2 (3.5-4.5) ng/ml; Ang2: 0.3 (0.1-0.5) vs 0.3 (0.2-0.6)).

Then, we asked whether differences in the antigenic concentration of C1-INH are associated with differences in VEGFs and Angs plasma levels. To this aim, we compared C1-INH type I patients with less than 25% or 25-50 % of normal C1-INH values. There were no differences in plasma concentrations of VEGF-A, VEGF-C, Ang1 and Ang2 between these groups (Fig. 3A-D). We also divided the patients according to their plasma levels of the C4 complement component (25% or 25-50 % of normal values), which is deficient in C1-INH-HAE patients and used as a screening test (4, 34). Again, we found no differences in plasma concentrations of VEGF-A, VEGF-C, Ang1 and Ang2 between these groups (Fig. 3E-H).

Plasma concentrations of VEGF-A, VEGF-C and Ang2 in patients with different attack frequency

Since the frequency of attacks is highly variable among C1-INH-HAE patients, we asked whether plasma levels of VEGFs and Angs could be used to identify patients at higher risk of recurrent attacks. Plasma of VEGF-A, VEGF-C and Ang2 (Fig. 4A-C), but not Ang1 (supplementary Fig. 3), were higher in patients with high frequency of attacks (>12/year) compared to patients with low frequency (<12/years). Interestingly, the Ang2/Ang1 ratio (an index of vascular permeability) was also increased in more severe patients (Fig. 4D).

We have previously demonstrated that plasma concentration of cleaved HK is higher in plasma of highly symptomatic C1-INH-HAE patients, than those with less frequent attacks(9). To further validate the usefulness of VEGFs and Angs plasma levels in identifying patients experiencing an higher number of attacks, we investigated possible correlations between plasma levels of cleaved HK and VEGFs and Angs. We found a positive correlation between plasma concentrations of both VEGF-A and Ang2 and that of cleaved HK (Fig. 4E, H). In addition, cleaved HK also correlated with the Ang2/Ang1 ratio (supplementary Fig. 4).

By contrast, there was no correlation between the plasma levels of both VEGF-C and Ang1 and cleaved HK (Fig. 4F, G).

DISCUSSION

Here, we demonstrate that C1-INH-HAE patients in remission have higher plasma levels of VEGFs and Angs compared to healthy control. Interestingly, plasma levels of VEGF-A, VEGF-C and Ang2 are even higher in C1-INH-HAE patients experiencing more than 12 attacks per year in the last year than in those who had less than 12 attacks. The relevance of these findings is supported by the correlation of plasma levels VEGF-A and Ang2 with those of cleaved HK, which we already demonstrated to distinguish in patients with a more severe disease phenotype(9). Hereditary angioedema is caused by a genetic mutation in the C1 esterase inhibitor gene (*SERPING1*) that is associated with a stable deficiency in C1-INH levels or function(35). Nonetheless, frequency and anatomical localization of angioedema attacks is highly variable among different patients and within the same patients from time to time, Thus, there is an increasing need for identifying biomarkers of disease activity.

More than 300 C1-INH-HAE causing mutations (Human Genome Mutation Database, <http://www.hgmd.cf.ac.uk/ac>) have been identified so far in *SERPING1*, but these genotypes do not correlate with clinical variants. In addition, C1-INH plasma levels are not good markers of disease severity and the same applies to C4, due to its genotype, which has high variability in the general population(36). The levels of the lectin complement pathway serine protease MASP-1 and its complex with C1-INH are reduced in C1-INH-HAE patients and seem to correlate with the degree of complement C4 consumption and disease severity(37). We already mentioned the evidence that plasma levels of cleaved HK increase

in patients with high frequency of attacks(9). Cleaved HK, measured by immunoblotting analysis after gel electrophoresis(30), is generated by activation of the contact system and can be assumed as indirect measurement of the release of bradykinin during such an activation (38). Our present results indicate that also VEGF-A and Ang2 mark C1-INH-HAE severity. It will be interesting to evaluate whether plasma levels of VEGF-A and Ang2 at time of diagnosis can predict the clinical severity (e.g. frequency of attacks) of angioedema.

Increased circulating levels of VEGF-A and Ang2 associated with higher vascular permeability have been observed in several diseases, namely systemic capillary leak syndrome (26) and sepsis(27, 28, 39). Combining these observations with the results of this study, we hypothesize that C1-INH deficiency and inflammatory stimuli create a variable, ongoing increase in vascular permeability that defines the threshold where triggers act for the development of angioedema attacks. We do not provide formal evidence of persistent higher vascular permeability in C1-INH-HAE patients. Microscopic anatomy of peripheral blood vessels and electron microscopy analysis of tissue biopsies could be tools to prove the existence of a “vascular pre-conditioning” priming endothelial cell to angioedema development.

Mice in whom *C1nh* gene has been knocked out *C1nh*^{-/-} have been established as a model of C1-INH-HAE. These mice do not spontaneously develop angioedema. However, they present a constitutive increase in vascular permeability, which is reversed by treatment with plasma derived human C1-INH (pdC1-INH), an inhibitor of pKK or a BK type 2 receptor (BK2R) antagonist(40). These therapeutic approaches efficiently reverse angioedema in patients and are now established treatments for C1-INH-HAE(41-43). It is tempting to speculate that these mice also have higher plasma levels of VEGFs and Angs that, together with bradykinin, contribute to increased vascular permeability. In this scenario, therapeutic

blockade of VEGFs and/or Angs may revert the increased vascular permeability and be employed for the prevention of angioedema attacks.

Several cell subsets produce VEGFs and Angs, including immune cells and endothelial cells(44, 45). It is still unclear the relative contribution of different cell subsets to the increased plasma levels of VEGFs and Angs that we have observed in C1-INH-HAE patients. In addition, further studies are required to assess whether the production of VEGFs and Angs by such cell subsets is modulated by mediators (e.g. bradykinin) involved in the pathophysiology of C1-INH-HAE.

In conclusion, we demonstrate that plasma levels of VEGFs and Angs are increased in patients with C1-INH-HAE, and they further increase in patients experiencing an higher number of angioedema attacks per year. Thus, VEGFs and Angs stand as candidate biomarkers of C1-INH-HAE severity. Based on this observation, we hypothesize that these factors along with an increased release of BK, induce a state of “vascular pre-conditioning” that may change the threshold for development of angioedema attacks. Pathways modulating “vascular pre-conditioning” could represent new targets for increasing resistance to angioedema recurrences.

STATEMENT OF CONFLICTS OF INTEREST

None

LIST OF AUTHOR CONTRIBUTIONS

Participate in research design: Loffredo S., Bova M., Suffritti C., Borriello F., Petraroli A., Cicardi M., Triggiani M., Marone G.

Conducted experiments: Loffredo S., Suffritti C., Zanichelli A., Borriello F.,

Performed data analysis: Loffredo S., Petraroli A., Varricchi G., Zanichelli A., Borriello F.

Wrote or contributed to the writing the manuscript: Loffredo S., Borriello F., Bova M., Cicardi M., Triggiani M., Marone G.

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TABLES

Table I – Patients' clinical characteristics

Characteristics		Healthy (n = 68)	Patients (n = 128)
Age (years)*		33 (27-45)	41 (23-52)
Gender male		39 (57%)	57 (45%)
Caucasian race		100%	100%
Unrelated families		NA	78
Age at onset (years)*		NA	6 (3-14)
C1-INH-HAE	Type I	NA	117 (91.4%)
	Type II	NA	11 (8.6%)
Prophylaxis	Acid tranexamic	NA	5 (4%)
	Attenuated androgens	NA	29 (22%)
Attack number per year (>12)		NA	41 (32%)

*Data are expressed as median values (interquartile ranges). Data were analyzed by using t test.

Table II – VEGFs and Angs plasma concentrations and gender in healthy and in C1-INH-HAE subjects.

	Healthy		C1-INH-HAE	
	female	male	female	male
VEGF-A (pg/ml)	0 (0-0)	0 (0-0)	3 (0-22)	3 (0-29)
VEGF-C (ng/ml)	0.2 (0.1-0.6)	0.2 (0.1-0.6)	0.7 (0.3-1.1)	0.7 (0.4-0.7)
Ang1 (ng/ml)	2.1 (1.5-2.9)	2.3 (1.3-2.9)	4.5 (3.5-5.7)	4.3 (3.3-5.5)
Ang2 (ng/ml)	0.3 (0.1-0.4)	0.3 (0.1-0.4)	0.4 (0.2-0.7)	0.5 (0.3-0.7)

*Data are expressed as median values (interquartile ranges). Data were analyzed by using t test.

FIGURE LEGENDS

Figure 1. Plasma concentrations of VEGF-A, VEGF-C, Ang1 and Ang2 in C1-INH-HAE patients.

Plasma VEGF-A (A), VEGF-C (B), Ang1 (C) and Ang2 (D) in controls (Healthy) and in patients with C1-INH-HAE in remission. Data are shown as the median (horizontal black line), the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 68 controls and 128 patients.

* p < 0.05.

Figure 2. VEGFs and Angs concentrations in patients with different functional activity of C1-INH.

VEGF-A (A), VEGF-C (B), Ang1 (C), and Ang2 (D) in two groups of patients: C1-INH activity less than 30% (N=98) and between 30% and 50% (N=30) of normal functional activity. * $p < 0.05$.

Figure 3. Relationship among levels of VEGFs and Angs and C1-INH or C4 protein levels.

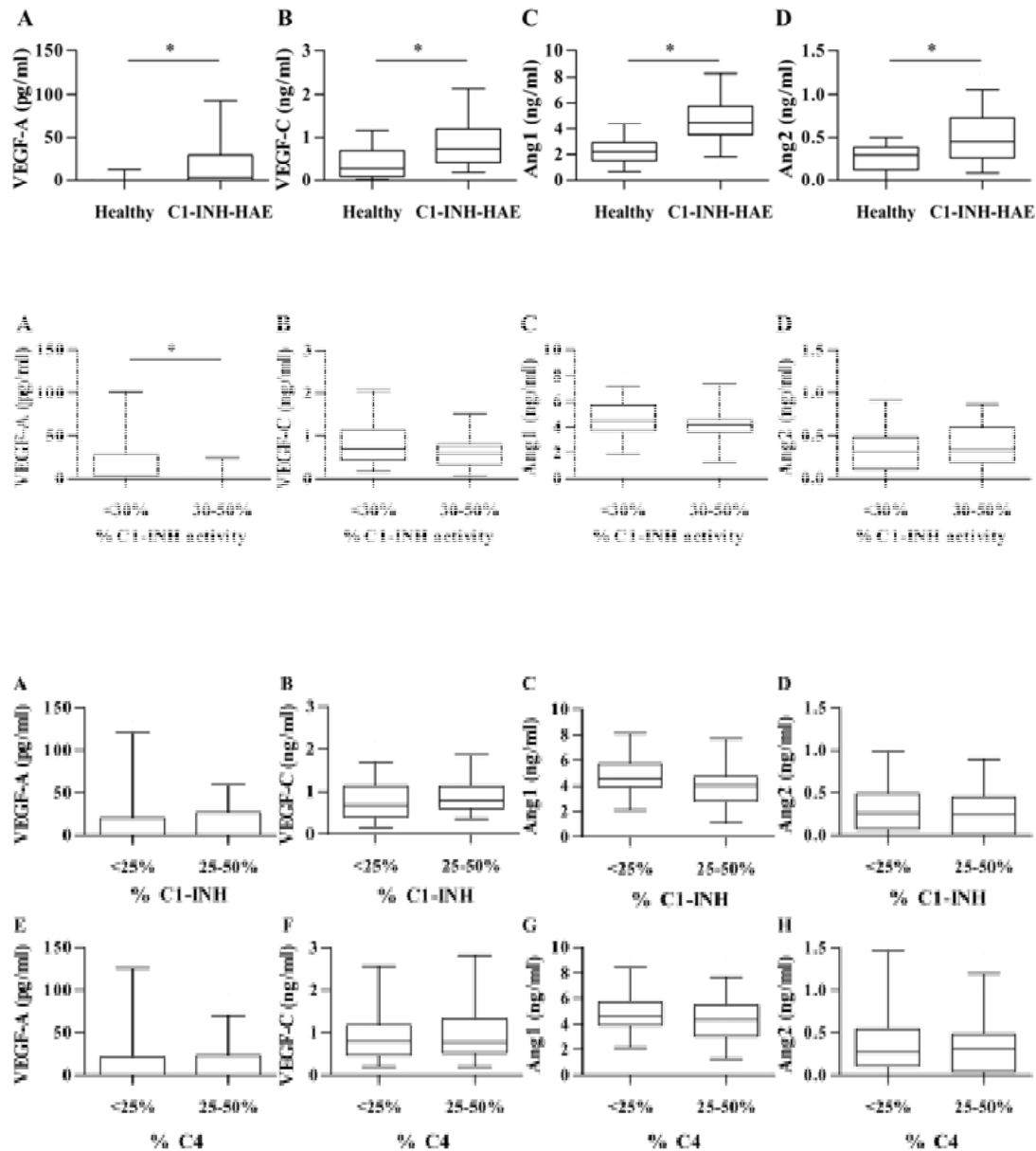
(A-D) Patients were divided in two groups: C1-INH type I patients with less than 25% or 25-50 % of normal C1-INH values.(E-H) Patients were divided in two groups: C1-INH-HAE patients with less than 25% or 25-50 % of normal C4 values. Concentrations of VEGF-A (A-E), VEGF-C (B-F), Ang1 (C-G) and Ang2 (D-H) are shown.

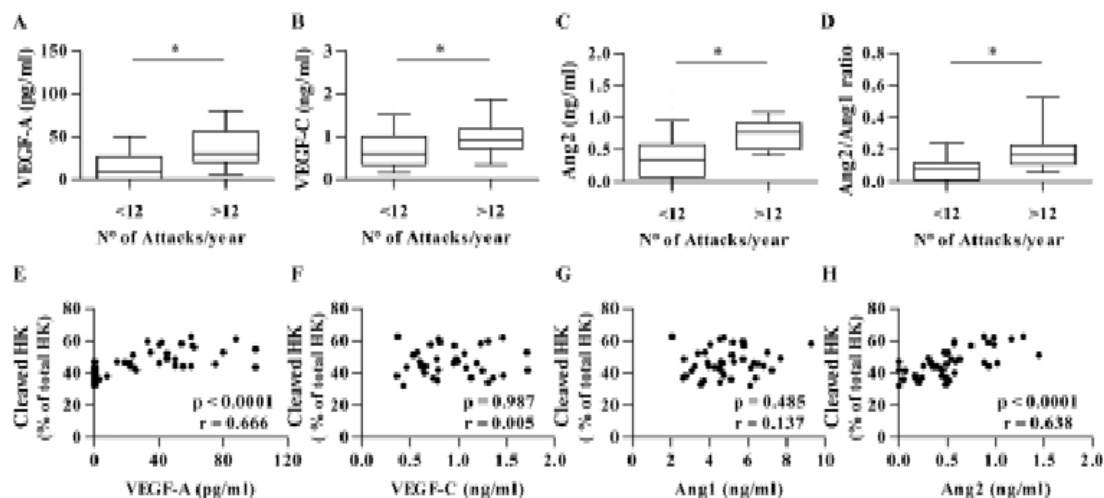
Figure 4: Relationships among VEGFs and Angs levels, attack frequency and severity biomarkers of angioedema.

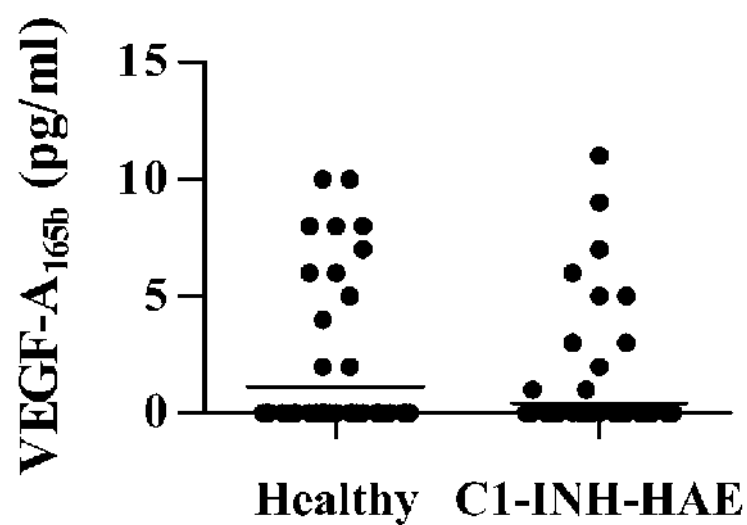
VEGF-A (A), VEGF-C (B), Ang2 (C), and Ang2/Ang1 ratio (D) were determined in 87 patients with low frequency (<12/year) and 41 patients with high frequency (>12/years) of attacks. Correlations between two variables: Cleaved high-molecular-weight kininogen (HK) and VEGF-A (E), HK and VEGF-C (F), HK and Ang1 (G), HK and Ang2(H) were assessed by Pearson 's correlation analysis and reported as coefficient of correlation (r). A p value ≤ 0.05 was considered statistically significant.. * $p < 0.05$.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

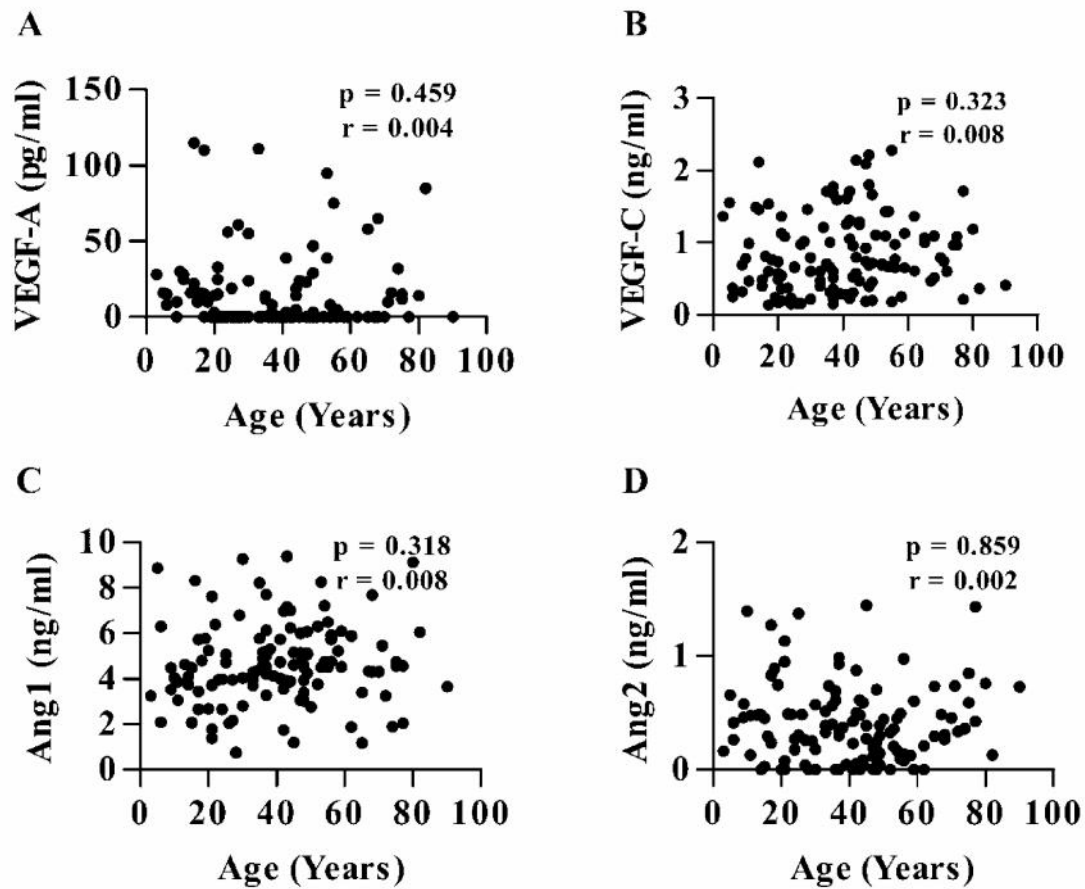






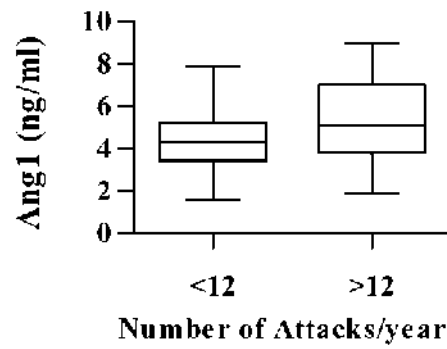
Supplementary Figure 1. Plasma concentration of VEGF-A_{165b} in C1-INH-HAE patients.

Plasma VEGF-A_{165b} in controls (Healthy) and in patients with C1-INH-HAE in remission was determined by ELISA. Horizontal bars depict the median value.



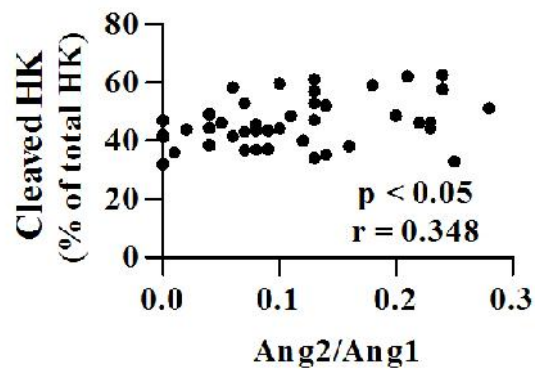
Supplementary Figure 2. VEGFs and Angs plasma concentrations and age of C1-INH-HAE patients.

VEGF-A (A), VEGF-C (B), Ang1 (C) and Ang2 (D) in patients with C1-INH-HAE were determined by ELISA. Correlation between levels of VEGF-A (A), VEGF-C (B), Ang1 (C) and Ang2 (D) and age (expressed as years) were assessed by linear regression analysis and reported as coefficient of determination (r^2). * $p < 0.05$.



Supplementary Figure 3: Relationships among levels of Ang1 and frequency of angioedema attacks.

Patients were divided in two groups: 87 patients with low frequency of attacks (<12/year) and 41 patients with high frequency (>12/years). Ang1 was determined by ELISA.



Supplementary Figure 4: Relationships among Ang2/Ang1 ratio and HK.

Ang2/Ang1 ratio and HK were determined in C1-INH-HAE patients . Correlations between two variables: Cleaved high-molecular-weight kininogen (HK) and Ang2/Ang1 ratio were assessed by Pearson 's correlation analysis and reported as coefficient of correlation (r). A p value < 0.05 was considered statistically significant.