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ROLE OF VASOACTIVE MEDIATORS IN HEREDITARY ANGIOEDEMA

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Abbreviations

AAE:	Acquired Angioedema
ACEI:	Angiotensin Converting Enzyme Inhibitors
ANGPT:	Angiopoietin
BAEC:	Bovine Aortic Endothelial Cells
BK:	Bradykinin
C1-INH:	C1 inhibitor
DAG:	Diacylglycerol
DMEM:	Dulbecco's Modified Eagle Medium
eNOS:	Endothelial nitric oxide synthase
FXII:	Factor XII
HAE:	Hereditary Angioedema
HK:	High Molecular Weight Kininogen
HSPG:	Heparan Sulfate Proteoglycans
KNG:	Kininogen
nlC1-INH-HAE:	Hereditary Angioedema with normal C1-INH levels
NO:	Nitric Oxide
NVC:	Nailfold Video-Capillaroscopy
PAF-AH:	Platelet Activating Factor
PCR:	Polymerase Chain Reaction
pdC1-INH:	Plasma-derived C1 inhibitor
PK:	Kallikrein
PLA2G2A:	Human $sPLA_2$ Group IIA
PLG:	Plasminogen
PPK:	Pre-kallikrein
RFU:	Relative Fluorescence Unit
rhC1-INH:	Recombinant C1 inhibitor
$sPLA_2$:	Secreted Phospholipases A_2
U-HAE:	Hereditary Angioedema with Unknown origin
VEGF-R:	Vascular Endothelial Growth Factor Receptor
VEGF:	Vascular Endothelial Growth Factor

ABSTRACT

Hereditary angioedema is a disabling, life-threatening condition caused by deficiency (type I) or dysfunction (type II) of the C1 inhibitor protein (C1-INH-HAE), mutation of genes encoding Factor XII (FXII-HAE), Angiopoietin-1 (ANGPT1-HAE) or by unknown factors (U-HAE) leading to bradykinin accumulation and recurrent episodes of edema attack. Vascular leakage is a complex process sustained by the coordinated production of several permeabilizing factors. It has been demonstrated the implication of Vascular Endothelial Growth Factors (VEGFs), Angiopoietins (ANGPTs), Secreted phospholipases A_2 ($sPLA_2$) and Platelet Activating Factor-Acetylhydrolase (PAF-AH) in vascular permeabilization.

In this study, we sought to analyze plasma levels of these mediators in C1-INH-HAE during remission and angioedema attack and in FXII-HAE, ANGPT1-HAE and U-HAE patients during remission phase. Plasma concentrations of VEGF-A, VEGF-C, ANGPT1 and ANGPT2 and enzymatic activities of $sPLA_2$ and PAF-AH are increased in patients with C1-INH-HAE during remission compared to healthy controls. In addition, FXII-HAE patients reported altered concentrations of VEGF-A whereas U-HAE patients of VEGF-A, VEGF-C and ANGPT1. By contrast, no alteration of these mediators was found in ANGPT1-HAE. VEGF-A and VEGF-C concentrations were not altered during attack compared to remission. By contrast, enzymatic activities of $sPLA_2$ and PAF-AH are decreased. Concentrations of ANGPT1, a vascular stabilizer, were increased during attacks compared to symptoms-free periods, whereas ANGPT2 levels were not altered.

In addition, nailfold video-capillaroscopy revealed structural and morphological alterations of capillaries of C1-INH-HAE patients.

Our results emphasize the complexity by which several vasoactive mediators are involved not only in the pathophysiology of C1-INH-HAE, but also during angioedema attacks and its resolution.

INTRODUCTION

1. ANGIOEDEMA: AN OVERVIEW

Angioedema is a disabling, life-threatening condition caused by subcutaneous and/or submucosal tissues edema due to a temporary increase in vascular permeability caused by the release of vasoactive mediator(s). Angioedema can occur in the absence of urticaria and can be broadly divided into histamine-mediated and non-histamine-mediated angioedema. Histamine-mediated angioedema can be allergic, pseudoallergic or idiopathic. Non-histamine mediated angioedema is largely driven by bradykinin (BK) and can be hereditary, acquired or drug-induced, such as with angiotensin-converting enzyme inhibitors (ACE-I)[1].

Heinrich Quincke first described the picture of angioedema in 1882[2]. William Osler remarked in 1888 that some cases may have a hereditary basis; he coined the term "hereditary angio-neurotic edema"[3].

It took 75 years from the accurate description of hereditary angioedema (HAE) by Osler, until a group of researchers headed by Donaldson (1963) disclosed the central role of C1 inhibitor (C1-INH) in angioedema pathophysiology[4]. Following years new angioedema types were described. The heterogeneity of clinical characteristics of angioedema made necessary a classification describing the various pathology expressions or different phenotypes. In 2014, a world-wide accepted classification was validated and published by the "*Hereditary Angioedema International Working Group*" under the aegis of the *European Academy of Allergy and Clinical Immunology* (EAACI)[5] and revised and updated in 2017 in order to add new hereditary angioedema subtypes[6]. The revised classification led to the identification of different inherited and acquired types of angioedema without wheals (*Picture 1*).

The angioedema with inherited pattern can be classified in six different phenotypes:

i. C1-INH-HAE (Hereditary Angioedema with C1-INH deficiency) caused by an antigenic and/or functional C1-INH deficiency;
ii. FXII-HAE (Hereditary Angioedema with Factor-XII mutation) characterized by normal C1-INH levels and caused by a mutation affecting *FXII*-gene;
iii. ANGPT1-HAE (Hereditary Angioedema with Angiopoietin-1 mutation) characterized by normal C1-INH levels and caused by a mutation affecting *ANGPT1*-gene;

iv. PLG-HAE (Hereditary Angioedema with Plasminogen mutation) characterized by normal C1-INH levels and caused by a mutation affecting *PLG*-gene;
v. KNG1-HAE (Hereditary Angioedema with Kininogen mutation) characterized by normal C1-INH levels and caused by a mutation affecting *KNG1*-gene;
vi. U-HAE (Hereditary Angioedema with unknown origin) characterized by normal C1-INH levels and unknown pathogenesis.

Angioedema with acquired pattern is characterized by absence of family history and late onset (older than 40 years old). To date, four sub-types have been described:

i. C1-INH-AAE (Acquired Angioedema with C1-INH deficiency) characterized by C1-INH deficiency and often associated to lymphoproliferative diseases;
ii. ACEI-AAE (Acquired Angioedema related to ACE-inhibitor therapy) caused by ACE-inhibitor therapy;
iii. IH-AAE (Idiopathic Histaminergic Acquired Angioedema) with unknown pathogenesis but antihistamine-responsive;
iv. InH-AAE (Idiopathic non-Histaminergic Acquired Angioedema) with unknown pathogenesis and non-responsive to antihistaminic treatment.

Even if different angioedema-types have been identified, many doubts still exist on pathophysiologic mechanisms to be clarified.

2. HEREDITARY ANGIOEDEMA WITH C1-INH DEFICIENCY

C1-INH-HAE is a rare dominant autosomal disorder characterized by recurrent episodes of tissue swelling (i.e., angioedema attack) involving the deeper layers of skin and/or submucosal tissue[6] and affecting between 1/10,000 and 1/50,000 individuals without distinction of ethnicity and gender[7].

Two forms of C1-INH-HAE are currently recognized:

- type I, involving 85% of patients, is caused by low antigenic and functional C1-INH;
- type II is characterized by normal antigenic but low functional C1-INH[6].

2.1. Pathophysiology of C1-INH-HAE

2.1.1. C1-inhibitor

C1-INH is a serine protease inhibitor (SERPING1) synthetized by hepatocytes and to a lesser extent by peripheral monocytes and involved in vascular homeostasis[8]. As a serpin, it regulates serine-proteases via an irreversible suicide substrate mechanism. The serpins undergo a profound change in conformation to entrap their target protease in an irreversible complex that inhibits and finally destroy the protease through a mechanism named "*molecular mousetrap"*[9]. *SERPING* gene encoding C1-INH is located at 11q12-q13.1 in humans and composed by 8 exons and 7 introns. The active site P1 is located within the exon 8, Arg 444. C1-INH is a single-chain, circulating protein, highly glycosylated mostly in N-terminal region. It has been described that partial or total suppression of N-terminal region increases the catabolism of the whole protein[10]. To date, more than 450 different mutations distributed across the entire *SERPING1* gene have been described, ranging from nucleotide substitutions to small insertions and deletions to large deletions and duplications. *De novo* mutations in *SERPING1* account for approximately 25% of cases with C1-INH-HAE[11]. Protein synthesis can be altered at any point depending on where the mutation occurs. Type I is caused by mutations that cause the production of truncated proteins or proteins that are not adequately configured and subsequently degraded. In the other hand, type II is generated by the replacement of a single amino acid mainly located in exon 8 which codes for the reactive center or for the two hinged regions. The exception is the Lys251/Lys273 mutation that determines a new glycosylation site far from the active site and alters the function of the protein itself[12]. The pathology has an autosomal dominant inheritance. Few examples of homozygous patients, mainly consanguineous, and only two cases of gonadal mosaicism have been described[13,14]. Heterozygous patients have plasma C1-INH concentrations ranging from 5 to 30% of normal levels. The explanation of this phenomenon is not completely clear, although for some mutations it has been found that they contribute to an increase in catabolism or a reduced secretion of normal proteins, causing a downregulation also of the normal allele[15-17]. In absence of family history, it must be assumed that it is a *de novo* mutation. This condition represents 20-25% of cases[18]. The lability of the gene is attributable to several factors: firstly its position, near the centromeric zone of the chromosome, an area with high mutagenicity; secondly, the high incidence of repetitive sequences, such as the *Alu*-rich sequences in the different introns and in exons 4 and 6 which represent *hot-spots* for

homologous recombination, CpG sequences mainly in the reactive site, and finally the presence in the same reactive site of DNA polymerase sequences. The main action of C1-INH is to prevent unnecessary activation of the complement classical pathway by acting on the proteases C1r and C1s, and to inhibit the MASP proteases involved in the lectin pathway[19]. It also exerts its activity on the contact system by preventing the activation of kallikrein and coagulation factor XII[20]. A lack of inhibition of factor XII and of kallikrein by C1-INH causes an increased activity of the high molecular weight kininogen (HK) leading to an increased production of BK, the main mediator of C1-INH-HAE symptoms. By contrast, the role of C1-INH on the coagulation system is limited to the inhibition of factors IX and XIa and of amplification of the coagulation cascade[21]. *In vitro* and *in vivo* tests showed a weak inhibition of C1-INH on plasmin and tPA, although during acute attacks it is possible to measure a significant formation of plasmin by measuring the plasmin-α2 antiplasmin complexes[22].

2.1.2. Kinin System and Bradykinin

Kinins are vasoactive proteins that play an important role in inflammatory processes[23]. The most important kinin is BK, a low molecular weight nonapeptide that is generated by the activation of the contact system. This system is composed of a substrate, the HK, and two zymogens: FXII and pre-kallikrein (PPK). The activation of contact system occurs when the two zymogens are cleaved in their active forms, respectively FXIIa and kallikrein (PK). Then, PK cleaves HK leading to the BK release.

HK is composed of 6 domains: domain 5 and domain 3 bind to the proteins of the endothelial membrane, respectively gC1qR (endothelial receptor of the globular heads of C1q) and cytokeratin 1 whereas domain 4 contains BK and the domain 6 binds the PPK[24]. It has been shown that FXII has a weak ability to self-activate, it modifies PPK into active compound PK that in turn produces[8].

The *in vitro* "contact" of FXII with negatively charged surfaces determines a conformational change which makes it susceptible to cleavage by PK and FXIIa itself[25].

Conversely, this mechanism is less clear *in vivo*; it seems that FXII can self-activate by binding to the gC1qR[26]. Recent studies have shown that one of the possible ways of activating the FXII-dependent contact system can be mediated by the heparin released by mast cells[27]. A further cleavage of the FXIIa allows the production of the fragment of FXII which is not able to activate the intrinsic coagulation system but is able to activate the PPK in PK and has the

ability to activate the first component of the complement system, C1r, get a link between the contact system and the complement system.

Kallikrein is a protein with a powerful proteolytic activity; it has several substrates including HK, FXII and FXIIa. Its ability to produce plasmin has been demonstrated by activating the fibrinolytic system both through the activation of the plasma pro-urokinase and through a direct activity on the plasminogen, although this second mechanism is less effective[28]. In addition, it has been also demonstrated a link between the contact system and the fibrinolytic system; *in vitro* studies have been shown that plasmin is able to enhance the generation of BK by activating FXII[29].

The contact system can also be activated by FXII factor independent manner. Other factors that activate PPK on endothelial cells have been found *in vivo*, such as HSP90 (heat shock protein 90) or a membrane protein called PCRP (prolylcarboxypeptidase), proteins released by activated endothelial cells[30,31].

BK has a short half-life of approximately 15 seconds and is rapidly degraded by endogenous metalloproteases such as angiotensin converting enzyme (ACE), carboxypeptidase N (CPN or Kinase I), aminopeptidase P and neutral endopeptidase (NEP or neprilisine)[32]. The biological effects of BK are exerted mainly through the activation of the B2 receptor which is constitutively expressed on endothelial cells or smooth muscle cells. After activation, the receptor is endocyted and resynthesized. The B1 receptor is induced by inflammation mediators (IL-1β and TNFα) and mainly binds a metabolite of BK (Des-Arg^9 bradykinin) produced by plasma carboxypeptidase N or cellular carboxypeptidase M; recent studies on endothelial permeability in animal models have been suggested a presumable role of these receptors during the late phase of angioedema attacks and the extension of their duration[33].

The B2 receptor is coupled to a G protein that transmits the intracellular signal by means of phospholipase C, causing an increase in intracellular calcium levels, the production of diacylglycerol (DAG) and the activation of protein kinase C[34]. The latter phosphorylates beta-catenin and leads to the internalization and the destruction of VE-cadherins, key proteins in the formation of endothelial adherens junctions. In addition, protein kinase C stimulates NO production by activating eNOS and phosphorylates the myosin light chain kinase by promoting the contraction of the actin within endocellular cytoskeleton. The result is the increase of the paracellular permeability between the endothelial cells which allows the fluids to move from the intravascular space to the tissue space[35].

The key experiments that led to the identification of BK as the main mediator in the pathogenesis of angioedema have been described by Davis[36] and Cugno *et al.*[37]. In 2002 Han *et al.* used B2 receptor knockout mice to demonstrate that the increase in permeability observed in angioedema with C1-INH deficiency was due to BK. Using the Evans Blue dye, they saw that the vascular permeability in mice with C1-INH-HAE was significantly increased compared to those with C1-INH-HAE and knockout for the B2 receptor[38].

In 2007, the first report was published demonstrating the efficacy of using a B2 receptor antagonist in the treatment of an acute attack of a patient with C1-INH-HAE[39].

Over the years, attempts have been made to measure BK levels and markers of the activation of the contact system in the plasma of patients with C1-INH-HAE. The dosage of plasma BK is made extremely difficult by pre-analytical factors such as low plasma concentration, its short half-life and its rapid generation and degradation during the sampling; and from analytical factors such as the interference of non-plasma proteins in the assay and the cross-reactivity with anti-BK antibodies with other molecules or with the products of bradykinin catabolism[40]. However, it has been shown that patients with C1-INH-HAE or AAE during angioedema attacks have higher BK plasma concentrations than during periods of remission and compared to patients with angioedema attacks responsive to antihistamine therapy[41]. The increase in BK levels during acute attacks is 2 to 12 times the normal limits in subjects with C1-INH-HAE[40]. Acute attacks were also associated with high levels of cleaved-HK indicating that the increase in BK is due to its increased production[21]. The levels of BK measured in the blood samples obtained from the angioedema arm compared to the levels measured in the contralateral unaffected arm were shown to be three to eight times higher demonstrating that a local production of bradykinin exists[42].

2.1.3. Role of endothelium in C1-INH-HAE

To date several questions still exist about this pathology that fascinate and are being studied. One of the most complex ones to answer is certainly the discrepancy that exists between a constant and unchanged reduced function of the C1-INH and the intermittent and unpredictable appearance of attacks, sometimes induced by microtraumas, stressing events, infections, exposure to cold and modifications of the hormonal aspect. Therefore, C1-INH deficiency is a necessary but not sufficient condition for the onset of angioedema.

Recent studies have shown that the endothelium can play an active role during angioedema attacks and that it can also be considered an important target in diagnosis and

therapy. Kajdacsi *et* al. showed that in patients with C1-INH-HAE during attacks there is a significant increase in endothelial activation markers such as von Willebrand factor, E-selectin and endothelin[43]. By contrast, in another study conducted by Czucz *et* al. these parameters seemed to return to normal range compared to controls during remission except for E-selectin which remains high even in the absence of attacks[44]. In the hypothesis that a possible endothelial dysfunction secondary to an inflammation could be responsible for the attack, Demirturk *et al.* showed that the plasma levels of some acute inflammatory mediators including IL-6, IL-8, IL-1β and TNFα were not increased in patients with C1-INH-HAE neither during attacks nor during baseline conditions compared to healthy controls. By contrast, eNOS concentrations significantly increased in patients with C1-INH-HAE both in basal conditions and during angioedema attacks, suggesting that their presentation does not depend on a permanent inflammatory state but that there is a state of vascular hyperpermeability due to the release of pro-inflammatory agents responsible for activating the eNOS/NO pathway[45].

The Vascular Endothelial Growth Factor - A (VEGF-A) is one of the actors of the eNOS/NO pathway activation. It is 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[46-48]. VEGF-A belongs to a family of growth factors that in humans consists of five separate gene products: VEGF-A, VEGF-B and PIGF are key regulators of blood vessel growth, and VEGF-C and VEGF-D modulate lymphangiogenesis[49,50]. VEGFs bind three receptor tyrosine kinases (VEGFR-1, VEGFR-2, VEGFR-3) expressed on blood and lymphatic endothelial cells[49] and modulate endothelial barrier structure and function[51]. In particular, VEGF-A regulates physiological and pathological blood vessel growth[52]. After the discovery of VEGF and its receptors in the late 1980s, the identification of two endothelial receptors with tyrosine kinase activity called Tie1 and Tie2 allowed the recognition of another pathway for regulating vascular permeability: the angiopoietin system[53,54].

Angiopoietins are a family of oligomeric glycoproteins whose main representatives are ANGPT1 and ANGPT2. ANGPT1 is widespread in the adult vascular system, mainly produced by pericytes but also by neutrophils and monocytes. In the other hand, ANGPT2 is not expressed in the tissues of healthy individuals. It is contained in the Weibel-Palade bodies of endothelial cells from where it is rapidly released at the sites of inflammation or where vascular remodeling is needed in response to a wide range of stimuli: thrombin, histamine,

VEGF, hypoxia and angiotensin-2. Both ANGPT1 and ANGPT2 mainly bind to the Tie2 receptor. The latter consists of an extracellular domain that binds at least 4 angiopoietin monomers, a small transmembrane region and an intracellular tyrosine-kinase domain responsible for the intracellular signal. Tie2 is extremely widespread throughout the vascular endothelium and appears to be constitutively phosphorylated even during the quiescence of the vessels, indicating an active role in maintaining vascular homeostasis. The cellular effects of ANGPT1 mediated by Tie2 are generally protective. The ligand inhibits endothelial apoptosis, promotes the migration of endothelial and smooth muscle cells and promotes the reorganization and growth of endothelial cells themselves. It also exerts an anti-inflammatory effect and reduces the endothelial permeability induced by VEGF promoting the stability of cell junctions[55]. ANGPT2 is a partial Tie2 agonist and at high concentrations competes with ANGPT1 for signal transduction mediated by the same receptor. ANGPT2 is currently considered an inducible inhibitor of ANGPT1-induced vascular quiescence[56,57]. It is noteworthy that upregulation of VEGF-A and increase in the ANGPT2/ANGPT1 ratio lead to increased permeability of blood–brain barrier[57,58]. In addition, increased concentrations of VEGF and/or ANGPTs have been found in different human disorders characterized by increased vascular permeability, namely systemic capillary leak syndrome[59] and sepsis[60,61].

Phospholipase A_2 (PLA_2) enzymes hydrolyze the fatty acid from membrane glycerophospholipids releasing arachidonic acid and lysophospholipid[62,63]. The superfamily of PLA_2 comprises different proteins that can be divided into six classes[63,64]. Secreted or extracellular PLA_2s ($sPLA_2$s) directly modulates endothelial cell migration and vascular permeability *in vitro*. The effects of $sPLA_2$s depend on their enzymatic activity and ability to engage different targets [i.e., PLA2R1, heparan sulfate proteoglycans (HSPGs), integrins][65-74]. They play critical roles in several pathophysiological processes. Indeed, $sPLA_2$s activate several immune cell subsets[68,73-76] and are expressed in inflamed tissues and tumors[63,77-79]. $sPLA_2$s modulate vascular permeability either by directly activating endothelial cells or by catalyzing the production/degradation of vasoactive molecules[80].

The PAF-AH family comprises extracellular and intracellular enzymes. In particular, extracellular or plasma-type enzyme is found in association with lipoprotein and catalyzes the deacetylation and inactivation of PAF[81]. It also displays an anti-inflammatory function through marked reductions in PAF-induced vascular leakage[81]. PAF-AH degrades not only PAF but also oxidatively fragmented phospholipids with potent biological activities and has been a target of many clinical studies in inflammatory diseases, such as asthma, sepsis, and vascular diseases[82].

2.2. Symptoms and Clinical features

The clinical course of C1-INH-HAE is described by recurrent and localized episodes of angioedema of the subcutaneous and submucosal tissue lasting from 2 to 5 days.

The age of onset of acute episodes of angioedema is variable, as well as the frequency and severity of attacks even between people of the same family, with the same mutation. However, characteristic features of the pathology that differentiate it from other types of angioedema can be outlined.

Symptoms typically appear between childhood and adolescence worsening in puberty and lasting throughout the patient's life. A retrospective analysis conducted by Bork on 209 C1-INH-HAE patients shows an average of the episodes onset of 11.2 years (range 1 and 40 years) and 50% of the patients have their first attack before 10 years[83]. After the first attack, patients have recurrent episodes with very variable symptom-free intervals but generally less than 12 months[84]. Untreated patients have been reported to have attacks every 7-14 days while patients on prophylactic treatment with danazol may experience a long symptom-free period, even ten years[83].

Several triggers can induce acute episodes such as exposure to cold, minor trauma, infections, emotional stress, treatment with estrogens, antihypertensive treatment (ACE inhibitors) or oral hypoglycemic agents such as gliptins[85]. Furthermore, the angioedema attack can be preceded by prodromes represented by erythema marginatum, asthenia, paresthesia, anxiety or change of mood. Symptoms will gradually worsen in the first 24 hours and then slowly resolve in 48-72 hours although swelling can persist for about 5 days. Typically, one district is involved but in some patients episodes may occur simultaneously involving multiple districts or closely spaced districts. Almost all patients have cutaneous involvement and recurrent abdominal pain while attacks affecting the larynx are rare although the latter occur in more than 50% of patients affected during their lifetime[86]. Skin angioedema mainly affects the extremities, the upper limbs more than the lower ones, followed by the face, genital organs and more rarely the trunk and neck. About 70-90% of patients report abdominal pain that results from edema of the abdominal walls or gastrointestinal tract. These symptoms can vary in intensity until they can become severe and characterized by hypovolemic shock. In these cases, instrumental tests can help in the differential diagnosis by showing free fluid in the peritoneum and/or interstitial edema of the mucosa although there are no characteristic signs of angioedema.

Abdominal attacks can sometimes occur with leukocytosis and hemoconcentration. Laryngeal edema is a rare but potentially fatal event. Data on 123 patients with C1-INH-HAE suggest that the first episode of laryngeal edema may occur at a later age (26.2 years) than in skin or abdominal attacks[87]. Local trauma such as dental interventions, endoscopy and intubation during general anesthesia can represent triggers of laryngeal edema. Although less common, other clinically important manifestations may include neurological, esophageal and urinary symptoms[88].

2.3. Diagnosis

A correct diagnostic evaluation is fundamental to allow an adequate treatment in order to improve the quality of life and preserve the patient from the fatal consequences of any attacks not adequately treated. The international WAO/EAACI guidelines suggest taking into account clinical and laboratory criteria[6]:

Clinical Criteria

1. Subcutaneous angioedema, non-itchy, non-erymatous, self-limiting, usually recurrent and long lasting (more than 12 hours), with no or little urticaria, sometimes preceded by a rash as erythema marginatum type;
2. Recurrent abdominal pain (often with vomiting and/or diarrhea) without other cause, spontaneously resolving in 12-72 hours;
3. Recurrent laryngeal edema;
4. Positive family history of C1-INH-HAE (although this may not be present in up to 25% of patients);
5. Onset of symptoms in childhood/adolescence;
6. Failure to respond to antihistamines, glucocorticoids, or epinephrine.

Laboratory Criteria

1. Antigenic levels of C1-INH <50% of normal in two separate assessments;
2. Functional activity levels of C1-INH <50% of normal in two separate assessments;
3. Low C4 levels;
4. Mutation of the *C1-INH* gene that impairs protein synthesis and/or function.

Measurements of serum/plasma levels of C1-INH function, C1-INH protein, and C4 are used to diagnose C1-INH-HAE type I and II (*Picture 2*). In type I C1-INH-HAE, which comprises about 85% of patients, both concentration and function of C1-INH are low. In type II C1-INH-HAE, C1-INH concentrations are either normal or elevated, whereas C1-INH function is reduced. C4 levels are usually low in C1-INH-HAE type I and II patients, but its sensitivity and specificity are limited. Abnormal results should be confirmed. Complement C3 and CH50 levels are expected to be normal and testing is usually not helpful. Sequencing of the *SERPING1* gene can be supportive in the diagnostic workup of some C1-INH-HAE type I and II patients (including prenatal diagnosis); however, biochemical C1-INH testing is effective and less expensive than genetic testing. DNA sequencing may miss mutations such as those creating cryptic splice sites. Genetic testing may be relevant in particular cases such as mosaicisms to allow for correct genetic counseling.

Diagnosis was defined by the presence of at least one of the two recommended clinical criteria and at least one laboratory criterion. In the presence of the only diagnostic criterion 4 and one of laboratory criterion, the patient is defined as "asymptomatic carrier".

2.4. Therapy

The therapeutic approach can be divided into four categories: prevention through behavioral measures, on-demand therapy for acute attacks, short-term prophylaxis carried out if it involves treatments that can induce attacks (dental, endoscopic procedures, etc.), and long-term prophylaxis, mainly in patients with frequent and severe attacks.

- *Behavioral measures* consist in avoiding stimuli that can trigger an acute attack, such as emotional and physical stress and taking drugs such as ACE inhibitors and estro-progestins;
- During the *acute attack*, as described in the current guidelines, the treatment must take place as early as possible and the first-line drugs are the plasma derived and recombinant C1-INH (pdC1-INH and rhC1-INH) and the B2 receptor antagonist of bradykinin (Icatibant)[6]. When these drugs are not available, SDP (Solvent Detergent treated Plasma) or fresh frozen plasma should be used, although in a small percentage of cases it has been seen that they can worsen the symptoms due to the presence of kininogen in the plasma[89];
- *Short-term prophylaxis* is indicated in conditions that can trigger the onset of acute attacks of angioedema, such as traumatism on oral cavity (endoscopies,

dental or surgical operations under anesthesia with or without intubation) or psychologically stressful interventions. C1-INH concentrate should be used for preprocedural prophylaxis, as close as possible to the start of the procedure. Dosage has yet to be fully established;

- *Long-term prophylaxis* is indicated in the event of more than 12 attacks of angioedema in a year, of moderate/severe intensity, or more than 24 days a year affected by episodes of angioedema, even of mild intensity. In this case, three classes of drugs are used: attenuated androgens, antifibrinolytics and pdC1-INH.

HAEGARDA was approved by the FDA in 2017 as the first subcutaneous C1-INH concentrate. A randomized, multicenter, double-blind, placebo controlled, phase III controlled clinical trial showed significantly reduced HAE attacks in patients who receive twice per week subcutaneous doses of HAEGARDA[90].

In recent years new therapeutic strategies have been developed. The goal is to find new long-term prophylactic treatments that can further reduce the dosage created by the pathology and the side effects related to the administration of parenteral drugs and attenuated androgens.

The new drugs for long-term prophylaxis are: *Lanadelumab*, a fully-human recombinant monoclonal antibody inhibitor of kallikrein (for subcutaneous use)[91-94], and *BCX7353*, a second-generation oral kallikrein inhibitor that is being studied as a once-daily medication[95].

3. ANGIOEDEMA WITH NORMAL C1-INH LEVELS

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 (FXII-HAE), ANGPT1 (ANGPT1-HAE), PLG (PLG-HAE) and KNG1 gene (KNG1-HAE)[96]. 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)[5,96-98]. All HAE types share similar clinical phenotypes with absence of wheals and they are non-responsive to H1-antihistamine therapy.

Mutations in *F12* gene (LRG_145, ENSG00000131187, OMIM #610619) encoding human coagulation FXII was the first identified gene variant leading to HAE with normal levels of C1-INH in plasma (FXII-HAE, OMIM # 610618)[99,100]. FXII-HAE phenotype is almost exclusively expressed by females[101,102]. 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[103]. Ivanov *et al.* demonstrated that FXII with Lys/Arg substitutions for Thr309 can be cleaved by thrombin and factor XIa generating the truncated species δFXII, which in turn activates kallikrein[104]. In ANGPT1-HAE the mechanism of angioedema implies that this mutation could impair the interaction of ANGPT1 with its endothelial membrane receptor Tie2, leading to a vascular leakage and angioedema[105]. It was recently found the c.807G>T, p.(Ala119Ser) *ANGPT1* mutation in a female patient with apparently non-hereditary recurrent angioedema[106]. No pathogenetic mechanism has been envisaged for HAE related to mutation in plasminogen and kininogen 1 genes.

AIM OF THESIS

Hereditary angioedema with C1-INH deficiency is the subtype of hereditary angioedema best understood from an epidemiological, pathogenetic, clinical and therapeutic point of view. However, it is characterized by inter- and intra- individual clinical variability, the mechanisms of which are still subject of debate and research.

In an attempt to elucidate the reasons of this heterogeneity, several studies have been conducted to search for a possible association between the genetic pattern and the clinical phenotype of the patients, unfortunately obtaining contradictory and not entirely convincing results.

Currently the research is aimed at identifying biomarkers that can correlate with the disease. The most promising resources appear to be plasma proteases, inflammatory mediators and endothelial cell-related factors.

In addition to C1-INH-HAE, other types of hereditary angioedema have been described (i.e., FXII-HAE, ANGPT1-HAE, U-HAE) and few data on biomarker's disease are available in literature.

The aim of my study focused on:

- The study about the role of mediators of the main endothelial pathways that regulate vascular permeability such as VEGFs, ANGPTs and phospholipases, in the pathophysiology of angioedema and evaluate their levels in patients with C1-INH-HAE during remission and during attack;
- The evaluation of the vessel features in angioedema patients during a symptom-free period using Nailfold Video-Capillaroscopy (NVC);
- The study about the role of VEGFs, ANGPTs and phospholipases and their levels in patients with FXII-HAE, ANGPT1-HAE and U-HAE patients during remission.

MATERIALS AND METHODS

Reagents

The following were purchased: bovine aortic endothelial cells (BAEC) (Thermo Fisher Scientific©, San Jose, CA, USA); bovine serum albumin, l-glutamine, antibiotic–antimycotic solution (10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 25 μg/ml amphotericin B), Heparinase I and III Blend from Flavobacterium Heparinum, DMEM and fetal calf serum (endotoxin level <0.1 EU/ml) (MP Biomedicals Europe, Illkirch, France). Antibody anti-VEGF-A, anti-ANGPT1, and anti-ANGPT2 (R&D System, Minneapolis, MN, USA). The recombinant human secreted PLA_2 group IIA (PLA2G2A) was prepared as described[107] and the inhibitor Me-Indoxam[108] and RO032107A[109] were obtained from Dr. Michael Gelb (University of Washington, Seattle, WA, USA). All other reagents were from Carlo Erba (Milan, Italy).

Blood Sampling

Blood was collected during routine diagnostic procedures and the remaining plasma sample was labeled with a code which was documented into a data sheet. 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 sodium citrate 3.2% as anticoagulant. After centrifugation (2000 g for 20 min at 22°), the plasma was divided into aliquots and stored at -80°C until used.

Study Population

We studied 128 adult patients with C1-INH-HAE in remission and 68 healthy controls followed at the University of Naples Federico II (Naples, Italy) and Milan Center of HAE (Milan, Italy) and 15 adult patients with C1-INH-HAE during remission and attack phase followed at the Semmelweis University (Budapest, Hungary). Clinical characteristics are reported in *Table 1*, *Table 2* and *Table 3*. The Ethical Committee of the University of Naples Federico II, University of Milan (protocol number: 216/16) and Semmelweis University of Budapest (protocol number: BPR/021/09599-7/2014) approved that plasma obtained during routine diagnostics could be used for research investigating the physiopathology of HAE and

written informed consent was obtained from patients in according to the principles expressed in the Declaration of Helsinki. The diagnosis of C1-INH-HAE was based on presence of at least one clinical and laboratory criteria[6]. Fifteen patients from Semmelweis University (Budapest, Hungary) were followed also during acute angioedema attack and their specific clinical characteristics are reported in *Table 2*. Regarding symptom-free samples, blood sampling was performed at least two weeks since the date of the last angioedema attack. While regarding samples during attack, blood sampling was performed within 6 hours after the onset of the symptoms.

Complement System Analysis

Plasma C1-INH was measured by radial immunodiffusion (NOR-Partigen, Siemens Healthcare Diagnostics, Munich, Germany). C4 antigen concentration in Italy was measured by radial immunodiffusion (NOR-Partigen) (the method is not specific for C4 fragments); in Hungary C4 was measured by turbidimetry (Roche Cobas Integra 800, Beckman Coulter Complement C4). The antibody employed in the Beckman Coulter C4 assay is directed against the common portion of the C4 molecule and it exhibits the same reactivity with C4 fragments as well as with the native molecule. 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 0.70–1.30 U C1-INH/ml (1 U 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 U C1 INH/ml (>70%). According to diagnostic criteria, all patients enrolled in this study had C1-INH functional activity lower than 50% of normal, positive family history, clinical symptoms of angioedema, low C4, normal C1q concentrations[6].

Contact System Analysis

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.*)[110]. Samples were loaded on a 9% SDS-PAGE. After electrophoretic separation, proteins were transferred from the gel to a polyvinylidene difluoride membrane using Bio-Rad Trans-Blot® TurboTM Transfer System (Bio-Rad

Laboratories, Hercules, CA, 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 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.

Assays of VEGFs and ANGPTs

Plasma levels of VEGF-A, VEGF-C, VEGF-D, ANGPT1 and ANGPT2 were measured using commercially available ELISA kits (R&D System, Minneapolis, USA) according to the manufacturer's instructions. The ELISA sensitivity is 31.1 – 2,000 pg/ml for VEGF-A, 62.5 – 4,000 pg/ml for VEGF-C, 31.3 – 2,000 pg/m for VEGF-D, 156.25 – 10,000 pg/ml for ANGPT1 and 31.1 – 4,000 pg/ml for ANGPT2.

Assays of Phospholipases

PLA_2 activity in plasma of C1-INH-HAE patients during remission and angioedema attacks was evaluated using commercially available kit (Life Technologies EnzChek® phospholipase A_2 assay) according to the manufacturer's instructions. The activity of PAF-AH enzymes was determined in plasma of patients and healthy controls by using PAF-AH assay kits (Cayman Chemical) according to the manufacturer's instructions.

ELISA for PLA2G2A

Human $sPLA_2$ group IIA (PLA2G2A) levels in plasma samples were determined by ELISA kit (Catalog No. MBS9303777, MyBioSource, San Diego, CA, USA). The concentration of PLA2G2A in plasma was tested in duplicate and determined against a standard curve for each ELISA assay. To evaluate the reliability of the assay we loaded 500 and 1,000 pg/ml of recombinant PLA2G2A in the ELISA wells and the spectrophotometer measured 397 and 886 pg/ml, respectively.

***In Vitro* Vascular Permeability Assay**

Endothelial cell permeability was assessed by *in vitro* vascular permeability assay kit (Life Technologies, Carlsbad, CA, USA). BAEC were seeded onto collagen-coated transwell filters (1 μm pore size) at the density of 7.5×10^4 cells/well in a 96-well receiver plate and incubated

at 37°C and 5% CO_2 for 72 hours. After this time, cells starvation step was performed by adding DMEM 0.5% FBS and incubation for 18 hours at 37°C, 5% CO_2. Plasma from patients with C1-INH-HAE was pre-incubated (20 min, 37°C) with Me-Indoxam (100 nM), RO032107A (100 nM), anti-VEGF-A (1 μg/ml), anti-ANGPT2 (1 μg/ml), anti-ANGPT1 (1 μg/ml), or control medium. BAEC were then pre-incubated (30 min, 37°C) with heparinase (0.4 U/ml) or control medium and then stimulated (18 hours, 37°C) with plasma of healthy controls, or with plasma of C1-INH-HAE patients or with the combination of C1-INH- HAE plasma with Me-Indoxam, RO032107A, anti-VEGF-A, anti-ANGPT2 and anti-ANGPT1. To evaluate vascular permeability, a high-molecular weight FITC-Dextran was added on top of the cells, allowing the fluorescent molecules to pass through the endothelial cell monolayer at a rate proportional to the monolayer's permeability. The extent of permeability was determined by measuring the fluorescence (485 nm excitation and 535 nm emission) using SunriseTM spectrofluorometer (Tecan) and RFU was calculated.

Nailfold Videocapillaroscopy

NVC (VideoCap® 3.0-D1, DS Medica, Milan, Italy) is a highly sensitive, inexpensive, simple, safe, and noninvasive imaging technique used in the morphological analysis of nourishing capillaries in the nailfold area[111].

It is performed by means of a microscope combined with a digital video camera put in direct contact with a nailfold[112].

The patients under investigation should initially remain seated in an acclimatized room for 15-20 min, with a set temperature of approximately 22-23°C. A drop of immersion (cedar) oil was placed onto the cuticle of the fingers to be analyzed in order to reduce any refractive defects and improve capillary visualization[112]. Patients were instructed not to remove their fingernail cuticles for one month to avoid microtraumas that could put the examination at risk. NVC was performed on the 4 fingers of both hands (excluding thumbs) with 200x magnification[113]. Angioedema patients underwent the procedure during a remission period (at least 8 days after an attack). NVC was also performed in two C1-INH-HAE patients during hand and abdominal attacks, respectively.

The following *quantitative* parameters were analyzed:

- *Intercapillary distance*: distance between two neighboring capillary loops, measured at the widest intercapillary space on the central capillary region;

- *Apical diameter*: distance from one external margin of the capillary loop to another on the apex (normal [8-25 µm], enlarged loop [>26 µm] or giant loop [>50 µm]);
- *Loop length*: «the distance between the apex of a capillary loop and the point where the capillary is no longer visible»[112];
- *Internal diameter*: the distance between the efferent and the afferent loop measured at the same level;
- *External diameter*: «the width of a capillary at its widest section»[112];
- *Capillary density*: «number of capillaries in a 1-mm length of the distal row of each finger»[112].

The following *qualitative* parameters were analyzed:

- *Capillary distribution*: organization of capillaries, scored as ordered (0), comma-like (1), irregular (2), and severely deranged (3)[114];
- *Capillary morphology*: the aspect of capillaries, scored as hairpin-like (0), mainly tortuous (1), mainly ramified (2), and severe alterations (3)[114].

Genotyping

Genotype analysis were performed at University Foggia. Genomic DNA was isolated from peripheral blood leukocytes according to standard protocols.

Mutational screening of *SERPING1*, *F12* and *ANGPT1* coding region and exon/intron boundaries was performed by direct DNA sequencing, as described elsewhere[105,115,116].

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* and *ANGPT1* as reported in ENSEMBL database (Wellcome Trust Sanger Institute, Cambridge, United Kingdom): *SERPING1* ENSG00000149131, *F12* ENSG00000131187, and *ANGPT1* ENSG00000154188. Briefly, polymerase chain reactions were carried out in 50 µl samples in 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 subject 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 that base. For viewing bases, assembly multiple samples and comparison to a reference sequence (alignment), the Sequencher v.4.7 tool (Gene Code, Corp.) was used. Variants causing HAE were described according to the Human Genome Variation Society recommendations (http://varnomen.hgvs.org/;v.10.01).

Statistical Analysis

Data were analyzed with the GraphPad Prism 7 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 paired two-tailed t-test or two-tailed Mann-Whitney test as indicated in figure legends. Correlations between two variables were assessed by Spearman's rank correlation analysis and reported as coefficient of correlation (r). Plasma concentrations of VEGFs and ANGPTs, the activity of $sPLA_2$ and PAF-AH are shown as the median (horizontal black line), the 25^{th} and 75^{th} percentiles (boxes) and the 5^{th} and 95^{th} percentiles (whiskers) of C1-INH-HAE patients and controls. Statistically significant differences were accepted when the p-value was ≤ 0.05.

RESULTS

1. Role of Vasoactive Mediators in C1-INH-HAE Patients in Remission

1.1. Plasma VEGFs and ANGPTs levels and sPLA$_2$ and PAF-AH activities in C1-INH-HAE patients in remission

Table 1 summarizes the clinical characteristics of 128 patients with C1-INH-HAE and 68 healthy controls. We evaluated the concentrations of different angiogenic and lymphangiogenic factors in patients with C1-INH-HAE in remission. *Figure 1A* and *1B* show that VEGF–A and VEGF–C plasma levels of patients with C1-INH-HAE were higher than healthy controls [VEGF–A: healthy controls 0 (0–0) *vs* C1-INH-HAE 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)]. By contrast, the other lymphangiogenic factor, VEGF–D was not modified [VEGF–D: 36 (12–91) *vs* 29 (0–144) pg/ml)] (*Fig. 1C*). Interestingly, both ANGPT2, which increases vascular permeability, and its inhibitor ANGPT1 were increased in patients with C1-INH-HAE compared with controls [ANGPT1: 2.2 (1.5–2.9) *vs* 4.5 (3.5–5.7); ANGPT2: 0.3 (0.1–0.4) *vs* 0.45 (0.3–0.7) ng/ml)] (*Fig. 1D* and *1E*). In addition, we assessed the PLA$_2$ enzymatic activity (likely attributable to sPLA$_2$) and PAF-AH in the plasma from 109 out of 128 C1-INH-HAE patients in the symptom-free period *vs* 68 healthy controls. *Figure 1F* and *1G* show that sPLA$_2$ and PAF-AH activities were increased in C1-INH-HAE patients in symptom-free period compared with controls [sPLA$_2$: 1.3 (0.6–1.8) *vs* 2.4 (1.3–3.0) U/ml; PAF-AH: 25 (0-46) *vs* 34 (0-54) nmol/min/ml]. No correlation was found between sPLA$_2$ and PAF-AH activities (r=0.03; NS) (data not shown).

Several sPLA$_2$s have been identified in mammals (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA, XIIB, and otoconin-95)[70], with PLA2G2A being the most represented in human serum and plasma[117-121]. Accordingly, we found increased levels of PLA2G2A protein in C1-INH-HAE patients compared with controls (*Fig. 2A*). The sPLA$_2$ enzymatic activity in plasma from C1-INH-HAE patients strongly correlated with PLA2G2A plasma levels (r=0.77; p<0.01) (*Fig. 2B*). The enzymatic activity was significantly inhibited by Me-Indoxam (an enzymatic inhibitor of several sPLA$_2$s)[108] and RO032107A (a specific PLA2G2A inhibitor)[109] in both C1-INH-HAE patients and healthy donors (*Fig. 2C*). Collectively, these results

indicate that the increased $sPLA_2$ enzymatic activity observed in the plasma from C1-INH-HAE patients is likely attributable to higher levels of PLA2G2A protein.

There was no difference in VEGFs and/or ANGPTs concentrations and $sPLA_2$ activity between male and female values in both controls and patients (*Fig. 3A-F*). It has been shown that estrogens decrease PAF-AH plasma levels in adult male and female rats, while progestins have the opposite effect[122]. However, no differences in PAF-AH activities were found between males and females in both controls and patients (*Fig. 3G*). Moreover, the age of patients and the concentration/activities of the different plasma mediators examined did not correlate (*Fig. 4A-G*).

1.2. VEGF-A concentration and $sPLA_2$ activity are inversely associated with functional activity of C1-INH

As patients with C1-INH-HAE may exhibit a spectrum of residual functional activity of C1-INH, we divided the patients according to their C1-INH activity. Ninety-eight 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 5A* and *5F* show that patients with the lowest functional activity of C1-INH had higher VEGF-A concentration and $sPLA_2$ activity than patients with higher functional activity of C1-INH [VEGF-A: 3 (0–27) *vs* 0 (0–0) pg/ ml); $sPLA_2$: 2.32 (0.1–8.25) *vs* 1 (0–4.6) U/ml]. By contrast, the concentrations of VEGF-C, VEGF-D, ANGPT1, ANGPT2 and PAF-AH activity did not differ between the two groups of patients (*Fig. 5B-E* and *5G*).

In C1-INH-HAE patients functional C1-INH levels are below 50% of normal value and C4 concentrations are usually reduced and can be used as a screening test[6]. We investigated whether differences in the complement component levels (C1-INH and C4) were associated with differences in VEGFs/ANGPTs levels and $sPLA_2$ and PAF-AH activity. To this aim, we compared patients with type I C1-INH with less than 25% and 25–50% of normal C1-INH values. There were no differences in plasma concentrations of VEGF-A, VEGF-C, VEGF-D, ANGPT1, ANGPT2 and PAF-AH activity between these groups (*Fig. 6A–E, 6G*). By contrast, $sPLA_2$ activity negatively correlated with C1-INH concentration (*Fig. 6F*).

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 patients with C1-INH-HAE and used as a screening test[123,124]. Again, we found no differences in plasma

concentrations of VEGF-A, VEGF-C, VEGF-D, ANGPT1, ANGPT2, $sPLA_2$ activity and PAF-AH activity between these groups (*Fig. 7A–G*).

1.3. Plasma levels of VEGF-A, VEGF-C, ANGPT1, ANGPT2 and $sPLA_2$ and PAF-AH activity in patients with different attack frequency

As the frequency of attacks is highly variable among patients with C1-INH-HAE, we asked whether plasma levels of VEGFs/ANGPTs and PLA_2 could be used to identify patients at higher risk of recurrent attacks. Plasma levels of VEGF-A, VEGF-C and ANGPT2 and PAF-AH activity (*Fig. 8A, B, E* and *H*) were higher in patients with high frequency of attacks (>12/year) compared to patients with low frequency (<12/years). By contrast, VEGF-D, ANGPT1 and $sPLA_2$ are not modified with attack frequency (*Fig. C, D* and *G*). Interestingly, the ANGPT2/ANGPT1 ratio (an index of vascular permeability) was also increased in more severe patients (*Fig. 8F*).

We have previously demonstrated that plasma concentration of cleaved HK (cHK) is higher in highly symptomatic C1-INH-HAE patients, than those with less frequent attacks[125]. Therefore, we analyzed the relationship among cHK and vasoactive mediators. We found a positive correlation between plasma concentrations of both VEGF-A and ANGPT2 and that of cleaved HK (*Fig. 9A, D*). In addition, cleaved HK also correlated with the ANGPT2/ANGPT1 ratio (data not shown). By contrast, there was no correlation between the plasma levels of both VEGF-C, ANGPT1, $sPLA_2$ and PAF-AH and cleaved HK (*Fig. 9B, C, E* and *F*).

1.4. C1-INH-HAE Plasma increases Endothelial Permeability

VEGF-A, ANGPT2 and $sPLA_2$ can modulate endothelial cell mobility and vascular permeability[57,59,80]. By contrast, ANGPT1 is a vascular stabilizer[55]. To gain mechanistic insight into the role of VEGF-A, ANGPT2 and $sPLA_2$s in C1-INH-HAE, we performed an *in vitro* vascular permeability assay by monitoring the leakage of dextran-FITC through a tight monolayer of BAEC[126]. Interestingly, plasma from angioedema patients in symptom-free period increased endothelial permeability compared with control plasma from healthy donors [128 (95–207) *vs* 52 (8.5–125) RFU] (*Fig. 10A*).

To assess whether our vasoactive mediators in plasma were responsible for this phenomenon, we incubated C1-INH-HAE plasma with blocking antibodies against VEGF-A, ANGPT1, and ANGPT2 or against PLA2G2A (Me-Indoxam and RO032107A). Anti-VEGF-

A and anti-ANGPT2 but not anti-ANGPT1 reduced the effect of C1-INH-HAE plasma without completely abolishing it (i.e., permeability levels were still higher compared with healthy donor plasma) (*Fig. 10B*). Interestingly, Me-Indoxam and RO032107A also reduced vascular permeability induced by C1-INH-HAE plasma [untreated 145 (126–227) *vs* Me-Indoxam 91 (34–114) RFU] (*Fig. 10B*). $sPLA_2$s also bind to HSPGs that may mediate some of their biological effects[127-129].

To evaluate a possible role for HSPGs, BAEC were treated with heparinase to eliminate surface HSPGs before stimulation with plasma[66]. Heparinase treatment of BAEC reduced the endothelial permeability induced by C1-INH-HAE plasma [untreated 145 (126–227) *vs* heparinase 117 (92–132) RFU] to a level comparable to Me-Indoxam and RO032107A (*Fig. 10B*).

2. Role of Vasoactive Mediators during C1-INH-HAE Attacks

2.1. ANGPT1 concentrations are increased whereas $sPLA_2$ and PAF-AH activities are decreased during C1-INH-HAE attack

Table 2 reports characteristics of C1-INH-HAE evaluated during angioedema attack. Different permeability factors in patients with C1-INH-HAE during angioedema attack and during remission were evaluated. VEGF-A concentrations (*Fig. 11A*) were not altered during acute attacks compared to symptom-free periods [attack-free 1 (0-8) *vs* angioedema attack 1 (0-10) pg/ml median values (interquartile ranges)]. The concentrations of the lymphangiogenic factors VEGF-C and VEGF-D during angioedema attack were similar to those during remission [VEGF-C: 341 (164-613) *vs* 358 (196-1042) pg/ml; VEGF-D: 27 (0-144) *vs* 30 (3-146) pg/ml] (*Fig. 11B, C*).

Similarly, we did not find modification in ANGPT2 levels during episodes of angioedema [185 (0-479) *vs* 170 (0-460) pg/ml] (*Fig. 11E*). By contrast, a significant increase of ANGPT1 concentrations was detected during the angioedema attack compared to remission phase [1578 (799-3052) *vs* 2518 (1096-6228) pg/ml] (*Fig. 11D*). In addition, the ANGPT2/ANGPT1 ratio was decreased in angioedema attack compared to symptom-free periods [0.09 (0-0.49) *vs* 0.06 (0-0.33)] (*Fig. 11F*).

$sPLA_2$ and PAF-AH activities were decreased in C1-INH-HAE patients during attack compared to symptom-free period [$sPLA_2$: symptom-free 3.0 (1.4-4.4) *vs* attack 1.2 (0.4-2) U/ml; PAF-AH: 32 (25–39) *vs* 19 (16–25) nmol/min/ml median] (*Fig. 11 G, H*).

3. Structural Capillaries Alteration in C1-INH-HAE Patients

3.1. Capillaroscopic Parameters in C1-INH-HAE patients compared to controls

Thirty-four C1-INH-HAE patients and 28 healthy controls underwent NVC (*Table 3*).

C1-INH-HAE patients showed significantly increased apical [28 (23-26) *vs* 22 (16-29) µm] (*Fig. 12A*), internal [22 (20-25) *vs* 20 (18-22) µm] (*Fig. 12B*), and external diameters [81 (65-91) *vs* 65 (55-73) µm] (*Fig. 12C*) compared to healthy controls, thus demonstrating the presence of enlarged capillaries. By contrast, loop length did not show any difference between the two groups [450 (330-556) *vs* 480 µm (350-590) µm] (*Fig. 12D*). In addition, both significantly increased intercapillary distance [216 (185-253) *vs* 190 (154-236) µm] (*Fig. 12E*) and decreased capillary density [4 (4-5) *vs* 5 (5-6) /mm] (*Fig. 12F*) indicate a reduction in the number of capillaries in C1-INH-HAE patients compared to controls. Capillary distribution was significantly more irregular [0 (0-1) *vs* 0 (0-0)] (*Fig. 12G*) and the morphology mainly tortuous [1 (0-1) *vs* 0 (0-0)] (*Fig. 12H*) in C1-INH-HAE patients versus controls. *Figure 13A-C* shows representative images of capillaroscopic parameters of three different patients with C1-INH-HAE. In particular, *Figure 13A* shows increased apical, internal, and external diameters, *Figure 13B* shows reduced capillary density, and *Figure 13C* shows irregular capillary distribution.

To evaluate whether NVC findings in C1-INH-HAE patients were related to pathology severity, we assessed the presence of correlations among capillaroscopic parameters and the severity score proposed by Bygum *et al.*[130]: no significant associations were found (data not shown).

However, a significant correlation was found with the frequency of attacks: C1-INH-HAE patients with ≥12 attacks/year (17/34 patients) had greater apical diameter with respect to those having <12 attacks/year. By contrast, other capillary characteristics were not correlated with the severity of disease (data not shown).

Similarly, the age of onset of C1-INH-HAE symptoms seemed to have no role in the onset of capillary alterations, as no significant correlations were found (data not shown).

Finally, to test the hypothesis that capillaroscopic parameters change in accordance with mediators known to regulate vascular permeability, we assessed whether capillaroscopic alterations were correlated with plasma levels of VEGF-A, VEGF-C, ANGPT1, and ANGPT2, but no significant correlations were found (data not shown).

A further exploratory analysis on a very small sample tested whether capillary alterations changed between remission and attack phases: NVC performed in only two C1-INH-HAE patients during hand and abdominal attacks, respectively, showed no changes compared to the remission phase (*Fig. 14A-H*).

4. Role of cHK and vasoactive mediators in angioedema with normal C1-INH levels in remission

To evaluate whether the altered levels of vasoactive mediators were specific only to C1-INH-HAE we measured plasma levels of cHK in samples from 72 healthy subjects (11 in sodium citrate and 61 with Protease inhibitor cocktail (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. 15A*). 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 than in U-HAE, but significantly higher than in normal subjects [50% (47-56) *vs* 33% (31-36); $p<0.01$] (*Fig. 15A*).

Then, 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 15* shows that VEGF-A (*panel B*) plasma levels of U-HAE patients were higher than in healthy controls [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. 15C) [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 B*) [0 (0-0) *vs* 0 (0-0.7) pg/ml], while VEGF-C concentration (*panel C*) was significantly higher [350 (192-442) *vs* 154 (97-211) pg/ml; $p<0.01$]. Interestingly, ANGPT1 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. 15D). In contrast, ANGPT2 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. 15E*). Moreover, *Figure 15* shows that the concentrations of VEGF-A

(*panel B*), VEGF-C (*panel C*), ANGPT1 (*panel D*), and ANGPT2 (*panel E*) 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 ANGPT2 concentrations (data not shown).

$sPLA_2$ activities, elevated in patients with C1-INH-HAE[131], showed no differences when measured in FXII-HAE, U-HAE and ANGPT-1 patients. Interestingly, the concentrations of these mediators did not differ between symptomatic and asymptomatic FXII-HAE patients (data not shown).

DISCUSSION

Several mediators play an important role in vascular permeability such as VEGFs, ANGPTs and components of PLA_2 superfamily (i.e., $sPLA_2$ and PAF-AH)[46-48,53,54,80,81].

Here, we demonstrate that patients with C1-INH-HAE in remission have higher plasma levels of VEGF-A, ANGPT1 and ANGPT2 compared with healthy controls. Among lymphangiogenic factors, VEGF-C but not VEGF-D levels are increased in C1-INH-HAE patients during remission compared to controls. In addition, enzymatic activities of PAF-AH and $sPLA_2$ are increased. Moreover, we showed that $sPLA_2$ activity detected in C1-INH-HAE is attributable to PLA2G2A isoform, the unique $sPLA_2$ detected in plasma[117,118,120]. Interestingly, plasma levels of VEGF-A, VEGF-C, ANGPT2 and PAF-AH activity are even higher in patients with C1-INH-HAE 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 of VEGF-A and ANGPT2 with those of cleaved HK, which we already demonstrated to distinguish in patients with a more severe disease phenotype[125]. Hereditary angioedema with C1-INH deficiency is caused by a genetic mutation in *SERPING1* gene that is associated with a stable deficiency in C1-INH levels or function[132]. Nonetheless, frequency and anatomical localization of angioedema attacks show highly intra- and inter-variability. Thus, there is an increasing need for identifying biomarkers of disease activity.

More than 450 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[116]. The levels of the lectin complement pathway serine protease MASP-1 and its complex with C1-INH are reduced in patients with C1- INH-HAE and seem to correlate with the degree of complement C4 consumption and disease severity[133]. The authors already mentioned the evidence that plasma levels of cleaved HK increase in patients with high frequency of attacks[125]. Cleaved HK, measured by immunoblotting analysis after gel electrophoresis[110], is generated by the activation of the contact system and can be assumed as indirect measurement of the release of bradykinin during such an activation[134]. Our present results indicate that also VEGF-A and ANGPT2 mark C1-INH-HAE severity. Increased circulating levels of VEGF-A and ANGPT2 associated with higher vascular

permeability have been observed in several diseases, namely systemic capillary leak syndrome[59] and sepsis[60,61,135]; in addition PAF-AH has been the target of many clinical studies in inflammatory disease, such as asthma, sepsis, and vascular disease[82]. Based on these results, we hypothesize that these factors along with an increased release of BK induce a state of 'vascular preconditioning' that may change the threshold for the development of angioedema attacks. To confirm the involvement of VEGFs, ANGPTs and $sPLA_2$ in angioedema vascular leakage, we assessed an *in vitro* vascular permeability assay. We show that plasma from C1-INH-HAE increases endothelial cell permeability compared to healthy controls. Anti-VEGF-A and anti-ANGPT2 but not anti-ANGPT1 reduced the effect of C1-INH-HAE plasma without completely abolishing it (i.e., permeability levels were still higher compared with healthy donor plasma). In addition, the addition of PLA2G2A-specific enzymatic inhibitor RO032107A or endothelial cell treatment with heparinase, an enzyme that degrades HSPGs, partially revert the leakage induced by plasma of patients. It is conceivable that PLA2G2A in C1-INH-HAE plasma binds to HSPGs on endothelial surface and increases endothelial cell permeability in a process that requires its enzymatic activity. These results are in keeping with the evidence that $sPLA_2$s together with VEGFs and ANGPTs can modulate endothelial cell permeability. Further studies are required to gain more insights into this model and define its relevance to C1-INH-HAE pathogenesis.

Instead the scenario during the angioedema attack seems to be completely different. In this study, we found no differences in VEGF-A, VEGF-C and ANGPT2 levels during acute attack compared to remission. Interestingly, a significant increase of ANGPT1 was detected during angioedema attack. ANGPT1 is an agonist of Tie2 receptor and plays an important role in vascular stabilization[55]. It has been recently reported that missense mutation of ANGPT1 gene is associated to a novel endotype of angioedema[105]. The mutation leads to a reduced binding of ANGPT1 to its Tie2 receptor caused by a lacking of ANGPT1 multimers formation[105] which results in vascular destabilization. Our data suggest that ANGPT1, a unique vascular stabilizer, may play an important role in restoring physiological vascular homeostasis during angioedema attack. This hypothesis is indirectly supported by decreased ANGPT2/ANGPT1 ratio, which is an index of vascular permeability.

Conversely, we observed decreased $sPLA_2$ and PAF-AH activities during angioedema attack. The explanation of this observation is still unclear. It is conceivable that decreased activity of PAF-AH could be due to its exhaustion in order to degrade PAF and other metabolites released during the angioedema attack or to its sequestration (binding to the surface and/or internalization), similarly to other $sPLA_2$[136] by activated endothelial cells

during the acute phase. Whatever the mechanisms, the alterations of $sPLA_2$ and PAF-AH during both remission and clinical attack suggest a possible involvement of these mediators in C1-INH-HAE.

To further confirm the central role of endothelium in C1-INH-HAE, we evaluated the structural alterations of capillaries in patients with angioedema.

Patients affected by C1-INH-HAE have capillaries characterized by significant quantitative and qualitative morphological alterations, i.e., increased apical, internal, and external diameters, reduced capillary density, and increased prevalence of irregular capillary distribution and tortuous morphology.

A derangement of capillary structure was observed in these patients: capillaries become progressively larger and less numerous.

It remains unknown whether those alterations trigger attacks or if, on the contrary, are caused by attacks. If the first hypothesis is confirmed by other studies, the pathogenesis of angioedema will be further elucidated as a kind of "vascular preconditioning" may predispose patients to angioedema attacks, and, consequently, the endothelium may become a new therapeutic target.

The presence of altered capillaries characterizes C1-INH-HAE patients. Preliminary data on two patients indicates no differences in capillaroscopic alterations between remission phase and attack, thereby suggesting that they are constitutive characteristics C1-INH-HAE patients. Some published studies report results regarding the presence of endothelial dysfunction in C1-INH-HAE patients.

The results obtained in terms of capillary differences led to the same conclusion about the presence of vascular preconditioning, as all except one (i.e., loop length) among the parameters analyzed were found to be significantly different with respect to controls. Due to the types of procedures used, it may be necessary to focus on the evaluation of histological aspects of the endothelium rather than on serum factors.

An endothelial dysfunction in hereditary angioedema (both due to C1-INH deficiency and FXII deficiency) was also found by Firinu *et al.*[137], who detected a lower reactive hyperemia index with non-invasive finger plethysmography in the fingertips in 24 HAE patients compared with 24 age- and gender-matched healthy controls. HAE patients also had higher plasma levels of asymmetric dimethylarginine (ADMA). However, these alterations had no correlations with disease severity.

On the contrary, the study of Nebenführer *et al.*[138] excluded the presence of endothelial dysfunction in a study involving 33 C1-INH-HAE patients and 30 healthy controls. The

technique used in that case was the flow-mediated dilation method measured at the brachial artery. The contrasting conclusions with respect to the study of Firinu *et al.* may arise from the different measurement techniques used and the heterogeneity of patient populations recruited[139].

Finally, the presence of many tortuous loops in capillaries was also found by means of NVC in a family affected by unknown hereditary angioedema (U-HAE)[105].

Data collected in our healthy subjects may contribute to the determination of normal values of capillaroscopic parameters, since in the literature, they are scarce. Our data are generally in line with those reported in the study carried out on the greatest sample size of healthy subjects[140].

NVC seems to be the most suitable method to analyze the endothelium in angioedema patients due to its features: it is simple to perform, non-invasive, repeatable, inexpensive, and highly sensitive. The nail bed is analyzed because of the easy approach with the probe and the fact that in this site, capillary vessels are parallel to the cutaneous surface[113].

This study has some limitations. First, the sample size was low. This is a common issue when a rare disease is considered. Second, even though most characteristics of patients and healthy subjects were similar, some of them were not (i.e., gender in the comparison ACEI-AAE patients/controls). However, we do not think these discrepancies affect the reliability of our results. Third, parameters were measured during attack in only two patients, thereby making the resulting measures less reliable. Due to the unpredictability of attack onset, it is not possible to plan such a procedure in the acute phase of this pathology.

In conclusion, our data suggest that C1-INH-HAE patients have important structural alterations of the capillaries, confirming the presence of an endothelial dysfunction in the angioedema, while NVC performed in two C1-INH-HAE patients during a hands and abdominal attack may indicate that capillaroscopic alterations are not related to the acute phase or to the site of the attack.

Finally, we reported the cohort of 105 patients with normal C1-INH levels (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[123]. The numbers suggest that frequency of nl-C1-INH-HAE is about 1/10 compared to that of C1-INH-HAE. Four different *F12* variants can lead to FXII-HAE[141-144], 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[145] and acts as a gain of function

mutation[104]. 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[18]. A 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[105].

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[99,100].

It has been demonstrated that contact system can be activated at blood drawing leading to a plasma kallikrein activation, as in C1-INH-HAE[146]. Massive cleavage of HK occurs unless blood is protected by direct collection in an anti-protease cocktail. It was reported that under anti-protease protection, plasma levels of cleaved-HK 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[21,125]. Baroso *et al.*[147] 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 cleaved-HK 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 cleaved-HK 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.

Finally, we measured VEGFs and ANGPTs levels 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. 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. In FXII-HAE and ANGPT1-HAE patients the relevance of these differences remains unclear.

In conclusion, our results emphasize the complexity by which several vasoactive mediators together with morphological/structural alteration of capillaries are involved not

only in the pathophysiology of various hereditary angioedema forms, but also during angioedema attacks and its resolution.

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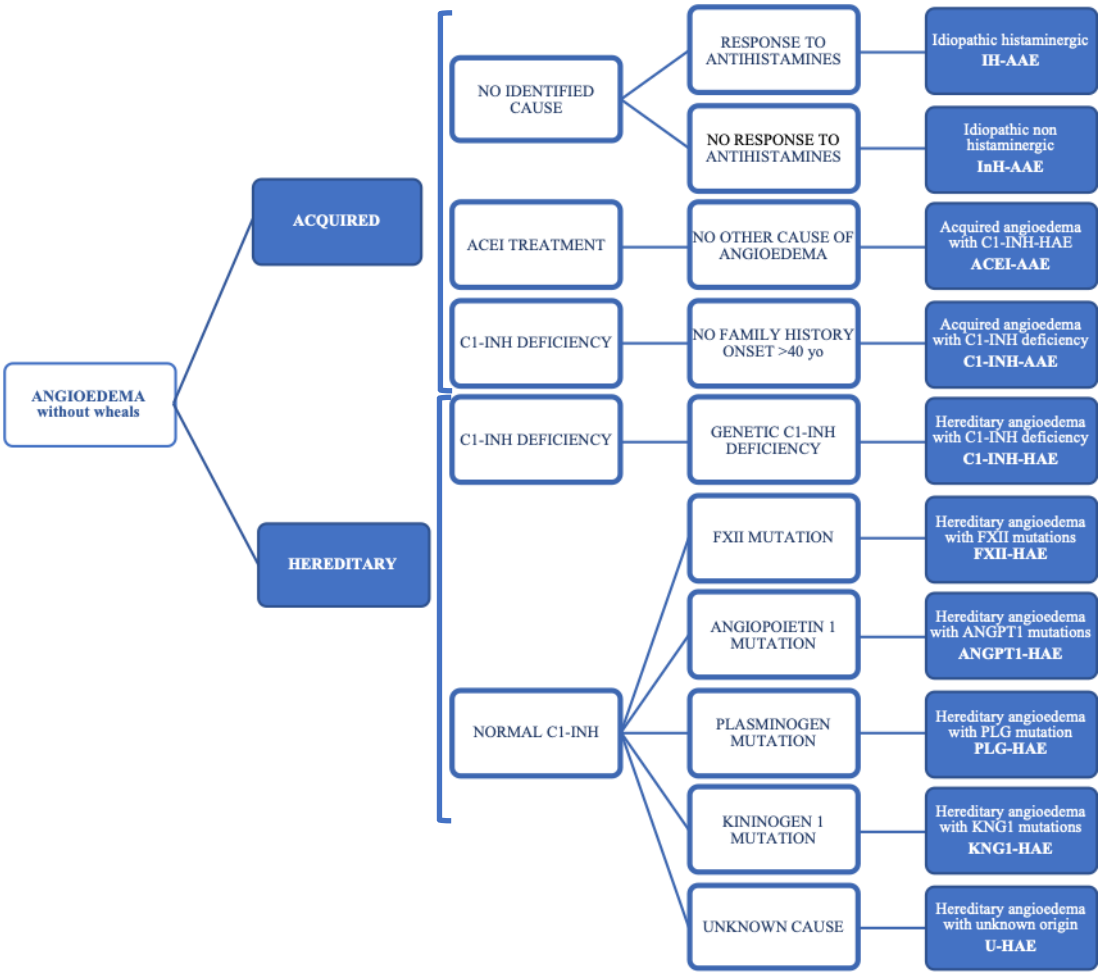
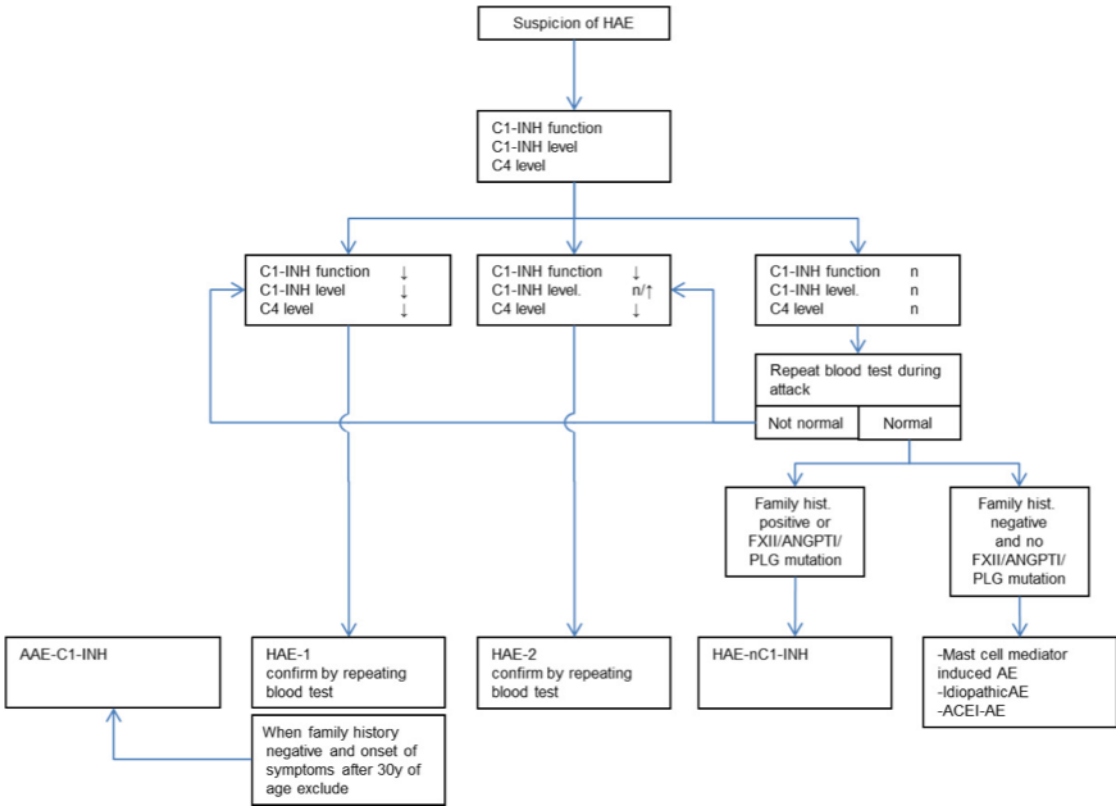
Pictures

Picture 1. Classification of angioedema without wheals.



Picture 2. Diagnosis of hereditary angioedema.



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Tables

Table 1. Characteristics of patients with C1-INH-HAE in remission and healthy controls.

Characteristics		Healthy donors (n = 68)	Patients (n = 128)
Age – yrs*		33 (27–45)	41 (23–52)
Gender male – no. (%)		39 (57%)	57 (45%)
Caucasian %		100%	100%
Unrelated families		NA	78
Age at onset – yrs*		NA	6 (3–14)
C1-INH-HAE – no. (%)	Type I	NA	117 (91.4%)
	Type II	NA	11 (8.6%)
Prophylaxis – no– (%)	Tranexamic acid	NA	5 (4%)
	Attenuated androgens	NA	29 (22%)
≥ 12 attacks per year – no. (%)		NA	41 (32%)

*Data are expressed as median values (interquartile ranges) and analyzed by using t-test. $p<0.05$

NA: not applicable

Table 2. Characteristics of patients with C1-INH-HAE followed during angioedema attack.

Characteristics		Patients (n = 15)
Age – yrs*		29 (18 – 49)
Gender male – no. (%)		4 (26.7%)
Caucasian %		100%
Unrelated families		15
Age at onset – yrs*		10 (1 – 18)
C1-INH-HAE – no. (%)	Type I	13 (86.6%)
	Type II	2 (13.4%)
Prophylaxis – no – (%)	Danazol	1 (6.7%)
	Tranexamic acid	1 (6.7%)
	Plasma-derived C1-INH	1 (6.7%)
≥ 12 attacks per year – no. (%)		5 (33.4%)

*Data are expressed as median values (interquartile ranges) and analyzed by using t-test. $p<0.05$

Table 3. Characteristics of patients with C1-INH-HAE and healthy controls underwent to NVC.

Characteristics		**Healthy donors (n = 28)**	**Patients (n = 34)**
Age – yrs*		40 (9–72)	39.5 (9–74)
Gender male – no. (%)		14 (50%)	17 (50%)
Caucasian %		100%	100%
Age at onset – yrs*		NA	6 (1–22)[†]
C1-INH-HAE – no. (%)	Type I	NA	31 (91.2%)
	Type II	NA	3 (8.8%)
Long Term Prophylaxis no. (%)	No Long Term Prophylaxis	NA	23 (67.6%)
	Attenuated androgens	NA	6 (17.6%)
	pd-C1-INH	NA	5 (14.7%)
Severity Score*		NA	7 (4.7–8)
≥ 12 attacks per year no. (%)		NA	17 (50%)
Attack Site – no. (%)			
Skin		NA	32 (94.2%)
Gastrointestinal Tract		NA	27 (79.5%)
Larynx		NA	19 (56%)
Genitalia		NA	8 (23.5%)
Comorbidities – no. (%)			
Hypertension		1 (7.7%)[‡]	3 (9%)
Dysthyroidism		0 (0%)[‡]	1 (3%)
Diabetes Mellitus		0 (0%)[‡]	0 (0%)
Body Mass Index (BMI)*		24 (21–26)[‡]	23.4 (15–40)

*Data are expressed as median values (interquartile ranges).

NA: Not Applicable

† Thirty-two patients were considered, as two of them were asymptomatic

‡ Data available for 13 subjects**.**

Figures

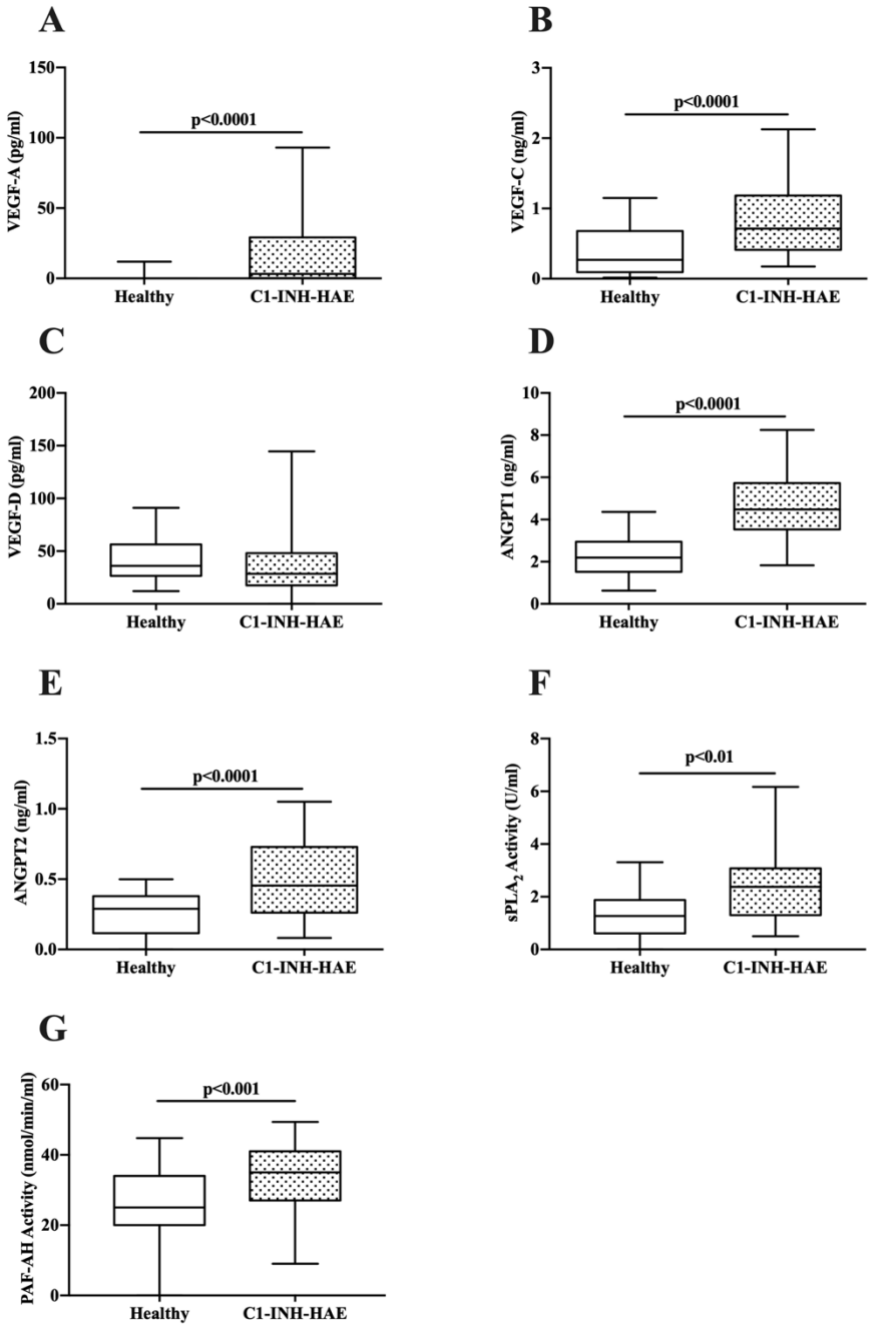

Figure 1. Plasma concentrations of VEGFs, ANGPTs and and enzymatic activities of sPLA2 and PAF-AH in C1-INH-HAE patients during remission.

VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 (E), sPLA2 activity (F) and PAF-AH activity (G) in controls (Healthy) and in patients with C1-INH-HAE in remission. Data are shown ad the median (horizontal black line), the 25th and 75th percentiles (boxes), and the 5th and 95th percentiles (whiskers) of 68 controls and 128 patients.

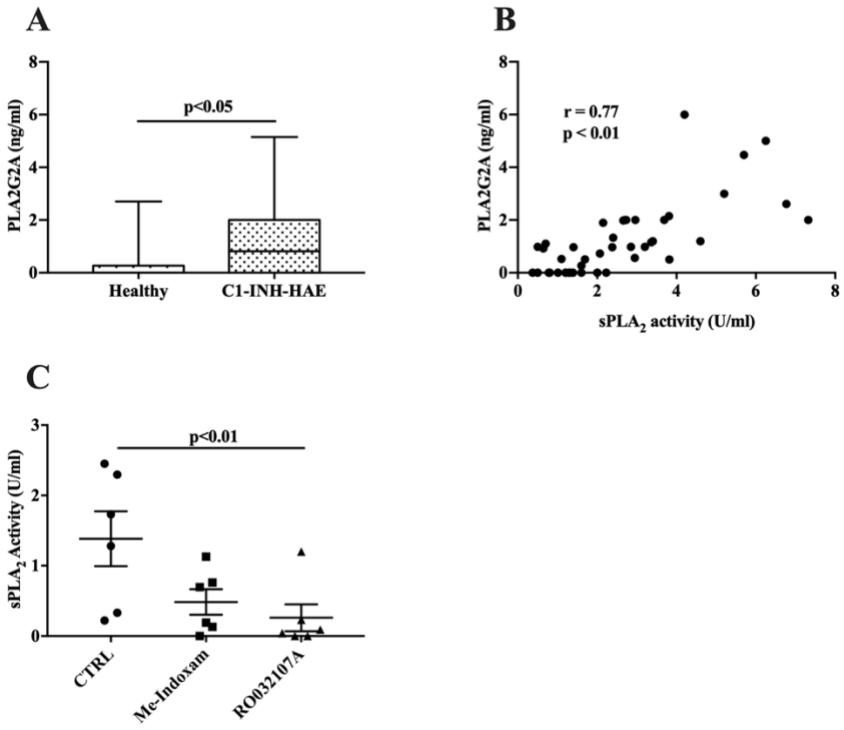

Figure 2. Plasma sPLA2 activity and PLA2G2A concentrations in C1-INH-HAE during remission and healthy controls.

(A) Data are shown ad the median (horizontal black line), the 25th and 75th percentiles (boxes), and the 5th and 95th percentiles (whiskers) of 36 controls and patients for PLA2G2A assessment. (B) Correlation between sPLA2 and PLA2G2A was assessed by Spearman's correlation analysis and reported as coefficient of correlation (*r*). (C) Plasma of patients with C1-INH-HAE was pre-incubated (20 min, 37°C) with Me-Indoxam (100nM) (black squares), RO032107A (100nM) (black triangles), or control medium (black circles). At the end of incubation, sPLA2 activity was evaluated.

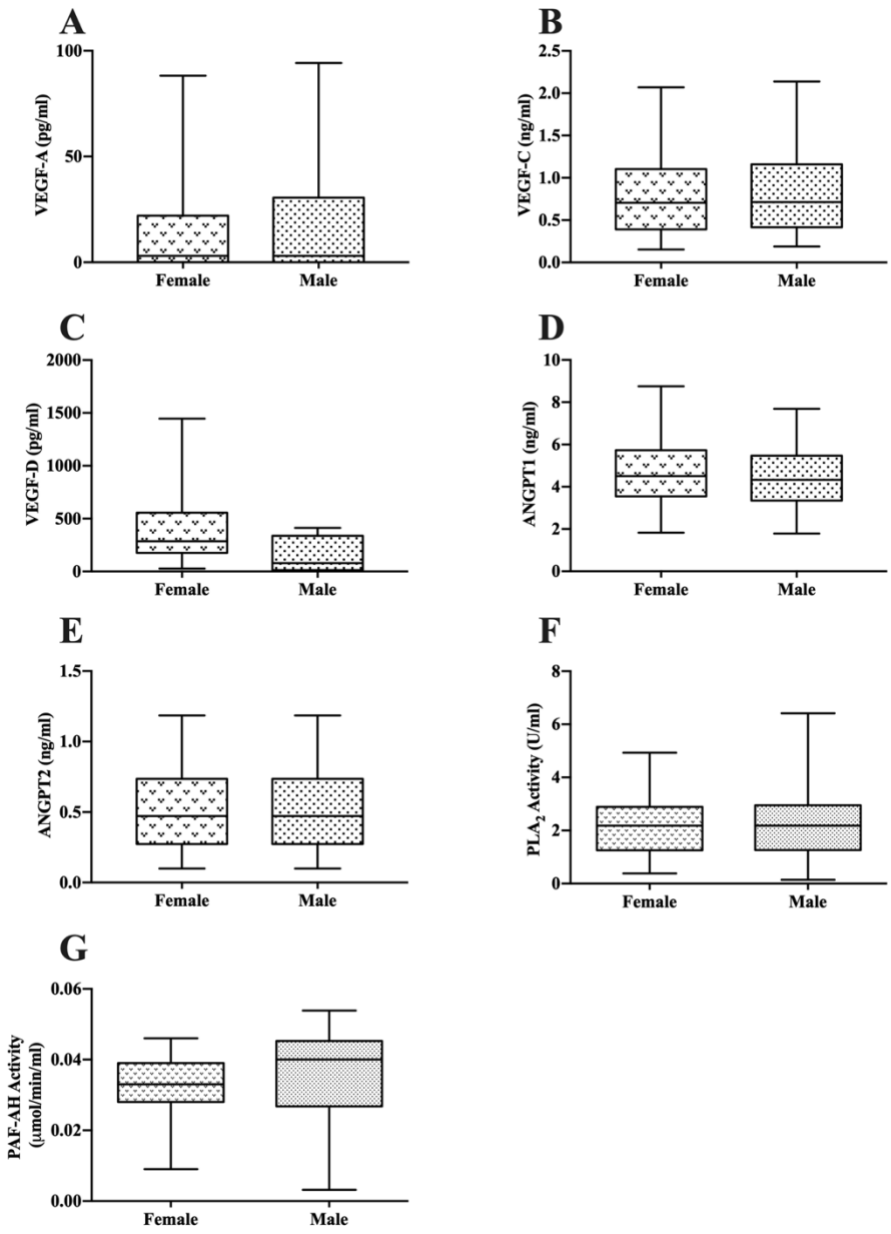

Figure 3. Relationship among plasma concentrations of VEGFs, ANGPTs and enzymatic activities of sPLA2 and PAF-AH and sex of C1-INH-HAE patients during remission.

Relationship between VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 (E), sPLA2 Activity (F) and PAF-AH Activity (G) and sex of 128 C1-INH-HAE patients in remission. Data are shown ad the median (horizontal black line), the 25th and 75th percentiles (boxes), and the 5th and 95th percentiles (whiskers) of female and male patients during remission.

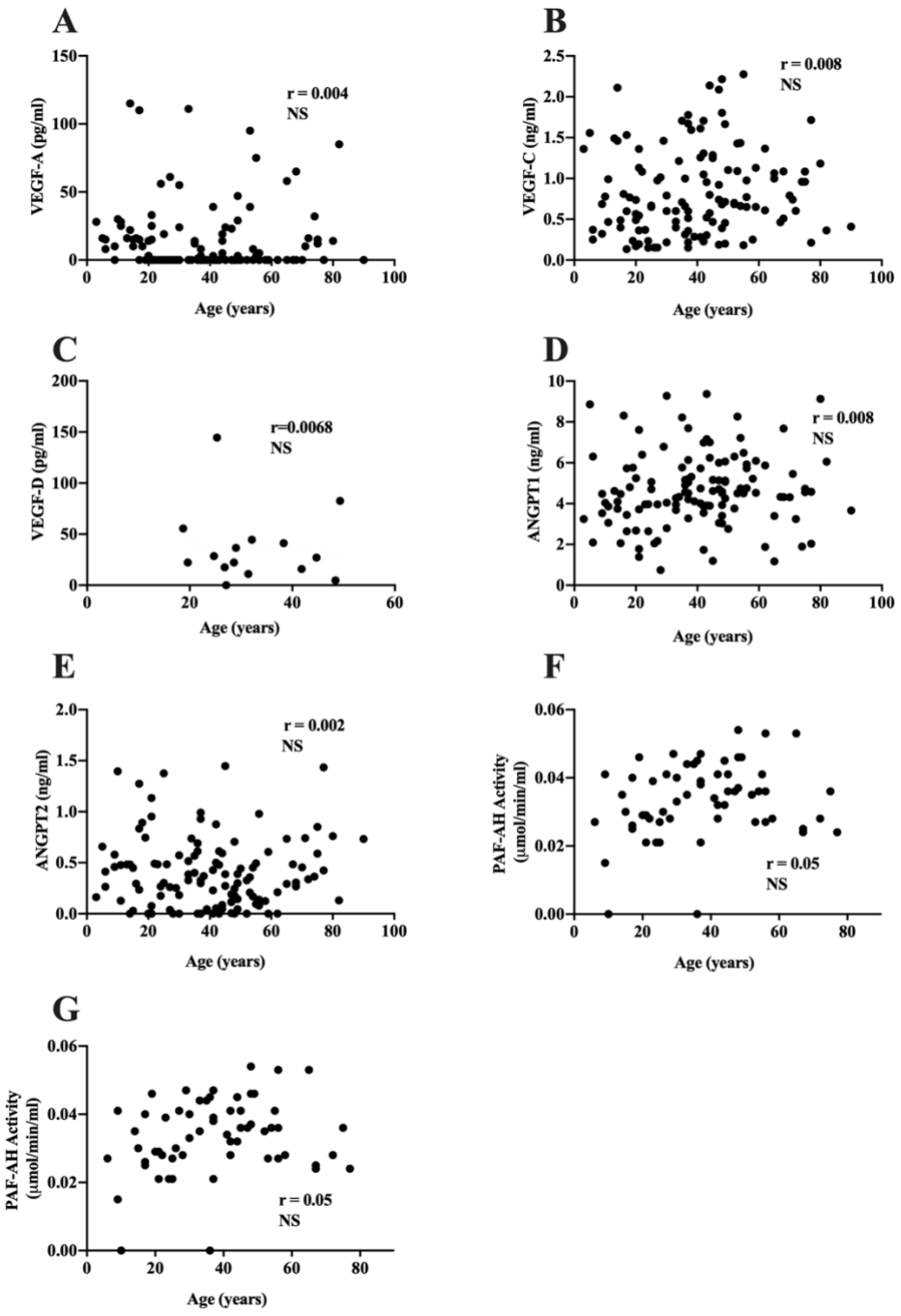

Figure 4. Correlation between VEGFs, ANGPTs, sPLA2 activity and PAH-AH activity and age of C1-INH-HAE patients during remission.

Correlation between plasma levels of VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 (E), sPLA2 activity (F) and PAF-AH activity (G) and age of 128 C1-INH-HAE patients during remission was assessed by Spearman's correlation analysis and reported as coefficient of correlation (*r*).

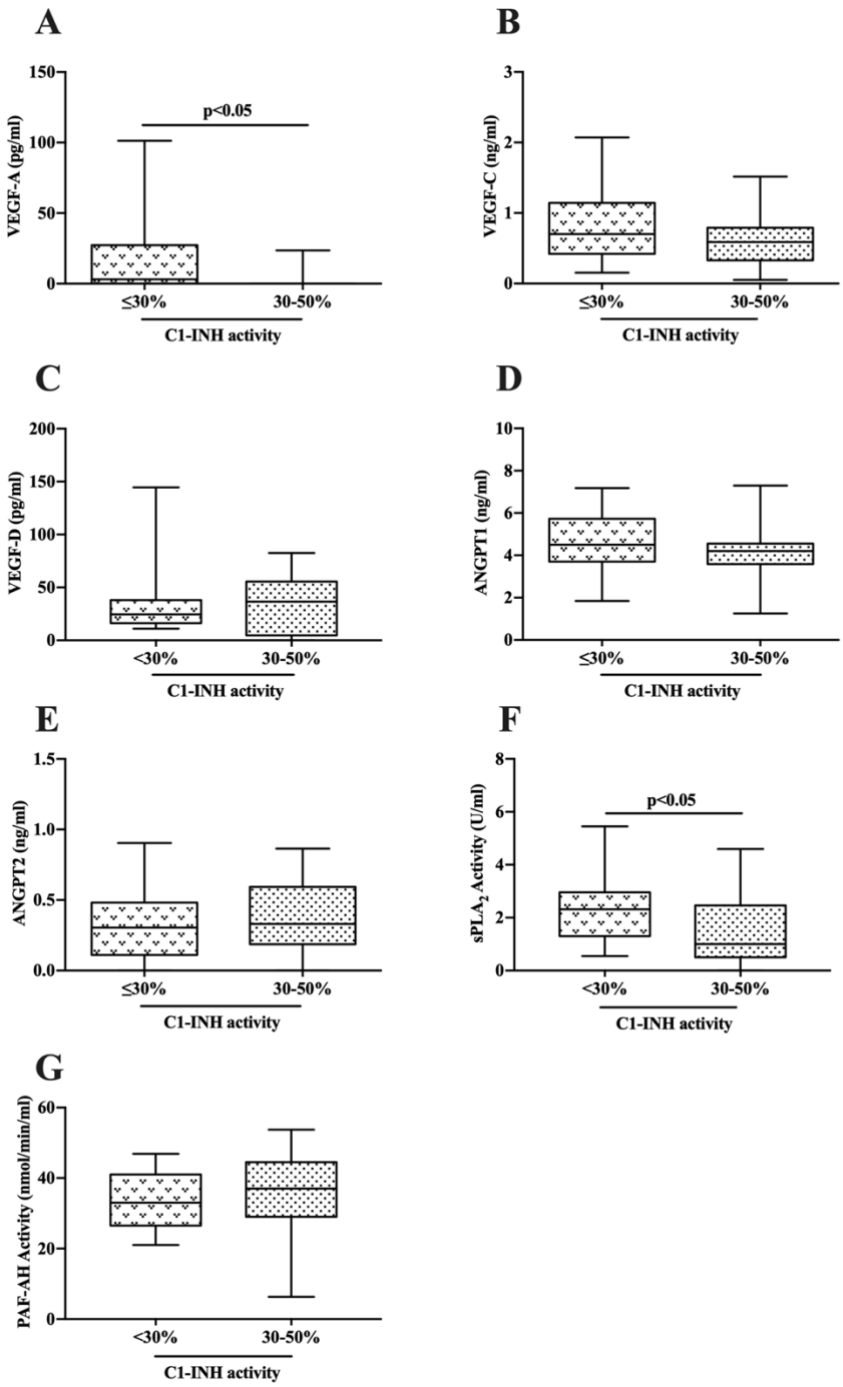

Figure 5. Relationship between VEGFs, ANGPTs, sPLA2, PAF-AH and functional activity of C1-INH in C1-INH-HAE patients.

Patients were divided in two groups: patients with <30% or 30-50% of normal C1-INH activity. Relationship between VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 concentrations (E), sPLA2 activity (F) and PAF-AH activity (G) and C1-INH activity.

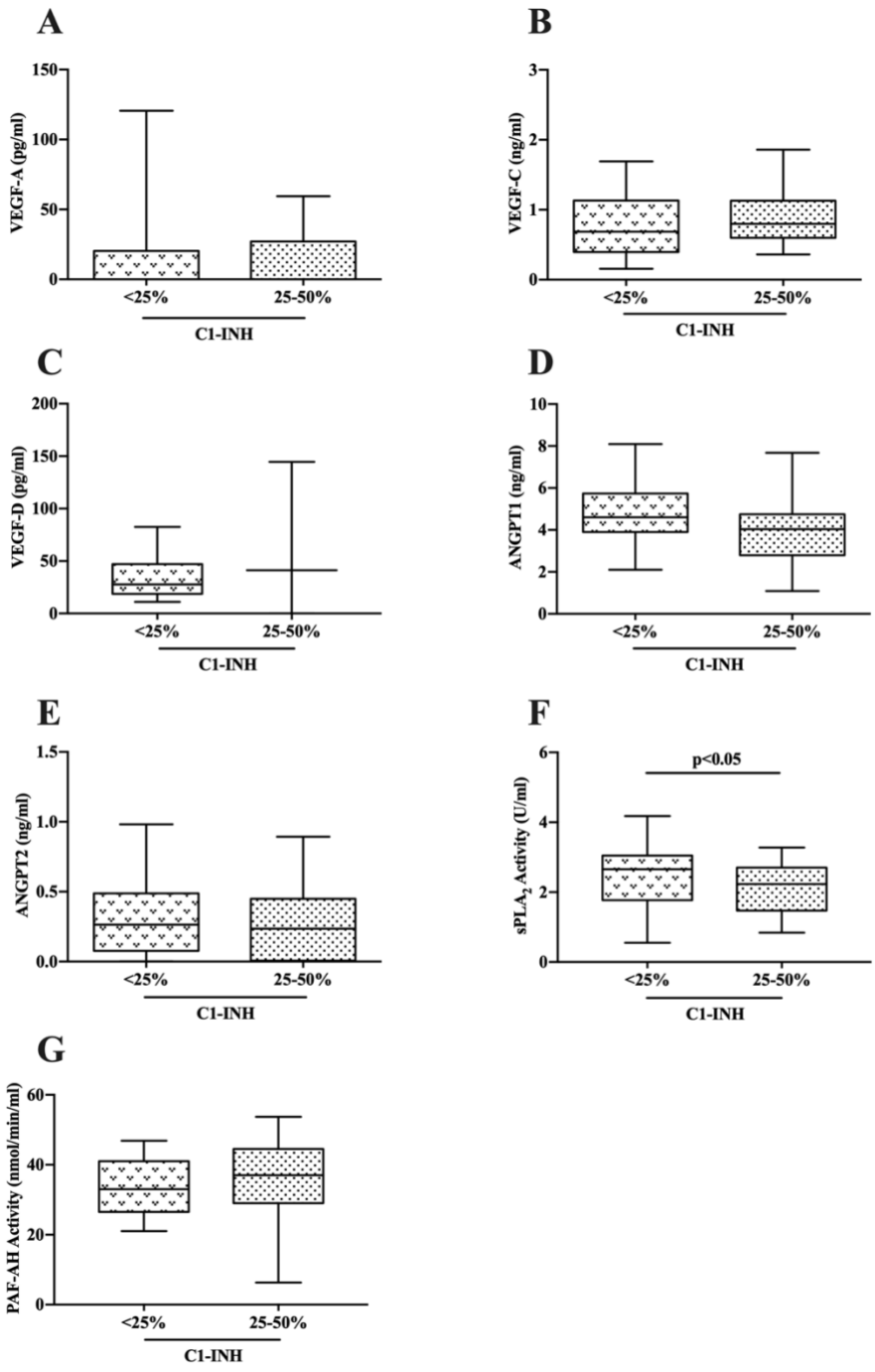

Figure 6. Relationship between VEGFs, ANGPTs, sPLA2, PAF-AH and antigenic C1-INH in C1-INH-HAE patients.

Patients were divided in two groups: C1-INH-HAE type I patients with less than 25 or 25-50% of normal C1-INH protein values. Relationship between VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 concentrations (E), sPLA2 activity (F) and PAF-AH activity (G) and antigenic C1-INH.

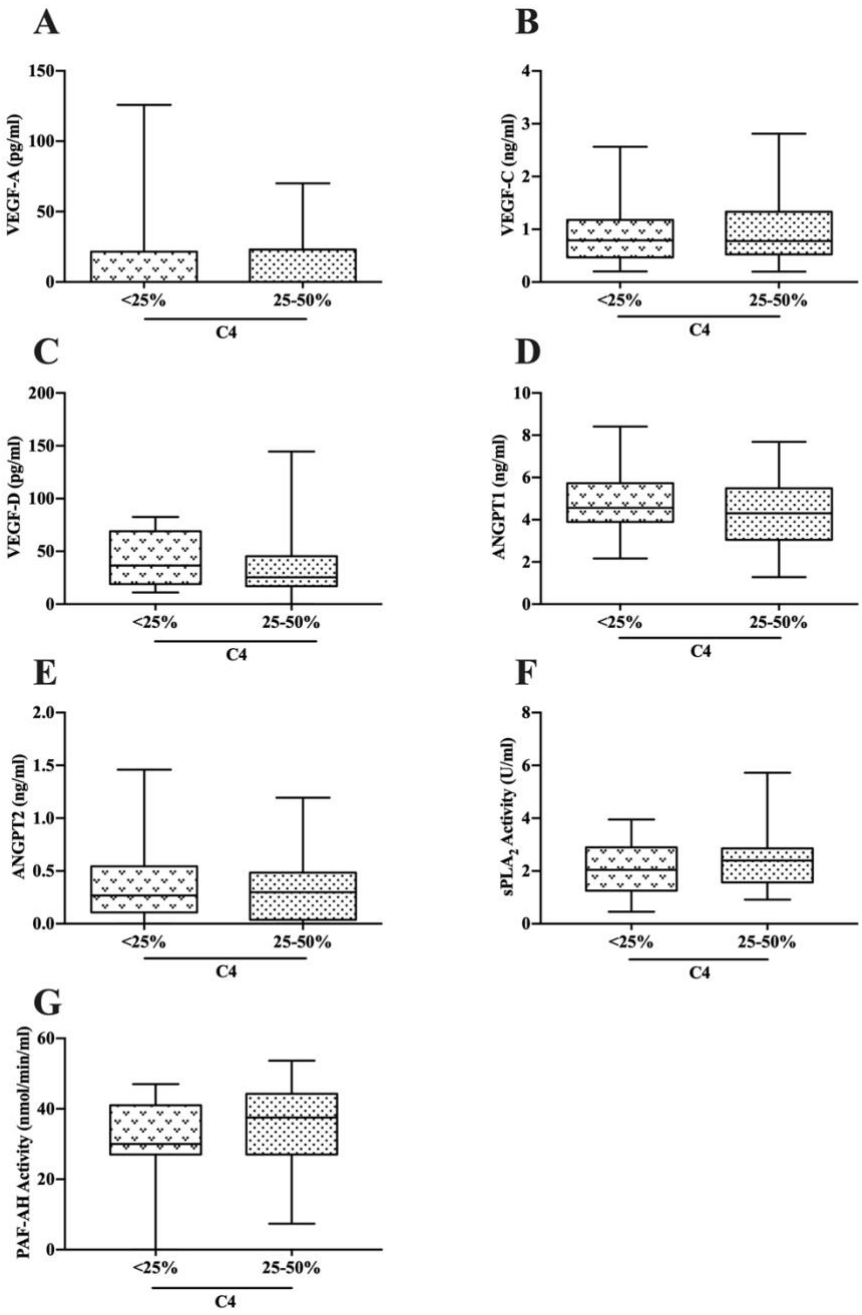

Figure 7. Relationship between VEGFs, ANGPTs, sPLA2, PAF-AH and C4 concentrations in C1-INH-HAE patients.

Patients were divided in two groups: C1-INH-HAE patients with less than 25 or 25-50% of normal C4 values. Relationship between VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 concentrations (E), sPLA2 activity (F) and PAF-AH activity (G) and C4 concentrations.

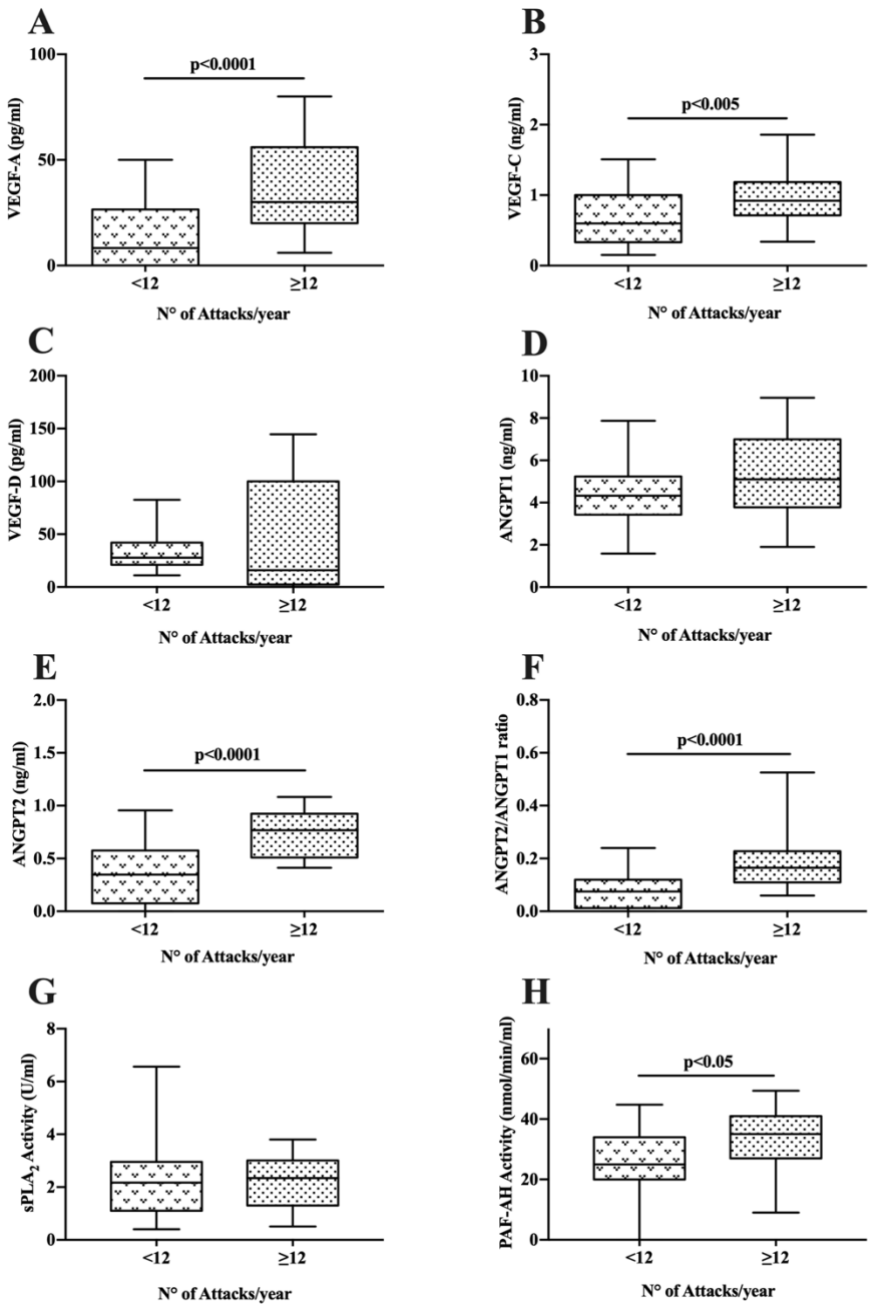

Figure 8. Relationship among plasma levels of VEGFs, ANGPTs and sPLA2 and PAF-AH activities and attack frequency.

VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 (E), ANGPT2/ANGPT1 Ratio (F), sPLA2 activity (G) and PAF-AH activity (H) were determined in 87 patients with low frequency (<12/year) and 41 patients with high frequency (>12/years) of attacks.

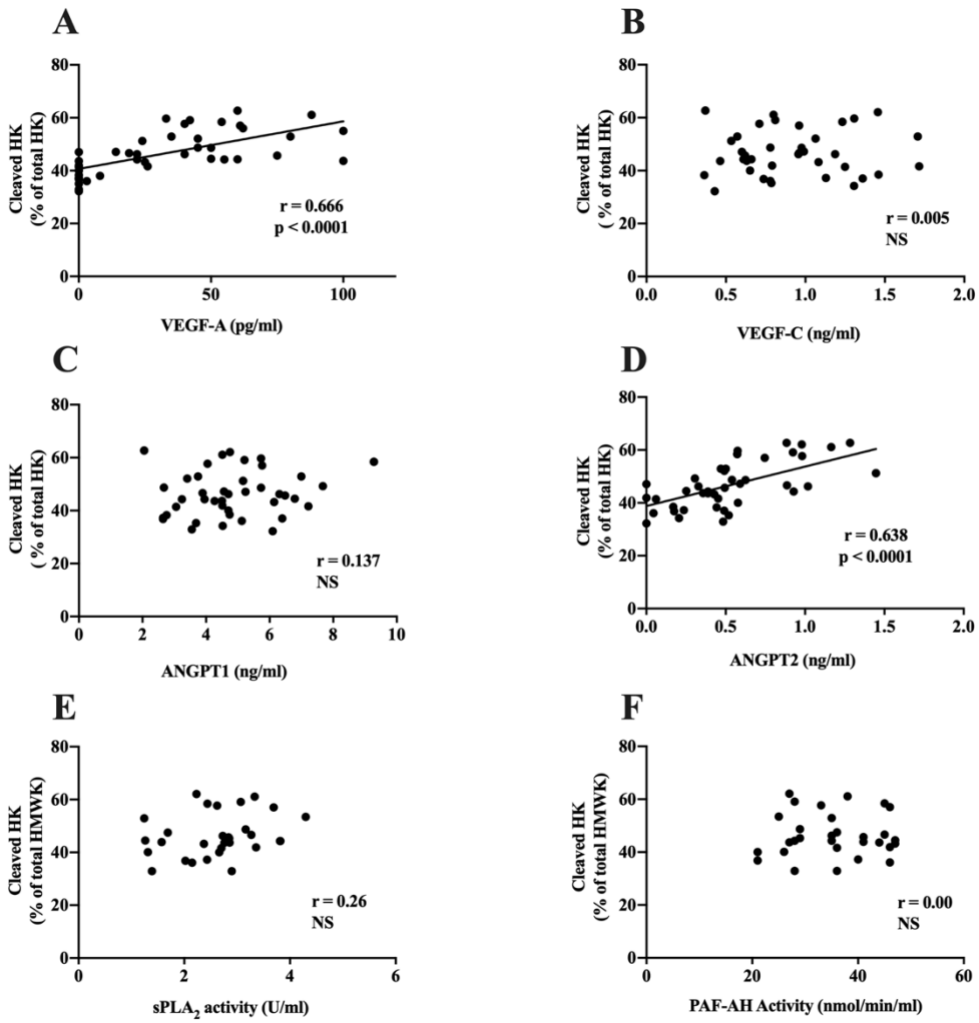

Figure 9. Relationship among plasma levels of VEGFs, ANGPTs and sPLA2 and PAF-AH activities and cleaved-HK.

Correlation between VEGF-A (A), VEGF-C (B), ANGPT1 (C), ANGPT2 (D), sPLA2 activity (E) and PAF-AH Activity (F) and cleaved-HK were determined 128 patients with C1-INH-HAE during remission and assessed by Spearman's correlation analysis and reported as coefficient of correlation (*r*).

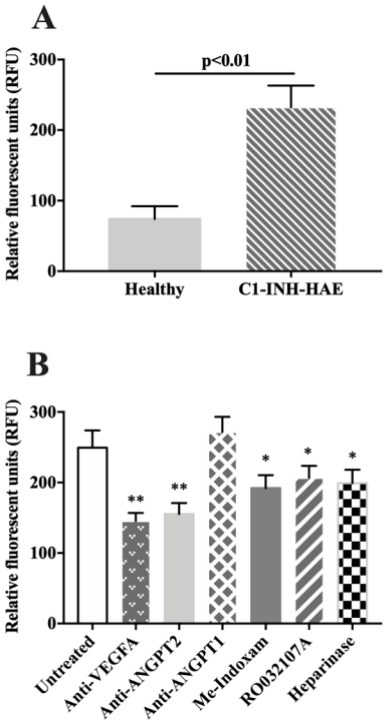

Figure 10. *In vitro* effects of plasma from healthy controls or patients with C1-INH-HAE on vascular permeability.

(A) Bovine aortic endothelial cells (BAEC) were incubated (18 h, 37°C) with plasma from healthy controls or from symptom-free patients with C1-INH-HAE. The *in vitro* vascular permeability was assessed as indicated in Section "Material and Methods". (B) Plasma of patients with C1-INH-HAE was incubated (20 min, 37°C) with anti-VEGF-A (1µg/ml), anti-ANGPT2 (1µg/ml), anti-ANGPT1 (1µg/ml), Me-Indoxam (100nM), RO032107A (100nM), or control medium. BAEC were then pre-incubated (30 min, 37°C) with heparinase (0.4. U/ml) or control medium and stimulated (18 h, 37°C) with plasma of C1-INH-HAE patients alone or with the combination of C1-INH-HAE plasma with inhibitors and then we evaluated vascular permeability. Data are shown in Relative Fluorescence Units (RFU).

**p*-value ≤ 0.05 and ** *p*-value ≤ 0.01 *vs* untreated plasma.

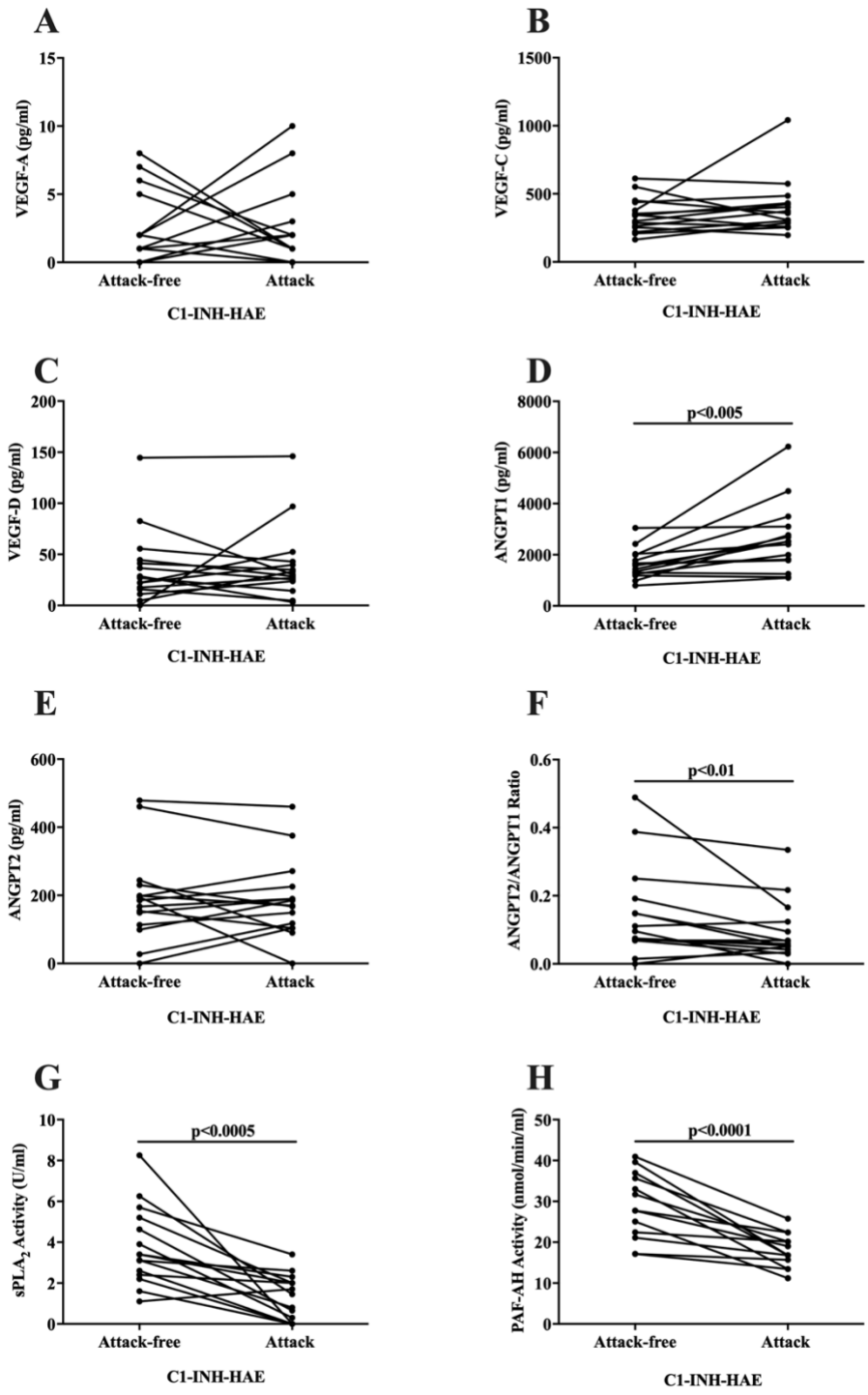

Figure 11. Plasma concentrations of VEGFs and ANGPTs in patients with C1-INH-HAE during remission and during the angioedema attack.

Plasma concentrations of VEGF-A (A), VEGF-C (B), VEGF-D (C), ANGPT1 (D), ANGPT2 (E), ANGPT2/ANGPT1 ratio (F), sPLA2 activity (G) and PAF-AH activity (H) in 15 patients with C1-INH-HAE during remission and angioedema attack. Data are shown as the median

(horizontal black line) of C1-INH-HAE patients o median (horizontal black line), the 25th an 75th percentiles (whiskers). A *p*-value ≤ 0.05 was considered statistically significant.

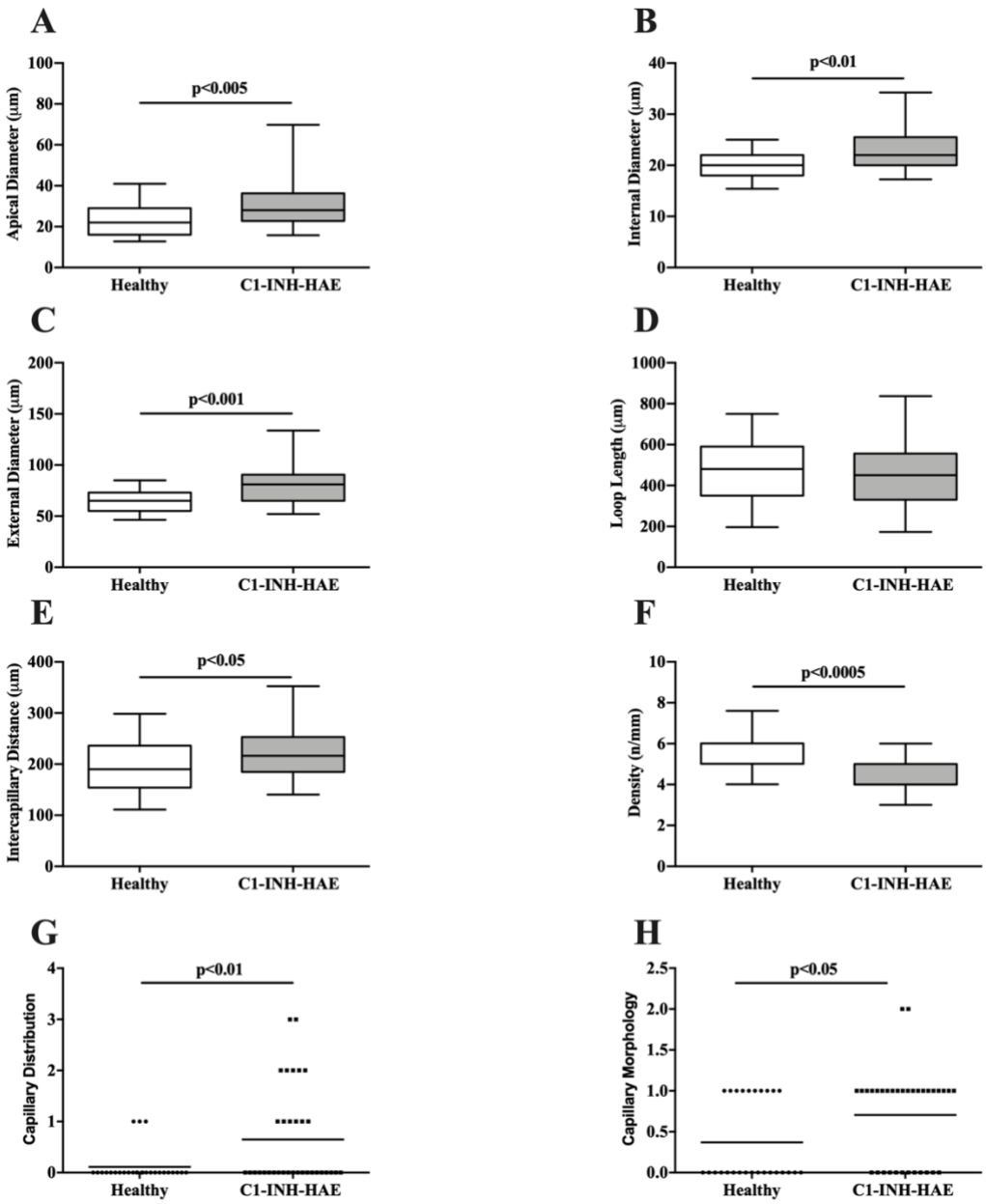

Figure 12. Capillaroscopic parameters in C1-INH-HAE patients and healthy controls. Apical (A), internal (B), external diameters (C), loop length (D), intercapillary distance (E), density (F), capillary distribution (G), and capillary morphology (H) were measured in 34 C1-INH-HAE patients in the symptom-free period and 28 healthy controls. Horizontal bars depict the median value (A-H), boxes the 25th and 75th percentiles, and whiskers the 5th and 95th percentiles (A-F).

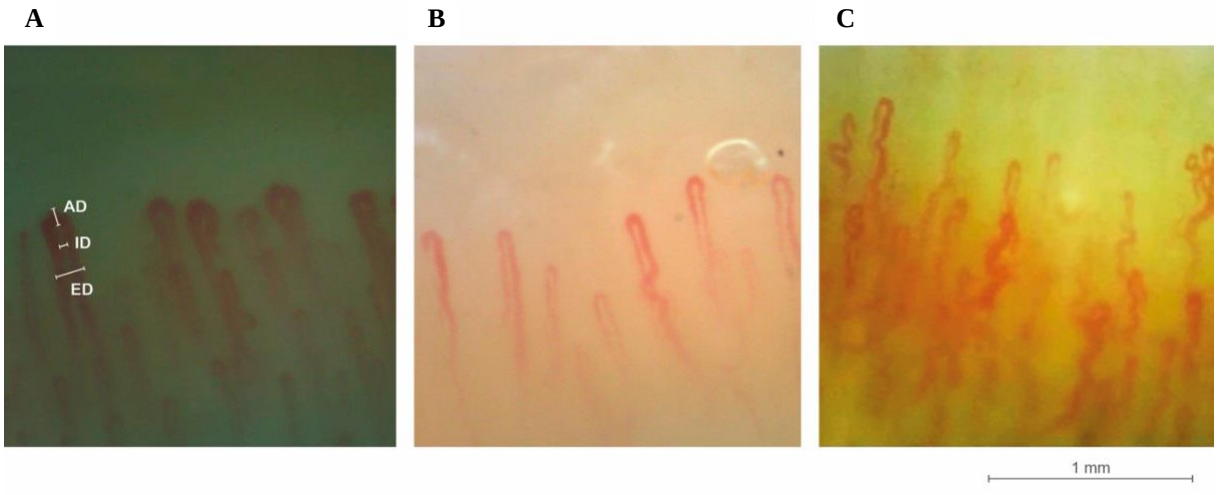

Figure 13. Nailfold Video-capillaroscopic Images in C1-INH-HAE patients.

Images from video-capillaroscopy (200x magnification) on recruited C1-INH-HAE patients showing apical (AD), internal (ID) and external (ED) diameters (A); capillary density (B); capillary distribution (C).

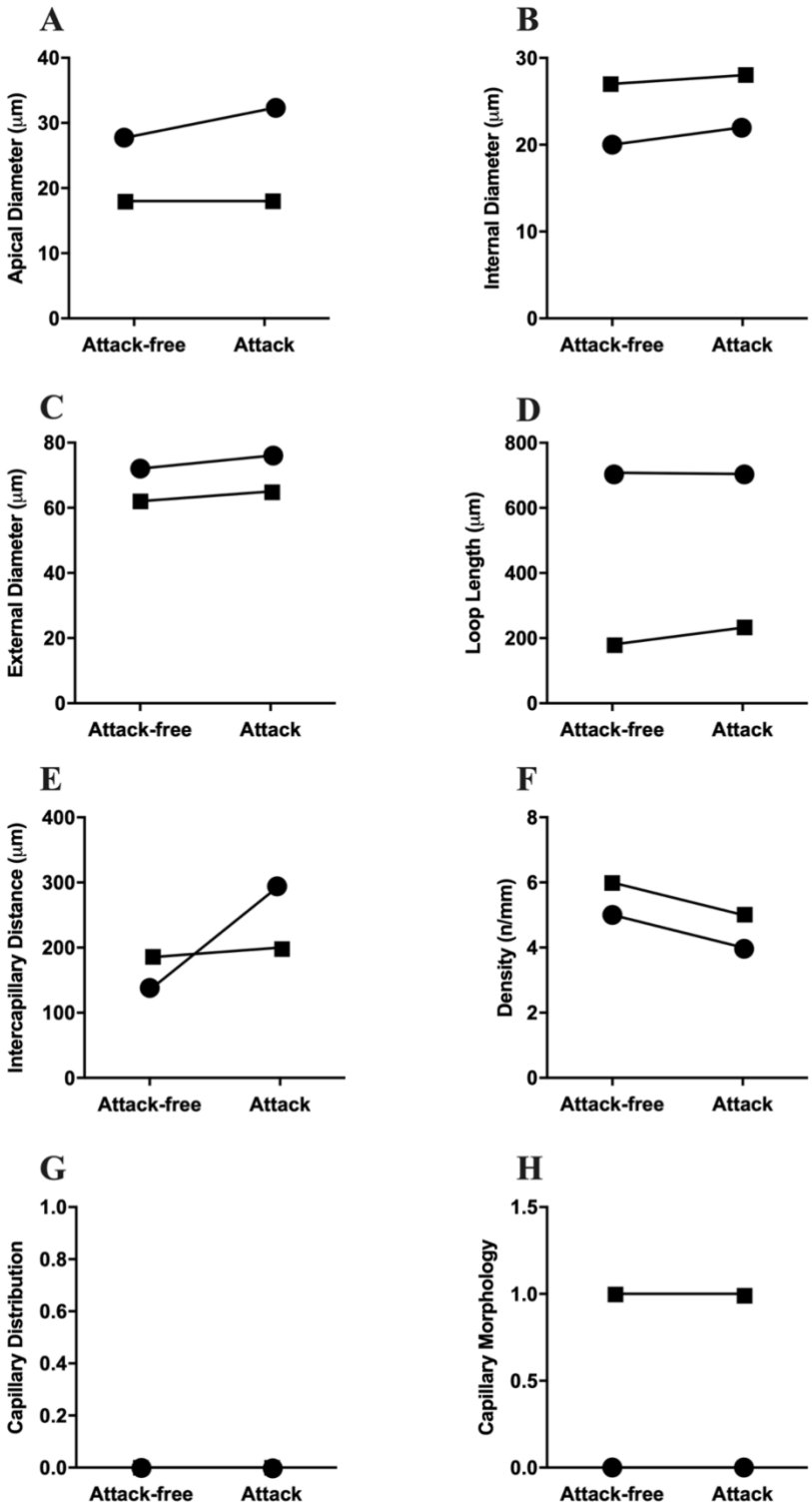

Figure 14. Capillaroscopic parameters in remission and attack periods in C1-INH-HAE patients.

Apical (A), internal (B), external diameters (C), loop length (D), intercapillary distance (E), density (F), capillary distribution (G), and capillary morphology (H) were determined in 2

patients with C1-INH-HAE in symptom-free period and during angioedema attack. Squares indicate data about the patient experiencing a gastrointestinal attack, while circles indicate data about the patient experiencing a hand attack.

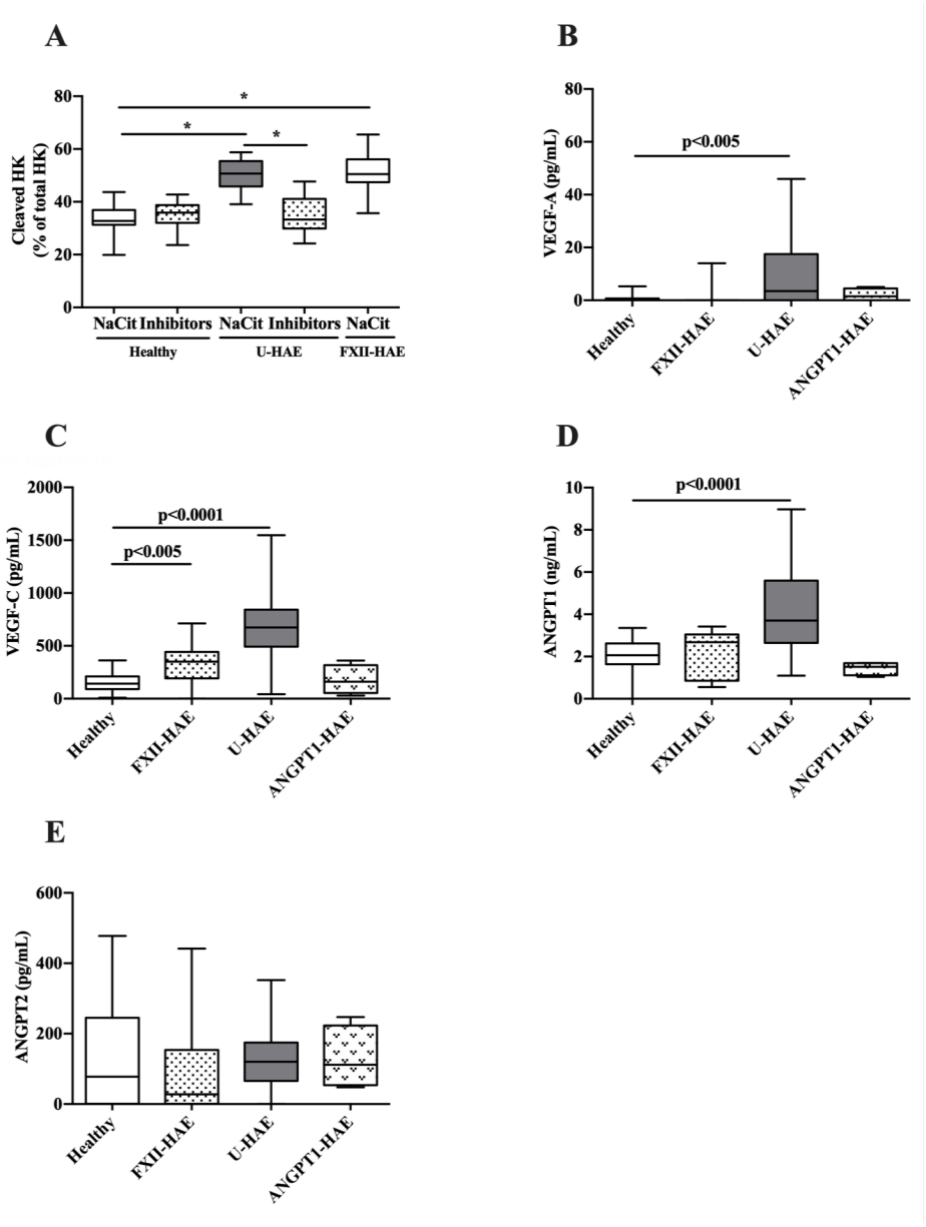

Figure 15. Levels of cleaved-HK, VEGFs and ANGPTs in FXII-HAE, ANGPT1-HAE and U-HAE.

(A) 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. Cleaved HK are shown as median (horizontal black line), the 25th and 75th percentiles (whiskers) of 72 healthy subjects (11 samples collected in NaCit and 61 with inhibitors), 19 FXII-HAE (samples collected in NaCit), 58 U-HAE (35 samples collected in NaCit and 23 with inhibitors. Plasma concentrations of VEGF-A (B), VEGF-C (C), ANGPT1 (D) and ANGPT2 (E) in healthy controls and 15 patients with FXII-HAE, 31 U-HAE and 4 ANGPT1-HAE in remission. *p<0.01

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