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Hereditary angioedema with normal C1 Inhibitor associated with Carboxypeptidase N deficiency

Denis Vincent, MD PhD,[1,2] Faidra Parsopoulou PharmD PhD,[3,4] Ludovic Martin, MD PhD,[5,6] Christine Gaboriaud, PhD,[7] Jacques Demongeot MD PhD,[8] Gedeon Loules, MSc,[4] Sascha Fischer, PhD,[9,10] Sven Cichon, PhD,[9,10] Anastasios E Germenis, MD PhD,[11] Arije Ghannam, MD PhD,[3*] Christian Drouet, PharmD PhD [8,12*]

[1] *Allergy and Internal Medicine Unit, University Hospital, Nîmes, France*

[2] *Centre de Référence des Angioedèmes (CREAK), France*

[3] *KininX SAS, Grenoble, France*

[4] *CeMIA SA, Larissa, Greece*

[5] *Dermatology Department, University Hospital, Angers, France*

[6] *Centre de Référence des Maladies Rares de la peau et des muqueuses d'origine génétique-Nord (MAGEC), filière FIMARAD, CHU Angers, Angers, France*

[7] *Univ. Grenoble Alpes, CEA, CNRS, IBS, Grenoble, France*

[8] *Univ. Grenoble Alpes, CHU Grenoble Alpes, Grenoble, France*

[9] *Human Genomics Research Group, Department of Biomedicine, University of Basel, Basel, Switzerland*

[10] *Institute of Medical Genetics and Pathology, University Hospital Basel, Basel, Switzerland*

[11] *Department of Immunology and Histocompatibility, University of Thessaly, Larissa, Greece*

[12] *Institut Cochin, Université Paris Cité, INSERM UMR1016, Paris France*

[*] Both authors share the senior authorship

Corresponding author: Christian Drouet, Université Paris Cité, INSERM UMR1016, CNRS UMR8104, Institut Cochin, 75679 Paris cedex 14, France

Email christian.drouet@inserm.fr

Conflict of interests

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ORCID

Sven Cichon https://orcid.org/0000-0002-9475-086X

Jacques Demongeot htpps://orcid.org/ 0000-0001-9518-9675

Christian Drouet https://orcid.org/0000-0003-1318-4278

Christine Gaboriaud http://orcid.org/0000-0001-5308-0516

Anastasios E. Germenis https://orcid.org/0000-0002-3315-6947

Arije Ghannam https://orcid.org/0000-0002-5045-0566

Abstract

Background: Hereditary angioedema (HAE) is a potentially life-threatening disorder characterized by recurrent episodes of subcutaneous or submucosal swelling. HAE with normal C1 Inhibitor (HAE-nC1-INH) is an under-diagnosed condition. Although the association with genetic variants has been identified for some families, the genetic causes in many patients with HAE-nC1-INH remain unknown. The role of genes associated with bradykinin catabolism is not fully understood.

Objective: We investigated the biological parameters and the genes related to kallikrein-kinin system (KKS) in families with a clinical phenotype of HAE-nC1-INH and presenting with a carboxypeptidase N (CPN) deficiency.

Methods: This study includes four families presenting with HAE-nC1-INH and CPN deficiency. Patients' clinical records were examined, biological parameters of KKS measured, genetics was analyzed by next-generation sequencing and Sanger sequencing. Predictive algorithms (HSF®, SIFT®, Polyphen-2®, MutationTaster®, ClinPred®) were used to classify variants as affecting splicing, as benign to deleterious, or as disease-causing.

Results: Patients presented with angioedema and urticaria, mainly on face/lips, but also with abdominal pain or laryngeal symptoms. Affected patients displayed low CPN activity –30 to 50% of median value in plasma. We identified three variants of the *CPN1* gene encoding the catalytic 55-kDa subunit of CPN at: c.533G>A, c.582A>G and c.734C>T. CPN deficiency associated with genetic variants segregated with HAE-nC1-INH symptoms in affected family members.

Conclusions: *CPN1* gene variants are associated with CPN deficiency and HAE-nC1-INH symptoms in four unrelated families. Genetic CPN deficiency may contribute to bradykinin and anaphylatoxins accumulation, with synergistic effects in angioedema and urticarial symptoms.

Highlights box

- Four families suffering from angioedema and urticaria were presenting with carboxypeptidase N deficiency.
- Carboxypeptidase N deficiency segregated with variants NM_001308.2:c.533G>A, c.582A>G and c.734C>T of the *CPN1* gene in homozygous or compound heterozygous carriers.

Abbreviations

ACE, Angiotensin-I converting enzyme

ACEi, Angiotensin-I converting enzyme inhibitors

ACMG, American College of Human Genetics

C1-INH, C1 Inhibitor

HAE-C1-INH, hereditary angioedema with C-INH deficiency

CPB2, plasma carboxypeptidase B

CPM, carboxypeptidase M

CPN, carboxypeptidase N

HAE-FXII, HAE with a gain-of-function of factor XII

HAE, hereditary angioedema

HK, high molecular weight kininogen

HAE-nC1-

INH, HAE with normal functional C1-INH

NGS, next-generation sequencing

HAE-PLG, HAE with plasminogen activation

HAE-U, HAE of unknown cause.

Keywords

urticaria, angioedema, hereditary carboxypeptidase N deficiency, *CPN1* gene

Introduction

Hereditary angioedema (HAE) is a potential life-threatening disorder; it is characterized by recurrent attacks of subcutaneous or submucosal swelling. It can be disabling, and it affects various areas of the body including face, the upper and lower limbs, tongue, uvula, lips, intestine and larynx.[1] The abdominal attacks are painful and often are accompanied by nausea, vomiting, and diarrhea.[1] Edema may become life-threatening if the upper respiratory airways are involved. Disease diagnosis can be challenging and patients are operated for un-necessary laparotomy in 27% of cases.[2]

HAE has been classified in different types: HAE with C1 inhibitor deficiency (HAE-C1-INH) resulting from variants in the *SERPING1* gene encoding C1-INH and HAE with normal C1-INH (HAE-nC1-INH), which includes variants in *F12*,[3,4] *PLG*,[5] *ANGPT1,*[6] *MYOF*,[7] *KNG1*[8] or *HS3ST6*.[9] Although the association with genetic variant(s) has been identified for some families, the genetic causes in many patients with HAE-nC1-INH are unknown. The role of genes associated with bradykinin catabolism in the aetiology of HAE is not fully understood.

Carboxypeptidase N (CPN, *alias* kininase I) is a zinc-metallopeptidase that specifically cleaves C-terminal basic residues, Arg and Lys, from biologically active peptides and proteins (reviewed in[10-12]). CPN has been shown to cleave and thereby regulate (i) kinins, *e.g.* bradykinin and Lys-bradykinin, generating *des*Arg^{9}-bradykinin and *des*Arg^{10}-Lys-bradykinin respectively,[13,14] with subsequent transformation of kinin B_2 receptor ligands into B_1 receptor ligands, (ii) anaphylatoxins,[15] *e.g.* C3a and C5a, with generation of inactivated *des*Arg-C3a and *des*Arg-C5a, and (iii) the chemokine stromal-derived factor-1α.[16] Thereby CPN exerts control over multiple mediators of inflammation, vascular permeability, chemoattraction, and leukocyte activation and trafficking. CPN is composed of a 55-kDa catalytic subunit, encoded by *CPN1*, and a non-catalytic regulatory subunit, encoded by *CPN2*. The CPN2 protein

protects the 55-kDa catalytic subunit from glomerular filtration, degradation or enzymatic inactivation.[17] CPN is biosynthesized by the liver in an active form and is present in plasma at a concentration of 30-40 mg/L.[6] This high CPN concentration has been implicated in supporting an essential protective role from chronic inflammatory conditions.[9,12,15]

As shown by Zhou et al,[18] mice lacking carboxypeptidase B2 (CPB2), CPN, or both plasma carboxypeptidases have enhanced vascular permeability implicating CPN deficiency in angioedema. Consequently, deficiency in either CPB2 or CPN activity should be considered in unexplained cases of HAE. Two cases of partial CPN deficiency have been documented (OMIM 603103), in patients presenting with angioedema or urticarial episodes: (i) A 65-year-old man with 21% of normal CPN activity and normal C1-INH, who presented with 11-year history of severe recurrent angioedema occurring almost 40 times a year. His medical history included allergy and asthma, with elevated histamine during attacks.[10,19] Genetic analyses revealed combined *CPN1* variants, NM_001308.2:c.[173dup(;)533G>A;p.(Gly178Asp)], which were suggested to cause the clinical phenotype.[20,21] (ii) A female with CPN deficiency and iatrogenic angioedema 2 years after starting treatment with an angiotensin-I converting enzyme inhibitor (ACEi), although the background for this case was not fully described. [22]

In this article, we present four families with HAE-nC1-INH clinical phenotype and CPN deficiency; the biological parameters and selected genes related to kallikrein-kinin system (KKS) were investigated.

Patients and methods

Study subjects.

Four unrelated families were included in an E-RARE-1 research program on HAE-nC1-INH; probands and several family members were investigated. All individuals tested had normal C1-INH and complement levels compared to healthy blood donors; a diagnosis of HAE-nC1-INH has been issued for family probands. Healthy blood donor samples served as controls.

Sampling procedures and laboratory methods

EDTA blood samples were used for genetic analyses. Citrated plasma samples were used to investigate bradykinin metabolism. Samples were collected outside the window of ACEi intake. Plasma samples were immediately frozen and kept at -80°C until analysis.

CPN activity was measured according to a protocol modified from Skidgel[23] using a FurylAcroyloyl-Ala-Lys substrate (Sigma-Aldrich, Saint Quentin Fallavier France) that does not cross-react with circulating CPB2. Aminopeptidase P (APP) activity was assessed as previously described[24] using *Abz*-Lys-Phe-Arg-Ser-Ser-Lys-Gln-EDDnp (ProteoGenix, Schiltigheim France). ACE activity was measured using the ACE kinetic® kit (Bühlmann Laboratories, Allschwil, Switzerland) and plasma kallikrein activity in line with our study methods was investigated using a H-D-Pro-Phe-Arg-*p*NA substrate (ProteoGenix).[25]

Genotyping

Genomic DNA was isolated using MagNA Pure® (Roche, Meylan, France). DNA samples from probands of families B and C were analyzed by next-generation sequencing (NGS) (Ampliseq® custom panel, Thermo Scientific, Waltham MA, USA), as described.[26] See Table S1, Supplementary material for the genes submitted to the analysis. 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

an 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 workflow. Primary data were analyzed using Ion Reporter® software (Thermo Scientific). Sanger sequencing of exon 3 (CPN1ex3_F: 5'-AGTATTCAATCTGAAACCTTCATTTTT-3', CPN1ex3_R: 5'-AGATGGCTTAGCAGTCTTTCTG-3') was used to confirm *CPN1* variants and to sequence of DNA samples.

Bioinformatics

In silico prediction tools specifically designed for mutation evaluation were performed using a number of software packages: HSF® (Human Splicing Finder; https://hsf.genomnis.com), SIFT® (Sorting Intolerant From Tolerant; https://sift.bii.a-star.edu.sg/), PolyPhen2® (Polymorphism Phenotyping v2; https://genetics.bwh.harvard.edu/pph2/), MutationTaster® (https://mutationtaster.org) and ClinPred® (https://sites.google.com/site/clinpred/). To determine minor allele frequency (MAF), we used the Genome Aggregation Database v3.1.1 (gnomAD®), available at URL gnomad.broadinstitute.org/. Clinical interpretation of genetic variants scored by ACMG-AMP 2015 guideline has been performed using InterVar® (https://wintervar.wglab.org/).

Post-analytical phase

We identified variant positions consistently with recommendations of Human Genome Variation Society and used terminology of angioedema in accordance with DANCE (definition, acronyms, nomenclature and classification of angioedema) initiative. Variant pathogenicity criteria were determined according to the American College of Medical Genetics (ACMG)[27] and variant pathogenicity was curated according to a recent International Consensus HAE genetics.[28]

Statistics

Individuals from 4 families were stratified by enzymatic activities of plasma kallikrein, CPN, APP and ACE. Reference interval was based on measurements of healthy blood donors, 98 male, 20-68y, and 101 female, 18-67y. The Kolmogorov-Smirnov statistical test of normality (D) provided a measurement of the divergence of sample distribution from the Gaussian distribution. The D score nearer to zero, the more likely distribution is normal. D values calculated for kallikrein and CPN activity in controls are ranging from 0.0376 to 0.1155, in line with data distribution not significantly deviating from a Gaussian distribution, making the Mann-Whitney test applicable in statistical analysis. The percentile distribution of the reference population has been developed for biological parameters, with position of median values for comparison of patient with healthy controls. Using nonparametric Mann-Whitney *U*-test (GraphPad Prism 8®) compared values of individual and healthy controls. A *p*-value less than 0.05 (typically ≤ 0.05) was statistically significant.

Ethics

All procedures were performed in accordance with the principles of the Helsinki declaration and French ethical policies governing the use of biological sample collections (Ministry of Health declaration #2008-634). Informed written consent for molecular genetics analysis was obtained from patients in the presence of the physician. Patients consented to participating in an investigation with biological assays. The Institutional Review Board at CECIC Rhône-Alpes-Auvergne, Clermont-Ferrand (IRB #5891), stated on 23rd August 2021 that the processing methods and data management met requirements. All data were processed anonymously.

Results

Clinical phenotypes

Clinical observations for all cases are listed in Table 1.

Family A, The proband II.1 presented with recurrent urticaria and peripheral angioedema triggered for the first time after stimulation for *in vitro* fertilization because of endometriosis. Symptom relief has been observed after taking 3g/day tranexamic acid and on-demand icatibant. Her mother I.2 and her brother II.2 were also symptomatic for angioedema with symptom relief after taking 3g/day tranexamic acid.

Family B, The proband II.1 was presenting with recurrent urticaria and episodic peripheral angioedema, with symptoms starting when the proband was under oral contraception. H1-antihistamines, even at the highest dose (20mg/day desloratadine), failed to relieve symptoms. Symptoms were relieved by 3g/day tranexamic acid and 10mg/day montelukast, and on-demand icatibant in case of severe episodes. The daughter III.1 presented with a similar phenotype to the mother, with symptoms starting when she was under anti-androgen treatment; she successfully responded to the same treatments as the mother. Four other family members - I.1, I.2, III.3 and IV.1 - presented also with same symptoms.

Family C, The proband III.2 presented with recurrent H1-antihistamine resistant urticaria episodes and abdominal attacks. Symptom relief has been observed after taking 3g/day tranexamic acid and on-demand icatibant. Two other family members - II.2 and III.1 - were also symptomatic.

Family D, The proband II.1 presented with cold urticaria and H1-antihistamine resistant angioedema (up to 20mg/day cetirizine). Symptoms were relieved by administration of 3-5g/day tranexamic acid and 10mg/day montelukast, plus on-demand icatibant. Both parents presented a moderate phenotype. The proband describes fatigue and stress as triggers of angioedema attacks.

The effectiveness of icatibant on the relief of severe episodes in all four families suggests at least partial involvement of bradykinin in the clinical phenotype.

All probands were born from non-consanguineous parents.

Biological characteristics of the patients

Antigenic C1-INH and function were in the normal range for all individuals. Plasma CPN activity was significantly below the reference interval for all symptomatic patients (Table 2). Plasma CPN activity when measured during the attacks was equivalent to levels measured in the intercritical period. Both APP and ACE activities were in the normal ranges (Table 2). Kinin catabolism enzyme activities were measured in all patient samples at various time points over the years of patient follow-up and remained unchanged throughout the study (not shown). Spontaneous kallikrein activity was in the normal range for all subjects. However, kallikrein activity in dextran sulfate-stimulated plasma samples from patients I.2, II.1, III.1 and IV.1 of family B were lower than the normal range, suggesting that these subjects have low proenzyme levels. Interestingly proband II.1 in family B had the variant c.689T>A on *KLKB1* gene, which may have contributed to the lower levels of proenzyme in this family (Table 2). An increased proportion of high molecular weight kininogen (HK) was cleaved in samples from proband II.1 in family B collected during angioedema attack compared to sample obtained during the intercritical period (not shown).

Genetic investigation

DNA from individuals from families B (II.1) and C (III.2 and III.1) were investigated by NGS; material from all the other individuals was subjected to Sanger sequencing. Figure 1 shows the pedigrees and the allele distribution revealed by these analyses. Within the four families, *CPN1* variants associated with clinical symptoms and low CPN activity. Table 3 summarizes the genetic findings and associated bioinformatics data recorded for genetic variants.

Family A. The proband II.1 is a female carrying compound heterozygous *CPN1* gene variants NM_001308.2:c.[533G>A];[734C>T]. The variants included a known variant c.533G>A;p.(Gly178Asp) and a new variant NM_001308.2:c.734C>T;p.(Thr245Met). Average frequencies of 3.4E-03 and 3.2E-05, respectively, were determined according to gnomAD; *i.e.* 1.09E-07 for the variant combination. The c.533G>A;p.(Gly178Asp) variant has been characterized as benign in ClinVar although it was suggested as pathogenic in the initial report[20] where it was correlated with functional CPN deficiency. The p.(Gly178Asp) and p.(Thr245Met) variants are both located in signature motifs - PM14-Zn carboxypeptidase for Gly_{178} and Zn-binding signature for Thr_{245}. These two residues are highly conserved residues across species and have corresponding positions in carboxypeptidase M (CPM),[29] a GPI-anchored carboxypeptidase expressed on endothelial cells (reviewed in[12,21,30]). The two variants are predicted to be deleterious (SIFT), damaging (ClinPred) and probably damaging (PolyPhen), polymorphic for p.(Gly178Asp) or disease-causing for p.(Thr245Met) according to MutationTaster (Table 3). The observations of both variations meet the ACMG criteria PS3, PS4, PM1, PM2, PP3, PP4, PP5, and BP6 specifically for p.(Gly178Asp), along with a pathogenic (recessive) characterization as evaluated by InterVar®.

Family B. The proband II.1 is a female carrying homozygous variants NM_001308.2:c.[533G>A];[533G>A];p.(Gly178Asp), with a frequency of 1.15E-05 for the homozygous combination – the same combination was detected in family D. The observations meet ACMG criteria PS3, PS4, PM1, PM2, PP3, PP4, PP5, and BP6, along with a pathogenic (recessive) characterization as evaluated by InterVar®. An additional variant was found in this family: NM_000892.3:c.689T>A;p.(Ile230Asn) in the *KLKB1* gene, located in the Apple 3 domain of KLKB1 (prekallikrein), predicted to be deleterious (SIFT), probably damaging (PolyPhen), disease causing (MutationTaster) and tolerated (ClinPred). P.(Ile230Asn) may be putatively responsible for the recurrent low proenzyme content detected in individuals I.2, II.1

(proband), III.1, and IV.1 (Table 2), recognized as a likely pathogenic (recessive) variant (frequency 1.8E-04) in a PK deficient individual;[31] it is not reported in ClinVar,

Family C. The female proband III.2 is homozygous for *CPN1* variant NM_001308.2:c.[533G>A];[533G>A];p.(Gly178Asp). Her sister III.1 is a compound heterozygous carrier of *CPN1* variants NM_001308.2:c.[533G>A];[c.582A>G]. Both individuals also carry a c.1299C>T;p.(His433=) variant, an SNP identified as rs61733667, not reported in ClinVar. The rare NM_001308.2:c.582A>G;p.(Glu194=) variant, with an average frequency of 5.8E-05 (gnomAD), is predicted to affect splicing by activation of a cryptic exon acceptor site (HSF 3.0), it is also unreported in ClinVar. The combination of both variants occurs at a frequency of 1.09E-07. The observations for c.582A>G meet the ACMG criteria PS3, PS4, PM2, PM3, PP3, and PP4, along with a characterization as pathogenic (recessive) as evaluated by InterVar®. Variant c.533G>A, as homozygous variant in combination with variant c.582G>A;p.(Glu194=), segregated with the clinical phenotype for patients II.2, III.1 and III.2, An additional variant was found in this family: NM_000505.3:c.-4T>C;rs1801020 polymorphism in the *F12* gene that has been recognized as a disease modifier in families with HAE-C1-INH[32] and with HAE-FXII.[33]

Family D. The male proband II.1 was found to be a homozygous carrier of NM_001308.2:c.[533G>A];[533G>A];p.(Gly178Asp) – the same combination as recorded in family B.

In conclusion, the clinical and genetic records from these four families, in line with the biological findings, are consistent with a hereditary CPN deficiency according to the following criteria:

a. Plasma CPN activity in proband samples significantly lower than 50% of the median value of healthy controls and below the lowest percentiles of a normal distribution of male and female controls (Table 2),

b. *CPN1* variants segregating in individuals presenting a clinical history of urticaria and angioedema,

c. c.533G>A;p.(Gly178Asp) variant inherited in an autosomal recessive pattern, suggested to be the disease-causing mutation when present in homozygous – proband II.2, family C and proband II.1, family D – or in compound heterozygous form, *e.g.* when combined with c.734C>T;p.(Thr245Met), for proband II.1, family A, or c.582A>G;p.(Glu194=), for patient III.1, family C (Figure 1); the frequency of variant combinations is in agreement with the expected occurrence of HAE-nC1-INH,

d. *CPN1* alleles combined with pathogenic variants/gene modifiers in genes involved in kinin metabolism, *e.g.* *KLKB1* and *F12* (Table 3), with potentially partial contributions to chronic symptomatology.

Discussion

This study presents four families with HAE-nC1-INH and CPN deficiency. Plasma CPN activity was significantly below the reference interval for all symptomatic patients with angioedema during critical and intercritical periods (Table 2). In these families, CPN deficiency is associated with combinations of *CPN1* variants, with c.533G>A when present on both alleles or in combination with c.582A>G or c.734C>T. The variants were transmitted as an autosomal recessive trait, and combinations of *CPN1* alleles co-segregated with angioedema clinical symptoms in patients. A high female to male ratio of CPN deficiency is in agreement with the observation of HAE-nC1-INH, including HAE-FXII.

Two major pathophysiological mechanisms of angioedema can be distinguished by the endotypes, with (i) mast cell activation and (ii) kinin-dependency.[34] Our findings are consistent with previous reports of CPN deficiency as described in two patients presenting with angioedema or/and urticarial episodes.[19,22] Whereas symptoms of *erythema marginatum*, with susceptibility to icatibant, are not uncommon in HAE-C1-INH patients during prodromal symptoms,[35-37] the urticarial episodes are very rare.[38] Besides the issue of angioedema- or urticaria-specific triggers of attacks for CPN deficient patients is difficult to address: Frequently these patients presented with urticaria concomitantly with angioedema symptoms. It could be hypothesized that, as kallikrein promote C5 and C3 cleavage and subsequent generation of anaphylatoxins,[39,40] triggers of KKS activation could generate both anaphylatoxin and kinin ligands with subsequent synergistic effects on the clinical phenotype.

A circulating inhibitor as responsible of decreased CPN activity has been excluded after dilution experiments of patient plasma in sample from healthy donors (not shown). Rather CPN deficiency was linked to a defective enzymatic activity or decreased level of the protein,[19] associated with three *CPN1* variants that have been shown to segregate with the clinical phenotype and a low CPN activity.

CPN mediates approximately 10% of the catabolism of bradykinin in plasma and CPN deficiency could contribute to the accumulation of bradykinin.[41] The observation that the affected probands from the 4 families obtain symptom relief after administration of icatibant, a B_2 receptor antagonist, implicates bradykinin in angioedema symptoms.

CPN deficiency was initially been attributed to a combination of *CPN1* variants, c.[173dup](;)[533G>A].[20] The three *CPN1* variants carried by individuals in these families were characterized with frequencies ranging from 3.2E-05 to 3.4E-03 (Table 3) and variant combination frequencies that are compatible with a rare disease. Missense variants on both alleles at one of the two positions highlighted in Figure 2, built from PDB #2NSM,[42] reduce - but not completely abrogate - catalytic activity, as seen in Table 2. Both Gly_{178} and Thr_{245} are strictly conserved throughout evolution[29] and are therefore likely to play a key role in preserving enzymatic structure and activity. Thr_{245}, located within α-helix-6, is close to the main binding-specificity pockets, next to the pepsin cleavage site (Arg_{238}-Arg_{239}), which enhances the catalytic efficiency of CPN.[42] A Thr to Met transition is predicted to destabilize the Pro_{225} position, and consequently the nearby loop - Asn_{223} and Asp_{227} - lining the pocket recognizing the Arg/Lys to be cleaved. The role of Gly_{178} has not yet been fully deciphered; a Gly to Asp transition may disrupt the conformation of the Gly_{177}-Gly_{178} stretch that stabilizes the structure after α-helices 4/5. Based on the predicted 3D-structure published,[42] missense variants at both these positions could affect binding of the regulatory CPN2 subunit. The carbohydrates are also displayed on the same face of the enzyme, and could possibly modulate catalytic activity of CPN1 subunit.

Additional *CPN1* variants were recently reported in HAE families in combination with other alleles, *e.g.* a NM_001308.2:c.931T>C;p.(Cys311Arg) variant, predicted to be deleterious, combined with NM_000301.3(PLG):c.988A>G;p.(Glu330Lys) in a HAE-PLG patient,[43] and a NM_001308.2:c.1219G>A;p.(Glu407Lys) variant combined with *TLR4*, *MASP1*, *PLAU*,

and *MPO* variants in a HAE-U patient.[26] Neither report documented biological data for CPN activity.

Low CPN activity has been implicated in protamine-reversal syndrome, a severe iatrogenic condition. Protamine, with its neutralizing properties of the effects of heparin, is given after extracorporeal circulation. But in patients with protamine-reversal syndrome, it can trigger a catastrophic reaction resulting in pulmonary vasoconstriction, bronchoconstriction, and systemic hypotension. As protamine is a potent CPN inhibitor, a decreased anaphylatoxin and kinin inactivation has been suggested to contribute to the syndrome.[44] More generally CPN has been recognized as a pleiotropic regulator of inflammation.[21,45,46] Furthermore, decreased plasma CPN activity has been identified as a risk factor for disease severity in HAE-C1-INH[47] and in HAE-FXII patients.[48]

CPN, more than CPB2, is a potent C3a and C5a inactivator.[12] The observation of urticaria in the CPN-deficient families described here is compatible with the above-described anaphylatoxin properties.[10] Plasma from *Cpn1*$^{-/-}$ mice failed to cleave the C-terminal Arg from C3a and C5a, emphasizing an important role that CPN plays in anaphylatoxin inactivation.[46] CPN has been demonstrated to protect from vascular leakage.[18] These observations are congruent with a regulation by CPN of the biologically active anaphylatoxins and kinins. In addition to the vascular effects, anaphylatoxins are non-immune activators of mast cell, with involvement in the pathophysiology of urticaria.

In addition, CPN may participate in plasminogen activation control. Through its catalytic action, CPN removes C-terminal Lys residues from cell surface proteins that act as plasminogen "receptors", the binding of plasminogen to C-terminal Lys residues on cell surface enhances its activation up to 1000-fold.[49] Consequently, CPN can down regulate plasminogen activation. Accordingly, it is tempting to speculate that when CPN activity is decreased in plasma, plasmin activity is likely to increase, leaving the KKS prone to rapid

activation. This scenario is compatible with our observation of HK cleavage in plasma during acute symptoms in patients' plasma with low CPN activity (not shown), leading to bradykinin production. The hypothesis could be consistent with the here reported positive response of CPN deficient patients to prophylaxis with tranexamic acid.

Mixed angioedema and urticaria phenotypes have already been recorded.[50,51] The present observations are in agreement with this phenotype. However the angioedema episodes described herein occurred in a reduced kinin catabolism condition, similarly to iatrogenic angioedema with ACEi and dipeptidyl-peptidase 4 inhibitors.[50] Some hereditary situations were provisionally included in an informal HAE-U group. Based on the results presented here, we suggest that CPN deficiency could characterize a group of HAE-CPN, with consequent challenges for patient treatment. Indeed, symptoms in these families do not respond to H1-antihistamines, and prophylaxis currently recommended for HAE must therefore be adapted.

In conclusion, our work implicates CPN mutations and enzyme deficiency in contributing to angioedema symptoms in HAE-nC1-INH. CPN deficiency could impair bradykinin catabolism and thereby increase B_2 receptor activation. As for other HAE-nC1-INH types, diagnosis of HAE-CPN requires concerted clinical, biological, and genetic investigation to decipher the dysregulation of the kallikrein kinin pathway.

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Author contributions

DV and LM recorded the individual clinical observations; CD coordinated the program, was involved in conceptualization, funding acquisition, and supervision; CD and AG were responsible for the methodology, planning and performing experiments, and writing the manuscript; CD, AG and JD performed the data analysis; FP, GL, SC, SF and AEG developed the molecular genetics studies and subsequent data curation; structural consequences of CPN variants were analyzed by CG; all authors have read and approved the final manuscript.

Data availability statement

Repository of variants. Variants have been included in the Global Variome shared LOVD database (https://databases.lovd.nl/shared/genes/CPN1).

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Legends to figures

Figure 1 (word count 60). Pedigrees of families A-D presenting with a CPN deficiency. Results of NGS and Sanger sequencing analyses. The *CPN1* variants c.533G/A, c.582G/A, c.734C>T, c.1299C>T and additional variants cosegregating with clinical symptoms are presented. Filled symbol: individual affected by recurrent angioedema possibly associated with urticarial lesions; empty symbol: asymptomatic subject; dashed symbol: non-investigated family member. Arrows indicate the probands of investigated families.

Figure 2 (word count 60). Expanded view of the carboxypeptidase N 3D-structure (PDB #2NSM) showing the position of CPN1 variants described in the study. The catalytic triad is labeled red, and the catalytic Zn^{2+}-binding site is indicated in blue. Residues lining the Arg/Lys binding pocket are labeled red, the Zn^{2+}-binding and catalytic residues are colored blue. Residues numbered according to positions in the mature protein.

Table 1. Clinical records. Age of onset and delay diagnostic are given in years (y) or months (mths)

Family ID	Patient	Sex	Symptoms§					Trigger	Age of onset	Delay diagnostic	Treatment Prophylaxis
			peripheral	abdominal	laryngeal	macroglossia	urticaria				
A	I.2	F	yes	yes	no	no	no	unknown	40y	1y	Tranexamic acid
	II.1‡	F	yes	yes	yes	no	yes	pressure pruritus; triptorelin	41y	2y	Tranexamic acid, Icatibant on demand
	II.2	M	yes	yes	yes	no	yes	unknown	-	-	Tranexamic acid
	II.3	F	yes	yes	yes	no	yes	unknown	-	-	None
	III.1	F	yes	yes	yes	no	yes	unknown	-	-	None
B	I.1	M	no	no	no	no	yes	unknown	22y	75y	None
	I.2	F	yes	yes	no	no	yes	none	27y	70y	None
	II.1‡	F	yes	yes	mild	no	yes	spontaneous and/or cold	30y	12y	Tranexamic acid, Montelukast, Icatibant on demand
	III.1	F	yes	yes	no	no	yes	unknown	25y	1y	Tranexamic acid

	III.3	F	no	no	no	no	yes	unknown	17y	1y	Montelukast, Icatibant on demand None
	IV.1	M	no	yes	no	no	yes	unknown	12y	6m	None
C	II.2	F	yes	yes	no	no	yes	cold	47y	6m	None
	III.1	F	no	yes	no	no	rare	unknown	17y	6m	None
	III.2‡	F	-	yes	no	no	yes	cold	15y	6m	Tranexamic acid, Icatibant on demand
D	I.1	M	no	yes	no	no	yes	unknown	16y	30y	None
	I.2	F	no	no	no	no	yes	unknown	12y	30y	None
	II.1‡	M	yes	yes	yes	rare	chronic urticaria	pressure; cold, fatigue	18y	14y	Tranexamic acid, Montelukast Icatibant or C1-INH concentrate on demand

‡Family proband.

§ Urticarial lesions in CPN deficient patients developed frequently, but not consistently, in association with angioedema attacks. An urticarial rash accompanied nearly 60% of symptomatic episodes of angioedema.

Table 2. Biological data. Enzymatic continuous variables in patient samples are presented as the mean ± 1SD. When compared with reference interval, significance of data is shown; **** for $p \leq 0.0001$, *** for $p \leq 0.001$, ** for $p \leq 0.01$, * for $p \leq 0.05$, *ns* when not significant (Mann-Whitney *U*-test). Reference intervals with medians generated in healthy donors are presented (95th percentile). Enzymatic activity outside the reference interval is highlighted in bold.

		Plasma kallikrein (V_{max}, nmol/min/mL)		CPN (V_{max}, nmol/min/mL)[1]	APP (V_{max}, nmol/min/mL)	ACE (IU)
		Spontaneous kallikrein activity	**Proenzyme activation**			
Reference population (5th-95th percentile interval; median)	male (n=98)	3.1–9.2 (5.8)	1,830-2,765 (2,253)	70.9–105.9 (92.0)	0.25-3.08 (1.04)	43-95 (69)
	female (n=101)	3.2–10.6 (6.4)	1,870-2,985 (2,351)	79.6–100.0 (90.7)	0.50-5.93 (1.59)	42-85 (62)
Family ID	**Patient**	**Sex**				
A	I.2	F		**65.1±11.1** (n=2)*		
	II.1‡	F	1.7	3,237±136 (n=2) *ns*	**27.2±0.9** (n=3)****	2.68±0.18 (n=3)	42±4 (n=3)
	II.2	M			**46.4±7.9** (n=2)***		
	II.3	F			82.5±6.8 (n=2) *ns*		
	III.1	F			86.7±3.8 (n=3) *ns*		
B	I.1	M	9.1	1,819±434 (n=2)*	**60.4±1.2** (n=2)***		
	I.2	F	5.7	**1,316±65** (n=2)**	**61.8±1.7** (n=2)**		
	II.1‡	F	7.7	**1,233±48** (n=2)**	**42.6±1.5** (n=2)***	0.82	53
	III.1	F	3.2	**1,156±43** (n=2)**	**62.4±4.8** (n=2)**	0.67	47±7 (n=2)
	III.3	F	10.6	1,896±15 (n=2) *ns*	**59.7±4.7 (**n=2)***	1.87	37.5±3.5 (n=2)
	IV.1	M	2.7	**1,185±106** (n=2)***	**50.7±4.2** (n=2)***	0.46	78
C	II.2	F	10.5	3,313	**38.4±7.6** (n=2)***		38.5±0.7 (n=2)
	III.1	F			**58.7±1.1** (n=2)***		

	III.2‡	F	10.1	2,808	**33.4±1.5** (n=2)***	0.90	41±1.4 (n=2)
D	I.1	M	3.9	3,010	**70.6±2.6** (n=2)*	1.56	43
	I.2	F	4.2	2,554	**63.7±0.9** (n=2)*	5.07	63
	II.1‡	M	8.4	2,883	**54.6±0.4** (n=2)***	1.89	75

‡ Family proband

[1] Lower threshold value 0.7 nmol/min/mL

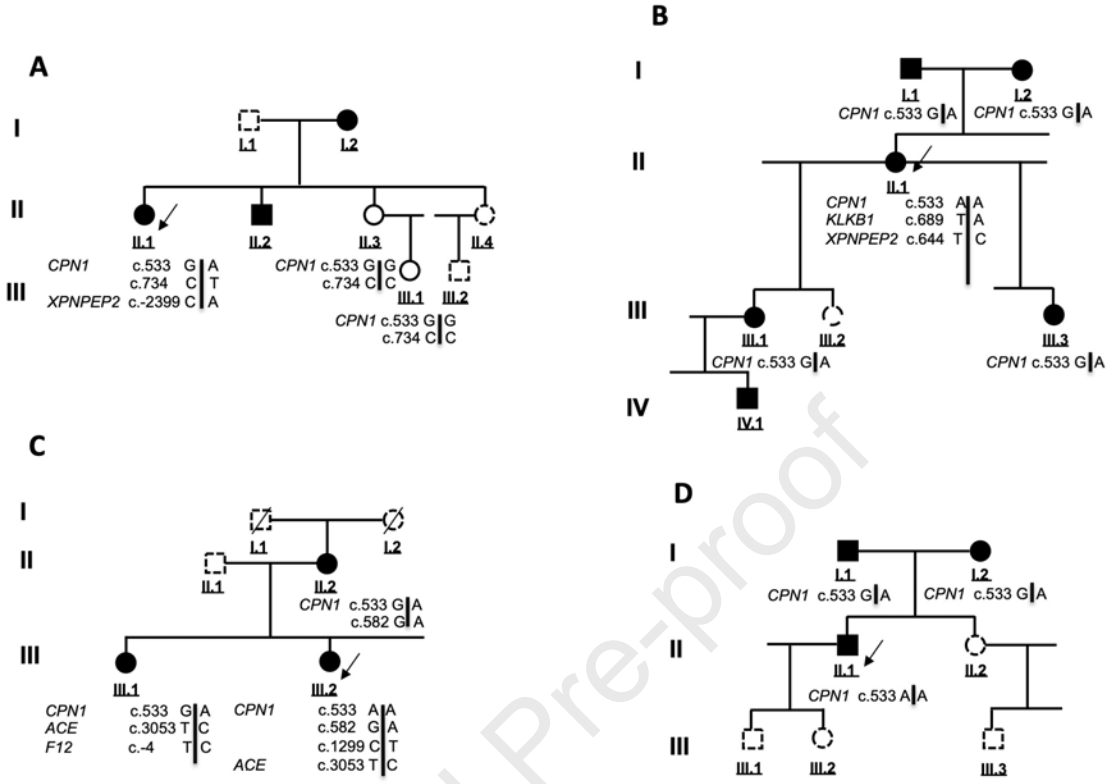
Table 3. Variants found in the 4 families presenting with CPN deficiency: bioinformatic analysis. The minor allele frequencies (MAF) detected in the World and European populations are indicated according to Genome Aggregation Database (gnomAD®). The results from four prediction algorithms applied to missense substitutions are summarised: SIFT (with score), PolyPhen-2 (with probability score), MutationTaster® and ClinPred® (with probability). The SIFT® and PolyPhen-2® algorithms give scores ranging from 0 to 1. A mutation is predicted as "deleterious" by SIFT® if its score is below 0.05; otherwise it is predicted as "tolerated". A mutation is predicted as "possibly damaging" by PolyPhen-2® if its score is greater than 0.15, and as "probably damaging" if it is greater than 0.85; otherwise it is predicted as "benign". The Mutation Taster® algorithm indicates the probability of an alteration being a polymorphism or a disease-causing alteration. The scores range from 0 to 1, with a score of 1 indicating a high security of prediction. ClinPred® incorporates machine-learning algorithms that use existing pathogenicity scores and benefits from normal population allele frequency. HSF 3.0® has been used as an algorithm for prediction of a synonymous variant.

Gene	Encoded protein	Protein function	OMIM[a]	Variant	MAF[b] (gnomAD[c])		Prediction algorithms				
					World	European	HSF 3.0[d]	SIFT[e]	PolyPhen-2[f]	MutationTaster[g]	ClinPred[i]
CPN1	Carboxypeptidase N, subunit 1	Kininase I	603103	c.533G>A p.(Gly178Asp) rs61751507	0.0034	0.0048		Deleterious (0.02)	Probably damaging (0.996)	Polymorphism (1.37E-07)	Damaging (0.997)
				c.582A>G p.(Glu194=) rs190183597	0.000058	0.000061	Affects splicing				
				c.734C>T p.(Thr245Met) rs3710700915	0.000032	0.0000309		Deleterious (0.00)	Probably damaging (0.998)	Disease causing (0.999)	Damaging (0.753)
				c.1299C>T p.(His433=) rs61733667	0.02721	0.03546					

	Associated variants										
ACE	Angiotensin-I converting enzyme	Kininase II	106180	c.3053T>C p.(Ile1018Thr) rs4976	0.00115	0.000037		Deleterious (0.00)	Probably damaging (0.999)	Disease causing (0.999)	Tolerated (0.099)
F12	Factor XII, *alias* Hageman factor	Kallikrein-kinin system (KKS)	610618	c.-4T>C rs1801020 common SNP	0.6522	0.348030					
HRH1	Histamine H1 receptor	Endothelial H1 histamine receptor	600167	c.42G>A p.(Met14Ile) rs79314450	0.0014	0.00053		Tolerated (0.058)	Possibly damaging (0.541)	Disease causing (0.946)	Tolerated (0.024)
KLKB1	Plasma prekallikrein	KKS	229000	c.689T>A p.(Ile230Asn) rs142420360	0.000180	0.0003406		Deleterious (0.00)	Probably damaging (0.999)	Disease causing (0.992)	Tolerated (0.315)
MASP2	Mannan-binding lectin associated protease 2	Complement convertase	613791	c.352C>T p.(Arg118Cys) rs147270785	0.00051	0.00064		Deleterious (0.02)	Benign (0.143)	Disease causing (0.999)	Tolerated (0.173)
MPO	Myeloperoxidase	Neutrophil /mast cell granule enzyme	606989	c.752A>G p.(Met251Thr) rs56378716	0.01259	0.01317		Deleterious (0.00)	Benign (0.032)	Disease causing (0.999)	Tolerated (0.088)
SERPINC 1	Antithrombin III	Control of coagulation, KKS and plasmin	107300	c.749C>T p.(Thr250Ile) rs144084678	0.00003	0.00005		Deleterious (0.00)	Probably damaging (0.993)	Disease causing (1.00)	Damaging (0.841)
SERPING 1	C1 Inhibitor	Control of KKS and complement	606860	c.1438G>A p.(Val480Met) rs4926	0.212	0.274		Tolerated (0.084)	Benign (0.034)	Polymorphism (0.999)	Tolerated (0.037)
XPNPEP2	Membrane X-Prolyl aminopeptidase (aminopeptidase P)	Membrane kininase	300145	c.-2399C>A rs3788853	0.2261	0.2233					
				c.644C>T rs138365897	0.00236	0.00349		Deleterious (0.01)	Possibly damaging (0.871)	Disease causing (0.995)	Tolerated (0.043)

KKS, kallikrein-kinin system; [a] OMIM, On-line Mendelian Inheritance in Man; URL omim.org/entry/; [b] MAF, Minor Allele Frequency;

[c] gnomAD, Genome Aggregation Database v2.1.1; URL gnomad.broadinstitute.org; [d] HSF 3.0, Human Splicing Finder system ; URL hsf.genomnis.com; [e] SIFT, Sorting Intolerant From Tolerant; URL sift.bii.a-star.edu.sg/; [f] PolyPhen-2, Polymorphism Phenotyping v2; URL genetics.bwh.harvard.edu/pph2/; [g] MutationTaster, URL mutationtaster.org; [i] ClinPred, URL sites.google.com/site/clinpred/



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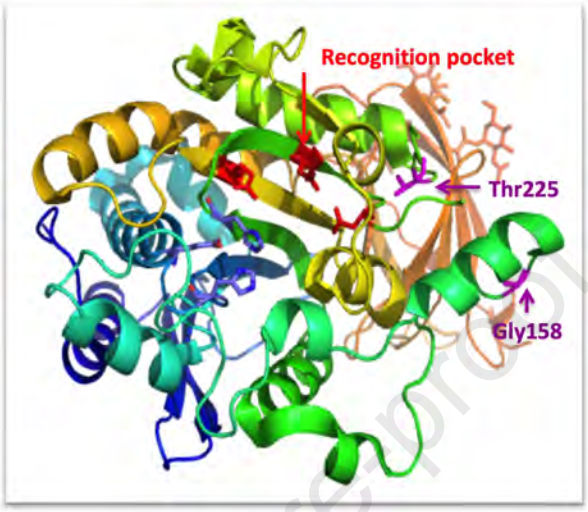

D Vincent et al - Figure 2