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► **To cite this version:**

Denise Ponard, Christine Gaboriaud, Delphine Charignon, Arije Ghannam, Ineke Wagenaar-Bos, et al.. SERPING1 mutation update: Mutation spectrum and C1 Inhibitor phenotypes. *Human Mutation*, 2020, 41 (1), pp.38-57. 10.1002/humu.23917 . hal-02429966

HAL Id: hal-02429966

<https://hal.science/hal-02429966>

Submitted on 24 Sep 2020

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Title

***SERPING1* mutation update: Mutation spectrum and C1 Inhibitor phenotypes**

Running title

Description and phenotype analysis of *SERPING1* variants

Authors

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/humu.23917.

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Conflict of Interest

DC and AG received funding from KininX. All the other authors declare no conflict of interest. ALL has received educational and advisory fees from Shire.

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DATA SHARING STATEMENT

Repository of novel variants. Novel variants (n=120) have been successfully introduced at Global Variome shared LOVD; URL

<https://databases.lovd.nl/shared/individuals/SERPING1>.

Abbreviations: C1Inh, C1 Inhibitor; C1-INH-HAE, HAE with C1Inh deficiency; EQT, exon quantitation technique; ESE, exonic splicing enhancers; ESS, exonic splicing silencers; HAE, hereditary angioedema; HAE-I/HAE-II/HAE-intermediate, HAE type I/type II/intermediate type; ISE, intronic splicing enhancers; ISS, intronic splicing silencers; KKS, kallikrein-kinin system; RCL, reactive centre loop; UTR, untranslated.

Abstract

C1 Inhibitor (C1Inh) deficiency is responsible for hereditary angioedema (C1-INH-HAE) and caused by variants of the *SERPING1/CIINH/C1NH* gene. C1Inh is the major control of kallikrein-kinin system. C1Inh deficiency leads to its uncontrolled activation, with subsequent generation of the vasoactive peptide bradykinin. This update documents 748

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different *SERPING1* variants, including published variants and additional 120 unpublished ones. They were identified as heterozygous variants (n=729), as homozygous variants in 10 probands) and as compound heterozygous variants (9 combinations). Six probands with heterozygous variants exhibited gonadal mosaicism. Probands with heterozygous (n=72) and homozygous (n=1) variants were identified as *de novo* cases. Overall, 58 variants were found at positions showing high residue conservation among serpins, and have been referred to as a mousetrap function of C1Inh: reactive centre loop, gate, shutter, breach, and hinge. C1Inh phenotype analysis identified dysfunctional serpin variants with failed serpin-protease association and a residual 105-kDa species after incubation with target protease. Regarding this characteristic, in conditions with low antigenic C1Inh, 74 C1-INH-HAE probands presented with an additional so-called intermediate C1-INH-HAE phenotype. The present update addresses a comprehensive *SERPING1* variant spectrum that facilitates genotype-phenotype correlations, highlighting residues of strategic importance for serpin function and for identification of C1Inh deficiency as serpinopathy.

Key words: C1 Inhibitor; *SERPING1*; mutational spectrum; hereditary angioedema; protease control; serpin; serpinopathy; structure-function relationship

1 BACKGROUND

C1 Inhibitor (C1Inh) is a multi-functional Serine protease inhibitor (serpin) that controls various Serine proteases in multiple plasmatic cascades, *eg.* contact phase, thereby limiting the production of the vasoactive peptide bradykinin [Davis et al., 2010]. C1Inh deficiency is responsible for hereditary angioedema (C1-INH-HAE). Today, C1-INH-

HAE is considered a heterogeneous disorder with a complex pathophysiology [Hofman et al., 2016]. Genetic defects of C1-INH-HAE patients have been recognized in the *SERPING1* gene, encoding C1Inh and located at 11q12-q13.1 in humans; pathogenic variants usually behaved in an autosomal-dominant character with incomplete penetrance and an uncertain prevalence estimated at 1:50,000. Haploinsufficiency can occur through a number of ways; some rare variants, *eg.* those found with as homozygous variants or as compound heterozygous variants may be quantitatively too weak to cause haploinsufficiency, are illustrated in section 4.1 with an example. In addition, *SERPING1* alleles affecting secretion of wild-type C1Inh protein in a dominant negative fashion are also introduced in section 4.1. In the original description of *SERPING1* gene defects, the variants have been distributed in a type 1 (HAE-I) where “C1-INH-HAE appears to result from failure to synthesize the esterase inhibitor”, and a type 2 (HAE-II), where “an abnormal, non-functional protein is synthesized” [Rosen et al., 1965]. These defects consisted of deletions/duplications [Stoppa-Lyonnet et al., 1987] and other variants [Agostoni et al., 2004; Maurer et al., 2018], and this genetic disease has been referred to as HAE due to C1Inh deficiency, *ie.* C1-INH-HAE, with MIM #606860. Affected patients presented with non-pruritic, subcutaneous, and submucosal episodes of localized swelling in the extremities, face, gut, or upper airways. Laryngeal oedema caused by upper respiratory mucosal involvement may lead to asphyxia [Agostoni et al., 2004]. Reduced plasma levels of C1Inh lead to enhanced activation of the kallikrein-kinin system (KKS) triggering accumulation of kinins and increased vascular permeability. Recent biological testing performed on C1-INH-HAE patients has suggested that C1Inh deficiency affects KKS control, more than that of C1 activation [Charignon et al., 2017].

C1Inh is a single-chain, highly glycosylated circulating protein of Mr 105kDa (Figure 1A). As a serpin (clade G), it regulates Serine proteases *via* an irreversible suicide substrate mechanism [Lomas et al., 2005]. Serpins are highly conserved proteins, with the serpin domain comprising three β -sheets (A-C) and nine α -helices (Figure 1B-E). By analogy with the archetypal serpin α 1-antitrypsin (A1AT), the reactive site is identified by a peptide bond between positions referred to as P1-P1' and is located in an exposed, flexible loop (RCL). C1Inh RCL extends from P15 to P5', with P15-P1 representing the affinity-based insertion sequence as strand 4A (s4A) into the central β -sheet A [Bos et al., 2002]. Serpins are unique in that the native molecule is not the most stable form, but a kinetically trapped intermediate [Patston et al., 1995]. Besides the native and cleaved forms, serpins can adopt additional, more stable conformations that have been shown to be biologically relevant. One of these is a latent, inactive form, adopted naturally by plasminogen activator inhibitor-1 and antithrombin-III, in which the uncleaved RCL is inserted into β -sheet A as s4A, and the C-terminal peptide stalk and s1C are reoriented along the side of the molecule, as shown for latent C1Inh [Beinrohr et al., 2007] (Figures 1C, -E). Multimeric conformations have also been identified in certain naturally occurring variants affecting the C1Inh mobile domains [Eldering et al., 1995; Madsen et al., 2014; Haslund et al., 2019]. Such variants render the molecule vulnerable to retention within the biosynthesis cell compartment, a hallmark of serpinopathies [Lomas et al., 2005]. *SERPING1* variant investigation has revealed several key genetic features, which have recently been reviewed [Germenis & Speletas, 2016]. More than 500 distinct variants are listed in the Human Gene Mutation Database (HGMD[®]; <http://www.hgmd.cf.ac.uk/>;

survey on 18th Feb 2018) [Stenson et al., 2017]. A C1Inh-specific mutational database of the *SERPING1* locus, with updated reports, has been described [Kalmár et al., 2005].

In this mutation update, we selected the peer-reviewed articles and HGMD[®] as base for known variants and documented 120 previously unpublished variants for reviewing the current state of our knowledge of pathogenic or likely pathogenic *SERPING1* variants. We also discuss variant positions of strategic importance in structure-function relationships in the ongoing international efforts toward the recognition of C1Inh deficiency as a conformational disease.

2 METHODS

SERPING1 variant extraction

All of the variants of *SERPING1* reported in peer-reviewed articles, locus-specific HAEdb (URL <http://hae.biomembrane.hu/>) or HGMD[®] and identified in subjects ascertained on the basis of a C1-INH-HAE diagnostic were included in the update. *SERPING1* genotypes were recorded from the original publications and adjustment of nomenclature of certain variations was made to ascertain that base pair and predicted protein sequence numbering were based on the *SERPING1* reference sequence NCBI #NM_000062.2 that should be taken for any variant with prefix “c.”.

Subjects

Unrelated C1-INH-HAE patients (n = 379), who met the international consensus Criteria [Maurer et al., 2018], were enrolled and subjected to molecular analysis of the *SERPING1* gene. Each diagnosis of C1-INH-HAE was supported by two separated

determinations of C1Inh functional levels [Drouet et al., 1988], which were mainly below 30% of the target value of a reference population. Reference values have been obtained from samples harvested from healthy blood donors (Etablissement Français du Sang, Grenoble France).

Biological testing

Antigenic C1Inh was quantified using nephelometry (BNII; Siemens Healthcare Diagnostics, Marburg, Germany); C1Inh function was measured using a chromogenic assay with C1s protease [Drouet et al., 1988] or contact-phase proteins as targets [Ghannam et al., 2015]. Anti-C1Inh immunoblot was performed on 7.5% SDS-PAGE under non-reducing conditions.

Genotyping

From samples of patients enrolled in the study, genomic DNA was isolated using a guanidine method, the MagNA Pure[®] System (Roche Life Science, Meylan, France), or the NucleoSpin Blood[®] kit (Macherey-Nagel, Düren, Germany) on a JANUS[®] Workstation (PerkinElmer). For variant analysis, the promoter sequence, the noncoding exon 1, as well as the 7 coding exons and intron-exon boundaries of the *SERPING1* gene (NCBI #NM_000062.2) were amplified from genomic DNA. Amplicons were then subjected to direct automated sequencing on an Applied Biosystems 3130[®] DNA Analyser (Life Technologies, Carlsbad, CA, USA). In cases of negative screening by Sanger, multiplex ligation-dependent probe amplification (MLPA) was subsequently performed to analyse genomic rearrangements using the SALSA MLPA P243[®] SerpinG1 kit and data were analysed using the COFFALYSER[®] software (MRC-Holland,

Amsterdam, NL). Genomic deletion of isolated or joint exons was confirmed by long-range PCR from the flanking exons using the Takara-LA DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) and analysis of the amplification products on 0.8% agarose gel. *SERPING1* variations were numbered starting from the first nucleotide of the initiation Methionine of the cDNA sequence (NCBI #NM_000062.2) according to the Human Genome Variation Society guidelines. For amino acid positions and, as is usual for serpins, we used historical numbering based on the 478-residue mature protein (without the 22 N-terminal residues of the signal peptide). For genomic positioning, the base assignment defined by Carter et al. [1991] was mentioned in order to enable comparisons with other nomenclatures for *SERPING1* (see the database HAEdb).

Human Gene Mutation Database (HGMD[®])

Identified *SERPING1* variants have been collected from the on-line HGMD[®], with additional unregistered variants, personal identifications and recent descriptions.

Bioinformatics

Bioinformatic analysis of all *SERPING1* missense variants identified in the probands we studied and of variants reported to HGMD[®] was performed using three different software packages: SIFT[®] (Sorting Intolerant From Tolerant; URL sift.bii.a-star.edu.sg/; [Sim et al. 2012]), PolyPhen2[®] (Polymorphism Phenotyping v2; URL genetics.bwh.harvard.edu/pph2/; [Adzhubei et al. 2013]) and MutationTaster[®] (URL mutationtaster.org; [Schwartz et al. 2014]). For determining Minor Allele Frequency (MAF) of some variants, we used the Genome Aggregation Database v2.1.1, acronym gnomAD (<http://gnomad.broadinstitute.org/gene/ENSG00000149131>).

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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 DC-2008-634). Informed written consent for molecular genetic analysis was obtained from patients. In addition, the last-enrolled patients provided their informed consent to participating in the investigation with biological assays, and blood donors (healthy controls) answered the standard questionnaire. The Grenoble University Hospital's Institutional Review Board (South-East committee V) stated that the supporting programs, sample collection and processing methods fulfilled the requirements. All the data were processed anonymously.

3 VARIANTS OF *SERPING1* IN C1-INHIBITOR DEFICIENCY

3.1 The spectrum of *SERPING1* variants causing C1-INH-HAE

Supplementary material displays the *SERPING1* alterations that were found in the HGMD[®] tables and the published reports and that we have observed in probands. As shown on Figure 2, the overall *SERPING1* variants comprise missense variants (n = 241; 32.1%) and nonsense variants (n = 66; 9.0%), compiled in Supp. Table S1, short deletions, duplications and delins variants (n = 272; 36.2%; Supp. Table S2), splice defects (n = 100; 13.6%; Supp. Table S3), large deletions and duplications (n = 62; 8.2%; Supp. Table S4) and a few variants affecting 5' untranscribed and 3' UTR sequences (n = 7; 0.9%; Supp. Table S5), amounting to a total of 748 distinct variants and including 120 novel variants. The latter and recurrent variants have been identified in 379 pedigrees that we investigated in the present study. The mature C1Inh N-terminal domain (residues 1-

112; Figure 1A) displayed nine missense variants (9/241; 3.7%), while its serpin domain (residues 113-478) presented almost all missense variants (232/241; 96.3%). This variant distribution was in a close relationship with C1Inh domains, with the few missense variants considered as disease-causing suggesting a non-functional N-terminal domain that is specific to C1Inh among serpins. This is in line with the independent biological properties of both domains. Overall, ten homozygous deficient probands were recorded, all but one were symptomatic, five with a missense variant, two with a splicing defect and one with a delins variant (Supp Table S7). Compound heterozygosity has been found in 9 pedigrees. Gonadal mosaicism has been reported in 6 probands and two young brothers were severely affected with asymptomatic parents [Guarino et al., 2006]. Probably these cases are underestimated because of non-systematically investigated. Overall, 73 C1Inh deficient probands presented with a *de novo* variant, *ie.* in a patient with the disease and no family history, with an absence of pathogenic variant detected in both parental DNA samples. As suggested by the repetitive occurrence of variant reports (Supp. Tables S1-3), some positions/regions of *SERPING1* gene could represent hotspots of gene alteration; of particular interest are the codons 1, 184, 271 to 272, 466 and 494.

Missense variants. In every case when it has been reported, a genetic variant was connected to a biological phenotype: serpin function of C1Inh, circulating species. While identified in relation to HAE-II phenotypes, the c.1198C>T;p.(Arg378Cys)/(Arg400Cys) mutant has been proved hypomorphic because of a tight control exerted over C1s protease, with a decrease in the constant of inhibition of $\approx 25\%$, more than over contact-phase activation, with $\approx 45\%$ [López-Lera et al., 2010; Ghannam et al., 2015], and a remittent serpin function [Caccia et al., 2018]. The Arg³⁷⁸ position was also recognised as

being involved in the polyanion potentiation of C1Inh function (Figures 1B, -D; Tables 1, -2). These observations are in line with control of C1s protease by polyanions, but not of factor XIIa or kallikrein [Wuillemin et al., 1996]. Conversely the c.452T>G;p.(Leu129Arg)/(p.Leu151Arg) has retained control of contact phase [Ghannam et al., 2015]. Glycosylation positions were disrupted at only three positions: Thr⁵⁴, with c.227C>T;p.(Thr54Ile)/(Thr76Ile), Asn²⁵⁰, with c.814_816delAAC;p.(Asn250del)/(Asn272del) and c.814_816dupAAC;p.(Asn250dup)/(Asn272dup) and Asn³³⁰, with c.1055A>T;p.(Asn330Ile)/(Asn352Ile). Glycosylation at the latter position has been found of strategic importance, as removal of the oligosaccharide resulted in a dramatic decrease of the expression yield [Rossi et al, 2010]. Missense variants were analysed so as to predict protein structure folding by means of three different bioinformatic methods (SIFT[®], PolyPhen2[®], and MutationTaster[®]). Conflicts between bioinformatics prediction methods are observed for some variants, *eg.* c.467C>A;p.(Ala134Asp)/(Ala156Asp), c.568A>G;p.(Lys168Glu)/(Lys190Glu), c.722C>G;p.(Arg219Pro)/(Arg241Pro), c.785T>C;p.(Ile240Thr)/(Ile262Thr) and c.1198C>T;p.(Arg378Cys)/(Arg400Cys), that were found to be either tolerant (SIFT[®]), probably damaging (PolyPhen2[®]), and disease causing (MutationTaster[®]), or *vice-versa*, for protein function and disease-causing predictions (Supp. Table S1). In addition to C1Inh variants associated with a loss of protease targeting, recurrent variants that have been established as disease-causing for years [c.1396C>A;p.(Arg444Ser)/(Arg466Ser), c.1397G>A;p.(Arg444His)/(Arg466His), and 1396C>G;p.(Arg444Gly)/(Arg466Gly)] were identified as tolerant or benign, because its cleavage-site property is not taken into account (Table 1). Analysis using

MutationTaster[®] attributed some HAE-II variants to polymorphisms, with affected pedigrees developing severe clinical phenotypes and patients presenting with C1Inh function below 30% of the target value. This apparent discrepancy could be pertaining to additional factors modifying clinical and biological phenotypes. The variants involved are c.1319C>T, p.(Ala440Val)/(Ala418Val); c.1378T>C, p.(Ser460Pro)/(Ser438Pro) and c.1399A>C, p.(Thr467Pro)/(Thr445Pro). However, distinguishing possibly harmless, non-pathogenic variants from *bona-fide* disease-causing ones by means of bioinformatic analyses alone might not be accurate enough to predict effects of these variants on serpin function.

Splicing defects. Variants affecting splice sites and those in splicing regulatory elements, incl enhancers and silencers, can impair pre-mRNA splicing, eventually leading to a severe phenotype, as suggested by Grodecká et al. [2017]. *SERPING1* is a naturally alternatively spliced gene, with in particular a highly variable abundance and a subtle regulation of exon 3 skipping [de la Cruz et al., 2012; Grymová et al., 2019]. Despite the availability of many prediction tools, prognosis of splicing affection is not trivial, especially when regulatory elements are involved. The presence of splicing defects was confirmed by exon skipping in 24 of non-canonical sequences using blood-derived patients' RNAs. In addition, the c.686-12A>G and c.1250-13G>A introduce *de novo* splice sites in introns 4 and 7, respectively. Splicing has been demonstrated to be affected by exonic variants, c.-21T>C (see above), by modifying the second nucleotide of exon 2, c.550G>A, c.550G>C and c.550G>T, by introducing substitutions on the last position of exon 3, c.685delG, with deletion of the last nucleotide of exon 4, and c.882C>G and c.884T>G, with substitutions close to the 3' end of exon 5, as observed on patients'

RNAs [Roche et al., 2005; Grodecká et al., 2017] and complemented by using a minigene construct [Grodecká et al., 2017].

Large deletions and duplications. These variants resulted from recombination promoted by two different *Alu* elements dispersed within *SERPING1* gene [Carter et al., 1991]. *Alu* sequences are movable repetitive elements that are responsible for nearly 10% of the human genome and may represent hotspots for non-homologous recombination that could cause various hereditary diseases. Nineteen *Alu* sequences have been detected within the seven introns of *SERPING1*, most of them are found in introns 4 and 6 [Germanis & Speletas, 2016]. These variants can be detected but not uncovered by MLPA, the reference technique for detection of large deletions and duplications/insertions [Kozłowski et al., 2008; Johnsrud et al., 2015]. Using MLPA could result in a false negative rate. Very recently, a precise mapping with identification of boundaries of multiexon deletion or duplication has been developed and standardized using a targeted next-generation sequencing platform [Loules et al., 2018], an Exon Quantitation Technique (EQT) [Nicolicht et al., 2019] or a combination of MLPA and direct sequencing of boundaries [Wong et al., 2019]. This accurate identification of 5' and 3' boundaries specifically establishes a deletion/duplication variant that differs or not from each other. Sixty-four variants of this category have been recorded in Supp. Table S4, but the boundaries have not been determined at the nucleotide level for most of them.

3.2 Polymorphisms

Many polymorphisms in *SERPING1* gene that are not causative for HAE could have an impact on the phenotype, as observed in some examples (Supp. Table S6). *SERPING1*

SNPs have been found to contribute to C1-INH-HAE with an incomplete penetrance and variations between alleles, *eg.* with deleterious consequence on *SERPING1* gene expression; Supp. Table S6 shows some variants partially or not described in the databases gnomAD Browser[®], 1000 Genomes Browser[®] or Variation Viewer[®]. (i) SNPs possibly affect splicing activities by introducing/disrupting intronic or exonic splicing regulatory elements [Wang & Burge, 2008], *eg.* c.52-130C>T (*rs1005510*), c.129G>T;p.(Gly21=)/(Gly43=) (*rs149573972*), c.285C>A;p.(Thr73=)/(Thr95=) (*rs886048400*), c.468C>T;p.(Ala134=)/Ala156=) (*rs150601964*), c.579G>A;p.Leu171=)/(Leu193=) (*rs763981414*), c.689T>C;p.Leu208Pro)/(Leu230Pro) (*rs281875171*), c.1218C>T;p.Ser384=)/(Ser406=) (*rs11229070*) or c.1257C>T (*rs775042436*). (ii) Exonic SNPs could be taken for pathogenic by the *in silico* applications SIFT[®] and Poly-Phen2[®], *eg.* c.461A>G;p.(Tyr132Cys)/(Tyr154Cys) (*rs281875168*), c.509C>T;p.(Ser148Phe)/(Ser170Phe) (*rs281875169*), c.550G>A;p.(Gly162Arg)/(Gly184Arg) (*rs281875170*), c.689T>C;p.(Leu208Pro)/(Leu230Pro) (*rs281875171*), c.695T>A;p.(Ile210Lys)/(Ile232Lys) (*rs281875172*), c.895T>C;p.(Trp277Arg)/(Trp299Arg) (*rs281875173*), c.1289T>A;p.(Leu408Gln)/(Leu430Gln) (*rs281875174*), c.1418T>G;p.(Val451Gly)/(Val473Gly) (*rs281875177*) or c.1475T>C;p.(Met470Thr)/(Met492Thr) (*rs978962357*). (iii) Pathogenic combinations of SNPs, *eg.* c.1030-20A>G (*rs2511988*) with c.922A>G;p.(Thr286Ala)/(Thr308Ala) (*rs1802212*) or with an exon 2 transition c.-21T>C (*rs28362944*), attributed to activate a cryptic acceptor site with exon 2 skipping, have been suggested to introduce a severe

phenotype when present in *trans* with a deleterious variant [Cumming et al., 2003; Duponchel et al., 2006]. (iv) Other ones were informative, *eg.* c.1438G>A;p.(Val458Met)/(Val480Met) (*rs4926*) previously reported not to affect serpin function.

3.3 Combined alleles

It should be highlighted that compound heterozygosity of *SERPING1* alleles with incomplete penetrance can cause pathogenicity as documented by the following examples. (i) Combined variant c.[5C>T(;1045C>T];p.[(Ala21Val)/(Ala2Val)(;);p.(Leu327Phe)/(Leu349Phe)] was associated with a pathogenic HAE-I phenotype by two daughters, while heterozygous parents are asymptomatic, with normal C1Inh function and expression. In a *cis* combination c.[49G>A(;953C>G]; p.[(Gly6Arg)/(Gly17Arg)(;);p.(Ser296*)/(Ser318*)], the c.49G>A;p.(Gly-6Arg)/(Gly17Arg) allele exacerbated functional impairment of C1Inh caused by c.953C>G;p.(Ser296*)/(Ser318*), with a severe phenotype of the carrier [Xu & Zhi, 2018]. With a *trans* combination of one recurrent and one novel C1Inh variant, c.[820A>G(;856C>T]; p.[(Ile252Val)/(Ile274Val)(;);p.(Arg264Cys)/(Arg286Cys)] and c.[856C>T(;1361T>A]; p.[(Arg264Cys)/(Arg286Cys)(;);p.(Val432Glu)/(Val454Glu)] were associated with HAE. (ii) Examples could be given by the above description of SNPs with c.-21T>C;*rs28362944* conferring severity when combined in *trans* with another *SERPING1* variant and c.1030-20A>G;*rs2511988*, also found associated with age-related macular degeneration [Kralovicova & Vorechovsky, 2009], when combined with c.922A>G;p.(Thr286Ala)/(Thr308Ala);*rs1802212*; some other examples are found

in Supp. Table S6. In addition to compound heterozygosity of different *SERPING1* variants, a combination of a non-pathogenic *SERPING1* variant and a variant in other HAE susceptibility genes can result in a severe C1-INH-HAE phenotype as shown by the following examples. (i) Combinations with other HAE susceptibility genes have been reported with a severe C1-INH-HAE phenotype, when *SERPING1* variants are combined with c.-46C/T genotype of the *F12* gene (*rs1801020*; MAF 0.472) [Bors et al., 2013; Speletas et al., 2015b] and c.51+3A>G variant of the *SERPING1* gene with c.1032C>A;p.(Thr309Lys)/(Thr328Lys) of the *F12* gene [Charignon et al., 2018]. (ii) In almost all symptomatic patients of a big pedigree presenting with C1Inh deficiency, multiple allele combinations have been identified with c.1369G>C;p.(Ala435Pro)/(Ala457Pro) variant of the *SERPING1* gene and *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)], *KNIG1* (c.533T>C;p.(Met178Thr) or c.591T>G;p.(Ile197Met)], *NOS3* (c.894T>G;p.(Glu298Asp) or c.2654G>T;p.(Arg885Met)] and *PRCP* (c.336A>T;p.(Glu112Asp)] alleles [Veronez et al., 2016]. These observations are congruent with the emerging concept where symptomatic manifestations of C1Inh deficiency should be, at least partially, determined by genetic variations in another/other gene(s) encoding protein(s) involved in kinin metabolism pathways.

3.4 *De novo* variants

Probands presenting with *SERPING1 de novo* variants were identified (n = 73), that might represent 5.6% of the probands (73/1294), a number that possibly may be related to the kindreds, but lower than that has been reported [Pappalardo et al., 2000; Germenis and Speletas, 2016]. These sporadic cases of angioedema were clinically and biochemically indistinguishable from those with a proven family story. Corresponding variants (missense/nonsense, short deletion/duplication/insertion, splicing defect, large deletion) were distributed between all the exons and exon/intron boundaries.

3.5 Unsuccessful findings in probands with C1-INH-HAE

Taken from our probands and using Sanger and MLPA methods, no *SERPING1* gene alterations have been identified in 24 pedigrees presenting with biological and clinical criteria of C1-INH-HAE (24/379; 6.3%). This percentage value is in agreement with other studies. As suggested by Speletas et al. [2014], a causative defect modifying C1Inh expression may be located in an intronic or untranslated region of the gene, and post-translational alterations affecting protein structure and/or function could explain unsuccessful molecular analyses in a few probands. As recently demonstrated by Nicolicht et al. [2019], EQT could be advantageously developed for detection of large rearrangements of *SERPING1*, with reports of their boundaries in C1INH-HAE patients, whereas no variants in *SERPING1* has been identified by conventional methods. Beside, analysis of transcripts could support the identification of variants in unsuccessful findings.

4 FUNCTIONAL PHENOTYPE

4.1 Haploinsufficiency of C1-INH-HAE

Most HAE-I patients are heterozygous for variants in the *SERPING1* gene and maintain production of functional C1Inh only from a single wild-type *SERPING1* allele. Surprisingly and rather than the 50% value that might be expected, plasma values of C1Inh are usually 5-30% of normal. This reduction could be caused by degradation/inactivation of the variant carrying allele by means that prevent (co-) transcription or abolish mRNA expression post transcriptionally. Laimer et al. [2006] proposed that C1Inh deficiency leads to a similar/analog dosage-dependent loss of C1Inh function induced by haploinsufficiency. A subsequent increased activation of some or all of the zymogens under C1Inh control and the subsequent higher catabolism of remaining C1Inh, a decreased production or compromised intracellular transport and segregation of the protein have been suggested to account for this phenomenon [Woo et al., 1985]. Regarding the genetic aspect with a transmission according to a dominant mode, some cases with biallelic *SERPING1* variants are contradictory. Pertaining to this hypothesis, most of homozygous probands carry non-recurrent variants, where heterozygosity has exceptionally been found associated with a clinical phenotype. Taking the c.1385T>G;p.(Ile440Ser)/(Ile462Ser) variant as an illustrative example, the clinical manifestations segregated in the pedigree according to an autosomal recessive mode of inheritance; both homozygotes were affected (Supp Table S7). Even if antigenic C1Inh and function were found lower than normal in the heterozygous individuals, only one in five carriers was pauci-symptomatic [Blanch et al, 2006]. The homozygous patients

exhibited low antigenic C1Inh and function because of its cleavage, consumption, or both, in concordance with their higher complement or KKS activation, but not attributed to a loss of c.1385T>G allele expression. Heterozygotes also presented with a cleaved C1Inh and a remaining C1Inh function measured in plasma attributed to the wild type allele: The c.1385T>G;p.(Ile440Ser)/(Ile462Ser) caused a lack of function but did not severely affect the secretion/function of the wild type allele, consistently with a slight C4 level reduction. Importantly, Haslund et al [2019] demonstrated that C1Inh variants encoded by a subset of C1-INH-HAE-causing *SERPING1* alleles affected secretion of wild-type C1Inh protein in a dominant negative fashion by triggering formation of protein-protein interactions between normal and mutant C1Inh. These alleles include c.508T>C, p.(Ser170Pro)/(Ser148Pro); c.530T>C, p.(Leu177Pro)/(Leu155Pro); c.566C>A, p.(Thr189Asn)/(Thr167Asn); c.707T>C, p.(Phe236Ser)/(Phe214Ser); c.773A>T, p.(Asn258Ile)/(Asn236Ile); c.871A>C, p.(Asn291His)/(Asn269His); c.1322T>C, p.(Met441Thr)/(Met419Thr); c.1342G>C, p.(Glu448Gln)/(Glu426Gln); c.1417G>A, p.(Val473Met)/(Val451Met); c.1427C>T, p.(Pro476Leu)/(Pro454Leu) (Supp. Table S1). Subsequent intracellular C1Inh aggregates were trapped in the endoplasmic reticulum, disrupting the expression of the wild-type protein.

4.2 Identification of an intermediate C1-INH-HAE type

The abundance of missense variants prompted a study aimed at investigating the possible expression of C1Inh alleles. In order to test this hypothesis, we took as a basis the early observation of a distinction between HAE-I and HAE-II developed by Rosen et al. [1965], where HAE-II diagnostic is retained when an abnormal, non-functional protein is

synthesized. Thus, C1-INH-HAE patient samples were investigated using anti-C1Inh immunoblot analysis. After incubation of a patient sample with target protease, a non-functional C1Inh variant product was identified by its *Mr* and ascribed to a biological deficient phenotype with failed serpin-protease association and a residual 105-kDa molecular species on SDS-PAGE (Figure 3). Seventy-three C1-INH-HAE probands presenting with low antigenic C1Inh and non-functional C1Inh variants have been identified, with 40 variants including 14 new ones (Supp. Tables S1 and -2). This type mimics HAE-I through low C1Inh levels, on the one hand, and HAE-II through expression of *SERPING1* variant, on the other hand. The corresponding C1Inh-deficient condition could be referred to as intermediate type, *ie.* HAE-intermediate, in agreement with Bors et al. [2013], and indicated as INT in Supp. Tables S1 and -S2. The variants associated with HAE-intermediate biological phenotype are distributed within exons 3-8 (Figure 4) and covers the entire serpin domain, with hot spots in identified structural domains, *ie.* mainly shutter, gate and breach (Figure 1B, -C).

Pertaining to the model developed by Haslund et al [2019], the HAE-intermediate type identifies some C1Inh deficiency situations where the intracellular non-functional allele is expressed, but circulating at low rate and mimicking a HAE-I phenotype. These variants with multimerisation ability are introduced in Class III of missense variants, as developed below and shown in Table 1. The effect of frameshifts, large deletions and duplications could be readily explained as being associated with HAE-I condition.

5 GENOTYPE-FUNCTIONAL PHENOTYPE

The trypsin-like Serine protease specificity of serpins is primarily determined by the nature of the residue at the P1 and P2 positions, *ie.* Arg⁴⁴⁴ and Ala⁴⁴³ for C1Inh. The control capacity of C1Inh is attributable to its ability to target a protease and to trap it according to the mousetrap model of serpins. As proposed by Bos et al. [2002], C1Inh requires adequate exposure of the RCL (Figure 1B), with the association constant (k_{on}) reflecting the rate of protease binding. The protease requires a fast insertion of the RCL into the central β -sheet after bonding between the active site Serine of the protease and the P1 Arg⁴⁴⁴ of C1Inh. The ability to keep the protease trapped is expressed with the dissociation constant (k_{off}). Bos et al. [2002] suggested that a molecule with a high k_{on} and a high k_{off} is not an inhibitor, but rather a suitable substrate. Subsequently, the *SERPING1* variants associated with HAE-II (and HAE-intermediate, as well) were categorized into three classes: (i) Class I included variants that lead to altered exposure of the active site, *eg.* variants at the P1 Arg⁴⁴⁴ and P2 Ala⁴⁴³, influencing target protease recognition;; (ii) Class II comprised variants leading to disturbed insertion of the RCL into central β -sheet, the serpin then becoming a substrate instead of an inhibitor with insufficient trapping mechanism, *eg.* proximal loop-reactive hinge mutants at the P14, P12, and P8 residues of C1Inh, *ie.* Val⁴³², Ala⁴³⁴, or Ser⁴³⁸ [Davis, 1997], and of the other serpins [Stein & Carrell, 1995; Irving et al., 2000], as well; (iii) Class III represented variants with spontaneous insertion of the RCL into the β -sheet A of the same or another molecule, producing a latent structure or polymers; *eg.* variants observed in the C-terminal hinge region [Eldering et al., 1995], or afflicting C1Inh at the shutter or the breach region [Madsen et al., 2014, Haslund et al. 2019]. As now being set for plasma

[Madsen et al., 2014] and within cell [Haslund et al., 2019], methods to identify C1Inh oligomers will introduce variants into Class III. Oligomerised C1Inh has been demonstrated to promote kallikrein-system activation [Madsen et al. 2015], subsequently accounting for an amplification process of kinin forming in C1-INH-HAE pathophysiology. Table 1 shows the phenotypic distribution of some *SERPING1* variants, mostly of the HAE-II and HAE-intermediate groups. By using the rating system in 3 classes, introduction of variants in 2 or 3 classes, *eg.* at positions Asn²⁵⁰, Arg³⁷⁸, Val⁴³², Ala⁴³⁴, Ala⁴³⁶, Ser⁴³⁸ (Table 1), suggests that there is no single mechanism underlying molecular dysfunction.

HAE-II phenotype is commonly attributed to a qualitative defect, and characterised by normal (or elevated) plasma C1Inh concentrations associated with low C1Inh function, and it traditionally accounts for 15% of all C1Inh-deficient cases. This condition has been shown to be associated with missense variants located mainly at exon 8 within the RCL (Figure 4A), as well as at two critical hinge regions upstream (proximal hinge) and downstream (distal hinge) of the RCL, but not in the shutter or the gate (Figures 1C, -D). Other ones have been observed in conditions of in-frame deletion/duplication variants at positions Ile¹⁷⁴-Tyr¹⁷⁷, Ile¹⁷⁴-His¹⁸⁵, Asn²⁵⁰, Lys²⁵¹, Ser²⁵⁸-Pro²⁶⁰, Val²⁶⁶, Val³⁰⁰, Met³⁰³ and Gly⁴³¹ (Supp. Table S2). Missense variants associated with an intermediate type distributed throughout exons 3 to 8 (Figure 4B).

6 STRUCTURE-FUNCTIONAL PHENOTYPE

6.1 Structure-functional phenotype associations

Native and latent C1Inh structures show how missense variants cluster in the 3-D space (Figures 1B, -C; respectively). Some variants are illustrated on the structure, in line with their distribution into three functional classes, as developed above (Table 1). Those that alter crucial positions maintaining correct protease specificity, *ie.* variants of Class I, were predominantly found around the RCL. This observation is pertaining to the substitutions of the P1 Arg⁴⁴⁴, and the adaptation of secondary C1s interaction site residues Gln⁴⁵³ and Phe⁴⁵⁵ (identified by He et al. [1997]), with the p.(Phe455Ser)/(Phe477Ser) variant. More intriguingly, the p.(Leu129Arg)/(Leu151Arg) variant, in a conserved position among serpins, exhibits a loss of C1s protease control, but an unaffected control of contact phase [Ghannam et al., 2015]. Beside, p.(Arg378Cys)/(Arg400Cys) displays a strongly decreased capacity to associate with kallikrein and retains a partial control of C1s protease [López-Lera et al., 2010; Ghannam et al., 2015]. Similarly the P2 p.(Ala443Val)/(Ala465Val) variant had diminished activity against C1r and C1s but normal activity against plasma kallikrein and factor XIIa [Zahedi et al., 1995; Zahedi et al., 1997]. Residues that prevent conversion to a substrate and disrupt serpin inhibitory capacity, *ie.* those of Class II, are located in the β -sheet C and the hinge region (Table 1). The corresponding C1Inh variants could be associated with the failed mousetrap functionality, either by inefficient or preliminary trapping. Examples are given with positions Pro³⁷⁷, Arg³⁷⁸ and Met³⁸⁷, located in the central β -sheet (or nearby, at start of helix I), with the molecular species p.(Pro377Ser)/(Pro399Ser),

p.(Arg378Cys)/(Arg400Cys) and p.(Met387Thr)/(Met409Thr) serving as a substrate, due to inefficient protease trapping, and subsequent serpin cleavage. Hydrophobic residues in the β -sheet C (Phe²⁹¹, Val³⁰⁰, Gly³²³) and a surface coil (Phe²⁸¹), corresponding to gate residues, have variants with inefficient trapping, suggesting their attribution into Class II. C1Inh RCL residues aligned with A1AT, P14, P12, P10 and P8 positions have short side-chains and are thought to permit efficient and rapid insertion of the RCL into the β -sheet A. Variants of hinge-region residues often convert inhibitory serpins into substrates (Table 1). The variant p.(Ala434Glu)/(Ala456Glu) is a good illustration, with (i) Ala⁴³⁴ substituted by a long side-chain residue and (ii) Ala⁴³⁴ aligning with the P12 residue of the serpins (Table 2). Besides, p.(Ala434Glu)/(Ala456Glu) was demonstrated to be susceptible to cleavage by C1s and contact phase proteases, but did not form complexes with them [Skriver et al., 1991]. Finally, variants anchoring the RCL and located in the distal hinge are critical for loop overinsertion or multimerisation and are introduced into Class III (Table 1), *eg.* p.(Pro454Leu)/(Pro476Leu) and p.(Pro476Thr)/(Pro498Thr) variants in conserved positions (Table 2). Interestingly, the observation of qualitative C1Inh defects opens the field for further investigations with unique opportunities to pinpoint the role of strategic residues in distinct protease control, latent structure or oligomerisation. The situation of Asn²⁵⁰ is specific, because it is located in a loop protruding and overlying the central β -sheet. Deletion and duplication of this residue are likely to alter the structure of this strategic central β -sheet (Figure 1D; Table 2), with possible subsequent oligomerisation, as observed for the Lys²⁵¹ deletion mutant [Zahedi et al., 1996](Figure 1E), but not conversion into a substrate (Table 1). Ala⁴³⁶ is a RCL residue (aligned to P10 in A1AT) whose side chain becomes buried upon RCL

incorporation (Figure 1E). P.(Ala436Thr)/(Ala458Thr) and p.(Ala436Val)/(Ala458Val) variants, unlike similar variants of other serpins, are not cleavable by target proteases. By increasing the interaction with Lys³⁰⁷ or Lys³⁰⁶, the RCL of p.(Ala436Thr)/(Ala458Thr) and p.(Ala436Val)/(Ala458Val) packed even better in the loop-inserted latent structure than in that of the wild-type residue [Beinrohr et al., 2007](Figures 1C, -E). Both variants favour overinsertion up to P10 with release of s1C from the β -sheet C likely to prevent RCL cleavage [Aulak et al., 1993a; Stein & Carrell, 1995]. Other examples of Class III variants could be p.(Pro454Leu)/(Pro476Leu), in the gate region, or p.(Thr167Asn)/(Thr189Asn), a variant in a position out of the functional regions of the serpins, and which was clearly shown to develop oligomers [Madsen et al., 2014]. The p.(Pro476Thr)/(Pro498Thr), analog of p.(Pro476Ser)/(Pro498Ser) described by Eldering et al. [1995] and Bors et al. [2013], that facilitates the interaction of Pro³⁹¹ with P8'-P11'-P12' residues, could promote a presumably latent variant and polymer formation [Beinrohr et al., 2007]. Surprisingly, the variant p.(Cys108Tyr)/(Cys130Tyr) in the N-terminus presumably lowers molecular stability and induces a latent conformation by removing a cysteine involved in an intramolecular disulphide bridge. Consistent observations of variants promoting oligomerisation/multimerisation would emphasise that C1-INH-HAE could undergo a serpinopathy archetypal process, similar to that causing most of serpin-related diseases. Furthermore, the great majority of the missense variants located in or in contact with the central β -sheet or afflicting the shutter region were found to be associated mainly with HAE-I. These are likely to promote oligomerisation, to compromise RCL functionality, or to cause protein instability or misfolding, and also to retain mutants within producing cells. The latter was demonstrated by Haslund et al

[2019] for some positions involved in the shutter region: Ser¹⁴⁸, Leu¹⁵⁵, Phe²¹⁴, Val²⁶⁶ and Asn²⁶⁹ (Figure 1B, -C; Table 1).

6.2 Lesson from variants at conserved positions in the serpin family

Certain mutated positions showed high residue conservation among serpins (Table 2); among 35 positions mapped onto the model of C1Inh structure, and referred to as a mousetrap function of C1Inh: RCL, gate, shutter, and breach (Table 2), 17 corresponded to buried positions, *ie.* accessible surface area $\leq 20\text{\AA}^2$, and 2 to exposed residues, *eg.* Asn²⁵⁰ and Arg³⁷⁸ (Figures 1D, -E). Residue substitutions at conserved positions have important consequences. Table 2 shows some examples: (i) destabilization of the central β -sheet, *eg.* p.(Phe147Ser)/(Phe169Ser), p.(Pro149Gln)/(Pro171Gln), p.(Val468Asp)/(Val490Asp) in the shutter, (ii) exposed positions of residues involved in strategic functions, *eg.* p.(Arg378Cys)/(Arg400Cys) with polyanions, (iii) disruption of RCL insertion into the central β -sheet, *eg.* p.(Ala434Glu)/(Ala456Glu). Interestingly, apparent correspondences of *SERPING1* variants have been found with those of the prototypical serpin A1AT, *eg.* p.(Ser148Pro)/(Ser170Pro), p.(Gly162Arg)/(Gly184Arg), p.(Met302Arg)/(Met324Arg), p.(Arg378Cys)/(Arg400Cys), p.(Glu429Lys)/(Glu451Lys) (with the Z allele of A1AT), p.(Pro454Leu)/(Pro476Leu) and variants at position Pro⁴⁷⁶.

A fine and precise molecular and structural understanding of the consequences of each variation is far beyond the scope of this update, because of the underlying complexity of the dynamical conformational changes and plasticity, which are key to both the function and dysfunction of serpins. In addition to the well-described key regions such as gate, breach and shutter, a cluster of variant positions can be observed at the 'bottom' of the

molecule, *eg.* including Thr¹⁶⁷ (Figure 1B, -C). An initial key to start to understand the possible influence of variants in this area is the fact that aggregation-prone intermediates are believed to form either during the folding process or by dynamic sampling of sheet-open states. This ‘bottom’ part of the molecule has been recently shown in the case of A1AT to display a lag phase in its folding kinetics [Tsutsui et al., 2012]. Furthermore, it also corresponds to more labile segments observed during local unfolding leading to transient sheet-open states, where destabilizing variants are more frequently observed [Krishnan & Gierasch, 2011].

7 *Serping1*-DEFICIENT MOUSE MODELS

To investigate the role of C1Inh *in vivo*, and to determine how bradykinin is involved in the induction of vascular permeability, C1Inh-deficient mice have been produced. *Serping1* gene was targeted 210 bp upstream of exon 7 [Han et al., 2002], or was inactivated by disrupting the donor splice site of intron 2 [Oschatz et al., 2009]. *Serping1*^{-/-} animals have increased vascular permeability, as demonstrated by the extravasation of Evans Blue dye. Another mouse model that mimics human HAE only at the metabolic level, with decreased plasma C1Inh and C4 levels, has been created using CRISPR/Cas9 technology with the introduction of a 10-nt deletion in exon 3 (c.159_168del; NCBI #NM_009776.3) [Qiu et al., 2019]. These mouse models do not reproduce the HAE phenotype, thus limiting its interest. Interestingly Farfara et al. [2019] knocked down circulating C1Inh in wild-type mice in order to understand the effect of plasma C1Inh on brain pathology *via* the vascular system, with observations pertaining to former clinical

observations with C1Inh deficiency described as angioneurotic oedema and the role of C1Inh as a gatekeeper to the brain *via* the neurovascular system.

8 CONCLUSIONS

Biological diagnostic. In line with the original description of HAE-II [Rosen et al., 1965], we suggest that HAE-II should be identified using a biological phenotype criterion detecting the production of a dysfunctional C1Inh protein that resulted in low C1Inh function, regardless of whether antigenic C1Inh levels were normal or reduced. In our study, patients displaying low antigenic and functional C1Inh associated with the variant expression were classified as HAE-intermediate. Anti-C1Inh immunoblot analysis should be recommended for qualifying any C1-INH-HAE associated with a missense variant, with an identification of any qualitative defect in allele product expression. Subsequently, HAE-II and -intermediate conditions were found in 30% of C1-INH-HAE patients of our series.

Genetic investigations. Several reasons for *SERPING1* allelic heterogeneity have been developed by Germenis and Speletas [2016]. A deeper knowledge of transcriptome signatures [López-Lera et al., 2013] is expected to be gained in the coming years, with a contribution of epigenetic elements, gene-gene or gene-environment that regulate gene expression and biological processes, as recently suggested by Maia et al. [2019]. This important issue will lead to a better understanding of C1-INH-HAE pathophysiology and interpretation of genetics of C1-INH-HAE. As suggested by Grodecká et al. [2017], interpretation of genotypes with variants in the introns and at the exon/intron boundaries should be recommended with demonstration of exon skipping.

Prospects in genetics and biology. The arrival of next-generation sequencing methods has dramatically improved the diagnosis of human genetic diseases. On the one hand and because of the massively parallel sequencing approach allowing more deep sequencing, the new technologies of second-generation sequencing are more sensitive to detect postzygotic variants [Xu et al., 2015], a condition where patients were sporadic cases, with two affected siblings or more and unaffected relatives in the same or previous generation. A good example is given by Ebo et al [2018] with genetic investigations of *SERPING1* and SNPs on peripheral and gonadal parent samples, in addition to the samples of index case and her sister. On the other hand, when applied to multiple alleles, next-generation sequencing can ascertain the abundance of allele combinations [Veronez et al., 2016; Loules et al., 2018]. Its precise incidence would be more systematically addressed by investigating the group of *de novo* variants. On the basis of data pertaining to structural biology and serpin function, identification of dysfunctional variants that shows a partial expression defect, *eg.* for HAE-intermediate patients, underlines the importance of accurate genotyping of patients with angioedema manifestations. Genotypes with missense variants must be interpreted in a close relationship with biological investigation fitting with C1Inh typing, including in HAE-intermediate situations. Any *SERPING1* variant reminiscent of dysfunctional phenotype of serpin with multimerisation or latency should be identified as serpinopathy.

Acknowledgments

Prof Joel Lunardi, Drs Julien Fauré and Gaelle Hardy, Laboratoire Biochimie et Génétique Moléculaire, CHU Grenoble Alpes, Grenoble, France, have contributed

importantly to identify the variants of the pedigrees recorded in the registry of the French Reference Center for Angioedema CREAK and we thank them warmly. We are grateful to the physicians of the CREAK network, the French National Centre for Angioedema, and to other people for having participated in the collaborative study and fruitful discussions: K Djenouhat, Algiers; L Martin, Angers; M Vigan and F Pelletier, Besançon; S Guez, Bordeaux; Y Ollivier, Caen; L Faivre, Dijon; D-A Moneret-Vautrin and E Beaudouin, Epinal; L Arlettaz, P Tamarcaz and M Gil, Geneva; C Jacquot, L Bouillet, I Boccon-Gibod, A Pagnier, Grenoble; A Leimgruber, Lausanne; J Gooi, Leeds; L de Lumley, Limoges; B Coppéré, Lyon; H Maillard, Le Mans; D Launay and I Pruvost, Lille; J-R Harlé, J-J Grob and S Gayet, Marseille; B Graffin, Metz; N Raison-Peyron, Montpellier; M Morisset and G Kanny, Nancy; PY Jeandel, Nice; D Vincent, Nîmes; C Delaunay, Niort; P Hutin, Quimper; O Fain and A Gompel, Paris; P Roblot, Poitiers; S Geny, Strasbourg; J Moreau, Toulouse. The authors are indebted to Dr B Favier and F Parsopoulou, Grenoble, and Dr L Truedsson, Lund, for helpful comments, to F Csopaki, V Reininger, P Sellier and M Allegret-Cadet, Grenoble, and to H Humeau, Angers, for their skilful assistance. We are also grateful to the physicians from Hospital La Paz and to those collaborating with Unidad de Inmunología from Hospital La Paz for providing the clinical samples included in this work: Dr. T Caballero, Madrid; Dr. C Marcos, Vigo; Dr. M Pedrosa, Madrid; Dr. R Cabañas, Madrid; Dr. MA Rico, A Coruña; Dr. M Guilarte, Barcelona; Dr. A Giacobino, Geneva; Dr. M. Corominas, Barcelona; Dr. V Rodrigo, A Coruña; Dr. J Figueroa, Las Palmas; Dr. C Colas, Zaragoza; Dr. M López, Madrid and Dr. D Rivero, Madrid.

Author contributions

CD coordinated the research program, with its conceptualization, funding acquisition, and supervision; CD and DP were responsible for the methodology, planning and performance of experiments, data analysis, and manuscript writing - review & editing; IWB, ALL, DR, MT and MLT developed the molecular genetics and subsequent data curation; CG introduced residue positions in the 3D-model and developed their interpretation; DC, AG, ALL, DP and MLT developed the biochemical experimental work; DC and DP conducted the statistical analyses; all authors have read and approved the final manuscript.

Funding

This work was supported by programs conducted within the French Programme Hospitalier de Recherche Clinique (promoter: CHU Grenoble; PHRC 1996, coordinator: Claude Jacquot; PHRC 2002, coordinator: Claude Jacquot #DGS:2002/0649), the European Framework Program 5 (acronym PreHAEAT; coordinator: Marco Cicardi), the KininX funding, the National Rare Disease Program from the French Ministry of Health (promoter: CHU Grenoble Alpes; National Reference Center for Angioedema CREAK) and Ministerio de Economía y Competitividad (AES2015: PI15/00255).

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Repository of novel variants. According to the Author Guidelines, novel variants (n=120) have been successfully introduced at Global Variome shared LOVD; URL <https://databases.lovd.nl/shared/individuals/SERPING1> according to the Author Guidelines.

Figures

FIGURE 1. Crucial regions ascribed to C1 Inhibitor (C1Inh) Serpin function. A. Schematic representation of secreted C1Inh. Residue numbering conforms to the mature protein; post-translational modifications and corresponding mRNA are shown. Branched symbols, *N*-glycosylation (n = 6); black circles, verified *O*-glycosylation sites (n = 7); gray circles, potential *O*-glycosylation (n = 7). S–S, disulfide bonds (n = 2). B. Overall structure of native C1Inh serpin domain, with the position of exemplary variants

described in Table 1 shown with side-chains in sticks. Five regions for the serpin function are presented on the 3D-model of C1Inh (PDB ID 5DU3; [Dijk et al., 2016]) presented using Pymol. The model starts at Phe¹⁰⁰ and lacks a great part of the N-terminal domain (residues 1-112). Strategic functional regions [Stein and Carrell, 1995] are indicated with (i) the *reactive site loop* (RCL), coloured purple, including Arg⁴⁴⁴ P1 and the *hinge region* (P15-P9), essential for protease recognition and RCL mobility and conformational transformation for its insertion as strand 4A (s4A), *ie.* S>R transition, after protease inhibition; (ii) the *central β -sheet A*, coloured red, with the *breach* region, located at its top and point of initial insertion of RCL, and the *shutter* domain, coloured dark blue, close to the centre 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, including s3C and s4C of β -sheet C. β -sheets B and C are coloured green and brown, respectively. Examples of *SERPING1* gene variants, mostly associated with the types II or intermediate, are indicated. C. Overall structure of the latent form of C1Inh. The same regions involved in the serpin function and examples of variants depicted in Figure 1B are presented on the 3D-model (PDB ID 20AY [Beinrohr et al., 2007]). D. Native form. Zoom showing the position of *SERPING1* variants in proximity to the areas involved in RCL insertion. Representative breach and shutter residues are labelled cyan and dark blue, respectively. Variant positions listed in Table 1 are framed in magenta, green and orange according to class I, II and III, respectively. E. Latent form. Zoom showing the same positions.

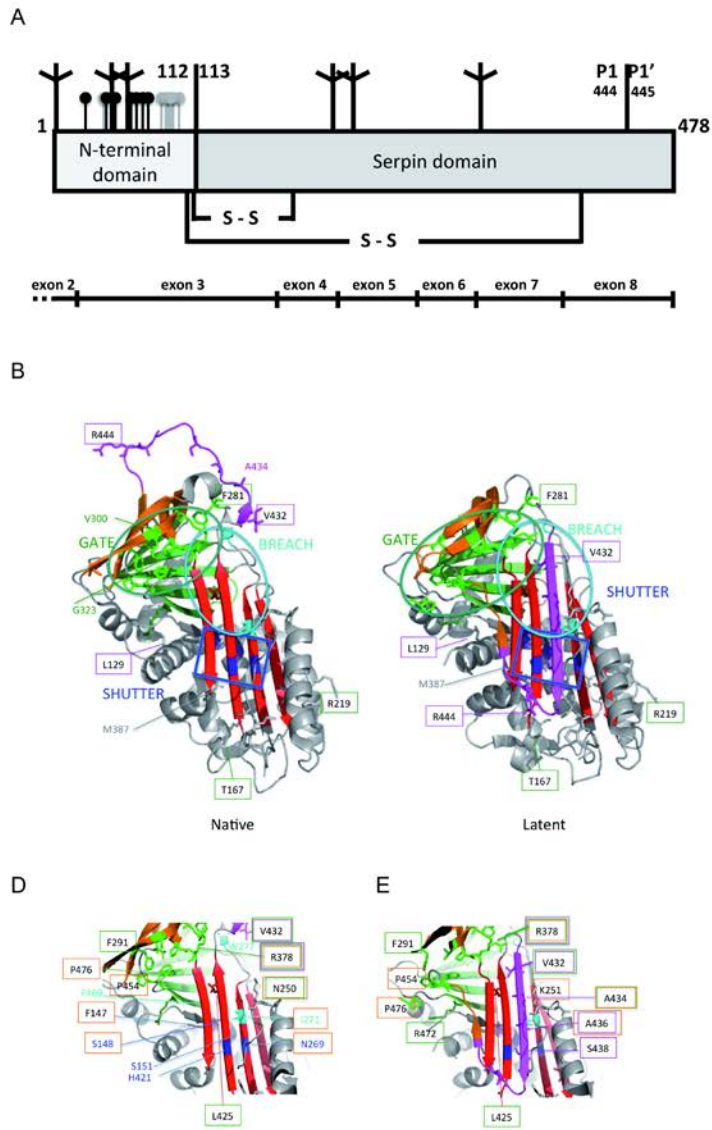


FIGURE 2. Non-polymorphic *SERPING1* variants distributed by variant type (n = 748). The overall *SERPING1* variants are compiled in Supp. Tables S1-5 according to specific variant groups and may be found online in the Supporting information. Two stop-loss variants have been included into the missense group.

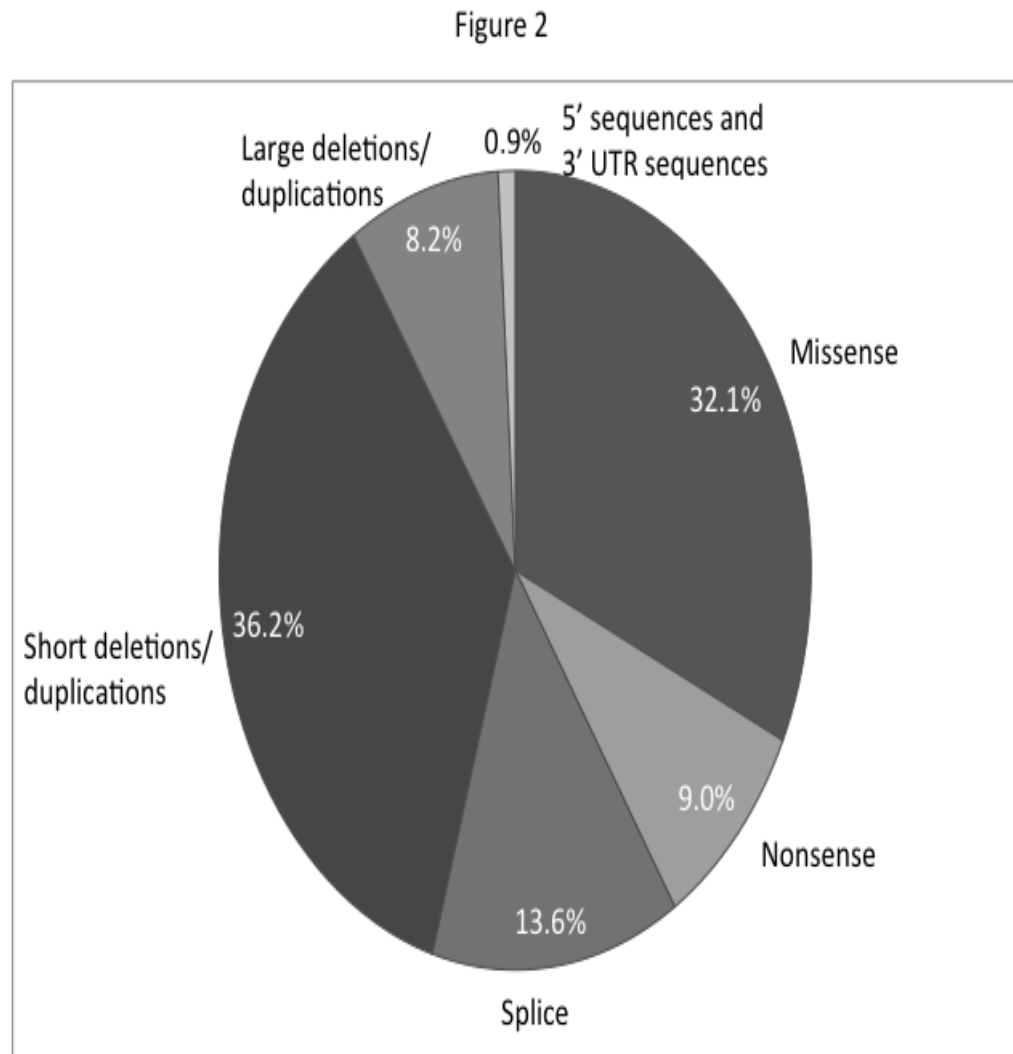


FIGURE 3. Circulating C1 Inhibitor (C1Inh) molecular species demonstrated by anti-C1Inh immunoblot. Plasma samples were collected from patients presenting with hereditary angioedema with C1Inh deficiency (regardless of whether during attack or remission period). The values of the corresponding antigenic C1Inh and C1Inh function assay using C1s protease or contact phase as targets are indicated for every individual. C1Inh, C1 Inhibitor; +C1s, addition of 0.1nM C1s protease to the sample followed by 15-min incubation at 37°C.

Figure 3

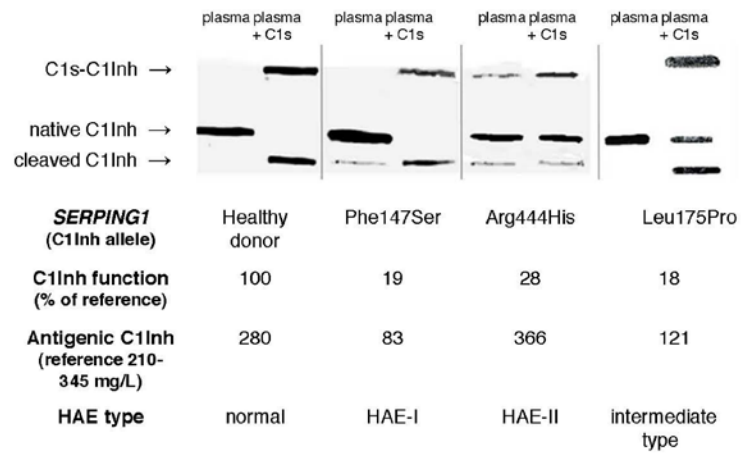


FIGURE 4. Distribution of variants associated with C1-INH-HAE of type II (panel A) and of intermediate type (panel B) in the *SERPING1* gene. Exons are numbered 1 to 8 and are drawn to scale in blue, with coding sequence in dark blue. The main protein domains are indicated in green. The *reactive site loop* (RCL) is indicated. Missense variants associated with intermediate type and type II are distributed according to their mature protein numbering.

Figure 4

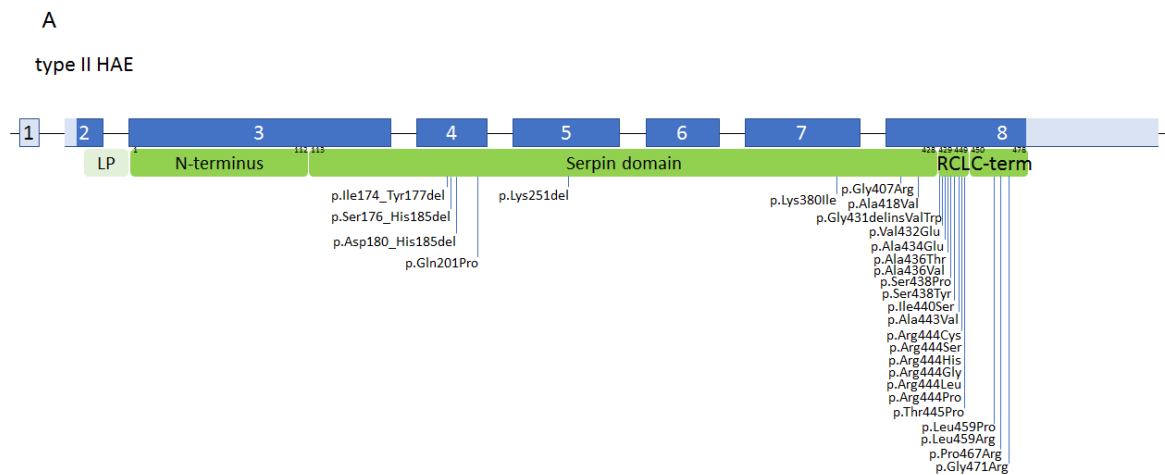


Figure 4

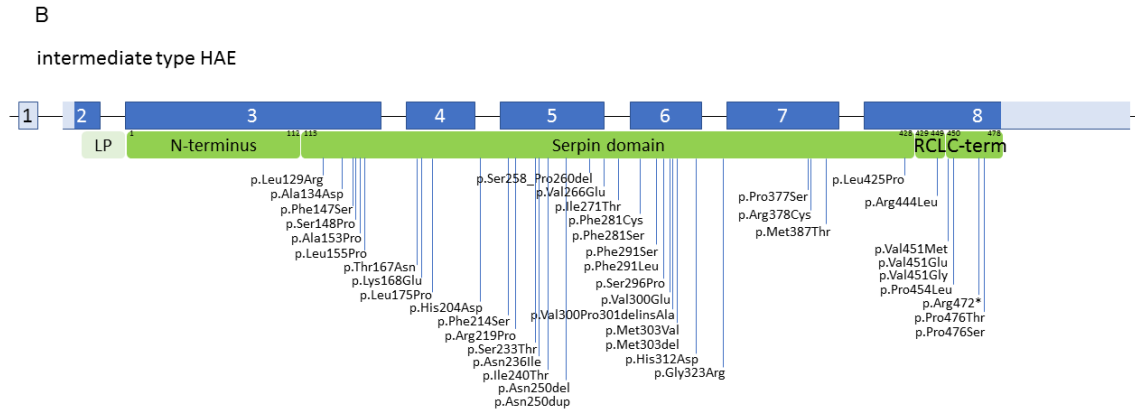


Table 1. Variants associated with type-II and intermediate-type hereditary angioedema phenotypes. Fifty-five *SERPING1* variants categorised into Class I (non-inhibitory C1Inh with altered exposure of the active site), Class II (disturbed insertion of the reactive centre loop, RCL, to properly develop Serpin-protease associations) and Class III (spontaneous self or mutual insertion of the RCL). For more detailed explanation, see Bos et al., [2002]; Beinrohr et al., [2007]. The results from three bioinformatic methods applied to missense substitutions of interest for structure are summarised: SIFT (with score), PolyPhen-2 (with probability score), and MutationTaster (with probability). Location of each of the residues in the Serpin structure is indicated. For an easy reference to the Serpin structure, the variant numbering is given according to the secreted protein sequence.

Biological phenotype	Class I	Class II	Class III	Prediction of phenotypic consequences		
				SIFT	PolyPhen-2	MutationTaster
Variants	p.(Leu129Arg) (α -helix A ⁷)	p.(Ala153Pro) (α -helix B)	p.(Cys108Tyr) (N-terminus) ^a	Intolerant (0.00)	Possibly damaging (0.785)	Disease causing (0.999)
	p.(Arg378Cys) (loop s2C/s6A)	p.(Lys168Glu) (α -helix C)	p.(Phe147Ser) ^a (s6B)	Tolerant (0.26)	Benign (0.003)	Polymorphism (0.999)
	p.(Val432Glu) (RCL P14 ⁵)	p.(Leu175Pro) (α -helix C)	p.(Ser148Pro) ^b (s6B)	Intolerant (0.00)	Probably damaging (0.999)	Disease causing (0.999)
	p.(Ala436Thr) (RCL P10 ⁶)	p.(Arg219Pro) (α -helix E)	p.(Leu155Pro) ^b (α -helix B)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
	p.(Ala436Val)	p.(Ile240Thr) (α -helix F)	p.(Thr167Asn) (α -helix C)	Intolerant (0.01)	Probably damaging (1.000)	Disease causing (0.999)
	p.(Ala443Val) (RCL P2)	p.(Asn250del) & p.(Asn250dup) (loop overlying the central β -sheet)	p.(Phe214Ser) ^b (α -helix E)	Intolerant (0.01)	Probably damaging (1.000)	Disease causing (0.999)
	p.(Arg444Cys) (RCL P1)	p.(Phe281Cys) (coil)	p.(Asn250del) & p.(Asn250dup) (loop overlying the central β -sheet)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
	p.(Arg444Gly)	p.(Phe281Ser)	p.(Lys251del) (α -helix F/s3A)	Tolerant (0.11)	Probably damaging (0.999)	Disease causing (0.778)
	p.(Arg444Ser)	p.(Phe291Leu) (s4C)	p.(Ser258_Pro260del) ^b (α -helix F/s3A)	Intolerant (0.00)	Benign (0.153)	Disease causing (0.999)
	p.(Arg444Leu)	p.(Phe291Ser)	p.(Val266Glu) ^b (s3A)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.982)
	p.(Arg444Pro)	p.(Ser296Pro) (s3C)	p.(Asn269His) ^b (s3A)	Intolerant (0.03)	Probably damaging (0.998)	Polymorphism (0.999)
	p.(Phe455Ser) (N-terminus of s4B) ^a	p.(Val300Glu) (s3C)	p.(Asn269Tyr)	Intolerant (0.00)	Probably damaging (0.992)	Disease causing (0.999)
		p.(Val300Pro301delinsA) (s3C)	p.(Ile271Thr) (s3A)	Intolerant (0.00)	Probably damaging (0.992)	Disease causing (0.999)
		p.(Met303Val) (s3C)	p.(Arg378Cys) (loop s2C/s6A)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.998)
		p.(Met303del)	p.(Ala434Glu) (RCL P12 ⁵)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
		p.(His312Asp) (s1B)	p.(Ala436Thr) (RCL P10 ⁶)	Intolerant (0.00)	Probably damaging (0.974)	Polymorphism (0.999)
		p.(Gly323Arg) (s2B)	p.(Ala436Val)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
		p.(Pro377Ser) (s2C)	p.(Ser438Tyr) (RCL P8 ^c)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
		p.(Arg378Cys) (loop s2C/s6A)	p.(Ser438Pro)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
		p.(Met387Thr) (α -helix I)	p.(Arg444His) (RCL P1)	Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
		p.(Val451Met) (loop	Intolerant		Disease causing	

		p.(Leu425Pro) (s5A)	s1C/s4B)	(0.00)	Possibly damaging (0.788)	(0.999)
		p.(Val432Glu) (RCL P14 ^c)	p.(Pro454Leu) (loop s1C/s4B)	Intolerant (0.00)	Probably damaging (1.000)	Polymorphism (0.999)
		p.(Ala434Glu) (RCL P12 ^c)	p.(Pro476Thr) (distal hinge region)	Intolerant (0.01)	Probably damaging (1.000)	Polymorphism (0.999)
		p.(Ser438Tyr) (RCL P8 ^c)	p.(Pro476Ser)	Intolerant (0.00)	Probably damaging (1.000)	Polymorphism (0.999)
		p.(Ser438Pro)		Intolerant (0.01)	Probably damaging (1.000)	Polymorphism (0.999)
		p.(Arg472*) (C-terminus of s5B)		Tolerant (0.14)	Probably damaging (1.000)	Polymorphism (0.999)
				Tolerant (0.12)	Probably damaging (1.000)	Disease causing (0.804)
				Intolerant (0.00)	Probably damaging (0.939)	Polymorphism (0.999)
				Intolerant (0.00)	Probably damaging (0.998)	Polymorphism (0.548)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.961)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.857)
				Intolerant (0.00)	Possibly damaging (0.673)	Disease causing (0.943)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.998)
				Intolerant (0.00)	Probably damaging (1.000)	Polymorphism (0.999)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
				Intolerant (0.02)	Probably damaging (0.978)	Disease causing (0.999)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
				Tolerant (0.12)	Probably damaging (0.995)	Disease causing (0.274)
				Tolerant (0.46)	Probably damaging (1.000)	Polymorphism (0.840)
				Tolerant (0.13)	Probably damaging (1.000)	Polymorphism (0.999)
				Tolerant	Probably	Polymorphism

				(0.99)	damaging (0.998)	(0.999)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.229)
				Intolerant (0.00)	Probably damaging (1.000)	Disease causing (0.999)
				Intolerant (0.05)	Probably damaging (1.000)	Disease causing (0.999)
				Intolerant (0.00)	Possibly damaging (0.663)	Disease causing (0.999)
				Intolerant (0.00)	Benign (0.101)	Disease causing (0.999)
				Intolerant (0.00)	Probably damaging (0.921)	Disease causing (0.999)
				Intolerant (0.00)	Benign (0.290)	Disease causing (0.999)
					Possibly damaging (0.666)	Disease causing (0.658)
					Benign (0.255)	Disease causing (0.999)
					Benign (0.197)	Disease causing (0.999)
					Benign (0.125)	Disease causing (0.999)
					Probably damaging (0.997)	Disease causing (0.999)
					Probably damaging (1.000)	Disease causing (0.999)
					Probably damaging (1.000)	Disease causing (0.999)
					Probably damaging (1.000)	Disease causing (0.999)
					Probably damaging (0.999)	Disease causing (0.999)

^a p.(Cys108Tyr)/(Cys130Tyr), p.(Phe147Ser)/(Phe168Ser) and p.(Phe455Ser)/(Phe477Ser) variants are associated with a HAE type I biological phenotype.

^b p.Ser148Pro/Ser170Pro, p.Leu155Pro/Leu177Pro, p.Phe214Ser/Phe236Ser, p.Val266Glu/Val288Glu and p.Asn269His/Asn291His variants develop a cellular pattern of aggregates with wild-type C1Inh [Haslund et al., 2018] and are associated with a HAE-I or a HAE-intermediate biological phenotype

^c P8, P10, P12 and P14 are referring to Stein & Carrell [1995], where position Ala⁴³⁴ of C1Inh attributed to P12 is supposed to be homologous to Ala³⁴⁷ in α 1-antitrypsin, a highly conserved position among serpins.

Table 2. Residue conservation. Variants at conserved amino acid positions in Serpin sequences. *SERPING1* gene variants, incl those described in supporting information Tables 1 and 2, distributed in strictly conserved positions among Serpin sequences. Possible intramolecular interactions in native or latent wild-type C1Inh between conserved residues have been suggested taking α 1-antitrypsin as the reference Serpin. For an easy reference to Serpin structure and to Serpin sequence alignments, the variant numbering is given according to the secreted protein sequence of C1Inh and A1AT.

C1 Inh residue ^a	Naturally occurring variant	Consensus residue ^b	Location in Serpin structure	Possible interactions of the C1Inh residue within native Serpin structure ^c
Phe ¹²⁷	p.(Phe127Val)	Phe (79%)	helix A	shutter, Phe ¹²⁷ packs against conserved Pro ¹⁴⁹ , Ala ¹⁵³ and Leu ¹⁷⁵
Asn ¹⁴⁴	p.(Asn144Ile)	Asn (87%)	β -sheet B (s1B)	shutter/gate, Asn ¹⁴⁴ makes extensive H-bond network of C1Inh C-terminus (474-478)
Phe ¹⁴⁷	p.(Asn144Lys)	Phe (84%)	β -sheet B (s6B)	
Ser ¹⁴⁸	p.P(he147Ser)	Ser (93%)	β -sheet B (s6B)	shutter; p.(Phe147Ser) variant is likely to destabilise the closure of a five-stranded central β -sheet, which in turn favors oligomerisation; apparent correspondence to p.(Phe52del) Mmalton in A1AT
Pro ¹⁴⁹	p.(Ser148Pro)	Pro (90%)	β -sheet B (s6B)	
Leu ¹⁵⁶	p.(Ser148Phe)	Leu (75%)	N-terminus of helix	shutter, Ser148Pro develops cell
Gly ¹⁶²	p.(Pro149Gln)	Gly (80%)		
Thr ¹⁶⁷	p.(Pro149Leu)	Thr (87%)		

Leu ¹⁷⁵	p.(Leu156Pro)	Leu (75%)	B	aggregates with wild-type C1Inh; apparent correspondence of p.(Ser148Phe) to p.(Ser53Phe) Siyama in A1AT with oligomers
Phe ²¹⁴	p.(Gly162Arg)	Phe (75%)	helix B	
Ile ²⁴⁰	p.(Gly162Trp)	Ile (83%)	turn helix B/helix C	shutter, forms tight turn; variants can perturb β -sheet A and so could result in the formation of loop-sheet polymers ; homologous to Pro ⁵⁴ of A1AT
Asn ²⁵⁰	p.(Gly162Glu)	Gly (75%)	helix C	
Ile ²⁵²	p.(Thr167Asn)	Ile (84%)	helix C	
Asn ²⁶⁹	p.(Leu175Pro)	Asn (85%)	helix E	shutter, buried hydrophobic residue, packs against conserved Leu ¹⁷⁵ , Ile ²⁴⁰ , Met ⁴¹⁹ and Phe ³⁹⁹ in native structure
Trp ²⁷⁷	p.(Leu175Val)	Trp (94%)	helix F	
Phe ²⁸¹	p.(Phe214Ser)	Phe (95%)	loop after helix F	forms tight turn, packs against conserved Phe ²¹⁴ in helix F; apparent correspondence of p.(Gly162Glu) to p.(Gly67Glu) Mineral Springs in A1AT
Thr ²⁸⁶	p.(Ile240Thr)	Thr (84%)	helix F	
Phe ²⁹¹	p.(Asn250del)	Phe (98%)	loop after helix F	
Val ³⁰⁰	p.(Asn250dup)	Val (80%)	loop after helix F	
Met ³⁰²	p.(Ile252Val)	Met (84%)	β -sheet A (s3A)	Thr ¹⁶⁷ makes H-bonds to Asp ³⁹⁸ and Asn ⁴⁰⁴ , stabilizing the long connection between helix I and s5A
Met ³⁰³	p.(Asn269His)	Met (86%)	β -sheet A (s3A)	
Leu ³²⁷	p.(Asn269Tyr)	Tyr (76%)	β -sheet A (s3A)	
Pro ³³⁸	p.(Trp277Arg)	Pro (93%)	loop s3A/s4C	shutter, buried hydrophobic, packing helix C to helix B, packs against conserved position in helix B (Leu ¹⁵⁶)
Pro ³⁷⁷	p.(Trp277Gly)	Pro (96%)	loop s3A/s4C	
Arg ³⁷⁸	p.(Phe281Cys)	Lys (72%)	β -sheet C (s4C)	packs against the conserved Gly ¹⁶² at the C-terminus of helix B and Leu ⁴⁰⁸ ; p.(Phe214Ser) develops cell aggregates with wild-type C1Inh
Leu ³⁸⁸	p.(Phe281Ser)	Leu (79%)	β -sheet C (s4C)	
His ⁴²¹	p.(Thr286Ala)	His (78%)	β -sheet C (s4C)	
Glu ⁴²⁹	p.(Phe291Leu)	Glu (91%)	β -sheet C (s3C)	shutter, packs into interface between helix F and central β -sheet (Phe ²⁰³ in native structure)
Ala ⁴³⁴	p.(Phe291Cys)	Ala (79%)	β -sheet C (s3C)	
Val ⁴⁵¹	p.(Val300Glu)	Phe (unknown)	β -sheet C (s3C)	highly exposed residue; makes H-bond to Ala ²⁴⁵ ; deletion or duplication strongly modify interactions of adjacent conserved positions in s3A (Lys ²⁵¹ , Ile ²⁵²)
Pro ⁴⁵⁴	p.(Met302Arg)	Pro (96%)	β -sheet C (s3C)	
Phe ⁴⁵⁵	p.(Met303Val)	Pro (96%)	distal hinge	shutter, H-bonding to Thr ²⁴⁸ ,
	p.(Met303Thr)	Phe (97%)		

Val ⁴⁶⁸	p.(Leu327Phe)	Leu (80%)	(s2B)	develops contact with Ala ⁴³⁶ in the latent form
Gly ⁴⁷¹	p.(Pro338His)	Gly (89%)	distal hinge	shutter, H-bonding to conserved positions in s5A (His ⁴²¹), helix B (Ser ¹⁵¹) and RCL at P8 ^d (Ser ⁴³⁸), in cleaved form; p.(Asn269His) develops cell aggregates with wild-type C1Inh
Pro ⁴⁷⁶	p.(Pro377Ser)	Pro (95%)	(s3B)	
	p.(Pro377Ala)		β-sheet C (s2C)	gate, buried hydrophobic, packs against conserved positions in a turn (Phe ²⁸¹) and s2B (Leu ³²⁷)
	p.(Pro377Leu)		loop s2C/s6A	
	p.(Pro377Arg)		helix I	gate, buried hydrophobic, packs against conserved positions in s3A (Trp ²⁷⁷) and s3C (Met ³⁰³)
	p.(Arg378Cys)		β-sheet A (s5A)	
	p.(Leu388Pro)		β-sheet A (s5A)	gate, H-bonding to conserved position in s5A (Glu ⁴²⁹)
	p.(His421Arg)		RCL	
	p.(Glu429Lys)		loop s1C/s4B	gate, buried hydrophobic, packs against conserved positions in s3C (Val ³⁰⁰), and distal hinge region (Pro ⁴⁵⁴ , Phe ⁴⁵⁵ and Pro ⁴⁷⁶ in native C1Inh; Val ⁴⁵¹ -Gln ⁴⁵² in latent structure); apparent correspondence of p.(Phe291Leu) to p.(Phe208Leu) in A1AT with pathogenic multimers
	p.(Ala434Glu)		loop s1C/s4B	
	p.(Val451Met)		β-sheet B (s4B)	gate, buried hydrophobic, packs against conserved positions in s4C (Phe ²⁹¹), s2C (Pro ³⁷⁷) and distal hinge (native Pro ⁴⁷⁶ ; latent Val ⁴⁵¹)
	p.(Val451Gly)		β-sheet B (s5B)	
	p.(Val451Glu)		β-sheet B (s5B)	gate, packs against conserved positions in s3A (Trp ²⁷⁷), loop s3A/s4C (Phe ²⁸¹), s2C (Pro ³⁷⁷), loop s2C/s6A (Arg ³⁷⁸); homologous to Met ²²⁰ in A1AT, with variants promoting structural misfolding
	p.(Pro454Leu)		distal hinge C-terminus	
	p.(Phe455Ser)			breach/gate, buried hydrophobic, packs against conserved positions in loop s3A/s4C (Phe ²⁸¹), s2C (Pro ³⁷⁷ , Arg ³⁷⁸) and s5A (Glu ⁴²⁹)
	p.(Phe455Leu)			
	p.(Val468Asp)			
	p.(Gly471Arg)			
	p.(Gly471Glu)			
	p.(Pro476Thr)			
	p.(Pro476Ser)			
	p.(Pro476His)			
	p.(Pro476Arg)			

	p.(Pro476Leu)		<p>breach, packs against conserved positions in s3A (Trp²⁷⁷) and s2B (Leu³³¹) or Val⁴³² for latent structure only</p> <p>gate, buried hydrophobic, packs against Leu³⁴⁵ and Met³⁴⁸, and conserved position in distal hinge (Pro⁴⁵⁴)</p> <p>gate, hinge at the junction between strands B and A, buried hydrophobic, packs against conserved positions in s4C (native Phe²⁹¹), s3C (Val³⁰⁰, Met³⁰²) and distal hinge (Phe⁴⁵⁵)</p> <p>gate, same hinge as above, exposed position, makes salt bridge to conserved position in s3C (Pro³⁰¹, Met³⁰³) and s5A (Glu⁴²⁹), participates in the polyanion binding site; position corresponding to Glu³⁴² in A1AT with variant p.(Glu342Lys), the Z allele promoting pathogenic multimers</p> <p>buried hydrophobic, packs against Asp³⁸⁶, Val⁴¹⁶, and conserved Phe³⁹⁹ and latent Ala⁴¹⁸ or native Met⁴¹⁹</p> <p>shutter, H-bonding to native Asn²⁶⁹ or latent Ser⁴³⁸; packs against conserved Ser¹⁴⁸ and Ile¹⁵²</p> <p>gate/breach, H-bonding to conserved Thr²⁸⁶, salt bridge to conserved and exposed electropositive Arg³⁷⁸, packs against conserved Met³⁰³, could be considered as a P17^d position in the N-terminus of the RCL; apparent correspondence to p.(Glu342Lys), Z allele in A1AT with pathogenic multimers</p> <p>P12^d residue (proximal hinge), where Ala>Glu substitution disrupts RCL insertion into the β-sheet A and is</p>
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				<p>likely to develop multimers</p> <p>gate; displaced during the RCL insertion</p> <p>gate; hinge; forms tight turn, the RCL flips for its insertion around this residue; packs against conserved position in s4C (Phe²⁹¹); apparent correspondence to p.(Pro369Leu) Mheerlen in A1AT with oligomers</p> <p>gate, buried hydrophobic, packs against conserved positions native Phe²⁹¹, Ile³³⁵, Val³³⁷, and Val⁴⁷³; corresponding position with Phe³⁷⁰ in A1AT</p> <p>shutter, buried hydrophobic, forms β-bulge in s5B, packs against conserved position in s6B (Ser¹⁴⁸)</p> <p>shutter</p> <p>gate, buried hydrophobic; in native structure packs against conserved positions in s4C (Phe²⁹¹), s3C (Val³⁰⁰) and s2C (Pro³⁷⁷); follows the hinge C-terminus at Arg⁴⁷²; apparent correspondence of p.(Pro476His) to p.(Pro391His) Yorzinuovi in A1AT with oligomers</p>
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^a Position numbering omitting signal peptide, according to indications in structural models (PDB entries 5DU3 and 2OAY).

^b High residue conservation (>70%) between aligned sequences of 219 Serpins, with the numbers in brackets indicating the percentage of shares [Irving et al., 2000].

^c Interactions described based on those reported for the human native C1 Inhibitor (PDB entry 5DU3), or latent C1Inh (PDB entry 2OAY). The functional strategic breach, shutter, gate and other structure features are indicated in Fig 1B.

^d P8, P12 and P17 are referring to Stein & Carrell [1995], where position Ala⁴³⁴ of C1Inh attributed to P12 is supposed to be homologous to Ala³⁴⁷ in A1AT.

A1AT, α 1-antitrypsin