Background

The C1-inhibitor (C1-INH) is the common, main regulator of the four major plasma enzymes (e.g. of the complement, kinin, blood coagulation, and fibrinolytic) systems of the blood. These systems undergo activation in the absence of C1-INH and this is accompanied by an increase in the concentration of the agents enhancing vascular permeability (bradykinin, in the first place), which act on the endothelial cells lining the blood vessels. In patients with Type I or Type II hereditary C1-INH deficiency (C1-INH-HAE), the effusion of intravascular fluid from the capillaries causes an edematous swelling in the subcutis and in the submucosa ('angioedema'). This may affect the subcutaneous tissues in any body region, but submucosal edema of the intestinal wall is similarly common and barely tolerable for the patients. The most serious condition is the involvement of the upper airways, because submucosal angioedema at this location may lead to suffocation without intervention. The site of the occurrence of the angioedematous symptoms is unpredictable and the episodes follow a fulminant, attack-like course.

Considering the central role of C1-INH in the pathomechanism of bradykinin-mediated angioedemas, one of the objectives of our research was (***A***) to develop a quantitative technique for determining the actual degree of activation, at a given point of time, of the activation pathways under the influence of regulation by C1-INH. The inhibitory function of C1-INH is shared among the active proteases of the four major plasma-enzyme cascade systems. Therefore, we assumed that by studying the concentrations of the protease/C1-INH complexes generated during enzyme-cascade processes and their relative ratios, the dominant activation pathway(s) responsible for the generalized C1-INH deficiency under physiological or pathological conditions could be identified with accuracy. The quantitative method for the determination of protease/C1-INH complexes was tested in *in vitro* activation systems, as well as in blood samples obtained from symptom-free or symptomatic C1-INH-HAE patients, as well as from healthy individuals. A part of the findings have already been published (1), and another manuscript (*see* attached) has been prepared (*Kajdácsi et al.: Simultaneous determination…submission in progress).* The remainder of the results is available as conference abstracts (2-5).

Most of the C1-INH present in the systemic blood circulation is produced by hepatocytes; however, a number of other cells also contribute to its biosynthesis. According to some authors, also human umbilical vein endothelial cells (HUVECs) can produce C1-INH under the effect of certain stimuli. If this is typical of all endothelial cells, then the latter may be capable of counteracting the enzymatic processes contributing to angioedema formation. However, our knowledge of other types of endothelial cells is limited. Therefore, our second main objective (***B***) was to clarify whether endothelial cells can produce C1-INH, to ascertain any differences among endothelial cells of diverse origin in this regard, and to identify

the effects inducing C1-INH production by these cells. In our assumption, the diverse origin of endothelial cells might underlie the differences observed in angioedema formation in various organs, and the sparing of the lung as well as of the kidney, for example. The findings on this subject have been published as a conference abstract (4).

A/ Simultaneous comparison of the concentrations of protease- C1-INH complexes

According to the relevant publications available so far, C1-INH can inhibit ten proteases, as well as it can form stable, covalent-bonded complexes with the active proteases. C1-INH is an exclusive inhibitor of active C1s and C1r (6, 7), as well as it is the major inhibitor of active MASP-1 and MASP-2 (8, 9), plasma kallikrein (10), active Factor XII (FXIIa) (11) and active Factor XI (FXIa) (12). It is a less potent inhibitor of thrombin (13), of plasmin (14), and of tissue-type plasminogen activator (t-PA) (15). Except for the latter, all other proteases have been previously implicated with the pathomechanism of C1-INH-HAE. We studied these nine proteases, by developing sensitive, sandwich ELISA techniques, we intended to determine the molar concentrations of the protease/C1-INH complexes characteristic of the individual activation pathways, along with the concentrations of antigenic and of functional C1-INH, in order to quantify the activation undergoing *in vivo*.

During the first half of this study, our workgroup produced or obtained from commercial sources, the necessary proteases, specific antibodies suitable for use with ELISA, ultra-pure active C1-INH (with molarity determined accurately by physical-chemical methods), and anti-C1-INH antibodies. The next step was to test these reagents – if any of these did not suit our purposes, we sought an alternative from a different manufacturer, or produced it ourselves as a last resort. This led to the production of monoclonal anti-MASP-1 and anti-C1-INH antibodies, and to the purchase of the nanobody recognizes cleaved and complexed C1-INH from the colleagues of the University Medical Center Utrecht. Using the appropriate active proteins and C1-INH, we produced *in vitro*, protease/C1-INH complexes of known molar concentration for use as standards during the ELISA tests for the measurement of the complexes. Although plasmin can form covalent-bound complexes with C1-INH or some of its fragments, it is known to degrade C1-INH (16-18). Having ascertained this to be the case, it became evident to us why no other work teams have been successful in developing a technique for the quantitative determination of plasmin/C1-INH complexes.

Once all the protease/C1-INH complexes have been produced (except plasmin/C1-INH), the next step was to develop and characterize sandwich ELISA protocols. An ELISA method for the detection of C1-INH (total antigenic C1-INH protein [$C1\text{-}INH_t$] and active C1-INH [$C1\text{-}INH_a$]) in molar concentration was

developed first (5). The next step was the development of the protease/C1-INH ELISAs for determining the levels of C1r/C1-INH, C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH, kallikrein/C1-INH, FXIIa/C1-INH, FXIa/C1-INH, and thrombin/C1-INH. These methods are described in more detail in the attached manuscript for publication.

From the outset, we have been collecting serum and anticoagulated plasma samples from healthy individuals and from C1-INH-HAE patients (as authorized by the ethical approval N° 3928/2014 for human research). The comparison of the samples did not reveal any meaningful differences except for the serum. Accordingly, in view of its ready availability and in storage stability, we chose EDTA-plasma samples for further use.

The simultaneous determination of C1-INH levels and of the eight protease/C1-INH complexes was performed in plasma collected from six healthy controls, from five symptom-free patients with Type I C1-INH-HAE, and from five Type II C1-INH-HAE patients. In the case of five out of these ten C1-INH-HAE patients, plasma samples were obtained also during edematous attacks. Besides, our study included the kinetic follow-up of an angioedematous episode in one Type I C1-INH-HAE patient, which yielded 12 plasma samples altogether over 96 hours. In the latter case, one of the six healthy individual served as control and just as with the subject of the kinetic follow-up blood sampling was performed at 6-hour intervals, over 24 hours. The observations made during the clinical course of this case, beginning from the symptom-free state through the onset of erythema marginatum (the only objective prodromal symptom of C1-INH-HAE) and then, the evolution and the spontaneous resolution of subcutaneous angioedema are now published (1). Although the findings of this single case cannot be generalized, it was an opportunity for making extraordinary observations. By testing the samples of healthy controls and patients, we sought answers to the following questions:

Primary questions:

1. What are the typical, molar concentrations of protease/C1-INH complexes in healthy individuals?
2. Do the concentrations of the individual protease/C1-INH complexes differ between symptom-free C1-INH-HAE patients and healthy controls?
3. If yes, then does this alter the relative order of the individual protease/C1-INH complexes in C1-INH-HAE patients?
4. Are there any differences between samples obtained from the patients during attacks or during symptom-free periods?
5. Is there a correlation between the severity of the angioedematous symptoms and protease/C1-INH levels?
6. Does the activation of any plasma-enzyme system precede the onset of symptoms?

Answers and conclusions:

1. Using a sensitive method, protease/C1-INH complexes can be detected – in nanomolar concentrations – even in healthy individuals. The measured concentration was the greatest in the case of C1r, C1s, and thrombin complexes (8 to 30 nM on average); moderate concentrations were measured for FXIIa and MASP-1 complexes (1 to 2 nM on average), whereas the concentrations of kallikrein, FXIa, and MASP-2 complexes were the lowest (0.1 to 1 nM).
2. The molar concentration of the kallikrein/C1–INH complex is greater in C1-INH-HAE patients than in healthy controls. The levels of MASP-2/C1-INH, FXIa/C1-INH, and thrombin/C1-INH complexes were higher, but only in patients with Type II C1-INH-HAE. When making a comparison using the ratios of the molar concentrations of active C1-INH, then the relative ratios of protease/C1-INH complexes are much higher in the patients than in the controls. However, the concentration of active C1-INH in the patients is as low as only 30% on average of that measured in healthy individuals.
3. Notwithstanding the differences observed between patients and controls, the order the individual protease/C1-INH complexes by their concentrations remained unchanged also in symptom-free patients.
4. Analyzing the samples obtained from the same five patients during HAE attacks and during symptom-free periods did not reveal differences among the concentrations of any of the complexes studied. It should be noted that several (12 to 130) days elapsed between the two samplings.
5. Remarkably, the most conspicuous relationship was seen in the case of the protease/C1-INH complexes measured during the kinetic follow-up of an HAE attack. In particular, the molar concentrations of the complexes of kallikrein, FXIIa, and FXIa proteases formed with C1-INH exhibited a definite peak. The levels of C1s, MASP-1, and MASP-2 complexes also increased compared to those measured 47 minutes earlier during the prodromal stage, and only the concentration of the thrombin/C1-INH complex decreased. The concentration of the kallikrein/C1-INH complex increased several-fold upon the onset of angioedematous manifestations and remained this high in the samples obtained 6 hours later when the patient recorded the maximum intensity of edematous symptoms during the course of hourly monitoring. By this time, the activation of all the other proteases returned to the baseline level. The plasmin/C1-INH complex could not be detected. However, during the kinetic follow-up of an angioedematous attack, when different methods were applied to study coagulation and fibrinolytic factors, D-dimers were elevated at the peak of the angioedematous symptoms, and this reflects the activation of the fibrinolytic system (3). Thus, as shown by the kinetic follow-up, proteases activation is a rather quick process, which takes place within one or two

hours and hence, elevated levels of the complexes cannot be detected later. Apparently, the studies comparing the properties of blood samples drawn during acute angioedematous episodes with those of samples obtained after a longer (several-day long) interval are unsuitable for the purposes of the research into HAE attacks.

6. The complement system undergoes activation (as shown by elevated C4a level) as early as during the prodromal stage – that is, before the onset of angioedematous symptoms, and C1-INH activity begins to decline as described by VESZELI *et al* (1). In another study, which also involved the kinetic follow-up of an angioedematous attack, we found elevated fibrin levels during the prodromal phase, and this suggests thrombin formation . Increasing the frequency of blood sampling from the prodromal phase onwards could possibly reveal further details. Implementing such a study appears feasible in the future because angioedema occurs right after the onset of erythema marginatum in a proportion of patients.

In addition to analyzing blood samples, we also studied the *in vitro* changes in the concentration of protease/C1-INH complexes in various activation systems.
In order to induce classical complement activation, serum samples were treated with heat-aggregated intravenous immunoglobulin (IVIG), whereas kaolin was used to activate the contact and the coagulation systems. The effect of activation on the level of protease/C1-INH complexes was evaluated also in the presence of surplus C1-INH and α2-macroglobulin (α_2M).

Our results show that as expected, the activation of the classical pathway of the complement system with heat-aggregated IVIG resulted in a several-fold increase in the production of C1r/C1-INH and C1s/C1-INH complexes. Heat-aggregated IVIG activated also the lectin pathway, and this was accompanied by a smaller but meaningful elevation of the levels of the MASP-1/C1-INH and MASP-2/C1-INH complexes. Complex formation of the other proteases (thrombin, FXIIa, and FXIa) was not influenced whatsoever by heat-aggregated IVIG. Concerning the kallikrein/C1-INH complex, we measured excessively high values also in the control serum samples and therefore, the changes in this parameter could not be interpreted.

The effect of kaolin activation was the greatest on the formation of kallikrein/C1-INH and MASP-1/C1-INH complexes – their concentrations exceeded the upper limit of the measurement range in both instances. The levels of the FXIIa/C1-INH and of FXIa/C1-INH complexes also increased several-fold in kaolin-activated samples; however, their concentrations were within the measuring range. Kaolin activation had the smallest effect on the increase of the levels of the complexes formed by the proteases thrombin and MASP-2 with C1-INH. The presence of surplus C1-INH or α_2-macroglobulin did not have a significant impact on complex-

formation by any instance, regardless of whether the α_2M has any role in the regulation of the given protease (*Figure 1*).

Conclusions: Our results are suitable for use in designing *in vitro* experimental systems intended as models for activation mechanisms. Heat-aggregated IVIG activates the complement system (not only the classical but also the lectin pathway – although to a lesser extent). Kaolin has no effect on the proteases of the classical pathway; however, it activates all other proteases regulated by C1-INH. The response of the levels of MASP-1/C-INH and of kallikrein/C-INH complexes to kaolin activation was similarly excessive. This may suggest that the triggers leading to the production of bradykinin might exert a substantial effect not only through kallikrein but also via the activation of MASP-1. This might have *in vivo* consequences, as active MASP-1 is known to contribute to the enhancement of vascular permeability by direct and by indirect mechanisms (19, 20).

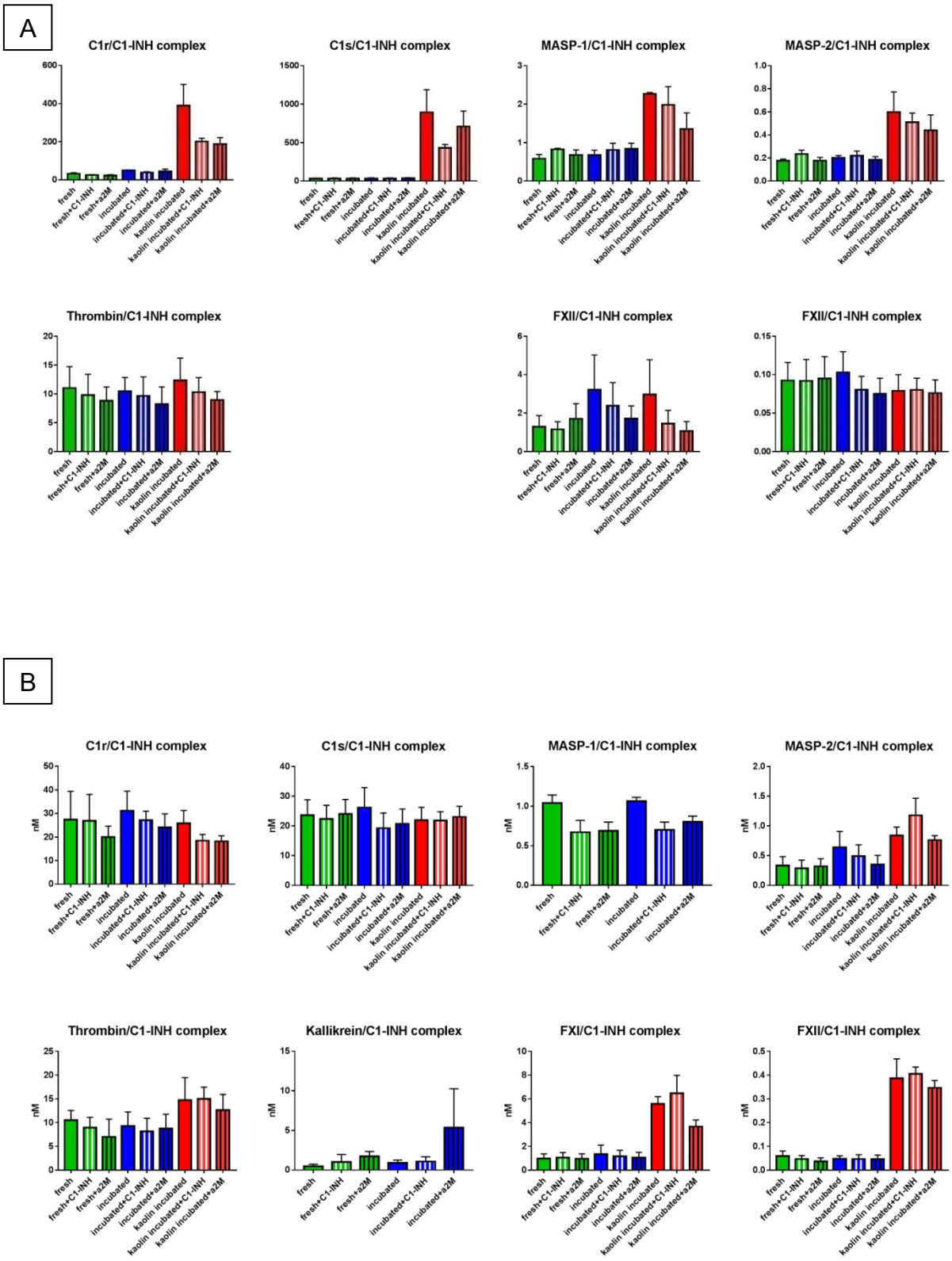

Figure 1. *Concentrations of protease/C1-INH complexes in serum activated by heat aggregated intravenous immunoglobulin (IVIG) or EDTA-plasma samples activated by kaolin.*
*Serum samples taken from three healthy volunteers (green) were activated by 20 mg/ml IVIG (9:1 (red), or PBS buffer (blue) at 37 °C, for 60 min (**A**). EDTA-plasma samples obtained from the same heathy individuals (green) were activated by 1% kaolin (1:1) (red), or PBS (blue) 37 °C,*

*for 5 min (**B**). Before activation serum or EDTA-plasma samples were completed with purified C1-INH (green and white banded) or α2-macroglobulin (green and black banded). In these samples the final concentration of the inhibitors were increased by two-times of the phisiological level. Int he case of kallikrein/C1-INH complex, we measured excessively high values also in the control serum samples and therefore, the changes in this parameter could not be interpreted (**A**).The response of the levels of MASP-1/C-INH and of kallikrein/C-INH complexes to kaolin activation was similarly excessive, therefore these results are not shown in the figure **B**.*

B/ Comparison of the C1-INH productions of different endothelial cells

We measured the mRNA expression of C1-INH in primary ECs, such as human umbilical vein and arterial ECs (HUVECs and HUAECs), human dermal microvascular ECs (HDMECs) and human glomerular ECs (GECs), as well as in EC line, human brain microvascular ECs (HCMEC-D3), and in HepG2 cell line as a positive control. After this we used different stimuli: thrombin (TR), BK, TGFβ, interferon gamma (IFNγ), and TR and BK together, and measured the changes of mRNA and protein levels of C1-INH after 24 and 48 hours. We used qPCR to measure mRNA levels, and an in-house ELISA to detect C1-INH protein production.

We found that all investigated ECs can produce C1-INH at mRNA level. Moreover, HUVEC and HDMEC produced and secreted C1-INH into the cell culture supernatant (2.41 +/- 0.34 ng/10^5 cells/48 hours for HUVEC, 0.496 +/- 0.018 ng/10^5 cells/48 hours for HDMEC). IFNγ treatment caused a significant increase in the expression of C1-INH at both mRNA and protein level in HUVECs and in HDMECs. Although TGFβ, and BK together with TR or TNF, and in some HUVEC lines BK alone could also induced the expression of C1-INH, these changes were minor compared to the effect of IFNγ *(Figure 2).*

Conclusions: All endothelial cell types produced C1-INH, which suggests that ECs can actively regulate the plasma serine protease cascades, thus the pathophysiological process of angioedema.

The difference between C1-INH expression upon stimulation with several potential trigger factors highlights that initiation routes of HAE attacks may implicate distinct predisposition for edema formation as well as to distinct efficiency to resolve the attacks. Taken together, we propose that the pathophysiology of HAE attacks may depend on the integrative function of bradykinin metabolism, C1-INH metabolism and the actual phenotype of endothelial cells (4). Preparation of manuscript is in progress, therefore we apply re-evaluation one year later.

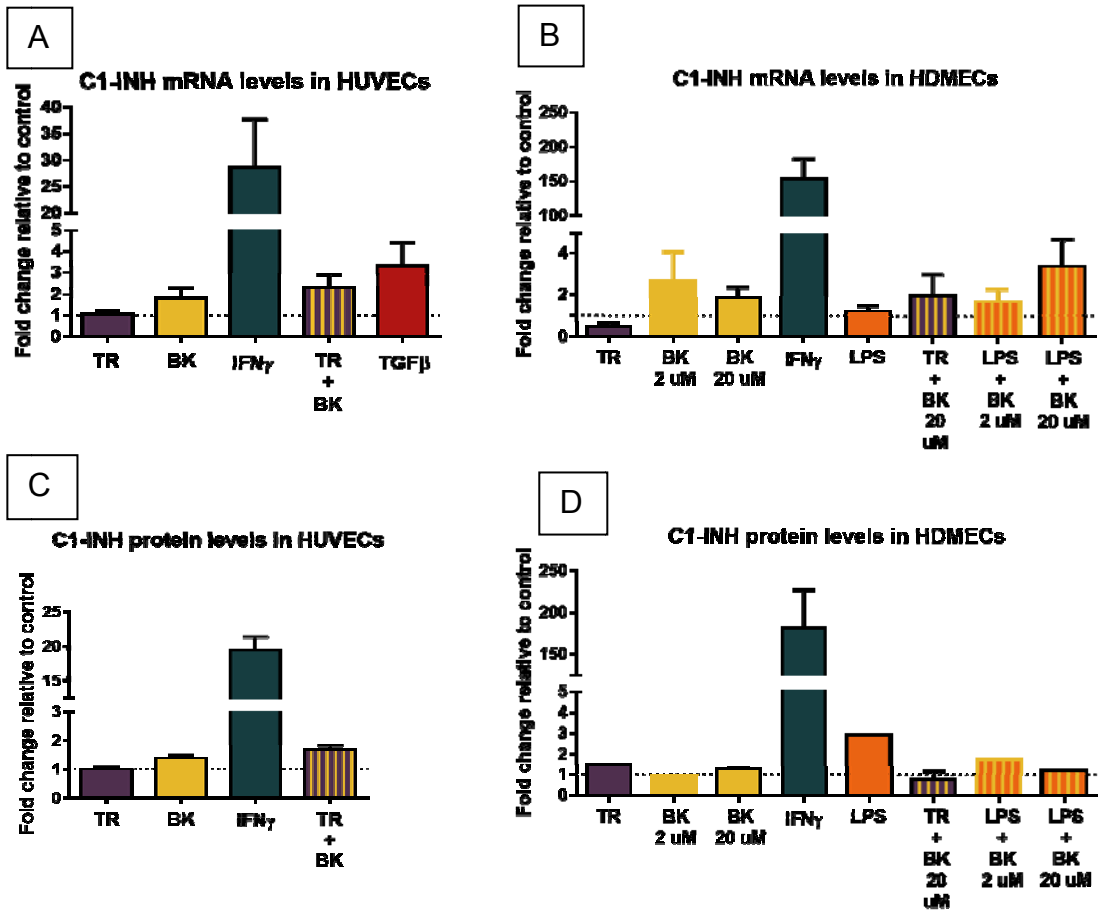

Figure 2. *The changes in C1- INH mRNA levels were measured in HUVECs (**A**) and HDMECs (**B**) after treatments. The C1-INH protein levels were measured in the supernatant of HUVECs (**C**) and HDMECs (). The results are shown as a fold change compared to the untreated cells (dotted line). Only IFNγ treatment shown significant changes both in C1-INH mRNA and protein levels.*

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Simultaneous determination of the complexes generated *in vivo* by the C1-inhibitor with C1r, C1s, MASP-1, MASP-2, kallikrein, FXIIa, FXIa, and thrombin

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Abstract

C1-inhibitor (C1-INH) is an important regulator of the complement, coagulation, fibrinolytic and contact systems. The quantity of protease/C1-INH complexes in the blood may well be proportional to the level of the *in vivo* activation of these four cascade-like plasma enzyme systems. Parallel determination of C1-INH-containing activation complexes would be important to understand the role of C1-INH in the regulation of plasma enzyme cascades in diseases like hereditary angioedema due to C1-INH deficiency (C1-INH-HAE).

We developed 10 in-house ELISAs for measuring the complexes of C1-INH formed with the following active proteases: C1r, C1s, MASP-1, MASP-2, kallikrein Factor XIIa, Factor XIa, and thrombin, as well as for measuring antigenic and functionally active C1-INH. We measured the levels of the complexes in EDTA plasma from 6 healthy controls, from 5-5 patients with type I or type II C1-INH-HAE during symptom-free periods and from 5 C1-INH-HAE patients during HAE attacks. We also measured the levels of these complexes in blood samples taken from one C1-INH-HAE patient during the kinetic follow-up of a subcutaneous angioedema attack, from the onset of the prodromal symptoms through the course of the edematous attack, until its spontaneous resolution.

Elevated kallikrein/C1-INH complex concentration suggests the activity of the contact system. The concentrations of FXIa/C1-INH, MASP-1/C1-INH, and MASP-2/C1-INH complexes were also elevated but only in type II C1-INH-HAE. We found no significant differences between the amounts of the complexes in samples obtained during attacks or in symptom-free periods. During the kinetic follow–up of an attack, the concentration of kallikrein/C1-INH complexes was the highest at the onset of edematous symptoms and the levels of additional protease/C1-INH complexes were elevated in this sample. The changes in the concentrations of the complexes follow rather rapid kinetics.

The quick changes in the amount of enzyme/C1-INH complexes during the kinetic follow-up study may suggest that a fast sampling schedule is needed if we want to compare the amounts of the complexes during HAE attacks. Simultaneous determination of molar concentrations, including those of eight protease/C1-INH

complexes, seems appropriate for studying the pathomechanism of C1-INH-HAE and of other disorders where the activation of complement, contact, coagulation, and fibrinolytic systems has a dominant role.

Introduction

The C1-inhibitor (C1-INH) belongs to the superfamily of serine protease inhibitors. With its mean plasma concentration of 0.25 g/L (3.5 μmol/L) (1), it is among the most abundant proteinase inhibitors present in the systemic blood circulation. It can inhibit a number of target enzymes and by way of its multi-functional role, C1-INH is the key regulator of the complement and kinin systems, as well as it is also involved in the control of blood coagulation and fibrinolysis (2). The hereditary or acquired deficiency of C1-INH leads to the occurrence of angioedematous symptoms affecting subcutaneous or the submucosal tissues (3, 4). Recognizing C1-INH deficiency is indispensable because, without appropriate treatment, angioedema involving the upper airways may cause suffocation (5). Hereditary angioedema resulting from the mutation of the *SERPING1* gene encoding the C1-INH protein (C1-INH-HAE) has two types, both exhibiting autosomal dominant inheritance. In Type I, this protein is produced by the non-mutated allele and hence, the concentration of C1-INH in the systemic circulation is usually lower than the half of the normal mean value. In Type II C1-INH-HAE, the concentration of C1-INH is normal owing to the simultaneous presence of the normal and of a non-functional protein, resulting in normal or increased C1-INH concentration. However, the activity of C1-INH is markedly decreased in both types of HAE (6). The decline of the plasma level of functional C1-INH below a critical value (7, 8) is entailed by the activation of the plasma enzyme systems regulated by this protein. These processes lead to the production of mediators that enhance vascular permeability and eventually, this causes the extravasation of plasma into – and the formation of angioedema in – the tissues (9). The key mediator is bradykinin, which is released from high-molecular-weight kininogen during the activation of the kinin system (10), but the role of additional factors cannot be excluded either. The direct effect of active MASP-1, generated during complement activation (11-13), as well as of thrombin, released upon the activation of the coagulation system (14) have been implicated.

C1-INH has been named after its inhibitory effect on the classical pathway of the complement system; however, it has been found to have a role in the inhibition of a number of additional plasma proteinases. C1-INH is the exclusive, natural inhibitor of the active C1s and C1r components of the complement system (15, 16). Further, it is

the major inhibitor of active MASP-1 and MASP-2 (17, 18) and of plasma kallikrein belonging to the kinin system (19), as well as of Factor XIIa (20) and of coagulation Factor XIa (21). C1-INH also inhibits thrombin (22). Moreover, it regulates the fibrinolytic system through the inhibition of plasmin (23), and of tissue-type plasminogen activator (t-PA) (24). Upon the activation of any of the cascade systems listed above, not only the active proteinase is inactivated, but also C1-INH is consumed during the inhibitory process. In particular and typical of serpines, the 'suicide' inhibitor C1-INH forms stable, larger molecular weight complexes – of 1:1 stoichiometry with monomeric and 2:1 stoichiometry – with dimeric proteases, in which both components are held together by covalent bonds, and both lose their activity (25, 26). The increased consumption of C1-INH may trigger an acute angioedematous attack. In this regard, it is not yet decided which plasma system(s) is/are responsible – by undergoing activation – for the onset of the attack. The presence of various proteinase/C1-INH complexes in the plasma reflects the activation of systems regulated by C1-INH. If the concentrations of the proteinase/C1-INH complexes were known, their relative ratios would give a clue to identifying the activation process in which C1-INH has been consumed. Our goal was to undertake a quantitative comparison of the degree of the activation of all C1-INH-regulated pathways, which underlies angioedematous symptoms. Further, we intended to characterize the pathomechanism of the acute episodes of angioedema by monitoring the concentrations of proteinase/C1-INH complexes over time. In order to ensure comparability, we have developed sensitive ELISA techniques, whereby the levels of the proteinase/C1-INH complexes can be expressed in a standardized fashion – that is, as molar concentrations. Using these new methods, we explored and quantified the differences in the activation of the proteases regulated by C1-INH, which have been found between healthy individuals and C1-INH-HAE patients with or without angioedematous symptoms. Furthermore, we performed a kinetic follow-up of these changes by monitoring the spontaneous course of an angioedematous attack, from the onset until the complete resolution of its symptoms.

Methods

Purified proteins and antibodies. The active proteases of high purity and the antibodies of the highest specificity – required for the development of sandwich ELISA tests – were obtained from commercial sources. These materials were tested and changed as necessary, as well as their cross-reactivity was minimized by altering the reaction conditions. Some reagents were not available; these we prepared ourselves. Active C1r, C1s, MASP-1, and MASP-2 were produced by the recombinant technique. In order to express the measured values as molar concentrations, ultra-pure, active C1-INH had to be obtained. Lyophilized Berinert P was dissolved in water as indicated by the manufacturer. The resulting solution contained glycine, NaCl, and Na-citrate in addition to the protein content. The solution is 6.5 mg/mL according to the manufacturer, but this concentration takes into account only the proteinaceous component of the C1-inhibitor preparation without the glycans. The concentration of the full glycoprotein is about 8.7 mg/mL. Berinert P solution was diluted with buffer “A” (100 mM NaCl, 10 mM NaP, pH=7.40) then applied to a Q Sepharose HP (GE Healthcare), 16 mm×100 mm column, and eluted with a 0-30% “B” gradient (100-370 mM NaCl) with 1 M NaCl, 10 mM NaP, pH=7.40 being used as buffer “B”. The chromatogram and the SDS-PAGE analysis are shown in *Figure 1*. The fractions of the middle of the main peak were combined. The concentration was determined using the extinction coefficient of 27 180 M^{-1} cm^{-1} (27) and the molecular mass of 71 kDa (28).

The quality of anti-C1-INH IgG obtained from the sera of rabbits immunized with C1-INH was further improved by affinity-purification. In the ELISA test for measuring the concentration of the kallikrein/C1-INH complex, we used 1B12 nanobody reacting with cleaved or complexed C1-INH. We prepared monoclonal anti-C1-INH IgG_2 and monoclonal anti-MASP-1 IgG_1 (these are not available from commercial sources) in mice for use in ELISA tests measuring complexes containing MASP-1 and MASP-2. Some of the reagents (recombinant C1s and anti-C1-INH) were biotinylated. The sources and the preparation of the reagents are detailed in *Tables 1 and 2*.

Protease/C1-INH complexes. The proteases prepared *in vitro* for the complexes were incubated in molar equivalent ratio with ultra-pure C1-INH for 120 minutes at 37 °C – except for thrombin, which was incubated with 8-fold excess of C1-INH. The

complexes were stored until use in 1% BSA-PBS solution, at minus 80 °C. The complexes were checked with reducing SDS-PAGE and densitometry was performed to determine, what percentage of the known quantity of proteases formed complexes with C1-INH. This ratio was then taken into account to adjust the baseline concentration of the complexes during their use as standards *(Table 1)*.

Table 1. *In vitro* preparation of the protease/C1-INH complexes used as standards.

Active proteases used for the preparation of complexes with C1-INH*	C1r (recombinant, in-house (29))	C1s (recombinant, in-house (29))	MASP-1 (recombinant, in-house (30))	MASP-2 (recombinant, in-house (30))	Kallikrein (Innovative Research)	FXII (Innovative Research)	FXI (Innovative Research)	Thrombin* (Merck)
Protease/C1-INH ratio in the end product	1:1	1:1	1:1	1:1	1:1	1:1	1:2	1:1
Rate of complex (%)	89	96	83	89	88	70	86	99

** Thrombin was incubated in the presence of heparin, with an 8-fold excess of C1-INH.*

In the case of plasmin (Innovative Research), we attempted to form complexes by adding it to C1-INH present in several-fold excess. Subsequently, it turned out that instead of forming a complex, plasmin cleaves C1-INH and hence, we failed to obtain a complex in this setup.

ELISAs. We used 96-well microtiter plates (Nunc® Maxisorp®) for every ELISA method; additional details on the material used are set out in Table 2. OD was determined with a microplate reader (Infinite® M1000 PRO, Tecan Group Ltd). Usually – except for kallikrein – the plates were covered with a protease-specific antibody and the complex content of the samples was determined by adding anti-C1-INH. In every ELISA test, the standards and the plasma samples were measured in parallel and then averaged upon interpretation. During complex ELISA, the protease/C1-INH complexes listed in Table 1 were used as standards in multiple steps and following linear dilution. Ultra-pure C1-INH was chosen as the standard for sandwich ELISA applied to determine the total quantity of antigenic C1-INH (C1-INH_t). To measure the quantity of active C1-INH (C1-INH_a), we adopted the working principle of the commercially available kit by Quidel – that is, the ELISA plate was

covered with streptavidin. In the case of the standard, the active C1-INH contained in the sample was applied to the plate following treatment with biotinylated, recombinant, active C1s. Of the latter, only those molecules were detected by adding anti-C1-INH, which had bound to biotin-C1s/C1-INH complexes. As our ultra-pure C1-INH standard contained literally 100-per-cent active protein, the molar concentration of the standard could be used to express C1-INH activity also as molarity.

Table 2. ELISA protocols for the measurement of protease/C1-INH complexes, total antigenic C1-INH ($C1\text{-}INH_t$) and active C1-INH ($C1\text{-}INH_a$).

ELISA	Coat	Plasma sample dilution	Secondary antibody	Detection (with TMB, Life Technologies) at 460/620 nm, end-point method)
C1s/C1-INH	Anti-human C1s (goat IgG, Quidel)	300x	Biotinylated anti-hu-C1-INH (rabbit IgG, in-house)	steptavidin-HRP (R&D)
C1r/C1-INH	Anti-human C1r (goat IgG, R&D)	1300x	Biotinylated anti-hu-C1-INH (rabbit IgG, in-house)	steptavidin-HRP (R&D)
MASP-1/C1-INH	Anti-human MASP-1 (monoclonal mouse IgG, in-house)	10x	Anti-human C1-INH (mouse IgG2a, in-house)	*GAM (Southern Biotechnology)
MASP-2/C1-INH	Anti-human MASP-2 (monoclonal rat IgG, Hycult Biotech)	10x	Anti-human C1-INH (mouse IgG2a, in-house)	*GAM (Southern Biotechnology)
kallikrein/C1-INH	Anti-human C1-INH (nanobody 1B12, (31))	32x	Anti-human kallikrein (sheep IgG, Cederlane)	RAS-HRP (Southern Biotechnology)
FXII/C1-INH	Anti-human FXII (affinity-purified goat IgG, Cederlane)	80x	Anti-human C1-INH (affinity-purified rabbit IgG, in-house	GAR-HRP (Southern Biotechnology)
FXI/C1-INH	Anti-human FXI (goat IgG, R&D)	160x	Biotinylated anti-human C1-INH (rabbit IgG, in-house)	steptavidin-HRP (R&D)
thrombin/C1-INH	Anti-human thrombin, (sheep IgG, Innovative Research)	160x	Affinity purified anti-human C1-INH (rabbit igG, in-house	GAR-HRP (Southern Biotechnology)
$C1\text{-}INH_t$	Anti-human C1-INH (affinity purified rabbit IgG, in-house)	10000x	Anti-human C1-INH (biotinylated rabbit IgG, in-house)	steptavidin-HRP (R&D)
$C1\text{-}INH_a$	Streptavidin (SIGMA)	6000x plasma, treated with 1.5 nM biotinylated C1s (recombinant in-	Anti-human C1-INH (rabbit IgG, in-house)	GAR-HRP (Southern Biotechnology)

		house, (29)		

**Kinetic measurement was done at 530/585 nm using AUR (Amplex Ultrared reagent, Thermo Fisher/Invitrogen) as substrate.*

Blood samples. In a proportion of the experiments, plasma samples anticoagulated with 10 nM EDTA were collected from 6 healthy individuals (5 females and 1 male – aged 28 to 62 years), from 5 patients with Type I, and additional 5 patients with Type II HAE (aged 21 to 70 years) during symptom-free periods. In five of the 10 patients, plasma samples were obtained also during angioedematous attacks, the properties of which are detailed in *Table 3*.

In the other stage of the experiments, EDTA-plasma samples were collected on 12 occasions altogether over 96-hour observation of the course of an angioedematous attack in a 56-year old female patient with Type I C1-INH-HAE. In this case, the occurrence of the angioedematous attack was expected, because similar episodes had been rather common in this patient also previously. The patient was symptom-free at the time when the initial and the last blood samples were obtained. During the period in between, erythema marginatum – the only objective prodromal symptom of C1-INH-HAE – developed first, followed by a subcutaneous attack of angioedema involving the left hand and then, the right thigh. The patient rated her symptoms by scoring on a visual analogue scale. For comparison, blood samples were collected on another occasion from a 56 year old healthy female as a control at 6-hour intervals, over 24 hours. Further details of this angioedematous attack are described in the paper by VESZELI *et al* (7). The blood samples were centrifuged immediately and stored in aliquots at minus 80 °C until use. All laboratory tests were performed on freshly thawed plasma samples.

Table 3. *The characteristics of the angioedema attacks.*

C1-INH-HAE type	Localization	Time from start of the attack to the blood sampling (hours)	Trigger factor
Type II	Abdominal	3	Stress, weather
Type I	Subcutaneous	9	Physical trauma
Type I	Subcutaneous	3	Not known
Type I	Abdominal & subcutaneous	2	Not known
Type I	Subcutaneous	4	Not known

Statistical analysis. Sandwich ELISA measurements were interpreted and the complex-to-active C1-INH ($C1\text{-}INH_a$) concentration ratios of the samples were

calculated using GraphPad Prism v5.00 (GraphPad Software Inc.) and Microsoft Excel 2010 software.

Results

Characterization of the ELISA tests. In observance of the protocols detailed in the *Methods* section, we measured the reliability parameters of the ELISA tests, i.e. detection limits and intra-/inter-assay variation coefficients (expressed as percentages). The detection limits were specified also in relation to the plasma concentrations of the individual proteases (based on literature data). The detection limits of the **C1-INH$_t$** and **C1-INH$_a$** ELISAs were calculated in relation to the corresponding C1-INH concentrations we had measured in healthy individuals. Because reliable systems with a low standard deviation are required in order for the comparisons to be accurate, we provide the details of the analytical parameters of the ELISA tests in *Table 4.*

Table 4. The specifications of the ELISA tests.

ELISA	Detection limit (pM)	Plasma concentration (nM) and [molecular weight (kDa)] of zymogen	Detection limit related to the plasma level of zymogen or C1-INH$_t$ or C1-INH$_a$ (%)	Intra-assay CV%	Inter-assay CV%
C1r/C1-INH	1.5	581 [173] (32)	1.4×10^{-4}	17.5	17.4
C1s/C1-INH	3.0	625 [160] (32)	5.0×10^{-4}	11.2	19.3
MASP-1/C1-INH	0.27	118 [186] (33)	2.9×10^{-4}	8.1	15.5
MASP-2/C1-INH	5.8	26,8 [148] (33)	8.6×10^{-2}	16.1	11.1
kallikrein/C1-INH	8.1	500 [88] (33)	1.6×10^{-5}		
FXII/C1-INH	2.1	400 [80] (33)	5.5×10^{-4}	7.5	9.0
FXI/C1-INH	0.5	31 [160] (33)	7.4×10^{-3}	11.2	14.9
TR/C1-INH	1.2	1670 [72] (33)	6.0×10^{-5}	10.9	19.5
C1-INH$_t$	5.9	3200 [71]	1.8×10^{-6}	6.0	8.0
C1-INH$_a$	80	2500 [71]	3.5×10^{-5}	5.7	12.1

The comparison of C1-INH$_t$ and C1-INH$_a$ concentrations. These two parameters were determined in every sample. In healthy controls, the concentration of C1-INH$_a$ was on average 70 to 75% of the total C1-INH protein concentration detectable by ELISA. A similar ratio was found also in the samples of patients with Type I C1-INH-HAE. Part of the difference may be attributed to the C1-INH component of the complexes, which formed upon the inactivation of active proteases – this may add to the concentration of C1-INH$_t$, but not of C1-INH$_a$. In this study, we did not determine the quantity of the additional forms of (inactive, cleaved, degraded) C1-INH, because expressing their molar concentrations would have been difficult owing to their variegated antigenic properties.

Plasma levels of C1-INH$_t$, C1-INH$_a$, and protease/C1-INH complexes in patients and in controls. Using our in-house C1-INH$_t$ ELISA test, we determined in 6 healthy individuals and in C1-INH-HAE patients (5 with Type I and 5 with Type II HAE) the total concentration of the C1-INH protein, as well as applied C1-INH$_a$ ELISA for measuring active C1-INH level. As expected, C1-INH concentration was markedly lower in samples from patients with Type I C1-INH-HAE than in controls, or in patients with Type 2 disease. In the latter, C1-INH$_a$ concentrations did not differ from those measured in the samples from healthy controls. However, the concentration of functionally active C1-INH was lower in both types of C1-INH-HAE than the C1-INH$_a$ levels found in healthy individuals *(Figure 2).*

We used the proprietary ELISA techniques to measure the molar plasma concentration of the complexes formed by C1-INH with eight proteases (FXIIa/C-INH, kallikrein/C1-INH, FXIa/C1-INH, thrombin/C1-INH és C1r/C1-INH, C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH). Compared with the controls, the molar concentration of kallikrein/C1-INH complexes was higher in patients with Type I, as well as in those with Type II C1-INH-HAE. The molar concentration of C1s/C1-INH and of MASP-2/C1-INH complexes was higher than in the controls only in C1-INH-HAE patients with Type II disease. The levels of FXIa/C1-INH, thrombin/C1-INH, and MASP-2/C1-INH complexes were not different from those of the controls; however, all were higher in patients with Type II than in those with Type I C1-INH-HAE. A comparison of the samples from patients and from the controls did not reveal any

meaningful differences between the molarity of FXIIa/C1-INH and of C1r/C1-INH complexes *(Figure 2)*.

Comparing the mean molar concentrations of the individual protease/C1-INH complexes among themselves, their order was similar in controls as well as in both types of C1-INH-HAE patients. The concentrations of the C1s/C1-INH, C1r/C1-INH, and thrombin/C1-INH complexes were the highest (8 to 36 nM). The levels of kallikrein/C1-INH and of FXI-C1-INH were lower by a magnitude (1 to 2 nM), whereas FXII/C1-INH, MASP-1/C1-INH és MASP-2/C1-INH were present in a concentration of <1 nM (*Figure 2*).

The ratios of individual protease/C1-INH complexes to C1-INH activity. Overall, the molar concentrations of the individual protease/C1-INH complexes added up to 65 nM on average in healthy controls. The mean concentration of the protease/C1-INH complexes was slightly higher in the patients – that is, 73 nm in Type I, and 84 nM in Type II C1-INH-HAE. As the concentration of C1-INH_a was much lower in the patients, the protease/C1-INH to active C1-INH ratio was higher in patients (14% in the samples from patients with Type I, and 16% in those from patients with Type II disease) compared to controls (3%) *(Figure 3)*.

Plasma levels of C1-INH_t, C1-INH_a, and protease/C1-INH complexes in patients during angioedematous attacks. The results discussed so far were obtained by testing samples from symptom-free C1-INH-HAE patients. In 5 patients of these (4 with Type I and 1 with Type II HAE) samples obtained during attacks were also available. The clinical properties of the attacks are shown in Table 3. The latency between the detection of the angioedematous symptoms and blood sampling was 4.2 (2-9) hours on average. The mean interval between samplings in the same patient during a symptom-free period or during an attack was 73 (15-130) days. Neither the antigenicity of the C1-INH protein nor the concentration of C1-INH determined based on its functional activity exhibited any marked changes in the samples obtained during an attack, compared with those measured in the samples from symptom-free periods *(Figure 4)*.

Kinetic follow-up of the levels of C1-INH and of the protease/C1-INH complexes during an attack. The levels of C1-INH_t, and C1-INH_a, as well as of the C1r/C1-INH,

C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH, kallikrein/C1-INH, FXIIa/C1-INH, FXIa/C1-INH, and thrombin/C1-INH complexes were measured in blood samples obtained during a subcutaneous attack experienced by a female patient with Type I C1-INH-HAE. Blood was drawn at the onset of the prodromal symptoms and at a 6-hour interval during the angioedematous attack. An additional, 'extra' sample was obtained when the patient herself perceived the onset of the angioedema. Although blood sampling had been originally scheduled at 6-hour intervals, the sample drawn at the onset of angioedema followed the previous one by a mere 47 minutes, and the next sample was obtained 5 hours and 13 minutes later. Thus, there were no less than three samples available from the periods before and after the onset of the attack. C1-INH_a levels were decreasing during this period and reached a nadir at the occurrence of angioedematous symptoms *(Figure 5).* At the time when the angioedematous symptoms were detected, the molar concentrations of the FXIIa/C1-INH and FXIa/C1-INH complexes reached a distinct peak, and the levels of C1s/C1-INH, MASP-1/C1-INH, and MASP-2/C1-INH complexes also increased compared with the concentrations measured 47 minutes earlier. The concentration of the kallikrein/C1-INH complex increased several-fold upon the detection of angioedematous manifestations and remained this high in samples obtained 6 hours later when the patient recorded the maximum intensity of edematous symptoms. The activation of all the other proteases had returned to baseline levels in the samples obtained 6 hours later. *(Figure 5)*.

Discussion

We consider the development of sensitive methods for the simultaneous assessment and relative comparison of the activation status of the enzyme-cascade systems controlled by C1-INH among the most important results of our study. We have developed the methodology for the concomitant determination of the molar concentrations of plasma protein complexes containing the C1-INH molecule – this makes the monitoring of the distribution of C1-INH in blood plasma possible. Our methods allow the separate determination of functionally active C1-INH, C1-INH present in complexes formed with target proteases (based on its antigenic property), and all C1-INH-containing proteins – based on their molar concentrations. By now, sensitive ELISA or RIA methods have been developed for the detection of nearly all target proteases (C1s, MASP-1, MASP-2, kallikrein, FXIIa, FXIa, and thrombin) that form complexes with C1-INH (21, 34-41). These have been used in research studies on this topic; however, the quantitative measurement of C1r/C1-INH and of plasmin/C1-INH complexes have never been performed so far. However, only a couple of the methods outlined above expressed the measured values as molar concentrations, and not as relative units (21, 36-38). Usually, the results were compared with the activity of an enzyme of known molarity that was added to normal, pooled plasma – complexes produced *in vitro* for use as standards have been prepared only rarely. The quantitative procedures did not allow the simultaneous determination of all protease/C1-INH complexes – the method of maximum capacity made the concomitant measurement of three different proteases (kallikrein, FXII, and FXI) possible (37, 42, 43). In the majority of cases, the question was what role do the different inhibitors (C1-INH, anti-thrombin, and α_2 macroglobulin) play in the regulation of the individual proteases. Our study focused on C1-INH and did not deal with other inhibitors. Notwithstanding this, we sought to include every protease of which C1-INH is an exclusive or privileged/dominant regulator (compared with other inhibitors), as well as the activation of which has been implicated in the pathomechanism of C1-INH-HAE. Accordingly, we undertook a complex study of the following nine protease/C1-INH complexes: C1r/C1-INH, C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH, FXII/C-INH, Kallikrein/C1-INH, FXI/C1-INH, thrombin/C1-INH és plasmin/C1-INH (the t-PA/C1-INH complex was omitted). Eventually, we succeeded in developing an ELISA method for the determination of eight

protease/C1-INH complexes (the only exception was plasmin/C1-INH). Data from the literature are discordant and suggest that plasmin is likely to cleave C1-INH with only negligible complex formation (44-46). However, it is common ground that C1-INH has only a subordinate role in the regulation of plasmin. The same applies to thrombin, which is also thought to form complexes with C1-INH on one hand and to undergo cleavage by the latter on the other (39). As gold standard, we used an ultra-pure C1-INH preparation, which contained active human C1-INH without impurities or inactive derivatives. Thus, the concentration of the active C1-INH in this standard corresponded to the molar concentration determined by physical-chemical methods, based on the conversion of the mass concentration of the protein. We used this high-purity, active C1-INH for preparing the different protease/C1-INH complexes for use as standards. ELISA methods allow testing complexes in the picomolar concentration range, and this makes it possible to detect rather small – several ten-thousandth – activation of the plasma proteases C1r, C1s, MASP-1, prekallikrein, FXII, and thrombin. In the case of MASP-2, and FXI, this ratio is higher – it is several hundredth to thousandth. We tested this method on samples from C1-INH patients and clarified several issues regarding protease/C1-INH complexes.

Compared with healthy individuals, the activity of the contact system is clearly enhanced in C1-INH-HAE patients, even during symptom-free periods. We measured higher molar concentrations of kallikrein/C1-INH complexes in these patients than in healthy controls. As indicated by the elevated level of MASP-2/C1-INH complexes, the activity of the lectin pathway of complement activation is also enhanced; however, we found this in only patients with Type II C1-INH-HAE. This may suggest a difference between the two types of C1-INH-HAE patients also in this respect because the concentration of complexes with certain proteases (FXIa, thrombin, MASP-1, and MASP-2) were higher in patients with Type II than in those with Type I disease.

Our observations made during the monitoring of an angioedema attack yielded remarkable, new results. As shown by the particularly long, kinetic follow-up of an acute angioedematous episode, the cascade systems controlled by C1-INH undergo intense activation, which is a quick process. At the onset of angioedematous manifestations (zero time point), the concentrations of all protease/C1-INH complexes were elevated compared with the values measured in the plasma sample

obtained a mere 47 minutes earlier. With regard to the stage following the onset of the angioedematous symptoms, we have no information on the kinetics of the changes in the levels of protease/C1-INH complexes. In particular, the next scheduled time for blood sampling occurred 5 hours and 13 minutes after the zero time point of our kinetic follow-up. By this time, the levels of the majority of the complexes returned to baseline, whereas the angioedematous manifestations were just reaching their peak. An exception was the molar concentration of the kallikrein/C1-INH complex, which at this time exhibited a further increase and a good correlation with the VAS score recorded by the patient. Overall, therefore, it appears that the analysis of blood samples obtained several hours after the onset of angioedematous symptoms hardly yields much information – at least on the concentrations of protease/C1-INH complexes – owing to the swiftness of these changes. This may explain why we have failed to detect in another five patients elevated concentrations of the protease/C1-INH complexes in blood samples drawn on a single occasion during an angioedematous attack, even when this was done (according to the records) as early as 2 hours after detecting the onset of the symptoms. The likely cause of this that not hours, but several days or months have elapsed between blood samplings during symptom-free periods or during attacks, and many circumstances relevant to plasma activation level may have changed during this period. We believe that in order to gain a more detailed insight into the pathomechanism of angioedematous attacks, blood sampling should be scheduled at much shorter intervals – at least every hour before and after the onset of the symptoms. With regard to the prodromal period preceding the attacks, the kinetic follow-up confirmed our earlier assumption (7, 47) that this phase does not correspond to a quiescent state, and the activation of the relevant processes begins as early as this time. It has turned out that a similar, quiescent state exists – both in healthy individuals and in C1-INH-HAE patients – except for the moment of the onset of angioedematous symptoms. In particular, ranking the complexes of C1-INH by their concentrations, we could not detect any meaningful difference characteristic of C1-INH-HAE patients – the order of the complexes was similar to that prevailing in healthy controls. The levels of C1r and C1s complexes were the highest (possibly because C1-INH is the exclusive inhibitor of these factors) followed by thrombin, kallikrein, FXIa, whereas the concentrations of the FXIIa, MASP-1, and MASP-2 complexes were the lowest. The baseline activation pattern of proteases, which is

characterized by complex formation with C1-INH, may be subject to a variety of influences, including the plasma concentration and C1-INH-reactivity of zymogen, the presence of other inhibitors, or the differences among clearance rates (e.g. this is slower in the case of kallikrein/C1-INH or C1s/C1-INH than with the FXIIa/C1-INH complex, while the intact C1-INH is cleared from the circulation much more slowly (48). This baseline activation pattern is overwritten – for a brief period – by certain triggering effects. Because the plasma enzyme systems can activate each other, monitoring a greater number of attacks with more frequent sampling might yield clues on the factor that launches the process of angioedema formation. Our study shows that the enhanced activation of the complement system begins as early as during the prodromal phase – as evidenced by the increase in the levels of C4a concentration (7). Compared with the molar concentration of active C1-INH present in the systemic circulation, the total concentration of all protease/C1-INH complexes represents only a minor fraction. This could lead to the conclusion that even lower C1-INH levels might be of sufficient capacity to inhibit target proteases and hence, the insufficient inhibitory capability of C1-INH does not have to be included in the circumstances listed above. The relative concentrations of protease/C1-INH complexes expressed as the ratio of active C1-INH were higher – for all kinds of complexes – in the patients than in healthy controls. However, even in C1-INH-HAE patients, only a fraction of active C1-INH concentration is consumed for the production of complexes detectable in the circulation. This relationship holds true also for blood samples obtained during angioedematous attacks. It cannot be excluded, however, that the protease/C1-INH complexes suggestive of activation are generated – temporarily – in much greater quantities in the fluid extravasating from the capillaries into the interstitial space of the tissues affected by angioedema formation. If this is the case, then for the sake of effectiveness, the inhibition of the protease cascade systems controlled by C1-INH should be more targeted both in time and in space. In particular, it should be present around the time of the onset of the attack or as early as in the prodromal period, and it needs to be focused on the tissues affected by angioedema.

The following limitations should be considered when interpreting our findings. Our study did not undertake the quantitative determination of the interaction between plasmin and C1-INH and hence, disregarded the changes occurring in the activation of the fibrinolytic system. Although we tried to analyze both types of C1-INH-HAE and

to elucidate the events of angioedematous attacks, we are aware that 6 controls, 5-5 symptom-free patients, and an additional 5 with HAE attacks constitute an insufficiently small study population. In order to obtain reliable results, in this stage of our research, we focused on the elimination of preanalytic bias upon every blood sampling and the storage of samples – this can hardly be achieved in large-scale routine studies. The kinetic follow-up of an angioedematous episode was implemented by monitoring a single attack of a single patient with Type I C1-INH-HAE –our conclusions drawn from this case may be overwritten by findings from a larger patient population. Nevertheless, our studies may be regarded as 'pioneering' in the respect that a similarly comprehensive characterization of the activation of the proteases under the control of C1-INH has never before been undertaken. It is unique also in that it has explored the kinetics of the regulation of the activation of serine proteases by C1-INH in a single clinical case. This we were able to do through great luck, as the subject of the kinetic follow-up tolerated the symptoms of subcutaneous angioedema well even without treatment, and this made it possible for us to observe the natural course of a spontaneous attack. The other lucky circumstance was that the patient detected the occurrence of angioedema on her hand 47 minutes after the scheduled initial blood sampling – and this resulted in obtaining three blood samples instead of two during this critical period. If this extra blood sample had not been drawn at the zero time point, we could not have observed any significant change in the levels of protease/C1-INH complexes.

In addition to C1-INH-HAE, studying the activation of the plasma enzyme systems controlled by C1-INH is important also in conditions other than C1-INH-HAE (including among others sepsis, cardiopulmonary bypass, SLE, polytrauma) and has already been undertaken, although by monitoring fewer parameters (36, 40, 41, 49). The methodology developed by us and described herein might allow more complex exploration of the pathomechanism of these disorders, as well as it may open the way for the research into further clinical domains.

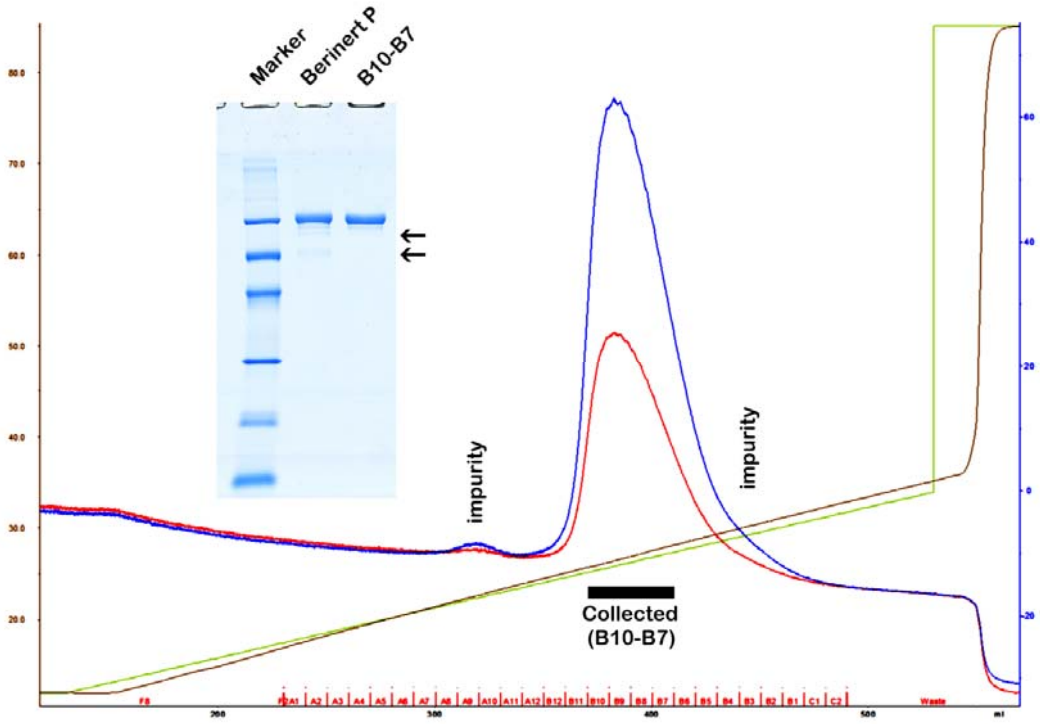

Figure 1. Purification of Berinert P (C1-inhibitor) by anion exchange chromatography. A black rectangle indicates the collected pure fractions representing the middle of the main peak. Blue, red, green and brown lines depict the absorbance at 280 nm, the absorbance at 254 nm, the gradient, and the specific conductivity, respectively. Removed impurities are indicated by labels on the chromatogram, and by arrows on the gel (insertion). SDS-PAGE was performed under reducing conditions. The marker (GE Healthcare Low Molecular Weight marker) is composed of 97, 66, 45, 30, 20.1 and 14.4 kDa proteins.

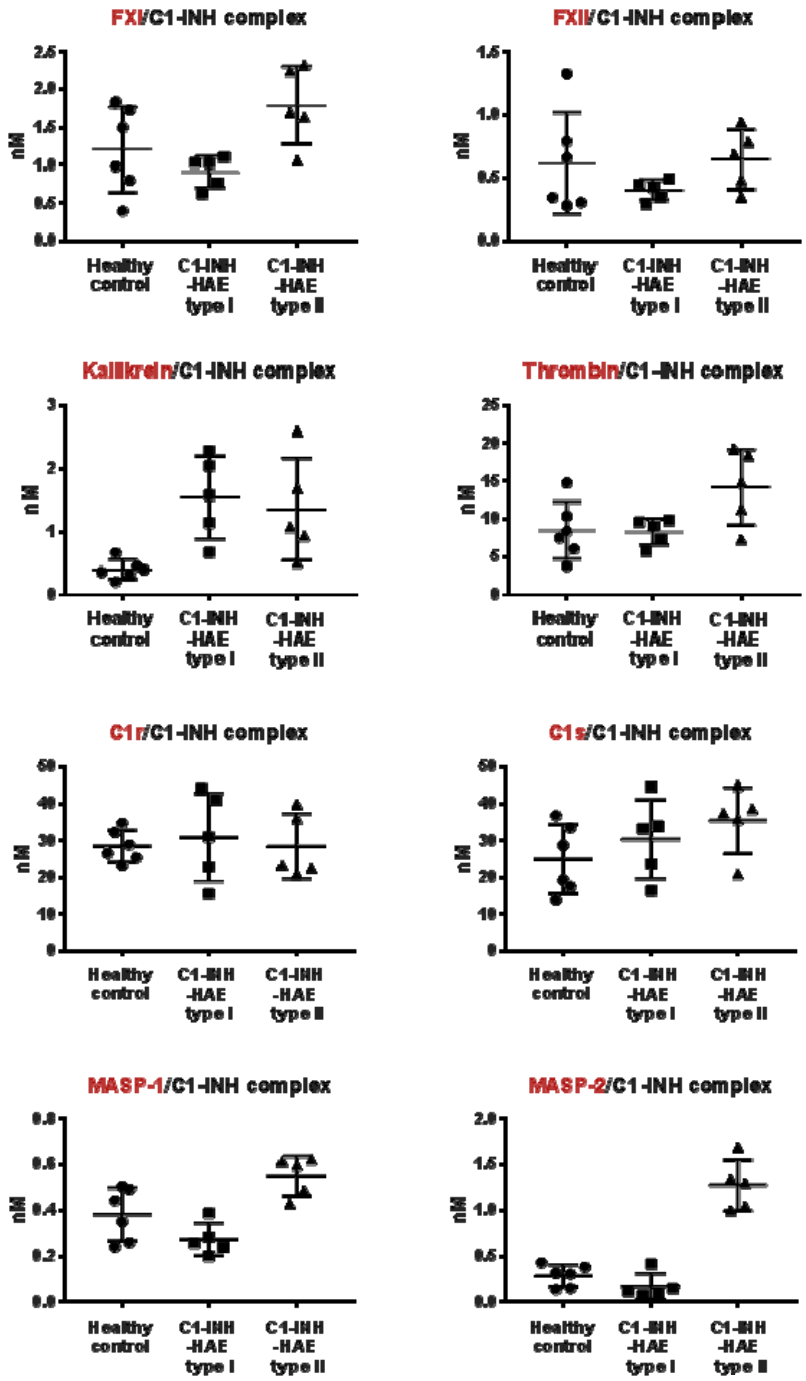

Figure 2. Molar concentrations of total antigenic C1-INH ($C1\text{-}INH_t$), functionally active C1-INH ($C1\text{-}INH_a$), and C1r/C1-INH, C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH, kallikrein/C1-INH, FXIIa/C1-INH, FXIa/C1-INH and thrombin/C1-INH complexes in EDTA-plasma samples taken from 6 healthy subjects, from 5 patients with C1-INH-HAE type I and from 5 patients with C1-INH-HAE type II.

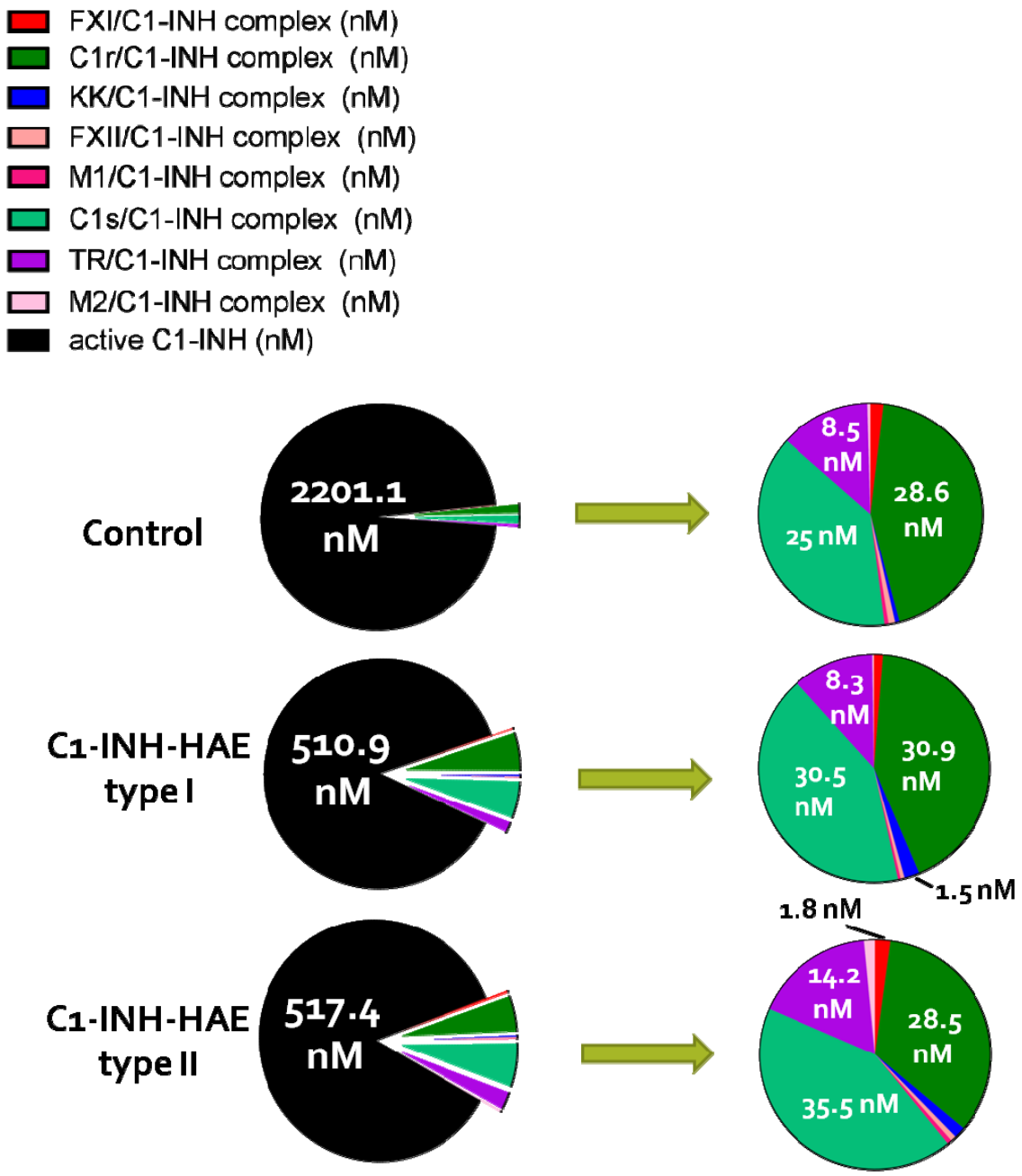

Figure 3. Relative average concentration of individual protease/C1-INH complexes to active C1-INH (C1-INH$_a$) in healthy controls and patients with C1-INH-HAE type I and C1-INH-HAE type II.

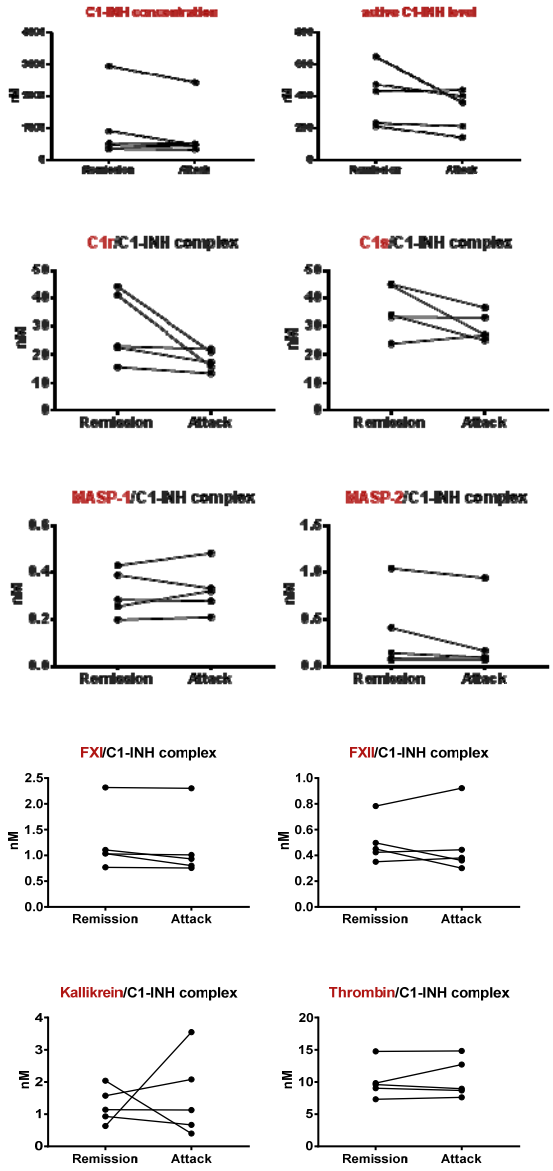

Figure 4. Molar concentrations of total antigenic (C1-INH$_t$) and functionally active C1-INH (C1-INH$_a$), and molar concentrations of C1r/C1-INH, C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH, FXIa/C1-INH, FXIIa/C1-INH kallikrein/C1-INH, thrombin/C1-INH complexes in patients with C1-INH-HAE. EDTA-plasma samples were obtained from the same patients during remission and edematous attack as well.

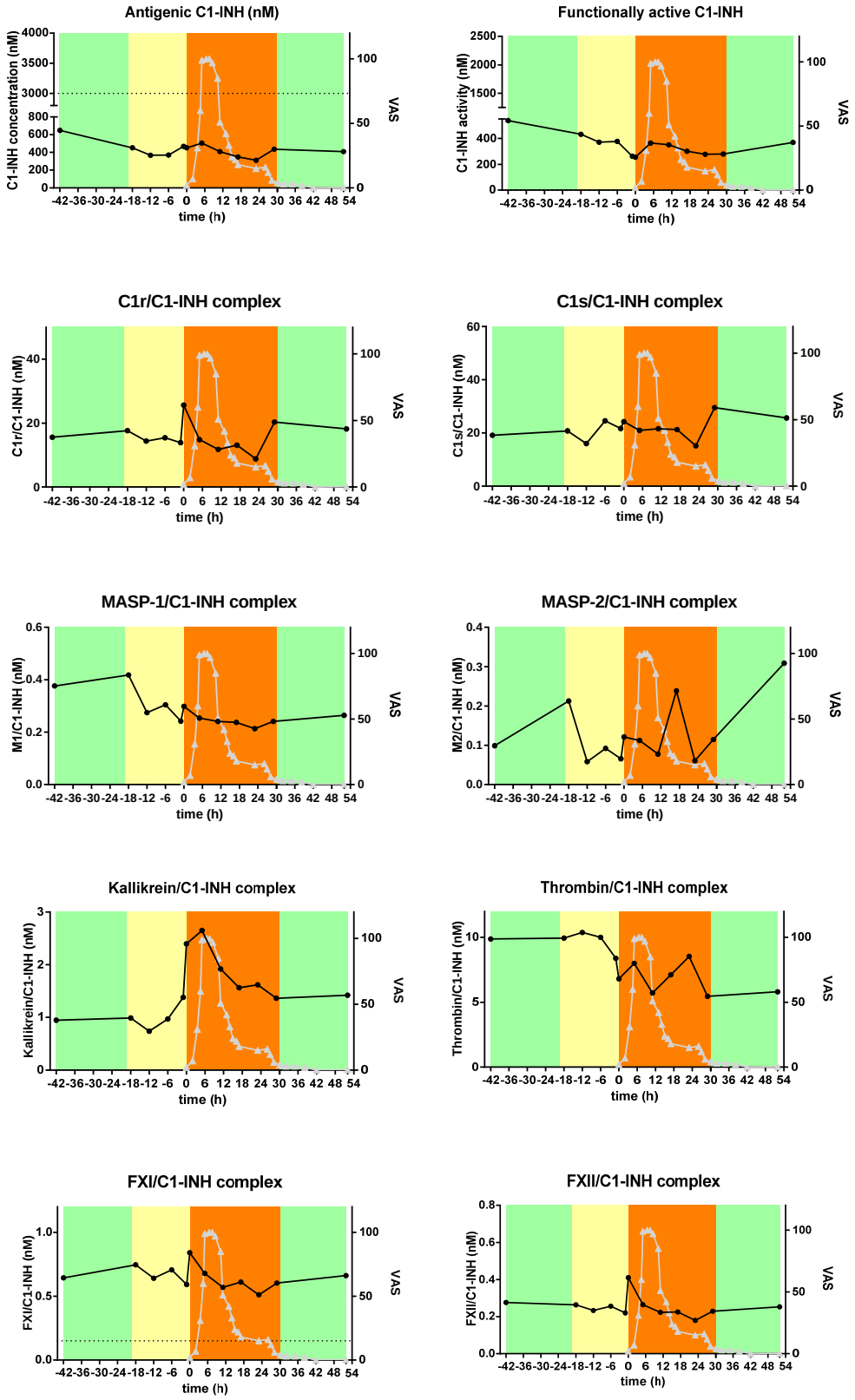

Figure 5. . Molar concentrations of antigenic and functionally active C1-INH and molar concentrations of C1r/C1-INH, C1s/C1-INH, MASP-1/C1-INH, MASP-2/C1-INH, Kallikrein/C1-INH, Thrombin/C1-INH, FXIa/C-INH, FXIIa/C1-INH complexes

(black dots with line) and the severity scores of angioedema symptoms (grey triangles with dotted line). Angioedema symptoms were assessed by the patient in a visual analog scale (VAS , 0-100 mm). Assymptomatic peroids are marked with green, prodromal phase with lemon and angioedematous phase with orange colors.

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