C1 INHIBITOR DEFICIENCY AND ANGIOEDEMA

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C1 INHIBITOR DEFICIENCY AND ANGIOEDEMA

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Editorial: C1 inhibitor deficiency and angioedema

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Editorial on the Research Topic
C1 inhibitor deficiency and angioedema

By Farkas H, Germenis AE, Longhurst H. (2022) Front. Allergy. 3: 1065400. doi: 10.3389/falgy.2022.1065400

The turn of the century was followed by a rapid expansion of our knowledge about C1-inhibitor (C1-INH) and angioedema. The role of C1-INH as the most important inhibitor in the kinin-forming cascade was further elucidated along with many other aspects of the production and regulation of bradykinin; genomics allowed a faster and more integrated analysis of the *SERPING1* gene as well as the recognition of five genes –beyond the *F12* gene– associated with angioedema with normal C1-INH (HAE-nC1-INH); and an abundance of therapeutic modalities were developed, perhaps higher than any other rare disease.

In the 23 years since its inception, the international C1 inhibitor Deficiency and Angioedema Workshop has been the premier forum for the presentation and discussion of this remarkable progress. Nine representative works presented in the most recent 12th C1 inhibitor Deficiency and Angioedema Workshop, held virtually, are included in this Research Topic.

In a detailed review article Kaplan et al. present the complex role of kininogens, and the substrates from which bradykinin is derived in hereditary angioedema (HAE), focusing on the multifaceted mechanisms by which high molecular weight kininogen simultaneously augments all the steps required for bradykinin formation and intrinsic coagulation and fibrinolysis, beyond being a substrate from which bradykinin is generated. These interacting mechanisms are intimately involved in manifestations of hereditary angioedema caused by C1 inhibitor deficiency, and beyond.

The diagnosis of hereditary angioedema is still challenging, especially in regard with special patient groups and/or the use of emerging approaches, i.e., genetics. Andrási et al. summarize their experience in the diagnostics of C1-inhibitor deficiency in pediatric age. The study presents a strategy for monitoring children in an Angioedema Center, emphasizes the importance of screening and performing complement and DNA tests not

just in peripheral blood, but also in cord blood to allow the early and proper diagnosis before the onset of clinical manifestations, even in cases with misleading complement result. Early diagnosis provides an opportunity to supply the patient with the appropriate treatment, as HAE attacks can occur at any age and their onset cannot be predicted in advance.

The wide and easy application of genomic techniques made intriguing the role of genotyping in the diagnosis of HAE due to C1-deficiency (HAE-C1-INH). Szabó et al. present their strategy on genetic work-up exploring the *SERPING1* gene and provide an overview of the mutations identified in a large cohort of Hungarian HAE-C1-INH patients. The combination of conventional and novel methods allowed them to unravel the genetic cause behind C1-inhibitor deficiency in each affected pedigree involving the identification of five, previously unreported variations. Detection and correct interpretation of disease causing *SERPING1* variants is of great importance in HAE-C1-INH as by facilitating correct and early diagnosis the patient's proper treatment, prognosis and quality of life can be improved.

Recognizing C1-INH features and *SERPING1* genetics together is a prerequisite for the curation of variant pathogenicity. With this aim, Drouet et al. reviewed all 809 reported variants of the SERPING1 gene in relation to the biological and structural features of C1-INH. This is largest study of the constellation of *SERPING1* variants found in nearly 1,500 HAE families, emphasizing that etiopathogenesis of HAE-C1-INH can be consistently predicted by C1-INH molecular analyses.

In regard with the diagnosis and management of HAE, pregnancy remains an interesting aspect of the disease. Considering the strong relevance of estrogens in HAE-nC1-INH, Gabriel et al. investigated the history of 45 pregnancies occurring in 26 HAE-nC1-INH patients. They found that the occurrence of abortion in HAE-nC1-INH was similar the expected rate for unaffected women. However, the first trimester of the pregnancy was more symptomatic for HAE-nC1-INH women. The authors concluded that although pregnancy could not be inputted as more dangerous for women with HAE-nC1-INH than the disease *per se*, a multidisciplinary approach, involving the obstetrician and other health care professionals when needed, would be beneficial.

The great variability of the clinical expression of HAE remains a serious, yet unmet problem. Serious efforts are made towards detecting biochemical and/or genetic biomarkers for diagnostic, prognostic and preventive use. Using a liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) platform of picomolar sensitivity developed for the analysis of eleven bradykinin (BK)-related peptides, Marceau et al. measured the presence of BK-related peptides in the plasma of patients with HAE-C1INH and HAE-FXII during remission, in order to examine whether some of these peptides might be biomarkers of these forms of the disease. According to their results, the concentrations of BK_{1-5}, BK_{2-9} and the sum of BK and its fragments could be used as biomarkers of HAE-C1-INH but not of HAE-FXII in remission.

On the other hand, accumulating evidence indicates that clinical variability of HAE-C1-INH is substantially attributable to modifier genes. To further examine this hypothesis, Parsopoulou et al. investigated the presence or absence of 18 functional variants of genes encoding proteins involved in the metabolism and function of bradykinin, in relation to three distinct phenotypic traits of patients with HAE-C1-INH, i.e., the age at disease onset, the need for long-term prophylaxis (LTP), and the severity of the disease. Their findings confirmed that variants other than the *SERPING1* causal variants, like *F13B-rs6003, PLAU-rs2227564, SERPINA1-rs28929474, SERPINA1-rs17580, KLK1-rs5515, SERPINE1-rs6092*, and *F2-rs1799963*, act as independent modifiers of HAE-C1-INH severity and could be tested as possible prognostic biomarkers.

Finally, as the treatment of HAE is considered, in their review article, Valerieva and Longhurst compare C1-INH replacement with newer therapies targeting the contact pathway. Both approaches have been shown to be effective for acute treatment and prophylaxis; with approved and investigational therapies showing therapeutic efficacy of inhibition of a number of targets in the contact pathway. These include (pre)kallikrein, the bradykinin B2 receptor and activated factor XII. A variety of therapeutic mechanisms, including small molecules, monoclonal antibodies, RNA silencing and genetic therapies are available or in development.

Accordingly, in their case series study, Zanichelli et al. report their real-life experience from HAE-C1-INH patients poorly controlled with their previous long-term prophylaxis or with difficult venous access, who received subcutaneous plasma-derived C1-inhibitor (pdC1-INH) at lower than the recommended doses. Their results indicate that, in patients with difficult venous access, in countries where pdC1-INH is not approved for subcutaneous administration, about half the recommended dose may be beneficial, although suboptimal results may be obtained, compared with licensed doses.

We hope that this Research Topic will provide a brief indication of the current state of the art and suggestions for further discussion during the forthcoming 13th C1 inhibitor Deficiency and Angioedema Workshop.

Author contributions

All authors contributed equally to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Picomolar Sensitivity Analysis of Multiple Bradykinin-Related Peptides in the Blood Plasma of Patients With Hereditary Angioedema in Remission: A Pilot Study

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Background: Hereditary angioedema (HAE) is a rare autosomal dominant disease; the most well understood forms concern the haplodeficiency of C1 esterase inhibitor (C1INH) and a gain of function mutation of factor XII (FXII). The acute forms of these conditions are mediated by an excessive bradykinin (BK) formation by plasma kallikrein.

Methods: A validated LC-MS/MS platform of picomolar sensitivity developed for the analysis of eleven bradykinin-related peptides was applied to the plasma of HAE-C1INH and HAE-FXII sampled during remission.

Results: In HAE-C1INH plasma, the concentrations of the relatively stable BK_{1-5} fragment (mean ± S.E.M.: 12.0 ± 4.2 pmol/L), of BK_{2-9} (0.7 ± 0.2 pmol/L) and of the sums of BK and its tested fragments (18.0 ± 6.4 pmol/L) are significantly greater than those recorded in the plasma of healthy volunteers (1.9 ± 0.6, 0.03 ± 0.03 and 4.3 ± 0.8 pmol/L, respectively), consistent with the previous evidence of permanent plasma kallikrein activity in this disease. Kinin levels in the plasma of HAE-FXII patients did not differ from controls, suggesting that triggering factors for contact system activation are not active during remission.

Conclusion: BK_{1-5}, BK_{2-9} and the sum of BK and its fragments determined by the sensitive LC-MS/MS technique are proposed as potential biomarkers of HAE-C1INH in remission while this was not applicable to HAE-FXII patients.

Keywords: hereditary angioedema, bradykinin, *F12* variant, *SERPING1* variants, LC-MS/MS

INTRODUCTION

Hereditary angioedema (HAE) is an autosomal dominant group of disorders that are determined by several gene variants proven or postulated to be permissive for bradykinin (BK) production, and possibly repressive for its degradation, with ensuing action on the endothelial BK B_2 receptors and localized edema of subcutaneous and submucosal tissues (1, 2). Many variants of the *SERPING1* gene encoding C1 esterase inhibitor (C1INH) with impaired expression or function cause the most common form of HAE (types 1 and 2, respectively). A rarer form of HAE with normal C1-INH levels is caused by mutation of genes encoding coagulation factor XII (FXII, *F12* gene) (3); further, several other causal gene variants have been identified, some plausibly associated with the kallikrein-kinin system (plasminogen *PLG* and kininogen *KNG1*) (1). The most common variants of FXII causing HAE-FXII introduce new sites of cleavage by plasmin that accelerate cleavage by this protease, a basis for a gain of function in the contact system (4). However, for many patients, no mutation or abnormalities have been yet found and they may differ in several aspects including gender distribution, genetics, symptoms, and impact of estrogens.

The nonapeptide BK, formed by plasma kallikrein from high molecular kininogen (HK), is inherently unstable in blood plasma with a half-life in the order of 30 sec (5, 6). Several peptidases located in plasma or expressed at the surface of endothelial cells hydrolyze BK in live rats: in decreasing order of importance, angiotensin-I converting enzyme (ACE), aminopeptidase P, and neutral endopeptidase (7). Dipeptidyl peptidase IV and arginine carboxypeptidases have only marginal roles in the inactivation of BK *in vivo*. Single, repeated, or combined actions of these peptidases potentially generate many BK fragments, some of which were identified by high pressure liquid chromatography (HPLC) from concentrated synthetic BK incubated in plasma, serum or other peptidase sources (8, 9). Notably, the BK_{1-5} fragment, generated by 2 successive hydrolytic reactions catalyzed by ACE, is relatively stable and reportedly accumulates, notably after the infusion of synthetic BK in humans (10). Plasma BK_{1-5} also increases in acquired angioedema associated with therapeutic ACE inhibition (11), as BK breakdown inhibition may also result in angioedema.

Using a validated liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) platform of picomolar sensitivity developed for the analysis of eleven bradykinin-related peptides (12–16), we addressed the presence of BK-related peptides in the plasma of patients with HAE-C1INH and HAE-FXII during remission. We hypothesized that some of these peptides, including BK_{1-5}, might be biomarkers of these forms of the disease, even if the buffering activity of kininases kept the patients symptom-free (kinin levels below a pharmacologic threshold for vascular effects).

MATERIALS AND METHODS

Human Subjects

The local ethical review board (Comité d'éthique de la recherche, CHU de Québec-Université Laval) granted ethical approval to carry out the study involving blood donations from adult healthy volunteers and HAE patients 16 years old or older from the Province of Quebec, Canada (file no. 2022-6044). Locally recruited healthy volunteers also participated under the same ethical approval. All subjects gave written informed consent. Patient characteristics are listed in **Tables 1**, **2** and their blood was withdrawn when in remission. HAE patients received various prophylactic treatments that were not interrupted during the study (**Table 2**). Six female and one male patients heterozygotes for p.Thr328Lys in FXII (also known as T309K in reference to mature FXII sequence; 1032C>A in *F12*) were recruited. They were of Mediterranean descent. All HAE-FXII subjects, except the completely asymptomatic young male (subject F2), were included in a previous study (6); the molecular diagnostic procedures were described in that report. All HAE-FXII female patients, except one (subject F6), suffered from an essentially estrogen-dependent form of the disease, during pregnancies, oral contraception, or estrogen administration for menopausal symptoms. None of these conditions were present at the time of blood sampling. HAE related to *SERPING1* variants (haplodeficiency of C1INH) concerned well characterized patients diagnosed using low antigenic C4 levels, functional C1-INH measurements and familial history.

Blood Sampling for Kinin Analysis

Blood of adult healthy volunteers and HAE patients was sampled in protease-inhibitor prespiked EDTA S-Monovettes® (Sarstedt, Nümbrecht, Germany). The applied seven-component protease inhibitor was previously shown to efficiently stabilize kinin levels *ex vivo* (14). In addition, a standardized procedure was applied for blood sampling to control impacts of blood sampling on generation of BK levels (16). In short, blood was sampled via butterfly needles with the aspiration technique. After blood sampling, kinins were centrifuged within 30 min and plasma was frozen and stored at −80 °C until analysis.

LC-MS/MS Determination of Kinins

Kinins were measured based on a validated LC-MS/MS platform (15) being extended specifically for the present study. In comparison, an enhanced lower limit of quantification was achieved and the extend of measured kinins was increased to eleven (BK, BK_{1-8}, BK_{2-9}, BK_{1-7}, BK_{1-5}, Hyp^3-BK, Hyp^3-BK_{1-8}, KD, KD_{1-9}, Hyp^4-KD, Hyp^4-KD_{1-9}). To prove the validity and collection of high-quality data, all measurements were handled within a quality control system. The determination was conducted according to Good Clinical Laboratory Practice. Concentrations of unknown samples were reported up to the detection limit; values below this threshold were set to zero.

Data Analysis

Numerical values are reported as means ± standard errors of the mean (S.E.M.), a distribution-free assessment of mean value uncertainty. Sets of values were compared with Kruskall-Wallis test (non-parametric ANOVA) followed by Dunn's multiple comparison test to compare selected pairs of values (Prism 5.0, GraphPad Software Inc., San Diego, CA).

TABLE 1 | Summary of the pilot study (all HAE patients seen in remission).

Group	Age ± S.E.M.	Number of subjects	Number of female subjects	Sum of BK and fragments ± S.E.M. (pmol/L) *
Healthy volunteers	53.8 ± 4.7	9	7	4.3 ± 0.8
HAE-FXII	37.6 ± 4.7	7	6	7.4 ± 1.5
HAE-C1INH	50.1 ± 6.4	9	7	18.0 ± 6.3

Statistics reported in **Figure 1.*

TABLE 2 | Characteristics of patients with HAE.

No.	Sex, age range	HAE type*	Prophylactic treatment**	Last dose of prophylaxis before sampling	Sum of BK and fragments (pmol/L)
H1	F, 61–65	1	Berinert	2 weeks	16.46
H2	F, 66–70	2	None		1.70
H3	F, 21–25	1	Haegarda	1 day	8.28
H4	M, 71–75	1	Berotralstat	14 h	64.39
H5	F, 26–30	1	Haergarda	1 day	3.54
H6	F, 51–55	1	Haegarda + Berinert	2 days	8.56
H7	M, 61–65	1	Berotralstat	14 h	27.56
H8	F, 41–45	1	None		16.20
H9	F, 31–35	1	Berinert	2 days	15.17
F1	F, 46–50	*F12* T328K	Tranexamic acid	2 h	11.82
F2***	M, 16–20	*F12* T328K	None		1.60
F3	F, 41–45	*F12* T328K	None		10.88
F4	F, 26–30	*F12* T328K	None		9.72
F5	F, 36–40	*F12* T328K	None		8.62
F6****	F, 51–55	*F12* T328K	None		6.23
F7	F, 36–40	*F12* T328K	None		3.05

**Types 1 and 2 are variants of C1INH haplodeficiencies. **Berinert and Haegarda are C1INH concentrates. ***Son of F1. ****mother of F4, F5, F7.*

RESULTS

Three groups of subjects were included in this pilot study: healthy volunteers and patients with HAE-FXII or HAE-C1INH (demographic data reported in **Table 1**). The concentrations of kinin peptides measured in the plasma of venous blood samples are illustrated in **Figure 1** for the 3 groups of subjects. The physiopathology of both forms of HAE involves plasma kallikrein that directly releases BK from HK (2). BK concentrations in all three groups are extremely low in the samples (<10 pmol/L, **Figure 1**), consistent with its short half life and catabolism by multiple peptidases. This finding is in line with measured values in a larger cohort of 24 healthy adults using the same LC-MS/MS method (data not yet published). Low levels were further observed for the primary BK metabolite generated by ACE, BK_{1-7}. However, BK_{1-5}, produced by a further reaction of BK_{1-7} with ACE, has a better stability (8–10) and is a potential biomarker of HAE-C1INH, as its concentration is significantly higher in this group than in controls (**Figure 1**). On the other hand, BK_{1-5} concentrations in HAE-FXII plasma did not differ significantly from those of healthy volunteers.

The metabolites of BK generated by alternate peptidases were examined. Arginine carboxypeptidases (carboxypeptidases N, M) produce BK_{1-8}; this peptide was not informative in the present study (**Figure 1**). Aminopeptidase P is the second-most important BK clearing peptidase *in vivo*, after ACE (7). The concentration of the resulting BK_{2-9} fragment, itself biologically inactive (17), is nevertheless the most discriminative biomarker of HAE-C1INH in the present study despite its very low concentration (**Figure 1**). BK_{2-9} remains mostly undetectable in the plasma of HAE-FXII patients. A further analysis was attempted by adding up the non-overlapping concentrations of BK, BK_{1-7}, BK_{1-5}, BK_{1-8} and BK_{2-9}, based on the assumption that their sum may represent the minimal kinin concentration generated by plasma kallikrein, despite their different half-life and the limited selection of peptides. The sums are significantly higher in the HAE-C1INH patients than in healthy volunteers, the values from HAE-FXII patients remaining similar to those of controls (**Figure 1**, **Table 1**).

A fair proportion of kininogen molecules in the circulation are affected by a post-translational hydroxylation of the proline residue that coincide with Pro^3 in the BK sequence (Pro^4 in kallidin = Lys-BK) (18, 19). Whether the hydroxyproline (Hyp)-containing kinin are preferentially released from either form of kininogens by either form of kallikreins is not clear at present, but the pharmacology of Hyp^3-BK does not quantitatively differ from that of BK for interaction with the human B_2 receptor (20, 21). However, the applied LC-MS/MS

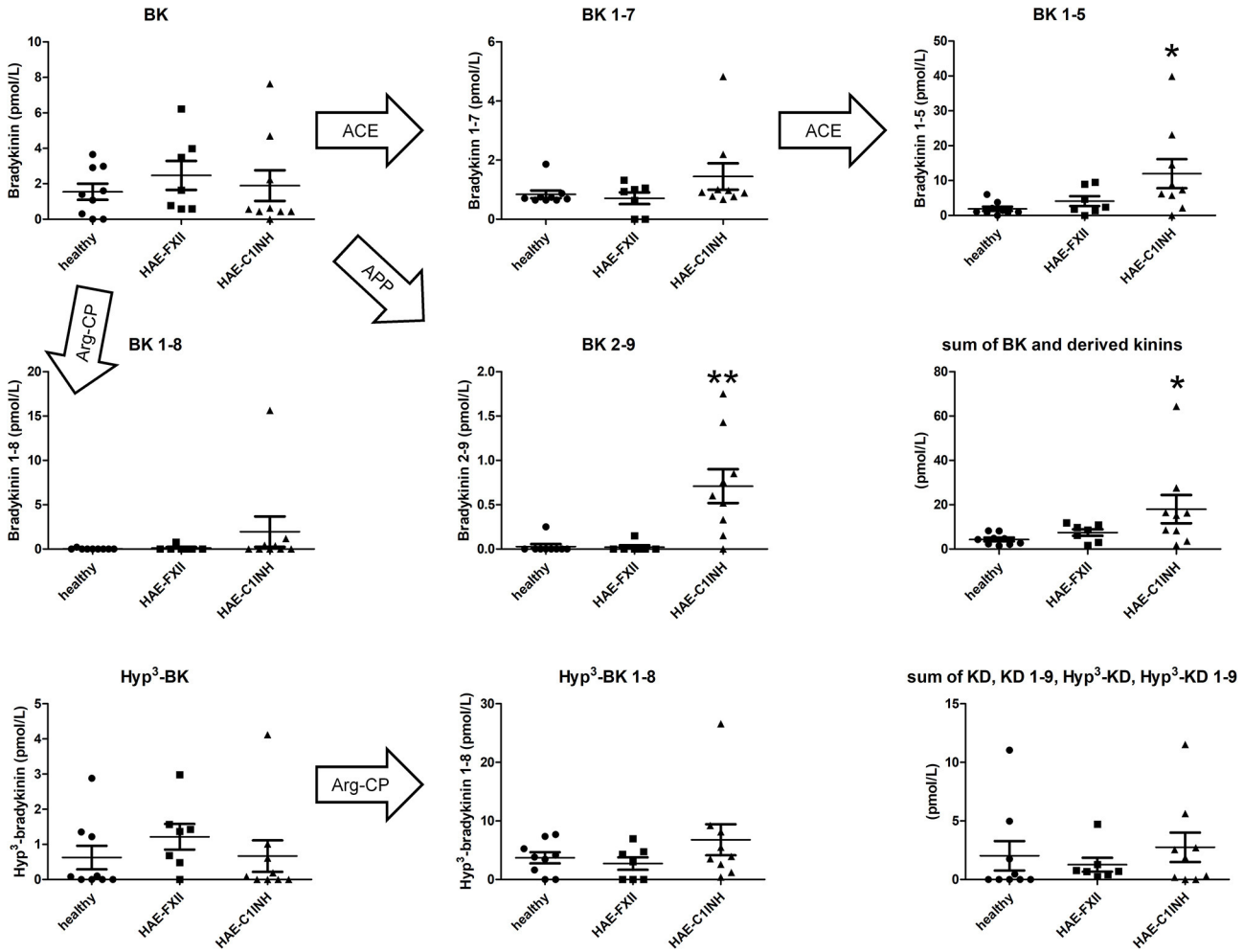

FIGURE 1 | Top rows: concentration of various BK-related peptides determined in the plasma of venous blood from healthy volunteers or patients with HAE-FXII or HAE-C1INH in remission (demographic data in **Table 1**). An additional panel (second row, right) shows the sum of BK and its fragments for each individual, presumed to represent the minimal concentration of kinins derived from plasma kallikrein activity. Bottom row: concentration of additional kinins. Individual values are shown. Horizontal bars are the mean, and the intervals the S.E.M. The Kruskall-Wallis test was applied to compare the effect of diagnostic categories. When significant, Dunn's multiple comparison test was applied to compare the values from each type of HAE patients to those of the healthy controls. $^{*}P < 0.05$; $^{**}P < 0.001$. Arrows indicate metabolic derivation of peptides from BK or Hyp^3-BK via the action of peptidases. ACE, angiotensin-I converting enzyme; APP, aminopeptidase P; Arg-CP, arginine carboxypeptidases; BK, bradykinin; Hyp, hydroxyproline; KD, kallidin (= Lys-BK); S.E.M., standard errors of the mean.

technique readily differentiate kinins possessing the Hyp residue owing to a different molecular weight. Validated tests for Hyp^3-BK and Hyp^3-BK$_{1-8}$ showed no differences between the 3 groups of subjects (**Figure 1**). Measurements of Hyp^3-BK$_{1-5}$ or Hyp^3-BK$_{2-9}$, possibly more discriminative, were not available.

Kallidin (KD = Lys-BK) is formed by the action of tissue kallikrein, mostly on low molecular weight kininogen (22). It is not believed that HAE-FXII or HAE-C1INH involve tissue kallikrein; rather, plasma kallikrein is a validated target for the development of prophylactic drugs such as lanadelumab, berotralstat and ecallantide for the prophylaxis of classical HAE attacks (23, 24). KD, Hyp^4-KD and their 1-9 fragments (with Arg^{10} removed) were measured in the plasma of the 3 groups of subjects. Concentrations generally remained very low and independent of the diagnostic category (the sums of the 4 peptides are illustrated in **Figure 1**).

Examination of sex or prophylactic treatments did not provide obvious explanations for extreme values of kinin concentrations in the patients with HAE group (**Table 2** reports individual values of the sum of BK and fragments).

DISCUSSION

Technological developments based on LC-MS/MS now supports the detection of several kinins with picomolar sensitivity and high reproducibility in biological fluids (12–16). The inclusion of biologically inactive fragments allows addressing the relative effects of various peptidases known to hydrolyze BK-related

peptides. The presence of cleaved HK in the blood of HAE-C1INH is well established during remission (25, 26) as well as a spontaneous plasma kallikrein activity (measured as the hydrolysis of synthetic HD-Pro-Phe-Arg-*p*NA) (27, 28). These results suggest a continuous formation of BK during remission in HAE-C1INH. While kinins are short-lived mediators *in vivo*, we hypothesized that the exquisitely sensitive LC-MS/MS detection of kinins might pick up bioactive kinins and their metabolites during remission of HAE-C1INH in a small sample of patients mostly under pharmacologic attack prophylaxis. It is a tribute to the detoxifying of multiple catabolic pathways that kinin concentrations in the venous blood were exceedingly small in all groups; even in the HAE-C1INH patients, the sum of BK and its fragments remained below a threshold for a biological activity mediated by the BK B_2 receptors. The concentration of the relatively stable metabolite BK_{1-5} generated from BK by ACE was significantly higher in these patients than that recorded in healthy volunteers, albeit with some overlap. The summed concentration of BK and its fragments was also significantly higher (**Figure 1**). An unexpected result was that the BK_{2-9} fragment, generated from BK by aminopeptidase P in a single step, behaved as a potential discriminative biomarker of HAE-C1INH, although the concentrations of BK_{2-9} were low in absolute values.

A relatively common form of HAE with normal C1INH involves a mutated FXII; the T328K or T329R substitutions introduce a new cleavage site for plasmin (4) and perhaps thrombin (29). Uncontrolled activation of plasma kallikrein is the consequence of this unconventional FXII cleavage. Although spontaneous cleavage of HK was anecdotally reported in HAE-FXII during remission (30), the plasma kallikrein enzymatic activity generally remained low; however, this activity definitely increased during attacks of HAE-FXII (27). We did not evidence increased concentration of kinin peptides in HAE-FXII patients in remission (**Figure 1**) and this must reflect the absence of triggering factors for the activation of the kallikrein-kinin system. The activation of the T328K mutant of FXII is postulated to occur during coagulation and fibrinolysis (4, 29), irrelevant processes in our patient sample, as well as the influence of estrogens. Indeed, attacks of HAE-FXII are particularly determined by the hormonal status, very rare in males and associated with estrogens as in pregnancies, oral contraception, and hormonal supplement for menopausal symptoms (31). On note, one of our female patients (subject F6) was never affected by attacks, although her 3 daughters were symptomatic in an estrogen-dependent manner in the past. These patients were included in a previous study where the patients' citrated plasma was incubated *ex vivo* and stimulated with recombinant tissue plasminogen activator to activate plasmin. Relative to control plasma, an explosive and rapid production of BK was observed in plasmas from all HAE-FXII patients including F6 (enzyme immunoassay of BK corroborated with signaling measurements in cells that expressed the human B_2 receptors) (6). These sharp differences in laboratory findings suggest that assessing *ex vivo* kinin formation is a promising complementary approach to investigate HAE with normal level of C1INH, especially if the very nuanced LC-MS/MS technique is exploited to quantify multiple kinin peptides.

KD is generated by tissue kallikrein (22); this form of secreted kallikrein is not relevant to the contact system and, therefore, to the physiopathology of the examined types of HAE. Of interest, the metabolite of KD generated by arginine carboxypeptidases, KD_{1-9} (or Lys-des-Arg^9-BK) is the optimal agonist of the human kinin B_1 receptor (32). The sum of KD and its fragments remained low and uninfluenced by HAE (**Figure 1**); such peptides may derive from alternate physiological or pathological processes. KD and KD_{1-9} were identified in nasal lavage fluid collected in healthy volunteers (13). However, the removal of the N-terminal Lys residue from these peptides by aminopeptidase N (CD13) (33) could potentially "contaminate" the concentration values of BK and its fragments to a small extent.

The limitations of the present pilot study are that the number of subjects is small, that the HAE patients were not seen during attacks and that many were under prophylactic treatments. Further, the detection of the most relevant fragments of Hyp^3-BK were not presently clinically validated.

In conclusion, the concentrations of BK_{1-5}, BK_{2-9} and the sum of BK and its fragments determined by a sensitive LC-MS/MS technique are proposed as potential biomarkers of HAE-C1INH in remission. This was not applicable to HAE-FXII, although excessive stimulated *ex vivo* BK generation was previously demonstrated. Future work based on the LC-MS/MS technique applied to *ex vivo* generation of multiple kinin peptides by standardized stimuli (6, 34, 35) is warranted to address the physiopathology of other HAE forms with normal C1INH level, especially because the spontaneous activity of tissue kallikrein is often low in these patients (27). The detection of multiple kinin metabolites is also ideal to address the effect of genetically determined deficiencies in kininase expression, postulated to interact with other causal genes for the severity of HAE states (1, 36).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study received approval by the local Ethical Review Board (Comité d'éthique de la recherche, CHU de Québec-Université Laval, file no. 2022-6044). Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

G-ER, JH, and FM identified and interacted with patients and organized sampling. TG, BB, FM, G-ER, JG, and HB participated to the experimental work. FM analyzed results and wrote the manuscript draft. All authors read and approved the final manuscript.

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BRIEF RESEARCH REPORT
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Pregnancy in Patients With Hereditary Angioedema and Normal C1 Inhibitor

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Background: HAE with normal C1 inhibitor (HAE-nC1-INH) has been identified as a bradykinin mediated angioedema. Estrogens are one of the main trigger factors. Pregnancy in HAE with C1 inhibitor deficiency showed variable course, however, few reports are available for HAE-nC1-INH. We evaluated the course of pregnancies in women diagnosed with HAE-nC1-INH.

Methods: Women with diagnosis of HAE-nC1-INH according to the following criteria: clinical manifestations similar to HAE-C1-INH, normal biochemical evaluation and family history were included. A questionnaire about pregnancies was applied after consent. Genetic evaluation for known mutations was performed in all patients.

Results: A total of 45 pregnancies occurring in 26 HAE-nC1-INH patients were evaluated (7/26 patients with *F12* variant). Spontaneous abortion was reported in 8/45 (17.8%) pregnancies. Onset of attacks started before the pregnancy in 18/26 patients; during the pregnancy in 2/26; and after the pregnancy in 6/26. HAE attacks occurred in 24/37 pregnancies (64,7%): during the 1st trimester in 41.7%; 2nd trimester in 12.5%; 3rd trimester in 20.8%; 1st and 3rd trimesters in 4.2% and during the whole pregnancy in 20.8%. Among 15/18 patients who had attacks before pregnancy, symptoms persisted with worsening in 9/15; improvement in 4/15; no change in 1/15, and no response in 1/15.

Conclusions: The occurrence of abortion in HAE-nC1-INH was similar to the expected for not affected women. The 1st trimester of the pregnancy was more symptomatic for HAE-nC1-INH women. Considering the strong relevance of estrogens in HAE-nC1-INH, pregnancy could worsen the course of disease.

Keywords: pregnancy, hereditary angioedema, hereditary angioedema with normal C1 inhibitor, FXII, mutation

INTRODUCTION

Hereditary angioedema (HAE) is a rare disease with autosomal dominant inheritance, characterized by recurrent episodes of subcutaneous and sub-mucosal edema attacks. HAE can be classified in HAE with C1 inhibitor deficiency (HAE-C1-INH) or HAE with normal C1 inhibitor (HAE-nC1-INH) (1). In both cases, angioedema occurs due to excessive activation of the plasma contact system, leading to increased levels of bradykinin and, consequently, an increase in vascular permeability and extravasation of fluids to the extravascular environment (2). The clinical manifestations of HAE-nC1-INH include angioedema attacks affecting extremities, face, tongue, genitals, abdomen, and upper airways. Unnecessary abdominal surgeries and asphyxia are severe complications related to inadequate treatment of HAE patients.

HAE-nC1-INH was first described in 2000 after the observation of angioedema without wheals affecting several women in the same family (3). Since then, genetic variants in the genes coding for Coagulation Factor XII (FXII), plasminogen, angiopoetin 1, kininogen 1, myoferlin, and heparan sulfatase have been described in HAE-nC1-INH patients; however, a subset of patients remain who do not have variants identified, a condition designated as HAE-unknown (HAE-U) (4).

In patients with HAE-nC1-INH with *F12* variants, estrogens have an important role. In a series of 57 patients from the French National Center of Reference for Angioedema, estrogen was associated with attacks in 36 of 38 symptomatic patients (5). In 24/36, exacerbation of symptoms occurred during pregnancy or associated with intake of estrogen-containing oral contraceptives (5). Recently, a systematic review identified clinical differentiators for genetic variants of HAE-nC1-INH. The findings reaffirmed the influence of estrogens mainly in FXII-HAE in comparison with other types of HAE-nC1-INH (6).

In women with HAE-C1-INH, pregnancy showed a variable course, worsening in different periods, suggesting that hormonal changes during the gestation were not the only factor influencing its course (7–9). There are only case reports about the pregnancy in HAE-nC1-INH women (10–19). Therefore, considering the close connection with estrogen and limited information on the course of pregnancies in women with HAE-nC1-INH, we aimed to evaluate the gestational period in these patients.

METHODS

We invited women previously enrolled in the Brazilian cohort of patients with diagnosis of HAE-nC1-INH to respond retrospectively about their pregnancies (20). Diagnosis was based on clinical symptoms, normal biochemical tests for HAE, and family history according to criteria established in 2012 (21). Genetic tests looking for variants in *F12*, *PLG* and *ANGPT1* genes were performed in all HAE-nC1-INH patients. A questionnaire was applied electronically to collect data on clinical characterization (age at onset of symptoms and diagnosis; clinical manifestations 1 year before the pregnancy and during each trimester of pregnancy; triggering factors, frequency, and severity of attacks); prophylactic and on demand treatment before and during the pregnancy and type of delivery. Only patients above 18 years of age were included. Patients presenting comorbidities which could worsen the pregnancy were excluded. Ethical Committee approved the protocol (CAAE: 98089218.4.0000.0082) and patients signed forms authorizing the study before data completion.

Abbreviations: ANGPT1, angiopoetin 1; FXII, Coagulation Factor XII; FXII-HAE, HAE with FXII mutation; PLG, plasminogen; HAE, Hereditary Angioedema; HAE-C1-INH, HAE with C1 inhibitor deficiency; HAE-nC1-INH, HAE with normal C1 inhibitor; HAE-U, HAE-unknown.

RESULTS

Twenty-six women with 37 pregnancies and 8 spontaneous abortions were enrolled in the study. Mean ages at onset of symptoms, at diagnosis, and at the start of pregnancy were; 34.6 ± 8.78 years; and 27.4 ± 5.28 years of age, respectively. Two out of eight abortions occurred in diabetic patients. *F12* mutation was identified in 7/26 (26.9%) women, and no patients with PLG or ANGPT1 mutation were detected. The development of symptoms occurred before the first pregnancy in 18/26 patients (mean age: 17.5 years old); during the first pregnancy in 2/26 (mean age 23.5 years old), and 6/26 after the pregnancy (mean age 26.2 years old). Among the six patients who developed symptoms after the first pregnancy, the mean time to onset of symptoms was 2.5 years. There was no difference in age at onset of symptoms according to presence of *F12* variant (19.8 ± 6.91 vs. 20.2 ± 5.95 years in the whole group).

From 37 analyzed pregnancies, 24 were characterized by angioedema attacks and 13 remained asymptomatic. During the pregnancy, attacks affected, preferentially, extremities in 16/24 (66.7%), abdomen in 10/24 (41.7%), and upper airways in 9/24 (37.5%). Besides abdominal pain, gastrointestinal symptoms including nausea in 7/10; vomiting in 6/10; abdominal distension in 5/10, diarrhea in 5/10, and cramps in 3/10 were also described. There was also the involvement of arms 7/24 (29.2%), legs 6/24 (25%), face 9/24 (37.5%), 2/24 neck (8.3%), tongue 4/24 (16.7%), lips 7/24 (29.2%), eyelids 7/24 (29.2%), and genitals 5/24 (20.9%). Additional symptoms referred during the attacks were arthralgia (11/24), headache (8/24), and difficulty urinating (1/24) (**Table 1**).

Triggering and/or worsening factors included emotional distress 20/23 (87%), trauma 17/23 (73.9%), infectious disease 6/23 (26%), cold weather 2/23 (8.7%), drugs (1/23) (4.3%), unknown 4/23 (17.4%), and no triggering factor identified in 1/23 patients (4.3%). One patient did not respond to this question.

Regarding timing of attacks during the pregnancy, the occurrence of attacks was in the first trimester in 10/24 (41.7%), second trimester in 3/24 (12.5%), third trimester in 5/24 (20.8%), first and third trimester in 1/24 (4.2%) and during the whole pregnancy in 5/24 (20.8%) (**Figure 1**). Fifteen patients had attacks both before and during the first pregnancy, which allowed us to make comparisons. Frequency of attacks worsened in 9/15 (60%), improved in 4/15 (26.7%),

TABLE 1 | Sites affected by HAE attacks before and during the pregnancy.

Sites	HAE-Unknown *n* = 20 (%)		HAE-FXII *n* = 4 (%)	
	Before pregnancy	During pregnancy	Before pregnancy	During pregnancy
Extremities	12 (80%)	8 (53.3%)	4 (100%)	2 (50%)
Arms	4 (26.7%)	3 (20%)	2 (50%)	2 (50%)
Legs	5 (33.3%)	3 (20%)	2 (50%)	2 (50%)
Face	13 (86.7%)	7 (46.7%)	4 (100%)	0
Neck	4 (26.7%)	2 (13.3%)	1 (25%)	0
Tongue	6 (40%)	2 (13.3%)	0	0
Lips	13 (86.7%)	5 (33.3%)	4 (100%)	0
Eyelids	8 (53.3%)	5 (33.3%)	3 (75%)	0
Genitals	6 (40%)	3 (20%)	3 (75%)	2 (50%)
Abdomen	8 (53.3%)	8 (53.3%)	2 (50%)	3 (75%)
Larynx	5 (33.3%)	4 (26.7%)	1 (25%)	0

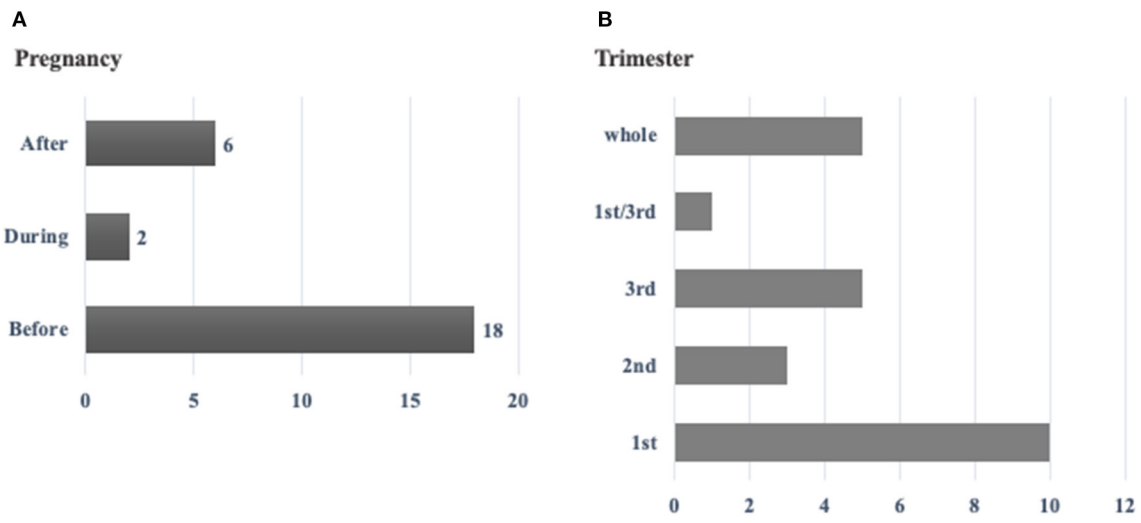

FIGURE 1 | (A) Development of HAE symptoms in 26 pregnant women before, during or after the pregnancy*. **(B)** Occurrence of HAE attacks in 24 pregnant women with HAE-nC1-INH according to the period of the pregnancy**. *Before pregnancy corresponds to the year before getting pregnant. During the pregnancy corresponds to the total period of pregnancy. After the pregnancy corresponds to the period of 6 months after delivery. **Whole period means that the women presented attacks during the whole pregnancy.

had no change in 1/15 (6.7%) and 1/15 (6.7%) did not respond. Also, 10/15 (66.7%) reported the same intensity of attacks, 3/15 (20%) reported higher intensity and 2/15 (13.3%) reported lower intensity. Concerning localization of attacks, extremities and abdomen were most affected 8/15 (53.3%) and involvement of upper airways was reported by 4/15 (26.7%).

Among patients with *F12* variant, 10 pregnancies from 7 women were analyzed. Attacks occurred before the pregnancy in 5/7 (71.4%) and after the first pregnancy in 2/ 7 patients (28.6%). Attacks occurred in 4/10 (40%) pregnancies, and 6/10 (60%) remained asymptomatic. Abdomen was affected in 2/4 (50%), extremities, legs, arms and genitals were affected in 2/4 (50%). Two patients had more attacks during the first trimester, one during the first and the third trimesters, and one during the whole pregnancy.

DISCUSSION

HAE-nC1-INH, first described in 2000, has been associated with variants in six genes, however, a subset of patients remains without a variant identified by genetic analysis, comprising the group designated as HAE-unknown (HAE-U). Only *F12* variants have been identified in Brazilian patients with HAE-nC1-INH so far, predominantly the missense c.983C>A (p.Thr328Lys) variant, found in 132/196 patients and relatives; and the c.971_1018D24del72 deletion which was found in only 2 patients (14, 20). Estrogen exposure related to use of estrogen-containing oral contraceptives [OC], menstruation, pregnancy, and hormone replacement therapy [HRT]) represents an important trigger or aggravating factor in HAE-FXII (22–24). This hormonal influence has not been observed in HAE-PLG patients (6). Our study evaluated pregnancies in women

with unknown variants and HAE-FXII, identified in 20% of the patients (25, 26).

The hormonal changes of pregnancy worsened the symptoms in about one third of our patients with HAE-C1-INH previously described (9). Estrogens can interact with most of the steps of the cascade generating bradykinin (2). The hormonal influence could partially explain the symptoms, and some women even report the onset of symptoms during their first pregnancy as it was reported by two patients of our cohort (27). However, the pathophysiology HAE-nC1-INH is not fully understood (24).

Previous reports revealed a variable course of HAE-C1-INH in pregnancy. Intensity and frequency of attacks may worsen, get better or remains the same (7–9, 23). Regarding pregnancies of women with HAE-nC1-INH, only case reports have been published and most of them reported worsening of symptoms during pregnancy (10–13, 15, 28). Although estrogens may increase severity of HAE-nC1-INH, the clinical expression is variable during pregnancy, even for the same patient (5). Our study revealed that the first trimester was the most challenging, with more attacks (41.7%), followed by the third trimester (20.8%). In 5 women, attacks were present during the whole pregnancy. This observation is similar to the published experiences in HAE-C1-INH, however, we had more frequent aggravated symptoms in the second trimester for pregnancies in HAE-C1-INH (7–9). Lower concentrations of C1-INH have been reported in HAE-nC1-INH during the pregnancy and therefore, the occurrence of symptoms could be similar in both situations (5). The difference between HAE-C1-INH and HAE-nC1-INH in our experience could be related to the higher hormonal influence in the second group or the facilitated access to the therapy afterwards. In addition, higher severity of attacks has been described more often in women with HAE-FXII, probably related to higher estrogen sensitivity in comparison with women with HAE-U (10).

Mechanical traumas due to the uterus growth and fetal movements were associated with abdominal attacks in HAE-C1-INH (8, 29), This association was also observed in HAE-nC1-INH (10, 11, 15). According to this theory, a higher frequency of abdominal attacks would be expected in the third trimester; however, symptoms were predominant in the first trimester in pregnancies of our cohort. It is important to emphasize that an HAE attack during the pregnancy may be misdiagnosed, and other obstetrical complications should be excluded (22). Experimental work in rats showed that bradykinin may increase uterus' contractility (30).

Subcutaneous edema affecting extremities and face predominated in the present study, as previously reported for HAE-C1-INH (9). However, upper airway obstruction was also reported by our patients, which could be of risk considering the reduced number drugs approved for gestational period. The emotional distress was reported as trigger factor by most of the women and the possibility of severe attacks had probably led to this consequence.

Although pregnancy was associated with onset and worsening of symptoms of HAE, few obstetric complications were reported by our patients. Severe complications, including fetal and neonatal death, have been previously reported in patients with HAE (5, 11). Recently, recurrent pregnancy loss was associated with MTHFR mutation in a patient with HAE-nC1-INH (18). We reported the follow up of a pregnancy in a patient with HAE-C1-INH and thrombophilia with good outcome (31). In the present study, the occurrence of spontaneous abortion was similar to the expected for non-affected women (25, 26), in contrast with our previous description in HAE-C1-INH pregnancies (9).

Pregnant patients with HAE should be assisted carefully, since the therapeutic options are limited during pregnancy, and experience with HAE-nC1-INH treatment is restricted to case reports or small case series. Two of our patients used pdC1-INH during the attacks and another one treated with FFP. Tranexamic acid was previously prescribed for HAE-C1-INH (9) and no pregnancies received prophylactic therapy in this cohort of HAE-nC1-INH. A management plan should be coordinated by an HAE specialist, with C1-INH concentrate available either in the maternity center or at home, for initiation of therapy as early as possible (22). The use of pdC1-INH during the pregnancy of HAE-nC1-INH women as long-term prophylaxis has been described without further complications (16). In Brazil, the access to pdC1-INH is restricted and it is not included among high-cost drugs which are provided by the government (31).

Clinical course, therapeutic options, outcomes, need for a close follow up with an HAE specialist, should be discussed in detail with women with HAE-nC1-INH who are pregnant or who want to become pregnant. A multidisciplinary approach, involving the obstetrician and other health care professionals when needed, would be beneficial. In addition, genetic counseling should be provided. Pregnancy could not be inputted as more dangerous for women with HAE-nC1-INH than the disease *per se*. In the present study, few women presented HAE-FXII, limiting the conclusion about a different course of the pregnancy in comparison with those with HAE-U, however, a strong relevance of estrogens in HAE-FXII has been observed. Prospective studies to assure appropriate management of HAE-nC1-INH in women during pregnancy are necessary.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Centro Universitario FMABC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NG and AG contributed to the study conception and design. The first draft of the manuscript was written by NG, LA, and AG. All

the authors contributed to data collection and read and approved the final manuscript.

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Overview of SERPING1 Variations Identified in Hungarian Patients With Hereditary Angioedema

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Background: Hereditary angioedema (HAE) due to C1-inhibitor (C1-INH) deficiency (C1-INH-HAE) is a rare autosomal dominant disorder, characterized by recurrent, unpredictable edematous symptoms involving subcutaneous, and/or submucosal tissue. C1-INH-HAE may be caused by more than 700 different mutations in the gene encoding C1-INH (*SERPING1*) that may lead to decreased protein synthesis or to functional deficiency.

Methods: Concentrations of C1-INH, C4, C1q, and anti-C1-INH antibodies, as well as functional C1-INH activity were determined in subjects suffering from edematous symptoms and admitted to the Hungarian Angioedema Center of Reference and Excellence. In those patients, who were diagnosed with C1-INH-HAE based on the complement measurements, *SERPING1* was screened by bidirectional sequencing following PCR amplification and multiplex ligation-dependent probe amplification. For detecting large deletions, long-range PCRs covering the entire *SERPING1* gene by targeting 2–7 kb long regions were applied.

Results: Altogether 197 individuals with C1-INH deficiency belonging to 68 families were identified. By applying Sanger sequencing or copy number determination of *SERPING1* exons, 48 different mutations were detected in 66/68 families: 5 large and 15 small insertions/deletions/delins, 16 missense, 6 nonsense, and 6 intronic splice site mutations. Two novel variations (p.Tyr199Ser [c.596A>C] and the duplication of exon 7) were shown to cosegregate with deficient C1-inhibitor level and activity, while two other variations were detected in single patients (c.797_800delinsCTTGGAGCTCAAGAACTTGGAGCT and c.812dup). A series of long PCRs was applied in the remaining 2 families without an identified mutation and a new, 2606 bp long deletion including the last 91 bp of exon 6 (c.939_1029+2515del) was identified in all affected members of one pedigree. In the remaining one family, a deep intronic *SERPING1* variation (c.1029+384A>G) was detected by a targeted next-generation sequencing panel as reported previously.

Conclusions: Sequencing and copy number determination of *SERPING1* exons uncover most pathogenic variants in C1-INH-HAE patients, and further methods are

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worth to be applied in cases with unrevealed genetic background. Since knowledge of the genetic background may support the establishment of the correct and early diagnosis of C1-INH-HAE, identification of causative mutations and reporting data supporting the interpretation on the pathogenicity of these variants is of utmost importance.

Keywords: C1-inhibitor, C1-INH-HAE, hereditary angioedema, long-range PCR, MLPA, mutation, sequencing, SERPING1

INTRODUCTION

Hereditary angioedema (HAE) due to C1-inhibitor (C1-INH) deficiency (C1-INH-HAE) is a rare autosomal dominant disorder, characterized by recurrent, unpredictable, nonpitting edematous symptoms involving the subcutaneous and/or submucosal tissues and showing intra- and interindividual variability. C1-INH-HAE episodes usually affect the extremities (arms, hands, feet, and legs), the face, lips, eyelids, bowels, and genitalia. In those rare cases when the edema evolves in the upper airways, the disease may lead to a potentially life-threatening condition within hours, without the proper treatment. Since the first pathogenic *SERPING1* mutation was described in 1987 (1), the presence of C1-INH-HAE was explained by more than 700 different mutations (2–4) in the gene encoding C1-INH (*SERPING1*) that can either cause decreased protein synthesis (C1-INH-HAE type I) or functional deficiency (C1-INH-HAE type II). The mutations in the background of C1-INH-HAE type II are usually missense mutations affecting the reactive center loop of C1-INH encoded in exon 8. On the other hand, variations leading to the development of C1-INH-HAE type I are quite heterogeneous and are distributed over the exons and introns of the entire *SERPING1* gene, in form of deletions or insertions of various sizes along with missense or nonsense substitutions, leading to the defect of C1-INH synthesis or secretion.

Molecular genetic testing is not considered as obligatory to confirm the diagnosis of C1-INH-HAE, though analyzing the segregation of novel *SERPING1* variants in case of available family members is recommended to confirm the pathogenicity and penetrance (5). Considering the facts that (1) *SERPING1* variants are predominantly associated with decreased functional C1-INH activity and that (2) C1-INH-HAE is inherited in an autosomal dominant pattern with high penetrance, novel *SERPING1* mutations need only in a few cases to be functionally characterized *in vitro*, when considering their pathogenicity (6, 7).

Establishing the correct and early diagnosis of C1-INH-HAE (including the screening of all symptomatic and asymptomatic first-degree relatives of the diagnosed patients) is of high importance for the affected subjects' proper treatment, prognosis, and quality of life. Furthermore, previously asymptomatic family members may – in the future – suffer unpredictable symptoms that require specific treatment. Knowledge of a pedigree's causative *SERPING1* mutation is also useful as this information may contribute to preimplantation and prenatal diagnosis or underpin diagnosis in cases with uncertain complement results that may occur in very early childhood (8).

Here, we explain our strategy on genetic work-up exploring the *SERPING1* gene and provide an overview of *SERPING1* mutations identified in Hungarian C1-INH-HAE patients over the past decades involving those published previously as well as new families with novel mutations.

MATERIALS AND METHODS

In the Hungarian Angioedema Center of Reference and Excellence, 197 individuals (110 female, 87 male, mean age 42.8 years) belonging to 68 families were identified with C1-INH deficiency. The diagnosis of C1-INH-HAE was established according to the international consensus criteria (9), based on the following complement measurements: serum concentration of C1-INH was determined by radial immunodiffusion (10), C4 level by immunoturbidimetry (Beckman Coulter, Brea, CA, USA), levels of anti-C1-INH antibodies and C1q were measured by ELISA (11, 12), whereas the functional C1-INH activity was analyzed by using a commercial kit (Quidel, San Diego, CA, USA) in the serum samples of subjects suffering from edematous symptoms.

In those patients, who were diagnosed with C1-INH-HAE based on the complement measurements, and also in their available family members, genomic DNA was isolated from peripheral or umbilical cord blood samples by the salting-out method (13).

Bidirectional DNA sequencing following PCR amplification was applied to screen the whole coding region of the gene encoding C1-inhibitor (*SERPING1*; OMIM #606860). Amplification of genomic DNA was carried out in 35 cycles with GoTaq G2 DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions (details of the reactions are available upon request) applying the primers listed in **Table 1**. Before sequencing, PCR products were purified with Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, Waltham, MA, USA) and sequencing was performed using the BigDye Terminator v3.1 Cycles Sequencing Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions with the sequencing primers specified in **Table 1**. After sodium acetate/ethanol purification, sequencing products were separated with an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Abbreviations: ACMG, American College of Medical Genetics and Genomics; CADD, Combined Annotation Dependent Depletion; C1-INH, C1-inhibitor; C1-INH-HAE, Hereditary angioedema due to C1-inhibitor deficiency; HAE, Hereditary angioedema; MLPA, Multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction; SIFT, Sorting Intolerant From Tolerant.

TABLE 1 | Primer sequences and PCR conditions applied in this study.

	Studied exon(s)	Primer	Product size (bp)	Applied enzyme	T_m
Primers for PCR	1–2	F: 5' TTGAGGAATAACGGAGGTGAG 3' R: 5' AGGAGGAGTAGGCTGAGAAAA 3'	1,278	GoTaq	59°C
	3	F: 5' GTACTAGCCAAGCAAGTGAGTC 3' R: 5' AGCAATCGTGCCTATTACATC 3'	1,082	GoTaq	59°C
	4	F*: 5' ATACCCTCCATTCCAGCCTGGTC 3' R*: 5' CTTCACCTGCTCTGCAGTCCATC 3'	368	GoTaq	59°C
	5–6	F: 5' CACCATGCCGTATTCACTAA 3' R: 5' AGGGTGGAAATACAGATGGAAG 3'or R: 5' TCCCTCCCTACTCATCAAAC 3'	798	GoTaq	59°C
	7	F: 5' TCAGTGGTGGAGTCAGGGTA 3' R: 5' CCAATGGGATAATAGCACCTAC 3'	631	GoTaq	59°C
	8	F: 5' CTGCCAGAGGGTACAGTATGT 3' R: 5' GAGATGGGAGGATTGTTTGA 3'	823	GoTaq	59°C
Primers for sequencing	1–2	F: 5' CCCCGTTCACCCCACCTACCA 3' or F: 5' TTGAGGAATAACGGAGGTGAG 3' R*: 5' GCCTGAAGGGTTAATCCTCAGCCA 3'			
	3	F: 5' TGGTGGTGGTTCTAAGACAGATT 3' R: 5' AGAGGCATGGCTTTGTAAGTG 3'			
	4	F*: 5' ATACCCTCCATTCCAGCCTGGTC 3' R*: 5' CTTCACCTGCTCTGCAGTCCATC 3'			
	5–6	F: 5' CTCAAATCGTGCTCATGGAA 3' R: 5' AGGGTGGAAATACAGATGGAAG 3' or R: 5' TCCCTCCCTACTCATCAAAC 3'			
	7	F: 5' TCAGTGGTGGAGTCAGGGTA 3' R: 5' CCAATGGGATAATAGCACCTAC 3'			
	8	F: 5' GGCAAACAAGGGAAGAGGAAG 3' R: 5' AGCCTGGGTGACAGATTGAGA 3'			
Primers for long PCR	1–3	F: 5' TGCACTGGAGCTGCCTGGTGA 3' R: 5' AGAGGCATGGCTTTGTAAGTG 3'	2,777	GoTaq	60°C
	3–5	F: 5' TGGTGGTGGTTCTAAGACAGATT 3' R: 5' GGAGGGTTGCTCTAATGCAG 3'	6,676	Phusion Flash	58°C
	5–7	F: 5' CACCATGCCGTATTCACTAA 3' R: 5' CCAATGGGATAATAGCACCTAC 3'	6,209	Phusion Flash	60°C
	7–8	F: 5' TCAGTGGTGGAGTCAGGGTA 3' R: 5' CACAGGGGTCAGAATCACCT 3'	5,289	Phusion Flash	62°C
	8	F: 5' GGCAAACAAGGGAAGAGGAAG 3' R: 5' TGCTAAAAACACCCTCCAAA 3'	6,310	Phusion Flash	60°C
Primers for the verification of exon 7 duplication	exon 7 MLPA probe hybridization site	F: 5' TACCAGGATCACCAAACTCAGAT 3' R: 5' CACAATCTGAGTTTGGTGATCCTG 3'		Phusion Flash	64°C

*Based on primer sequences published in (14).
F, forward; R, reverse.

In order to detect large deletions or duplications in the *SERPING1* gene, multiplex ligation-dependent probe amplification (MLPA) was performed applying the SALSA MLPA P243-A3 or P243-B1 SERPING1-F12 probemixes (MRC Holland, Amsterdam, The Netherlands). Data were analyzed using the Coffalyser.Net™ MLPA analysis software (MRC Holland, Amsterdam, The Netherlands) according to the manufacturers' instructions.

A series of long-range PCRs amplifying 2–7 kb sequences was performed with Promega GoTaq G2 DNA polymerase (Promega, Madison, WI, USA) or Phusion Flash PCR Master Mix (Thermo Scientific, Waltham, MA, USA) in cases of unresolved genetic background and their family members using the primers listed in **Table 1**.

The identified genetic variations were named according to the Human Genomic Variation Society (HGVS) recommendations (15) and for cDNA nucleotide numbering the reference sequence of *SERPING1* (ID: NM_000062.3) was used. Interpretation of sequence variants was based on the criteria established by the American College of Medical Genetics and Genomics (ACMG) (16). The possible functional effect of an identified novel rare variation was assessed using *in silico* prediction tools, such as Sorting Intolerant From Tolerant (SIFT) (http://siftdna.org/www/Extended SIFT chrcoords

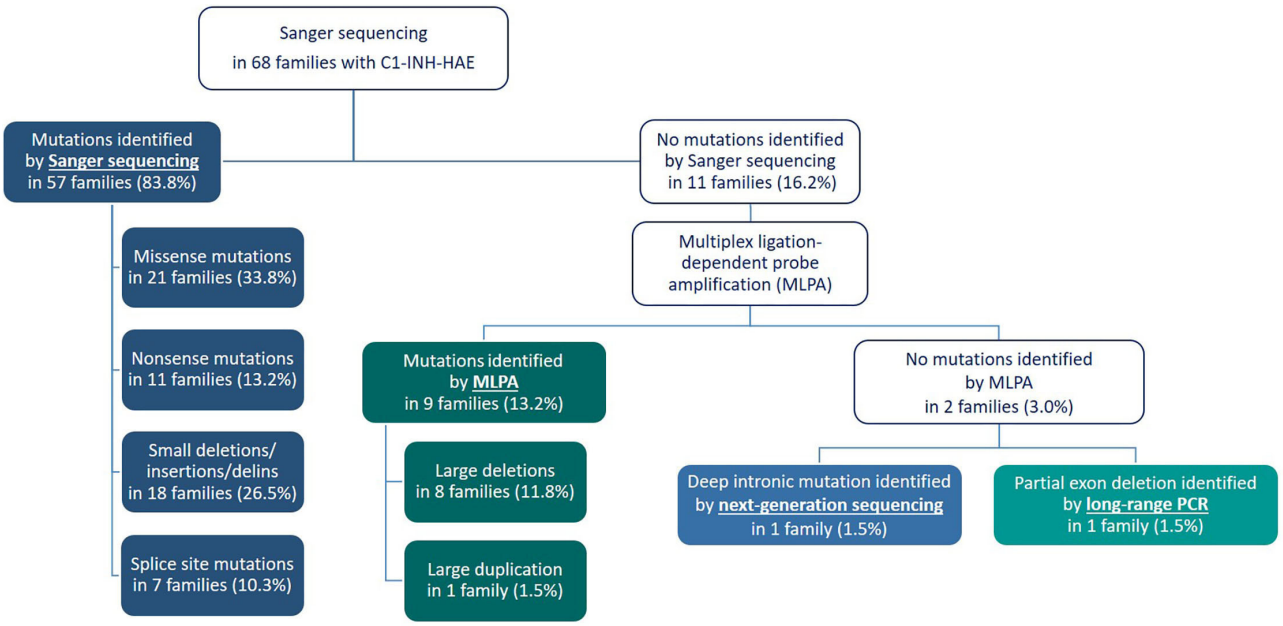

FIGURE 1 | Flow chart illustrating the algorithm for molecular genetic testing in the Hungarian Angioedema Center of Reference and Excellence.

submit.html)(17), PolyPhen (version 2) (http://genetics.bwh.harvard.edu/pph2/) (18), PROVEAN (http://provean.jcvi.org/genomesubmit.php) (19), CADD (https://cadd.gs.washington.edu/) (20), Mutation-Taster (http://mutationtaster.org) (21), or Human Splicing Finder (22) (version 3.1; http://www.umd.be/HSF3/~[21]).

RESULTS

By applying Sanger sequencing to screen coding exons and exon–intron boundaries of the *SERPING1* gene, 43 different mutations were identified in 57 of the studied 68 families (**Figure 1**). Alterations changing the coding region of *SERPING1* included 10 small deletions, 4 small duplications, 1 delins, and 22 substitutions, the latter resulting in 16 missense and 6 nonsense variations, while 6 mutations influenced the intronic splice sites (**Tables 2**–**5**). All the cases (13 patients in 5 families) with C1-INH-HAE type II carried the p.Arg466Cys missense variation.

Among the detected substitutions, one novel (c.596A>C) was identified that causes a tyrosine to serine amino acid change at codon 199 (Tyr199Ser) located in the enzymatically active serpin domain. The potential effect of this missense variation was estimated by five different prediction programs, with 4 out of the 5 programs predicting the change to be disease causing and one to be neutral (SIFT: damaging [score: 0.049]; PROVEAN: deleterious [score: −5.03]; Mutation Taster: disease-causing [probability: 0.67]; CADD PHRED-like score: 23.1; Polyphen-2: benign [score: 0.250] [sensitivity: 0.91; specificity: 0.88] by the HumDiv model). Available family members were also tested for the carrier state of the mutation, and the affected sister and their mother were found to be heterozygous for this substitution and both of them showed impaired complement results (low C4 with deficient C1-INH function and level).

A novel single base duplication (c.812dup) was identified in a patient with low C1-INH level and activity. By the insertion of an adenine in exon 5, this mutation causes frameshift and introduces a premature stop codon (p.Asn271Lysfs*34) in the mRNA. Two of the applied prediction tools were suitable for estimating the potential effect of a duplication; both indicated a deleterious effect for this variation with high probability (Mutation Taster: disease-causing [probability: 1]; CADD PHRED-like score: 26.3). No other rare variation was detected in the patient, who had no available family members.

A complex variation with the deletion of 4 nucleotides and the insertion of 24 bp (c.797_800delinsCTTGGAGCTCAAGAACTTGGAGCT) was also identified that causes a frameshift and premature termination of protein synthesis at the amino acid position 266 (p.Val266Alafs*). The patient who carried this mutation did not have any other rare variation in the *SERPING1* gene and her complement measurements showed deficient C1-inhibitor level as well as below-normal C4 level. Segregation could not be verified in this case as no family members were available for testing.

In those patients in whom no sequence alteration was found with Sanger sequencing or the pathogenicity of the identified variation was not undoubtedly supported, copy number of *SERPING1* exons was studied by MLPA (**Figure 1**). A part of our patient group was analyzed previously with Southern blot technique combined with relative quantification of *SERPING1* exons using real-time PCR and SybrGreen detection (24), and the MLPA provided concordant result in each case. Five different

TABLE 2 | Missense and nonsense mutations identified in the *SERPING1* gene.

Affected exon	cDNA position	Protein position	Number of affected families	Number of affected patients	References
2	c.1A>G	p.Met1Val	1	2	(23)
3	c.65C>G	p.Ser22*	1	2	(7)
3	c.94C>T	p.Gln32*	3	12	(24)
3	c.253G>T	p.Glu85*	1	1	(3)
3	c.389G>A	p.Cys130Tyr	2	6	(24)
3	c.425T>C	p.Leu142Ser	1	3	(25)
3	c.503C>A	p.Ala168Asp	1	2	(26)
4	c.553G>C	p.Ala185Pro	1	6	(3)
4	c.596A>C	p.Tyr199Ser	1	3	-
4	c.667C>T	p.Gln223*	1	3	(24)
5	c.728T>C	p.Leu243Pro	1	1	(27)
5	c.752T>G	p.Leu251Arg	1	2	(3)
6	c.911A>G	p.Asp304Gly	1	3	(3)
6	c.988T>G	p.Tyr330Asp	1	6	(25)
7	c.1180A>C	p.Thr394Pro	1	1	(28)
7	c.1223A>T	p.Asp408Val	1	1	(24)
8	c.1396C>T	p.Arg466Cys	5	13	(29)
8	c.1418T>A	p.Val473Glu	1	1	(24)
8	c.1423C>T	p.Gln475*	1	1	(3)
8	c.1478G>A	p.Gly493Glu	1	1	(30)
8	c.1480C>T	p.Arg494*	4	6	(28)
8	c.1493C>G	p.Pro498Arg	1	1	(24)

TABLE 3 | Small deletions/insertions/delins identified in the *SERPING1* gene.

Affected exon	cDNA position	Protein position	Number of affected families	Number of affected patients	References
3	c.106_107del	p.Ser36Phefs*21	1	2	(28)
3	c.249del	p.Asp84Metfs*64	1	1	(31)
3	c.392_393del	p.Ser131*	1	1	(24)
3	c.435_476del	p.Leu146_Ala159del	4	26	(24)
5	c.705del	p.Phe236Leufs*2	1	4	(3)
5	c.797_800delinsCTTGGAGCTCAAGAACTTGGAGCT	p.Val266Alafs*20	1	1	-
5	c.812dup	p.Asn271Lysfs*34	1	1	-
6	c.982del	p.Lys328Argfs*13	1	1	(32)
7	c.1106del	p.Asn369Alafs*28	1	5	(24)
7	c.1127dup	p.Ser377Phefs*48	1	1	(33)
7	c.1147dup	p.Met383Asnfs*42	1	2	(3)
8	c.1356_1357del	p.Val454Glyfs*18	1	2	(33)
8	c.1357_1382dup	p.Ile462Glyfs*123	1	1	(24)
8	c.1391_1392del	p.Val464Glyfs*8	1	3	(33)
8	c.1466del	p.Pro489Leufs*87	1	2	(33)

deletions involving one or more *SERPING1* exons were identified all of which were reported previously in C1-INH-HAE patients (**Table 5**). MLPA results showed three copies of exon 7 in case of each available affected members of a large family (**Figure 2**) and as this mutation was not reported previously, patients from this family were further investigated in order to verify the duplication of exon 7 with an independent method. Since MLPA indicated an extra copy of the site recognized by the exon 7 probe, forward and reverse PCR primers (listed in **Table 1**) were designed for this region to amplify the sequence between the duplicated

TABLE 4 | Intronic mutations identified in the *SERPING1* gene.

Affected intron	cDNA position	Number of affected families	Number of affected patients	References
1	c.51+1G>A	2	7	(23)
3	c.550+1G>A	1	3	(24)
3	c.550+2dup	1	5	(24)
3	c.550+5G>A	1	1	(7)
4	c.686-3C>G	1	12	(7)
5	c.889+1G>A	1	3	(2)
6	c.1029+384A>G	1	4	(34)

TABLE 5 | Large deletions/duplication identified in the *SERPING1* gene.

Affected exon	Variation	Number of affected families	Number of affected patients	References
4	exon 4 deletion	5	9	(35)
6	c.939_1029 +2515del	1	2	-
7	exon 7 deletion	1	4	(33)
7	exon 7 duplication	1	9	-
7–8	exon 7–8 deletion	1	2	(23)
1–8	exon 1–8 (whole gene) deletion	1	6	(35)

exon 7 probe binding sites. Applying this PCR, an app. 4 kb product was generated in samples of each affected individual of the corresponding family but not from healthy controls (data not shown). Complement measurements showed deficient C1-INH activity and level in the available members with edematous symptoms, while normal complement results were obtained from the analyzed healthy relatives (**Figure 2**).

In the case of two families, MLPA showed two copies for each studied exon and a normal sequence was retrieved from Sanger sequencing of exons and exon–intron boundaries in the patients. However, these methods have limitations and may overlook certain mutations, such as deletions that does not involve the recognition site of any of the MLPA probes or mutations that affect one or both of the PCR primer binding sites. To overcome this problem, a series of long-range PCRs was applied that cover the whole *SERPING1* gene with 2–7 kb long products. As **Figure 3** shows an extra, smaller band was observed in case of the long PCR amplifying the region of exon 5–7 and sequencing of this product revealed that the patient carries a 2606 bp long deletion including the last 91 bp of exon 6 and 2515 bp of intron 6 (c.939_1029+2515del). This particular long-range PCR was applied to analyze the available family members of the patient showing that his affected father also carried this deletion but PCR performed from the samples of his symptom-free mother and aunt (the father's sister) revealed only wild-type products. In agreement with this, deficient C1-inhibitor activity and level were detected only in the index patient and his father.

In the remaining one family, a deep intronic *SERPING1* variation (c.1029+384A>G) was detected by a targeted next-generation sequencing (NGS) panel as reported previously (34). In order to verify this variant with Sanger sequencing and to screen for its carrier state in new patients, a new reverse primer was designed for the previously used forward one and since then applied in the diagnostic work-up (**Table 1**).

Besides the mutation identified as pathogenic, two rare variations were detected in two families, a sense variant (p.Leu251=) in the two affected members of a family with exon 4 deletion and a rare missense polymorphism (p.Arg366His) in a patient without available family members, who also carried the p.Glu85* nonsense mutation (3).

As a probable causative variation was detected in each of the studied Hungarian families, the diagnosis based on complement measurements was verified by genetic testing in each of the patients. Knowledge of the *SERPING1* mutation allowed the verification of C1-inhibitor deficiency in 24, at the time of the analysis yet symptom-free individuals. These subjects included 11 children under the age of 1 year, when complement levels (especially C4) may be lower than the normal adult levels in healthy children as well (36). Among them, 5 were newborns, in whom the analyzed DNA was isolated from the umbilical cord. Moreover, exclusion of C1-INH-HAE was achieved from the umbilical cord DNA of 4 offspring including two cases with ambiguous complement results (below normal C4 and C1-INH level with normal C1-INH function).

DISCUSSION

Based on the current guidelines, molecular genetic testing is not obligatory as a first-line diagnostic approach in case of patients with angioedema, as most of the cases can be diagnosed based on the clinical picture, complement laboratory findings (functional and antigenic levels of C1-inhibitor, concentrations of C4, C1q, and autoantibodies against C1-inhibitor) as well as family history (if available) (9). However, it is still of high importance to report the identified variations that (1) have a clear damaging effect on protein function/level, or that (2) are segregating with the disease or that (3) are confirmed to be pathogenic by functional studies, as their publication or inclusion in disease-specific databases may help further investigators to decide about their pathogenicity.

Here, we report the mutational spectrum of a large cohort of Hungarian C1-INH-HAE patients diagnosed in the Hungarian Angioedema Center of Reference and Excellence, the national center caring all Hungarian C1-INH-HAE patients. This is an update of the whole Hungarian cohort diagnosed in the past almost 50 years including families with previously published genetic background (3, 24, 32–34, 37, 38), but extended with further family members and also newly diagnosed patients with the identified *SERPING1* mutations. By applying conventional molecular genetic methods such as Sanger sequencing and

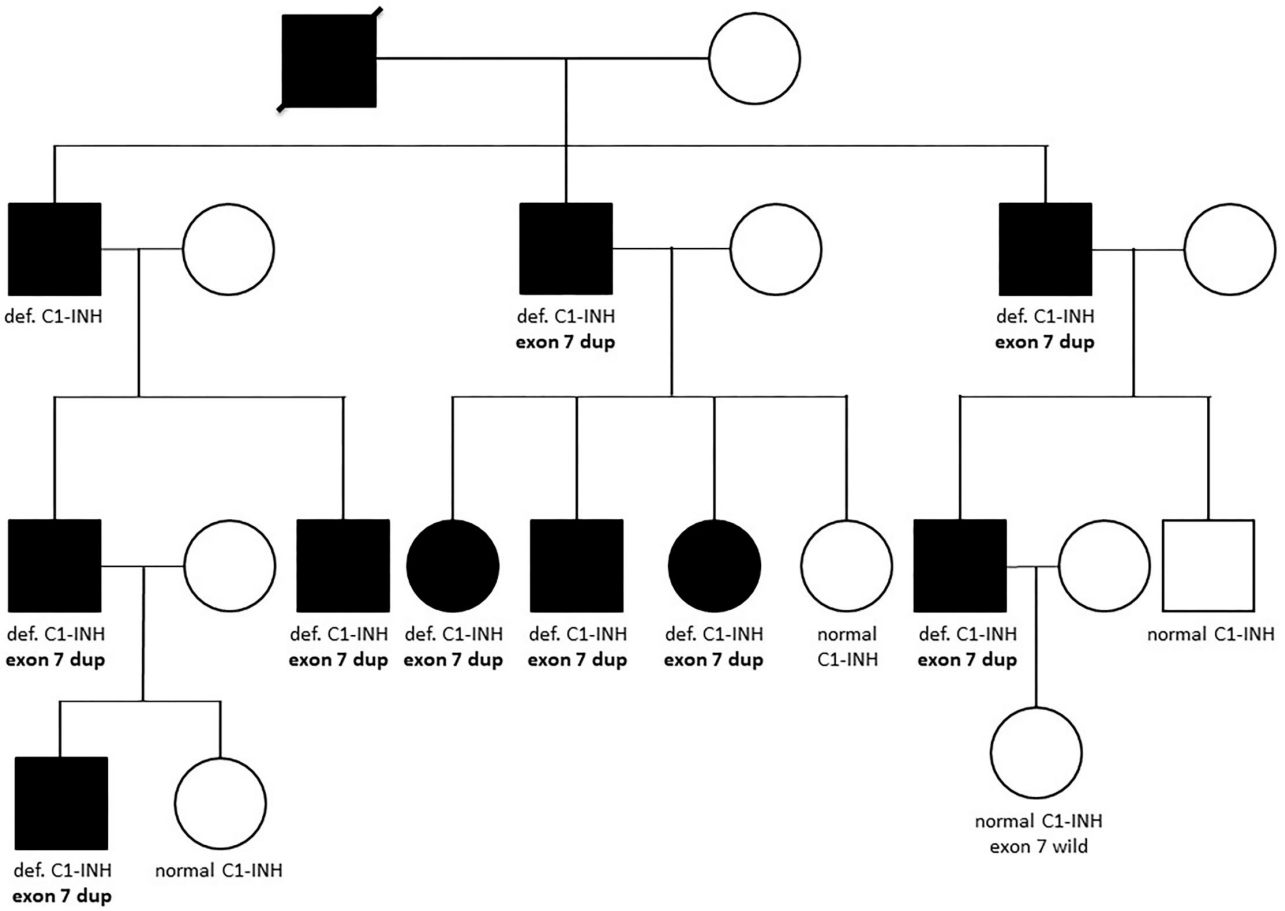

FIGURE 2 | Family tree of a patient with the novel *SERPING1* exon 7 duplication. Affected members of the family are depicted with dark symbols, while all available results of complement and genetic measurements are denoted below the symbols. Def. or normal C1-INH means deficient or normal activity and level of C1-inhibitor, respectively. "Exon 7 dup" refers to the heterozygous carrier state of the duplication of *SERPING1* exon 7, while individuals denoted as "exon 7 wild" does not carry this variation.

MLPA, a pathogenic *SERPING1* variation could be explored in almost all families: C1-INH deficiency was caused by a missense or nonsense mutation in 47.1% (32/68), by a small deletion, insertion, or delins in 26.5% (18/68), by a large deletion or duplication in 13.2% (9/68) or by an intronic variation in 10.3% (7/68) of the analyzed pedigrees.

Among missense variations one novel was identified, an adenine to cytosine substitution in exon 4, causing a tyrosine to serine change at codon 199 of the C1-inhibitor protein. This novel substitution was not found in international databases studying large populations with different ethnicity (gnomAD: https://gnomad.broadinstitute.org/; 1,000 Genomes project: https://www.internationalgenome.org/; NHLBI GO Exome Sequencing Project: https://evs.gs.washington.edu/EVS/). Applying five prediction tools to assess its pathogenicity, ambiguous results were retrieved, however, with a dominance of damaging predictions (4 out of 5). The pathogenic role of this p.Tyr199Ser variation is supported by several factors, one of which is that the two carrier children in the family have recurrent angioedema attacks and their complement parameters are typical of C1-INH-HAE. Coinheritance of deficient C1-inhibitor level and activity with the mutation was also observed as the mother with insufficient level and activity of C1-inhibitor is a mutation carrier as well. However, segregation of the mutation with symptoms was not clearly confirmed as she only once had an attack of suspicious abdominal pain, but never experienced subcutaneous edematous episodes. The pathogenicity of the identified p.Tyr199Ser variation located in the helix-rich region of the serpin domain is also underpinned by a previous study that described a pathogenic tyrosine–asparagine change at this position (p.Tyr199Asn), indicating the importance of the tyrosine at codon 199 (7).

Another novel single nucleotide variation, the insertion of an adenine (c.812dup) was detected in a patient without available family members who had decreased level of C1-INH and C4 as well as deficient functional activity of C1-INH. The pathogenicity of this mutation is clear, as by the duplication of one base it shifts the reading frame and results in the generation of a premature termination codon in the formed mRNA.

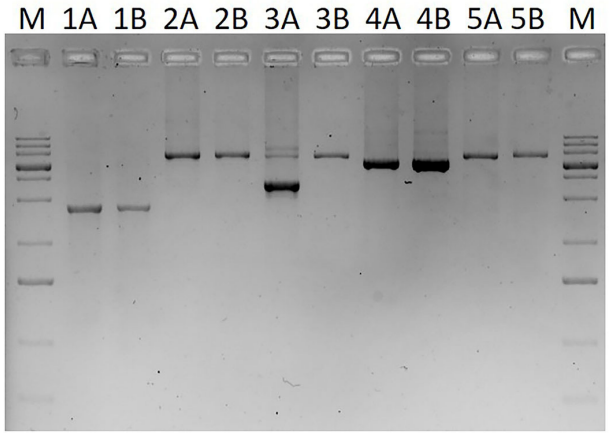

FIGURE 3 | Image of agarose gel (1%) electrophoresis of long-range PCR products covering the whole *SERPING1* gene. M: GeneRuler 1 kb plus DNA ladder (Thermo Scientific, Waltham, MA, USA). PCR products in lines involve the following exons: lines 1: exons 1–3 (from the binding site of the MLPA probe specific for exon 1), lines 2: exons 3–5, lines 3: exons 5–7, lines 4: exons 7–8, lines 5: exon 8 (plus an extra 1375 bp downstream from the 3'-end of exon 8). Samples marked with A (1A–5A) are derived from a C1-INH-HAE patient, samples with B (1B–5B) are from a healthy control.

In our C1-INH-HAE cohort, five gene rearrangements were identified by MLPA: four previously published large deletions involving 1 to 8 exons and a duplication of exon 7 which was not reported previously. The presence of this novel copy number variation was verified by long-range PCR in each affected individual of the corresponding family; moreover, this large pedigree allowed the detection of clear cosegregation of the mutation with C1-INH-HAE symptoms and deficient complement levels. Besides, amplification of an app. 4 kb product by applying forward and reverse primers specific for the MLPA exon 7 probe hybridization site supported the close (within gene) location of the extra copy of this site.

After performing the analysis of *SERPING1* gene with Sanger sequencing and MLPA, two families (2.9% of the pedigrees analyzed by our group) still remained in which no mutation could be identified. This is in agreement with previous studies (3, 27) and a meta-analysis of Ponard et al. who described that no *SERPING1* gene alterations could be identified using Sanger and MLPA methods in 24 families fulfilling the clinical and laboratory criteria of C1-INH-HAE (24/379; 6.3%) (2). Patients in these two families were further analyzed by a series of long PCRs that was developed in order to detect medium-sized alterations that involve only a small part of an exon and not affect hybridization sites of the MLPA probes. These kinds of mutations are not detected by MLPA and if the binding site of the forward or reverse PCR primer is also deleted, it won't be detected by Sanger sequencing either as only the wild-type allele will be amplified from the other chromosome. Long-range PCR is a widely accepted method to investigate large gene rearrangements, and was applied by several groups in the genetic diagnosis of HAE as well (3, 27, 39). These PCRs were usually designed to amplify extra-large products (sometimes more than 10–15 kb) in order to capture large deletions, or even deletion of the whole gene; however, capacity of these methods to detect deletions affecting only a few hundred or thousand nucleotides is limited. By applying the MLPA technique, we were able to detect large rearrangements; therefore, in this study, we rather focused on smaller deletions and applied PCR primers to amplify 2–7 kb products. With this method, a partial deletion was detected involving the last 91 bp of exon 6 and 2,515 bp of intron 6 in the two symptomatic cases but not in two healthy members of the corresponding family. Functional relevance of this mutation is obvious either by causing the deletion of 91 bp from the coding sequence resulting in reading frameshift or by abolishing the exon–intron boundary impairing correct splicing. Moreover, the clear cosegregation of this mutation with deficient C1-INH function and level as well as edematous symptoms also confirms its pathogenicity.

In one family, the standard approaches failed to uncover the disease-causing *SERPING1* alteration; therefore, a NGS platform targeting the entire *SERPING1* gene was performed that resulted in the identification of a deep intronic variation (c.1029+384A>G) as published in details previously (34). Intronic regions were initially considered to be mostly nonfunctional and variations found deep within the introns (i.e., in a distance of more than 100 base pairs away from the exon–intron boundaries) were disregarded as pathogenic alterations having any functional consequences. However, with the advent of whole-genome sequencing a few novel deep intronic variants were identified in *SERPING1* that showed a clear association with the symptoms of C1-INH-HAE based on recent studies, supporting the functional importance of the intronic sequences (6, 40). As Hujová's study nicely presented the *SERPING1* c.1029+384A>G mutation results in *de novo* donor splice site creation and subsequent pseudoexon inclusion (6).

In each affected family of our C1-INH-HAE cohort of almost 200 subjects, we were able to detect a variant that was classified as "pathogenic" or "potentially pathogenic" based on the criteria of ACMG (16). Identifying the disease-causing *SERPING1* variation could be highly useful in those cases where the clinical diagnosis of C1-INH-HAE is highly suspected but the complement test results are inconclusive. This occurs frequently before or upon the first presentation of HAE-like symptoms (such as unexplained gastrointestinal symptoms) in children with a positive family history of C1-INH-HAE, where the functional and antigenic C1-INH levels are not reliable parameters of C1-INH-HAE before the first year of age, as their reference ranges are significantly lower compared to the adult reference ranges (8, 9, 41). Knowledge of the causative mutation allowed us to perform genetic analysis immediately after birth from umbilical cord samples of newborns with a parent suffering from C1-INH-HAE. In five cases, sequencing or copy number analysis of the corresponding exon(s) indicated inheritance of the *SERPING1* mutation, while it excluded C1-INH-HAE in four newborns (including two cases with uncertain complement results). Besides, determination of the mutation carrier state verified the diagnosis in further 6 children under the first year of age when complement measurements may be inconclusive.

Considering the routinely used molecular genetic approaches such as bidirectional sequencing of *SERPING1* exons and

the exon/intron boundaries along with the copy number determination of *SERPING1* exons, these are able to explore most of the disease-causing variations in C1-INH-HAE patients. However, in those families where complement laboratory studies suggest the diagnosis of C1-INH-HAE but no *SERPING1* mutation can be identified by the conventional methods used in a diagnostic laboratory, or in case the detected missense *SERPING1* variation is supposed to be benign according to previous publications, databases or *in silico* prediction tools, further unique techniques should be applied in order to explore the pathogenic alteration in the background of the disease. Discovering *SERPING1* variations in C1-INH-HAE patients and reporting data that contribute to the correct interpretation of the pathogenicity of these variations are of utmost importance since knowledge of the genetic background may support the establishment of the correct and early diagnosis of C1-INH-HAE promoting the patient's proper treatment, prognosis, and quality of life.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Research Council of the Ministry of Human Capacities in Hungary (approval number: 55381-1/ 2015/EKU) and the institutional review board of the Semmelweis University, Budapest. Written informed consent to patients, or participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ES, DC, and ÁS: performed genetic analyses, participated in writing, and technical editing of the manuscript. NA: collected patient's data. LV: managed complement measurements. HF: provided and cared for study patients. The final version of the article was read and approved by all the contributors. All authors contributed to the article and approved the submitted version.

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REVIEW
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SERPING1 Variants and C1-INH Biological Function: A Close Relationship With C1-INH-HAE

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Hereditary angioedema with C1 Inhibitor deficiency (C1-INH-HAE) is caused by a constellation of variants of the *SERPING1* gene (n = 809; 1,494 pedigrees), accounting for 86.8% of HAE families, showing a pronounced mutagenic liability of *SERPING1* and pertaining to 5.6% *de novo* variants. C1-INH is the major control serpin of the kallikrein–kinin system (KKS). In addition, C1-INH controls complement C1 and plasminogen activation, both systems contributing to inflammation. Recognizing the failed control of C1s protease or KKS provides the diagnosis of C1-INH-HAE. *SERPING1* variants usually behave in an autosomal-dominant character with an incomplete penetrance and a low prevalence. A great majority of variants (809/893; 90.5%) that were introduced into online database have been considered as pathogenic/likely pathogenic. Haploinsufficiency is a common feature in C1-INH-HAE where a dominant-negative variant product impacts the wild-type allele and renders it inactive. Small (36.2%) and large (8.3%) deletions/duplications are common, with exon 4 as the most affected one. Point substitutions with missense variants (32.2%) are of interest for the serpin structure–function relationship. Canonical splice sites can be affected by variants within introns and exons also (14.3%). For noncanonical sequences, exon skipping has been confirmed by splicing analyses of patients' blood-derived RNAs (n = 25). Exonic variants (n = 6) can affect exon splicing. Rare deep-intron variants (n = 6), putatively acting as pseudo-exon activating mutations, have been characterized as pathogenic. Some variants have been characterized as benign/likely benign/of uncertain significance (n = 74). This category includes some homozygous (n = 10) or compound heterozygous variants (n = 11). They are presenting with minor allele frequency (MAF) below 0.00002 (i.e., lower than C1-INH-HAE frequency), and may be quantitatively unable to cause haploinsufficiency. Rare

benign variants could contribute as disease modifiers. Gonadal mosaicism in C1-INH-HAE is rare and must be distinguished from a *de novo* variant. Situations with paternal or maternal disomy have been recorded ($n = 3$). Genotypes must be interpreted with biological investigation fitting with C1-INH expression and typing. Any *SERPING1* variant reminiscent of the dysfunctional phenotype of serpin with multimerization or latency should be identified as serpinopathy.

Keywords: C1-INH-HAE, C1 Inhibitor, *SERPING1* gene, genetic variation, serpin function, serpinopathy, angioedema, hereditary–diagnosis

INTRODUCTION

Knowledge on angioedema has been first aimed at unraveling the pathophysiology of hereditary angioedema (HAE) with C1 Inhibitor (C1-INH) deficiency, namely, C1-INH-HAE (OMIM #106100; ORPHANET #91,378), a rare genetic disorder that is inherited as an autosomal-dominant trait in most cases. This prototypical condition has been shown to be bradykinin (BK) mediated by a clinical response to a specific BK receptor antagonist (1). Its prevalence is low, from 1/50,000 to 1/100,000, without known ethnic differences (2). HAE pathogenesis has been progressively deciphered from patient observations and basic investigations, showing that HAE results from variants in the *SERPING1* gene in association with the dysfunction of C1-INH (3). When the function of C1-INH has failed, circulating kallikrein–kinin system (KKS) is insufficiently controlled, with subsequent prekallikrein to plasma kallikrein conversion and the production of BK, a vasoactive peptide that causes increased vascular permeability with an activation of the B2 BK receptor. Together with KKS, complement and fibrinolysis are under C1-INH control, all systems sharing many inflammatory features are mediated by the vascular system (4). **Figure 1** shows the central position of C1-INH in the control of kinin forming systems and the additional companions that could be affected by pathogenic variants.

Complement activation is not documented as it is directly involved in HAE pathophysiology. However, plasma kallikrein has been recognized as a pro-convertase, with anaphylatoxin production (6). In addition to anaphylatoxin generation, complement proteases promote plasminogen activation (7), with plasmin production that in turn triggers KKS. This latter proteolytic system directly cleaves circulating high molecular weight kininogen, subsequently generating BK production. Reversely, plasmin has been demonstrated to activate the key complement proteins C3 and C5 (8). These observations provide arguments for an interplay between complement, KKS, and fibrinolysis (4, 9), sustaining inflammation with a pivotal control by C1-INH. Furthermore, medications targeting the KKS or a B2 BK receptor have been developed and opened a way to understand HAE pathogenesis (10).

An important issue in C1-INH-HAE is the relationship between the systemic plasmatic changes in the KKS activation process and the local effect of BK accumulation in angioedema attacks (11). A failed C1-INH function has been considered as a causative participant. However, other contributors might be critical for the disease severity risk [e.g., kinin catabolism (12) and neutrophil inflammatory mediators (13, 14)] or for an angioedema phenotype of upper airway [e.g., EBV infection (15)]. The contribution of peripheral blood mononuclear cells to the HAE clinical phenotype has been questioned (16). In practice, novel genetic strategies are emerging, resulting in the characterization of a combination of common variants in *SERPING1* and in other genes involved in kinin pathway and metabolism (17).

Many observations of families carrying *SERPING1* variants and associated C1-INH data have been collected. This study aims to figure out the state-of-the art of *SERPING1* genetics, with an advantage for C1-INH-HAE diagnostic and its relationship with C1-INH expression and function. Recognizing C1-INH features and *SERPING1* genetics together is a prerequisite for the curation of variant pathogenicity.

C1-INH DEFICIENCY

The diagnosis of HAE with C1-INH deficiency (C1-INH-HAE) is established on a decreased C1-INH function. Rosen et al. distinguished a type-1 HAE (HAE-1), where C1-INH-HAE results from the failure to synthesize the protein, from a type-2 HAE (HAE-2) where an abnormal, dysfunctional protein is synthesized (18). HAE-2 is commonly identified from the data presenting with normal, or elevated, antigenic C1-INH in serum (19). However, many dysfunctional missense variants with a low antigenic C1-INH have been shown to be expressed together with the normal allele (20); they are characterized as HAE-2. Sharing same clinical presentation, diagnostic and pathophysiology, both types are also sharing the same recommendations for treatment options (10). This suggests that a distinction between HAE-1 and HAE-2 should not be relevant for physician tasks, but could be valuable for a structure–function relationship in the identification of pedigrees.

Substantial misdiagnoses and the delayed diagnoses of C1-INH-HAE are common. A significant obstacle for diagnosis is its low prevalence, high phenotypic variability, and incomplete

Abbreviations: ACMG, American College of Medical Genetics; BK, bradykinin; C1-INH, C1 Inhibitor; C1-INH-HAE, Hereditary angioedema due to C1 Inhibitor deficiency; HAE, Hereditary angioedema; HAE-1, Hereditary angioedema type I; HAE-2, Hereditary angioedema type 2; KKS, Kallikrein–kinin system; MAF, Minor Allele Frequency; nC1-INH-HAE, HAE with normal C1-INH function; RCL, Reactive center loop; SRE, Splicing regulatory element; VUS, variant of uncertain significance.

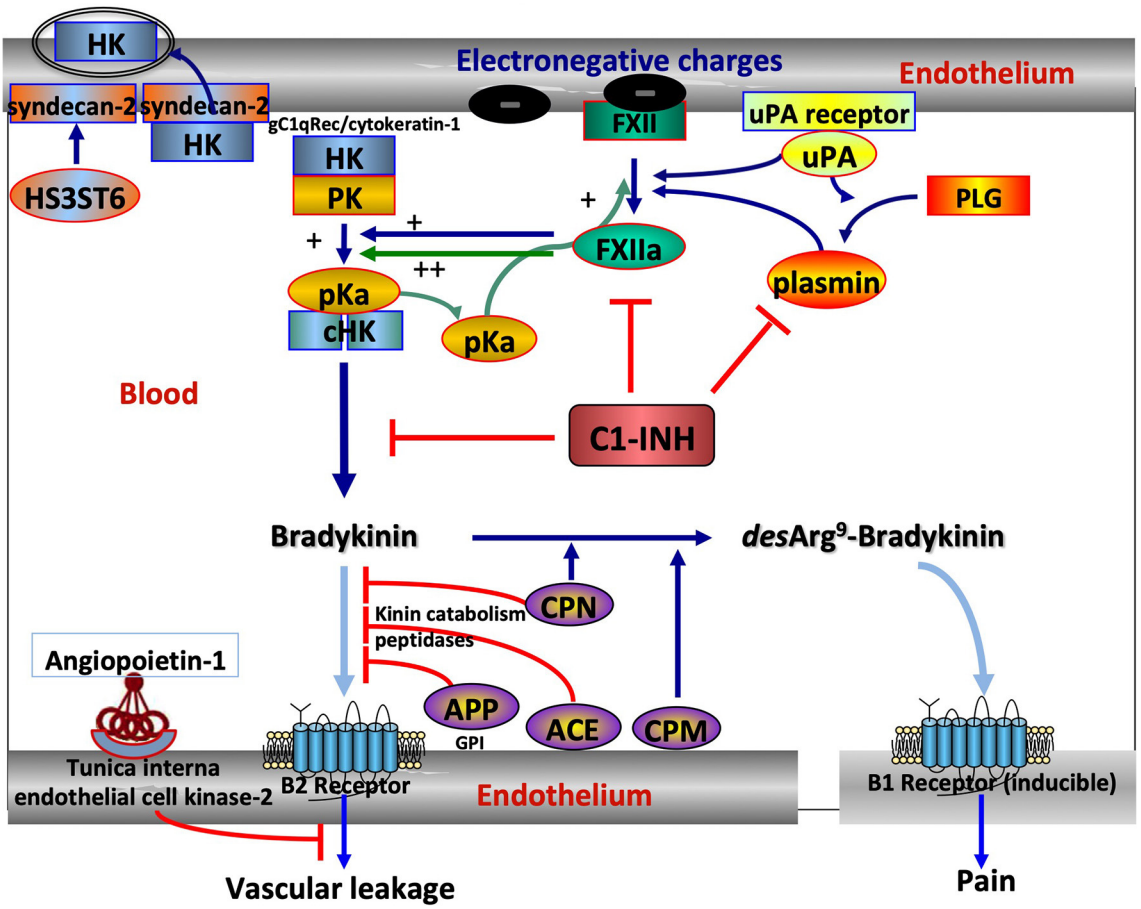
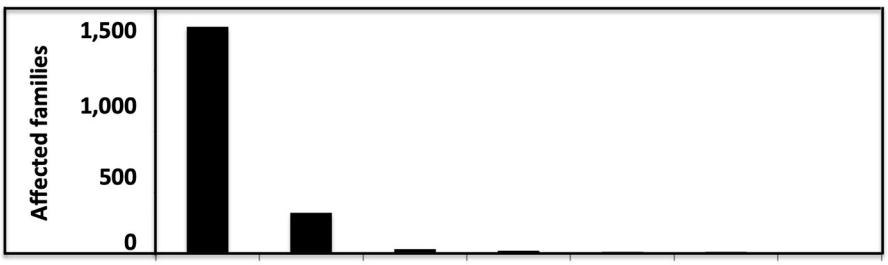


FIGURE 1 | The central position of C1 Inhibitor (C1-INH) within the kallikrein–kinin system (KKS) and its companions. KKS activation is triggered when FXII becomes activated into FXIIa on binding to an activator onto an electronegative membrane. KKS activation results in HK cHK and bradykinin (BK). The serpin C1-INH controls both activation and activity of KKS, with zymogen to enzyme conversion of FXII and pK. C1-INH also controls the reciprocal activation of FXII and pK by plasmin in an interconnected amplification (5). ANGPT1 is a secreted protein ligand for tunica interna endothelium kinase-2, a receptor expressed in growing vascular endothelial cells. ANGPT1 targets key mechanisms contributing to the maintenance of endothelium function by inhibiting the effects of permeability enhancing agents, including BK, protecting from extensive permeability. H3ST6 is involved in HK docking on the endothelial cell surface, preventing HK binding to gC1q receptor and cytokeratin-1 and engaging in kinin forming. Aminopeptidase P (APP) and Angiotensin-I converting enzyme (ACE) are the membrane peptidases that inactivate BK, whereas carboxypeptidases M and N transform a B2 ligand into a B1 ligand. What this scheme means for understanding hereditary angioedema (HAE). *SERPING1* variants display a markedly reduced control function of C1-INH toward KKS and plasmin. *F12* and *PLG* variants are shown to increase FXII and plasminogen (PLG) activation, respectively, and *KNG1* variants more susceptible to HK cleavage with BK production. *ANGPT1* variants showed reduced capacity to bind its natural receptor, with less control of BK-dependent vascular leakage. Because of incomplete heparan-sulfate modification of syndecan-2 by H3ST6, *H3ST6* variants were less able to take up HK *via* endocytosis into the endothelial cell and more HKs entering into the KKS process. Dark blue arrows indicate activation, green arrows the amplification loop, light blue arrows the ligand–receptor interactions, and red lines indicate an inactivation of BK function. FXII, Factor XII; pK, prekallikrein; pKa, plasma kallikrein; HK, High-molecular-weight kininogen; cHK, cleaved HK; ANGPT1, angiopoietin-1; H3ST6, Heparan-sulfate-glucosamine 3-*O*-sulfotransferase 6; uPA, urokinase-type plasminogen activator.

penetrance, with the failed suspicion of angioedema symptoms, in particular abdominal attacks (21). This emphasizes the need to identify patients with HAE and HAE families to improve disease management and patient outcome (22).

Hereditary angioedema due to C1 Inhibitor deficiency shares a common kinin dependency with other HAE situations: F12-HAE, PLG-HAE, KNG1-HAE, ANGPT1-HAE, and HS3ST6-HAE, but not with MYOF-HAE, U-HAE (**Figure 1**). The distribution of HAE conditions is shown in **Figure 2**. With 1,494 affected families recorded so far, C1-INH-HAE represents a predominant HAE condition (86.8%). Variants of these HAE-associated genes have been characterized with autosomal-dominant transmission and incomplete penetrance. Some rare *SERPING1* variants have been found in affected families with a recessive transmission (Section Recessive Variants), and *de novo* variants have been identified in 5.6% of the probands (20). These C1-INH-HAE features prompt biologists to perform the most accurate assays of the function of C1-INH for analytical HAE diagnostics.



	SERPING1	*F12*	*PLG*	*ANGPT1*	*KNG1*	*MYOF*	*H3ST6*
No of affected families	1,494	186	33	3	2	1	1
No of variants[1]	809	4	3	3	2	1	1
% of affected families	86.8	10.8	1.9	0.002	0.001	0.0005	0.0005

FIGURE 2 | The distribution of variants responsible for HAE and characterized as pathogenic/likely pathogenic, with a number of affected families. Pathogenic/likely pathogenic variants have been characterized in agreement with the American College of Medical Genetics (ACMG) criteria or as declared by authors in the observations. [1]Variants were characterized as pathogenic/likely pathogenic.

The Function of C1-INH and Its Analysis in Plasma

C1 Inhibitor is a multifunctional serine protease inhibitor (serpin) that controls various serine proteases involved in multiple plasmatic cascades [e.g., KKS, thereby limiting the production of the vasoactive peptide BK (23)]. C1-INH is a single-chain, highly glycosylated circulating protein of M*r* 105 kDa (nonreducing conditions) and 478 aminoacid residues (24).

As a serpin (clade G), it regulates serine proteases *via* an irreversible suicide-substrate mechanism as a set mousetrap (25). C1-INH consists of 3 β-sheet structures, 9 α-helices, and a Reactive Center Loop (RCL) located at the top of the central β-sheet (**Figure 3A**). RCL serves as a bait region for specific proteases. The target protease recognizes and cleaves the P1-P1′ scissile bond in the RCL, with an insertion of the hinge and RCL into the central β-sheet A as an additional strand 4A. This drives the covalently bound protease to the base of the serpin molecule (26, 27). After this conformational change, the serpin adopts a thermodynamically stable conformation, with an irreversible inhibition of the protease. C1-INH-protease complexes are then cleared by the liver (28). This remarkable conformational change represents a common feature for serpins and illustrates a conformational pathology (25, 29). C1-INH also directly interacts with native C1 to prevent the autoactivation of C1 (30), thereby inhibiting its own consumption.

Laboratory identification of C1-INH-HAE is challenging with a primary interest for physicians, with the need (i) to recognize a C1-INH dysfunction and (ii) to decide on a molecular diagnosis, e.g., for patients with HAE without family history or with records of inconsistent biochemical measurements.

C1-INH Inhibitor circulates in the plasma (0.21–0.35 g/L), corresponding to a nearly 100% serpin function measurement. Two types of C1-INH function assays are available, the residual enzymatic C1s protease/KKS activity (chromogenic assay) or the C1-INH-protease complex formation using an ELISA. The chromogenic assay is more sensitive than the ELISA for detecting a low C1-INH function and is often preferred (31, 32).

The genetic basis of C1-INH-HAE means that family history represents a starting point for diagnosis. So, all family members must be strongly encouraged to be tested when a C1-INH-HAE has been diagnosed in a relative.

C1-INH Expression Criteria: Biological Phenotype

Variants commonly characterized as HAE-2 from the only normal antigenic C1-INH criterion are found within the RCL (i.e., variants affecting the positions Ala^{443}-Arg^{444} in the mature sequence; **Figure 3A**) and at the positions Gln^{201} and Lys^{251} (20). Many missense variations meet the Rosen's criterion of HAE-2 (18) and affect the functionality after protein misfolding. When an abnormal, nonfunctional protein is synthesized, circulating C1-INH presents with a remnant 105-kDa species after incubation with equimolar C1s protease on the anti-C1-INH immunoblot (20). This analytical strategy is helpful to identify the molecular features of any missense variant, with HAE-2 characteristics, including latent and oligomerized forms (33).

Haploinsufficiency

As C1-INH-HAE is inherited as a dominant disorder in heterozygous cases, with one normal allele, antigenic C1-INH should theoretically be 50%. However, common observations recognized that C1-INH values are <35% of normal (i.e., from analytical threshold to 35%); attacks of angioedema are likely to occur when functional C1-INH levels are within this range (34).

Haploinsufficiency is common in C1-INH-HAE, where a dominant-negative variant product impairs the expression of normal allele. Haploinsufficiency may occur either through a *trans*-inhibition of normal protein expression (e.g., in an intracellular retention subsequent to intermolecular aggregates),

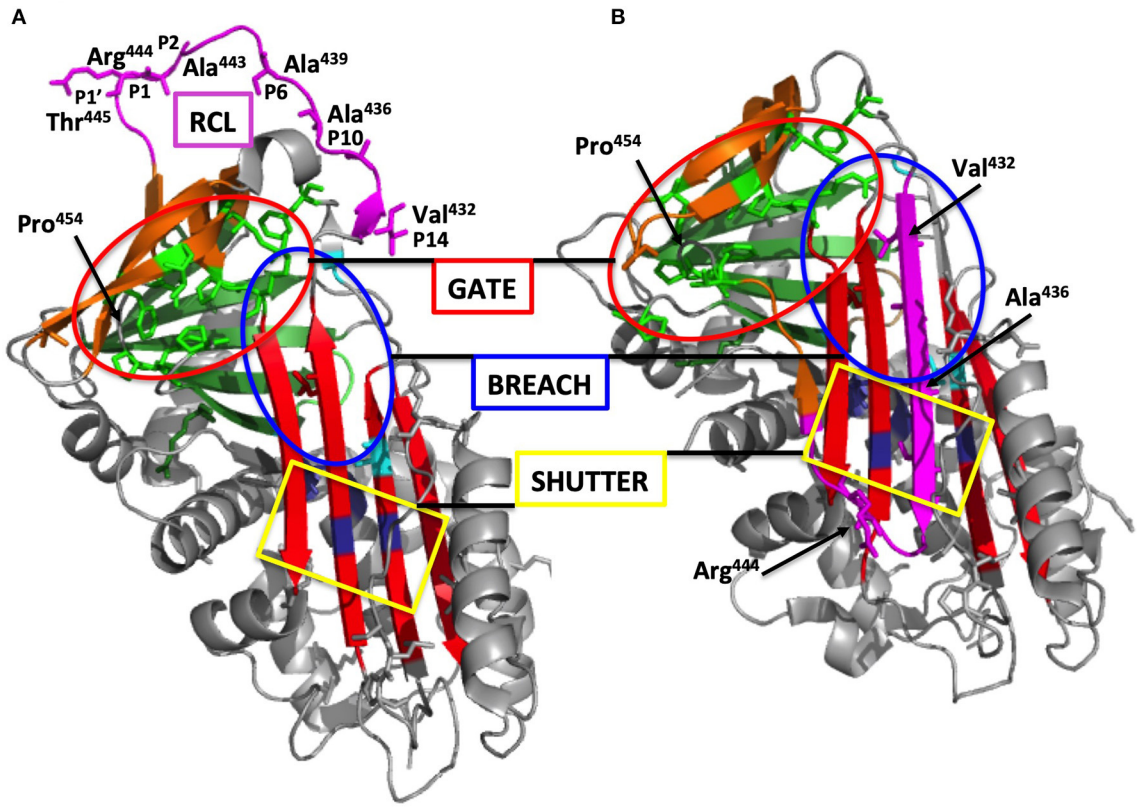


FIGURE 3 | Overall structure of C1-INH. **(A)** Native C1-INH serpin domain, with positions of strategic residues shown with side chains in sticks. Five regions for the serpin function are presented on the 3D-model of C1Inh (PDB ID 5DU3) presented using Pymol. The model starts at Phe^{100} and lacks a great part of the N-terminal domain (residues 1-112). Strategic functional regions are indicated with (i) the *reactive site loop* (RCL), colored purple, including Arg^{444} P1 and the *hinge region* (P15-P9), essential for protease recognition, RCL mobility and conformational transformation for its insertion as strand 4A (s4A) (i.e., S to R transition, after protease inhibition); (ii) the *central β-sheet A*, colored red, with the *breach* region, indicated by a blue ellipse, located at its top and point of initial insertion of RCL, and the *shutter* domain, with a yellow rectangle, close to the center of β-sheet A, that, with *breach*, assists sheet opening and accepts the insertion of conserved *proximal hinge* s4A between s3A and s5A; (iii) the *gate*, highlighted with green sticks and targeted with a red ellipse, including s3C and s4C of β-sheet C. β-sheets B and C are colored green and brown, respectively. **(B)** Latent form of C1-INH. The same regions involved in the serpin function but in a nonfunctional conformation where RCL is kept inside the central β-sheet A structure with additional s4A colored purple and remaining inaccessible to target proteases (PDB ID 20AY). Residue numbering was done according to mature C1-INH protein. Pictures were drawn by Dr. Christine Gaboriaud, Institut de Biologie Structurale, Grenoble France.

or with decreased C1-INH production due to altered epigenetic control (Section Dominant-Negative Effect).

Decreased C1-INH function in plasma could be caused by high catabolism of remaining C1-INH was demonstrated in a 95-kDa species in patient plasma (20). This molecular distribution is in line with an increased proteolysis, rather than with a haploinsufficiency.

Limits of Biological Testing

Transient decreases of C1-INH function are recurrently found in *F12*-c.983C>A;p.(Thr328Lys) female carriers, with angioedema attacks precipitated by estrogen intake or pregnancy. This decrease is consistent with C1-INH proteolysis by activated KKS (35).

Variant identification could be essential for the HAE diagnosis of patients presenting with symptoms, but also with the biological features of AAE-C1-INH or autoimmunity and with the lack of HAE family history, as reported by Veronez (36).

Homozygous and Compound Heterozygous Probands Carrying *SERPING1* Variants

Hereditary angioedema due to C1 Inhibitor deficiency dominant trait, though with incomplete penetrance in heterozygous probands, early suggested that homozygosity for *SERPING1* variants may be embryonically lethal despite the fact that no signs of increased developmental lethality are present in HAE cohorts (37). In the last 15 years, several HAE pedigrees evidencing a recessive manner of inheritance have been documented and to date account for 10 true homozygous probands and 11 compound heterozygote alleles segregating in nine pedigrees. In general, *SERPING1* variants with recessive behavior represent a minor percentage of the total (5.8%) and are commonly classified by pathogenicity prediction tools such as "benign," "likely benign," or "variant of uncertain significance (VUS)." They are rare in the general population, usually presenting with minor allele frequencies (MAFs) below 0.0001. No *SERPING1*

region has been recognized as specifically linked to a recessive feature, the variants being distributed all over the gene. No specific mutation categories are statistically associated with homozygosity, with two promoter, six missense, one splicing, and one indel variants (**Supplementary Table S1**).

The variants c.[-(163)C>T] and c.[-(161)A>G] are located in the promoter region of *SERPING1* and disrupt a putative CAAT box located at −62 bp from the origin of transcription (38–40). These two variants provide a hypothetical mechanism for their pathogenicity based on the defective transcription of *SERPING1*. The disruption of CAAT promoter sequences hampers the transcription of the affected alleles due to a defective binding of the RNA polymerase II and other nuclear-binding factors. However, in the case of *SERPING1*, there exists no direct evidence of the functionality of its CAAT sequence and therefore additional mechanisms may be postulated (41). The remaining recessive *SERPING1* variants reported affect the coding sequence of the gene and account for one variant in the 5′ untranslated region and six variants distributed through exons 4, 7, and 8. The variant c.-21T>C in the second nucleotide of exon 2 was reported as a probable cause of the disease in a homozygous proband with a HAE-1 phenotype (42). Due to its relatively high MAF (0.03), it has been reported as a polymorphic or disease-modifying allele (38, 43–45).

The seven remaining homozygous variants located in the coding sequence are associated with a HAE-1 phenotype in symptomatic homozygotes and with a HAE-2 (most commonly) or an asymptomatic presentation in heterozygous carriers. An illustrative example of this segregation of phenotypes is the c.1385T>G;p.(Ile462Ser) variant. In the original pedigree described by Blanch et al., HAE manifested exclusively in one of the two homozygous siblings as a HAE-1 phenotype while all their heterozygous relatives remained asymptomatic despite presenting with C4 consumption and a low C1-INH function (37). Interestingly, the homozygous probands from this family, as well as those with the c.[1198C>T];p.(Arg400Cys) (46) and c.[1379C>T];p.(Ser460Phe) (47) variants, presented with a very low or an undetectable antigenic C1q, thus reminding the phenotype of some patients with acquired angioedema with C1-INH deficiency. This acquired C1q deficiency is not an invariant trait in patients with C1-INH homozygous deficiency but pertains to the specific functional impairments of some recessive variants (47). Other variants have been found in homozygous carriers (**Supplementary Table S1**): c.[440T>A];p.(Val147Gln), c.[668A>C];p.(Gln223Pro), c.1202T>C];p.(Ile401Thr), and [c.646_647delAinsTCAGTGTCGTG], and the latter is characterized as a *de novo* variant (48).

Compound heterozygosity of *SERPING1* alleles with highly variable and incomplete penetrance is also a cause of C1-INH-HAE, with pedigrees presenting with uncommon symptomatic individuals (**Supplementary Table S1**); one or both allele(s) has (have) been characterized as VUS/benign variant(s).

Digenic Variants

On the other hand, the combined presence of a *SERPING1* variant and a variant in another HAE susceptibility gene is now recognized as a cause of the disease. Examples of such situations are the combination of *SERPING1* variants with the *F12*-c.-4C>T polymorphic variant (rs1801020; MAF 0.472) (49, 50) and that of the c.513 + A>G *SERPING1* variant with the *F12*-c.1032C>A;p(Thr328Lys) pathogenic variant presenting HAE symptoms since 7 years of age with a severe phenotype (51).

A curious case shows an interesting pattern of the localization of swellings in a family composed by patients carrying (i) the variant c.1480C>T;p.(Arg494*) in *SERPING1* in heterozygosis, (ii) the variant c.988A>G;p.(Thr328Lys) in *PLG* in heterozygosis, or (iii) both (52). Individuals carrying only the *SERPING1* mutation or the combination of *SERPING1* and *PLG* mutations presented abdominal pain and edema of the extremities (hands and feet) more frequently when compared to the patients carrying only the *PLG* variant. Records of patients with PLG-HAE present with a higher proportion of attacks affecting the tongue and face, and less abdominal attacks (53), which was confirmed in this family case study (52). The presence of *SERPING1* and *PLG* pathogenic variants demonstrated a combination of symptoms but was not enough to prove an increase in the severity of the disease phenotype.

In an attempt to explain and correlate the variability in the manifestation of symptoms in C1-INH-HAE with other genes, Veronez et al. (54) evaluated 45 *SERPING1* mutation carriers and 15 healthy relatives from 26 families. The authors analyzed 15 genes entangled in the function of KKS and metabolism of associated enzymes and ligands/receptors using a next-generation sequencing (NGS) panel, and a total of 211 different variants were identified in the 15 genes analyzed. *BDKRB2* and *CPM* presented a large number of variants in untranslated regions, whereas *ACE*, *CPM*, and *NOS3* genes presented a higher number of variants directly affecting amino acid sequence. Despite the large amount of variants identified, no specific variant was significantly associated to any of the clinical symptoms affecting the patients (facial, abdominal, extremities, upper airways, and genitalia), indicating that the modulation of HAE symptoms could require a more complex regulation, probably involving pathways beyond the KKS, epigenetics, and environmental factors.

Aiming to uncover the genetic basis of nl-C1-INH-HAE, Loules et al. also applied a custom NGS platform to analyze 55 genes related to KKS involved in angioedema pathogenesis (55). Patients with normal antigenic C1-INH were evaluated in the study using patients with C1-INH-HAE as control, deciphering the presence of common variants that could modulate the patient clinical phenotype. Although the frequency of variants per gene was comparable between HAE with normal C1-INH function (nC1-INH-HAE) and C1-INH-HAE, variants of the *KNG1* and *XPNPEP1* genes were detected only in patients with nC1-INH-HAE. The authors concluded that alterations in some genes (e.g., *KNG1*) could play a role in the complex trait of HAE. These results emphasize the importance of modulator genes in HAE clinical expression with a better understanding of disease pathophysiology. This observation possibly drives the discovery of new therapeutic targets and provides useful indicators for disease clinical management.

Polygenic/Oligogenic Conditions

Recently, the results suggest that polygenic situation is common in C1-INH-HAE and can significantly influence the penetrance of the disease, as exemplified by Veronez et al. (56). Almost all symptomatic patients from a pedigree presenting with C1-INH deficiency due to the c.1369G>C;p.(Ala457Pro) *SERPING1* variant carry multiple allele combinations with *ACE* [c.970C>T;p.(Arg324Trp)], *ENPEP* [c.638A>G;p.(Gln213Arg)], *KLK1* [c.433G>C;p.(Glu145Gln) or c.556A>G;p.(Lys186Glu)], *KLKB1* (c.428G>A;p.(Ser143Asn) or c.1679G>A;p.(Arg560Gln)], *KNG1* (c.533T>C;p.(Met178Thr) or c.591T>G;p.(Ile197Met)], *NOS3* (c.894T>G;p.(Glu298Asp) or c.2654G>T;p.(Arg885Met)] or *PRCP* (c.336A>T;p.(Glu112Asp)] alleles (56). This genetic complexity is pertaining with the present understanding of HAE as a pathology caused by an overactivation of the KKS and/or by a kinin accumulation.

DISTRIBUTION OF VARIANTS

The *ensembl* database displays 8,574 variants for the *SERPING1* gene (URL www.ensembl.org; ID ENSG00000149131; retrieved on October 28, 2021), with a homogeneous distribution along the 21,785 bases (GRCh38/hg38; latest assembly). Only 809 have been registered as pathogenic or likely pathogenic and 50 as VUS, according to the published observations and fulfilling the American College of Medical Genetics (ACMG) recommendations (57, 58). These variations are recorded in genetic databases (e.g., LOVD, tracked October 28, 2021; ClinVar, tracked November 2, 2021). **Supplementary Table S2** shows the distribution of pathogenic/likely pathogenic variants.

Missense Variants

Taking into account pathogenic/likely pathogenic/VUS variants, the distribution of missense variants is highly unbalanced between the mucin-like N-terminal domain (8/112 aminoacid residues; i.e., 7.1% of residues are affected) and the serpin domain (241/366; 65.8%); six variants have been recognized within the signal peptide sequence (6/22; 27.2%). This is congruent with a great impact of any modification of the peptide sequence within the serpin domain on C1-INH dysfunction. This prompts the data curator to a special attention for the interpretation of a missense variant with functional structures of strategic importance for serpin biology: gate, shutter, breach, hinge region, and less importantly, polyanion-binding domain (**Figure 3A**).

Missense variants (32.2% of all disease-causing variants) must be considered in association with mutant allele expression and C1-INH function. Most of the variant products are expressed, fulfilling criteria of a HAE-2 or an intermediate type, and have been distinguished between class I (i.e., altered exposure of the active site), class II (i.e., disturbed insertion of the RCL), and class III (i.e., conformational transition with spontaneous self or mutual insertion of the RCL) (20, 59) (**Figures 2**, **3**); the latter could shape a M* serpin conformation prior to the formation of extending chains of ordered and thermodynamically stable polymers, a characteristic of serpinopathy, or C1-INH species taking a stable and pathological latent form (**Figure 3B**) (25).

Large Deletions/Insertions

Large deletions/duplications are likely to result from the recombination between *Alu* repeat sequences present in most introns of the *SERPING1* gene (60), and account for 8.3% of all disease-causing variants in the *SERPING1* gene (19; LOVD). The 7 introns contain 19 *Alu* repeat sequences, and a high density of *Alu* repeats has been reported in introns 3, 4, and 6. This is considered as a hotspot for nonhomologous recombination resulting in deletions or duplications, with exon 4 as the most affected (22, 60). Different approaches, including Southern blot analysis (61), fluorescent multiplex PCR (62–64), and long-range PCR (22, 65), have been used to detect those variants, while MLPA represents the reference technique for the detection of large deletions and insertions (22, 64, 65). The disadvantage of those methods is that it cannot precisely locate the boundaries of the insertion/deletion. To determine a precise location, time-demanding combination of previously mentioned methods with direct sequencing of the boundaries must be performed (64–66). Recently, a targeted NGS platform has been developed and validated to simultaneously detect the large deletions/insertions providing at the same time both exact size and location of the deletions/insertions (67). Up to now 64 large deletions/insertions in the *SERPING1* gene have been reported (19; LOVD), but for the majority, the exact size and location of boundaries remain unknown.

Small Deletions/Insertions

Small deletions/duplications/insertions are abundant in *SERPING1* variants (36.2%) and mostly with subsequent frameshift and characterized as pathogenic/likely pathogenic.

Variants Affecting Splicing

Splicing affecting variants typically have a deleterious effect on protein expression and/or function. They can result in exon skipping (or even multiple exon skipping), intron retention, *de novo* splice site creation, cryptic splice site usage, or a combination of 2 or more of these effects. All mentioned options were demonstrated to occur in *SERPING1* variants (68).

Variants at Intron/Exon Boundaries

Splicing mutations comprise 14.3% of *SERPING1* mutations, and 47% of them are located in conserved canonical positions (±1, ±2) of splice sites adjoining all exons of the gene (20) (**Supplementary Table S5**). According to HGMD and LOVD databases, 35 substitutions and 13 deletions and insertions changing canonical positions have been described as HAE-1-causing variants. Pathogenicity of these variants is well established and easily assessed by *in silico* prediction tools.

The evaluation of potentially pathogenic splicing variants in other than canonical positions is more difficult and considerably less reliable using *in silico* prediction tools; especially of those affecting splicing regulatory elements (SRE). Importantly, various procedures have been applied to prove their effect on splicing, from analyses of *SERPING1* variant transcript from patients' blood-derived RNA samples or from minigene splicing assays (62, 67).

Siddique et al. referred for the first time the correlation of an intronic variant with HAE in a family carrying c.10291 + G>T (69). The function of the variant could not be proven as the mRNA appeared to be normal. However, the wild-type mRNA had a relative abundance of ≈50%, suggesting that the mutant replicate was not converted to a stable mRNA and rapidly degraded.

Up to now, 31 potentially pathogenic variants in noncanonical intronic positions of splice sites have been described. Whereas pathological impact of some of these variants has been well established based on functional tests or affirmative results of multiple prediction tools and/or the presence in several patients, other variants require further analysis of their functional significance. Illustrative examples are given by c.686-12A>G (44, 70), creating a *de novo* splice site in intron 4 (68) (**Figure 4**), c.52-10T>A in intron 2 after the observation of alternative short transcript species (72), and c.1250-13G>A in intron 7 after an *in silico* analysis (40).

Consequences of several new splicing variants already reported (20) could be thus evaluated as pathogenic/likely pathogenic, and the effect of others (e.g., c.890-8C>G) remains to be further established.

Interestingly, Colobran et al. detected c.6852 + T>A in intron 4. Functional studies of the mRNA demonstrated that this variant leads to the omission of exon 4 (73). Exon 4 consists of 135 bp (i.e., 45 codons); the lack of exon 4 corresponds to an in-frame deletion. Thus, the mutant allele could produce a protein lacking 45 amino acids. The levels of the mutant mRNA have been found to be very low compared to the wild-type, indicating that the mutant mRNA was degraded by one of the three surveillance pathways. In addition, the bioinformatic tool RNAfold® predicted a modification of the secondary structure. These data are consistent with the degradation of the mutant mRNA *via* the no-go degradation (NGD) pathway, which is associated with secondary structural features.

In ≈85% of cases, a G base is located in the fifth nucleotide of an intron (74). Variants at the +5 position are thought to significantly reduce the binding at the 5′ splice site to the complementary site at the U1snRNP particle, one of the first steps in the complex process of mRNA splicing (75). Consequently, the immediately preceding exon is omitted, followed by the activation of a deviating 5′ splice site and complete retention of introns (76). Alternatively, variants at this site may result in a reduced quantity of wild-type mRNA, or qualitative defects, by omitting an exon, activating a cryptic splice site, or creating a new splice site. The variant c.-22-19_-22-4del was detected in intron 1, predicted as pathogenic by the bioinformatic tool MutationTaster®, but marked as VUS, until a functional study and/or other evidence proves its pathogenic significance (77). At c.550-5 in intron 3, both variants c.551-5T>A and c.550T>G have been characterized as pathogenic due to its location near the 3' splice site (**Supplementary Table S5**). The c.890-14C>G variant has been detected in intron 5 (78) and the c.1250-13G>A variant in intron 7 (40, 79), the latter reducing the possibility of splicing at a rate similar to a variant located at the canonical positions. Other examples are displayed in **Supplementary Table S5**: c.515 + G>A, c.5505 + G>C, c.5505 + G>A, c.12495 + G>A, and c.12495 + G>T.

Exonic variants can also affect splicing. The impact of several exonic variants on splicing has been functionally evaluated using the RNA analysis. Three substitutions in the last nucleotide of the exon 3 (position c.550), the deletion of the last nucleotide of the exon 4 (c.685del) (**Figure 5**), and substitutions in the proximity of the 3′ end of the exon 5 (c.882C>G, c.884T>G) have been shown pathogenic (63, 68). Moreover, the exonic variant c.-21T>C activates a cryptic acceptor site and causes exon 2 skipping to a certain extent (80) and might be linked to a more severe phenotype when occurring in *trans* with another pathogenic variant (20, 80).

Deep-Intronic Variants (>20 nt Upstream/Downstream of the Exon)

Near the 3' splice site, a pattern of polypyrimidines (C or T, 5–40 nucleotides) is usually located between the branch point and the 3′ splice site. As stated in Section Variants at Intron/Exon Boundaries, consequences of new variants within introns need additional consideration, in particular, the influence of the c.6853 + 1G>A variant (72).

Four variants have been detected deeper within the *SERPING1* gene and have been associated with HAE, two being considered as deep-intronic variants (i.e., located ≈100 bp from the intron–exon junction). The variant c.1029 + 84G>A in intron 6 is recurrent, detected in the affected families, and thought to affect the transcriptional process (44, 81–84). The bioinformatics analysis predicted that c.1029 + 384A>G results in a *neo*-5' splice site with the incorporation of a pseudo-exon into the transcript. The inclusion of the pseudo-exon leads to an early termination codon and possibly to the destruction of the produced mRNA by cell control mechanisms, which could potentially explain the reduced amount of proteins. The functional study performed by RT-PCR finally proved the above prediction (83). Vatsiou et al. detected the variant c.-22-155G>T in intron 1 in four patients (85). The *in silico* analysis predicted that the variant c.-22-155G>T causes a deleterious effect on the gene and degradation of the mutated transcript by the mRNA surveillance pathways; barring this variant was demonstrated by the loss of heterozygosity on the cDNA level. The variant was classified as pathogenic, in line with ACMG-AMP 2015 guidelines (e.g., Intervar) (57).

INHERITANCE OF VARIANTS

Dominant-Negative Effect

Dominant-negative involves a change of the function; the disease is not caused due to the loss of protein function, but happens due to a change in protein function or in disequilibrated mRNA species between alleles. The dominant-negative variant acts antagonistically in the wild-type allele by impairing its expression or by biochemically interacting with the normal gene product and interfering with C1-INH function.

A first pioneering investigation on C1-INH transcripts has shown a "*trans*" inhibition of the normal allele by variant mRNA or protein (86). The authors studied C1-INH expression in

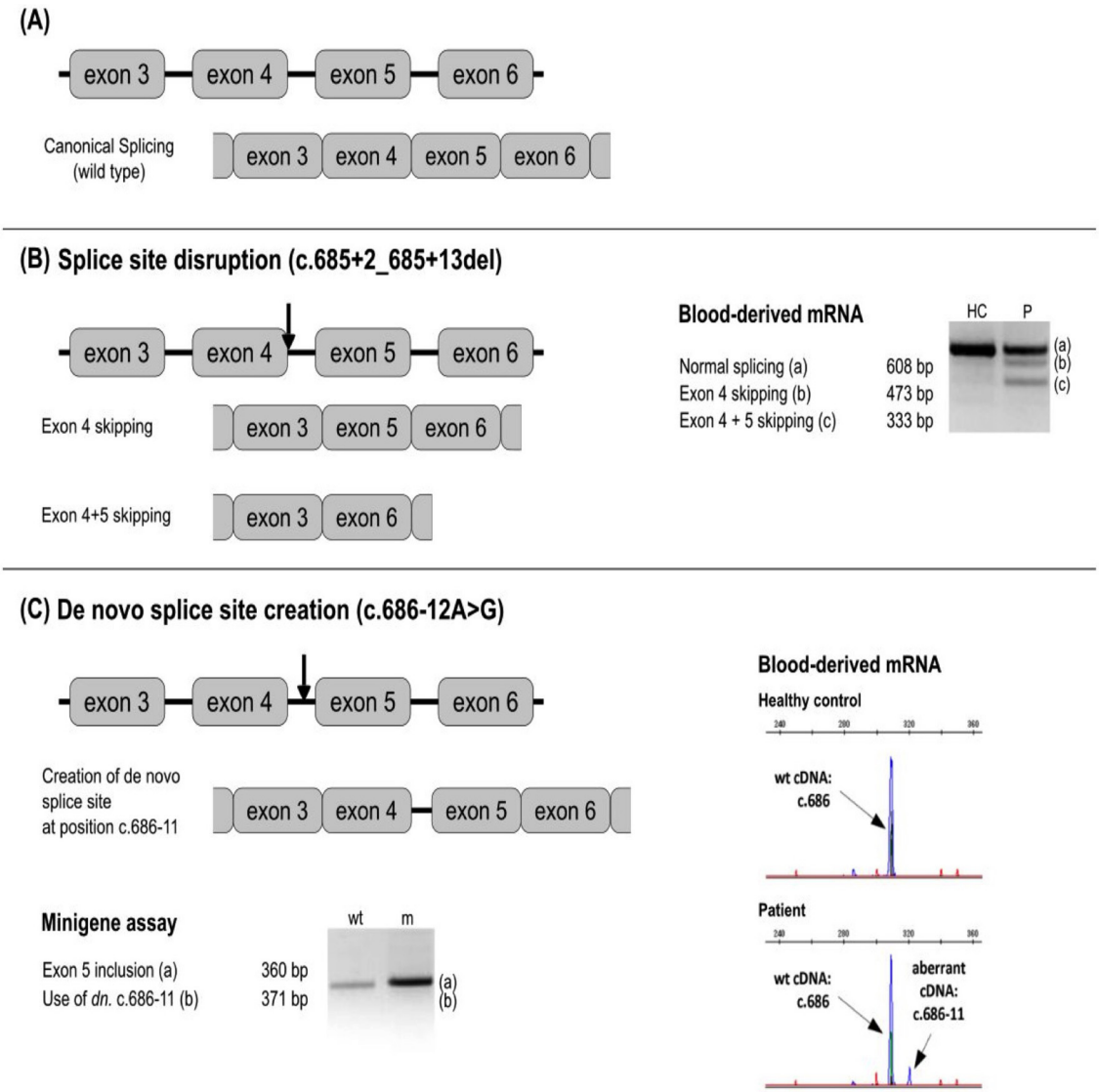


FIGURE 4 | Examples of a splicing variant analysis. Next to the respective splicing defect schemes, the results of blood-derived patients' RNA analysis and/or minigen analysis are shown as RT-PCR amplicons visualized by agarose or capillary gel electrophoresis. Arrows depict approximate location of the analyzed variants. **(A)** No splicing defect. **(B)** Splice site disruption. The variant c.685 + 2_685 + 13del leads to the formation of new transcripts with exon 4, and exons 4 + 5 skipped. **(C)** New splice site creation. The variant at c.686-12 leads to the splice site gain at the position c.686-11 and the inclusion of intron 4 in the transcript. *In silico* prediction is helpful to identify splicing-affecting mutations prior to functional assays (71).

fibroblasts in which the mutant and wild-type mRNA and protein could be distinguished because of the deletion of exon 7. In patient cells, the wild-type mRNA was expressed at ≈50% of normal, whereas the mutant mRNA was 17% of normal. Rates of synthesis of both normal and mutant proteins were 11% and 3% of normal, respectively (i.e., lower than predicted from the mRNA levels), suggesting a *trans*-inhibition of normal allele by a variant product. A substantial reduction of both mRNA as well as C1-INH protein expression has also been shown using two allele-specific PCR in a carrier of the variant c.*101_*254del, a 155-bp deletion 100 bp downstream of the stop codon in exon 8 (87).

An elegant demonstration of dominant-negative effect of variants has been provided by Haslund et al. (88), where the mutated C1-INH species affected wild-type C1-INH in a dominant-negative manner with intracellular/plasmatic C1-INH aggregates and subsequent reduction in functional C1-INH. C1-INH encoded by a subset of HAE-causing *SERPING1* alleles disturbed the secretion of normal C1-INH protein in a dominant-negative fashion by triggering the formation of protein–protein interactions between normal and mutant C1-INH. The authors observed large intracellular C1-INH aggregates that were trapped in the endoplasmic reticulum.

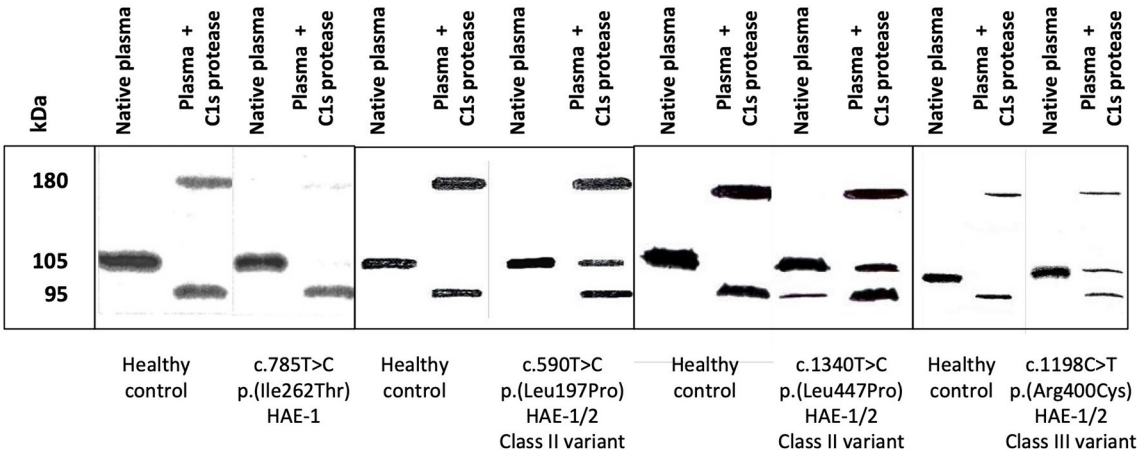


FIGURE 5 | Circulating molecular species were displayed by an anti-C1-INH immunoblot assay. Citrate-plasma samples were collected from patients presenting with hereditary angioedema with C1 Inhibitor deficiency (C1-INH-HAE). Native plasma and plasma submitted to a 15-min incubation at 37°C with 0.1 nM C1s protease (plasma + C1s protease) were analyzed as described in (20). Four assays are displayed corresponding to healthy controls and patients with C1-INH-HAE: one type-I HAE (HAE-1) variant and three HAE-1/-2 variants, distributed in classes II and III. HAE-1/-2, C1-INH-HAE satisfying Rosen's criterion (18), with the expression of both alleles and presenting with a low antigenic C1-INH. C1-INH, C1 inhibitor.

Interestingly, the transfection of wild-type *SERPING1* constructs into cells was able to remove the block in normal C1-INH secretion (88). Polymerogenic variants have been recognized with a location in proximity to/within the insertion site of RCL in C1-INH (i.e., shutter and hinge/gate regions; **Supplementary Table S3**), for example within α-helix C, c.566C>A;p.(Thr189Asn), β-sheet 3A, c.878T>C;p.(Ile293Thr) (33), α-helix F, c.838_846del;p.(Leu281_Ser283del) (88). The first variant prone to oligomerization has been recognized with c.1372G>A;p.(Ala458Thr) within RCL (89) and c.818_820del;p.(Lys273del) affecting the inter-domain α-helix F/β-sheet 3A (90). More recently, an additional variant p.(Ser150Phe) targeting α-helix A has been shown to be stably expressed within the cultured cells and not secreted into the medium at all. The mutant C1-INH significantly prevented the secretion of wild-type C1-INH, with its degradation within the cytoplasm through an interaction with the mutant protein (91). Observations of protease-resistant mutant C1-INH species suggest circulating latent C1-INH species that could also represent stable and low energetic conformations (**Figure 3B**, **Supplementary Table S3**) (20, 92).

Recessive Variants

Some variants have been shown to circulate in a 50% expression of normal C1-INH allele, with an absence of haploinsufficiency (**Supplementary Table S4**). They do not segregate with symptomatic carriers. Homozygous carriers have been recognized as symptomatic for angioedema or these variants may have a disease-modifying effect (Section Homozygous and Compound Heterozygous Probands Carrying *SERPING1* Variants).

The recurrent c.1198C>T;p.(Arg400Cys) variant has been studied for its remittent expression (93). It is responsible for a temporary drop of C1-INH function with an enhanced effect in a homozygous state to express a HAE phenotype (recessive inheritance; 45). Once correctly folded, C1-INH^{Arg378Cys} is secreted as an active, although quite unstable, monomer. However, it could bear a folding defect, occasionally promoting protein oligomerization and interfering with the secretion process. Environmental factors (i.e., temperature, pH, and oxidative stress), which could apply even in situations of mild physical stress, like hyperthermia or metabolic acidosis, have been demonstrated *in vitro* to affect the stability and the function of C1-INH^{Arg378Cys}.

Rare variants of *SERPING1* yielding deficient inhibitory activity toward complement C1 proteases but not toward the KKS proteases result in paucisymptomatic HAE. These have been characterized within the RCL [e.g., variations at the P2 position c.1394C>T;p.(Ala465Val) (94), at the P6 position c.1382C>T;p.(Ala461Val) (95, 96)], or out of the RCL [e.g., c.452T>G;p.(Leu151Arg) (20, 97)]. The position P2 in C1-INH supports protease specificity, with a significant reduction in rate constants for the reaction of C1-INH^{Ala443Val} with C1r, but not with C1s, FXIIa, plasma kallikrein or plasmin (96). However, C1-INH^{Ala439Val} (i.e., a variation at P6) displays a moderate decrease in control of C1s, compared with the wild-type protein (96).

Uniparental Disomy

Gonadal mosaicism situations have rarely been detected in C1-INH-HAE; this condition must be distinguished from a *de novo* variant. The identification of mosaicism is important in establishing the disease diagnosis, assessing recurrence risk and genetic counseling. The pathogenic variant could be inherited from a maternal or paternal allele.

From a Maternal Allele

A c.[597C=/>G] condition has been found in a family in which only both sons, but not the parents, show clinical and laboratory

findings typical of HAE, with allele segregation demonstrated using DHPLC (98). c.597C>G variant has not been detected in DNA derived from buccal cells, urinary cells, hair roots, and cultured fibroblasts from the mother, whereas it has occurred on the maternal transmitted chromosome.

From a Paternal Allele

(1) Family 1. A c.[69_139=/del] has been shown in a family where three sons, but not the parents, show clinical and laboratory findings typical of HAE, with demonstrated allele segregation (99). c.69_139del variant has not been detected in DNA derived from somatic cells from the father and the mother, whereas it has occurred on the paternal transmitted chromosome.
(2) Family 2. A c.[536C=/>T] has been observed in a family in which only both sisters, but not the parents, show clinical and laboratory findings typical of HAE, with allele segregation demonstrated using Sanger sequencing (100). c.536C>T variant has not been detected in DNA derived from lymphocytes from the father and the mother, whereas it is present on the DNA prepared from the sperm of the father and on the paternal transmitted chromosome.

NONCAUSAL VARIANTS AS GENE MODIFIERS

The combination of genetic variants may explain the variability in the manifestation of symptoms in C1-INH-HAE, in which nonpathogenic variants in diverse genes may confer susceptibility to a more severe phenotype when associated to pathogenic mutations in *SERPING1*. Within *SERPING1*, the c.-21T>C variant has been recognized as a disease modifier (Sections Homozygous and Compound Heterozygous Probands Carrying *SERPING1* Variants and Variants at Intron/Exon Boundaries). Beyond the known pathogenic mutations in *F12* gene that are described as causative for HAE (c.983C>A, c.983C>G, c.971_10182 + 4del, c.892_909dup), a common polymorphism, c.-4T>C (*F12*-46C/T) is demonstrated to influence the severity of disease in patients with C1-INH-HAE (50). This variant was associated with a delay in the onset of symptoms and with a decreased necessity to use long-term prophylaxis therapy (50, 101). A further study confirmed low FXII serum levels in patients with C1-INH-HAE carrying the T allele and found that asymptomatic patients presented the T allele in a higher frequency compared to symptomatic ones (102). Another study sequenced the exonic and regulatory regions (5′-UTR and 3′-UTR) of *F12* gene from 161 C1-INH-HAE and 191 HAE-nC1-INH, and found 6 *F12* polymorphisms in patients with C1-INH-HAE and 9 in patients with HAE-nC1-INH, including rare and first described variants (103). Variants such as *F12*-c.1768T>G;p.(Cys590Gly), which affect the catalytic domain of *F12* in a hotspot previously associated with protein deficiency, could be beneficial to HAE genetics. However, more studies are needed to establish any protective association of this variant in HAE (54).

DATA CURATION

In silico Rating

Although the genetic screening of the *SERPING1* gene has been facilitated by recent high-throughput technologies that have been available for massive DNA sequencing, clinical classification of the detected variants remains challenging. *SERPING1* variants could be divided into categories according to their possible pathogenicity:

- All variants (nonsense, frameshift, splicing, and large defects) with structural changes or misfolding of the protein, likely associated with a deleterious impact.
- Missense variants and changes affecting untranslated sequences in the 5′ or 3′ ends, both lacking strong evidence regarding their pathogenicity.

Pathogenicity supporting evidence provided by large pedigrees is required, such as functional analyses, population data, *in silico* predictions, and segregation family studies. Bioinformatic tools (e.g., SIFT®, PolyPhen®, Mutation Taster®) have been developed based on evolutionary conservation, the type of amino acid change, and the position within a functional domain, allele frequency. The certainty with which any detected variant is considered clinically relevant falls within a spectrum, ranging from pathogenic to unrelated to the phenotype.

Germenis et al. introduced a specific customization of ACMG criteria for *SERPING1* to improve variant interpretation of *SERPING1* variants (58). Every detected variant should be assessed with respect to its presence in public, internal and disease-specific databases, population data, computational predictions, *in vivo* and *in vitro* test results, evidence of segregation, and allelic and variant-specific information. The ACMG criteria that are supporting or tolerable must be applied to the abovementioned evidence, whenever possible, resulting in variant classification in one category (i.e., benign, likely benign, VUS, likely pathogenic, and pathogenic) (57). Variants and supporting data should be submitted to public databases upon classification, new evidence that may alter the initial variant assessment and favoring further exchanges between submitters. Online bioinformatic tools can be helpful for determining pathogenicity (e.g., Genetic Variant Interpretation Tool, InterVar, Varsome).

About variants detected in introns, many potential SRE-affecting variants fall into the category of so-called "variants of unknown significance" (104). To distinguish between pathogenic/likely pathogenic mutations and harmless non-splicing-affecting variants, medical geneticists are encouraged to investigate *in vitro* on transcript distribution from patient RNA samples or, although less reliable, to use *in silico* predictions (105). An estimate of exon susceptibility to be skipped or to activate nearby cryptic splice sites can be possible by SRE predictions. An evaluation of their reliability and potential use in clinical diagnostic settings has been developed by Grodecká et al. (71).

Functional Analysis of Serpin Function

Establishing a dysfunctional C1-INH protein is a prerequisite for further genetic analysis. The common C1-INH function testing should be advantageously completed by an analysis of circulating species for missense variants.

When variants are recognized in an intronic region out of a noncanonical splice sequence, a transcription analysis of an RNA sample extracted from blood nucleated cells is recommended for a functional distinction between a benign and a pathogenic/likely pathogenic variant. Cultured monocytes provide RNA samples of good quality for a downstream analysis; however, the distribution of transcripts could be different from that extracted from blood nucleated cells. A specific approach taking into account a possible NMD of transcripts with premature stop codon should be used to minimize a risk of missing aberrant transcript when evaluating RNA extracted from patients' blood cells (83).

Structural Analyses With Genotype to Phenotype Correlation; Conserved Positions Among Serpins

C1 Inhibitor controls target proteases as a suicide-substrate where RCL displays the scissile bond Arg^{444}-Thr^{445} (i.e., P1-P1′; **Figure 3A**). The N-terminal region of RCL is conserved among inhibitory serpins, maintaining proper RCL mobility and loop insertion. However, the sequences adjacent to the cleavage site in the RCL, P6-P4′ (i.e., Ala^{439}-Val^{448}), are highly variable between the different serpins and considered to be a major determinant of serpin specificity (106). In this region, some variants have been recognized as benign/likely benign (e.g., those at the positions Ala^{439}, Val^{442}, or Val^{448}); variants at nonadjacent positions have been characterized as pathogenic because a mutant on the hinge/RCL sequence packs favorably in the loop-inserted latent structure (e.g., those at the positions Ala^{436} or Pro^{454} located in the proximal and distal hinge, respectively; **Figure 3B**). Importantly because of a lack of the suicide-substrate property of C1-INH, all variants at position Arg^{444} are interpreted as pathogenic, inconsistently with commonly used *in silico* predictions.

If the protruding structure of the RCL makes it more accessible for an interaction with target proteases, it also favors the native serpin to be in a stressed thermodynamically metastable conformation M*. However, polymerized C1-INH represents a stable and low energetic conformation, which can be achieved upon some missense mutations (107). Variants have been recognized as favoring the thermodynamically stable conformation, with susceptibility to spontaneous loop-sheet polymerization or latent serpin species, as described in Section Inheritance of Variants (**Supplementary Table S3**). These variants, whose feature makes it recognized in the class III identified in Section Missense Variants, should be included as HAE-2. **Figure 5** shows an example with the p.(Arg400Cys) variant.

Frequency of Variants Within Global or Selected Population

Pertaining to the characterization of pathogenic criteria with C1-INH-HAE incidence of 0.00002, submitters are invited, whenever possible, to consider MAF (i.e., MAF in a global/selected population) (57).

Figure 6 shows a short algorithm displaying the position of biological and genetic analyses within a diagnostic algorithm.

Contribution to the Field Statement

To our knowledge, this is the largest study taking an overlook of the constellation of *SERPING1* variants found in nearly 1,500 HAE families. This study emphasizes that etiopathogenesis of C1-INH-HAE could be consistently implemented by C1-INH molecular analyses.

Misfolding and polymerization/latentization of the mutated serpins are at the base of a group of conformational diseases collectively known as serpinopathies (25, 29, 107). Likewise, missense mutations in *SERPING1* can cause polymerization/latentization, with an impaired secretion or a failed cleavage by target protease, that lead to C1-INH deficiency and C1-INH-HAE (20, 33, 88, 89).

Recording *SERPING1* genetics together with biological data on serpin function and C1-INH transcripts, for intronic variants out of canonical sequences, should contribute to a high quality value for national registries and for open databases.

CONCLUSION AND PERSPECTIVE

Hereditary angioedema due to C1 Inhibitor deficiency has been first characterized as a monogenic disease; however, cumulative arguments on a contribution of additional alleles make clear that clinical variability of C1-INH-HAE is substantially attributed to modifier genes. As suggested by Veronez et al. (36), even if pharmacogenomic associations are very difficult to prove in a rare disease, recommendations will assist the physician for an optimal treatment option for individual patients.

A pathogenic model of C1-INH-HAE has been proposed with a KKS activation in a systemic activation process, where fluid-phase activation of the KKS generates BK, associated with local manifestations after an interaction with locally expressed endothelial kinin receptors (11). The model provides an explanation for why symptoms can occur at multiple sites during an attack and why HAE attacks respond well to modest increases of circulating C1-INH function. The recent observations of multiple allele combinations and of abnormal kinin metabolism are congruent with this model, making C1-INH a strategic component with the participation of additional parameters in patient clinical phenotype.

Functional studies of modulating factors, acting on systemic activation or on activator-bound process, should combine with the discovery of new mutations, with genotype to biological phenotype associations and including a large number of patients.

The document presented here describes the biological and structural features of C1-INH deficiency in relation to groups of *SERPING1* variants. As well as inherited serpinopathies, namely,

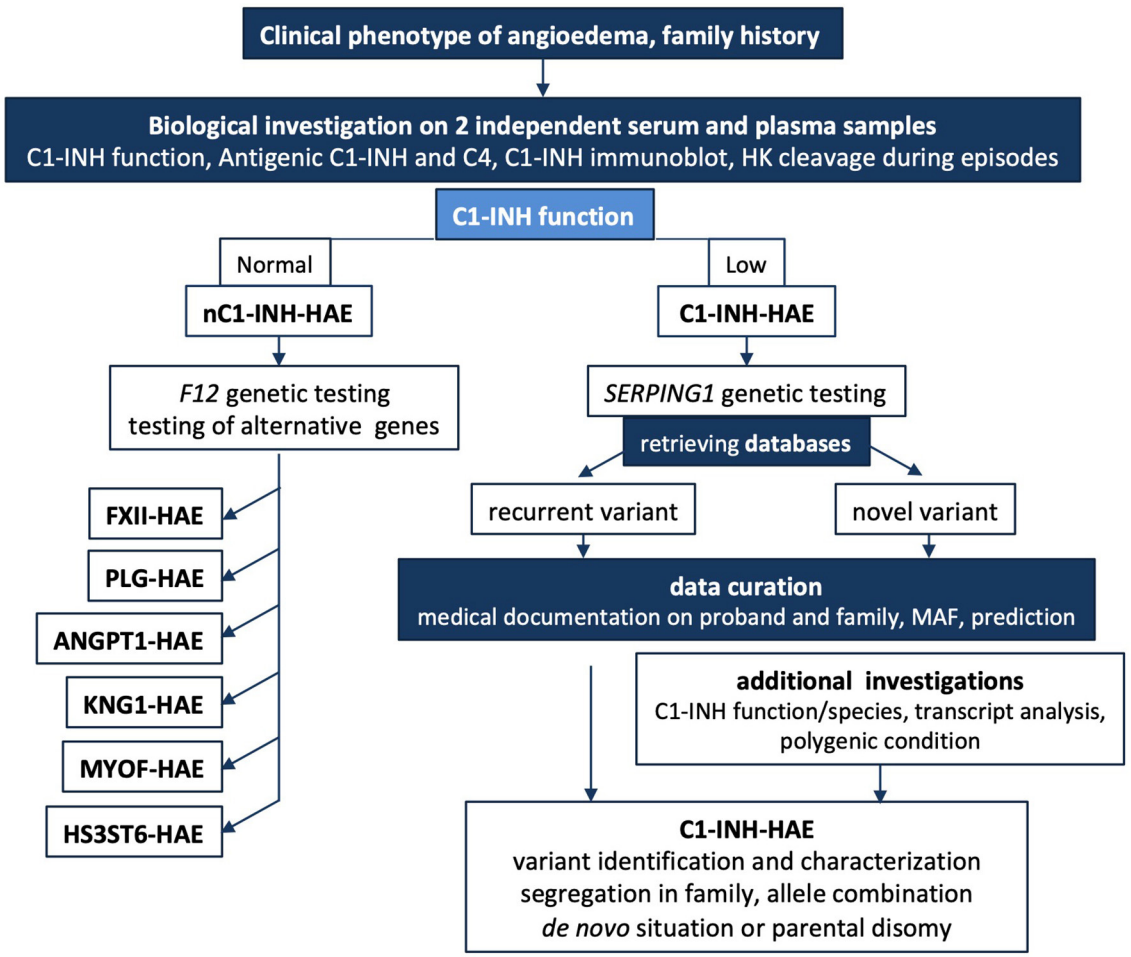


FIGURE 6 | Short algorithm for C1-INH-HAE. The medical algorithm of Caballero et al. (108) is supplemented for biological and genetic additional testings. In cases where there is a family history of HAE or the clinical history is highly suggestive of HAE, biological investigation should begin with an assessment of the established C1-INH function after 2 independent determinations on plasma; this mandatory testing can be completed by C1-INH molecular species and in some instances by high molecular weight kininogen cleavage to establish the involvement of KKS. A low antigenic C4 in serum could contribute to biological diagnostic, but false positives should be considered because *C4A* and *C4B* null alleles are common. A normal C1-INH function could suggest a nC1-INH-HAE and *F12* genetic study should be performed. When unsuccessful, alternative genetic testing could be developed. *SERPING1* genetic testing can confirm a C1INH-HAE diagnosis. Medical geneticists investigate a possible *de novo* mutation or a parental disomy on additional DNA analyses. In case of intronic variant detection, transcript distribution is investigated for noncanonical sequences. Data curation is mandatory for every novel variant submitted to sequence interpretation according to ACMG guidelines (57, 58). C1-INH-HAE, hereditary angioedema with C1-INH deficiency; nC1-INH-HAE, hereditary angioedema with a normal C1-INH function; F12-HAE, with gain-of-function of FXII; PLG-HAE, with gain-of-function of plasminogen; ANGPT1-HAE, with altered angiopoietin-1; KNG1-HAE, with high molecular weight kininogen susceptible to cleavage; HS3ST6-HAE, with altered heparan sulfate-glucosamine 3-*O*-sulfotransferase 6; MYOF-HAE, with altered myoferlin; HK, high molecular weight kininogen; MAF, minor allele frequency.

α1-trypsin deficiency, could be considered as ideal candidates for gene therapy, strategies for C1-INH-HAE treatment have been suggested. Based on the argument that HAE should be viewed primarily as a metabolic liver disorder, new therapeutic approaches to C1-INH-HAE have been outlined by Ameratunga et al. (109). Given the very high costs of treating HAE, the authors have considered that gene therapy as curative option may become feasible in the next decade.

In a next future, epigenetics and environmental factors should be considered in the molecular identification of C1-INH-HAE with the characterization of individual severity risk factors as well.

DATABASES AND BIOINFORMATICS SUPPORTS

- ClinVar: www.ncbi.nlm.nih.gov/clinvar/
- Ensembl: http://www.ensembl.org, a centralized resource for geneticists
- Exonic splicing enhancers: ESEfinder release 3.0
- Genetic Variant Interpretation Tool: http://www.medschool.umaryland.edu/Genetic_Variant_Interpretation_Tool1.html
- Human Splicing Finder, version 3.1: www.umd.be>hsf
- InterVar: https://wintervar.wglab.org/evds.php

- LOVD: Leiden Open Variation Database; databases.lovd.nl/shared/variants/SERPING1
- Varsome: https://varsome.com/,InterVar, wintervar.wglab.org.

AUTHOR CONTRIBUTIONS

CD, AL-L, AGh, ML-T, SC, DP, MR, TF, CV, JP, and AGe wrote sections or subsections of this manuscript. DP and AGh prepared **Figure 5**. TF and HG prepared **Figure 4**. FP prepared **Supplementary Tables S2**, **S3**. HG prepared **Supplementary Table S5**. CD, AL-L, AGh, ML-T, SC, DP, MR, TF, CV, JP, and AGe revised the last version of this manuscript. CD supervised the writing of the manuscript and the selection of the figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.835503/full#supplementary-material

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BRIEF RESEARCH REPORT
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Real-Life Experience With Subcutaneous Plasma-Derived C1-Inhibitor for Long-Term Prophylaxis in Patients With Hereditary Angioedema: A Case Series

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Hereditary angioedema due to C1-inhibitor deficiency (C1-INH-HAE) is characterized by swelling attacks that may be even life-threatening. To reduce the frequency of attacks, some patients need a long-term prophylaxis (LTP). In addition to the intravenous administration, plasma-derived C1-inhibitor (pdC1-INH) has been proved effective also if administered subcutaneously at the dose of 120 IU/kg/week. In this case series, we collected from clinical records data about 5 patients with poorly controlled C1-INH-HAE with the registered LTPs or with difficult venous access, referred to the angioedema center in Milano (Italy), who received it at lower doses, i.e., 42.86–65.22 IU/kg/week. All the patients experienced a reduction in the attack rate, ranging from 29.67% to 96.53% compared with a control period with a different LTP or with no LTP. For one patient, the comparison was made with a period when he received s.c. pdC1-INH 2 (with poor outcomes) instead of 3 times a week, which made the patient experience a decrease in the attack rate from 5.26 to 1.12 attacks/month. Observation periods varied between 2.6 and 47.97 months. Two patients reported adverse events, which were localized at the infusion site and mild in severity. In conclusion, subcutaneous pdC1-INH represents an alternative therapeutic choice according to the physician's judgment for selected patients with HAE poorly controlled with registered LTPs. In patients with difficult venous access, in countries where pdC1-INH is not approved for subcutaneous administration, about half the recommended dose may be beneficial, although suboptimal results may be obtained.

Keywords: plasma-derived C1-inhibitor, hereditary angioedema, subcutaneous use, attack frequency, dose/weight ratio

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INTRODUCTION

Hereditary angioedema due to C1-inhibitor deficiency (C1-INH-HAE) is characterized by recurrent attacks of swelling that may be life-threatening in cases of laryngeal involvement. Treatment strategies for HAE include on-demand therapy to rapidly resolve angioedema symptoms, short-term prophylaxis (STP), to prevent attacks when a patient is exposed to known triggers, and long-term prophylaxis (LTP), to decrease the frequency and severity of attacks (1).

Treatment must be individualized to provide optimal care and normalize quality of life. Whereas on-demand therapy is required for all patients, LTP is used as needed for individual patients.

Among the products for LTP, plasma-derived C1-inhibitor concentrate (pdC1-INH) administered intravenously was shown to reduce the frequency of acute attacks by 50% compared with placebo in a study published in 2010 (2). Despite the LTP with pdC1-INH, the patients continued to experience, on average, 6.26 attacks in 12 weeks (2), which were treated with additional rescue infusions of pdC1-INH.

Subcutaneous (s.c.) administration of Berinert® (CSL Behring) has been studied in Phases 2 and 3, and open label extension COMPACT trials and shown to reduce the frequency of attacks compared with placebo (3–5). Unlike intravenous administration, s.c. administration maintained the plasma C1-INH activity levels continuously above ∼40% of normal (3), which is the threshold known to have a clinically meaningful effect on preventing HAE attacks (6). In Europe, s.c. Berinert® is licensed as LTP at a dose of 60 international units (IU) per kg of body weight by s.c. injection two times weekly (7, 8).

In Italy, Berinert® for s.c. use has not yet been registered. Patients with severe disease and frequent attacks are prescribed other LTPs, such as intravenous pdC1-INH, androgens, and tranexamic acid. When data were collected, neither lanadelumab nor berotralstat was available in Italy. For patients experiencing frequent breakthrough attacks despite LTP or having poor venous access, an unmet need for prophylaxis is present (3).

METHOD

Patients with poorly controlled C1-INH-HAE with the registered LTPs or with difficult venous access, referred to the angioedema center in Milano (Italy), were treated with plasma-derived C1-inhibitor (Berinert®) registered for on-demand treatment at the dose of 20 IU/kg, administered subcutaneously as LTP. The dose of s.c. pdC1-INH injection was lower than the recommended dose for subcutaneous use (which is not approved in Italy). We collected relevant data and report the results.

Demographic data and clinical history (frequency and treatment for attacks) were retrieved from the clinical records. The severity of attacks was categorized as mild if no interference with activities of daily living was experienced, moderate in case of partial interference, and severe for complete incapacity. The patients were tested for functional and antigenic C1-INH. C1-INH-HAE type 1 was diagnosed when both functional and antigenic C1-INH were $\leq$ 50% of normal (9).

Although the dose/weight ratio per administration is generally used in clinical studies, we chose to calculate the dose/weight ratio per week in order to allow comparisons among patients receiving a different number of administrations per week.

RESULTS

In this analysis, 5 patients with C1-INH-HAE type 1 were observed.

The dose/weight ratio of s.c. infusions weekly ranged from 42.86 to 65.22 IU/kg (**Table 1**).

The frequency of attacks/month during LTP decreased in three patients (2, 4, and 5) and remained substantially stable in one patient (1) compared to the period when they were not on LTP with s.c. C1-INH. Patient 3 did not initially experience an improvement in the frequency of attacks during LTP with s.c. C1-INH administered 2 times/week (the dose weight ratio = 42.86 IU/kg per week). Therefore, the frequency of administration was increased to 3 times/week (the weekly dose/weight ratio = 64.28 IU/kg per week) and the frequency of attacks decreased from 5.26 to 1.12 attacks/month. No patient was attack free.

TABLE 1 | Patients' data.

					Period without s.c.Berinert®		Period with s.c.Berinert®			
Pt	**Sex**	**Age[a]**	**Weight (kg)**	**Type of HAE**	**Type of LTP/no LTP**	**Frequency of attacks/month (n. months of observation)**	**Dosage of s.c.Berinert®**	**Frequency of attacks/month (n. months of observation)**	**Dose/weight ratio per week (IU/kg)[b]**	**Reduction in attack rate**
1	F	23	60	1	No LTP	3 (10.78)	1,500 IU/2 tpw	2.11 (5.68)	50	29.67%
2	F	45	70	1	i.v.Cinryze® 1000 IU/2 tpw	3.46 (2.6)	1,500 IU/3 tpw	0.12 (25.49)	64.29	96.53%
3	M	60	70	1	s.c.Berinert® 1500 IU/2 tpw	5.26 (1.71)	1,500 IU/3 tpw	1.12 (3.58)	64.28[c]	78.71%
4	F	45	69	1	No LTP	8.80 (5.91)	1,500 IU/3 tpw	4.30 (9.76)	65.22	51.14%
5	F	49	70	1	p.o.Tranex® 1 g per 3 die	2.46 (15.41)	1,500 IU/2 tpw	0.44 (47.97)	42.86	82.11%

[a] *age at the LTP start.*

[b] *120 IU per kg per week is the total recommended dose (60 IU/kg two times weekly).*

[c] *42.86 in the control period.*

LTP, long-term prophylaxis; Pt, patient; tpw, times per week.

TABLE 2 | Laboratory findings and clinical history.

	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5
Functional C1-INH (nv 70-130%)	9%	16%	0%	23%	20%
Antigenic C1-INH (nv 70-115%)	9%	25%	-	25%	18%
Age at diagnosis of HAE	6	32	24	8	19
Clinical history	Tonsillectomy	• Anemia • Appendectomy • Venous embolism and thrombosis of deep vessels in lower extremities • Neoplasms • Pulmonary embolism and infarction	• Diaphragmatic hernia • Hyperplasia of prostate • Diseases of esophagus, stomach, and duodenum	• Esophageal reflux • Asthma • Constipation • Other operations in the abdominal region • Diseases of esophagus, stomach, and duodenum • Acute/chronic viral hepatitis • Nephritis, nephrotic syndrome, and nephrosis • Diabetes mellitus	• Bulimia • Depressive disorder • Anxiety

C1-INH, C1-inhibitor; LTP, long-term prophylaxis; nv, normal values; Pt, patient.

TABLE 3 | Severity of attacks.

	Pt 1		Pt 2		Pt 3		Pt 4		Pt 5	
	No LTP	LTP with s.c.Berinert®	LTP with i.v.Cinryze®	LTP with s.c.Berinert®	LTP with s.c.Berinert® 2 tpw	LTP with s.c.Berinert® 3 tpw	No LTP	LTP with s.c.Berinert®	LTP with p.o. Tranex®	LTP with s.c.Berinert®
Severe	100%	100%	22.22%	66.67%	77.78%	25%	–	26.19%	31.58%	23.80%
Moderate	0%	0%	77.78%	33.33%	22.22%	75%	–	14.28%	28.95%	71.43%
Mild	0%	0%	0%	0%	0%	0%	–	59.52%	39.47%	4.76%

LTP, long-term prophylaxis; Pt, patient; tpw, times per week.

Additional data about the patients, laboratory findings, and attack characteristics are shown in **Tables 2**, **3**, respectively.

Our cohort previously received attenuated androgens, but they were discontinued because of a lack of effectiveness or adverse events.

Adverse events during s.c. administration of C1-INH were reported by 2 patients, which included erythema at the injection site and mild itching. In both cases, the adverse events were not severe and resolved spontaneously.

During LTP with s.c. C1-INH, breakthrough attacks were mostly treated at home by patients with s.c. icatibant or intravenous (i.v) C1-INH as rescue therapy. In one patient, breakthrough attacks were treated with i.v. C1-INH by health care professionals in the hospital (**Table 4**).

DISCUSSION

The patients showing a reduced frequency of attacks received a mean of ∼60 IU/kg per week, which is lower than the recommended weekly dose of 120 IU per kg (8). It should be noted that, in the COMPACT study (4), even the dosage of 40 IU/kg two times weekly, i.e., 80 IU/kg per week, significantly reduced the frequency of attacks compared to placebo.

The patients included in this analysis had severe disease and required LTP. Androgens were previously prescribed in our patient cohort and were discontinued because of a lack of effectiveness or adverse events. One patient switched from LTP with tranexamic acid, which was ineffective. Another patient switched from prophylaxis with i.v. C1-INH (Cinryze®), discontinued because of breakthrough attacks and poor venous access.

TABLE 4 | Treatments used for attacks and hospital access.

	Pt 1		Pt 2		Pt 3		Pt 4		Pt 5	
	No LTP	LTP with s.c.Berinert®	LTP with i.v.Cinryze®	LTP with s.c.Berinert®	LTP with s.c.Berinert® 2 tpw	LTP with s.c.Berinert® 3 tpw	No LTP	LTP with s.c.Berinert®	LTP with p.o. Tranex®	LTP with s.c.Berinert®
Icatibant	85.71%	91.67%	0%	0%	22.22%	0%	0%	0%	100%	100%
pdC1-INH (Berinert®)	28.57%[a]	8.33%	0%	100%	77.78%	100%	100%	85.71%	0%	0%
pdC1-INH (Cinryze®)	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%
Untreated	0%	0%	0%	0%	0%	0%	0%	14.28%	0%	0%
No. of attacks requiring ER/hospital access (%)	0%	0%	100%	100%	11.11%	0%	0%	9.52%	0%	0%

[a] *3 attacks were treated with both on-demand treatments.*

ER, emergency room; LTP, long-term prophylaxis; pdC1-INH, plasma-derived C1-inhibitor; Pt, patient; tpw, times per week.

Therefore, s.c. C1-INH was prescribed to this patient cohort. The dose that proved effective in reducing the number of attacks was ∼60 IU/kg per week.

The adverse events reported in this study are similar to those reported in the clinical trials COMPACT (4) and COMPACT-OLE (5), i.e., local site reactions.

The data presented in this paper are affected by some limitations. Control periods without s.c. C1-INH were usually shorter than those with s.c. C1-INH. As the attack frequency might vary during patients' lives because of unpredictable factors [i.e., stress (10)], longer observation periods could be useful to better evaluate disease severity. A wash-out period from one LTP period to another was not considered. This is a case series reporting findings obtained in 5 patients, without a control group, as we never meant to conduct neither a clinical trial nor an observation study. The therapeutic results were suboptimal, as expected, and the number of treated patients was too small to draw definite conclusions. A multicenter study with more patients would be more informative.

Far from willing to recommend a lower dosage of s.c. C1-INH, we aimed to report our real-life experience. In conclusion, even half the recommended dose of subcutaneous C1-INH may be beneficial, although suboptimal results may be obtained. These results may be useful for physicians facing patients with difficult venous access in countries where pdC1-INH is not registered for subcutaneous administration or in the absence of availability of other new drugs for long-term prophylaxis such as lanadelumab and berotralstat.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Diagnosing Pediatric Patients With Hereditary C1-Inhibitor Deficiency—Experience From the Hungarian Angioedema Center of Reference and Excellence

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Background: Hereditary Angioedema with C1-inhibitor deficiency (C1-INH-HAE) is a rare disease characterized by recurrent subcutaneous and/or submucosal edematous (HAE) episodes, which may occur at any age. The mean age of the symptom onset is 10–12 years. Diagnostic protocols differ by age group and family history.

Methods: We retrospectively analyzed clinical and laboratory data (C4-, C1-INH concentration and function) from 49 pediatric patients diagnosed with C1-INH deficiency at our Angioedema Center between 2001 and 2020. Moreover, we analyzed the connection between complement parameters and symptom onset.

Results: From the 49 pediatric patients [boy/girl: 23/26, the average age of diagnosis: 6.7 years (min: 0-max: 18.84)], the majority (36/49, 73%) was diagnosed as the result of family screening. Of all the enrolled patients, 34% (17/49) experienced symptoms before the diagnosis. During the observational period, 33% (16/49) of the patients remained asymptomatic, while 33% (16/49) became symptomatic. The average age at symptom onset was 7.8 years (min: 0.5–max: 18). Only 27% (13/49) of pediatric patients were diagnosed after referrals to our center because of typical symptoms. From those patients diagnosed with family screening, 4/36 experienced symptoms at or before the time of the diagnosis. In the case of five newborns from the family screening group, umbilical cord blood samples were used for complement testing. In the case of 3/36 patients, the first complement parameters did not clearly support the disease, but the presence of the mutation identified in the family verified the diagnosis. Complement results were available from 11 patients who became symptomatic during the observational period. Complement parameters 1 year prior to and after the onset of symptoms were compared, and significantly lower concentrations of C1-INH ($p = 0.0078$) were detected after the onset of symptoms compared to the preceding (symptom-free) period.

Discussion: The majority of pediatric patients were diagnosed as a result of family screening before the onset of symptoms. Early diagnosis allows supplying the patients with special acute treatment for HAE attacks, which may occur at any time. Our results highlight the importance of DNA analysis in pediatric patients in case of a known mutation in the family, and an ambiguous result of complement testing.

Keywords: hereditary C1-inhibitor deficiency, diagnosis, pediatric, umbilical cord blood, complement and genetic testing

INTRODUCTION

Hereditary angioedema developing with C1-INH deficiency (C1-INH-HAE) is a rare autosomal dominantly inherited disease, where the estimated prevalence is 1:50,000. It is characterized by recurrent subcutaneous and/or submucosal edematous episodes. The clinical appearance of this disease is diverse: from completely asymptomatic patients to patients experiencing angioedematous attacks on a monthly basis or even more frequently. Symptoms can occur at any age, but, in half of the patients, the first symptom occurs before the age of 18 years (1, 2). The average time of the appearance of symptoms is 10–12 years (3). Since this is a rare disease, health care practitioners tend not to recognize it; therefore, the diagnosis is often late, and almost half of the patients have at least one misdiagnosis in their medical history prior to the correct diagnosis. In childhood, angioedema occurring on the gastrointestinal submucosa is one of the most common symptoms, which may mimic the clinical picture of acute abdomen. A rare but life-threatening manifestation of the disease is angioedema occurring in the upper airways, which, in childhood, may cause suffocation in a short time due to the smaller airway diameter of children (1, 3). These factors significantly increase disease burden with frequent emergency department admission, unnecessary therapies, and surgical procedures, moreover, raise the risk of death from upper-airway obstruction (1, 4). Bradykinin has a role in the pathomechanism of C1-INH-HAE. This mediator is responsible for vasodilation and increased vascular permeability, which causes the development of edema. Due to this, HAE attacks do not respond to conventional antihistamines, glucocorticosteroids, or adrenaline used in histamine-mediated angioedema. Instead, C1-INH-HAE requires special treatment, which is targeted to supplement the missing protein, inhibit the formation of bradykinin, or inhibit bradykinin's effect on bradykinin 2 receptors (2, 5).

In 2017, an international consensus was published on the diagnosis and care of C1-inhibitor-deficient individuals under the age of 19 years (3). In pediatric patients, diagnosis is based on complement measurement, similar to that in adults. Low C1-inhibitor and C4 concentration, besides a low C1-inhibitor function, support the diagnosis of the first type of the disease (C1-INH-HAE type I). If the C1-INH concentration is normal or even increased but the functional activity of the protein is decreased and the level of C4 is low, then the second type of the disease is suspected (C1-INH-HAE type 2). In newborns, the level of C4 and C1-INH may be decreased because the complement system is not matured at this age. Therefore, to establish a diagnosis based on complement testing, at least two congruent complement results verifying C1-INH deficiency are necessary, out of which the second test needs to be done after the child has turned 1 year old. If the mutation causing the disease in the family is known and genetic testing is available, the analysis of the *SERPING1* gene encoding the C1-inhibitor protein may provide an opportunity for the diagnosis, especially under the age of 1 year, when ambiguous complement results may occur. In the case of newborns, it is possible to perform complement and genetic testing from umbilical cord blood (UCB), and if there is a family member diagnosed with C1-INH-HAE, until the exclusion/confirmation of the disease, C1-inhibitor deficiency should be presumed, even in asymptomatic cases (3, 6, 7).

This study aimed to assess the process of diagnosing C1-INH deficiency in children under 19 years (pediatric patients) in the Hungarian Angioedema Center of Reference and Excellence from approximately the last 20 years. We examined the advantages of the applied methods (complement measurement and genetic testing) in clinical practice and the time of appearance of clinical symptoms and their connection to complement parameters.

MATERIALS AND METHODS

We retrospectively analyzed clinical and laboratory data from 49 Hungarian patients who were diagnosed with hereditary C1-inhibitor deficiency during their childhood (<19 years of age). Clinical data, such as the reason for specialized medical investigation (family screening or typical symptoms), age at diagnosis, and symptom appearance, together with laboratory data (complement parameters, identified genetic variation), were collected from the National Database of Hereditary Angioedema. All the patients included in the study were diagnosed, managed, and followed up regularly at the Hungarian Angioedema Center of Reference and Excellence between 2001 and 2020. The study protocol was approved by the institutional review board of the Semmelweis University of Budapest, and informed consent was obtained from the participants in accordance with the Declaration of Helsinki.

Complement testing was performed in all cases. Two types of samples were used for complement testing: peripheral blood

Abbreviations: C1-INH, C1-inhibitor; C1-INH-HAE, hereditary angioedema with C1-inhibitor deficiency; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunoassay; HAE, hereditary angioedema; MLPA, multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction; UCB, umbilical cord blood.

and umbilical cord blood (if it was available). The C1-inhibitor activity was measured with a commercially available ELISA kit (Quidel, San Diego, US), while radial immunodiffusion was used to assess C1-inhibitor concentration. The C4 complement levels were detected by turbidimetry (Cobas Integra 400 analyzer; Roche, Switzerland).

If the C1-INH functional level was lower than the normal range together with a low C4 level, and the patient had low C1-INH concentration, the diagnosis of HAE with C1-INH deficiency type I was established. Patients with low C1-INH function, along with a normal or elevated C1-INH concentration, were diagnosed as having C1-INH-HAE type II. All symptom-free newborns or infants with a parent or sibling with C1-INH-HAE were considered to have C1-INH deficiency until this diagnosis was excluded, and these patients were supplied with special emergency treatments.

Analysis of the *SERPING1* gene was performed in each case. Isolated DNA samples were analyzed by bidirectional Sanger sequencing following PCR amplification, and the presence of copy-number alterations (deletions or duplications) in the *SERPING1* gene was detected by using multiplex ligation-dependent probe amplification (MLPA), applying the SALSA MLPA P243 SERPING1 kit (MRC-Holland, Amsterdam, The Netherlands).

Moreover, we analyzed the connection between complement parameters (C4-, C1-INH concentration and function) and the symptom onset. The patients who became symptomatic during the follow-up period and had complement results in the preceding 1 year and after symptom appearance were included. Besides, C1-INH concentration was compared in 8 pediatric patients who were asymptomatic and had complement parameters in the year after the diagnosis. Paired sample *t*-tests were used for statistical analyses. The level of statistical significance was set at 0.05.

RESULTS

Of the 49 pediatric patients, 23 were boys and 26 were girls. The average age at the time of diagnosis was 6.7 years (min: 0-max.: 18.84, median: 5). The average follow-up period in our center was 8.83 years (min.: 0.21- max.: 19.42, median: 7.9). Two out of 49 patients had C1-INH-HAE type 2; the other 47 patients had C1-INH-HAE type 1. Regarding the starting point of the diagnosis, the majority of the pediatric patients (36/49, 73%) were diagnosed as the result of family screening. The other group of patients (13/49, 27%) was diagnosed after referrals to our center because of typical symptoms. In the case of 2/13 patients, the disease was not present in any available family members. While, in 11/13 cases, family screening following the diagnosis verified the presence of further affected family members.

The average age at symptom onset was 7.8 years (min.: 0.5–max.: 18, median: 6.5). Of all the enrolled patients, 34% (17/49) experienced symptoms before the diagnosis. During the follow-up period, 33% (16/49) of the patients stayed asymptomatic, while 33% (16/49) became symptomatic. From the 36/49 group (the family screening group), only 4/36 experienced symptoms at or before the time of the diagnosis. In the case of symptomatic patients, the diagnostic delay was 3.5 years on average (min.: 0–max.: 11.7, median: 3).

Of all the enrolled pediatric patients, 11 were newborns (all term babies) or infants when they were diagnosed (<1 year of age). In the case of five newborns, UCB samples were used; in the other six cases, peripheral blood serum was tested to establish the diagnosis. In all five cases where UCB samples were analyzed, the complement parameters clearly confirmed the diagnosis: in 4 cases, typical results for C1-INH-HAE type 1, and, in 1 case, typical findings for C1-INH-HAE type 2 were detected. In the remaining 6 infants, peripheral blood serum was tested, and complement measurements provided uncertain results in 2 cases; in both cases, C1-INH function was in a normal range, with low levels of C4 and C1-INH in one case (Patient 1), while, in the other case (Patient 2), C1-INH concentration was also in a normal range. In these two cases, genetic analysis verified the presence of a pathogenic *SERPING1* variation. In the case of Patient 1, the C1-INH function stayed in the normal range in repeated measures (in a yearly checkup), and no HAE attack occurred during the observational period. In the case of Patient 2, C1-INH-HAE type 2 was diagnosed, and, later on, (when he was older than 1 year of age), low functional activity of C1-INH was detected. Patient 2 had his first HAE attack at the age of 5. One other child (more than 1 year of age at the time of diagnosis) had normal complement results; C1-INH function, -concentration, and C4 concentration were in a normal range; however, the known *SERPING1* mutation in the family could be detected with genetic testing. In this patient, a year after the diagnosis C1-INH function, - concentration were found to be low; however, C4 levels still stayed in the normal range.

Of the 16 patients who became symptomatic, 11 had been sampled in the 1 year before and after symptom appearance. We compared complement parameters measured in samples obtained prior to and after the occurrence of symptoms. The C1-INH concentrations were significantly lower (before occurrence median: 0.05; IQ range: 0.05; after occurrence median: 0.03; IQ range: 0.05; $p = 0.0078$) after the onset of symptoms in these patients (**Figure 1**). In the case of C4 and C1-INH functions, no significant changes could be detected. Sixteen patients remained asymptomatic through the follow-up period; 8 of them were analyzed at diagnosis and a year after as well. In these patients, no significant change was found in C1-INH concentration in samples collected before and 1 year after diagnosis (at diagnosis median: 0.055; IQ range: 0.0625; after diagnosis median: 0.07; IQ range: 0.0625; $p = 0.4219$) (**Figure 2**).

DISCUSSION

Here, we summarized our experience of the past 20 years in diagnosing children with C1-INH-HAE. Processing the clinical and laboratory data of pediatric patients diagnosed with C1-INH deficiency in our Angioedema Center between 2001 and 2020, we found that 65% of the pediatric patients were diagnosed as a result of family screening without preceding edematous episodes. The mean age at the first appearance of the symptoms in the case

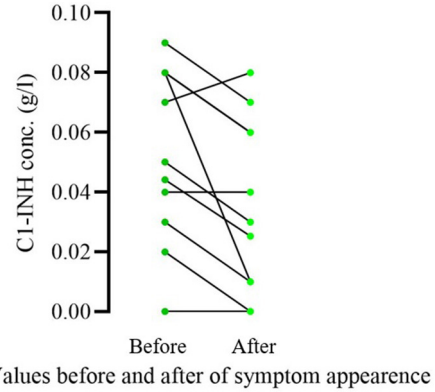

FIGURE 1 | Comparison of C1-INH concentration prior vs. after the onset of symptoms in pediatric patients diagnosed as a result of family screening. Only patients who became symptomatic during the follow-up period and had complement results in the 1 year before and after symptom appearance were included in this analysis (11/16). Significantly lower C1-INH concentrations were detected ($p = 0.0078$) after the onset of symptoms in these patients.

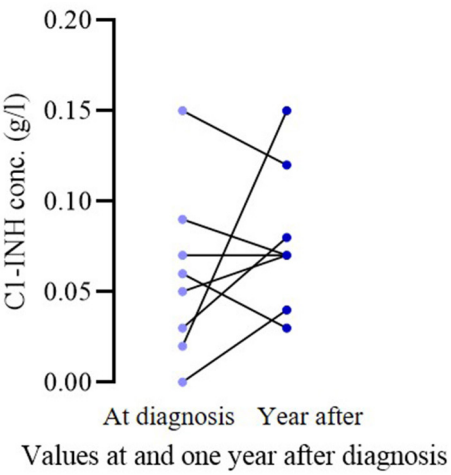

FIGURE 2 | Comparison of C1-INH concentration at diagnosis vs. a year after in asymptomatic pediatric patient. In the case of 8 asymptomatic patients, the C1-INH levels at diagnosis and in the year after were compared. No significant change could be detected ($p = 0.4219$).

of our patients was 7.8 years, which is lower than the average 10 years found in the literature. It is known that there may be significant differences between different populations in the appearance of symptoms, so, for example, in the case of Chinese patients, the first symptoms typically occur at the age of 20–30 years (3, 8). The median delay in the diagnosis was 3 years in our study, which is considerably lower than the value described in the literature (median of 8.5 years) (9). During the surveillance period, 33% of our patients remained asymptomatic; a similar percentage of asymptomatic children was found in an Italian survey as well (10).

Based on previous literature data, the concentration of C1-INH, its function, and the level of C4 may be lower than the applied reference range (determined by analyzing healthy adults) even in healthy children under the age of 1 year, which may cause a diagnostic difficulty in some cases (6, 11, 12). However, in a study by Pedrosa et al. published in 2016, only the level of C4 seemed not to be reliable in this age group. They studied nine pediatric patients under the age of 1; out of whom, four turned out to have C1-INH deficiency. In all four cases, the C1-INH function and concentration were low, while, in the five children who did not have C1-INH deficiency, these values were in the normal range. However, in 4/5 cases in the non-deficient group, the value of C4 was under the lower reference limit (7). Analyzing complement results in our cohort revealed that, in the case of 2/11 patients under the age of 1 year, the first complement test showed normal C1-INH function, but, since the mutation of the *SERPING1* gene was known in the family, the affected *SERPING1* exon was analyzed, and the presence of the disease-causing mutation was verified. Moreover, there was one child in the age group older than 1 year, in case of whom the first complement results were also normal, but, since the mutation of the *SERPING1* gene was known in the family, molecular genetic testing was applied that confirmed the diagnosis of C1-INH-HAE. These results highlight the importance of DNA analysis in pediatric patients with positive family history and a known mutation in the family.

In a paper by Nielsen et al. in 1994, C1-INH deficiency was proved with complement testing performed from UCB samples (6). There are no further published data analyzing the reliability of UCB samples for the diagnosis of C1-INH deficiency. In our study, the diagnosis of five newborns was established based on the complement parameters measured from UCB; all five samples had unambiguous complement results, verifying the presence of the disease, which was confirmed by genetic testing.

In the case of 11 patients whose symptoms developed during the follow-up period and had a complement result in 1 year, preceding the appearance of the first symptoms, we found that the concentration of C1-INH was significantly lower in the blood samples taken after the appearance of HAE symptoms compared to the values measured prior to the appearance of the symptoms. A similar decrease could not be observed as regards the C1-INH concentration of asymptomatic patients, when comparing their levels at the diagnosis and 1 year later, excluding the possibility of an age-related decrease in symptomatic patients. These results suggest that further decrease of C1-INH levels may favor the appearance of HAE symptoms or HAE symptoms are accompanied by a decrease in C1-INH concentration. This is in agreement with previous results, showing a connection between the C1-INH level and the initiation of HAE attacks (13, 14).

We aimed to study and evaluate the diagnostic process of the patients with pediatric C1-INH deficiency followed up in the Hungarian Angioedema Center of Reference and Excellence. Our results emphasize the importance of family screening and newborn screening in the establishment of an early diagnosis, ideally prior to the appearance of symptoms, as this provides an opportunity to offer adequate treatment modalities before unnecessary hospitalization and medical procedures happen.

Although we have limited data, our few cases suggest that the earliest diagnosis can be established safely from UCB samples. It should be highlighted that, in case of a positive family history, when the *SERPING1* mutation is known, genetic testing is a safely applicable and recommended method.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

NA: research topic development, data collection, and the wording of the article. HF: research topic development, article creation, and review of the article. DC and ÁS: genetic analyses. LV: complement measurements. ÁS, ZB, BV, DC, and LV: article creation and review. The final version of the article was read and approved by all the contributors. All authors contributed to the article and approved the submitted version.

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Searching for Genetic Biomarkers for Hereditary Angioedema Due to C1-Inhibitor Deficiency (C1-INH-HAE)

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Existing evidence indicates that modifier genes could change the phenotypic outcome of the causal *SERPING1* variant and thus explain the expression variability of hereditary angioedema due to C1-inhibitor deficiency (C1-INH-HAE). To further examine this hypothesis, we investigated the presence or absence of 18 functional variants of genes encoding proteins involved in the metabolism and function of bradykinin, the main mediator of C1-INH-HAE attacks, in relation to three distinct phenotypic traits of patients with C1-INH-HAE, i.e., the age at disease onset, the need for long-term prophylaxis (LTP), and the severity of the disease. Genetic analyses were performed by a validated next-generation sequencing platform. In total, 233 patients with C1-INH-HAE from 144 unrelated families from five European countries were enrolled in the study. Already described correlations between five common functional variants [*F12*-rs1801020, *KLKB1*-rs3733402, *CPN1*-rs61751507, and two in *SERPING1* (rs4926 and rs28362944)] and C1-INH-HAE severity were confirmed. Furthermore, significant correlations were found between either the age at disease onset, the LTP, or the severity score of the disease and a series of other functional variants (*F13B*-rs6003, *PLAU*-rs2227564, *SERPINA1*-rs28929474, *SERPINA1*-rs17580, *KLK1*-rs5515, *SERPINE1*-rs6092, and *F2*-rs1799963). Interestingly, correlations uncovered in the entire cohort of patients were different from those discovered in the cohort of patients carrying missense causal *SERPING1* variants. Our findings indicate that variants other than the *SERPING1* causal variants act as independent modifiers of C1-INH-HAE severity and could be tested as possible prognostic biomarkers.

Keywords: C1-inhibitor deficiency, genetic biomarkers, functional variants, hereditary angioedema, long-term prophylaxis, next-generation sequencing, severity score

INTRODUCTION

Hereditary angioedema due to C1-inhibitor deficiency (C1-INH-HAE), an autosomal dominant disorder with recurrent attacks of edema spontaneously developing in any body location, is characterized by a large heterogeneity in its clinical expression, including the age at disease onset, the number and triggers of attacks, the severity and localization of edema, and prodromal signs and symptoms (1, 2). These features show variable expressivity, i.e., it may vary even among members of the same family carrying the same *SERPING1* causal mutation, which, at present, is unpredictable and largely unexplained.

Accumulating evidence indicates that modifier genes could change the phenotypic outcome of the variant at the primary mutation in the target *SERPING1* gene, and thus explain disease expression variability (3). Understanding genetic modification phenomena will obviously improve our ability to better manage the disabling and potentially fatal manifestations of C1-INH-HAE. To this aim, we investigated here the relationship between parameters associated with disease course severity and common functional variants in genes involved in the metabolism of bradykinin, the main mediator of angioedema attacks in patients with C1-INH-HAE.

PATIENTS AND METHODS

This study included 233 patients (104 men and 129 women, mean age 40 years, range 2.5–85) with C1-INH-HAE (217 type I, 16 type II) from 144 unrelated families (16 Bulgarian, 23 German, 30 Greek, 43 Hungarian, and 32 Polish). All patients were previously genotyped for *SERPING1* mutations. Demographic, clinical, and molecular data of the patients are presented in **Table 1**. Patients' medical records were reviewed and data regarding the age at disease onset and the long-term prophylaxis (LTP) were recorded. For patients not receiving long-term prophylactic treatment, the severity score Cutaneous Abdominal Laryngeal Score (CALS) was calculated according to the equation "CALS=1*Cutaneous+2*Abdominal+3*Laryngeal last year attacks".

DNA samples were analyzed in a validated next-generation sequencing (NGS) platform (Ampliseq custom panel, Thermo Scientific, Waltham MA, USA), as previously described (4, 5). Briefly, DNA libraries were constructed for each sample using the Ion AmpliSeq Library Kit 2.0 (Thermo Scientific) and indexed with a unique adapter using the Ion Xpress barcode adapter kit (Thermo Scientific). Template preparation, enrichment, and chip loading were carried out on the Ion Chef System (Thermo Scientific). Sequencing was performed on S5XL on 520 and 530 chips, using the Ion 510, Ion 520, and Ion 530 Kit-Chef (Thermo Scientific). All procedures were performed according to the manufacturer's instructions. Base calling, demultiplexing, and alignment to the hg19 reference genome (GRCh37) of the raw sequencing data were performed in Torrent Suite 5.10 software (Thermo Scientific, Waltham, MA, USA) using the default parameters. Variant calling was performed by the VariantCaller v.5.8.0.19 plug-in and coverage analysis by the CoverageAnalysis v.5.8.0.8 plug-in in Torrent Suite 5.10. All variants were annotated on Ion Reporter Software (Thermo Scientific).

We investigated the presence or absence of 18 common functional variants (allele frequency $\geq$1%) in relation to three distinct phenotypic traits of patients, i.e., the age at disease onset, the need for LTP, and the severity of the disease based on the CALS score. The investigated variants were variants of genes encoding proteins involved in the metabolism and function of bradykinin, the main mediator of C1-INH-HAE attacks, which were chosen based on their effect on protein activity, their frequency, and the coverage that could be achieved by the platform (**Table 2**). The local institutional review boards approved this study, and written informed consent was obtained from each individual or an accompanying relative.

STATISTICAL ANALYSIS

Categorical variables were analyzed with Fisher's exact test. Normality of continuous variables was assessed with Kolmogorov–Smirnov test. Normally distributed data were analyzed with Student's *t*-test and one-way ANOVA as appropriate. Skewed data were analyzed with nonparametric methods (Mann–Whitney test or Kruskal–Wallis test as appropriate). Given the fact that our patient population consisted of correlated subjects (members of individual families), we implemented generalized estimating equations (GEE), an extension of the generalized linear model that accounts for the within-subject correlation. GEE was used to model the relationship between explanatory variables (polymorphisms, sex, etc.) and response variables (age at disease onset, need for long-term treatment, CALS severity). Age at disease onset and CALS severity score were modeled as continuous variables in linear GEE models and the need for long-term treatment was entered as a binary variable in logistic GEE models. In all GEE models, an unstructured correlation structure was used, and the Quasi-Likelihood Information Criterion (QIC) was used for model selection. Data analysis was performed with SPSS 17.0 (IBM Corporation, NY, 2008). For all analyses, alpha was set at 0.05 (two-tailed). In cases in which multivariable analysis was more appropriate, such analysis was performed with the dependent variable, including the age at disease onset, the LTP or the CALS, and with those of the variants presenting significant associations in univariable analysis fitted as independent variables. This type of analysis was performed for two groups of C1-INH-HAE patients (a) independently of the *SERPING1* variant and (b) for patients carrying a missense variant in *SERPING1* (n = 69). The common functional variants *SERPINA1*-rs121912714, *SERPINA1*-rs28929474, and *MPO*-rs56378716 were not detected in any patient of the missense group.

RESULTS AND DISCUSSION

The allele frequency of the selected SNPs in our cohort did not differ significantly from the Global Allele Frequency (GMAF) and the European Non-Finnish Allele Frequency (ENFMAF) as recorded by GnomAD v2.1.1 (**Supplementary Table 1**).

TABLE 1 | Demographic, clinical, and molecular data of the C1-INH-HAE patients.

Clinical data	Total	Greek	Polish	German	Hungarian	Bulgarian
No (patients, families)	233, 144	31, 30	47, 32	23, 23	113, 43	19, 16
Sex (male/female)	104/129	17/14	18/29	11/12	48/65	10/9
Age at analysis(median, range)	40.0 (2.5–85)	35.0 (2.5–67)	44.0 (25–85)	42.0 (13–81)	39.0 (9–82)	47.0 (8–81)
Age at onset(median, range)	12.5 (0.5–73)	9.0 (1–31)	17.0 (1–73)	10.0 (3–19)	12.0 (0.5–53)	11.0 (1–50)
HAE Type (I/II)	217/16	31/0	44/3	22/1	102/11	18/1
Longterm treatment(Yes/No/NA)	79/141/13	11/16/4	3/42/2	6/10/7	55/58/0	4/15/0
CALS Severity(median, range)	26.74 (0–238)	24.72 (4–69)	43.95 (0–238)	Missing data	10.74 (0–88)	41.11 (6–103)
***SERPING1* defects**						
Regulatory, n (%)	1 (0.43%)	0 (0.00%)	0 (0.00%)	1 (4.35%)	0 (0.00%)	0 (0.00%)
Missense mutations, n (%)	69 (29.61%)	6 (19.36%)	20 (42.55%)	8 (34.78%)	33 (29.20%)	2 (10.53%)
Nonsense mutations, n (%)	33 (14.16%)	5 (16.13%)	5 (10.64%)	2 (8.70%)	17 (15.04%)	4 (21.05%)
Splice defects, n (%)	22 (9.44%)	4 (12.90%)	1 (2.13%)	3 (13.04%)	12 (10.62%)	2 (10.53%)
Small deletions or insertions (frameshift alterations), n (%)	46 (19.74%)	11 (35.48%)	2 (4.26%)	6 (26.08%)	24 (21.24%)	3 (15.78%)
Large deletions or insertions, n (%)	28 (12.02%)	1 (3.23%)	7 (14.89%)	2 (8.70%)	16 (14.16%)	2 (10.53%)
Deep intronic, n (%)	2 (0.86%)	2 (6.45%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Unidentified defects, n (%)	32 (13.74%)	2 (6.45%)	12 (25.53%)	1 (4.35%)	11 (9.74%)	6 (31.58%)

Similarly, the prevalence of the polymorphisms did not differ significantly between examined patient groups from different countries (**Supplementary Table 2**).

The correlations found between functional variants and the age at disease onset, the LTP, or the severity score of the disease are summarized in **Table 3**. Five common functional variants had been previously correlated with C1-INH-HAE severity – *F12*-rs1801020, *KLKB1*-rs3733402, *CPN1*-rs61751507, and two in *SERPING1* (rs4926 and rs28362944).

The presence of the C allele of the *F12*-rs1801020 (c.-4T>C) was significantly correlated with an increase in disease severity. More precisely, independently of the type of *SERPING1* mutations, homozygotes (CC) and heterozygotes of this variant present a mean disease severity score higher by 28.21 ($p < 0.001$) and 18.69 ($p = 0.002$) units of the CALS severity score, respectively. Similarly, homozygotes (CC) and heterozygotes (CT) of this variant carrying *SERPING1* missense variants present a mean disease severity score higher by 25.48 ($p = 0.002$) and by 13.88 ($p = 0.003$) units of the CALS severity score, respectively, compared with CALS score in patients lacking the polymorphism. Our results agree with evidence provided by Bors et al. (6) who suggested that the carriage of the T allele of the *F12*-rs1801020 variant is independently associated with a less severe C1-INH-HAE clinical phenotype. Moreover, this result is in agreement with Rijavec et al. (7) who have shown that the C allele and the CC genotype were represented more in symptomatic patients, compared to asymptomatic. These authors suggested that carriers of the CC genotype have a 25-fold greater risk of developing the disease compared to those carrying the TT genotype. In our study, *F12*-rs1801020 displays a robust linear trend among the ordinal categories (homozygosity-heterozygosity-absence), indicating that the T allele provides a protective effect in regards to angioedema severity. Moreover, univariable analysis in patients with missense *SERPING1* mutations uncovered a significant 5-year delay at disease onset in heterozygous (TC) compared to homozygous (CC) patients ($p < 0.001$), a result that agrees with our previous findings (8). These effects could be explained by the findings of Kanaji et al. (9). These authors observed different levels of FXII in plasma, depending on the genotype. Even though both alleles were equally transcribed in hepatocytes of heterozygotes, the cDNA containing the T allele was producing less FXII *in vitro* than the one containing the C allele. Therefore, the presence of the variant is affecting the efficiency of translation.

In regards to the carriage of the C allele of the *SERPING1*-rs28362944 (c.-21T>C) variant, the probability of the need for LTP was found increased by 4.2-fold ($p = 0.02$) and 2.5-fold ($p = 0.012$) among patients with C1-INH-HAE who were carrying missense *SERPING1* mutations and independently of the *SERPING1* variation, respectively. The variant had been previously characterized as likely pathogenic when in a homozygous state (49, 50), despite that no correlation between this variant and the biochemical values of C1-INH function or the clinical severity score has been reported by other investigators (51). Interestingly, however, Duponchel et al. (52) have proposed the variant as a modifier of disease severity as they had found that the variant yields low but significant levels of exon 2 skipping in transfected cells. Therefore, this allele may contribute, at the RNA level, to more severe forms of C1-INH-HAE. In accordance, Cumming et al. (54) reported an increased disease penetrance in carriers of c.-21T>C when the variant allele presented in *trans* with the *SERPING1* mutation. Unfortunately, segregation analysis in our cohort could not be performed in many cases due to a lack of available family members.

The *SERPING1*-rs4926 (c.1438 G>A, p.Val480Met) had been predicted as deleterious and possibly damaging according to bioinformatic tools, because the highly conserved amino acid (Val) is important for the folding of the C1-INH protein into its native conformation (53). However, it is a well-documented common variant and characterized by different groups as benign

TABLE 2 | Selected common functional variants.

Gene	Protein	dbSNP	Nucl. change	aa change	*OMIM	References
F12	Factor XII	rs1801020	c.-4T>C	NA (5'UTR)	610,619	(6–9)
F13A1	Factor XIII Subunit A	rs5985	c.103G>T	p.Val35Leu	134,570	(10–13)
F13B	Factor XIII Subunit B	rs6003	c.344G>A	p.Arg115His	134,580	(14)
F2	Factor II	rs1799963	c.*97G>A	NA (3'UTR)	176,930	(15, 16)
CPN1	Carboxypeptidase N	rs61751507	c.533G>A	p.Gly178Asp	603,103	(17, 18)
A2M	Alpha-2-Macroglobulin	rs669	c.2998A>G	p.Ile1000Val	103,950	(19)
KLK1	Kallikrein 1	rs5515	c.230G>A	p.Arg77His	147,910	(20, 21)
KLKB1	Plasma Kallikrein B (Prekallikrein)	rs3733402	c.428G>A	p.Ser143Asn	229,000	(22)
MASP2	Mannan-binding lectin serine protease 2	rs72550870	c.359A>G	p.Asp120Gly	605,102	(23, 24)
MPO	Myeloperoxidase	rs56378716	c.752T>C	p.Met251Thr	606,989	(25, 26)
PLAU	Urinary plasminogen activator (urokinase, plasminogen activator)	rs2227564	c.422T>C	p.Leu141Pro	191,840	(27–29)
SERPINA1	Serine protease inhibitor, clade a, member 1 (a1-antitrypsin)	rs28929474	c.1096G>A	p.Glu366Lys	107,400	(30–36)
		rs17580	c.863A>T	p.Glu288Val		(37–40)
		rs121912714	c.839A>T	p.Asp280Val		(41–43)
SERPINE1	Serine protease inhibitor, clade e, member 1 (Nexin, PAI-1)	rs6092	c.43G>A	p.Ala15Thr	173360	(44)
TLR2	Toll-like receptor 2	rs5743708	c.2258G>A	p.Arg753Gln	603028	(45)
SERPING1	Serine protease inhibitor, clade g, (C1-INH)	rs28362944	c.-21T>C	NA (5'UTR)	606860	(46–52)
		rs4926	c.1438G>A	p.Val480Met		(53–55)

in public databases. Independently of the *SERPING1* causal mutation, a significant 3.6-year ($p = 0.018$) and a trend toward 6.3-year ($p = 0.058$) delay at the age of disease onset was found in heterozygous (GA) and homozygous (AA) carriers, respectively. Functional studies by Cumming et al. (54) found no detectable effect of this variant on C1-INH structure, function, stability, plasma levels, or disease expression. However, the authors did not exclude a consequence of the variant on other functions of C1-INH as a modulator of the coagulation and kinin release pathways.

Homozygosity (AA) for *KLKB1*-rs3733402 (c.428G>A, p.Ser143Asn) in carriers of a missense *SERPING1* variant was significantly associated with 7-year earlier disease onset ($p = 0.029$) and increased disease severity by 30.45 units in CALS score ($p < 0.001$) compared to GG carriers. This result is in accordance with the results of a previous study by Gianni et al. (22). However, this is an unexpected finding. The *KLKB1*-rs3733402 variant locates in Apple domain 2 of the heavy chain where prekallikrein (PK) binds to high-molecular-weight kininogen (HMWK). The resulting reduced formation of the PK-HMWK complex interferes with optimal PK activation and reduces bradykinin formation and plasma kallikrein protection from inhibition by C1-INH.

Heterozygosity for *CPN1*-rs61751507 (c.533G>A, p.Gly178Asp) in carriers of missense *SERPING1* variants were independently associated with a 98% decrease in the probability of LTP ($p = 0.017$). In the past, *CPN1*-rs61751507 has been once associated with HAE when found in compound heterozygosity with a rare frameshift mutation in *CPN1*-exon 1 (17, 18). The effect of this variant observed in our study might be related to the substitution of the Gly^{178} residue of CPN the significance of which is underlined by the fact that it has been conserved in diverse species and is also conserved among most members of the human carboxypeptidase family.

Apart from our above findings that were confirmatory of previously described correlations between functional variants and parameters of the C1-INH-HAE severity, a series of novel correlations were uncovered in this study. The variants *F13B*-rs6003, *PLAU*-rs2227564, and *SERPINA1*-rs28929474 were found significantly correlated with the severity of C1-INH-HAE, independently of the *SERPING1* mutational status. Precisely, heterozygosity (GA) for *F13B*-rs6003 (c.344G>A, p.Arg115His) was correlated with decreased disease severity by 11.84 ($p = 0.024$) units of the CALS score. *F13B*-rs6003 has been considered benign concerning the FXIII subunit B deficiency. However, this variant has been characterized as a risk factor for venous thrombosis attributed to the substitution of Arg^{115} that prevents the dissociation between the A and B subunits of FXIII after activation by thrombin (14).

Carriage of T allele in homozygosity (TT) for *PLAU*-rs2227564 (c.422T>C, p.Leu141Pro) was found correlated with decreased disease severity by 13.67 units ($p = 0.004$) of the CALS score. Urokinase, encoded by *PLAU*, is an activator of plasminogen, which plays a significant role in the activation of the kinin-kallikrein system and the generation of bradykinin. The amino acid change p.Leu141Pro is located within the kringle domain of urokinase at the junction between two β-pleated sheets. The presence of the T allele does not appear to affect the activity of urokinase, but the zymogen containing Pro^{141} binds fibrin aggregates less efficiently than the one containing Leu^{141}, suggesting a possibility of altered extracellular urokinase localization or stability (28).

The presence of the A allele of *SERPINA1*-rs28929474 (c.1096G>A, p.Glu366Lys) was correlated with increased disease

TABLE 3 | Summary of the correlations of common functional variants with patients' phenotype – age at disease onset, need for LTP, CALS severity score.

	SNP *(Gene)*	Nucl. change	Genotype	Age at disease onset		Need for LTP		CALS	
All *SERPING1* variants	*SERPING1*-rs4926	c.1438G>A	GA	+3.6 years	$p = 0.018$	–		–	
			AA	+6.3 years	$p = 0.058$	–		–	
	SERPING1-rs28362944	c.-21T>C	TC and CC	–		2.5-fold	$p = 0.012$	–	
	F12-rs1801020	c.-4T>C	CT	–		–		+18.69	$p = 0.002$
			CC	–		–		+28.21	$p < 0.001$
	F13B -rs6003	c.344G>A	GA	–		–		−11.84	$p = 0.024$
	SERPINA1-rs28929474	c.1096G>A	GA and AA	–		–		+80.16	$p = 0.003$
	PLAU -rs2227564	c.422T>C	TT	–		–		−13.67	$p = 0.004$
Missense *SERPING1* variants	*SERPINA1*-rs17580	c.863A>T	AT	−8 years	$p < 0.001$	–		–	
	KLKB1-rs3733402	c.428G>A	AA	−7 years	$p = 0.029$	–		+30.45	$p < 0.001$
	KLK1-rs5515	c.230G>A	GA	+8.95 years	$p = 0.05$	–		−16.79	$p = 0.029$
	SERPINE1-rs6092	c.43G>A	AA	+8.4 years	$p = 0.009$	–		–	
	SERPING1-rs28362944	c.-21T>C	TC	–		4.2-fold	$p = 0.02$	–	
	CPN1-rs61751507	c.533G>A	GA	–		98% probability decrease	$p = 0.017$	–	
	F12-rs1801020	c.-4T>C	CT	+5 years	$p < 0.001$	–	–	+13.88	$p = 0.003$
			CC	CT *comp. to CC*		–	–	+25.48	$p = 0.002$
	F2-rs1799963	c.*97G>A	GA	–		–		−25.97	$p = 0.017$

severity by 80.16 units ($p = 0.003$) of CALS score. *SERPINA1*-rs28929474, commonly known as the Z allele of a_1-antitrypsin (A1AT), is five times less effective than the normal M allele as an inhibitor of neutrophil elastase. It forms polymers in the lung that can be chemoattractants for neutrophils, thereby increasing inflammation (31–33), while it alters the global structural dynamics of A1AT (34). When found in a homozygous state, the Z allele is responsible for 95% of all clinical cases of A1AT deficiency, and in compound heterozygosity with the S allele, it is associated with 20–50% risk for emphysema (35, 36).

In addition to the above correlations detected between functional variants and disease severity, independently of *SERPING1* variation, further correlations were uncovered only in carriers of missense *SERPING1* variants. Heterozygosity (AT) for *SERPINA1*-rs17580 (c.863A>T, p.Glu288Val) was correlated with an 8-year earlier age at disease onset ($p < 0.001$) compared to AA carriers. *SERPINA1*-rs17580, commonly known as the S allele of A1AT, causes reduced cellular secretion of A1AT because the newly synthesized protein is degraded intracellularly before secretion (37). The S allele is not disease causing. Even homozygous carriers do not present the common expressions of A1AT deficiency. However, it represents a risk factor when in compound heterozygosity with the Z allele. Such compound heterozygotes are relatively frequent due to the high frequency of this allele. However, this compound heterozygosity was not detected among our patients.

Another functional variant related to disease severity in *SERPING1* missense carriers is *SERPINE1*-rs6092 (c.43G>A, p.Ala15Thr). Homozygous carriers (AA) of *SERPINE1*-rs6092 displayed a significantly higher mean age at disease onset by 8.4 years ($p = 0.009$). The variant has been previously characterized as likely benign for plasminogen activator inhibitor-1 (PAI-1) deficiency, but functional studies in a heterozygous patient showed activity at about 70%. Zhang et al. (44) suggested that the change from a hydrophobic non-polar amino acid (Ala) to a hydrophilic polar amino acid (Thr) in the hydrophobic core region (h-region) of the signal peptide of the protein may disturb its function.

Moreover, in the same group of C1-INH-HAE patients, heterozygous carriers (GA) of the gain-of-function mutation *F2*-rs1799963 (c*97G>A) had a significant decrease in the disease severity by 25.97 units in CALS score ($p = 0.017$) compared to GG carriers. According to Gehring et al. (15), this variant probably affects the generation of prothrombin. Finally, the *KLK1*-rs5515 (c.230G>A, p.Arg77His) variant was correlated with both the mean age at disease onset and the disease severity. Heterozygous carriers of this variant were presented with an 8.95-year later age at disease onset ($p = 0.05$) and with decreased disease severity by 16.79 units of the CALS score ($p = 0.029$). This variant had been characterized as a loss-of-function polymorphism resulting in reduced kallikrein activity. Slim et al. (20) detected 50–60% lower urinary kallikrein activity to carriers, while in studies concerning branchial artery function (21), which exhibited arterial dysfunction. Interestingly, this is an inverse correlation

to what has been detected for the *KLKB1*-rs3733402 variant, as it is described above.

CONCLUSION

Our study provides clear evidence that variants other than the *SERPING1* causal variants act as independent modifiers of C1-INH-HAE severity and could serve as possible prognostic biomarkers. The next step is the validation of detected correlations in a large cohort of patients. Such a study would best be performed by a global consortium of angioedema centers such as the ACARE network (56) to allow for the inclusion of a diverse and sizable population of clinically well-characterized patients. Enrolling large cohorts of patients could also allow examining the different effects possibly exerted by variants of modifier genes on carriers of different kinds of causal *SERPING1* mutations (nonsense, frameshift, large defects, etc.) with different impacts on C1-INH production, structure, and function. Furthermore, functional studies on the effect of these variants could shed light on missing parts of the pathogenesis of the disease. Finally, our results indicate that functional variants of genes involved in pathways other than contact activation system/kallikrein kinin system but recently recognized as participating in C1-INH-HAE pathogenesis (e.g., endothelial cells) should be investigated.

DATA AVAILABILITY STATEMENT

The data have been submitted to European Variation Archive (EVA) EMBL-EBI (https://www.ebi.ac.uk). The accessions associated with submission are: Project: PRJEB51008, Analyses: ERZ5253849.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local institutional review boards, and written informed consent was obtained from each individual or an accompanying relative. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FP performed genetic analyses, analyzed the results, and wrote the manuscript. GL performed genetic analyses and critically read the manuscript. MK analyzed the results. DC designed the study and collected clinical data. GP, FP, MMag, AV, MS, and KO collected clinical data. MZ, AS, MMau, MS, and HF critically read and commented on the manuscript. AG designed and supervised the study, critically read and revised the manuscript. All authors approved the submitted version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.868185/full#supplementary-material

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The complex role of kininogens in hereditary angioedema

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Human high molecular weight kininogen (HK) is the substrate from which bradykinin is released as a result of activation of the plasma "contact" system, a cascade that includes the intrinsic coagulation pathway, and a fibrinolytic pathway leading to the conversion of plasminogen to plasmin. Its distinction from low molecular weight kininogen (LK) was first made clear in studies of bovine plasma. While early studies did suggest two kininogens in human plasma also, their distinction became clear when plasma deficient in HK or both HK and LK were discovered. The light chain of HK is distinct and has the site of interaction with negatively charged surfaces (domain 5) plus a 6th domain that binds either prekallikrein or factor XI. HK is a cofactor for multiple enzymatic reactions that relate to the light chain binding properties. It augments the rate of conversion of prekallikrein to kallikrein and is essential for the activation of factor XI. It indirectly augments the "feedback" activation of factor XII by plasma kallikrein. Thus, HK deficiency has abnormalities of intrinsic coagulation and fibrinolysis akin to that of factor XII deficiency in addition to the inability to produce bradykinin by factor XII-dependent reactions. The contact cascade binds to vascular endothelial cells and HK is a critical binding factor with binding sites within domains 3 and 5. Prekallikrein (or factor XI) is attached to HK and is brought to the surface. The endothelial cell also secretes proteins that interact with the HK-prekallikrein complex resulting in kallikrein formation. These have been identified to be heat shock protein 90 (HSP 90) and prolylcarboxypeptidase. Cell release of urokinase plasminogen activator stimulates fibrinolysis. There are now 6 types of HAE with normal C1 inhibitors. One of them has a mutated kininogen but the mechanism for overproduction (presumed) of bradykinin has not yet been determined. A second has a mutation involving sulfation of proteoglycans which may lead to augmented bradykinin formation employing the cell surface reactions noted above.

KEYWORDS

angioedema, bradykinin, kininogen, kallikrein, vascular permeability

Introduction

When one considers the plasma pathway(s) for the production of bradykinin, a role for kininogen was determined many years before any role for factor XII or prekallikrein was discerned and bovine proteins were purified and at least partially characterized before the corresponding human proteins were identified. Defined as substrates from

which bradykinin is derived, the role of the various kininogens in actually producing bradykinin *in vivo*, is far more complex. This review article is based on a lecture given at the Hereditary Angioedema Conference in Budapest, Hungary in 2020, and includes updates representing the most recent discoveries since then.

The earliest history: How many kininogens are there?

While it may seem obvious to any worker in the bradykinin "field" or those involved with the pathogenesis and/or treatment of hereditary angioedema (i.e., C1 inhibitor deficiency) that the answer is two kininogens, this was not always clear, and if one studies rodent kininogens, particularly in the rat, the correct answer is three! Nevertheless, workers in Japan purified two kininogens from bovine plasma and did find them to differ in molecular weight, hence the names low molecular weight kininogen (LK) and high molecular weight kininogen (HK) (1, 2). Those working with human plasma questioned whether the smaller form is a degradation product of the larger or perhaps the larger represents an aggregate of the smaller, based in part because early purifications suggest just one species (3). Other workers disagreed, and felt that two forms were more likely based on two observations, namely, (A) two kininogens of differing size were identified upon fractionation of human plasma and (B) partially purified kininogen preparations showed different kinetics i.e., different values for K_m, V_{max}, and/or K_{cat} when digested either by a plasma kallikrein preparation or tissue kallikrein (4, 5). The larger form seemed to be a preferential substrate of plasma kallikrein (6).

The answer emerges: There are two human kininogens and one of them is a clotting factor

Experiments of nature came to the rescue with the discovery of three plasmas that were deficient in one or both kininogens and were named for the patients affected; namely, Fitzgerald, Williams, and Flaujeac (7–9). These plasmas failed to produce bradykinin when "activated" by a negatively charged surface, e.g., kaolin, which was routinely used in the 1960's and 1970's. However, the partial thromboplastin time (PTT) was as abnormal as factor XII deficiency (8) and factor XII-dependent fibrinolysis was also markedly prolonged. The patients were generally well and Mrs. Williams, in particular, was identified when her pre-operative laboratory values were assessed prior to an elective cholecystectomy and her plasma failed to clot. William's plasma turned out to have a deficiency of all kininogens, both LK and HK (8) while Flaujeac plasma had a selective deficiency of HK (9). Subsequently, the defect in Fitzgerald plasma was confirmed to be due to HK deficiency. Our studies of the Williams' plasma are summarized below.

It was quickly determined that Williams plasma lacked kininogen. At that time (1975), we had factor XII fragment (β-FXIIa) and preparations of active kallikrein. Incubation of William's plasma with these enzymes produced no bradykinin. We had no source of purified kininogen, however, heating normal plasma to 61 °C for 2 h. destroyed all enzymes needed to produce bradykinin, kininogen was still viable, so that incubation with plasma kallikrein produced bradykinin while incubation with factor XIIf did not (prekallikrein had been destroyed). Thus, the addition of heated plasma to Williams plasma and the addition of either factor XIIf or kallikrein not only produced bradykinin but also corrected the coagulation defect (abnormal PTT. By that time, (1975), Jack Pierce had preparations of kininogens that differed in size and susceptibility to plasma kallikrein (the larger ones) or tissue kallikrein (the smaller ones) except for one, which was small but was mainly cleaved by plasma kallikrein (5, 8). When samples of each were numbered and studied blindly, all the larger ones corrected the William's plasma defects, but the small ones did not, except for the one "small" preparation that was susceptible to plasma kallikrein (8). His chromatographic separations were so sensitive, that he had numbered 15 kininogens that sorted into these two main groups, and later once we purified LK and HK, we realized that he was discerning the carbohydrate heterogeneity of these glycoproteins. The one small protein that functioned as HK was probably an HK cleavage product that may actually exist in plasma (10).

From these results, suggesting two kininogens, HK and LK, with all the functions associated with HK, we proceeded to purify kininogens from 10 L of human plasma, facilitated by the identification of HK by correction of the PTT of Williams plasma and an anti-kininogen antibody reactive with any kininogen (11). The isolated proteins had single bands on disc gel electrophoresis. The HK preparation was cleaved with plasma kallikrein and the kinin-free protein was reduced and alkylated and fractionated by Sephadex G200. Peaks identified as heavy chain and light chain (Figure 1A) were assayed for the ability to correct the PTT of Williams plasma and all the activity was in the light chain (8). Similarly, the fibrinolytic defect was also reversed by the addition of a light chain but not a heavy chain. While the molecular weights calculated were 66,000 and 37,000 respectively, urea-disc gel electrophoresis revealed light chain "activity" at 56,000 and 37,000 and the larger was converted to the smaller representing an additional cleavage of the light chain without loss of function (Figure 1B).

Assembly of bradykinin-forming proteins in plasma

During the course of these studies, prekallikrein was purified (12) and was found to have a molecular weight of 80,000.

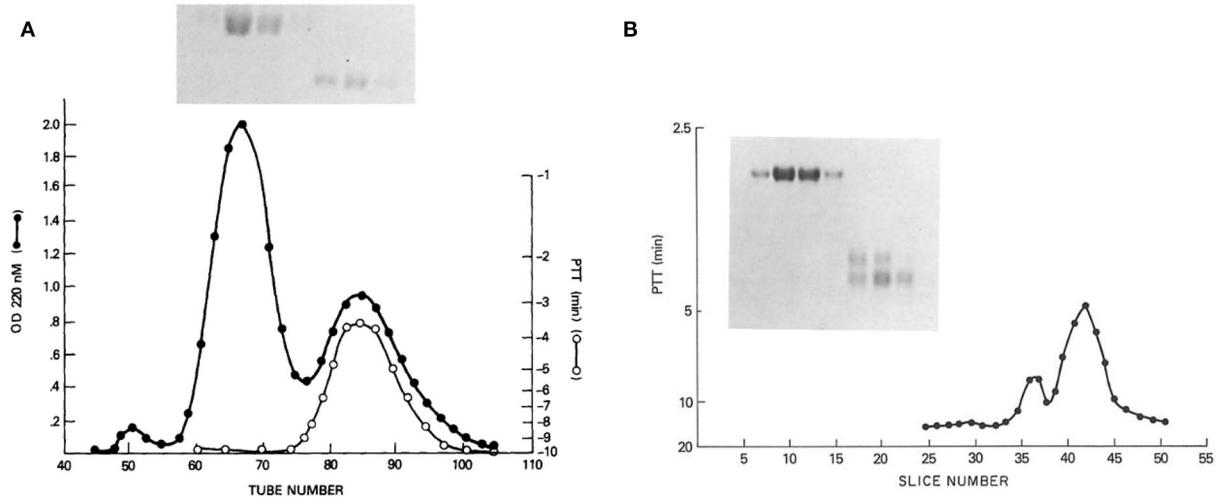

FIGURE 1
(A) Sephadex G-200 gel filtration of reduced kinin-free HMW kininogen. The absorbance at 280 nm is shown (black line) in addition to the ability of fractions to correct the partial thromboplastin time of HK kininogen-deficient plasma (white line). Above (inset) is shown the SDS-PAGE pattern obtained after electrophoresis of 100 μl of tubes 60, 65, 70, and 75 (heavy chain), and electrophoresis of 100 μl of a 10-fold concentration of tubes 80, 85, and 90 (light chain). **(B)** Urea disc gel electrophoresis of samples taken from the Sephadex G-200 gel filtration of reduced kinin-free HWM kininogen [in this figure **(A)**]. A 100 μl of tubes 60, 65, 70, and 75 followed by 100 μl of a 20-fold concentration of tubes 80, 85, and 90 were applied. A replicate gel was sliced, each slice was eluted with 0.2 ml phosphate-buffered saline, and the eluates were assayed for their ability to correct the partial thromboplastin time of HMW kininogen-deficient plasma. The peaks of coagulant activity seen at slices 36–38 and 40–44 correspond to the two fainter light chain bands seen on the right side of the gel.

Conversion to kallikrein requires a single cleavage within a disulfide bridge at an Arg-Ile bond so that the active enzyme has a heavy chain disulfide-linked to a light chain (actually two forms of the light chain because of carbohydrate heterogeneity) but no change in total molecular weight, i.e., 80,000 Kd. Earlier, there was a publication by Nagasewa et al. who fractionated whole plasma and determined the molecular weight of prekallikrein to be 280,000 (13). We surmised that this difference in size could be due to the binding of a protein in plasma, and HK seemed to be a reasonable candidate. We determined a molecular weight of 285,000 for prekallikrein in normal plasma (14), and next fractionated Williams plasma on Sephadex G200. The value was 120,000 (typical of gel filtration −80,000 on SDS gel electrophoresis). We concluded that prekallikrein circulates bound to HK. We then used I^{125} labeled prekallikrein to prove the point. When added to Williams plasma, the radioactivity traveled with a molecular weight of 120,000. When Williams plasma was reconstituted with purified HK, ^{125}I prekallikrein was added, and the plasma was then subjected to gel filtration, the ^{125}I prekallikrein was then found at 300,000 indicating interaction with HK to form a complex (14). The percentage of binding depends on the concentration of the reactants and the binding constant. Subsequent analysis revealed that about 75% of prekallikrein is bound, so up to 25% can be present free (15). We assessed the molecular weight of factor XII throughout these experiments and found it to be the same in plasma as that of the purified protein, and concluded that it circulates unbound to other plasma constituents. Given the findings that the light chain derived from kinin-free HK possesses all of the clotting and fibrinolytic potential of HK, we compared the binding of prekallikrein to the purified heavy and light chains, and binding to the light chain but not the heavy chain was affirmed (16).

We next turned our attention to factor XI, even though it had a known role in the generation of bradykinin. Factor XI has four tandem repeats of about 90 amino acids each along the N-terminus of the protein that are homologous to repeats that is only in prekallikrein (17). Thus, they are more closely related to each other structurally than any other plasma protein. So, we repeated the aforementioned experiments with factor XI in place of prekallikrein. Factor XI is dimeric (18, 19) i.e., two 80,000 Kd subunits are disulfide-linked, so its molecular weight purified, or in Williams plasma is 160,000. However, its molecular weight in normal plasma is over 400,000 (20), and we could observe the increase in the apparent size of ^{125}I-factor XI when added to Williams plasma reconstituted with HK by gel filtration. Again, binding was to HK-derived light chain and not a heavy chain (16, 20). Although the plasma concentration of factor XI is less than prekallikrein (7 μg/ml vs. 25 μg/ml), about 99% of factor XI circulates bound to HK indicating a more avid attachment. Further, the dimeric structure of factor XII indicates that it is possible for one molecule of factor XI to bind two molecules of HK, one for each subunit. Figure 2 is a fractionation of normal plasma depicting the factor XI-HK complex as the largest, followed by the prekallikrein HK complex. The second

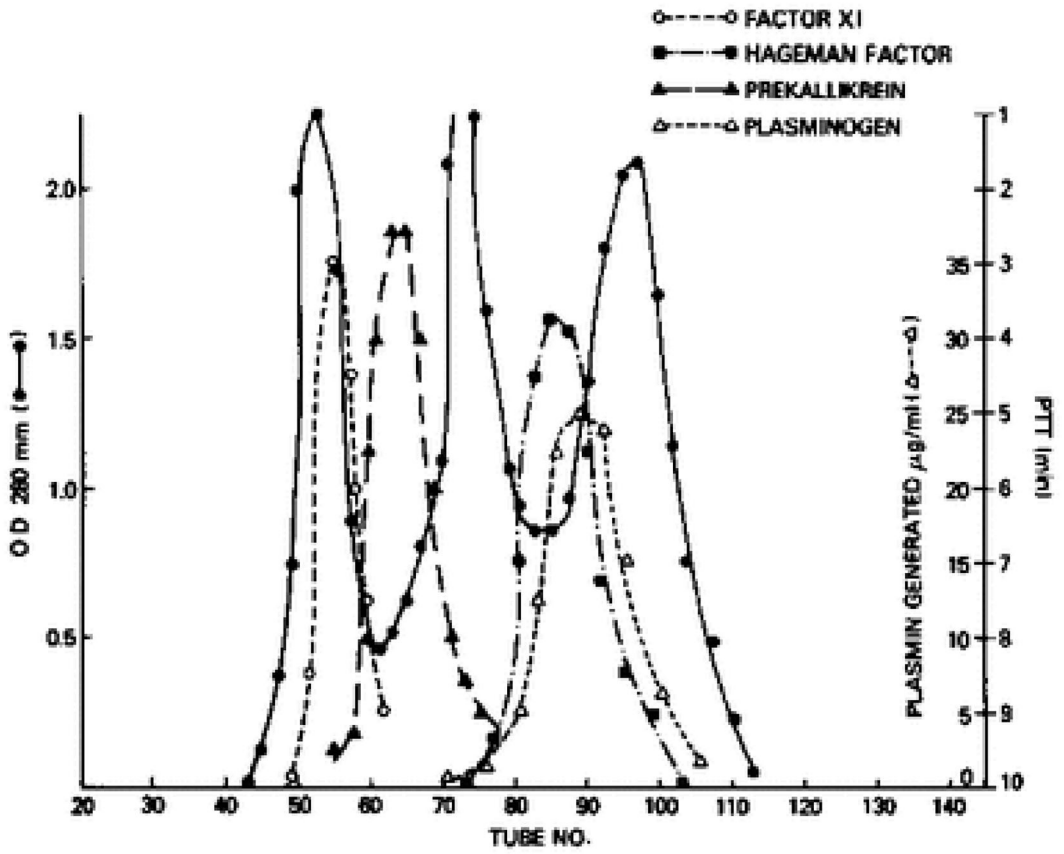

FIGURE 2
Gel filtration of human plasma on Sephadex G-200. The OD 280 indicates 3 protein peaks. The location of factor XI, prekallikrein, factor XII (Hageman factor) and plasminogen was identified immunologically. The molecular sizes of factor XI and prekallikrein reflect the binding of each to HK.

protein peak is IgG, which marks 160,000. The factor XII and plasminogen peaks are shown along the ascending limb of the third protein peak, albumin, at 60,000.

The amino acid sequences required for binding of prekallikrein and factor XI are not identical although there is some overlap. Factor XI binds to residues 185–242 of the light chain of HK (21) and prekallikrein binds to residues 185–224 (22). There is no effective competition for binding within plasma because the HK concentration at 80 μg/ml, when converted to moles present, is sufficient (theoretically) to bind virtually all the factor XI and prekallikrein present.

The domain structure of the HK proteins

The domain structure of HK is quite complex and clearly assembled from exons that may have evolved from smaller proteins with very varied functions. The N-terminal 4 domains are shared with LK and encompass the heavy chain (domains 1–3) and the bradykinin sequence plus an additional 10 amino acids (domain 4). The first 3 domains are homologous to each other and are derived from smaller entities known as cystatins (23) whose ancestors are still smaller proteins known as stefins (24). The cystatins are cysteine protease inhibitors, e.g., they inhibit papain or cathepsins B, H, and L (25). Domains 2 and 3 of the heavy chain both retain this enzyme inhibitory activity. Domain 1, while still homologous, lacks inhibitory function. This is depicted in Figure 3, which includes all six HK domains, with domains 1–3 indicated by "cysteine protease inhibitor." Domain 4 includes the bradykinin amino acid sequence plus 10 amino acids. This is the point of divergence of HK and LK, so they are exactly alike for domains 1–4. Domains 5 and 6 are unique to HK and are responsible for all of its functions in "contact activation," i.e., the plasma bradykinin-forming cascade. Domain 5, also known as the histidine-rich region, is the main site for interaction of HK with negatively charged substances/surfaces when used to activate the cascade *in vitro* such as kaolin, dextran sulfate, or ellagic acid. Domain 6 is critical and has the site(s) for interaction with prekallikrein

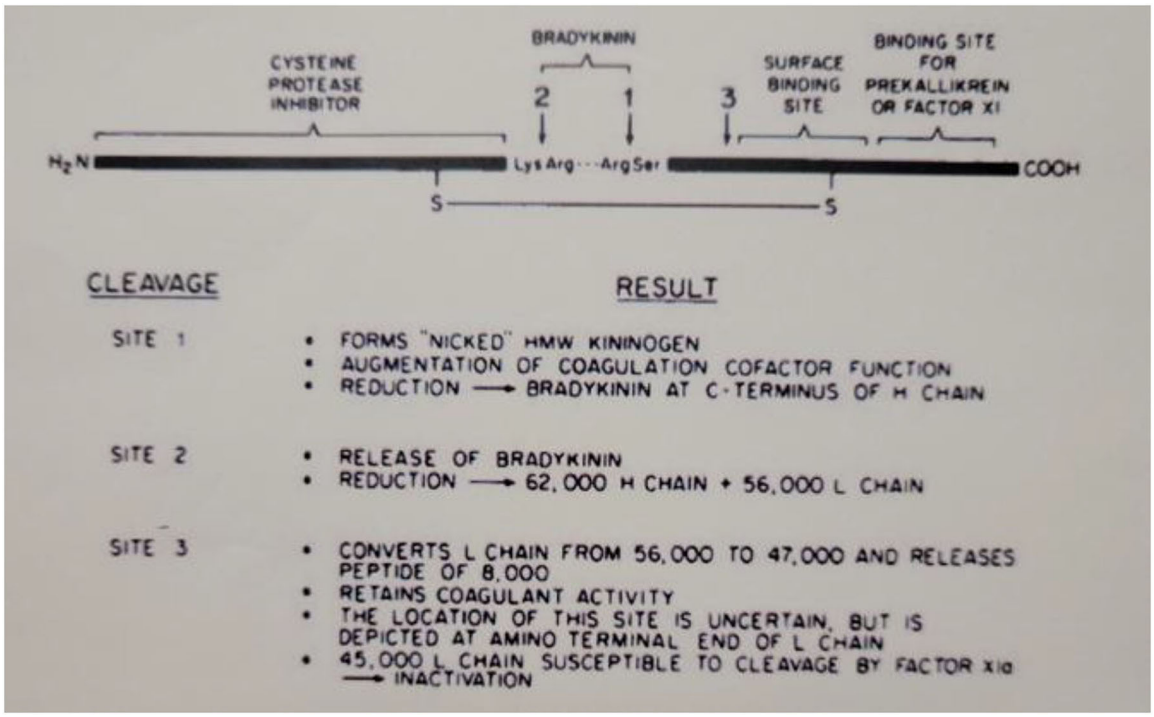

FIGURE 3
Depiction of the structure of human HWM kininogen and the consequences of cleavages at the sites indicated. The amino-terminal portion (heavy chain of cleaved kininogen) has the cysteine protease inhibitor activation. The light chain of cleaved HWM kininogen serves as the cofactor function in coagulation, fibrinolysis, and initiation of bradykinin formation.

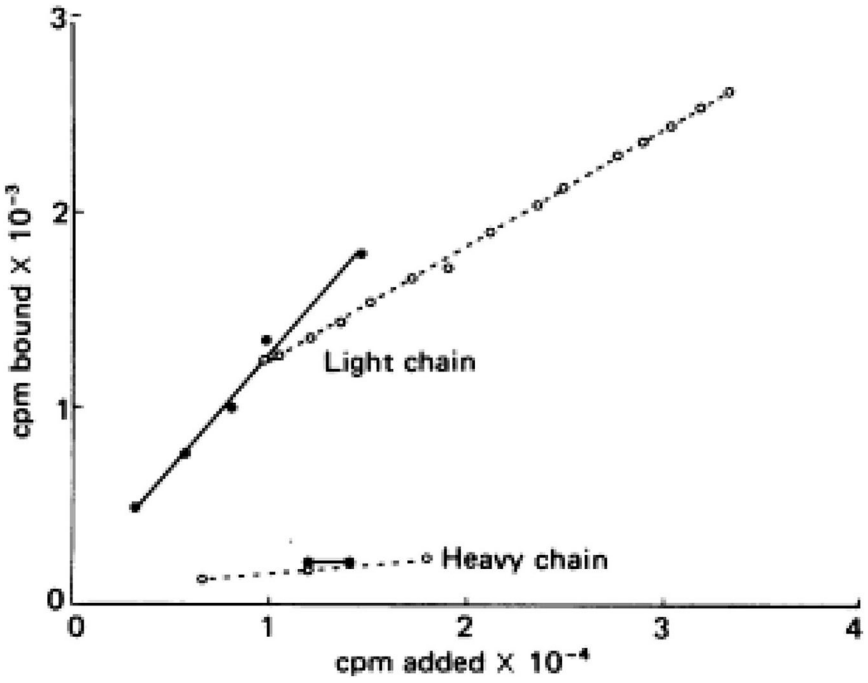

FIGURE 4
Addition of ^{125}I—factor XI (solid lines) or ^{125}I—prekallikrein (dotted line) to surface-bound light chain or heavy chain purified from HK. The counts bound (vertical) per moles added (horizontal) are plotted.

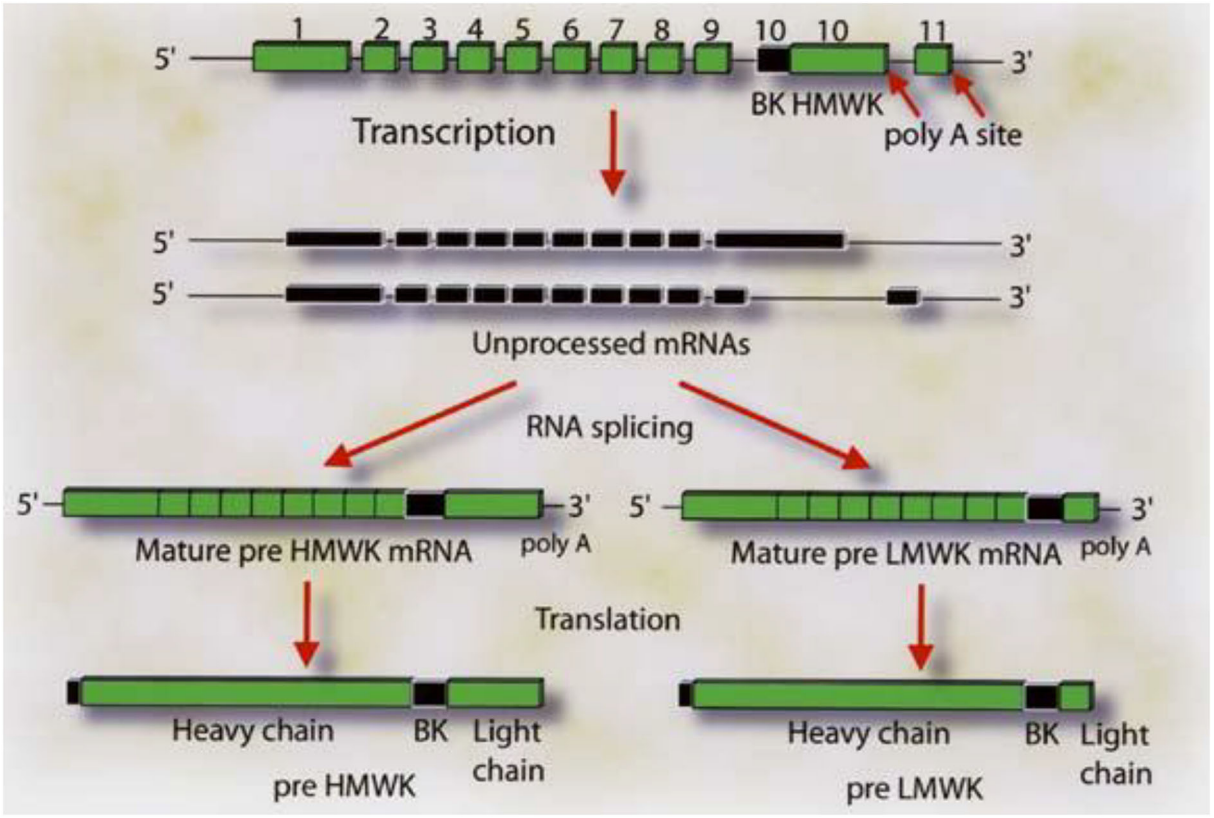

FIGURE 5
The gene structure for HK and LK. The boxes labeled 1–9 represent the exon coding for the heavy chain of HK and LK. Exon 10 codes for the bradykinin sequence and the light chain of HK. The respective mRNA's are assembled by alternative spicing events in which the light chain sequences are attached to the 3′ end of the 10 amino acid sequence C-terminal to bradykinin. Attachment of exon 10 in its entirety produces HK. Splicing of exon 10 (blackened portion) to exon 11 produces LK. Reproduced from Kitamura et al. (27).

and factor XI. The entire amino acid sequence analysis and domain structure for high and low molecular weight kininogens have been published (26, 27). One of the main *in vivo* binding interactions of HK is with vascular endothelial cells (discussed below), and here there are binding sites both in domain 3 in addition to domain 5 (28, 29).

When bradykinin-free HK is reduced and alkylated, the heavy chain contains domains 1–3 and the light chain has domains 5 and 6 plus the 10 C-terminal amino acids of domain 4. Thus, the light chain possesses all of the cofactor functions of HK, i.e., augmentation of the rate of various enzymatic reactions (30) as discussed below.

HK is a co-factor for intrinsic coagulation, fibrinolysis, and bradykinin formation

While plasma lacking HK cannot produce bradykinin by activation of factor XII (i.e., there is no substrate for plasma kallikrein), it becomes evident why coagulation and fibrinolysis are about as abnormal as they are in factor XII-deficient plasma. Four issues need to be considered (A) HIK augments the rate of conversion of prekallikrein to kallikrein (30). Since the kallikrein-feedback activation of factor XII is rate limiting for factor XII activation (31, 32), there is a slower rate of factor XII activation. (B) When negatively charged surfaces (kaolin, dextran sulfate) initiate the cascade, factor XI activation is totally dependent on attachment to the surface *via* HK; it is not activated in the fluid phase. Thus, factor XI is not activated and intrinsic coagulation does not proceed. (C) Intrinsic fibrinolysis is complex and dependent on multiple enzymes to convert plasminogen to plasmin. These include factor XIIa (33), plasma kallikrein (34), and factor Xia (35) plus kallikrein activation of the small amount of prourokinase in plasma to urokinase (36). Activation of all these enzymes, including prourokinase is retarded by the above consequences of HK deficiency. (D) The fourth consideration is more subtle. It is evident that the kallikrein feedback is impaired if kallikrein formation is slowed by HK deficiency. However, it has been shown that

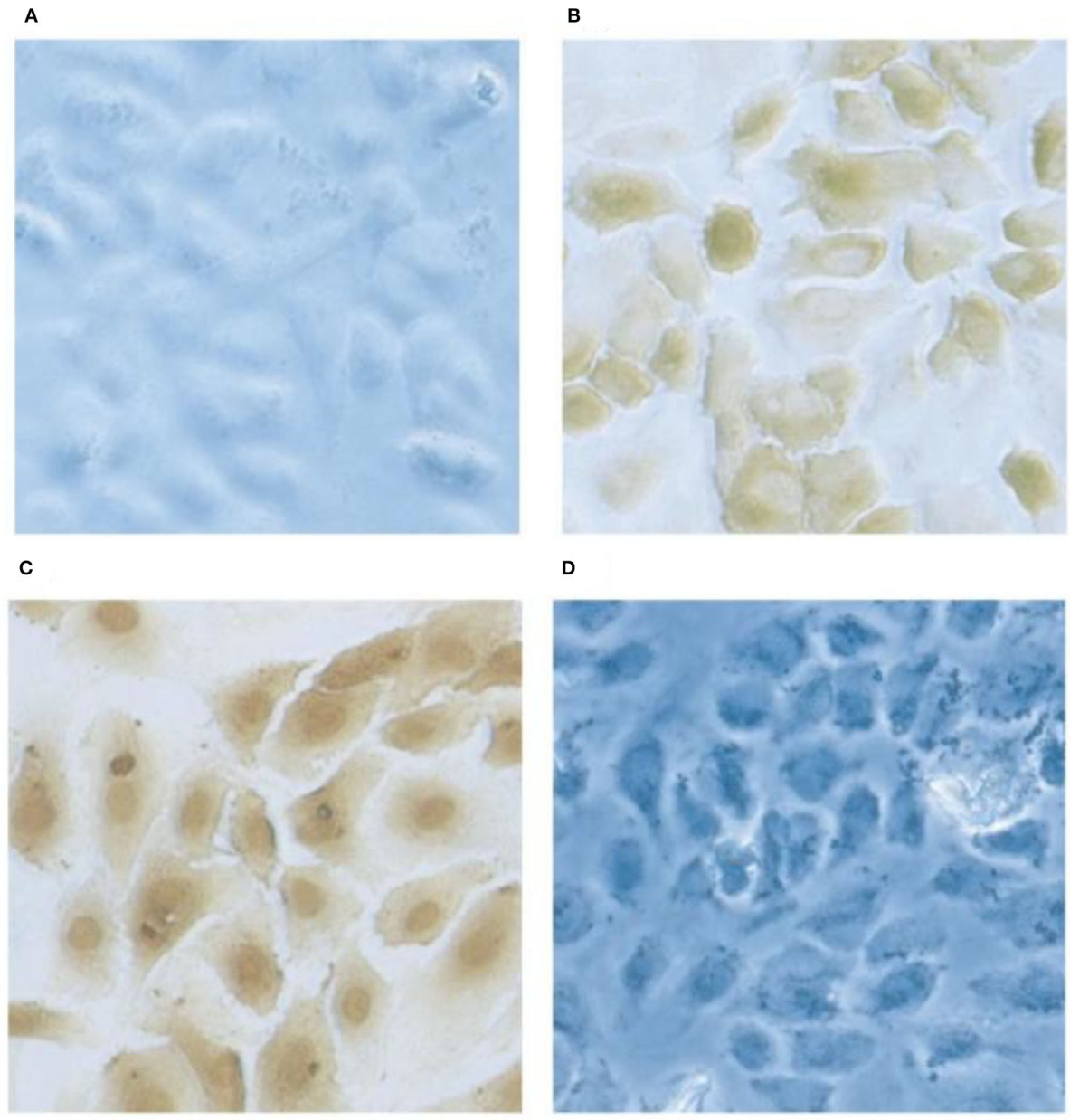

FIGURE 6
Localization of gC1qR on HUVEC's by immunochemical staining. Monolayer cultures of HUVECs on slides are fixed using 4% formaldehyde. The cells were first probed with rabbit anti gC1qR antibody and subsequently with horse radish -labeled goat anti-rabbit IgG. The gC1qR was visualized by 3,3-diaminobenzidine. Preimmune rabbit IgG staining showed no signal **(A)** and the anti-gC1qR antibody stained at the cell surface in non-permobilized cells **(B)** while in permimobilized cells **(C)**, the perinuclear and nuclear regions were prominently stained. In **D**, the cells were treated as in **(B)** but the antibody was pretreated with excess recombinant gC1qR.

the cleavage of surface-bound factor XII by kallikrein (already formed) is augmented upon the addition of HK (31). The most likely explanation is that kallikrein, in the absence of HK, binds firmly to the negatively charged surface, thus limiting its ability to activate factor XII. However, binding the HK-prekallikrein complex occurs *via* HK, so that some of the kallikreins thus formed can dissociate from HK and as it dissociates can pass along the cell surface to activate multiple factor XII molecules (37, 38).

Virtually all the above reactions, except bradykinin formation, are corrected with purified light chains derived from HK, while LK has no activity. An example of such light

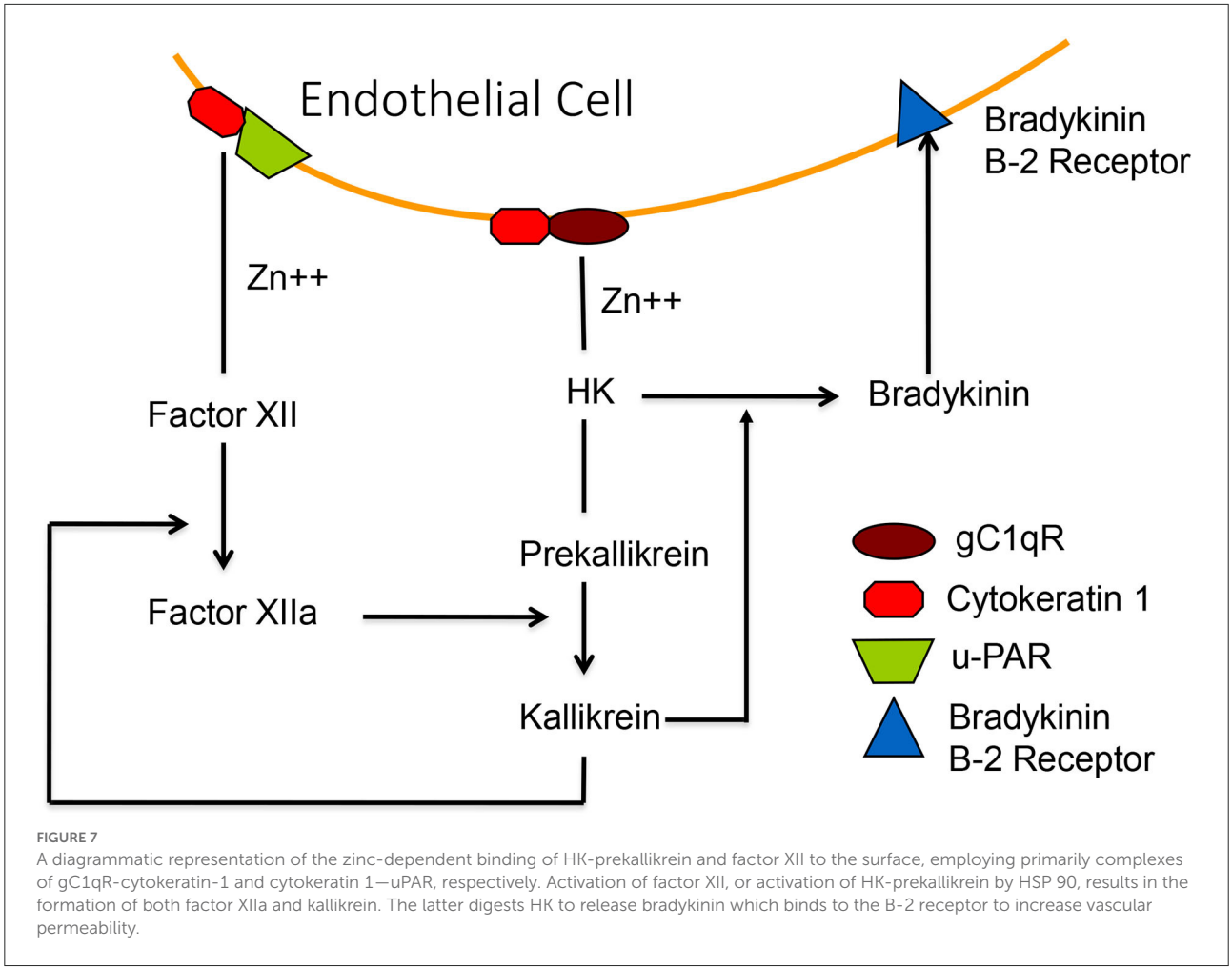

FIGURE 7
A diagrammatic representation of the zinc-dependent binding of HK-prekallikrein and factor XII to the surface, employing primarily complexes of gC1qR-cytokeratin-1 and cytokeratin 1—uPAR, respectively. Activation of factor XII, or activation of HK-prekallikrein by HSP 90, results in the formation of both factor XIIa and kallikrein. The latter digests HK to release bradykinin which binds to the B-2 receptor to increase vascular permeability.

chain function is shown in Figure 4 which depicts the binding of ^{125}I light chain to prekallikrein and factor XI with ^{125}I heavy chain as the negative control. The exon/intron structures of HK and LK (27) are shown and contrasted in Figure 5. Multiple exons are typically linked to produce one domain. The critical point is that the mechanism of alternative splicing accounts for the difference in the C-terminal portion (or light chains) of HK and LK. The splice site is within exon 10. Then either exon 11 is attached to produce the small light chain of LK, or the entire exon 10 is attached to produce the light chain of HK, and accounting for domains 5 and 6 of HK.

Interaction of HK with endothelial cells

Factor XII and HK each bind to the surface of endothelial cells where activation of the various cascades can take place. Three binding proteins have been described (gC1qR, cytokeratin 1, and u-PAR-(urokinase plasminogen activation receptor) (39–41) which are assembled as bimolecular complexes consisting of gC1qR-cytokeratin 1 and cytokeratin 1-u-PAR (42). When binding to an individual protein is studied, HK binds to gC1qR via domain 5 (39) (and as such competes for the binding site with factor XII) but, HK binds to cytokeratin 1 by domain 3 (43) thus both domains 3 and 5 may be involved upon interaction with the gC1qR-cytokeratin 1 complex. The staining of human umbilical cord vascular endothelial cells (HUVEC) for the presence of surface gC1qR is shown in Figure 6. Although HK binds to u-PAR, the interaction is relatively weak and an HK affinity column failed to bind u-PAR from solubilized endothelial cell membranes from which gC1qR and cytokeratin 1 were readily isolated (43). Binding to the cell surface can be inhibited with a combination of antibodies directed to gC1qR plus cytokeratin 1 and in our hands, this can account for about 85% of total binding (43). The urokinase plasminogen activator receptor (along with gC1qR) is a major binding protein for factor XII (44).

The crystal structure for binding factor XII and/or HK to a trimer of gC1qR has been solved (45) including an allosteric

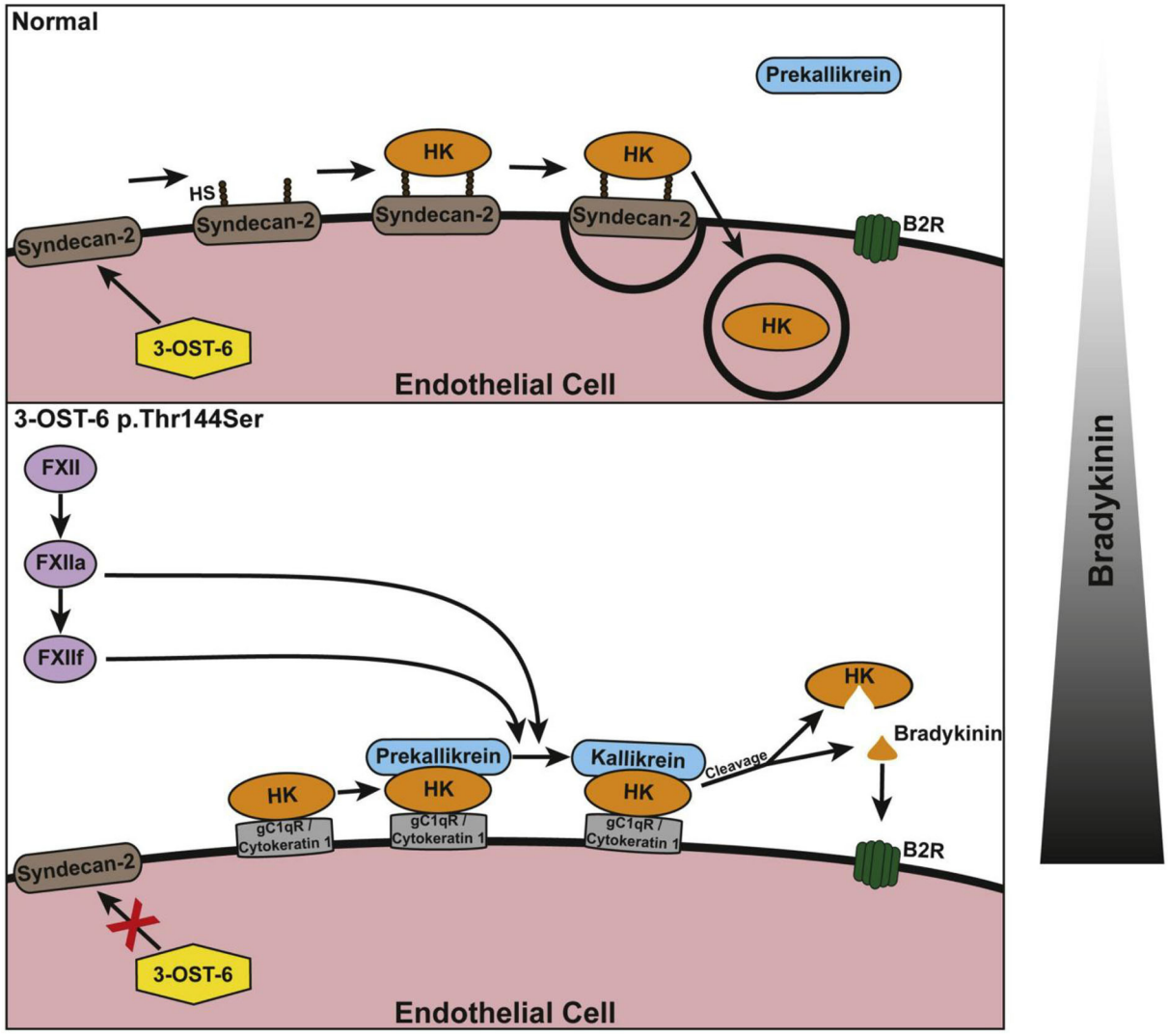

FIGURE 8
Schematic of the suggested involvement of 3-OST-6 in HK docking on the endothelial cell surface. The upper panel shows the normal (wild type) situation with HK being taken up *via* endocytosis due to interaction with heparan sulfate (HS)-containing proteoglycans. This prevents cleavage and bradykinin production. The lower panel shows the mutant situation: Because of incomplete HS modification, HK interacts with alternative interaction partners on the cell surface. This does not result in endocytosis and allows for HK cleavage and increased bradykinin production. The bar at the right indicates a shift in the balance of bradykinin production. Reproduced with permission: Bork et al. (60).

effect of zinc, which is required for binding HK and factor XII. It has been shown that HK, once bound to the cell surface, can be cleaved by adding kallikrein to release bradykinin (46) and one could activate the HK-prekallikrein complex, as bound to cells, even in the absence of factor XII. We purified a protein, heat shock protein-90 (HSP90) (47), which is secreted by endothelial cells and interacts with the complex of HK-prekallikrein (but not prekallikrein alone) to form a trimolecular complex which stoichiometrically converts the prekallikrein to kallikrein. The kallikrein thus formed can then activate factor XII under normal conditions. HSP 90 protein secretion can be induced by estrogen, interleukin 1, and TNFα, and also induces secretion of urokinase, which can bind to u-PAR and convert plasminogen to plasmin (48). Thus, the fibrinolytic cascade is also activated. Figure 7 is a depiction of the binding of factor XII and HK-prekallikrein to HUVEC as well as cell-surface reactions leading to bradykinin formation.

Apart from the aforementioned proteins, interactions of factor XII and HK with proteoglycans such as syndecan (49, 50) are also capable of modulating bradykinin formation (51), however, the effect of binding is inhibitory. The authors postulated that detachment from such binding is required for activation to proceed. Thus, we have two differing views of the mechanism(s) for factor XII and HK interaction with endothelial

cells. These are not mutually exclusive, and it is likely that both are relevant but the contributions of one vs. the other and the circumstances that might affect HK and factor XII binding to protein complexes vs. proteoglycan are not known. We made one attempt to show evidence of a role for proteoglycan (sulfated mucopolysaccharide-protein complexes) in HK binding, and we approached it by enzymatically removing sulfates (which are thought to be essential for binding) and then checking the interaction of HK with the cell surface. It was unchanged (52). Thus, binding to the protein complex thesis was confirmed, and the proteoglycan contribution could not be quantitated. Nevertheless, a new form of HAE (discussed below), calls attention to a role for proteoglycan which, as implied above, may be inhibitory.

HAE with normal C1INH

HAE with normal C1 INH has six forms thus far defined. Angiopoietin 1 and myoferlin mutations appear to be gain of function mutations that augment the effects of bradykinin upon endothelial cell receptors but do not appear to increase bradykinin formation (53, 54). The more common factor XII mutation (55) focuses on an augmented rate of factor XII activation (56, 57), by plasmin or thrombin, producing a truncated factor XII followed by cleavage by kallikrein (or plasmin) to activate it. A plasminogen mutation, however, appears to generate a mutant plasmin (glu^{311} plasmin) which directly cleaves kininogens, HK and LK, to produce bradykinin (58). Thus, factor XII and prekallikrein have been bypassed and a new, different enzyme produces bradykinin. There is one family with mutant HK, which in contrast to Williams plasma, has a mutation that leads to augmented bradykinin formation. That mechanism is thus far unknown (59). And finally, there is a family with a mutation in the heparin sulfate 3-0-sulfatransferase-6 gene that leads to HAE where there are two unproven assumptions: (A) that it leads to overproduction of bradykinin – this seems likely to be proven correct, and (B) the mutation diminishes sulfation of cell surface proteoglycan (60), but instead of decreasing bradykinin production, it leads to the opposite effect. With this information at hand, observations that shed light on this process include the ability of proteoglycans to assemble components (including HK) of the kinin-forming cascade (61), mediate endocytosis of HK with plasma prekallikrein to lysosomes (62), and then inhibit that endocytosis once bradykinin is released (63). A regulatory function for proteoglycan is thereby demonstrated. This is separate from the possible activation of factor XII by interaction with highly sulfated proteoglycan. It has been proposed that binding to proteoglycan (syndecan) is important at baseline and inhibits bradykinin formation and that with a defect in sulfation (as is depicted in Figure 8), the bimolecular protein complexes described above come into increased relevance (60) with activation of the cascade as a consequence. However, the protein complexes also appear to be significant at baseline (43). Thus, studies of the binding of HK and factor XII are needed that consider both binding to protein complexes and to proteoglycan at the surface of vascular endothelial cells in the same experiment. Nevertheless, the working hypothesis is that the mutation may eliminate an inhibitory effect of proteoglycan binding on bradykinin formation. These last two mutations (like the plasminogen mutation) may lead to a new understanding of the many routes by which *in vivo* bradykinin formation can occur.

Concluding comments

There are data involving cleaved HK as an anti-adhesion molecule, and domain 5 of HK as an inhibitor of angiogenesis, and tumor formation, that I have not addressed (64, 65). The focus is on the multifaceted mechanisms by which HK simultaneously augments all the steps required for bradykinin formation and intrinsic coagulation and fibrinolysis, beyond being a substrate from which bradykinin is generated. These hopefully illuminate the ways in which this multi-domain protein is intimately involved in hereditary angioedema caused by C1 inhibitor deficiency, and beyond.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Treatment of hereditary angioedema—single or multiple pathways to the rescue

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Hereditary angioedema (HAE) is a rare disease caused by mutations in the *SERPING1* gene. This results in deficient or dysfunctional C1 esterase inhibitor (C1-INH) and affects multiple proteases involved in the complement, contact-system, coagulation, and fibrinolytic pathways. Current options for the treatment and prevention of HAE attacks include treating all affected pathways *via* direct C1-INH replacement therapy; or specifically targeting components of the contact activation system, in particular by blocking the bradykinin B_2 receptor (B2R) or inhibiting plasma kallikrein, to prevent bradykinin generation. Intravenously administered plasma-derived C1-INH (pdC1-INH) and recombinant human C1-INH have demonstrated efficacy and safety for treatment of HAE attacks, although time to onset of symptom relief varied among trials, specific agents, and dosing regimens. Data from retrospective and observational analyses support that short-term prophylaxis with intravenous C1-INH products can help prevent HAE attacks in patients undergoing medical or dental procedures. Long-term prophylaxis with intravenous or subcutaneous pdC1-INH significantly decreased the HAE attack rate vs. placebo, although breakthrough attacks were observed. Pathway-specific therapies for the management of HAE include the B2R antagonist icatibant and plasma kallikrein inhibitors ecallantide, lanadelumab, and berotralstat. Icatibant, administered for treatment of angioedema attacks, reduced B2R-mediated vascular permeability and, compared with placebo, reduced the time to initial symptom improvement. Plasma kallikrein inhibitors, such as ecallantide, block the binding site of kallikrein to prevent cleavage of high molecular weight kininogen and subsequent bradykinin generation. Ecallantide was shown to be efficacious for HAE attacks and is licensed for this indication in the United States, but the labeling recommends that only health care providers administer treatment because of the risk of anaphylaxis. In addition to C1-INH replacement therapy, the plasma kallikrein inhibitors lanadelumab and berotralstat are recommended as first-line options for long-term prophylaxis and have demonstrated

Abbreviations

AE, adverse event; B1, bradykinin B_1; B2R, bradykinin B_2 receptor; bw, body weight; CI, confidence interval; C1-INH; C1 esterase inhibitor; HAE, hereditary angioedema; EAACI, European Academy of Allergy and Clinical Immunology; FAST, For Angioedema Subcutaneous Treatment; HAE-nl-C1INH, hereditary angioedema with normal C1-INH; HMW, high molecular weight; IL, interleukin; IMPACT, International Multicenter Prospective Angioedema C1-INH Trial; IQR, interquartile range; IV, intravenous; LTP, long-term prophylaxis; MASP, mannose-binding lectin–associated serine proteases; OR, odds ratio; pdC1-INH, plasma-derived C1 esterase inhibitor; rhC1-INH, recombinant human C1 esterase inhibitor; SC, subcutaneous; SAE, serious adverse event; SE, standard error; WAO, World Allergy Organization.

marked reductions in HAE attack rates. Investigational therapies, including the activated factor XII inhibitor garadacimab and an antisense oligonucleotide targeting plasma prekallikrein messenger RNA (donidalorsen), have shown promise as long-term prophylaxis. Given the requirement of lifelong management for HAE, further research is needed to determine how best to individualize optimal treatments for each patient.

KEYWORDS

bradykinin B_2 receptor antagonist, complement C1 inhibitor protein, hereditary angioedema, kallikreins, prophylaxis

Introduction

Hereditary angioedema (HAE) is a rare and potentially life-threatening genetic disease that can cause recurrent episodes (attacks) of nonpruritic swelling of the skin, affecting the extremities (e.g., hands, feet), face, and genitals, as well as submucosal swelling of the gastrointestinal and upper respiratory tracts (1–3). Approximately 85% of patients have type I HAE (type I C1-INH-HAE), which is characterized by a deficiency in C1 esterase inhibitor (C1-INH) levels (2–4). Type II HAE (type II C1-INH-HAE) accounts for about 15% of cases and is associated with abnormal function of C1-INH in the presence of normal C1-INH levels. These 2 types of HAE are caused by mutations in the *SERPING1* gene, which encodes C1-INH (2). A third type of HAE, in which both levels and function of C1-INH are normal (HAE-nl-C1INH) (2, 5), is associated with specific genetic mutations (i.e., *F12*, *ANGPT1*, *HS3ST6*, *PLG*, *MYOF*, and *KNG1*), although many patients with HAE-nl-C1INH have no currently identified genetic mutation (2, 6, 7). HAE substantially impairs patient health-related quality of life, disrupts daily activities, and adversely affects social and professional and/or academic functioning (3, 8–11). Additionally, HAE is associated with increased rates of anxiety and depression, likely related to the unpredictability of symptoms and the associated emotional and physical stress (8–11). Severe HAE attacks require immediate intervention and may necessitate an emergency department visit or hospitalization, adding to the disease burden (11, 12).

The ultimate goal of HAE treatment is to achieve complete disease control (i.e., prevent all HAE attacks) and normalize patients' quality of life and daily functioning (12). For those patients who are unable to achieve complete disease control, therapy aims to reduce the number of HAE attacks and improve quality of life. Pharmacologic management of HAE consists of on-demand (acute) treatment of HAE attacks, short-term prophylaxis delivered prior to procedures or events anticipated to trigger HAE symptoms, and routine, long-term prophylaxis to prevent HAE attacks (2, 13). On-demand therapy aims to minimize morbidity and prevent mortality during an HAE attack, while long-term prophylaxis is broadly deemed valuable for helping to optimize patients' quality of life and daily functioning. Minimizing the burden of treatment and associated adverse effects is also an important consideration, especially in patients receiving long-term prophylaxis (12, 13). In recent years, the range of pharmacologic treatment options for the management of HAE has expanded considerably to encompass agents with more convenient routes of administration that facilitate self-management, as well as medications with different mechanisms of action that target distinct components of the pathways involved in HAE (14). Multiple treatment options offer the opportunity to individualize therapy, provide an opportunity for both prophylaxis and on-demand therapy using synergistic mechanisms of action, and minimize the disease burden. This narrative review describes the biologic pathways of importance in HAE and provides an overview of therapies that target these pathways to prevent and treat HAE attacks.

Pathways of importance in HAE

The plasma contact system is composed of the enzymes factor XII and plasma prekallikrein and is involved in the generation of the inflammatory peptide bradykinin and in blood coagulation (15). This system, together with the nonenzymatic co-factor high molecular weight (HMW) kininogen, comprises the kallikrein–kinin pathway (15, 16). Factor XII is activated by a number of mechanisms to generate factor XIIa (activated factor XII), which then cleaves kallikrein from prekallikrein. Kallikrein further activates factor XII in a positive feedback loop and also cleaves the HMW kininogen, releasing bradykinin (**Figure 1**) (5, 16). Bradykinin plays a role in blood coagulation, fibrinolysis, and vasodilation and is the primary mediator of enhanced vascular permeability during an HAE attack (5, 15, 16). Additionally, cleavage of factor XIIa by kallikrein results in release of the active protease factor XIIf, which activates the classical complement pathway (16).

Under healthy conditions, C1-INH inhibits several proteases, including factors XIIa and XIIf and plasma kallikrein, as well as components of the early classical complement pathway (**Figure 1**) (5, 16). In types I and II

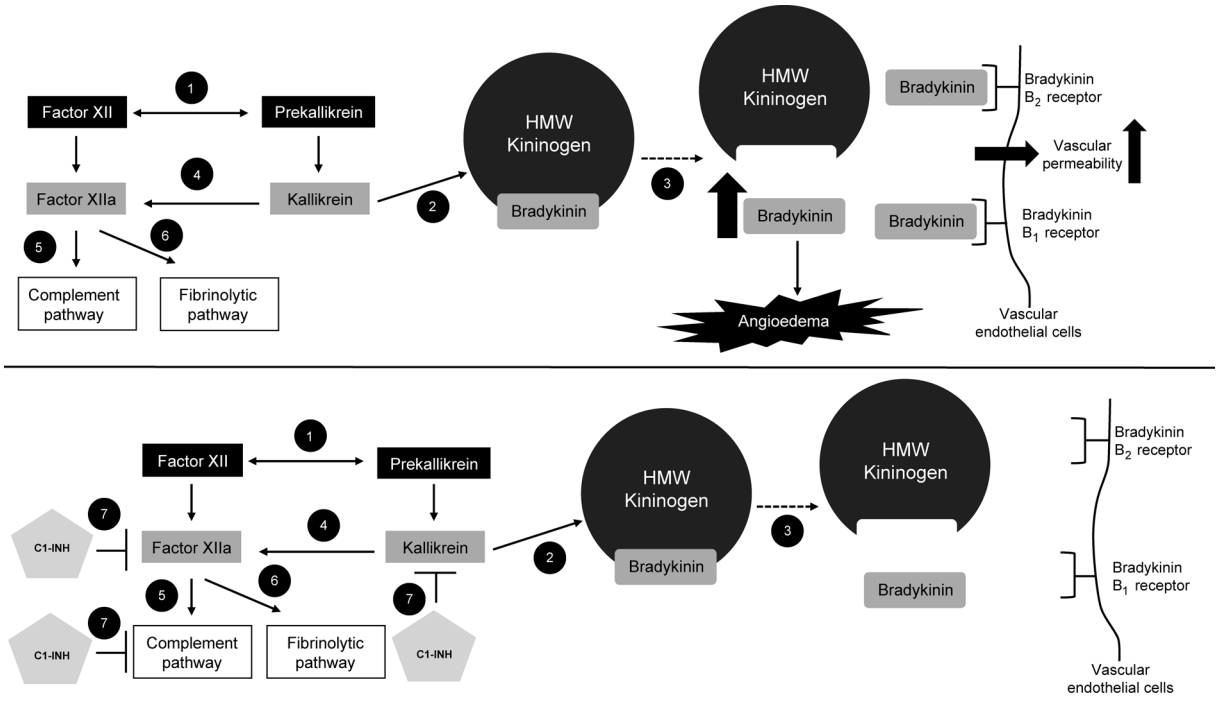

FIGURE 1
Dysregulation of signaling pathways in HAE. (1) When activated by trace amounts of factor XIIa, plasma prekallikrein and factor XII cleave each other to generate kallikrein and factor XIIa. (2) Kallikrein cleaves HMW plasma kininogen, leading to (3) the release of bradykinin. (4) Plasma kallikrein cleaves factor XIIa, leading to (5) activation of complement and (6) fibrinolytic pathways. In the top figure, the increase in bradykinin levels results in angioedema. Bradykinin binds bradykinin B_1 and B_2 receptors on vascular endothelial cells, leading to an increase in vascular permeability. In the bottom figure, (7) C1-INH inhibits factor XIIa, the complement pathway, and kallikrein, thus leading to a decrease in bradykinin production and reduced activation of bradykinin B_1 and B_2 receptors on vascular endothelial cells. *C1-INH, C1 esterase inhibitor; HAE, hereditary angioedema; HMW, high molecular weight. Figure created with data from Cicardi M, et al. J Allergy Clin Immunol Pract. (2018) 6(4):1132–41; and Zuraw BL. N Engl J Med. (2008) 359(10):1027–36* (5, 16).

HAE, C1-INH deficiency or dysfunction causes an increase in bradykinin levels because of dysregulation of the plasma contact system (5, 16). Unabated generation of bradykinin, resulting from insufficient C1-INH regulation of factor XIIa and kallikrein, leads to angioedema. Plasma kallikrein cleaves factor XIIa, leading to activation of the complement cascade; activation of complement results in the cleavage of C5 (to the anaphylatoxin C5a) and formation of the complement fragment C4a, which increases endothelial permeability (17). Cytokines can also affect endothelial permeability, with proinflammatory cytokines (e.g., interleukin [IL]-1, IL-4, IL-6, IL-8, IL-13) increasing permeability and anti-inflammatory cytokines (e.g., IL-1Ra, IL-10) inhibiting permeability (17). While evidence supports a key role of the bradykinin B_2 (B2) receptor in angioedema, upregulation of the bradykinin B_1 (B1) receptor during stress, trauma, or infection may influence susceptibility to angioedema, and prolonged B1 signaling may be involved in sustaining swelling during an HAE attack (16, 18). Thus, it is unclear whether blockade of both B1 and B2 receptors may be needed to completely prevent vascular leakage and associated swelling (18).

The lectin pathway is an activator of the complement cascade, which is mediated, in part, through mannose-binding lectin–associated serine proteases (MASPs) (19). MASP-1 is able to directly cleave HMW kininogen to generate and release bradykinin. C1-INH is an important regulator of the lectin pathway *via* inhibition of MASP-1 and MASP-2. Thus, dysregulation of MASPs may play a role in the pathophysiology of HAE by contributing to elevated bradykinin levels. The fibrinolytic pathway is also activated by factor XIIa (**Figure 1**), leading to conversion of plasminogen to plasmin; plasmin subsequently cleaves fibrin, leading to fibrin degradation (5). This pathway plays a greater role in some types of HAE-nl-C1INH pathophysiology, but also contributes to the endothelial dysfunction of types I and II HAE (20).

Pathways important in C1-inhibitor deficiency in HAE may also play a role in autoimmunity and neoplasms. A Swedish population-based cohort study reported that patients with HAE were at increased risk of autoimmune disease compared with individuals in the general population (odds ratio [OR], 1.6; 95% confidence interval [CI], 1.2–2.4); the risk of developing systemic lupus erythematosus was significantly

greater in patients with HAE compared with individuals without HAE (OR, 71.9; 95% CI, 8.8–586.7) (21). While this study did not find an increased risk of cancer in patients with HAE vs. the general population (OR, 0.9; 95% CI, 0.6–1.4) (21), a retrospective review of medical records in Italy reported that neoplasms were the most common cause of mortality in patients with HAE, compared with cardiovascular disease as the most common cause of mortality in the general population (22). However, future studies are warranted to examine the role of HAE pathways in autoimmune disease and cancer.

Multiple pathway therapies

C1-INH replacement therapy

C1-INH products provide a direct replacement for the low levels or low functional activity of C1-INH in patients with type I or II HAE, respectively (13). Accordingly, administration of C1-INH replacement therapy during an HAE attack restores regulation of the cascade systems producing bradykinin by inhibiting the same targets as endogenous C1-INH (i.e., plasma kallikrein, factors XIIa and XIIf, and elements of the complement pathway, including MASP-1; **Figure 1**) (5, 13, 16).

On-demand therapy

The World Allergy Organization (WAO)/European Academy of Allergy and Clinical Immunology (EAACI) guidelines recommend treatment with intravenous (IV) C1-INH as soon as possible after onset of an HAE attack (13). Intravenously administered plasma-derived C1-INH (pdC1-INH; Berinert; CSL Behring LLC; Kankakee, IL) and recombinant human C1-INH (rhC1-INH; Ruconest; Pharming Healthcare Inc.; Warren, NJ; **Table 1**) (23–37) are efficacious and well tolerated as on-demand treatment of HAE attacks (**Table 2**) (38–41). In a phase 2/3, randomized, double-blind, placebo-controlled trial ($n = 124$), pdC1-INH (Berinert) 20 U/kg provided a significantly faster onset of symptom relief of abdominal or facial attacks compared with placebo (median, 0.5 vs. 1.5 h, respectively; $P = 0.002$) and a significantly shorter median time to complete resolution of symptoms (4.9 vs. 7.8 h, respectively; $P = 0.02$; **Table 2**) (38). These data were supported by an open-label extension trial ($N = 57$) of pdC1-INH 20 U/kg as on-demand treatment (median follow-up, 24 months; **Table 2**) (39). Another pdC1-INH product (Cinryze; ViroPharma Biologics LLC; Lexington, MA) administered as on-demand treatment also showed significantly faster onset of symptom relief compared with placebo (40) (**Table 2**) but is not approved for acute treatment of HAE in the United States or European Union (**Table 1**).

Two similarly designed randomized, double-blind, placebo (saline)-controlled trials evaluated rhC1-INH 50 U/kg ($n = 12$) or 100 U/kg ($n = 29$) as on-demand treatment for HAE attacks (**Table 2**) (41). Compared with placebo, rhC1-INH significantly reduced the time to onset of symptom relief (50 U/kg: median, 122 vs. 495 min, respectively; $P = 0.01$; 100 U/kg: median, 66 vs. 495 min, respectively; $P < 0.001$), as well as the time to minimal symptoms (50 U/kg: median, 247 vs. 1,210 min, respectively; $P = 0.001$; 100 U/kg: median, 266 vs. 1,210 min, respectively; $P < 0.001$). The median time to onset of symptom relief with IV C1-INH replacement therapies has varied in relation to trial design, product formulation, and dosing, with a range of 0.4–1.2 h for pdC1-INH and approximately 1.0–2.0 h for rhC1-INH (**Table 2**) (38, 39, 41). Review of these trials suggests that there is a dose response, with higher doses providing earlier onset of relief, as well as relief in a greater percentage of patients (42).

Short-term prophylaxis

Surgical and dental procedures and other medical interventions (e.g., diagnostic procedures) may trigger an attack in patients with HAE (43–46). WAO/EAACI guidelines recommend short-term prophylactic therapy for patients with HAE undergoing these types of procedures, with C1-INH replacement therapies considered the first-line option for short-term prophylaxis (13). Patient-specific emotional triggers may also precipitate an HAE attack, so WAO/EAACI guidelines also suggest that short-term prophylaxis be considered prior to exposure to an anticipated stressful life event.

No C1-INH formulation is currently approved in the United States for short-term prophylaxis, although pdC1-INH (Berinert and/or Cinryze) is approved for this indication in the European Union and Japan (**Table 1**). Evidence for the efficacy and safety of C1-INH replacement therapy for short-term prophylaxis is limited to retrospective and observational analyses, including from patient registries (**Table 3**) (40, 46–54). Data from a retrospective study ($N = 137$) reported that patients ≥2 years of age with HAE who received short-term prophylaxis with pdC1-INH (Berinert) prior to medical procedures experienced a decrease in post-procedure HAE attacks compared with the number of attacks they experienced before being diagnosed with HAE (**Table 3**) (46). It is noteworthy that patients in this study received pdC1-INH 500 U, which is half the dose noted by WAO/EACCI guidelines (1,000 U or 20 U/kg) for short-term, pre-procedure prophylaxis with pdC1-INH (13, 46). Registry data also indicated a low cumulative HAE attack rate within 3 days of pdC1-INH (Berinert) administration as short-term prophylaxis (47). Numerical trends suggested greater efficacy with weight-based doses of ≥15 IU/kg or absolute doses ≥1,500 IU. A recombinant preparation of C1-INH has also proved effective. A retrospective study ($N = 51$; 92% with type

TABLE 1 Approved treatments for hereditary angioedema.

Treatment	Route of administration	FDA-approved indication and dose	EMA-approved indication(s) and dose	MHLW-approved indication(s) and dose
C1-INH replacement products				
pdC1-INH (Berinert)	IV	Acute treatment in pediatric patients and adults (20 IU/kg)	Acute treatment (20 IU/kg) and short-term prophylaxis (pediatric patients: 15–30 IU/kg; adults: 1,000 IU)	Acute treatment and short-term prophylaxis (<50 kg: 500 U; >50 kg, 1,000–1,500 U)
pdC1-INH (Cinryze)	IV	Long-term prophylaxis in patients aged ≥6 y (children 6–11 y: 500 U q3–4 d; adolescents ≥12 y and adults: 1,000 U q3–4 d)	Acute treatment (2–11 y [10–25 kg]: 500 IU; 2–11 y [>25 kg: 1,000 IU]; ≥12 y: 1,000 IU) and short-term prophylaxis in patients aged ≥2 y (2–11 y [10–25 kg]: 500 IU <24 h; 2–11 y [>25 kg]: 1,000 IU <24 h; ≥12 y: 1,000 IU <24 h); long-term prophylaxis in patients aged ≥6 y (6–11 y: 500 IU q3–4 d; ≥12 y: 1,000 IU q3–4 d)	Not approved
rhC1-INH/Conestat alfa (Ruconest)	IV	Acute treatment in adolescents and adults (<84 kg: 50 U/kg; ≥84 kg: 4,200 U)	Acute treatment in patients aged ≥2 y (<84 kg: 50 U/kg; ≥84 kg: 4,200 U)	Not approved
pdC1-INH (Haegarda [US]/ Berinert [EU])	SC	Long-term prophylaxis in patients aged ≥6 y (60 IU/kg q3–4 d)	Long-term prophylaxis in adolescents and adults (60 IU/kg)	Not approved
Bradykinin B2 receptor antagonist				
Icatibant (Firazyr)	SC	Acute treatment in patients aged ≥18 y (30 mg)	Acute treatment in patients aged ≥2 y (patients 2–17 y: 12–25 kg, 10 mg; 26–40 kg, 15 mg; 41–50 kg, 20 mg; 51–65 kg, 25 mg; >65 kg, 30 mg; adults: 30 mg)	Acute treatment in adults (30 mg)
Plasma kallikrein inhibitors				
Ecallantide (Kalbitor) (33)	SC	Acute treatment in patients aged ≥12 y (30 mg)	Not approved	Not approved
Lanadelumab (Takhzyro) (34, 35)	SC	Long-term prophylaxis in patients aged ≥12 y (300 mg q2–4 wk)	Long-term prophylaxis in patients aged ≥12 y (300 mg q2–4 wk)	Long-term prophylaxis in patients aged ≥12 y (300 mg q2–4 wk)
Berotralstat (Orladeyo) (36, 37)	Oral	Long-term prophylaxis in patients aged ≥12 y (150 mg once daily)	Long-term prophylaxis in patients aged ≥12 y (150 mg once daily)	Long-term prophylaxis in patients aged ≥12 y (150 mg once daily)

EMA, European Medicines Agency; FDA, US Food and Drug Administration; IV, intravenous; MHLW, Ministry of Health, Labour and Welfare (Japan); pdC1-INH, plasma-derived C1 esterase inhibitor; SC, subcutaneous.

I HAE) demonstrated that short-term prophylaxis with rhC1-INH was efficacious for increasing the percentage of medical and dental procedures that remained attack-free compared with procedures in which no short-term prophylaxis was administered (**Table 3**) (48). However, rhC1-INH is currently not approved for prophylaxis (**Table 1**) (28).

Long-term prophylaxis

WAO/EAACI guidelines recommend consideration of long-term prophylaxis for all patients with type I or II HAE, taking into consideration patients' preferences, disease activity, HAE impact on quality of life, availability of health care resources, and inability to achieve adequate control of symptoms with appropriate on-demand therapy (13). Patients with HAE should be evaluated for long-term prophylaxis at each office or telemedicine visit or at least annually because these factors can vary over time. C1-INH replacement (e.g., pdC1-INH) is recommended as a first-line, long-term prophylactic therapy (13). C1-INH products approved in the United States and the European Union for routine, long-term prophylaxis include IV pdC1-INH (Cinryze) and a subcutaneous (SC) formulation of pdC1-INH (Haegarda; CSL Behring LLC; **Table 1**).

A double-blind, placebo-controlled, crossover trial ($N = 22$) determined that pdC1-INH (Cinryze) 1,000 U IV administered every 3 to 4 days as long-term prophylaxis significantly reduced the HAE attack rate compared with placebo during a 12-week period (difference, 6.5 attacks; $P < 0.001$; **Table 3**) (40). In addition, HAE attack duration was significantly decreased for patients receiving pdC1-INH prophylaxis compared with those receiving placebo (2.1 vs. 3.4 days, respectively; $P = 0.002$; **Table 3**). An open-label prophylaxis trial ($N = 146$) of pdC1-INH 1,000 U (Cinryze) every 3–7 days for up to 2.6 years found a 90% decrease from baseline in the mean number of attacks [4.7 attacks/month (baseline) vs. 0.5 attacks/month]

TABLE 2 Summary of clinical trials of C1-INH replacement therapy as on-demand treatment for HAE attacks.

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
pd C1-INH (Berinert)	Craig TJ, et al. (38) IMPACT1 August 2005–December 2007 R, DB, PBO-C, ph 2/3 study Pts ≥6 y with type I or II HAE with single abdominal or facial attacks	pdC1-INH 10 U/kg bw IV ($n = 39$) pdC1-INH 20 U/kg bw ($n = 43$) IV PBO ($n = 42$)	Time to onset of symptom relief[a]: Mean (SD): pdC1-INH 10 U/kg: 7.5 h (10.5) pdC1-INH 20 U/kg: 3.9 h (8.2) PBO: 10.3 h (11.5) Median (range): pdC1-INH 10 U/kg: 1.2 h (0.2–24.0) pdC1-INH 20 U/kg: 0.5 h (0.2–24.0) PBO: 1.5 h (0.2–24.0) $P = 0.002$ (20 U/kg vs. PBO) Time to complete resolution of HAE symptoms: Mean (SD): pdC1-INH 10 U/kg: 216.1 h (494.2) pdC1-INH 20 U/kg: 81.8 h (314.3) PBO: 125.1 h (382.8) Median (range): pdC1-INH 10 U/kg: 20.0 h (0.5–1,486.2) pdC1-INH 20 U/kg: 4.9 h (0.5–1,486.2) PBO: 7.8 h (0.3–1,486.2) $P = 0.02$ (20 U/kg vs. PBO)	pdC1-INH 10 U/kg ($n = 39$) pdC1-INH 20 U/kg ($n = 46$) PBO ($n = 41$) Any AE ≤4 h of start of tx: pdC1-INH 10 U/kg, 25.6% ($n = 10$); pdC1-INH 20 U/kg, 19.6% ($n = 9$) vs. PBO 43.9% ($n = 18$) Tx-related AEs: 20.5% ($n = 8$), 10.9% ($n = 5$), vs. 19.5% ($n = 8$), respectively SAEs/AEs leading to discontinuation: 0 (all groups) Most common AEs[b]: Muscle spasms: 10.3% ($n = 4$), 2.2% ($n = 1$), vs. 4.9% ($n = 2$) Pain: 10.3% ($n = 4$), 2.2% ($n = 1$), vs. 2.4% ($n = 1$) Nausea: 2.6% ($n = 1$), 6.5% ($n = 3$), vs. 12.2% ($n = 5$) Diarrhea: 2.6% ($n = 1$), 0, vs. 9.8% ($n = 4$)
	Craig TJ, et al. (39) (IMPACT2) August 2005–February 2010 OL extension of IMPACT1 Pts ≥6 y with type I or II HAE who previously participated in IMPACT1 ($n = 57$; 1,085 attacks) with attacks at any body location	pdC1-INH 20 U/kg bw IV Median study duration: 24 mo (range, 0–51 mo) Pts received tx for median 7 attacks (range, 1–184 attacks)	Per-attack analysis: Median (range) time to onset of symptom relief[a]: 0.4 h (0.05–497.0[c]) Median (range) time to complete resolution of HAE symptoms: 14.3 h (0.2–497.0[c]) Single dose effective for 1,073/1,085 HAE attacks (99%) Per-pt analysis: Median time to onset of symptom relief[a]: 0.46 h (95% CI, 0.39–0.53; range, 0.2–497.0[c]) Median time to complete resolution of HAE symptoms: 15.5 h (95% CI, 11.6–21.6; range, 0.6–497.0[c])	AEs by no. of pts ($n = 57$)/no. of attacks ($n = 1,085$): Any AE: 43.9% ($n = 25$)/5.4% ($n = 59$) Tx-related AEs: 14.0% ($n = 8$)/0.8% ($n = 9$) SAEs: 1.8% ($n = 1$)[d]/<0.1% ($n = 1$)[d] AEs leading to discontinuation: 1.8% ($n = 1$)/<0.1% ($n = 1$) Most common AEs[c]: Headache: 8.8% ($n = 5$)/0.7% ($n = 8$) Nasopharyngitis: 5.3% ($n = 3$)/0.3% ($n = 3$)
pdC1-INH (Cinryze)	Zuraw BL, et al. (40) 14 March 2005–18 May 2007 R, DB, PBO-C Pts ≥6 y with single HAE attacks (abdomen, external genitalia, extremities, face, throat)	pdC1-INH 1,000 U/10 ml ($n = 36$) PBO ($n = 35$)[e]	Median time to onset of symptom relief: pdC1-INH 1,000 U: 2 h PBO: >4 h Estimated success rate ratio, 2.4 (95% CI, 1.2–5.0; $P = 0.02$) Pts with time to relief ≤4 h: 21/35 (60%) vs. 14/33 (42%; $P = 0.06$) Median time to complete resolution of symptoms (after second dose of study drug [pdC1-INH: $n = 23$; PBO: $n = 28$]): 12.3 vs. 25.0 h ($P = 0.004$)	pdC1-INH 1,000 U/10 ml ($n = 36$) PBO ($n = 35$) ≥1 AEs: 17% ($n = 6$) vs. 20% ($n = 7$) Possibly tx-related AEs pdC1-INH: Rash at injection site: 2.8% ($n = 1$) PBO: Contact dermatitis: 2.9% ($n = 1$) Tetany: 2.9% ($n = 1$)
rhC1-INH Ruconest	Zuraw B, et al. (41) R, DB, C 2 independent trials with similar design, entry criteria, endpoints Pts ≥12 y (US/Canada) or ≥16 y (Europe)	rhC1-INH 50 U/kg bw IV[f] ($n = 12$) rhC1-INH 100 U/kg bw IV ($n = 29$) PBO ($n = 29$)	Time to onset of symptom relief: Median (range): rhC1-INH 50 U/kg: 122 min (72–136) $P = 0.01$ vs. PBO rhC1-INH 100 U/kg: 66 min (61–122) $P < 0.001$ vs. PBO PBO: 495 min (245–520)	rhC1-INH 50 U/kg ($n = 12$) rhC1-INH 100 U/kg ($n = 29$) PBO ($n = 29$) Any AE ≤90 d of tx administration: rhC1-INH 50 U/kg, 33% ($n = 4$); rhC1-INH 100 U/kg, 24% ($n = 7$), vs. 48% ($n = 14$) Tx-related AEs: 0, 3% ($n = 1$), vs. 10% ($n = 3$) SAEs: 0, 3% ($n = 1$), vs. 10% ($n = 3$)

(*continued*)

TABLE 2 Continued

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
				AEs leading to discontinuation: 0 (all groups) Most common AEs[b]: Headache: 0, 10% ($n = 3$), vs. 14% ($n = 4$) Back pain: 8% ($n = 1$), 0, vs. 0 CRP: 8% ($n = 1$), 0, vs. 0 Erythema: 8% ($n = 1$), 0, vs. 0 Pruritus: 8% ($n = 1$), 0, vs. 0 Tooth abscess: 8% ($n = 1$), 0, vs. 0 UTI: 8% ($n = 1$), 0, vs. 0

AE, adverse event; bw, body weight; C, controlled; C1-INH, C1 esterase inhibitor; CRP, C-reactive protein; DB, double-blind; HAE, hereditary angioedema; IMPACT, International Multicenter Prospective Angioedema C1-INH Trial; IV, intravenous; OL, open-label; PBO, placebo; PBO-C, placebo-controlled; pdC1-INH, plasma-derived C1 esterase inhibitor; ph, phase; pts, patients; R, randomized; rhC1-INH, recombinant human C1 esterase inhibitor; SAE, serious adverse event; tx, treatment; UTI, urinary tract infection.

[a]Time from the start of treatment to the onset of symptom relief, as determined by patients' responses to a standard question posed at appropriate time intervals for as long as 24 h after the start of treatment.

[b]In any group, the most common AEs.

[c]One patient was discontinued from the study after receiving treatment for an abdominal attack, after genetic testing did not confirm an HAE diagnosis. The time to complete resolution of HAE symptoms for this patient's attack was 497 h, which was included in the data analysis.

[d]One patient (later determined to not have HAE) had 2 SAEs considered unrelated to treatment.

[e]After treatment, 3 patients were determined to not have had a true HAE attack (pdC1-INH [$n = 1$]; PBO [$n = 2$]).

[f]Patients in US or Canada only.

(49). During that trial, 140 patients underwent complement testing to assess C1-INH antigen levels and functional activity at baseline (immediately prior to treatment) and 1 h after treatment. Baseline functional activity was inversely correlated with HAE attack frequency (i.e., lower baseline C1-INH activity was predictive of an increased attack rate during treatment; $R = 0.2$; $P = 0.01$); however, no significant relationship was observed between postinjection C1-INH function and attack frequency ($R = 0.09$; $P = 0.3$) (49).

In a phase 3, randomized, double-blind, crossover trial ($N = 90$), a significant decrease in the mean frequency of attacks was observed with SC pdC1-INH 40 or 60 IU/kg, administered twice weekly for 16 weeks, compared with placebo (**Table 3**; $P < 0.001$ for both doses vs. placebo) (50). In that trial, 76% and 90% of patients were responders (i.e., ≥50% decrease in the number of attacks vs. placebo) with pdC1-INH 40 and 60 IU/kg, respectively. An open-label extension trial ($N = 126$) supported these results, with >90% of patients in the pdC1-INH 40- and 60-IU/kg treatment groups achieving a ≥50% reduction from baseline in HAE attacks (**Table 3**) (51). Pharmacokinetic modeling indicated that pdC1-INH administered SC exhibits a more consistent exposure and a lower peak-to-trough ratio compared with pdC1-INH given IV, which may account for the improved efficacy of SC pdC1-INH observed in clinical trials (50, 55).

A prospective, observational trial of long-term HAE prophylaxis with IV pdC1-INH (Berinert), which is not currently approved for this indication, reported that more than half of the patients (8/14) experienced a reduction in attack frequency during the last 12 months of treatment (mean duration, 9 years) compared with the attack frequency before initiation of long-term prophylactic therapy (**Table 3**) (52). However, 5 of the 14 patients experienced an increase in attack frequency, even with increased pdC1-INH dosing, and 1 patient discontinued participation in the trial after 5 years because of attack frequency (after receiving pdC1-INH 1,000 U daily for 1.5 years). One hypothesis for the increase in HAE attack rate during the trial was that frequent IV injections may be activating the plasma contact system, either directly or indirectly, leading to an increase in underlying disease activity that is otherwise masked by the effectiveness of pdC1-INH administration (52). Patients in this trial received pdC1-INH at least once weekly for a mean of 9 years (range, 4–19 years). Cycling from increased C1-INH levels immediately after treatment to a decrease in C1-INH levels several days post-treatment may have lowered the threshold for activation of the plasma contact system (52). An observational registry trial ($N = 47$) of long-term HAE prophylaxis with pdC1-INH (Berinert; mean 9.2 months of treatment per patient) showed that more than two-thirds of patients (68.1%) experienced ≥1 attack within 7 days of receiving IV treatment (**Table 3**) (53).

rhC1-INH is also not approved as long-term prophylaxis of HAE (**Table 1**) but proved efficacious for this use in a phase 2, randomized, double-blind, placebo-controlled crossover trial (**Table 3**) (54). When administered once or twice weekly for 4 weeks, rhC1-INH reduced in a statistically significant manner the mean number of attacks, as well as the attack frequency, compared with placebo (54).

TABLE 3 Summary of clinical trials of C1-INH replacement therapy as short-term or long-term prophylaxis for HAE attacks.

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
pdC1-INH (Berinert)	**Short-term prophylaxis**			
	Farkas H, et al. (46) Retrospective analysis of pts with angioedema before HAE dx, and prospective analysis of the same pts after HAE dx receiving STP for medical procedures	STP with pdC1-INH 500 IU IV pre-procedure ($n = 137$) 20 pediatric pts (mean age, 12.2 y; range, 2.2–17.3 y) 117 adults (mean age, 41.3 y; range, 18.5–81.2 y)	HAE attacks decreased with pdC1-INH vs. pre-STP: 9% (5/54) vs. 58.7% (74/126)	No tx-related AEs No discontinuations
	Magerl M, et al. (47) Retrospective analysis of Berinert registry (US and Europe) 2010–2014	STP with pdC1-INH (median dose per infusion, 14.6 [range, 3.6–33.9] IU/kg or 1,000 [range, 500–3,500] IU) 79 patients (mean age, 42.4 y; range, 8–76 y)	Cumulative HAE attack rate after STP (attacks per infusion): Within 1 d: 0.04 (95% CI, 0.015–0.088) Within 2 d: 0.06 (95% CI, 0.028–0.115) Within 3 d: 0.11 (95% CI, 0.061–0.174)	6 AEs reported in 5/79 patients (6.3%), 2 (both headache) of which were considered tx related
	Long-term prophylaxis			
	Bork K and Hardt J (52) Prospective observational study End December 2008 Pts with type I or II HAE ($n = 19$) Mean age at end of study: 51.8 y (range, 35–78)	LTP with pdC1-INH IV ≥ once/wk Mean (SD) weekly dose: Tx onset: 1,253 U (641 U; range, 500–2,300 U) Tx end: 2,564 U (1,835 U; range, 500–7,000 U) Mean (SD) tx duration: 9 y (4.2); range, 4–19 y	8/14 pts had fewer attacks with LTP 12 mo after starting tx vs. pre-LTP 2 pts symptom-free 6 pts had 0.2–3.5 attacks/mo 5/14 pts had increase in attack frequency from beginning to end of study: Mean (SD) attacks/mo: beginning, 1.9 (0.8) vs. end, 9.7 (10.2) 1/14 pts discontinued after 5 y because of attacks	Not reported
	Craig T, et al. (53) MC, observational patient registry (US and Europe) 2010–2014 47 pts w/mean age 44.8 y (range, 13–79 y)	Total LTP duration for 47 pts: 430.2 mo Mean LTP duration: 9.2 mo/pt pdC1-INH IV dosing: Median absolute dose: 1,000 IU (range, 500–3,000 IU)	Pts with ≥1 attack within 7 d of tx: 68.1% (32/47) HAE attack rates among pts receiving pdC1-INH: 5.2 attacks/pt 0.06 attacks/infusion 0.6 attacks/mo Interval between pdC1-INH administration and attack: Mean (SD): 73.7 h (32.5 h) Median (range): 72.0 h (0–166.4 h)	Pts with ≥1 AE: 34.0% (16/47) Tx-related AEs: 3.7% (3/81) Not serious: Noncardiac chest pain ($n = 1$) Postinfusion headache ($n = 1$) SAE: Deep vein thrombosis ($n = 1$) SAEs not related to tx ($n = 3$): GI hemorrhage Severe HAE attack UTI No AE-related discontinuations
pdC1-INH (Cinryze)	**Long-term prophylaxis**			
	Zuraw BL, et al. (40) DB, PBO-C, crossover Pts ≥6 y with ≥2 HAE attacks/mo (moderate or severe attacks affecting abdomen, face, or external genitalia)	Two consecutive 12-wk tx periods pdC1-INH 1,000 U/10 ml ($n = 11$) or PBO ($n = 11$) administered IV q3–4 d	Mean number of HAE attacks during tx period (pdC1-INH vs. PBO): 6.3 vs. 12.7 (i.e., ~2 vs. 4/mo) Mean difference in attack rates vs. PBO: 6.5 (95% CI, 4.2–8.7; $P < 0.001$) Mean (SD) duration of attacks (pdC1-INH vs. PBO): 2.1 (1.1) d vs. 3.4 (1.4); $P = 0.002$	AEs possibly related to pdC1-INH: Fever: 8.3% (1/12) Lightheadedness: 8.3% (1/12) Definitely related: Pruritus and rash: 8.3% (1/12)
	Zuraw BL and Kalfus I (49) OL, single arm Pts ≥1 y with ≥1 HAE attack/month, or any laryngeal attack Mean age: 36.5 y (range 3–82)	pdC1-INH 1,000 U administered IV q3–7 d at study site ($n = 146$) LTP duration up to 2.6 y	Number of HAE attacks/mo at baseline vs. during the study: Mean (SD): 4.7 (5.2) vs. 0.5 (0.8) 90% decrease from baseline Median (IQR): 3 (2–4) vs. 0.19 (0.0–0.64) 93.7% decrease from baseline	No AE-related study discontinuations 2 deaths not related to study drug (pulmonary arterial embolization of foreign material from IV injection of oral medication [$n = 1$]; HCC [$n = 1$]) 99/101 SAEs not related to pdC1-INH (2 of unknown relationship: musculoskeletal chest pain, major depression)

(*continued*)

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TABLE 3 Continued

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
pdC1-INH (Haegarda)	**Long-term prophylaxis**			
	Longhurst H, et al. (50) COMPACT trial R, DB, PBO-C, crossover, ph 3 Pts ≥12 y with type I or II HAE w/ ≥4 attacks in 2-mo period ≤3 mo before screening Number of HAE attacks in 3 mo before screening, mean (SD): pdC1-INH 40 U/kg or 60 U/kg: 10.8 (6.7) or 8.8 (6.4) (i.e., ~3 to 4/mo)	Two 16 wk tx periods: pdC1-INH 40 IU/kg bw SC ($n = 45$), or pdC1-INH 60 IU/kg bw SC ($n = 45$), followed by PBO, or vice versa, administered twice weekly	Number of HAE attacks per month: pdC1-INH 40 IU/kg ($n = 43$) vs. PBO ($n = 44$): 1.2 vs. 3.6 ($P < 0.001$; within-pt difference, −2.4) Mean/median decrease in attacks vs. PBO: 55%/89% pdC1-INH 60 IU/kg ($n = 43$) vs. PBO ($n = 42$): 0.5 vs. 4.0 ($P < 0.001$; within-pt difference, −3.5) Mean/median decrease in attacks vs. PBO: 84%/95% Pts with response (≥50% decrease in number of attacks with pdC1-INH 40 IU/kg or 60 IU/kg, vs. PBO): 76% or 90% Use of rescue medication/mo: pdC1-INH 40 IU/kg vs. PBO: 1.1 vs. 5.6 ($P = 0.02$; within-pt difference, −4.4) Mean/median decrease in use of rescue medication vs. PBO: 70%/89% pdC1-INH 60 IU/kg vs. PBO: 0.3 vs. 3.9 ($P < 0.001$; within-pt difference, −3.6) Mean/median decrease in use of rescue medication vs. PBO: 89%/100%	pdC1-INH 40 IU/kg ($n = 43$) pdC1-INH 60 IU/kg ($n = 43$) PBO ($n = 86$) AEs with onset ≤24 h of administration Any AE: pdC1-INH 40 IU, 67% ($n = 29$); pdC1-INH 60 IU/kg, 70% ($n = 30$), vs. PBO 66% ($n = 57$) Any tx-related AE: 33% ($n = 14$), 35% ($n = 15$), vs. 26% ($n = 22$) Any SAE: 2% ($n = 1$), 0, vs. 2% ($n = 2$) Any tx-related SAE: 0, 0, vs. 1% ($n = 1$) AEs leading to discontinuation: 0, 5% ($n = 2$), vs. 1% ($n = 1$) Nonserious AEs ≥5% of pts: Injection-site reaction: 28% ($n = 12$), 35% ($n = 15$), vs. 24% ($n = 21$) Dizziness: 9% ($n = 4$), 0, vs. 1% ($n = 1$) Upper respiratory tract infection: 7% ($n = 3$), 7% ($n = 3$), vs. 7% ($n = 6$) Hypersensitivity: 5% ($n = 2$), 7% ($n = 3$), vs. 1% ($n = 1$) Nasopharyngitis: 2% ($n = 1$), 19% ($n = 8$), vs. 7% ($n = 6$) Fatigue: 2% ($n = 1$), 2% ($n = 1$), vs. 7% ($n = 6$) Back pain: 2% ($n = 1$), 2% ($n = 1$), vs. 6% ($n = 5$)
	Craig T, et al. (51) OL extension of COMPACT trial R, OL, ph 3 December 2014–December 2017 Pts ≥6 y with type I or II HAE w/ ≥4 attacks in 2-mo period before enrollment into COMPACT trial or treatment-naïve pts Number of HAE attacks in 3 mo before screening, mean (SD): pdC1-INH 40 IU/kg: 12.8 (8.4) pdC1-INH 60 IU/kg: 12.7 (10.2) Overall, mean of 4.3 attacks/mo in the 3 mo before study entry	pdC1-INH 40 IU/kg bw SC ($n = 63$) pdC1-INH 60 IU/kg bw SC ($n = 63$)	745 HAE attacks (observation period of up to 2.7 y) Number of HAE attacks/mo (mean [SD]): pdC1-INH 40 IU/kg: 0.4 (0.7) pdC1-INH 60 IU/kg: 0.5 (0.9) Number of HAE attacks/y (median [range]): pdC1-INH 40 IU/kg: 1.3 (0.0–40.6) pdC1-INH 60 IU/kg: 1.0 (0.0–48.0) Time-normalized reduction in HAE attack rate from baseline (mean [SD]): pdC1-INH 40 IU/kg: −6.8 (−9.8 to −3.8) pdC1-INH 60 IU/kg: −6.8 (−10.9 to −2.7) Pts with response (≥50% decrease in number of attacks with pdC1-INH 40 IU/kg or 60 IU/kg) 94% or 92%	pdC1-INH 40 IU/kg ($n = 63$) pdC1-INH 60 IU/kg ($n = 70$)[a] Any AE: pdC1-INH 40 IU/kg, 89% ($n = 56$); pdC1-INH 60 IU/kg, 83% ($n = 58$) Events (events/patient-year): pdC1-INH 40 IU/kg, 948 (11.3); pdC1-INH 60 IU/kg, 849 (8.5) Any SAE: pdC1-INH 40 IU/kg, 6% ($n = 4$); pdC1-INH 60 IU/kg, 7% ($n = 5$) Any tx-related SAE: 0 (both groups) AEs leading to discontinuation: pdC1-INH 40 IU/kg, 0; pdC1-INH 60 IU/kg, 1% ($n = 1$) Most common AEs (pdC1-INH 40 IU/kg, pdC1-INH 60 IU/kg) Nasopharyngitis: 19% ($n = 12$), 30% ($n = 21$) Injection-site pain: 27% ($n = 17$), 14% ($n = 10$) Injection-site erythema: 16% ($n = 10$), 17% ($n = 12$) Headache: 16% ($n = 10$), 14% ($n = 10$) Injection-site bruising: 14% ($n = 9$), 10% ($n = 7$) UTI: 13% ($n = 8$), 11% ($n = 8$)

(*continued*)

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TABLE 3 Continued

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
rhC1-INH (Ruconest)	**Short-term prophylaxis** Valerieva A, et al. (48) Retrospective study Pts with HAE (51 pts received rhC1-INH) Median age 44 y (range, 17–73) Procedures (e.g., medical/dental) with rhC1-INH prophylaxis ($n = 70$) vs. procedures with no prophylaxis ($n = 26$)	Median rhC1-INH IV dose: 3,075 IU (range, 2,100–4,200 IU) Administered median 60 min pre-procedure	rhC1-INH vs. no rhC1-INH Attack-free procedures 2 d post-procedure: 97.1% vs. 23.1% 7 d post-procedure: 88.6% vs. 19.2%	No AEs in the 70 treated procedures
	Long-term prophylaxis Riedl MA, et al. (54) R, DB, PBO-C, crossover, ph 2 Pts ≥13 y with HAE w/ ≥4 attacks/mo for 3 consecutive mo before study initiation ($n = 32$) Mean (SD) attacks ≤3 mo: 17.9 (7.2) Median (range) attacks ≤3 mo: 14.5 (12–33)	Three 4-wk tx periods, with 1-wk washout before crossover: rhC1-INH 50 IU/kg bw (pts <84 kg) or 4,200 IU (pts ≥84 kg) once or twice weekly IV, vs. PBO	Mean (SD) number of HAE attacks in each 4-wk tx period ($n = 31$): Once weekly, 4.4 (3.2) Twice weekly: 2.7 (2.4) PBO: 7.2 (3.6) Mean differences vs. PBO: −2.8 ($P = 0.0004$) and −4.4 ($P < 0.0001$), respectively Mean decrease in attack frequency (once weekly, twice weekly vs. PBO) ($n = 31$): 34.9% and 63.3% Pts with clinical response (≥50% decrease in number of attacks with rhC1-INH vs. PBO): Once weekly tx: 13/31 (42%) Twice weekly tx: 23/31 (74%)	rhC1-INH once weekly ($n = 29$) rhC1-INH twice weekly ($n = 29$) PBO ($n = 28$) Any AEs: once weekly, 45% ($n = 13$); twice weekly, 34% ($n = 10$) vs. PBO 29% ($n = 8$) Tx-related AEs: 0, 7% ($n = 2$), vs. 0 SAEs: 0, 3% ($n = 1$), vs. 0 AEs occurring in ≥5% pts: Nasopharyngitis: 10% ($n = 3$), 0, vs. 7% ($n = 2$) Headache: 7% ($n = 2$), 17% ($n = 5$), vs. 0 Anxiety: 7% ($n = 2$), 0, vs. 0

AE, adverse event; bw, body weight; C1-INH, C1 esterase inhibitor; COMPACT, Optimal Management of Preventing Angioedema with Low-Volume Subcutaneous C1-Inhibitor Replacement Therapy; DB, double-blind; dx, diagnosis; GI, gastrointestinal; HAE, hereditary angioedema; HAE-nC1, normal hereditary angioedema; HCC, hepatocellular carcinoma; IQR, interquartile range; IV, intravenous; LTP, long-term prophylaxis; MC, multicenter; OL, open-label; PBO, placebo; PBO-C, placebo-controlled; pdC1-INH, plasma-derived C1 esterase inhibitor; ph, phase; pts, patients; R, randomized; rhC1-INH, recombinant human C1 esterase inhibitor; SAE, serious adverse event; SC, subcutaneous; STP, short-term prophylaxis; tx, treatment; UTI, urinary tract infection.

[a]Included 7 patients whose treatment was uptitrated from 40 IU/kg to 60 IU/kg and were included in both treatment arms in the safety population.

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Single pathway therapies

Single pathway therapies that are currently available for the treatment of HAE include the SC administered B2 receptor antagonist icatibant (Firazyr; Takeda Pharmaceuticals America, Inc.; Lexington, MA), the SC administered plasma kallikrein inhibitors ecallantide (Kalbitor; Dyax Corp., a Takeda company; Lexington, MA) for attacks or lanadelumab (Takhzyro; Dyax Corp; Lexington, MA) for prophylaxis, as well as the oral plasma kallikrein inhibitor berotralstat (Orladeyo; BioCryst Pharmaceuticals, Inc.; Durham, NC; **Table 1**), also for prophylaxis. By blocking the effects of bradykinin at the B2 receptor (**Figure 2**), icatibant has been shown to reverse the vascular permeability observed in C1-INH gene knockout mice (56) and to decrease bradykinin-induced vasodilation in the forearms of healthy volunteers (57). Treatment with icatibant also decreased bradykinin levels in patients with HAE, but changes in plasma bradykinin levels were not directly related to the degree of symptom relief (58). Working upstream from icatibant, plasma kallikrein inhibitors block the binding site of kallikrein to prevent cleavage of HMW kininogen and subsequent bradykinin release and also reduce further activation of factor XIIa to disrupt the positive feedback loop that would otherwise lead to increased kallikrein production (**Figure 2**) (16, 33, 59, 60).

On-demand therapy for HAE attacks

In addition to IV C1-INH replacement therapy, WAO/EAACI guidelines recommend IV ecallantide or icatibant as on-demand treatment of HAE attacks (13). Both agents are approved in the United States for this indication (**Table 1**), while icatibant is approved in the European Union (61) and Japan (62).

In two phase 3 randomized, double-blind trials (For Angioedema Subcutaneous Treatment [FAST]-1 [$N = 56$] and FAST-2 [$N = 74$]), icatibant 30 mg SC was administered for the treatment of acute HAE attacks. Icatibant reduced the median time to clinically significant relief of the patient's index symptom compared with oral tranexamic acid 3 g daily for 2 days (FAST-2; 2.0 vs. 12.0 h, respectively; $P < 0.001$) and also reduced the median time compared with placebo, although the difference was not statistically significant (FAST-1; 2.5 vs. 4.6 h; $P = 0.1$; **Table 4**) (63–70). Furthermore, in both trials, icatibant significantly reduced the median time to initial symptom improvement by both patient assessment and investigator assessment. In a third phase 3 trial, icatibant 30 mg SC ($n = 43$) was significantly more efficacious than placebo ($n = 45$) for reducing the median time to onset of symptom relief for nonlaryngeal attacks (2.0 h vs. 19.8 h, respectively; $P < 0.001$) (65).

In a phase 3, randomized, double-blind trial ($N = 72$), on-demand treatment with ecallantide 30 mg SC significantly improved the median treatment outcome score (patient-reported composite comprising sites of symptoms, symptom severity, and response to treatment) compared with placebo (**Table 4**; $P = 0.004$) (66). Further, a greater percentage of patients who received ecallantide experienced significant improvement ("a lot better or resolved") in overall response vs. placebo (50% vs. 33%, respectively) (66). Results of another phase 3, randomized, double-blind trial ($N = 96$) noted a significant improvement in treatment outcome score 4 h after dosing with ecallantide compared with placebo ($P = 0.003$) and an overall response maintained through 24 h ($P = 0.02$; **Table 4**) (67). Although anaphylaxis was not reported during these 2 trials (66, 67), a retrospective database analysis

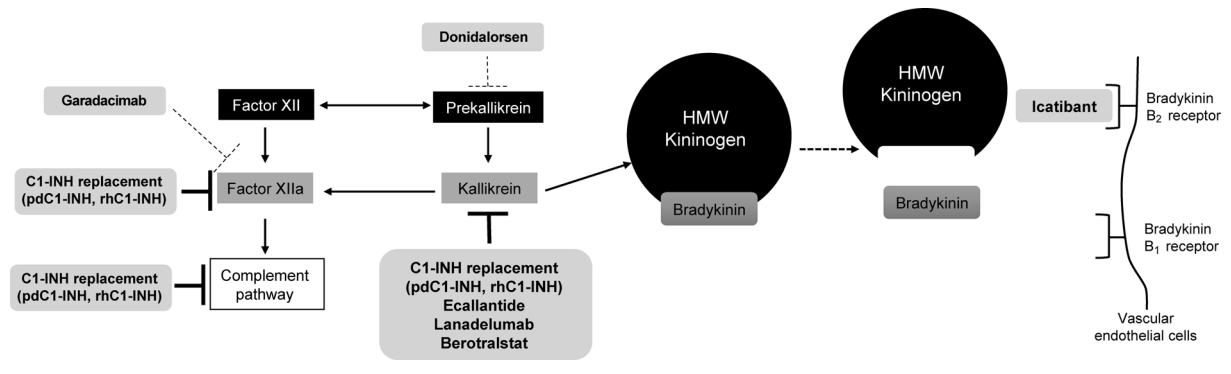

FIGURE 2
Therapeutic targeting of pathways in HAE. Inhibitors of the pathway are shown in light gray shaded boxes at their respective sites of action. Investigational therapies are shown with dashed lines at their target sites. *C1-INH, C1 esterase inhibitor; HMW, high molecular weight; pdC1-INH, plasma-derived C1-INH; rhC1-INH, recombinant human C1-INH. Figure created with data from Cicardi M, et al. J Allergy Clin Immunol Pract. (2018) 6(4):1132–41; Zuraw BL. N Engl J Med. (2008) 359(10):1027–36; and Fijen LM, et al. Clin Rev Allergy Immunol. (2021) 61(1):66–7* (5, 14, 16).

TABLE 4 Summary of clinical trials of bradykinin B_2 receptor antagonists and kallikrein inhibitors as on-demand or prophylactic treatment of HAE attacks.

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
On-Demand Treatment				
Bradykinin B_2 Receptor Antagonist				
Icatibant (Firazyr)	Cicardi M, et al. (64) FAST-1 and FAST-2 DB, R, C, ph 3 Pts ≥18 y with type I or II HAE (abdominal or cutaneous attacks)	FAST-1: Icatibant 30 mg SC ($n = 27$) PBO ($n = 29$) FAST-2: Icatibant 30 mg SC ($n = 36$) Oral tranexamic acid 3 g/d for 2 d ($n = 38$)	FAST-1: icatibant vs. PBO FAST-2: icatibant vs. tranexamic acid Median (IQR) time to clinically significant relief of index symptom: FAST-1: 2.5 h (1.1–6.0) vs. 4.6 h (1.8–10.2; $P = 0.1$) FAST-2: 2.0 h (1.0–3.5) vs. 12.0 h (3.5–25.4; $P < 0.001$) Pts (95% CI) with clinically significant relief of index symptom 4 h after start of tx: FAST-1: 67% (46–84) vs. 46% (28–66; $P = 0.2$) FAST-2: 80% (63–92) vs. 31% (16–48; $P < 0.001$) Median (IQR) time to first symptom improvement (pt assessed) FAST-1: 0.8 h (0.5–2.0) vs. 16.9 h (3.2–NA; $P < 0.001$) FAST-2: 0.8 h (0.4–1.4) vs. 7.9 h (1.1–NA; $P < 0.001$) Median (IQR) time to first symptom improvement (investigator assessed) FAST-1: 1.0 h (0.8–2.0) vs. 5.7 h (2.0–11.2; $P < 0.001$) FAST-2: 1.5 h (0.7–3.0) vs. 6.9 h (4.0–13.8; $P < 0.001$)	FAST-1: icatibant ($n = 27$) vs. PBO ($n = 29$) FAST-2: icatibant ($n = 36$) vs. tranexamic acid ($n = 38$) Any AEs: FAST-1: 44% ($n = 12$) vs. 66% ($n = 19$) FAST-2: 53% ($n = 19$) vs. 42% ($n = 16$) Tx-related AEs: FAST-1: 15% ($n = 4$) vs. 3% ($n = 1$) FAST-2: 14% ($n = 5$) vs. 11% ($n = 4$) SAEs: FAST-1: 0 vs. 0 FAST-2: 11% ($n = 4$) vs. 3% ($n = 1$) Injection-site reaction: FAST-1: 96% ($n = 26$) vs. 28% ($n = 8$) FAST-2: 97% ($n = 35$) vs. 26% ($n = 10$)
	Lumry WR, et al. (65) FAST-3 MC, R, DB, PBO-C, ph 3 Pts ≥18 y with type I or II HAE (abdominal, cutaneous, or laryngeal attacks)	Icatibant 30 mg SC ($n = 46$) vs. PBO ($n = 47$) 3/46 icatibant and 2/47 PBO pts had laryngeal attacks Tx administered ≤6 h of attack	Median (95% CI) time to onset of symptom relief (icatibant vs. PBO): Nonlaryngeal attacks[a]: 2.0 h (1.5–3.0) vs. 19.8 h (6.1–26.3; $P < 0.001$) Laryngeal attacks[b]: 2.5 h (1.3–3.0) vs. 3.2 h (1.0–5.4) Median (95% CI) time to first symptom improvement (pt-assessed) Nonlaryngeal attacks: 0.8 h (0.5–1.0) vs. 3.5 h (1.9–5.4; $P < 0.001$) Laryngeal attacks: 1.0 h (0.6–2.5) vs. 2.1 h (0.5–3.9) Median (95% CI) time to first symptom improvement	Icatibant 30 mg SC ($n = 46$) PBO ($n = 46$) ≥1 AE: 41.3% ($n = 19$) vs. 52.2% ($n = 24$) ≥1 drug-related AE: 10.9% ($n = 5$) vs. 6.5% ($n = 3$) ≥1 SAE: 0 vs. 6.5% ($n = 3$) ≥1 tx-related SAE: 0 vs. 0 AE-related deaths: 0 vs. 2.2%[c] ($n = 1$)

(*continued*)

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TABLE 4 Continued

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
			investigator-assessed) Nonlaryngeal attacks: 0.8 h (0.6–1.3) vs. 3.4 h (2.6–6.0; $P < 0.001$) Laryngeal attacks: 1.0 h (0.6–2.5) vs. 2.2 h (0.5–3.9)	
Kallikrein Inhibitor				
Ecallantide (Kalbitor)	Cicardi M, et al. (66) EDEMA3 trial 8 December 2005–10 February 2007 MC, R, DB, PBO-C, ph3 Pts ≥10 y with HAE (GI, laryngeal, peripheral attacks)	Ecallantide 30 mg SC ($n = 36$) PBO ($n = 36$) Pts observed for ≥4 h after tx	TOS[d] at 4 h post-tx (ecallantide vs. PBO): Mean (SD): 46.8 (59.3) vs. 21.3 (69.0) Median (range): 50.0 (−100.0 to 100.0) vs. 0 (−100.0 to 100.0; $P = 0.004$) Pts with significant improvement in overall response ≤4 h: 50% ($n = 18$) vs. 33% ($n = 12$)	Ecallantide 30 mg SC ($n = 36$) PBO ($n = 36$) ≥1 AE: 56% ($n = 20$) vs. 33% ($n = 12$) ≥1 tx-related AE: 11% ($n = 4$) vs. 14% ($n = 5$) SAEs: 8% ($n = 3$) vs. 6% ($n = 2$) Most common AEs: Headache: 11% ($n = 4$) vs. 6% ($n = 2$) Diarrhea: 8% ($n = 3$) vs. 0 Pyrexia: 8% ($n = 3$) vs. 0 Nasopharyngitis: 6% ($n = 2$) vs. 3% ($n = 1$) Tachycardia, not otherwise specified: 6% ($n = 2$) vs. 3% ($n = 1$) Nasal congestion: 6% ($n = 2$) vs. 0 No AE-related mortality or study discontinuations
	Levy RJ, et al. (67) EDEMA4 trial DB, R, PBO-C, ph 3 Pts ≥10 y with type I or II HAE (any attack location)	Ecallantide 30 mg SC ($n = 48$) PBO ($n = 48$) Pts observed for ≥4 h after tx	TOS[d] at 4 h post-tx (ecallantide vs. PBO): Mean (SD): 53.4 (49.7) vs. 8.1 (63.2) Median (range): 50.0 (−66.7 to 100) vs. 0 (−100.0 to 100.0; $P = 0.003$) Pts maintaining significant improvement in overall response through 24 h: 44% ($n = 21$) vs. 21% ($n = 10$; $P = 0.02$)	Ecallantide 30 mg SC ($n = 48$) PBO ($n = 48$) ≥1 AE: 17% ($n = 8$) vs. 40% ($n = 19$) Most common AEs: Nausea: 6% ($n = 3$) vs. 2% ($n = 1$) Headache: 4% ($n = 2$) vs. 10% ($n = 5$) Dizziness: 4% ($n = 2$) vs. 2% ($n = 1$) Abdominal pain: 2% ($n = 1$) vs. 4% ($n = 2$)
Prophylactic Treatments				
Kallikrein Inhibitors				
Lanadelumab (Takhzyro)	Banerji A, et al. (68) HELP trial MC, R, DB, *P*, PBO-C, ph 3 Pts ≥12 y with type I or II HAE with ≥1 attack/q4 wk	Lanadelumab (26 wk tx) 150 mg SC q4 wk ($n = 28$) 300 mg SC q4 wk ($n = 29$) 300 mg SC q2 wk ($n = 27$) PBO ($n = 41$) Run-in period attack rate (mean attacks/mo [SD]): Lanadelumab 150 mg q4 wk: 3.2 (1.8) 300 mg q4 wk: 3.7 (2.5) 300 mg q2 wk: 3.5 (2.3) PBO: 4.0 (3.3)	Mean (95% CI) number of attacks/mo Lanadelumab 150 mg q4 wk: 0.5 (0.3–0.7) 300 mg q4 wk: 0.5 (0.4–0.8) 300 mg q2 wk: 0.3 (0.1–0.5) PBO: 2.0 (1.6–2.4) Mean difference (95% CI) in number of attacks/mo vs. PBO 150 mg q4 wk: −1.5 (−1.9 to −1.1; $P < 0.001$) 300 mg q4 wk: −1.4 (−1.8 to −1.0; $P < 0.001$)	Lanadelumab 150 mg q4 wk ($n = 28$) 300 mg q4 wk ($n = 29$) 300 mg q2 wk ($n = 27$) PBO ($n = 41$) Any AE: 150 mg q4 wk, 89.3% ($n = 25$); 300 mg q4 wk, 86.2% ($n = 25$); 300 mg q2 wk, 96.3% ($n = 26$), vs. PBO 75.6% ($n = 31$) Any tx-related AE: 60.7% ($n = 17$), 48.3% ($n = 14$), 70.4% ($n = 19$), vs. 34.1% ($n = 14$) Injection site pain: 42.9% ($n = 12$), 31.0% ($n = 9$), 51.9% ($n = 14$), vs. 26.8% ($n = 11$) Any SAE: 0, 10.3% ($n = 3$), 3.7% ($n = 1$), vs. 0

(*continued*)

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TABLE 4 Continued

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
			300 mg q2 wk: −1.7 (−2.1 to −1.3; $P < 0.001$)	Any AE leading to discontinuation: 0, 3.4% ($n = 1$), 0, vs. 2.4% ($n = 1$)
	Banerji A, et al. (69) OL extension trial Pts ≥12 y with type I or II HAE with ≥1 attack/q4 wk Planned treatment duration: 924 d	Rollovers from HELP trial ($n = 109$): Lanadelumab 300 mg (Day 0; last visit HELP trial); treatment paused until first HAE attack (dose-and-wait stage), at which point patients received second dose lanadelumab 300 mg and continued lanadelumab 300 mg q2 wk (regular dosing stage) Non-rollovers ($n = 103$): lanadelumab 300 mg q2 wk Mean (SD) lanadelumab exposure: 29.6 (8.2) months; 92.5% of patients completed ≥12 mo	Reduction in attack rate Overall: 87.4% Rollovers (starting from regular dosing stage/second dose, not including first HAE attack): 92.4% Non-rollovers (starting from Day 0): 82.0% Attack-free (overall): Mean (SD), % days: 97.7% (6.0%) Mean, (SD), duration: 14.8 mo (415.0 d) ≥6 mo: 81.8% ≥12 mo: 68.9%	Total ($N = 212$) Any AE: 97.2% ($n = 206$) Treatment-related AE: 54.7% ($n = 116$) Any SAE: 9.9% ($n = 21$) Any treatment-related SAE: 0 Any severe AE: 17.9% ($n = 38$) Any investigator-reported AESIs, including hypersensitivity reactions and disordered coagulation (hypercoagulability events and bleeding events): 6.1% ($n = 13$) Any hospitalizations due to AEs: 9.9% ($n = 21$) Any AE leading to discontinuation: 2.8% ($n = 6$) Most common AEs: Injection site pain: 47.2% ($n = 100$) Viral URTI: 42.0% ($n = 89$) Upper respiratory tract infection: 25.9% ($n = 55$) Headache: 24.5% ($n = 52$) Injection site erythema: 17.0% ($n = 36$) Arthralgia: 12.7% ($n = 27$)
Berotralstat (Orladeyo)	Zuraw B, et al. (63) APeX-2 trial, part 1 14 March 2018–10 April 2019 (first 24 weeks of treatment) MC, R, DB, *P*, PBO-C, ph 3 Pts aged ≥12 y (US and Canada) or ≥18 y (Europe) with type I or II HAE with ≥2 HAE attacks requiring treatment or causing functional impairment in first 56 d of run-in period	Berotralstat orally once daily for 24 wk 110 mg ($n = 41$) 150 mg ($n = 40$) PBO ($n = 40$, 39 dosed) Mean (SD) baseline HAE attacks/28-d period over screening period of up to 70 d (investigator confirmed) Berotralstat 110 mg: 2.97 (1.36) 150 mg: 3.06 (1.56) PBO: 2.91 (1.12)	Mean number of investigator-confirmed attacks/mo over 24 wk Berotralstat 110 mg: 1.65 150 mg: 1.31 PBO: 2.35 Attack rate ratio (95% CI) relative to PBO Berotralstat 110 mg: 0.70 (0.51–0.95), $P = 0.024$ 150 mg: 0.56 (0.41–0.77), $P < 0.001$	Berotralstat 110 mg ($n = 41$) Berotralstat 150 mg ($n = 40$) PBO ($n = 39$) Any AE: 83% ($n = 34$)/85% ($n = 34$)/77% ($n = 30$) Any SAE: 2% ($n = 1$)/0/8% ($n = 3$) Tx-related AEs: 0 (all groups) AE leading to discontinuation: 7% ($n = 3$)/3% ($n = 1$)/3% ($n = 1$) Most common AEs (berotralstat 110 mg/berotralstat 150 mg/PBO): URTI: 32% ($n = 13$)/30% ($n = 12$)/28% ($n = 11$) Nausea: 15% ($n = 6$)/15% ($n = 6$)/18% ($n = 7$) Abdominal pain: 10% ($n = 4$)/23% ($n = 9$)/10% ($n = 4$) Vomiting: 10% ($n = 4$)/15% ($n = 6$)/3% ($n = 1$) Diarrhea: 10% ($n = 4$)/15% ($n = 6$)/0 Headache: 7% ($n = 3$)/10% ($n = 4$)/5% ($n = 2$) Back pain: 2% ($n = 1$)/10% ($n = 4$)/3% ($n = 1$)
	Wedner HJ, et al. (70) APeX-2 trial, part 2 14 March 2018–25 September 2019 (first 48 wk of treatment) MC, R, DB, *P*, ph3	Pts taking berotralstat in part 1 continued same dose; pts taking PBO re-randomized to berotralstat 110 or 150 mg once daily (all blinded) Berotralstat once daily for 48 wk 110 mg ($n = 37$)	Mean (SEM) number of investigator-confirmed attacks/month at 48 wk: Berotralstat 110 mg: 1.35 (0.33) Berotralstat 150 mg: 1.06 (0.25)	Berotralstat 110 mg ($n = 41$) Berotralstat 150 mg ($n = 40$) Results up to wk 48: Any AE: 92.7% ($n = 38$)/95.0% ($n = 38$) Any SAE: 2.4% ($n = 1$)/2.5% ($n = 1$)

(*continued*)

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TABLE 4 Continued

Therapy	Study and inclusion criteria	Treatments	Efficacy outcome(s)	Safety
	Pts aged ≥12 y (US and Canada) or ≥18 y (Europe) with type I or II HAE with ≥2 HAE attacks requiring treatment or causing functional impairment in first 56 d of run-in period	150 mg ($n = 37$) PBO for 24 weeks, then berotralstat for 24 wk 110 mg ($n = 17$) 150 mg ($n = 17$) Mean (SE) baseline attack rate (attacks/mo): Berotralstat 110 mg: 2.97 (0.21) Berotralstat 150 mg: 3.06 (0.25) Mean (SE) attack rate at week 24 (before berotralstat) PBO → berotralstat 110 mg: 2.39 (0.41) PBO → berotralstat 150 mg: 2.56 (0.61)	PBO → berotralstat 110 mg: 1.25 (0.32) PBO → berotralstat 150 mg: 0.57 (0.23)	Tx-related SAE: 0 (both groups) AE-related discontinuation: 9.8% ($n = 4$)/7.5% ($n = 3$) Investigator-identified rash 0/5.0% ($n = 2$) Most common AEs (berotralstat 110 mg/ berotralstat 150 mg): URTI: 36.6% ($n = 15$)/52.5% ($n = 21$) Nausea: 19.5% ($n = 8$)/20.0% ($n = 8$) Abdominal pain: 9.8% ($n = 4$)/30.0% ($n = 12$) Dyspepsia: 9.8% ($n = 4$)/12.5% ($n = 5$) Diarrhea: 12.2% ($n = 5$)/17.5% ($n = 7$) Vomiting: 9.8% ($n = 4$)/15.0% ($n = 6$) Headache: 9.8% ($n = 4$)/12.5% ($n = 5$) Flatulence: 7.3% ($n = 3$)/7.5% ($n = 3$) Back pain: 4.9% ($n = 2$)/12.5% ($n = 5$) Gastroesophageal reflux disease: 9.8% ($n = 4$)/5.0% ($n = 2$)

AE, adverse event; AESI, adverse event of special interest; APeX-2, Angioedema Prophylaxis 2; C, controlled; DB, double-blind; EDEMA, Evaluation of DX88's Effects in Mitigating Angioedema; FAST, For Angioedema Subcutaneous Treatment; GI, gastrointestinal; HAE, hereditary angioedema; HELP, Hereditary Angioedema Long-term Prophylaxis; IQR, interquartile range; MC, multicenter; NA, not available; *P*, parallel group; PBO, placebo; PBO-C, placebo-controlled; ph, phase; pts, patients; R, randomized; SAE, serious adverse event; SC, subcutaneous; TOS, treatment outcome score; tx, treatment; URTI, upper respiratory tract infection; VAS, visual analog scale.

[a]Symptom relief defined as 50% decrease from pretreatment in VAS-3 score; VAS-3 is a 3-symptom composite visual analog scale score for cutaneous and/or abdominal attacks that includes scores for skin swelling, skin pain, and abdominal pain.

[b]Symptom relief defined as 50% decrease from pretreatment in VAS-5 score; VAS-5 is a 5-symptom composite visual analog scale score for laryngeal attacks that includes scores for skin swelling, skin pain, abdominal pain, difficulty swallowing, and voice change.

[c]Myocardial infarction.

[d]Score is a composite of patient-reported outcomes related to site(s) of symptoms, baseline symptom severity, and treatment response. Scores range from +100 (significant improvement in symptoms) to -100 (significant worsening of symptoms).

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of ecallantide clinical trials identified 8 of 230 patients (3.5%) with hypersensitivity reactions consistent with anaphylaxis (71). All of these events occurred within 1 h of ecallantide administration, and no cases of anaphylaxis occurred after the first exposure (71). Accordingly, the US ecallantide prescribing information contains a boxed warning regarding the potential for anaphylaxis and states that only health care providers should administer the medication (33).

Long-term prophylaxis

The plasma kallikrein inhibitors lanadelumab and berotralstat are both recommended by WAO/EAACI guidelines as first-line options for long-term prophylaxis (13) and are approved in the United States, European Union, and Japan for this indication (**Table 1**). A phase 3 trial ($N = 125$) demonstrated that the 3 different lanadelumab SC dosing regimens examined (150 mg every 4 weeks, 300 mg every 4 weeks, or 300 mg every 2 weeks) all significantly reduced the mean number of attacks experienced by patients each month compared with placebo ($P < 0.001$ for all comparisons) over the 26-week treatment period (**Table 4**) (68). Patients who completed the trial ($n = 109$) or new patients ($n = 103$) were eligible for an open-label extension trial of lanadelumab 300 mg SC every 2 weeks (**Table 4**) (69). Long-term treatment with lanadelumab reduced the mean HAE attack rate by 87.4% overall. During treatment, patients were attack-free for 97.7% of days on average, and the mean duration of the attack-free period was >14 months. More than 80% of patients remained attack-free for ≥6 months and 69% were attack-free for ≥12 months.

In part 1 (a 24-week, placebo-controlled phase) of a phase 3, randomized, double-blind trial ($N = 121$), berotralstat 110 and 150 mg once daily significantly reduced the mean number of HAE attacks per month compared with placebo ($P = 0.024$ and $P < 0.001$, respectively; **Table 4**) (63). The attack rate ratio relative to placebo (95% CI) was 0.70 (0.51–0.95) for berotralstat 110 mg and 0.56 (0.41–0.77) for berotralstat 150 mg. Both doses of berotralstat significantly reduced the rate of HAE attacks in patients with ≥2 attacks per month at baseline (110 mg, $P = 0.04$; 150 mg, $P = 0.005$), but only berotralstat 150 mg significantly reduced the HAE attack rate among patients with <2 attacks per month at baseline ($P = 0.009$). In part 2 of this trial, berotralstat-treated patients in part 1 continued their assigned double-blind dose, and placebo-treated patients were re-randomized to double-blind berotralstat 110 mg or 150 mg once daily (**Table 4**) (70). Among patients who continued on berotralstat, the mean (SE) monthly attack rates declined from 2.97 (0.21) at baseline to 1.35 (0.33) at week 48 in the berotralstat 110-mg group and from 3.06 (0.25) at baseline to 1.06 (0.25) at week 48 in the berotralstat 150-mg group. For placebo-treated patients who switched to berotralstat, the decrease in HAE attack rates was similar to that observed in part 1 of the trial in the berotralstat treatment groups (**Table 4**) (70).

Investigational treatments

Garadacimab (CSL312) is a human recombinant monoclonal antibody that inhibits factor XIIa and thus acts on the complement pathway in addition to the kallikrein-kinin pathway (**Figure 2**) (72). It has been shown to potently inhibit bradykinin formation in plasma samples from patients with HAE and to reduce edema in animal models (72). Data have been published from a phase 2, randomized, double-blind trial (73). Patients were randomly assigned to receive an IV loading dose (placebo or garadacimab 40, 100, or 300 mg) followed by SC administration (placebo [$n = 8$], garadacimab 75 mg [$n = 9$], garadacimab 200 mg [$n = 8$], or garadacimab 600 mg [$n = 7$]) on day 6 and then every 4 weeks for 12 weeks. During the 12-week SC treatment period, the median number (interquartile range) of monthly attacks was 4.6 (3.1–5.0) in the placebo group, 0.0 (0.0–0.4) in the garadacimab 75-mg group, 0.0 (0.0–0.0) in the garadacimab 200-mg group, and 0.3 (0.0–0.7) in the garadacimab 600-mg group. Compared with placebo, garadacimab 75 mg, 200 mg, and 600 mg significantly reduced the median attack rate by 100% ($P = 0.0002$, *post hoc* analysis), 100% ($P = 0.0002$), and 93% ($P = 0.0003$), respectively. The most common adverse events were injection-site reactions (reported by 25%, 11%, 13%, and 57% of patients in the placebo and garadacimab 75-, 200-, and 600-mg groups, respectively). No serious adverse events, anaphylaxis, or thromboembolic events were reported. Phase 3 trials are ongoing.

Administration of an antisense oligonucleotide targeting plasma prekallikrein messenger RNA (**Figure 2**), which has been shown to prevent bradykinin formation (74), has been reported for 2 patients with severe bradykinin-mediated forms of HAE (75). The patients were initially treated with the parent antisense oligonucleotide IONIS-PKK_{Rx} SC for 12 to 16 weeks, followed by treatment with a ligand-conjugated form of the oligonucleotide, donidalorsen (formerly named IONIS-PKK-L_{Rx}) 80 mg SC every 3 to 4 weeks for 7 to 8 months. IONIS-PKK_{Rx} SC dosing was 200 mg once weekly, with optional dose loading in the first 2 weeks. If the 2 patients had breakthrough HAE attacks, dosing could be increased to 300 mg after 6 weeks and to 400 mg after 12 weeks. During IONIS-PKK_{Rx} treatment, the mean monthly attack rate decreased from 1.2 to 0.2 attacks (patient 1) and from 7.9 to 1.0 attacks (patient 2). When patients were switched to donidalorsen (IONIS-PKK-L_{Rx}), the mean monthly attack rate was 0 (patient 1) and 3.4 (patient 2). Plasma prekallikrein levels were reduced with both IONIS-

PKK_{Rx} and donidalorsen (IONIS-PKK-L_{Rx}) treatment. Both patients experienced injection-site reactions.

A phase 2, randomized, double-blind, placebo-controlled trial was conducted to further examine the efficacy and safety of once monthly SC donidalorsen 80 mg in adults with HAE (76). During the ≤8-week study run-in period, the 20 patients (donidalorsen [$n = 14$]; placebo [$n = 6$]) included in the trial experienced a mean 2.7 HAE attacks/month (range, 1.0 to 5.6) (76). Donidalorsen significantly reduced the mean number of monthly attacks compared with placebo (0.2 vs. 2.2, respectively; 90% difference; $P < 0.001$) (76). Donidalorsen decreased plasma prekallikrein activity from baseline by 61% (76). The most commonly reported adverse events with donidalorsen were headache (14%) and nausea (7%); no patients in either treatment group experienced serious adverse events or discontinued the trial due to adverse events (76).

Discussion

Several options are available as on-demand treatment and as short-term or long-term prophylaxis of HAE attacks (2). Direct replacement with C1-INH products (e.g., pdC1-INH and rhC1-INH) treats all affected pathways, whereas single-target (pathway) therapies affect different components of the kallikrein-kinin system. Approved treatments for HAE vary in terms of indication(s), age limitations, route of administration, and frequency of dosing. C1-INH replacement therapies have a well-established efficacy and safety profile, given their long-term history of use in HAE (77), and newer agents are now being incorporated into HAE treatment regimens (78). Data for the C1-INH replacement therapies support dose-dependent efficacy, including the achievement of a zero-rate attack frequency (50, 54). Notably, the efficacy of C1-INH products for long-term prophylaxis was generally demonstrated in populations with a more severe HAE phenotype (on-placebo or pretreatment attack rate ∼4–7 attacks/month) (40, 50, 54) than in similar trials of single-pathway treatment options (∼2–4 attacks/month) (**Tables 3**, **4**) (63, 68). However, the phase 2 trial of garadacimab, an agent that targets both contact and fibrinolytic pathways, included patients with a more severe phenotype (mean 3.5–7.5 attacks/month) (73).

Although on-demand and prophylactic C1-INH treatments are effective for many patients with HAE (**Tables 2–4**), some patients may experience an increase in frequency of attacks while taking prophylactic therapy. Some authors have suggested that patients may develop tolerance to prophylactic therapy over time (52, 53). However, alternative explanations must be considered. For example, many patients lacking easy access to on-demand treatment do not treat every attack, and a percentage of attacks may remain untreated for other reasons (9, 79), so an increase in C1-INH usage may merely reflect better access to therapy and better patient education rather than tolerance induction. Prospectively collected observations based on patients receiving higher doses of prophylactic C1-INH do not suggest an increase in attack frequency and, if anything, show improved symptom control during 30 months of treatment, which disputes the theory of tolerance induction with C1-INH replacement therapy (51).

Long-term response to treatment may be dependent on baseline C1-INH levels in each patient (49). This is likely true for prophylaxis with C1-INH replacement therapies, but this hypothesis has not been specifically studied. Differences in study design, particularly in baseline or placebo HAE attack rates, treatment dosing, and route and frequency of administration, and a lack of head-to-head comparisons, make it difficult to evaluate any such relationship. For single-pathway therapies, genetic polymorphisms and perhaps environmental factors affecting other relevant pathways may be the most important predictors, along with factors affecting the degree of inhibition of the contact pathway target. Again, more research and better biomarkers are needed to determine the optimal approach for the individual patient. Given the lack of head-to-head clinical trials and differences among studies in patient populations, methods, and outcomes, which limit the ability to compare across trials, research is also needed to directly compare therapies in patients with HAE. Because both HAE therapies and a failure to control HAE symptoms have pharmacoeconomic implications (e.g., direct and indirect costs, including health care utilization), more data are needed about individual determinants of treatment response and the relative efficacy of HAE therapies (i.e., on-demand, short-term, and long-term prophylaxis) to enable delivery of an optimized treatment strategy for each patient.

In view of the wide variety of potentially relevant pathways regulated by C1-INH, it is perhaps surprising that several therapies that target only the contact pathway can produce a high level of control of angioedema (**Figure 2**). This reflects the central role of the contact pathway in angioedema generation and the multiple feedback loops between the contact pathway and other pathways implicated in angioedema generation. Further, new targeted agents in development for HAE have shown promise, albeit in small populations (73, 75).

In conclusion, some therapies for HAE target multiple pathways (i.e., C1-INH replacement therapy), while others target a single pathway (e.g., kallikrein inhibitors). Future research is needed to optimize therapeutic strategies and, in particular, to compare long-term outcomes both for angioedema control and for other consequences of C1-inhibitor deficiency, such as autoimmunity. In the meantime, health care providers and patients should establish an individualized management strategy that considers on-demand treatment and short-term prophylaxis, as well as long-term

prophylaxis in appropriate patients, to minimize the disease burden of this condition.

Contribution to the field statement

Hereditary angioedema (HAE) is a rare genetic disease that causes unpredictable, recurrent episodes of skin/mucosal swelling (called attacks) that can affect multiple locations, such as the hands, feet, gastrointestinal tract, other intra-abdominal organs, face, and upper respiratory tract. This condition substantially impairs patient-related quality of life and interferes with their ability to function at home, work or school, or socially. Management of HAE aims to treat or, preferably, prevent attacks, improving quality of life. Swelling during HAE attacks is mediated by bradykinin. Different pathways contribute to bradykinin generation. Almost all cases of HAE are caused by deficiency or dysfunction of C1-esterase inhibitor (C1-INH), which under normal conditions regulates different pathways to inhibit bradykinin production. C1-INH replacement therapy has been used for decades to treat or prevent HAE attacks. While complete disease control with C1-INH replacement is possible, some patients experience breakthrough attacks. More recently available agents target a single pathway—the contact pathway—to prevent bradykinin generation or block the effects of bradykinin. We review the mechanism of action, efficacy, and safety of approved therapies, as well as investigational agents; consider the therapeutic potential of single-pathway treatment options vs. agents with broader effects; and identify gaps in research.

Author contributions

This work was developed from a poster presented at the 12th C1-Inhibitor Deficiency and Angioedema Workshop, June 3–6, 2021 (virtual). Both authors have contributed to the conception, development, drafting, and finalization of the manuscript. Both authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AV has received consultancy/speaker honoraria/meeting sponsorship from, or collaborated in research with, Pharming Group NV, Takeda/Shire, Sobi, CSL Behring, and Pharvaris. HJL has consulted for, acted as speaker for, or collaborated in research with the following: Adverum, BioCryst, CSL Behring, GSK, Intellia, Ionis, KalVista, Pharming, Pharvaris, and Takeda.

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