

Activation of the ficolin-lectin pathway during attacks of hereditary angioedema

Dorottya Csuka, PhD,^{a,b} Lea Munthe-Fog, PhD,^b Estrid Hein, MSc,^b Zsuzsanna Zotter, MD,^a Zoltán Prohászka, MD, PhD, DSc,^a Henriette Farkas, MD, PhD, DSc,^a Lilian Varga, PhD,^{a*} and Peter Garred, MD, PhD, DSc^{b*} *Budapest, Hungary, and Copenhagen, Denmark*

Background: The activation of plasma enzyme systems is insufficiently controlled in hereditary angioedema due to the deficiency of C1-inhibitor (C1-INH) (HAE-C1-INH). Recently, it was suggested that the ficolin-lectin pathway (ficolin-LP) might play a more dominant role than the mannose-binding lectin-lectin pathway in the pathomechanism of HAE-C1-INH. **Objective:** Because the role of the ficolin-LP during edematous attacks is still enigmatic, we analyzed its activity during such episodes.

Methods: Thirty-five patients with HAE-C1-INH, who have experienced severe attacks on 106 occasions, were enrolled. We analyzed blood samples drawn during attacks, and obtained 35 samples from the same patients during symptom-free periods. The serum levels of ficolin-2, ficolin-3, MASP-2, ficolin-3/MASP-2 complex, C1-INH, and C4, as well as the extent of ficolin-3-mediated terminal complement complex (FCN3-TCC) deposition, were measured using ELISA-based methods.

Results: Levels of MASP-2 and of the ficolin-3/MASP-2 complex were elevated ($P < .0001$ and $.033$, respectively), whereas that of FCN3-TCC was lower ($P < .0001$) during attacks than during the symptom-free period. During symptom-free periods, FCN3-TCC deposition was significantly related to concentrations of ficolin-3 ($R = 0.2778$; $P = .0022$), antigenic C1-INH ($R = 0.3152$; $P = .0006$), and C4 ($R = 0.5307$; $P < .0001$). Both ficolin-3 and MASP-2 levels correlated inversely with the time from the onset of the attack until blood sampling.

Conclusions: There is a marked heterogeneity of the pathomechanism and development of hereditary angioedema attacks in different patients. Our results suggest that the activation of the ficolin-LP may deplete the innately low level of C1-INH and thus, it may contribute to the uncontrolled activation of plasma cascade systems, and thereby to edema formation. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Hereditary angioedema, C1-inhibitor, ficolins, MASPs, lectin pathway, edematous attack

In humans, soluble pattern recognition molecules (such as mannose-binding lectin [MBL], ficolin-1, ficolin-2, ficolin-3, and collectin-11), acting in concert with MBL-associated serine proteases (MASP-1 and MASP-2), activate the lectin pathway of the complement system.^{1,2} According to preliminary studies, MASP-3 as well as the nonenzymatic proteins MAP-1 and sMAP are also involved in the regulation of this activation.³⁻⁵ The binding of MBL/ficolins to the carbohydrate molecules (mannose, *N*-acetyl-glucosamine, fucose) present on the surface of bacteria, viruses, and other microorganisms, as well as of dead host cells, activates MASP-2. The latter cleaves the complement components C4 and C2, leading to the formation of the terminal complement complex (TCC).⁶⁻⁸ Both the C1-inhibitor (C1-INH) and alpha-2-macroglobulin play a dominant role in the regulation of lectin pathway activation because they form complexes with MASP-1 and MASP-2⁹⁻¹¹ although they are not able to bind to MASP-3.¹² Considering the prominent role of C1-INH in the regulation of lectin pathway activation, we have studied previously whether the deficiency of C1-INH has a role in the activation of the MBL-lectin pathway or of the ficolin-lectin pathway in hereditary angioedema due to C1-inhibitor deficiency (HAE-C1-INH). Furthermore, we explored its possible effect on the pathomechanism of this disorder. Previous studies conducted in symptom-free patients showed that the levels of antigenic and of functional C1-INH, C4, C2, and MASP-2, as well as the total activity of the MBL-lectin pathway, are suppressed in this disorder.¹³ However, the MBL level was not related to any of the clinical indicators, and even MBL-MASP-2 activity correlated only with the quantity of the plasma-derived C1-INH (pdC1-INH) concentrate administered.¹⁴ However, our previous observation implies that the ficolin-mediated activation of the lectin pathway in HAE-C1-INH might be important. In particular, ficolin-2, ficolin-3, MASP-3, and MAP-1 levels were closely correlated with clinical indicators of the severity of HAE-C1-INH (ie, annual attack number, the number of attacks occurring in different locations, and the quantity of pdC1-INH concentrate administered). Our findings suggested that the severity of the disease is inversely related to ficolin levels. That is, lower ficolin-2 and

From ^athe 3rd Department of Internal Medicine, Semmelweis University, Budapest; and ^bthe Laboratory of Molecular Medicine, Faculty of Health Sciences, Department of Clinical Immunology, Rigshospitalet, University of Copenhagen, Copenhagen.

*These authors contributed equally to this work.

This research was realized in the frames of TÁMOP 4.2.4. A/1-11-1-2012-0001 "National Excellence Program – Elaborating and operating an inland student and researcher personal support system" (grant no. A1-EPFK-14-0092). The project was subsidized by the European Union and cofinanced by the European Social Fund. The equipment and the infrastructure of the research was supported by the Országos Tudományos Kutatási Alapprogramok (OTKA) 100886 (HF) grant, the Novo Nordisk Research Foundation, the Svend Andersen Foundation, the Danish Medical Research Council, and the Capital Region of Denmark.

Disclosure of potential conflict of interest: D. Csuka has received travel support from Viropharma, CSL Behring, and Shire Human Genetic Therapies, Inc. H. Farkas has received research support from the Hungarian Scientific Research Fund and has consultant arrangements with and has received travel support from Shire Human Genetic Therapies, Inc, Pharming, Viropharma, and CSL Behring. L. Varga has received travel support from CSL Behring and Shire Human Genetic Therapies, Inc. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 18, 2013; revised April 26, 2014; accepted for publication May 28, 2014.

Corresponding author: Dorottya Csuka, PhD, Faculty of Medicine, 3rd Department of Internal Medicine, Semmelweis University, H-1125 Budapest, Kútvölgyi út 4, Hungary. E-mail: csuka.dorottya@med.semmelweis-univ.hu. 0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2014.05.030>

Abbreviations used

C1-INH:	C1-inhibitor
FCN3-TCC:	Ficolin-3-mediated terminal complement complex
HAE-C1-INH:	Hereditary angioedema due to the deficiency of C1-inhibitor
LMM:	Linear mixed model
MBL:	Mannose-binding lectin
pdC1-INH:	Plasma-derived C1-INH
TCC:	Terminal complement complex

ficolin-3 levels are typical of patients with more severe disease, and this implies the consumption of these components during the activation process.¹⁴ Because of the deficiency of C1-INH, the C1-complex, as well as MASP-1 and MASP-2, may undergo uncontrolled activation, which leads to the increased cleavage of C4.^{13,15,16} Both MASP-1 and MASP-2 may be activated and their levels may be elevated during episodes of stress and/or in response to infections. The latter may trigger edematous attacks, at least in a proportion of patients with HAE-C1-INH.¹⁶⁻²⁰ It seems reasonable that excessive activation of the lectin pathway—initiated by the ficolins, and leading to C1-INH consumption by MASP-1 and MASP-2—might throw the regulation of C1-INH off-balance, and thereby lead to edematous attacks via the consumption of C1-INH.

To analyze this issue, our aim was to study the levels of proteins of the ficolin-lectin pathway and its activation in the same patients with HAE-C1-INH, both during symptom-free periods and during edematous attacks. We also investigated whether the ficolin-lectin pathway undergoes activation during edematous attacks, and studied possible changes in ficolin-lectin pathway parameters that characterize attacks occurring in different locations.

METHODS**Patients with HAE-C1-INH**

Thirty-five patients with HAE-C1-INH (12 men and 23 women; median age, 38 years; 25th-75th percentiles, 22-50 years), 29 with HAE-C1-INH type I and 6 with HAE-C1-INH type II, were enrolled in our study. In each patient, HAE-C1-INH was diagnosed according to the accepted clinical and laboratory criteria (positive family history, clinical symptoms of angioedema, low functional C1-INH level, low C4 level, normal C1q).¹⁶ Human plasma-derived C1-INH concentrate (Berinert; CSL Behring, Marburg, Germany) was used as an acute treatment for edematous attacks, or as short-term prophylaxis. All patients received pdC1-INH concentrate in every instance to relieve the most severe forms of edematous episodes (ie, upper airway, abdominal, facial, genital, and severe limb edema, predominantly).

The “symptom-free samples” were collected during the annual control visits. Furthermore, 106 samples in total were collected from the patients during edematous attacks, before administering the pdC1-INH concentrate (at least 1, but no more than 17 “during-attack samples” were obtained per patient). The severity and the location of edematous attacks, as well as the time from the onset until blood sampling, were recorded in the Hungarian HAE Registry.

Submucosal versus subcutaneous attacks

Submucosal edema in the upper airways may progress to involve the larynx within hours and thus cause suffocation. In the gut, the edema of the intestinal wall may mimic the clinical symptoms of an acute abdominal “catastrophe,” such as nausea, vomiting, colicky pain, and watery diarrhea, after the attack.

During the episode, abdominal ultrasound depicts free peritoneal fluid and in a large proportion of cases, it also confirms edema of the intestinal wall.

Subcutaneous edema most commonly affects the extremities, face, eyelids, neck, torso, gluteal region, and external genitals. The ensuing swelling is painless and it is not accompanied by erythema and pruritus because the edema involves the deeper cutaneous layers containing fewer mastocytes and sensory nerve endings. On the face, subcutaneous edema may extend to involve the mucosa of the upper airways. On the neck, it can cause complications through compression, whereas the subcutaneous swelling of the chest may be accompanied by pericardial or pleural effusion.

Healthy controls

The control group consisted of 54 healthy adults (21 men and 33 women; median age, 33 years; 25th-75th percentiles, 21-58 years). All the subjects had been referred for routine medical check-up, and they had volunteered for the study by giving informed consent. The healthy controls did not have any known disease, or receive medicinal products at the time of blood sampling. C1-INH deficiency was excluded by complement testing in all healthy subjects. Patients with HAE-C1-INH and controls were not statistically different as regards age and sex distribution.

Blood sampling

Serum and EDTA-plasma samples obtained from patients with HAE-C1-INH both during symptom-free periods and during attacks were stored at -70°C until processing. Peripheral blood samples were also drawn from healthy subjects, as prescribed by the study protocol. The latter was approved by the Institutional Review Board of Semmelweis University of Budapest, and informed consent was obtained in accordance with the Declaration of Helsinki.

Measurement of the lectin pathway parameters

The levels of ficolin-2,²¹ and ficolin-3,²² as well as of the ficolin-3/MASP-2 complex²³ were determined by using standard sandwich ELISA techniques, using mAbs specific for each molecule. Biotinylated antibodies were added to the second layer, and streptavidin/horseradish peroxidase complexes were used for detection. All samples were tested in duplicate against a standard serum pool with known content of each analyte.

Ficolin-3-mediated terminal complement complex (FCN3-TCC) deposition was measured as described previously by Hein et al.²⁴ In brief, acetylated BSA was immobilized in Maxisorb ELISA plates and used as a ficolin-3 ligand. To block any interference from the classical pathway or the alternative pathway,²⁵ full serum samples were preincubated with sodium polyanethole sulfonate. Serum samples were diluted 1:25 in barbital buffer containing 0.05% Tween-20 and incubated on the plate for 45 minutes at 37°C . Thereafter, mouse-anti-human TCC was applied for 2 hours at room temperature and then, rabbit-anti-mouse-horseradish peroxidase was added to the wells as secondary antibody, for 1 hour at 37°C . Finally, the plates were developed using ortho-phenylenediamine substrate and the OD was determined at 490/630 nm.

Commercial kits were used to quantify the levels of MASP-2 in EDTA-plasma samples. We used a solid-phase ELISA based on the sandwich principle (Hycult Biotech, Inc, Uden, The Netherlands), described by Møller-Kristensen et al.²⁶ We measured the functional activity of C1-INH in serum samples (Quidel, San Diego, Calif) according to the manufacturer's instructions.

To measure the concentration of C4, radial immunodiffusion was performed using polyclonal rabbit anti-human C4c (Dako, Glostrup, Denmark) and human serum protein calibrator (Dako).

Statistical analysis

Statistical calculations were performed with Prism for Windows (version 5.02; GraphPad Software, Inc, San Diego, Calif; www.graphpad.com) and SPSS (version 13.0; SPSS, Inc, Chicago, Ill). The Mann-Whitney *U* test was used to compare 2 independent groups, whereas the Wilcoxon test was chosen to compare the symptom-free and during-attack results of the same

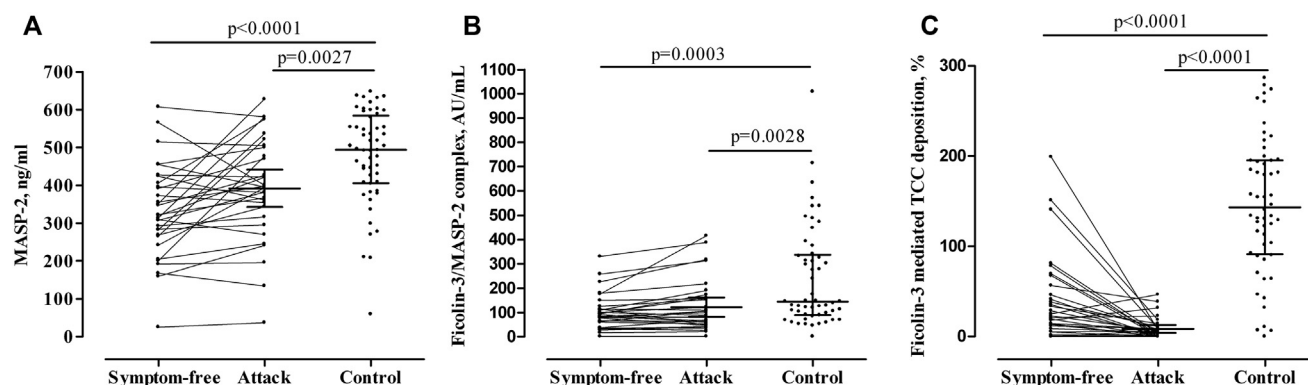


FIG 1. The levels of the ficolin-lectin pathway parameters (A, MASP-2; B, Ficolin-3/MASP-2 complex; C, Ficolin-3 mediated TCC deposition) during symptom-free periods and attacks in the same patients with HAE-C1-INH and in healthy controls. Patients who had several attacks are represented by mean values. Control vs “symptom-free” or “during attack”: Mann-Whitney *U* test.

patients. If a patient had several “during-attack samples,” a mean value was calculated and used for paired statistical comparison (Fig 1). To take into account all attack samples, a linear mixed model (LMM) was also fitted to estimate changes between the symptom-free and during-attack states of the same patients. In this model, the symptom-free or during-attack type of sample was included in the LMM as a fixed effect, whereas patient ID and the number of subsequent attacks were included as random effects, to adjust for correlation among repeated measures within subjects. Different measures of the lectin pathway were each included as a dependent variable in the LMM.

When calculating correlations using Spearman rho, each patient was characterized by a randomly selected value for each parameter. All the statistical analyses were 2-tailed, and $P < .05$ was considered to represent a significant difference, or correlation.

RESULTS

The levels of the ficolin-lectin pathway parameters and complement activation products during symptom-free periods and attacks in the same patients

To analyze the role of the ficolin-lectin pathway in the development of edematous attacks, we determined the native components of the ficolin-lectin pathway, as well as its functional activity (based on the FCN3-TCC deposition) in samples collected from the same patients during symptom-free periods and attacks (patients who had several attacks are represented by mean values in Fig 1).

Interestingly, the median level of MASP-2 was elevated during attacks than during symptom-free periods of the same patients (paired *t* test, $P = .0056$). The level of MASP-2 was significantly lower both in symptom-free samples (320.0 [265.9-406.0] ng/mL; $P < .0001$) and in during-attack samples (396.3 [316.2-500.4] ng/mL; $P = .0027$) than in those from healthy subjects (494.6 [406.6-581.4] ng/mL) (Fig 1).

A mild but significant elevation was observed in the level of the ficolin-3/MASP-2 complex during attacks than during the symptom-free period (87.8 [46.6-160.5] AU/mL vs 84.5 [36.9-114.7] AU/mL; paired *t* test, $P = .0261$). Healthy subjects were characterized by higher levels of the ficolin-3/MASP-2 complex (144.9 [89.87-336.2] AU/mL), as compared with the symptom-free ($P = .0003$) and during-attack periods ($P = .0028$) of the patients (Fig 1).

In contrast, FCN3-TCC deposition was markedly decreased during attacks than during the symptom-free period (5.1%

TABLE I. The levels of the ficolin-lectin pathway parameters during symptom-free periods and attacks in the same patients with HAE-C1-INH

Dependent variable	Estimate*	95% CI		P value*
		Lower bound	Upper bound	
Ficolin-2 (μg/mL)	−0.458	−0.511	−0.406	.206
Ficolin-3 (μg/mL)	−0.502	−0.967	−0.038	.781
MASP-2 (ng/mL)	56.568	54.667	58.469	<.0001
Ficolin-3/MASP-2 complex (AU/mL)	16.058	15.492	16.624	.033
FCN3-TCC deposition (%)	−22.916	−25.344	−20.489	<.0001

The LMM was used to simulate random effects and repeated measures to detect differences between the “symptom-free” and the “during-attack” states of the same patients, including all measured parameters.

Values in boldface represent significant differences between the “symptom-free” and the “during-attack” states.

*For the difference between the “during-attack” and the “symptom-free” samples.

[0.5-9.4] vs 23.2% [8.8-56.6]; paired *t* test, $P = .0002$). Furthermore, the median level of FCN3-TCC in patients was significantly reduced both in the symptom-free ($P < .0001$) and in the during-attack ($P < .0001$) states than in healthy subjects (143.4% [91.9-195.3]) (Fig 1).

To analyze the changes in complement parameters in patients during attacks in comparison with reference (without attack) samples, a mixed linear model was applied. This model permits the inclusion of all the data obtained during various attacks of the different patients (random effects) and even allows for the presence of missing data. After performing the LMM analysis, we found the same measures between the symptom-free and during-attack states (fixed effect) because they were statistically significantly different, which confirms the results of the previous paired *t* test (Table I).

There was no difference between the ficolin-2 (5.2 [3.6-8.0] μg/mL vs 5.3 [3.4-6.9] μg/mL; paired *t* test, $P = .5867$) or ficolin-3 levels (20.1 [13.9-27.9] μg/mL vs 18.8 [14.6-28.9] μg/mL; paired *t* test, $P = .7853$) measured in the symptom-free or in the during-attack state of the same patients with HAE-C1-INH.

We could not detect significant differences among attacks of subcutaneous, submucosal, or mixed location in respect to any of the lectin pathway parameters.

Despite the significant changes in the parameters (Fig 1 and Table I), we observed some heterogeneity among the patients,

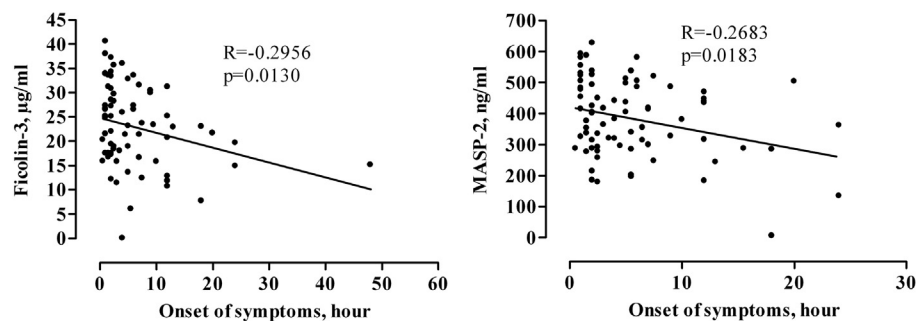


FIG 2. Correlations between time from the onset of the symptoms to blood sampling, and the parameters of the ficolin-lectin pathway. In many patients, multiple values of the studied parameters were available. Of these, we selected one at random (from each patient) for use in the statistical analysis of the individual parameters. Spearman rank correlation coefficient was calculated.

which may be explained by the different trigger factors or mechanisms leading to edema formation. MASP-2 concentrations increased (in 71.4% [25 of 35] of the patients), did not change (5.7% [2 of 35]), or decreased (22.9% [8 of 35]) during attacks than during the symptom-free period. Similarly, the changes in ficolin-3/MASP-2 complex levels during attacks were inconsistent (increase in 65.7% [23 of 35], no change in 14.3% [5 of 35], whereas a decrease in 20% [7 of 35] of the patients) when compared with the symptom-free period. The extent of FCN3-TCC deposition decreased in 88.6% (31 of 35), did not change in 2.8% (1 of 35), or increased in 8.6% (3 of 35) of the patients during attacks compared with the symptom-free period.

Furthermore, we identified the following patterns during attacks experienced by the study patients: (a) MASP-2 concentration increased along with the level of the ficolin-3/MASP-2 complex during attacks in 19 patients; (b) MASP-2 concentration increased and the extent of FCN3-TCC deposition decreased during attacks in 23 patients; and (c) the concentration of the ficolin-3/MASP-2 complex increased and the extent of FCN3-TCC deposition decreased during attacks in 20 patients. Therefore, most of the patients were characterized by the signs of lectin pathway activation during attacks.

Correlations among the ficolin-lectin pathway parameters and other complement components

We analyzed whether there is a correlation between the ficolin-lectin pathway parameters and further complement components in patients with HAE-C1-INH, either during the symptom-free period or during attacks. The different correlation patterns identified during symptom-free periods and during attacks suggest that the C1-INH-controlled segment of the ficolin-lectin pathway undergoes rearrangement during edematous episodes. See further details related to this section in this article's Online Repository at www.jacionline.org.

Correlations between the time from the onset of the symptoms to blood sampling, and the parameters of the ficolin-lectin pathway

We analyzed whether there is any relationship between the lectin pathway parameters and the time from the onset of the edematous attack until blood sampling. Interestingly, both ficolin-3 ($R = -0.2956$, $P = .0130$) and MASP-2 ($R = -0.2683$,

$P = .0183$) correlated inversely with this period (Fig 2). If we omitted the longer time points (>20 hours in case of ficolin-3 and >18 hours in case of MASP-2), the correlation between the time interval and the ficolin-3 ($R = -0.2837$; $P = .0173$) or MASP-2 ($R = -0.2703$; $P = .0198$) levels remained significant.

DISCUSSION

We have described previously that both the MBL-lectin pathway¹³ and the ficolin-lectin pathway¹⁴ undergoes activation in HAE-C1-INH, even during symptom-free periods. Furthermore, we have detected a remarkable, inverse relationship between the markers of the severity of HAE-C1-INH and ficolin levels during our previous study. However, the possible role of the ficolin-lectin pathway in the development of edematous attacks has not yet been investigated.

Our study—the first to analyze blood samples obtained during symptom-free periods and during attacks from a large number of the same patients with HAE-C1-INH—has shown that the edematous attacks may be accompanied by the activation of the ficolin-lectin pathway. We observed a mild but significant elevation in the levels of MASP-2 and of the ficolin-3/MASP-2 complex during attacks than during the symptom-free period of the same patients. Although we refrained from drawing a far-reaching conclusion about the elevation in levels of MASP-2 seen during the attacks, we considered it important to present this finding to provide a more comprehensive view on the corresponding changes in the lectin pathway. However, the biological importance or background of this observation is still unknown and further studies are needed to explain this phenomenon. Our previous experiments have shown that increasing the concentration of acetylated BSA (specific activator of ficolin-3) induced a dose-dependent elevation in the level of the ficolin-3/MASP-2 complex.²³ Thus, we may presume that the presence of a specific activator agent induces the elevation in ficolin-3/MASP-2 level during attacks, at least in a proportion of the cases (eg, in attacks triggered by infection). Moreover, the extent of FCN3-TCC deposition was smaller during attacks than during the symptom-free period of the same patients, and this also suggests the activation of the ficolin-lectin pathway during attacks. When measuring FCN3-TCC deposition, the samples are activated *in vitro* by a specific ligand of ficolin-3 (acetylated BSA) and then, *in vitro*-induced TCC deposition is analyzed. Therefore, low levels of FCN3-TCC deposition suggest that the ficolin-lectin pathway has already been activated *in vivo*

and only minor, residual activation remains possible *in vitro*. Taken together, the elevation in the ficolin-3/MASP-2 complex and the decreased extent of FCN3-TCC deposition suggest that the ficolin-lectin pathway may be activated during attacks. There is a marked heterogeneity of the pathomechanism and development of hereditary angioedema attacks in different patients, and in a proportion of the patients the ficolin-lectin-MASP pathway may play an important role.

Interestingly, we found an inverse relationship between ficolin-3, as well as MASP-2, levels and the time from the onset of the attack to blood sampling. It means that the longer the time elapsed from the onset of the symptoms the lower is the level of the above-mentioned parameters. This relationship remained significant if patients with longer time intervals were excluded from the analysis. However, further studies are needed to determine whether the activation of the ficolin-lectin pathway is a cause or a consequence of the edematous attacks. For this purpose, it would be interesting and informative to perform serial measurements of the above parameters during attacks in individual patients.

Although the activation of the lectin pathway during edematous attacks has not been studied until now, Nielsen et al²⁷ performed a comprehensive study on the activation of the classical complement pathway during such episodes. Interestingly, they detected increased SC5b-9 formation during attacks than during symptom-free periods. This may suggest that the complement system undergoes activation during attacks, in agreement with our findings.

Because the low level of FCN3-TCC may result from the decreased levels of both C4 and MASP-2, we introduced a new quantitative method for measuring the concentration of the ficolin-3/MASP-2 complex. Thus, we can now predict the activation potential of the ficolin-lectin pathway regardless of the levels of downstream components.²³ This new method is a useful add-on to the functional FCN3-TCC deposition test introduced earlier by Hein et al.²⁴ Because the level of the ficolin-3/MASP-2 complex has not yet been studied in other disorders, our results may contribute to a better understanding of its role in a model of the dysfunctional regulation of C1-INH.

On the basis of our findings, we presume that edematous attacks may be triggered or influenced by a yet undefined activating agent that contributes to the activation of the ficolin-lectin pathway.

When present in relatively high concentrations, the activation of ficolin-bound MASP-1 and MASP-2 may contribute—through consumption—to the depletion of the innately low level of functional C1-INH. This may have a role in the uncontrolled activation of the systems regulated by C1-INH, as well as in the ensuing edema formation. Furthermore, MASP-1 plays a role in the cleavage of high-molecular-weight kininogen (even in the absence of kallikrein) and in the release of bradykinin. This may represent a novel link between the complement system and the pathomechanism of edema formation, based on previous studies by Dobó et al.²⁸ Moreover, elevated MASP-1 and MASP-2 levels have been found during episodes of stress and/or infection, both of which are known initiators of hereditary angioedema attacks.¹⁸ Further studies would be useful to analyze the role of MASP-1 and/or MASP-2 (and eventually of their complexes formed with C1-INH) in the symptom-free and during-attack samples of the same patients. This could yield more comprehensive and detailed information on the role of these serine-proteases in the pathomechanism of hereditary angioedema.

Clinical implications: Activation of the ficolin-lectin pathway may deplete the innately low level of C1-INH and it may contribute to the uncontrolled activation of plasma cascade systems, and thereby to edema formation.

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CORRELATIONS AMONG THE FICOLIN-LECTIN PATHWAY PARAMETERS AND OTHER COMPLEMENT COMPONENTS

During the symptom-free period, patients with HAE-C1-INH exhibited the strongest positive correlations between the extent of FCN3-TCC deposition and the concentrations of C4 ($R = 0.5307$; $P < .0001$), the ficolin-3/MASP-2 complex ($R = 0.4670$; $P < .0001$), MASP-2 ($R = 0.4194$; $P < .0001$), antigenic C1-INH ($R = 0.3152$; $P = .0006$), C3 ($R = .3226$; $P = .0022$), or ficolin-3 ($R = 0.2778$; $P = .0022$). Furthermore, a significant

positive correlation was observed between the levels of the ficolin-3/MASP-2 complex and of MASP-2 ($R = .6132$; $P < .0001$).

During attacks, the most prominent, positive correlations were found between the levels of the ficolin-3/MASP-2 complex and of functional C1-INH activity ($R = 0.6059$; $P = .0028$) or the levels of MASP-2 ($R = 0.5911$; $P = .0005$). Furthermore, ficolin-2 showed a significant positive correlation with the functional C1-INH activity ($R = 0.4640$; $P = .0257$) during attacks.