Università degli Studi di Milano

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PhD Thesis:

"Plasma levels of bradykinin and cleaved high molecular weight kininogen in patients with idiopathic angioedema during acute attack"

Tutor: Prof. Massimo Cugno

Coordinator: Prof. Massimo Del Fabbro

PhD candidate: Dr. ssa Federica Depetri matr.R12453

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1. Introduction

Angioedema is defined as a circumscribed non-pitting edema of the subcutaneous tissues involving lips, face, neck, extremities and/or submucosal tissues of oral cavity, larynx and gut. According to the involved site, angioedema may represent a life-threatening condition (e.g. larynx angioedema) or, when gut is affected, it can be very painful and mimick an acute abdomen Angioedema is caused by a localised increase of vascular permeability due to the accumulation of endogenous inflammatory compounds without a frank inflammatory process [1] Angioedema frequently tends to recur and may occur as part of urticaria, which is characterized by two symptoms: wheals (that is edema of superficial skin layers), and angioedema (that is edema of deep skin layers) (Figure 1) [2].

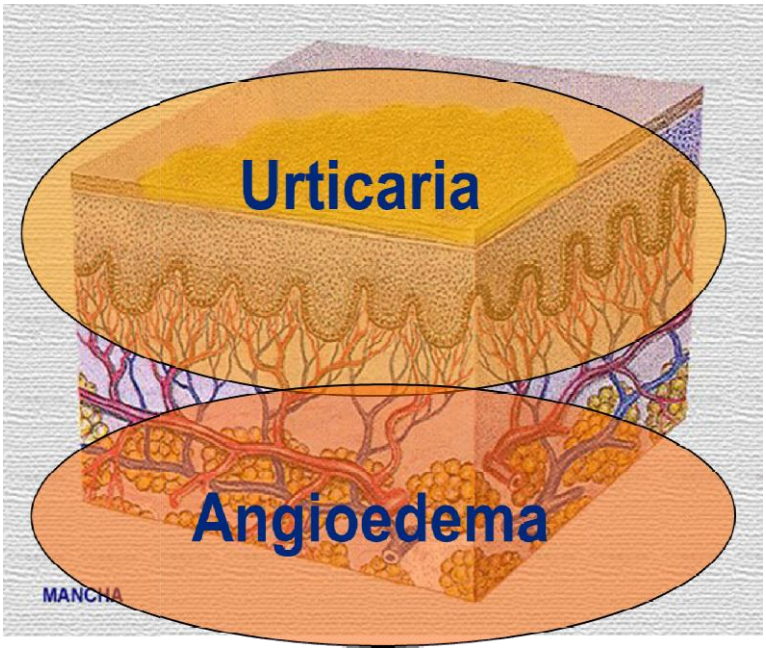

Figure 1. Skin involvement in urticaria (superficial increase of vascular permeability) and in angioedema (deep layer involvement).

Angioedema may be hereditary or acquired. Hereditary forms are due to genetic mutations of C1-INH or rarely of other factors, such as factor XII, plasminogen, angiopoietin-1 and kininogen 1. Acquired forms may be of allergic origin (histaminergic angioedema), generally associated with other manifestations of anaphylaxis (wheals), or non-allergic (nonhistaminergic angioedema). Other forms of acquired angioedema may be drug-induced (mainly by angiotensin converting enzyme

inhibitors and non steroidal anti-inflammatory drugs) or complement-mediated (due to an acquired deficiency of C1-inhibitor). However, sometimes a specific cause can not be defined and thus angioedema remains idiopathic. In clinical practice, the majority of angioedema cases are associated with allergic reactions and in such cases patients frequently have an history of recent exposure to a specific substance (i.e., food, drug, stings) [3]. In the various forms of angioedema, different mechanisms contribute to the pathogenesis of the increase of vascular permeability. Among a number of mediators, histamine and bradykinin are the best documented (Figure 2) [4].

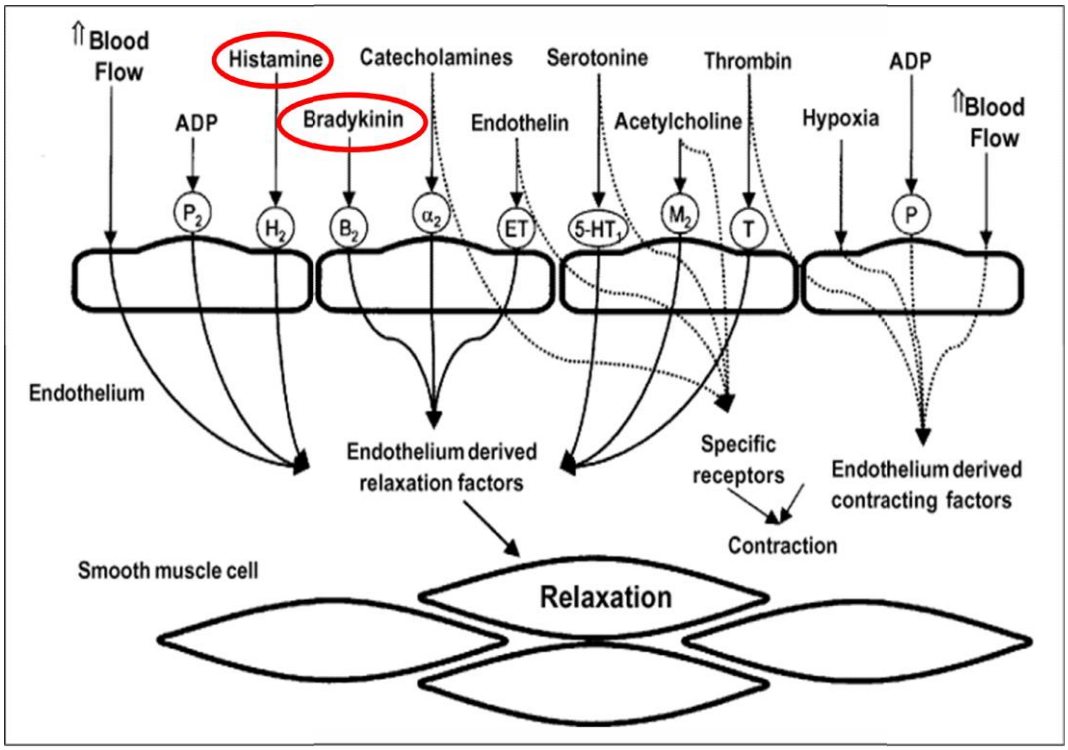

Figure 2. Vasoactive substances involved in increase of endothelial permeability.

On the basis of the underlying pathogenetic mechanism, angioedema can be classified into 3 major categories: 1) histaminergic angioedema, 2) bradykinin-mediated angioedema (e.g. hereditary angioedema [HAE], ACE inhibitor induced angioedema, and acquired C1 inhibitor deficiency angioedema) and 3) idiopathic forms, whose causes are still unknown (4,5) (Figure 3).
Distinguishing bradykinin-mediated angioedema from histamine-mediated forms is an important challenge faced by the clinician because it is critical for its treatment.

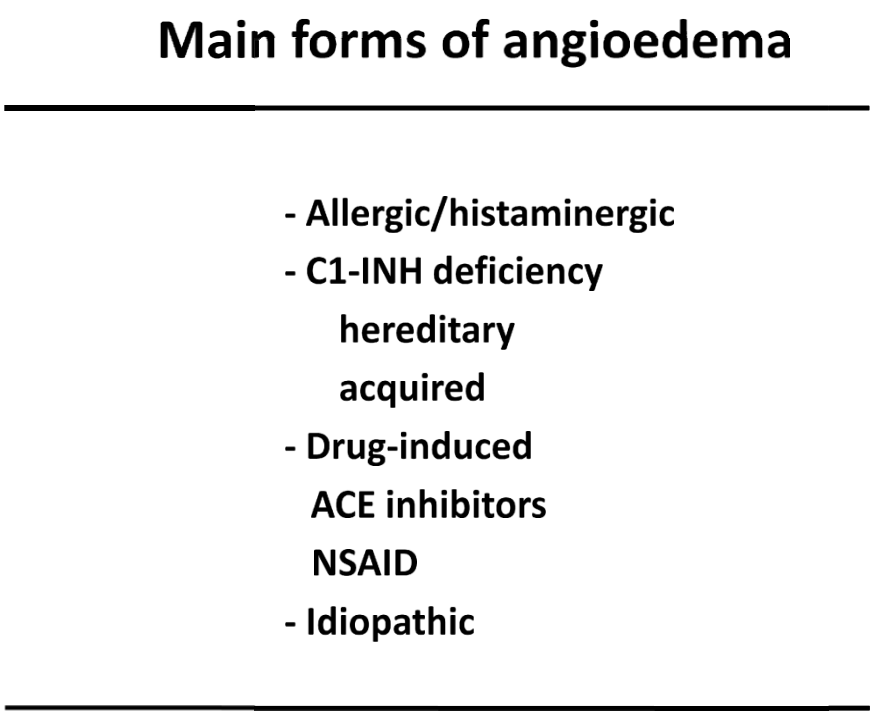

Figure 3. Main forms of angioedema obeserved in clinical practice.

2. Histamine-mediated angioedema

Histamine-mediated angioedema is the most common and is subdivided into acute and chronic forms according to the duration of symptoms (acute less than 6 weeks, chronic more than 6 weeks). Histaminergic angioedema is further classified as with or without urticaria. Histaminergic angioedema usually responds to antihistamines, corticosteroids, or epinephrine, whereas bradykinin-mediated angioedema requires medications targeting this peptide and/or its pathway [5].

Acute histaminergic angioedema

Allergic (Immunoglobulin E-mediated) angioedema

In this form of histaminergic angioedema, the activation of mast-cells occurs following the release of several mediators, including histamine, leukotrienes, heparin, platelet activating factor, and cytokines, and leads to various symptoms, such as flushing, generalized pruritus, and bronchospasm whereas abdominal pain and vomit may manifest when allergens are ingested. Patients typically experience angioedema and urticarial symptoms within 30 to 60 minutes after allergen exposure. Specific foods (eg, peanuts, tree nuts, shellfish, milk, egg and soy), insect venom, medications (eg, penicillins), and environmental allergens are common triggers producing immediate symptoms. Often, symptoms resolve within 24 hours and usually will recur after repeated exposure to the allergen or to cross-reacting allergens. Sometimes, the allergic reaction can progress to anaphylaxis which may result in death without appropriate treatment [6]. Allergic angioedema is caused by the production of antigen(Ag)-specific immunoglobulin E (IgE) molecules in predisposed individuals after an initial exposure to this antigen. After secretion by B-lymphocytes, most Ag-specific IgE bind to their high affinity FcεR1 receptors on the surface of mast cells and basophils, whereas remainder circulates in the serum. When the patient is reexposed to the Ag, bound IgE molecules recognize specific proteins on the Ag, binding them, and bringing the IgE molecules closer together on the cell surface, a term called “cross-linking.” Cross-linking activates intracellular tyrosine kinases and protein kinase C, increasing intracellular calcium. This cascade degranulates mast cells and basophils with subsequent release of histamine, tryptase, and chymase, which comprise the “early phase” of the reaction and begins transcription of specific cytokines (eg, interleukin [IL]-1, IL-2, IL-5, IL-6, IL-8, IL-9, IL-13), chemokines, and growth factors (eg, vascular endothelial growth factor) that initiate the “late-phase” reaction. Histamine binds to selective H1-receptors, inducing vasodilation and increasing blood flow and vascular permeability of the submucosal or subcutaneous capillaries, and/or postcapillary venules. The result is increased plasma extravasation to nearby tissues, producing angioedema [7,8].

2.1.1.2 . Acute nonallergic Mast-Cell–Mediated Angioedema

Acute urticaria and angioedema occur less frequently from direct mast cell activation or a non-IgE-mediated process. Triggers include some medications and infections, but more frequently, the cause remains unidentified. Some reactions can occur within 1 hour after exposure to the precipitant, for example, drugs (eg, opiates, vancomycin, nonsteroidal anti-inflammatory drugs) or radiocontrast dye [9]. Although mast cells are typically activated by cross-linking of their FcεRI and IgE-Ag complexes, there are alternative pathways. Anaphylatoxins C3a and C5a, the human antibacterial peptide B-defensin, and substance P can degranulate mast cells. In addition, murine studies have demonstrated that influenza A–specific IgG2a, IgG2b, and IgG1 induced mast cell degranulation. After mast cell activation, the subsequent inflammatory cascades are similar to IgE-triggered events. [10].

Chronic histaminergic angioedema

Histamine-mediated angioedema with chronic urticaria

Angioedema is present in up to 50% of patients with chronic urticaria. Chronic urticaria is defined "inducible" or physical urticaria when symptoms are secondary to specific triggers (ie, UV light, cold, exercise, pressure, water, vibration), and "spontaneous" when it develops without identifiable provocation [11].

Physical urticaria

Physical urticarias is a heterogeneous subgroup of inducible urticaria in which wheals are provoked by specific physical stimuli, such as mechanical pressure (ie, rubbing, vibration), thermal stimuli (ie, cold or heat), or sunlight. Angioedema is usually limited to the stimulus site. The severity of physical urticaria varies and in some cases it can be potentially life threatening (ie, airway swelling after a cold exposure in patients with cold induced urticaria) [12]. Dermographism is the most common form of physical urticaria, which presents with raised urticarial wheals on the site of the skin after scratching or stroking. Triggers of dermographism include itching dry skin, resulting in linear urticarial lesions, and rubbing of clothing, or direct pressure upon the skin (for example, sitting on a chair) [13]. The pathophysiology of physical urticaria/angioedema is not fully

understood. Mast cell involvement has been suggested by increased serum histamine in patients with dermographism or aquagenic urticaria [14].

Chronic spontaneous urticaria with angioedema

The clinical manifestation of chronic spontaneous urticaria is similar to that of chronic inducible urticaria, but in this case there is no identifiable trigger. A lowered mast cell activation threshold is central to the underlying pathophysiology of chronic spontaneous urticaria. In approximately one-third of patients, there are IgG autoantibodies targeted to either IgE or the α subunit of the high-affinity IgE receptor (FcεRIa), which then activates mast cells and basophils [14]. Recently also an autoallergic mechanism has been described with IgE directed against a number of autoallergens [15].

Histamine-mediated angioedema without urticaria

This condition is also defined as idiopathic histaminergic acquired angioedema and indicates patients with recurrent episodes of angioedema without urticaria, whose symptoms are controlled by antihistamines and in whom there is no identifiable cause such as allergy or physical stimuli. [16]. These patients typically present with angioedema developing rapidly, which reaches maximum swelling within 6 hours and resolves in 24 hours. The face is the most commonly affected site, but the disease may involve the extremities and the gastrointestinal mucosa [17].

Evaluation and diagnostic approach of patients with histaminergic angioedema

Initial evaluation of a patient with angiodema should starts with the assessment of the level of consciousness and vital signs (i.e. blood pressure, heart rate, oxygen saturation and peripheral perfusion) while skin examination may detect the presence of edema and/or urticaria. If there are signs of significant airway compromise, intubation should be considered. Most patients with acute angioedema have normal hemodynamic parameters, whereas several patients may be critically ill. Both histaminergic and non histaminergic angioedema can potentially cause hypovolemic shock due to the shift of fluids in various site of the body and/or acute respiratory failure [18]. After airway and hemodynamic stabilisation, a directed patient's history is essential to determine whether angioedema may be histamine or bradykinin mediated. A recent ingestion of food should be investigated with particular attention to those more commonly allergenic (eg, peanut, tree nuts, fish, shellfish, milk and egg). An IgE-mediated reaction typically occurs within one hour after ingestion. Also medication history should focus on recent assumption of antibiotics, NSAIDs, or ACE-I. The recurrence of urticaria with angioedema favors a histamine-mediated process. Moreover, asking

patients about the duration of symptoms and their response to treatment (antihistamines and/or steroids) is helpful to suggest a histamine-mediated disorder, whereas a lack of response suggests a bradykinin-driven process [2] (Figure 4)

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Histamine-mediated	Bradykinin-mediated
Recognizable triggers such as insect stings, food, medications	Not accompanied by urticaria
Onset of swelling is rapid and often accompanied by urticaria and itching	History of recurrent swelling or unexplained, recurrent abdominal pain
Can affect any area of the body, although the facial area, throat, and larynx are more common	Family history of angioedema
Progression to anaphylaxis is possible	Ongoing treatment with angiotensin-converting enzyme inhibitor
	Progression to anaphylaxis is possible

Cicardi M et al., J Investig Allergol Clin Immunol. 2016; 26(4):212-21.

Figure 4. Histamine-mediated and bradykinin-mediated angioedema.

Treatment of histaminergic angioedema

The treatment of acute histamine-mediated angioedema is driven by symptoms. For patients presenting with airway compromise and/or a systemic reaction (ie, hypotension, tachycardia, gastrointestinal symptoms), intramuscular epinephrine is required. [18]. Antihistamines are administered during an acute histamine-mediated episode of angioedema/urticaria of any severity. Glucocorticoids are often administered to prevent late-phase reactions that can occur 4 to 6 hours after the initial response. In cases in which patients have required epinephrine, they must be prescribed and taught how to use a self-injectable epinephrine before discharge. Patients should also be referred to an allergist upon discharge who can follow up for evaluation of IgE sensitizations and continued management [19].

3. Bradykinin-mediated angioedema

Generation and catabolism of bradykinin

Bradykinin and its related kinins are small peptides with several biologic effects, including vascular permeability. Kinins are generated from the cleavage of kininogen, a large plasma protein that contains bradykinin (Figure 5).

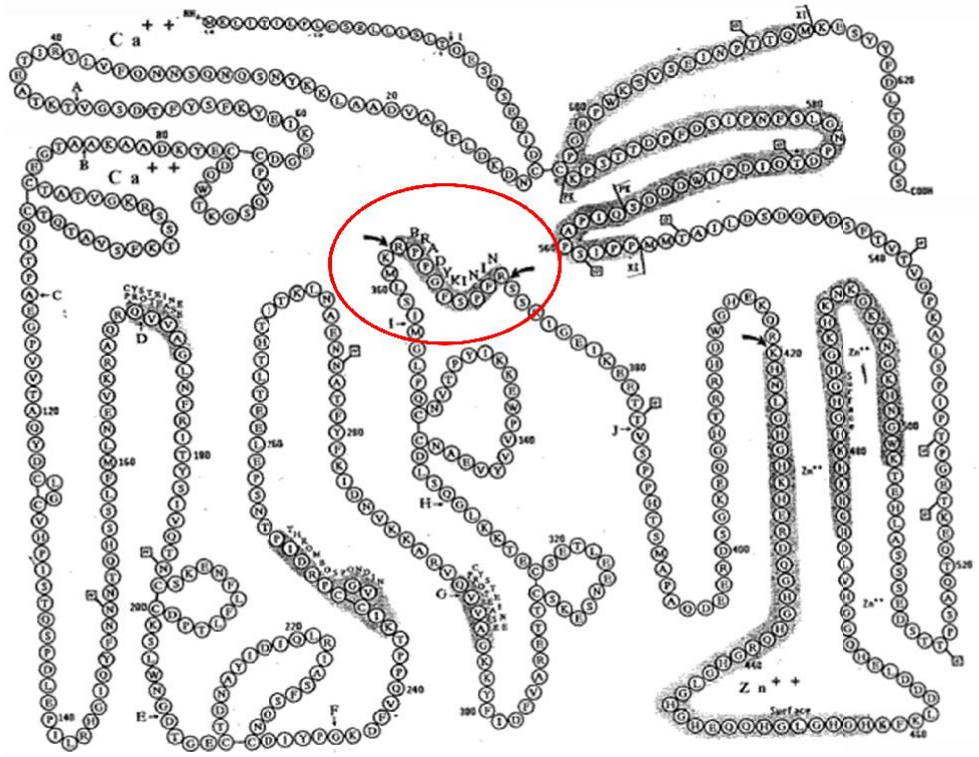

Figure 5. Schematic representation of the molecules of high molecular weight kininogen containing the nonapeptide bradykinin.

The protease releasing kinin from kininogen is called kallikrein. There are two distinct kinin-generating systems: the plasma contact system and the tissue kallikrein system [20] (Figure 6). The plasma contact system consists of 3 plasma proteins: coagulation factor XII (FXII), plasma prekallikrein, and high molecular weight kininogen (HMWK). When activated, FXII and prekallikrein reciprocally cleave each other to generate the active proteases FXIIa and plasma kallikrein. Plasma kallikrein then cleaves HMWK, releasing bradykinin. Plasma kallikrein and FXIIa are inhibited by C1-inhibitor, the protein that is deficient in type I and type II HAE [21]. The

tissue kallikrein system consists of a large family of tissue kallikreins, which are not closely related to plasma kallikrein. Most tissue kallikreins cannot generate kinins. Tissue kallikrein cleaves low-molecular-weight kininogen, releasing kallidin, also called Lys-bradykinin. Kallidin consists of ten amino acids, one more than bradykinin and can be transformed in bradykinin by the action of an aminopeptidase that cleaves lysine residue at its N-terminus. Tissue kallikrein is not regulated by C1-inhibitor [22], but it is inhibited by kallistatin (Figure 6). Bradykinin is catabolized by several peptidases (also called kininases), of which the most important is angiotensin-converting enzyme (ACE) [23].

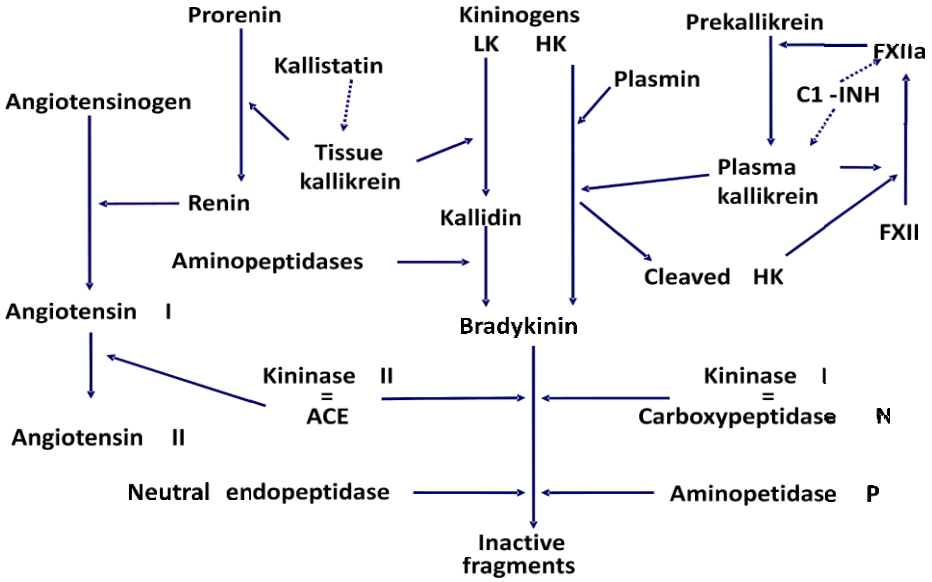

Figure 6. Simplified representation of the kinin system.

Bradykinin receptors and their effect on vascular permeability

Kinins carry out their biologic effects through two kinin receptors: bradykinin B1 receptor and bradykinin B2 receptor. Bradykinin and Lys-bradykinin bind to and activate the bradykinin B2 receptor, which is constitutively expressed on many cells including endothelial cells. On binding, the receptor is activated and transduces a signal cascade. Following activation, the receptor is desensitized, endocytosed and resensitized. The bradykinin B1 receptor is activated by desArg(10)-kallidin or desArg(9)-bradykinin, which are metabolites of kallidin and bradykinin, respectively. In contrast to B2 receptor, the bradykinin B1 receptor is not expressed in normal tissue but it is rapidly induced by tissue injury or after treatment with bacterial endotoxins, such as lipopolysacharide or

cytokines [23]. Angioedema is caused by a transient increase in vascular permeability that is largely determined by the pore size between endothelial cells, and is normally tightly controlled through the dynamic opening and closing of interendothelial junctions [24]. An increase in the pore size allows fluid to move more freely across the endothelial barrier, from the higher pressure vascular space into the lower pressure interstitial space. The major junctional component limiting permeability is the adherens junction, which maintains the permeability barrier through homotypic associations between transmembrane vascular-endothelial cadherin (VE-cadherin) molecules. Signaling cascades downstream from the kinin receptors phosphorylate VE-cadherin, resulting in internalization and degradation of VE-cadherin and contraction of the actin cytoskeleton. These changes increase the effective pore size and the permeability. Signaling from kinin receptors also stimulates the release of the vasodilator nitric oxide, contributing to endothelial dysfunction and enhanced vascular permeability [25] (Figure 7).

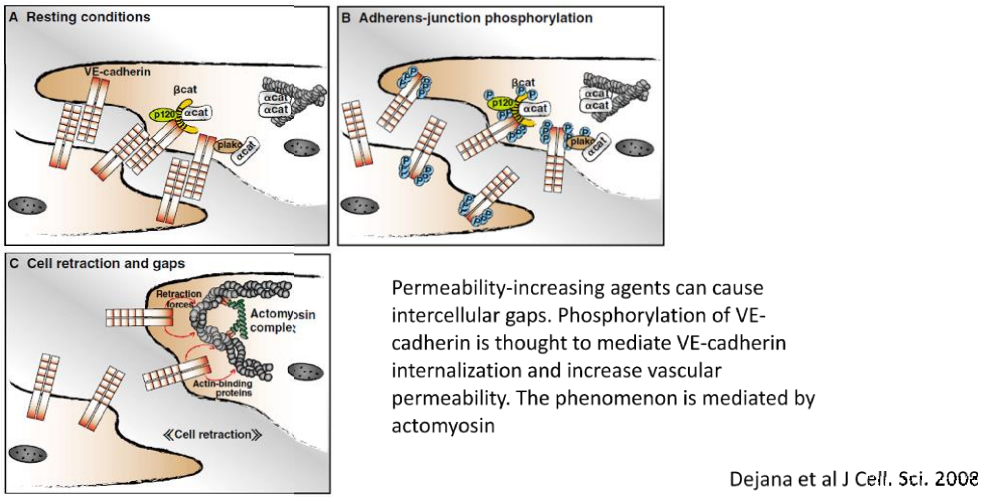

Figure 7. Functional modifications of endothelial junctions that increase vascular permeability.

Bradykinin dysregulation in angioedema

The nature of the bradykinin dysfunction that results in angioedema varies among the different angioedema types. C1-inhibitor blocks the plasma bradykinin-forming cascade binding the active site of kallikrein and inhibiting the generation of bradykinin from HK. Whenever C1-inhibitor is deficient, an increase in bradykinin generation occurs. Hereditary angioedema (HAE) may be due to a defect of the synthesis of C1-inhibitor (Type I HAE) or the the synthesis of a dysfunctional C1-inhibitor (Type II HAE). In the case of type I HAE, C1-inihibitor levels are low, whereas type II

HAE is associated with normal or near-normal C1-inihibitor protein levels but reduced C1-inihibitor function due to a dysfunctional protein. [26,27]. In patients with acquired C1-inhibitor deficiency, C1-inhibitor is secreted normally but it is catabolized at a rate faster than it can be synthesized. In most of these patients, the increased catabolism of C1-inhibitor appears to result from an autoantibody against C1-inhibitor, an underlying malignancy, or both [28]. C1-inhibitor regulates various serine proteases, including early proteases of the classical and lectin complement systems, contact system protease (plasma kallikrein and FXIIa), coagulation factor XIa, and plasminogen [29]. A defect of C1-inhibitor leads to excessive activation of the contact system and enhanced bradykinin generation because plasma kallikrein and FXIIa are not adequately regulated (see Figure 6). As previously described, angiotensin converting enzyme (ACE) is the primary peptidase responsible for inactivation of bradykinin. ACE inhibitors block the inactivation of bradykinin by ACE, resulting in a decrease of bradykinin catabolism [30].

Forms of bradykinin-mediated angioedema

Bradykinin-mediated angioedema comprises a group of conditions that includes hereditary C1-inhibitor deficiency angioedema, acquired C1-inhibitor deficiency angioedema, hereditary angioedema with normal C1-inhibitor levels and ACE inhibitor induced angioedema. Each type of bradykinin-mediated angioedema, can be distinguished from the others also according to C1-inhibitor, C4 and C1q plasma levels and to the presence or absence of autoantibodies to C1-inhibitor.

Hereditary C1-inhibitor deficiency angioedema

Among the hereditary types of angioedema, the best characterized form is due to C1-inhibitor deficiency, caused by mutations in one of the two alleles of the C1-inhibitor gene on chromosome 11 [3]. These mutations produce a defective C1-inhibitor resulting in two clinically similar phenotypic variants: quantitative deficiency (HAE type I, 85 % of cases) or functional deficiency (HAE type II, 15 % of the cases). In both cases, the disfunction of C1-inhibitor causes an uncontrolled activation of the complement system with uncontrolled activity of C1, consumption of C4 and C2, and generation of vasoactive mediators. Patients with HAE type 1 have low C4 levels, low C1-inhibitor functional and quantitative levels, and a normal C1q whereas patients with HAE type 2 have low C4 levels, low C1-inhibitor functional levels, and normal or high C1-inhibitor quantitative level, with a normal C1q level [3]. No clinical differences are associated with these variants. Edema usually lasts 2–5 days, with the most commonly involved areas being the skin, the gastrointestinal tract, and the upper respiratory airways. Sometimes, prodromes (subjective and objective signs and symptoms) can precede the attack by many hours and seem to be correlated to attacks in severity, location, and degree of dysfunction [44].

Hereditary angioedema with normal C1-inhibitor levels

In 2000, an unexpected form of HAE was described. This type of HAE was characterized by normal C1-inhibitor antigenic and functional levels [31,32]. Some years later, in a minority of these patients, a mutation in exon 9 of the Factor XII was found [33]. This variant appears to have a higher prevalence in the female gender and lacks of family history. Hormonal factors have been shown to play a role in this form (i.e., use of contraceptives, mainly estrogens, menses, pregnancy, ovulation), as well as common triggers as trauma or stress) [34]. Excluding patients with HAE with FXII mutations, there are 3 other forms of HAE with normal C1-inhibitor levels: the largest, in which genetic etiology remains unknown, and the 3 recently described genotypes involving angiopoietin-1 (ANGPT1), plasminogen (PLG) and kininogen 1 (KNG1) genes [35,36,37].

Acquired C1-inhibitor deficiency angioedema

Deficiency of C1-inhibitor can also be acquired. This diagnosis is made in the absence of a family history of angioedema and the absence of deficiency of C1-inhibitor. In this form, symptoms start after the fourth decade of life and patients often present low levels of C1q and circulating autoantibodies to C1-inhibitor [38,39]. Functional and usually antigenic C1-inhibitor and C4 levels are low. In contrast to C1-inhibitor HAE, in the acquired form, low C1q values and anti-C1-inhibitor antibodies are often detected [40]. Facial edema is the most frequent involved site, followed by peripheral/abdominal attacks and oral mucosa and/or glottis/pharyngeal attacks [41]. More than 50 % of C1-inhibitor acquired angioedema patients have or will develop a hematological disorder, including monoclonal gammopathy of uncertain significance or B cell malignancies, which seems to be directly implicated in the pathogenesis of angioedema, through the aggressive consumption of C1- nhibitor by the neoplastic lymphatic tissues and/or the formation of anti-C1-inhibitor neutralizing autoantibodies [42,43].

Acquired Angioedema Related to Angiotensin-Converting Enzyme (ACE) Inhibitors

Angioedema is recognized to be a rare complication of ACE inhibitor therapy in less than 0.5% of Caucasian patients but its prevalence appears to be 3-4.5 fold higher in black subjects. This type of angioedema has a higher prevalence in female patients and usually occurs over 65 years of age [45,46]. As decribed above, ACE inhibitors cause an inhibition of bradykinin catabolism by its main catabolic enzyme ACE. These patients present normal C1-inhibitor levels. Symptoms are

more likely to occur early close to the initiation of ACE inhibitor therapy, but they can develop up to several years from the beginning of therapy [47,48]. Symptoms can persist even after discontinuation of ACE inhibitor therapy, as demonstrated by the long-term follow-up of 111 patients, which showed that 46 % had angioedema recurrences even after ACE inhibitor withdrawal [49]. The oral cavity is the most common localization, including the tongue, uvula, pharynx, larynx, and neck [50]. Abdominal involvement can be present but is probably underdiagnosed [51].

Idiopathic nonhistaminergic angioedema

Patients affected by idiopathic nonhistaminergic angioedema tend to have recurrent attacks of angioedema not associated with urticaria or with previous history of allergy. In this form antihistamine do not reduce the severity of attacks nor prevent their recurrence. Facial involvement is present in most patients, while abdominal and upper airways' involvement have a lower rate of presentation. All patients with idiopathic nonhistaminergic angioedema present normal C1-inhibitor and C4 levels [52].

4. Treatment of bradykinin-mediated angioedema

A number of agents have been developed for the treatment of HAE. The therapeutic approach to C1-inhibitor HAE is based on the periodic nature of this disease, with strategies to revert or to prevent attacks by blocking release or activity of bradykinin. Acute attacks can be treated using C1-inhibitor replacement, inhibitors of plasma kallikrein, and blockade at the bradykinin B2 receptors. Prophylaxis is based on attenuated androgens and infusion of plasma-derived C1-inhibitor concentrates. The initial approach involves on-demand treatment, which must be replaced by long-term prophylaxis when it fails to significantly improve the patient's quality of life. Three C1-inhibitor concentrates (Berinert, Cinryze, and Ruconest), the bradykinin B2 receptor antagonist icatibant, and the kallikrein inhibitor ecallantide are licensed for treatment of acute attacks. When it became clear that C1-inhibitor deficiency was the genetic defect in HAE patients, replacement therapy with fresh frozen plasma was the first rational therapeutic approach [54] and this treatment is still used when alternatives are not available. In the early 1970s, preparations of C1- inhibitor partially purified from pooled human plasma became available. The main advantages of this option over fresh frozen plasma were: 1) to provide the deficient protein devoid of the enzymatic substrates that sustain angioedema; 2) to infuse much lower volumes in a shorter period of time; and

3) to guarantee higher safety levels in terms of viral transmission [55]. Treatment with C1-inhibitor concentrates replace the deficient or dysfunctional C1-inhibitor in patients with hereditary angioedema type I and II [56-57]. At present, two C1-inhibitor plasma-derived concentrates (Cynrize® ViroPharma [Europe only] and Berinert® CSL Behring) and one recombinant (Ruconest® Pharming Group NV [Europe only]) are available. Ecallantide (Kalbitor; Dyax Corp, Cambridge, Mass) is a genetically engineered inhibitor to plasma kallikrein, it blocks kallikrein activity which in turn inhibits the cleavage of high-molecular-weight kininogen to bradykinin [56-57]. Ecallantide has been approved to treat acute attacks of type I and II HAE in patients 16 years and older [59,60]. However, severe hypersensitivity reactions, including anaphylaxis, have been described [55]. Icatibant is a potent selective B2 receptor antagonist, it is the only agent that targets the action of bradykinin directly, and its effectiveness in patients with HAE has been demonstrated [53]. The safety and tolerability of icatibant are good, although transient local injection site reactions can occur whereas allergic reactions have not been reported [61].

In patients who experience frequent attacks and in whom on-demand therapy cannot adequately control the disease, regular therapy to prevent episodes of angioedema should be considered. Attenuated androgens and plasma derived C1-inhibitor have proven to be effective for long term prophilaxis. The use of antifibrinolytic agents for long term prophylaxis is supported by findings from randomized trials [62]. Cinryze is currently the only C1-inhibitor concentrate approved for long term prophilaxis [63]. Garadacimab (CSL Behring) is a fully human, immunoglobulin G4 monoclonal antibody targeting activated FXII, intended to prevent attacks in patients with C1-inhibitor-deficient hereditary angioedema. Garadacimab significantly reduced the number of monthly attacks versus placebo and was well tolerated in phase 2 trial [64]. Lanadelumab is a subcutaneously administered fully human monoclonal antibody that acts as a potent and specific inhibitor of active plasma kallikrein. This agent is approved in the United States and the European Union for the prevention of HAE attacks in patients 12 years of age or over, and is recommended as a first-line option for long term prophilaxis by the International/Canadian Hereditary Angioedema Guideline and the US HAE Association Medical Advisory Board 2020 guidelines for the management of hereditary angioedema. This agent demonstrated sustained efficacy and acceptable tolerability with long term use in HAE patients [65]. Acquired C1-inhibitor deficiency angioedema patients may benefit by treatment of the associated disease, by chemotherapy and other cytoreducing approaches (radiotherapy, surgery, antibodies to specific cell populations) or by acting directly on the angioedema symptoms by preventing their recurrence or reversing them on appearance [66]. Control of angioedema recurrences and treatment of acute attacks is performed using attenuated androgens, antifibrinolytic agents and C1-inhibitor concentrate, as in HAE.

However, patients with the acquired defect respond differently to these treatments; antifibrinolytic agents tend to be more effective than androgens in prophylaxis and some patients might become refractory to C1-inhibitor concentrates [67]. Ecallantide has also been used successfully to treat angioedema attacks in two acquired angioedema patients refractory to C1-INH concentrate [68]. No medication is currently approved for ACE inhibitor induced angioedema, but in a small retrospective analysis in patients with ACE inhibitor angioedema treated with either icatibant (n = 8) or standard care (steroids and antihistamine), time to complete symptom relief was 4 h and 33 hours, respectively [69]. However, these data have not been confirmed in two subsequent studies [70,71]. Idiopathic nonhistaminergic angioedema continues to be a difficult condition to manage. In 2014, Cicardi et al and the Hereditary Angioedema International Work Group published a summary article discussing the various forms of angioedema and available treatment options. The report noted the limited existing literature discussing idiopathic non-histaminergic angioedema and the treatment summary stated that there is no conclusive evidence for the effective treatment of attacks [16]. Zingale et al proposed a diagnostic flowsheet for angioedema without urticaria and recommended to start antihistamines first and then, if symptoms are unresolved, to initiate tranexamic acid therapy [72]. Tranexamic acid is an antifibrinolytic agent that displaces plasminogen from fibrin, resulting in inhibition of fibrinolysis. Its mechanism of action includes inhibition of the proteolytic activity of plasmin and decreased activation of complement and consumption of C1 esterase inhibitor, which might explain its effectiveness in the treatment of angioedema. Tranexamic acid is relatively inexpensive and it is generally well tolerated. However, as the underlying cause of idiopathic angioedema is unknown, a pharmacologic mechanism for treatment cannot be predicted [73].

5. Aim of the study

The pathogenesis of idiopathic angioedema is still unknown as it is not related to allergies, not associated with drugs and it does not have the characteristics of C1-inhibitor deficiency angioedema. Idiopathic angioedema is a relevant problem because it does not respond to common antihistamines and corticosteroid therapies. The study of bradykinin in these patients and in those with chronic urticaria and angioedema may open new diagnostic and therapeutic perspectives, but until now, the evaluation of bradykinin *in vivo* has always presented huge methodological problems for which reliable data are not available [74]. The main problems are related to the low plasma concentration of bradykinin (fmol/ml), its short half-life (15-20 seconds) and the easy generation and degradation during sample collection and handling [74,75]. To solve these problems, our group has developed a rigorous method that allows to completely block the generation and degradation of bradykinin *in vitro* and therefore to have a photograph of what is happening *in vivo* at the time of sampling. With this method we were able to demonstrate the involvement of bradykinin in C1-inhibitor-deficiency angioedema and ACE inhibitor related angioedema [75,76]. To date, the meticulous blood sampling procedure for bradykinin assessment had been possible only in the context of our specialised laboratory. In the present study, instead, a specifically trained researcher was able to meticolously collect biological samples operating in the emergency department. Thus, the aim of our study was the evaluation of the mediator of angioedema (bradykinin) and of the marker of its generation (cleaved high molecular weight kininogen) during maximum disease activity, i.e. at the exact moment the patient afferes to the emergency department.

6. Patients and methods

Patients

We studied 9 patients with idiopathic angioedema (4 men and 5 women; age range, 34 to 85 years), during acute attacks. All patients presented to the Emergency Department of Ospedale Maggiore of Crema for angioedema reporting also previous episodes without known causes or family history of angioedema. All patients gave their written consent on the use of their blood samples in an anonimous form for research purpouse. The study was approved by Ethics Committee Valpadana of ASST Ospedale Maggiore Crema (No. 104, 22 march 2019) and was carried out in conformity with the 2013 revision of the Declaration of Helsinki and the code of Good Clinical Practice.

Blood was drawn by venous sampling with minimal stasis and collected in silicone-coated Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing 0.13 mol/L of trisodium citrate. For the evaluation of high molecular weight kininogen, tubes containing an inhibitor cocktail (100 mM benzamidine, 400 g/ml hexadimethrine bromide, 2 mg/ml soybean trypsin inhibitor, 263 M leupeptin and 20 mM aminoethylbenzenesulfonylfluoride) dissolved in acid-citrate-dextrose (100 mM trisodium citrate, 67 mM citric acid, and 2% dextrose, pH 4.5) were used to prevent in vitro activation of the contact system [77,78]. The tubes were centrifuged at 2,000g for 10 minutes at room temperature and the plasma aliquots were stored in polystyrene tubes at -80 °C until the tests were performed. For bradykinin measurement, venous blood (5 mL) was collected in pre-cooled syringes containing various protease and peptidase inhibitors to obtain final concentrations of 21 μmol/L aprotinin, 73 μg/mL egg trypsin inhibitor chicken albumin, 305 μg/mL hexadimethrin bromide, 4.5 mmol/L 1.10-phenanthroline and 4.5 mmol/L edetic acid. Blood samples were quickly transferred into pre-cooled polypropylene tubes and centrifuged at 2 °C and plasma aliquots were stored in polystyrene tubes at -80 °C until the tests were performed.

Methods

Cleaved high molecular weight kininogen was evaluated in plasma by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis, using a method previously validated by our group [79]. After electrophoretic transfer of the proteins from the gel onto a polyvinylidenedifluoride membrane (Immobilon; Millipore Corp, Milford, MA), the high molecular weight kininogen was identified with goat-specific polyclonal antibody (Nordic, Tilburg, The Netherlands) and visualized with a biotinylated rabbit anti-goat antibody (Sigma Chemical Co, St Louis, MO). The apparent molecular weights of the proteins were estimated against high molecular weight protein markers from Bio-Rad Laboratories, Hercules, CA. With this method, the native HK appears as a band with Mr 130,000 and the cleaved one is represented by two bands with Mr 107,000 and 98,000. The density of the bands was evaluated by computerized image analysis (Image Master; Pharmacia, Uppsala, Sweden). The amount of cleaved kininogen (sum of the bands with Mr 107,000 and 98,000) was expressed as a percentage of the total amount of kininogen (sum of the three bands).

Bradykinin levels were evaluated in plasma with an enzyme immunoassay method which involves adsorption on microplates of an anti-bradykinin antibody and evaluation of the competition between the bradykinin contained in the samples and a fixed amount of biotinylated bradykinin which is then highlighted with streptavidin (Human Bradykinin EIA, RayBiotech, Inc. Peachtree Corners, GA, USA).

C1-inhibitor and C1q antigens were measured by mean of radial immunodiffusion (RID, NOR-Partigen, Siemens Healthcare Diagnostics, Munich, Germany).

C1-inhibitor function was assessed as the capacity of plasma to inhibit the esterase activity of exogenous C1s measured on a specific chromogenic substrate by means of a commercially available kit (Technoclone GmbH, Vienna, Austria).

7. Statistical analysis

Because of non-normal distribution results were reported as medians and ranges (minimum to maximum), and nonparametric methods were used to assess statistical differences between groups. The significance level was set at $p=0.05$. The Spearman correlation coefficient was calculated to assess relationships between variables. The data were analyzed using the SPSS PC statistical package, version 27 (IBM SPSS Inc., Chicago, IL, USA).

8. Results

Demographic, clinical and laboratory characteristics of patients are reported in Table 1.

All patients presented to the emergency department because of acute angioedema involving the face in 5 patients, lips in 3 patients and both abdomen and legs in one patient. Urticaria was present in two patients. An intercurrent infectious event was evident in 3 patients (acute pharyngitis, acute cystitis and acute sinusitis). Three patients suffered from a chronic disease (Crohn's disease, hypercholesterolemia and chronic kidney disease). Concerning the causes of angioedema, no patient had known allergy or C1-inhibitor deficiency and no patient was on ACE inhibitor therapy or non-steroidal anti-inflamatory drugs. Moreover, all patients had experienced previous episodes of angioedema whereas no patient had familiary history of the disease.

Table 1. Demographic, clinical and laboratory data of patients with acute idiopathic angioedema.

Patient	Age (years)	Sex	Angioedema localization	Urticaria	Associated diseases	C1-INH Activity (%)	C1-INH Antigen (%)	C1q (%)
1 (C.L.)	60	M	face	no	none	91	111	103
2 (C.R.)	57	M	lips	no	chronic kidney disease	115	127	103
3 (D.D.)	57	F	face, tongue	no	none	123	142	97
4 (G.A.)	36	F	abdominal pain, feet	yes	acute cystitis	135	127	98
5 (L.V.)	32	F	face	no	depressive syndrome, acute sinusitis	139	121	98
6 (P.A.)	40	M	face	no	Crohn's disease	93	127	97
7 (R.GP)	59	M	lips	no	acute pharyngitis	86	111	108
8 (S.V.)	57	F	face	yes	none	103	142	114
9 (T.G.)	83	F	face, lips	no	hypercholesterolemia	134	159	108

In normal subjects, SDS-PAGE followed by immunoblotting analysis of HK showed a major band of M_r 130,000 which represents native HK, and a faint one of M_r 107,000, which represents a catabolic cleavage product (Figure 8, upper panel). After *in vitro* activation of the contact system with kaolin (K), normal plasma showed the disappearence of the band of M_r 130,000, an increase of the band of Mr 107,000 and the appearence of the band of M_r 98,000. The last two bands represent cleaved HK (Figure 8, lower panel). Angioedema patients showed an increase of cleaved HK, evidenced by a greater intensity of the band of M_r 107,000 and/or the appearence of the band of M_r 98,000 (Figure 8, lower panel). The levels of cleaved HK (Figure 9, left panel) were significantly higher in angioedema patients (median 27.1 %, range 22-61.3 %) than in normal subjects (median 20.5 %, range 9.5-23.4 %) ($p = 0.0001$).

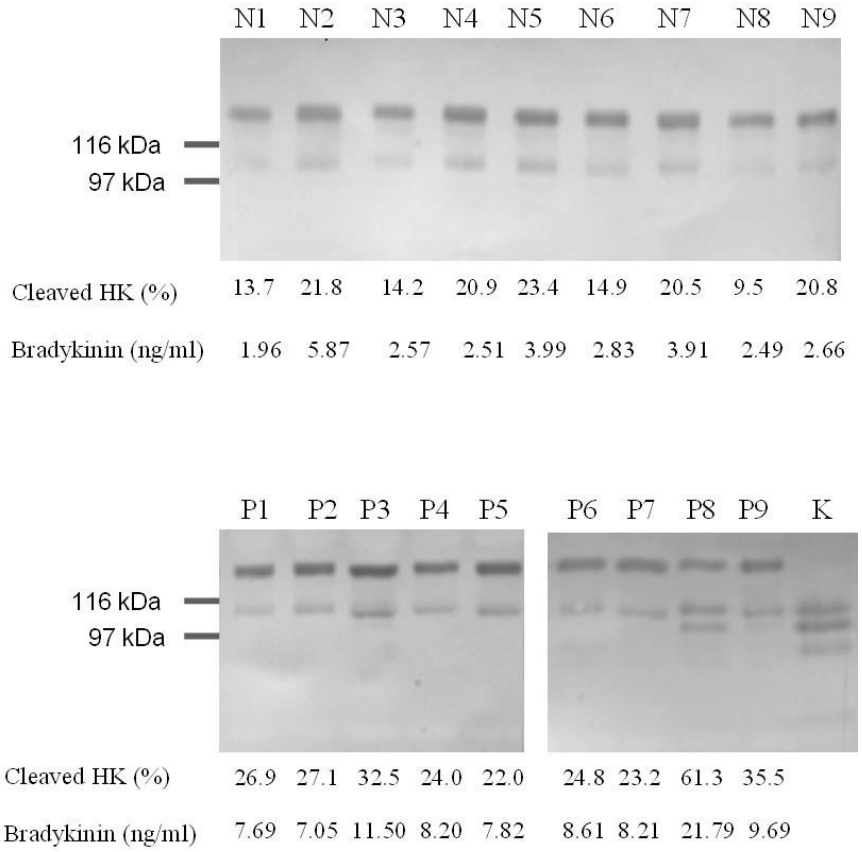

Figure 8. Cleaved high molecular weight kininogen (HK) and bradykinin levels in patients with idiopathic angioedema during acute attacks and in controls.

Bradykinin levels (Figure 9, right panel) were higher in angioedema patients (median 8.21 ng/mL, range 7.05-21.79 ng/mL) than in normal subjects (median 2.83, range 1.96-3.99) (p=0.0001). Bradykinin levels were directly correlated with levels of cleaved high molecular weight kininogen both in normal subjects and in angioedema patients (r =0.893, p=0.0001) (Figure 10).

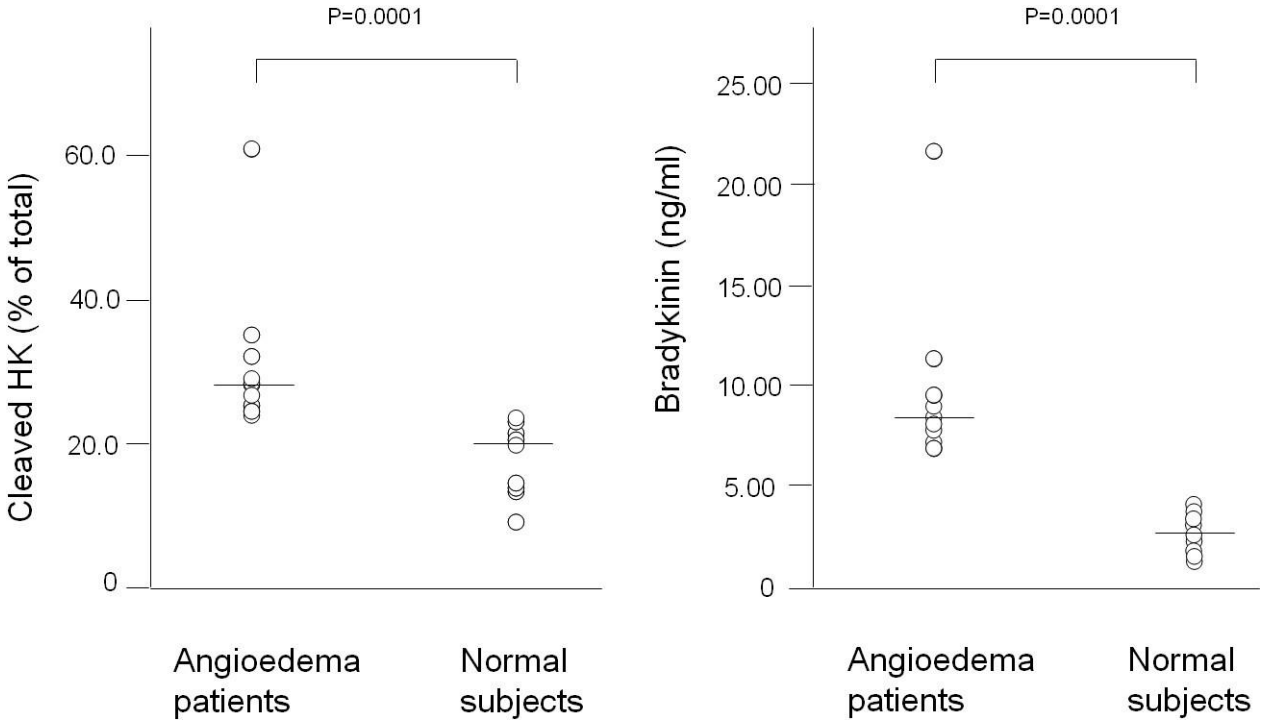

Figure 9. Plasma levels of cleaved high molecular weight kininogen (HK) and bradykinin in patients with idiopathic angioedema during acute attacks and in normal subjects.

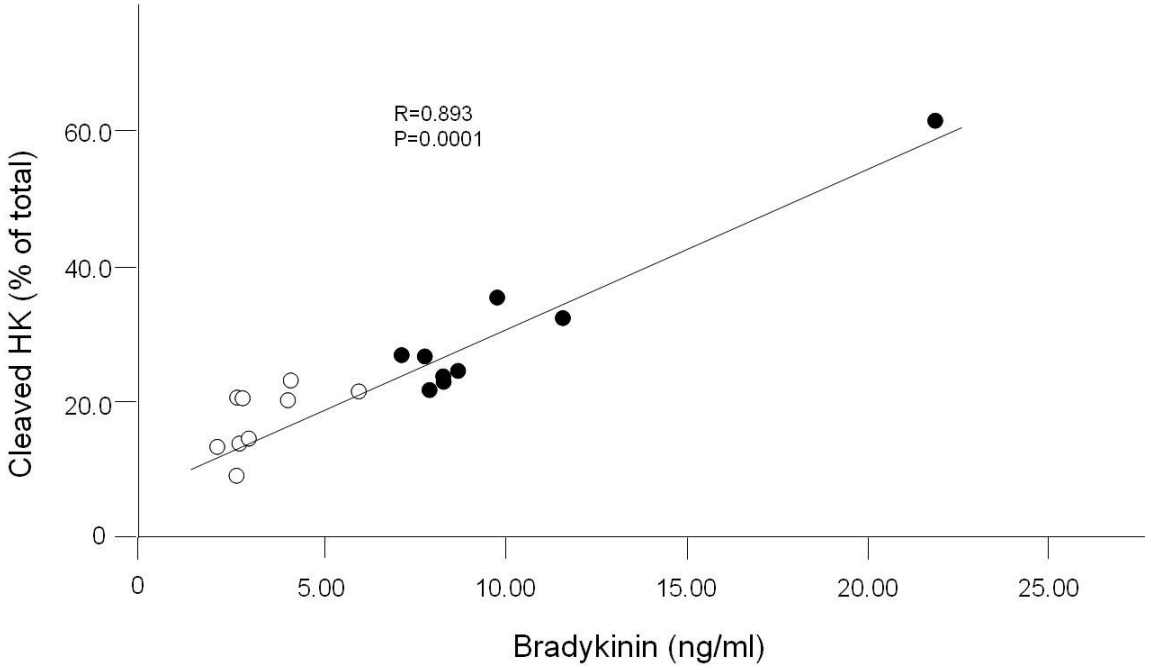

Figure 10. Correlation between plasma levels of cleaved high molecular weight kininogen (HK) and of bradykinin in 9 patients with idiopathic angioedema during attacks (full circles) and in 9 normal subjects (empty circles).

After evaluation in the Emergency Department, patients were treated with sistemic steroids and antihistamine and, on the basis of oral or tongue involvement, also with epinephrine (Table 2). The time of complete resolution of symptoms (reported by the patients after discharge at home) had a median of 30 hours (range 15-48 hours). In patients with higher bradykinin levels (over the median levels of 8.21 ng/ml) had a time of resolution of 36 hours (range 27-48 hours) compared to patients with lower bradykinin levels (below the median levels of 8.21 ng/ml) in which the time of response had a median of 16 hours (range 15-30 hours) (p=0.035). The time to resolution was longer in patients with higher levels of cleaved HK (median 30 hours, range 15-48 hours) than in patients with lower levels of cleaved HK (median 28.5 hours, range 15-36 hours) but. the difference did not reach statistical significance.

Table 2. Responses to therapy in patients with acute idiopathic angioedema.

Patient	**Angioedema localization**	**Therapy**	**Symptoms resolution (hours from onset)**	**Bradykinin (ng/ml)**
1 (C.L.)	face	Steroid, antihistamine	15	7,69
2 (C.R.)	lips	Steroid, antihistamine	17	7,05
3 (D.D.)	face, tongue	Epinephrine, antihistamine, steroid	36	11,5
4 (G.A.)	abdominal pain, feet	Steroid, antihistamine	30	8,2
5 (L.V.)	face	Epinephrine, antihistamin e, steroid	15	7,82
6 (P.A.)	face	Steroid, antihistamine	27	8,6
7 (R.GP)	lips	Steroid, antihistamine	36	8,21
8 (S.V.)	face	Steroid, antihistamine	48	21,79
9 (T.G.)	face, lips	Steroid, antihistamine	30	9,69

9. Discussion

In our study, we found increased levels of bradykinin and cleaved high molecular weight kininogen during acute attacks in patients with recurrent angioedema not due to allergies, C1-inhibitor deficiency, ACE inhibitor treatement and non steroidal-antiinflamatory drugs. All patients were treated with sistemic steroids and antihistamine but the response to treatment was better in patients with lower levels of bradykinin. Our data suggest that the kinin system is involved at least in some cases of idiopathic angioedema and may influence the response to therapy.

Presently, idiopathic angioedema is still a difficult condition to manage; indeed, there is no conclusive evidence for an effective treatment of attacks due to limited existing literature on the subject. There have been 20 articles and case reports published on the clinical aspects of patients with idiopathic angioedema [73]. Although the cause of idiopathic angioedema is unknown, all authors agree to start with intravenous steroids, intramuscular antihistamine and eventually epinephrine but what can be used when these fail remains a major problem. Intravenous tranexamic acid has been proposed in acute attacks but it is effective only as prophylactic treatment. Cicardi et al. described 25 patients with idiopathic angioedema who improved with prophylactic treatment with tranexamic acid [17], an antifibrinolytic agent that is analogous to epsilon-aminocaproic acid but has fewer side effects. These agents can control angioedema in C1-inhibitor deficiency, because they block the activation of plasmin, which faciliates the release of the vasopermeability mediator bradykinin [80]. Patients with acquired C1-inhibitor deficiency, in whom these agents are particularly effective, had high fibrinolytic activity [81]. Tranexamic acid was given to patients with idiopathic nonhistaminergic angioedema because of their similarities to patients with C1-inhibitor defects. Although theoretically the block of fibrinolysis may be a prothrombotic condition, to date, there is no evidence of a thrombogenic effect of tranexamic acid in these patients. [17]. In addition, some medications used for HAE, such as human plasma derived or recombinant C1 inhibitor (inhibitor of factor XIIa and kallikrein), icatibant (bradykinin blocker), and ecallantide (inhibitor of

kallikrein) have been reported to be successful in few acute episodes of idiopathic angioedema [73]. The efficacy of the pharmacological block of the kinin system in some patients with idiopathic angioedema, suggests that bradykinin plays a role in this condition. Indeed, our data are in agreement with this view because our patients with idiopathic angioedema showed a response to treatment that was inversely related to the levels of bradykinin and cleaved high molecular weight kininogen. Thus, the more the kinin system is activated, the worse the response to treatment.

The clinical picture of our patients was similar to that of the 201 patients with idiopathic angioedema described in the literature [73]. Most patients presented angioedema of face and lips and in some cases of some other body parts such as throat or abdomen. All patients reported more than 3 attacks per year, with most having monthly episodes. The typical length of the angioedema episodes was 36 to 72 hours until resolution. All patients had undergone treatment with multiple high-dose antihistamines. Sixty percent of patients tried additional medication, including, but not limited to, corticosteroids and epinephrine, but only a small decrease or remission of symptoms was reported.

The main limitations of our study are related to the small number of subjects due to the low incidence of idiopathic angioedema, and to the fact that we have not had the opportunity to evaluate patients during remission because the resolution of symptoms was reported by the patients after discharge at home. However, the strength of our study lies in the meticulous blood sampling procedure, performed by a trained researcher in the emergency department; this allowed to solve all the preanalitycal problems of bradykinin measurement providing reliable results.

In conclusion, in patients with idiopathic angioedema, our study showed increased levels of bradykinin and cleaved HK during acute attacks; such levels are correlated each other and are inversely correlated with the response to treatment. Our data suggest that parameters indicating the involvement of the kinin system may help in the choice of therapy, especially since effective drugs that block the kinin system are currently widely available.

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