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Genetics of Hereditary Angioedema Revisited

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Abstract Contemporary genetic research has provided evidences that angioedema represents a diverse family of disorders related to kinin metabolism, with a much greater genetic complexity than was initially considered. Convincing data have also recently been published indicating that the clinical heterogeneity of hereditary angioedema due to C1 inhibitor deficiency (classified as C1-INH-HAE) could be attributed at least in part, either to the type of *SERPING1* mutations or to mutations in genes encoding for enzymes involved in the metabolism and function of bradykinin. Alterations detected in at least one more gene (*F12*) are nowadays considered responsible for 25 % of cases of hereditary angioedema with normal C1-INH (type III hereditary angioedema (HAE), nlC1-INH-HAE). Interesting data derived from genetic approaches of non-hereditary angioedemas indicate that other immune pathways might be implicated in the pathogenesis of HAE. More than 125 years after the recognition of the hereditary nature of HAE by Osler, the heterogeneity of clinical expressions, the genetics of this disorder, and the genotype-phenotype relationships, still presents a challenge that will be discussed in this review. Large scale, in-depth genetic studies are expected not only to answer these emerging questions but also to further elucidate many of the unmet aspects of angioedema pathogenesis. Uncovering genetic biomarkers affecting the severity of the disease and/or the effectiveness of the various treatment modalities might lead to the prevention of attacks and the optimization of C1-INH-HAE management that is expected to provide a valuable benefit to the sufferers of angioedema.

Keywords Genetics · Hereditary angioedema · *SERPING1* gene · *F12* gene

Introduction

Rapid changes in the treatment of hereditary angioedema (HAE) have accelerated during the past decade, owing to the research which led to a better understanding of the pathogenesis and the genetics of the disease. Under the light of these advances, HAE is nowadays considered a heterogeneous disorder with a complex pathophysiology. Two decades after the initial recognition that mutations of the *SERPING1* gene encoding for C1 inhibitor (C1-INH) are responsible for HAE due to C1-INH deficiency (C1-INH-HAE) [1, 2], a new clinically indistinguishable type of HAE was described associated with mutations in the *F12* gene encoding for coagulation factor XII (FXII; Hageman factor (FXII-HAE)) [3]. Despite the fact that *SERPING1* was defined the primary gene encoding to C1-INH production, in a small proportion of patients with C1-INH-HAE, no *SERPING1* alterations can be detected even after meticulous analysis of the coding region [4–6]. Moreover, the vast majority of patients with a clinical history suggestive of HAE but with normal C1-INH function have no mutation in the genes associated with HAE (*SERPING1, F12*; unknown HAE, U-HAE) [7, 8]. Interestingly, accumulating evidence indicates that defects in genes encoding proteins involved in kinin generation or its catabolism, other than *SERPING1* and *F12*, may be involved in the pathogenesis of HAE and may modify its clinical expression [9, 10]. Thus, nearly a century after the recognition of the hereditary nature

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of HAE, the genetics of this disorder presents with a challenging complexity that will be thoroughly discussed in this review.

C1-INH-HAE Genetics

The Allelic Heterogeneity of C1-INH-HAE

SERPING1 gene (serpin peptidase inhibitor, clade G (C1 inhibitor), member 1) (OMIM no. 606860; GenBank NM_000062.2) extends over a 17,159-bp genomic region located on chromosome 11q12-q13.1 (Fig. 1). The gene comprises eight exons and seven introns, presents an unusual promoter with no TATA sequence but a TdT-like initiator and a polypurine-polypyrimidine tract, and gives rise to a messenger RNA (mRNA) product of 1827 bp excluding the poly(A) tail [11]. By sequence analogies, the encoded protein, C1-INH, was identified as a member of the protein superfamily of serine protease inhibitors (Serpin). Its reactive site, identified as P1 site by analogy with α_1-antitrypsin, is located in exon 8 at Arg444. C1-INH is highly glycosylated (26–33 % of the total mass). The glycosylated sites are located within a stretch of 100 amino acids at the N-terminal end of the molecule and appear to be irrelevant to the protein function, although removal of the sugars increases its catabolic rate [12]. The N-terminal end represents the non serpin-like domain that is highly divergent among different species suggesting that they accept extensive variations both in primary sequence and in glycosylation pattern (Fig. 2) [14–16]. Over 450 different *SERPING1* mutations related with C1-INH-HAE have been detected up to now, scattered over all exons and exon/intron boundaries of the gene with higher concentration in exon 8, 5, and 6 and low concentration in the aminoterminal end.

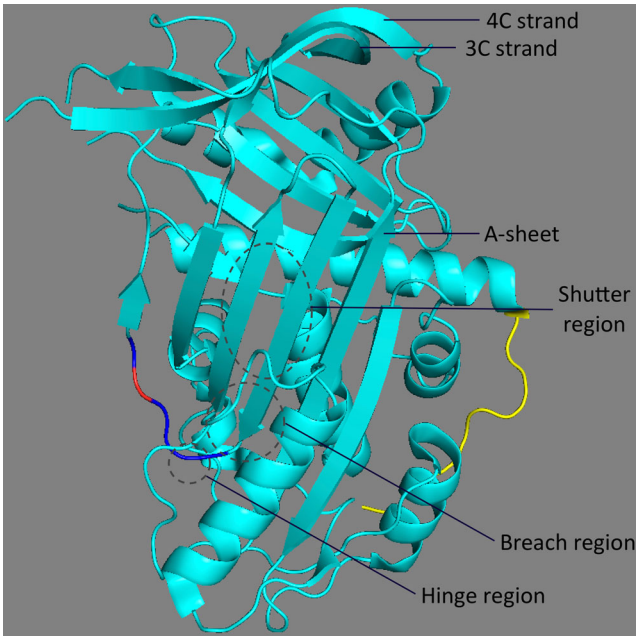

Fig. 2 The five crucial regions for the proper function of C1-INH are presented on the crystal structure of the C-terminal serpin domain (residues 113–478) of recombinant non-glycosylated C1-INH (PDB 2OAY) [13] generated using PyMOL Molecular Graphics System; in *yellow*, a small portion of the N-terminal domain (residues 97–112). The *blue color marks* the *reactive-center loop* (RCL), inside of which the residue Arg444 is shown in *red*. The P15-P9 portion of the RCL is called the *hinge region* that is essential for the RCL mobility. At the top of the A β-sheet is the *breach region* and near its center is the *shutter domain*. The *gate region* is composed of s3C and s4C strands. The breach, shutter, and gate regions drive the initial insertion and passing of RCL in the β-sheet A of the serpin. Missense mutations affecting these regions of the protein are most probably pathogenic

Most of them are listed in the Human Gene Mutation Database (Online Mendelian Inheritance in Man—OMIM ID 106100) [17] and a database specific to this disease

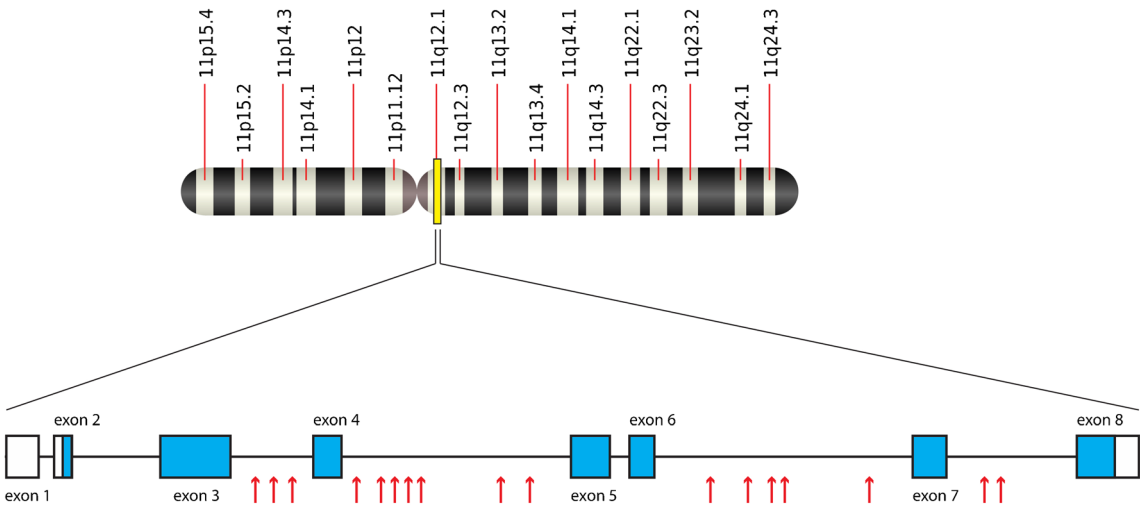

Fig. 1 The location of the *SERPING1* gene on chromosome 11 and its structure. *Black lines connecting boxes* represent introns, *white boxes* represent untranslated regions (UTRs), and *arrows* denote the *Alu* repeats

(HAEdb, hae.enzim.hu) [18]. Recently retrieved data from HAEdb show that the majority of C1-INH-HAE-related *SERPING1* mutations are missense mutations (34 %) followed by frameshift alterations and small indels (31 %), large gene rearrangements (17 %), splice-site defects (10 %), nonsense mutations (7 %), and regulatory mutations (1 %). Most of these mutations including nonsense mutations and indels lead to premature truncation of protein synthesis causing a translational frame shift. Usually, mRNAs with premature stop codons are eliminated by nonsense-mediated mRNA decay [19]. If truncated proteins are translated at all, they are non-functional and/or are rapidly degraded. Mutations resulting in the production of truncated or misfolded proteins that are not secreted efficiently occur throughout the gene and are responsible for the common type 1 C1-INH-HAE. This C1-INH-HAE type accounts for 80–85 % of all cases and is defined by reduced plasma C1-INH concentrations leading to low C1-INH function.

Single amino acid substitutions, located mainly at exon 8 that decode the reactive center as well as two critical hinge regions upstream (proximal hinge) and downstream (distal hinge) the reactive site of C1-INH, lead to the less common but clinically indistinguishable type 2 C1-INH-HAE (15–20 % of cases). The majority of these mutations represent mutations located in residue Arg444, according to traditional amino acid numbering [20], which corresponds to residue Arg466 according to HUGO recommendations (where the methionine at position 1 has the number 1, while in traditional system the number −22) [21]. Only one mutation, a deletion of Lys251/Lys273 that creates a potential new glycosilation site, causing type 2 C1-INH-HAE has been detected far from the reactive-center region [22]. All these mutations lead to the production of a dysfunctional C1-INH protein that results in low C1-INH function despite normal levels of antigenic C1-INH [23].

C1-INH-HAE is transmitted as an autosomal dominant trait [1, 2]. Very rare cases of homozygous C1-INH-deficient patients have been described, mostly in consanguineous parents [24–26], while two cases of gonadal mosaicism have also been reported [27, 28]. However, on the contrary to what is expected in a dominantly inherited disease, heterozygous patients present with C1-INH plasma levels ranging from 5 to 30 % of the normal level [29]. The mechanisms that prevent the remaining wild-type allele from generating C1-INH plasma levels around 50 % are yet to be elucidated. This discrepancy has been attributed to an increased catabolic rate [30] and/or a decreased rate of C1-INH synthesis, with concomitant downregulation of the normal allele at least in some mutations [31–33]. At last, it is worthy to be mentioned that *SERPING1* exhibits also a unique kind of phenotypic heterogeneity. Beyond mutations associated with C1-INH-HAE, this gene harbors a number of single nucleotide polymorphisms (SNPs), which have been associated with other diseases. One of the few common missense variants in the coding region, the polymorphism rs4926 [c.1438G>A, p.Val480Met], is associated with nasal carriage of *Staphylococcus aureus* [34], while six common intronic SNPs (especially the rs2511989) are strongly associated with age-related macular degeneration (AMD) in Caucasians [35]. Among them, the rs2511988 [c.1030-20A>G], located into intron 6, upstream of the 3′ splice site of exon 7, has been proposed to be more prone to functional alterations [36]. However, such an association was not confirmed in Chinese [37] and Japanese populations [38]. Similarly, a *SERPING1* mutation (replacement of Ala443 with Val) affecting complement regulation but preserving kallikrein inhibitory activity has been described in one family. None of the members of this family had angioedema but were presented with systemic lupus erythematosus (SLE) possibly due to acquired deficiency in C2 and C4 [39].

The Mutagenic Lability of *SERPING1*

The pronounced allelic heterogeneity as well as the fact that approximately 20–25 % of all unrelated C1-INH-HAE cases have no apparent family history of C1-INH-HAE (i.e. sporadic cases) and present *de novo SERPING1* mutations with the same mutational spectrum as the familial cases [40], make the *SERPING1* gene a prime example of mutagenic lability. Investigation of *SERPING1* mutations has revealed several key features about the DNA itself, as well as protein structure-function relationships that may explain this phenomenon [41, 42]. *SERPING1*'s proximity to the centromere is expected to be the main reason for its high mutation rate, since centromeric regions are considered the most rapidly evolving compartments in the eukaryotic genome. Another feature of the *SERPING1* gene to which its high mutation spectrum has been attributed, is the high incidence of DNA repetitive elements. The 7 introns of the gene contain 17 *Alu* repeat sequences [43] which represent "hotspots" for non-homologous recombination events that may cause partial deletions or duplications of the gene [44] (Fig. 1). Large mutations related to clusters of *Alu* repeats in various orientations, particularly in introns 4 and 6, represent a major source of genetic instability of the *SERPING1* gene. An additional structural feature of the *SERPING1* gene potentially responsible for the higher than average mutation rates is the high frequency of CpG sites. Like *Alu* elements, CpG sites also represent mutational hotspots as they are prone to spontaneous deamination. The CpG dinucleotide in the first two positions of codon 444/466 encoding the central arginine (CGC) of the reactive center of C1-INH seems to be a frequent target for recurrent amino acid substitutions [45]. Moreover, the region encoding the reactive center, like in some other Serpins, contains both primary and secondary structure DNA polymerase-pause sites, which enhance its rates of mutation and evolution.

Interestingly, some of *SERPING1* alterations represent extremely rare mutagenesis events. Such alterations are the dinucleotide mutation g.8502_03TC>AA resulting in the missense mutation V233E [46], as well as the recently described substitution of two consecutive nucleotides TC to AA resulting in a termination codon (F225X) [47]. Dinucleotide substitution is an extremely rare mutagenesis mechanism in eukaryotes usually resulting in a missense mutation. Considering other genes and diseases, causative dinucleotide substitutions have been described in less than 1 % of Factor IX gene associated with hemophilia B, in Keratin 10 gene causing annular epidermolytic ichthyosis, and in the RET proto-oncogene resulting in the aggressive type 2B of multiple endocrine neoplasia syndrome (MEN-2B) [47].

The Pathogenicity of Single Amino Acid Substitutions

How mutations causing single amino acid substitutions almost invariably result in a loss of C1-INH inhibitory activity remains as yet an intriguing enigma. Evidently, misfolding of serpins resulting in serpin polymerization that causes retention or impaired secretion of the mutated protein is a common molecular event highly relevant to pathologic processes of serpinopathies [48]. Actually, there are reports of C1-INH oligomerization which leads to lose of its inhibitory activity, like in most serpins with proximal-hinge mutations. As such, point mutations have been described affecting the C-terminal region relative to the reactive center causing partial C1-INH secretion due to intracellular multimerization [49]. Similarly, polymerization has been observed when the C1-INH molecule carries a mutation in the reactive-center proximal hinge [50]. However, contrary to other serpinopathies, these mutants are not converted to a substrate but exist in both monomeric and multimeric forms. Moreover, no conformation-dependent consequences of an intracellular accumulation of C1-INH polymers indicating misfolded-protein stress have been related so far to C1-INH mutations, other than its deficiency in the plasma.

More recently, Madsen et al. [51] demonstrated the presence of polymerized C1-INH in the plasma of C1-INH-HAE patients carrying certain *SERPING1* mutations. The pathogenetic role of these polymers remains as yet unclear. However, given the inability of polymers to control target proteases and the potent activation of the kallikrein kinin system induced by misfolded proteins [52], it is probable that they can potentiate the formation of bradykinin. Unequivocally, further research is required towards a better understanding of the ability of C1-INH mutants to form and accumulate intracellularly polymers that are secreted thereafter into the blood, or to assemble polymers in the blood stream, in order to reveal the molecular mechanisms of the extended phenotypic variability of C1-INH-HAE [53].

Another interesting point in regard with the pathogenicity of single amino acid substitutions is related to the differential control exerted by certain C1-INH mutants (R378C and I271T) on the protease C1s as opposed to kallikrein [25, 54]. It is worth to be noticed here that commonly used methods using C1s as target are unable to effectively evaluate C1-INH function in such cases, where the use of a newly introduced method based on a contact phase protease target is preferable [54].

Notwithstanding the above explanations, questions are still emerging in regard to the causal nature of all the reported *SERPING1* missense mutations. C1-INH structure-function correlates do not always explain satisfactorily the linkage between specific mutations and the disease phenotypes. Unfortunately, appropriate functional or segregation studies cannot be carried out due to the large number of private mutations and the unavailability of relatives. Regarding functional studies, this complexity is further accentuated by findings suggesting that some mutations may have different consequences in different cell types [55].

The Molecular Analysis of *SERPING1* Gene

Currently, the molecular analysis of *SERPING1* initiates with the prioritized amplification of all exons and the exon/intron boundaries by PCR and the detection of mutations by direct sequencing. If no mutation were found by sequencing, or in case a detected missense mutation would be proven benign for protein function by bioinformatic tools, further analysis for the identification of large gene rearrangements is performed [4]. For this purpose, two different techniques are widely used, namely the long-range PCR and the multiplex ligation-dependent probe amplification (MLPA). In long-range PCR, segments of the *SERPING1* gene encompassing at least 7–15 kb are amplified using common oligonucleotide primers, specific Taq polymerase mixes, and long-time PCR conditions [4, 5, 56]. MLPA is a high-throughput, sensitive technique for detecting copy number variations in genomic sequences [57] usually performed by a commercially available kit (SALSA MLPA probemix P243-A2 SERPING1 kit, MRC-Holland, Amsterdam, The Netherlands). Targeted analysis by simple and widely accessible methods, like polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), is not applicable in *SERPING1* molecular studies; such molecular approaches are useful in the molecular studies of families with known *SERPING1* mutations by modifying the DNA sequence in order to be recognized by restriction enzymes.

The cumbersome and time consuming molecular analysis of *SERPING1* gene prevents sometimes the detection of the causal genetic defect due to an incomplete study of the gene. Assuming that a mutation detected after sequencing the initially examined exon(s) of the gene is the causal one, further

analysis is usually not taking place. This practice, however, hampers the identification of mutations that possibly coexist in positions next to the initially analyzed or large gene rearrangements. In order to clarify such a case, we had recently submitted to bioinformatics analysis all missense mutations deposited in HAEdb and we found that at least one of them was benign, thus making its causative effect disputable. The reanalysis of *SERPING1* in this case uncovered an additional genetic damage that was deleterious for the protein function (nonsense mutation), obviously representing the causative alteration [4]. This case also indicates that multiple single amino acid substitutions might coexist in the same patient with as yet unknown consequences to both C1-INH production and function and possibly to the disease phenotype.

Besides the above, serious questions regarding the adequacy of conventional molecular analysis in uncovering the causal genetic alterations arise from the fact that in about 5 % of C1-INH-HAE cases no mutation could be detected in the coding region of *SERPING1* [4–6]. This finding indicates that, in some patients, a causative defect modifying C1-INH expression may be located in an intronic or an untranslated region of the gene. Actually, such functional intronic alterations have been recently reported [55, 58, 59]. In any case, the comprehensive analysis of *SERPING1* must be affirmed before attributing the disorder to a possibly deleterious effect of a detected missense mutation and before reporting the result of a genetic analysis as negative.

Bearing in mind the above arguments and shortcomings, it remains elusive whether the presence of all *SERPING1* alterations considered so far responsible for HAE, are isolated or are they expressed in tandem with either functional alterations on genes involved in the function or degradation of bradykinin, or whether they display *SERPING1* polymorphisms and mutations with as yet unknown functionality.

Genotype-Phenotype Correlations in C1-INH-HAE

The clinical expression of C1-INH-HAE is characterized by a great variability. Features of the disease, like the age of disease onset, frequency and triggers of attacks, severity and localization of edema, prodromal signs and symptoms, and the need for long-term treatment, vary broadly even among members of the same family sharing the same mutation [23, 60, 61]. Some patients suffer frequent, life-threatening attacks, while others experience a mild disease course, whereas as much as 14 % of carriers of *SERPING1* mutation may remain symptom free throughout their entire lives [56, 62]. Evidently, about 5 % of the adult C1-INH-deficient patients are identified only after their child has been diagnosed, and despite carrying and transmitting the mutation to their children, may remain asymptomatic. Therefore, the factors influencing this heterogeneous clinical expression represent one of the oldest and unsolved mysteries of the disease that needs to be solved since the optimal therapy is still based on individualization of patients' history [63]. Following the initial detection of *SERPING1* mutations and their consequences on the complex structure of C1-INH, it was assumed that certain genetic alterations might result in specific disease phenotypes. The very first evidence for such a non-linear genotype-phenotype correlation was derived from studies indicating that the presence of one mutated allele affects mRNA transcription and protein secretion, leads to downregulation of the normal allele and, finally, to C1-INH plasma levels ranging from 10 to 30 % of normal [32, 33, 64]. In fact, C1-INH levels are not correlated to the clinical presentation of the disease irrespective of the type of *SERPING1* mutation. Almost all heterozygotes present with plasma C1-INH concentration lower than the expected 50 %. In isolated reports of homozygous C1-INH-HAE patients, suffering from a severe clinical disease course, their heterozygous family members were symptom free [24, 25, 65], which offers further evidence to support a possible relationship between certain mutation types and disease phenotype. Over the recent decades, the increasing number of mutations associated with a low prevalence of the disease did not allow the confirmation of a strong correlation between certain *SERPING1* mutations and the clinical phenotype of C1-INH-HAE. Thus, it was generally accepted that there is little correlation between the type of *SERPING1* mutations and disease phenotype [53, 66]. Subsequent studies attempted to correlate the type of *SERPING1* mutations with the severity of the disease but reached to conflicting results [9, 67, 68]. Interestingly, however, in the largest study ever in this field, Speletas et al. [4] examined 265 C1-INH-HAE patients from 4 European countries (including the study cohort of one of previous studies [9]) and showed that patients carrying *SERPING1* missense mutations have a significantly lower probability of manifesting C1-INH-HAE attacks before age 10 than those with all other *SERPING1* defects (i.e., regulatory, nonsense, splice defects, frameshift, large indels). Bearing in mind that early onset of C1-INH-HAE symptoms is predictive of higher severity of the disease course [69, 70], carriage of missense mutations may predict milder HAE-C1-INH clinical course. In accordance to these results, a similar genotype-phenotype correlation has been very recently reported in a small cohort of Serbian patients [71].

The intra-familial heterogeneity of C1-INH-HAE prompted further studies aiming to investigate the effect of common *SERPING1* polymorphisms and/or of alterations in genes encoding other proteins involved in the pathogenesis of the disease. So far no correlation was found between the severity of C1-INH-HAE and the p.V480M polymorphism (c.1438G>A, rs4926) in the *SERPING1* gene [72, 73], while conflicting results were obtained regarding the association with the C allele of another common *SERPING1* polymorphism (c.-21T>C, rs28362944) [74, 75]. However, Duponchel et al. [55] showed that transfections with the minigene construct carrying the C

variant at position c.-21 consistently yielded a weak product lacking exon 2 in the transfected cell lines, suggesting that this allele might contribute to the lower expression of the normal protein and, hence, to the occurrence of more severe disease. Similarly, older studies attempting to correlate polymorphisms in other proteins with C1-INH-HAE severity had failed to provide definitive results. The interesting finding of Blasko et al. [76] that the biannual attack rate is lower in patients carrying three or four copies of *C4B* gene encoding the B isotype of the C4 protein, has not been confirmed as yet. An common functional polymorphism of the *BDKR2* gene encoding B2 bradykinin receptor, which is located in the 5′UTR of the gene and characterized by the absence of 9-base pair repeat sequencing (B2R-9 allele, rs71103505), has been associated with increased C1-INH-HAE severity only in one study where it was found to be consistently present in the most symptomatic cases (21 patients) [77]. Finally, no correlation was found between the severity of C1-INH-HAE and the functional polymorphisms in the genes of bradykinins' B1 receptor (*BDKR1*), in the *ACE* gene or in the gene of mannose-binding lectin (*MBL2*) [78, 79].

Recently however, Bors et al. [9] reported a strong correlation between the severity of the disease and the *F12*-46C/T polymorphism (c.-4C/T, rs1801020). Thereafter, Speletas et al. [10] confirmed this result analyzing 258 C1-INH-HAE patients from 113 unrelated European families (including the study cohort of Bors et al. [9]). In this study, the carriage of *F12*-46C/T polymorphism was found strongly associated with a 7-year delay in disease onset, and significantly but negatively associated with the need for long-term treatment, both independently of the type of *SERPING1* mutations. The *F12*-46C/T polymorphism is located in the promoter region of the *F12* gene, four nucleotides before the initiation codon (ATG, Methionine), creating a new initiation codon (ATG) for transcription of the mRNA and a frameshift that produces a truncated protein. The T allele destroys the Kozak's consensus sequence (GCCAGCCATGG) for translation initiation signaling and prevents proper recognition of the translation initiation site [80, 81]. Thus, the above finding could be attributed to the effect that the polymorphism has on the synthesis of FXII and, secondarily, on the production of bradykinin downstream the contact system. Another interesting feature of C1-INH-HAE is that the proportion of the various types of disease-related gene alterations was recently found to be different between different geographical regions [4]. Missense mutations and small indels represent 30 and 28 %, respectively, of all *SERPING1* alterations observed in Hungarian patients, while the corresponding percentages among Romanian patients are 50 and 14 %. Taken together with the fact that the disease may follow a variable course in the different life periods of the same patient, this finding indicate a possible implication of environmental factors either in the mutagenesis or in the epigenetic regulation of the *SERPING1* gene.

Genetics of FXII-HAE

nlC1-INH-HAE (type III HAE) is a new entity described in 2000 simultaneously by Bork et al. [82] and Binkley and Davis [83]. This rare kind of inherited (and familial) angioedema is exhibited by similar clinical manifestations to C1-INH-HAE, but with otherwise normal plasma levels of functional C1-INH, and without causal mutations at the *SERPING1* locus [82]. In some of the affected females, clinical angioedema attacks can be attributed to exposure to estrogens (i.e., contraceptives, hormone replacement therapy). A subset of about 300 reported cases of (≈25 % of all estrogen-dependent HAEs) have been attributed to alterations in the *F12* gene (OMIM no. 610619) [84]. The *F12* gene has been mapped to chromosome 5 (5q33-qter) and comprised 13 introns and 14 exons covering 12 kb [85]. Several loss-of-function mutations in the *F12* gene associated with FXII deficiency (Hageman's disease) have been described [86]. Beyond these, four *F12* alterations have been identified so far which, according to their co-segregation patterns, were assumed to be causal for HAE with normal C1-INH function. Firstly, two distinct missense mutations located on the exon 9 have been described, resulting in threonine-to-lysine (Thr328Lys) and threonine-to-arginine (Thr328Arg) substitutions. Moreover, a large deletion of 72 bp (c.971_1018+24del72) located at the exon 9/intron 9 border was identified in two unrelated families of Turkish origin that is involved in the proline-rich region of the FXII in which the above two substitutions are located [87, 88]. More recently, a duplication of 18 bp (c.892_909dup) causing the repeated presence of six amino acids (p.298–303) in the same region of factor XII was also described in a Hungarian family [89]. All the above four mutations are located in the proline-rich linker peptide between the Kringle and trypsin-like serine protease (Tryp-SPc) domains of the FXII protein.

The Thr328Lys substitution has been identified in clinically affected and symptom-free individuals from numerous families [3, 90], whereas the Thr328Arg replacement has been found only in two German families [3]. After the discovery of these mutations, it was suggested that they confer a putative gain of FXII function leading to upregulation of contact system activation and therefore may account for increased bradykinin formation. Actually, patients with FXII-HAE who are carriers of the Thr328Lys mutation present with an increased amidolytic FXII activity, but with normal plasma levels of the protein [91]. On the contrary, however, Bork et al. [92] have shown that there is no difference between FXII-HAE patients with the Thr328Lys mutation and their healthy probands neither in FXII surface activation by silicon dioxide nor in kallikrein-like activity with and without activation by dextran sulfate, indicating that the Thr328Lys mutation does not cause a gain of function of FXII. Thus, the role of the *F12* mutations in the generation of FXII-HAE and the

underlying pathophysiology of this HAE type remains still poorly understood. Notwithstanding, the response of some patient's attacks to bradykinin receptor antagonist (icatibant) [93] supports the assumption that contact pathway dysregulation is involved, with bradykinin being the primary mediator of this type of angioedema. Interestingly, after examining a large cohort of women with anti-histamine-resistant angioedema, negative family history and normal C1-INH activity, Bork et al. [94] have also found that 3.4 % of them carried the Thr328Lys mutation in a heterozygous form, indicating that FXII-HAE can exist even in unaffected family members. More recently, Gelincik et al. [95] reported two more cases of idiopathic non-histaminergic angioedema (InH-AAE) carrying *F12* mutations (c1681-1G/A—intron 13 and c1027G/C—exon 10). Although the above data may indicate a putative a gain-of-function mutations in patients with FXII-HAE, both of these mutations have been previously identified in association with FXII deficiency. Moreover, it was confirmed that one of the detected alterations was a de novo mutation. It remains to be elucidated whether these alterations are actually causal and are not coincident with some other protein damage.

The Special Case of ACEi-AAE

The pathophysiology of angiotensin-converting enzyme inhibitor-associated angioedema (ACEi-AAE) is not fully known, but several lines of evidence indicate that a blockade of the bradykinin and substance P degradation by ACE (kininase II), and perhaps impaired metabolism of bradykinin and des-Arg(9)-bradykinin by aminopeptidase P (APP) might be the primary mechanism of this disorder [96]. As both the frequency and the severity of ACEi-AAE appear to be rising over the past several decades, efforts have been made towards uncovering genetic markers that could predict the risk of ACE inhibitor-exposed individuals. Through a pedigree analysis and a case–control study, Duan et al. [97] found that the genetic SNP variant encoding for APP, C-2399A (rs3788853) for *XPNPEP2*, is indeed associated with a reduction in APP activity and a higher incidence of ACEi-AAE. Subsequently, Woodard-Grice et al. [98] pointed out that this SNP has been associated with APP activity and with the emergence of ACEi-induced AAE in men, but not in women. More recently, Cilia La Corte et al. [99] identified, including the above, 3 SNPs (c.-2399C-A, c.-1612G-T, c.-393G-A) that are significantly associated with plasma APP activity, the ATG haplotype of which was associated with decreased plasma APP activity and contributes to the development of ACEi-AAE. Moreover, the above mentioned rs71103505 polymorphism along with another SNP located in 5′UTR of *BDKRB2* gene, namely the rs1799722 (g.96671139C>T, c.-191C>T, also known as C-58T) have been also associated with ACEi-AAE [100], in accordance with the previous observation of Lung et al. [77] regarding the correlation of rs71103505 polymorphism with the severity of C1-INH-HAE. The −9/+9 polymorphism (rs71103505) has been associated with bradykinin-mediated vasodilation, as well as bradykinin-mediated tissue plasminogen activator (t-PA) release in men, during ACE inhibition [101]. More recently, Pare et al. [102] by both a replicated genome-wide association study (GWAS) and a candidate gene approach including the largest reported sample of ACEi-AAE and ACE inhibitor-exposed controls, was unable to confirm the above correlation. On the contrary, they detected an association between increased risk of ACEi-AAE and a polymorphism in intron 1 of the gene encoding for neprilysin (*MME*, rs989692). Neprilysin (neutral endopeptidase, EC 3.4.24.11 (NEP)) is a membrane-bound metallopeptidase that colocalizes with ACE and metabolizes a number of vasodilator and vasoconstrictor peptides, including bradykinin [103]. Most interesting, however, were the findings of this study implicating inflammatory pathways in the pathogenesis ACEi-AAE. Consistent associations were detected between ACEi-AAE and polymorphisms in two other genes involved in immune regulation, i.e. the rs500766 polymorphism in the *PRKCQ* gene encoding for protein kinase C θ (PKCθ) and the rs2724635 polymorphism in the ETS variant gene 6 (*ETV6*), also known as TEL (or translocation ETS leukemia). PKCθ is involved in the activation of T lymphocytes [104]. ETV6 is a transcriptional repressor that is disrupted by translocation in 26–47 % of cases of childhood pre-B-cell acute lymphocytic leukemias, but also in some cases of T cell leukemias [105, 106]. ETV6 also regulates interleukin 18 (IL-18), IL-10, and IL-4 cytokines [106, 107] involved in the clonal expansion of Th1 (IL-18 and IL-10) and Th2 (IL-4) subsets of $CD4^+$ helper T cells [108].

A Link Between HAE and Autoimmune Diseases

The possible involvement of immune regulation in the pathogenesis ACEi-AAE, as indicated by the above study, could imply that immune-mediated mechanisms may be implicated in HAE. Despite the fact that the link between autoimmune manifestations and HAE remains debated [109], Triggianese et al. [110] recently reported that the prevalence of autoimmune diseases among their 143 HAE patients as high as 4.2 %. Moreover, they claim that HAE patients express enhanced production of autoantibodies due most probably to an increased activation of B cells, found to be associated with a high expression of TLR-9. In favor of the involvement of immunoregulatory mechanisms in HAE, is another piece of evidence indicating that HAE patients have higher levels of circulating IL-17, and growth factors FGFb and GM-CSF compared with healthy individuals, which

becomes further elevated during clinical attacks [111]. Currently, no further genetic data are available to demonstrate a possible linkage between autoimmunity and HAE.

The Role of Kinin Catabolic Enzymes Deficiencies

Carboxypeptidase N (CPN) is an important enzyme that regulates biologically active peptides including complement anaphylatoxins and kinins by removing carboxy-terminal arginine or lysine [112]. In 2004, a unique form of HAE has been described associated with CPN deficiency [113–115]. The proband of the affected family displayed an 11-year history of angioedema attacks occurring about once weekly and lasting 24 h. Plasma CPN activity that was as low as 20 % of normal, remained unchanged during attacks, concurrently with increase in C3a and histamine levels. Several family members were clinically affected, expressing a combination of angioedema or chronic urticaria, as well as hay fever and asthma, and had slightly depressed serum CPN, suggesting an autosomal recessive inheritance. More than two decades after the publication of this case, Cao and Hegele [116] sequenced the *CPN1* gene which encodes the catalytic subunit of CPN, in the archival genomic DNA of the proband and identified three mutations. The first was a frameshift alteration in exon 1 due to a single G insertion at nucleotide 385 (385fsInsG) that should result in a truncated protein with little to no active site. This mutation was not present in the 128 normal Caucasians screened, and therefore was assumed to be extremely rare. The second was a missense mutation in exon 3 that predicted substitution of aspartic acid for the wild-type glycine at amino acid 178 (Gly178Asp) which is a highly conserved amino acid in different species and many human carboxypeptidases. This mutation had an allelic frequency of 0.0078 among the 128 normal Caucasian subjects, suggesting that it was also rare. This aspartic acid 178 mutation may produce a protein product with reduced activity, thereby explaining why this individual had 21 % CPN activity compared with 50 % that would be expected in a single allelic exon 1 frameshift mutation. The third polymorphism, in intron 1, did not affect the coding sequence, suggesting that it did not contribute to the CPN deficient phenotype. Interestingly, the *CPN1* harbors five nonsynonymous mutations and several genomic alterations (especially in 3′ UTR-region), associations and functional consequences of which are still unknown. More recently, Willemse et al. [117] while exploring the role of CPN in the development of ACEi-AAE, identified a patient with a complete carboxypeptidase N deficiency (3 % of the normal serum CPN activity). The patient suffered from a moderate facial angioedema attacks, 2 years after initiating an ACEi, and her attacks ceased subsequent to discontinuation of the drug and H1-antihistaminics. In a series of 162 patients with suspected bradykinin-mediated angioedema, Dessart et al. [118] detected 21 % with non-iatrogenic defective kininase activity and 30 % with idiopathic increased kinin formation. Isolated or combined deficiencies in three major enzymes involved in kinin degradation (kininases), namely CPN, ACE, and aminopeptidase P (APP) were found. Seven out of their 20 C1-INH-HAE patients, had additionally a significant deficiency in one or several kinin catabolic enzymes (three of APP, one of CPN, and three of ACE). Two patients with an ACE (kininase II) deficiency were related, while one asymptomatic individual with both deficiency of C1-INH and APP was detected. A kinin catabolism deficiency was detected in five out of the six patients with ACEi-AAE, normal C1-INH and no *F12* gene mutation (two of ACE deficiency and three of APP associated to the SNP c.-2399C>A of the *XPNPEP2* gene). Moreover, 34 patients with normal C1-INH had a kinin catabolism deficiency, with enzymatic activities below the low reference value. Of these, 10 displayed CPN, 15 APP, and 12 ACE deficiencies associated with the SNP c.-2399C>A of the *XPNPEP2* gene.

Future Implications

The emerging picture for 2016 is that HAEs represent a family of diverse disorders of the contact system-kinin (mainly bradykinin) metabolism, with a much greater genetic complexity than the discovery of *SERPING1* gene could offer. Recent achievements provide convincing evidences that many of the obscure issues, like the genetic damage(s) underlying U-HAE and normal C1-INH-HAE, and the detection of genetic markers of disease severity, are close to be elucidated. In order to achieve this goal, large-scale studies and the use of contemporary genetic analysis approaches, like genome-wide association studies or next-generation sequencing, are required. However, neither a detailed genetic study nor any other isolated approaches can effectively address the complexity characterizing the clinical expressions of HAE. Classifying and diagnosing patients at specific clinical risks can be reached only by using advanced tools, which will combine clinical, genetic, transcriptomic, and biochemical approaches. Precision medicine is a much promising tool towards the achievement of this goal [119]. Once a satisfactory level of predictability was reached, it will allow to introduce appropriate modifications in the current prognostic and therapeutic approach. This will certainly upgrade the prevention and timely management of the disease, reduce life-threatening conditions, and improve patients' quality of life and minimize of the burden of the disease. Moreover, the performance of robust clinical trials for drugs under investigation, which at the moment presents great difficulties due to the

peculiar nature of the disease, will be facilitated. Beyond these, the accumulated evidence indicates that, in the near future, genetics will also facilitate understanding many of the unresolved aspects of HAEs pathogenesis. In depth genetic studies will uncover disease associations with mutations in genes encoding proteins other than those apparently involved in bradykinin metabolism and function. Possible associations of this kind might give answers in unexplained issues of the disease or might suggest the implication of additional pathways in its pathogenesis. For example, more than 30 years ago it was observed that HAE is provoked by olfactory stimuli [120], while recent evidence indicates that HAE patients present an impaired sense of smell [121]. The physical closeness of *SERPING1* gene span to a large olfactory receptor cluster on chromosome 11q13 [122] cannot exclude a high recombination frequency and the possibility of this olfactory region to be in linkage disequilibrium with *SERPING1*, with its mutations exerting functional roles on *SERPING1*-linked olfactory receptor cluster and vice versa [109]. Similarly, a genome-wide screen might localize high-affinity estrogen response elements (EREs) in genes encoding for proteins involved in HAE pathogenesis and shed light to the gender hormone-mediated effect that remains one of the most complex features of HAE. EREs are specific DNA sequences mediating the stimulation of target genes expression in response to estrogens. Over 70.000 EREs have been identified in human genome [123]. The *F12* promoter contains at position −44/−31 a palindrome similar, but not identical, to an ERE together with four hemisite EREs between positions −1314 and −608 [124]. The presence of these elements underlies the mechanism by which estrogens enhance FXII concentrations in plasma, and possibly is associated with estrogen-mediated features of not only the FXII-HAE but, to a different degree, of all HAE types. Unknown, however, remains the role of EREs localized within a range of −10 to +5 kb from mRNA 5′-ends in *SERPING1* gene as well as in other HAE-related genes, like *C4B* and *MBL2* [125].

Diagnostic Issues

Genetic testing is not included in the first-line diagnostic approach recommended for patients with angioedema, since the vast majority of angioedema cases can be diagnosed on the basis of family history, clinical picture, and complement tests (antigenic and functional levels of C1-INH, C1q, and C4) [126]. However, these may not always indicate the appropriate form of angioedema, while genetic testing can be proven a valuable tool for improving diagnostic accuracy and, finally, the adequacy of patient care [127]. In general, genetic testing might be useful in case of high clinical suspicion of HAE where the complement tests are inconclusive. Such typical examples are doubtful cases necessitating differentiation between the hereditary and the acquired forms of angioedema. More interesting is the confirmation of diagnosis upon the first presentation of the disease in children with a positive family history of C1-INH-HAE. It has been shown that patients with early onset of the disease have more severe disease course than those with later onset [69, 70]. Moreover, since gastrointestinal symptoms are a common presenting symptom in children with C1-INH-HAE, there is a greater need for proper differential diagnosis [128]. Notwithstanding that the clinical appearance of the disease is very unusual before the second year of age, the antigenic and functional C1-INH levels are not reliable indicators of C1-INH-HAE at this age, as their reference ranges are much lower compared with the adult reference ranges [129, 130]. Therefore, the confirmation of C1-INH-HAE diagnosis before the second year of age, might be complemented by genetic analysis. Moreover, bearing in mind that missense *SERPING1* mutations [4] and the carriage of the *F12*-46C/T functional polymorphism [10] are significantly associated with the age at C1-INH-HAE onset, genetic analysis of the offspring of an affected parent might contribute to better genetic counseling. Detection of *F12* mutations is the only test to date to confirm the diagnosis of FXII-HAE. Identification of pre-symptomatic individuals in pedigrees with an established diagnosis of FXII-HAE should be a priority in order to avoid exogenous triggers, such as estrogens (e.g., oral contraceptives in young women) and avoid fatal attacks. Pregestational genetic testing for specific mutations by chorionic villous sampling or amniocentesis might be helpful in prenatal diagnosis of C1-INH-HAE or FXII-HAE. In established pregnancies, it could be considered only in cases of an affected parent carrying a known genetic defect in *SERPING1* or *F12* gene but has ethical implications. Since not all genetic defects of the *SERPING1* gene detected in HAE patients are pathogenic, prenatal diagnosis must be considered only when the parent's mutation is undoubtedly disease-causing [4]. Given that *SERPING1* or *F12* defects may result in a nonfatal and manageable disease in the offspring, it should be considerd that modern advances in therapy have significantly improved the quality of life of the patients. Prenatal diagnosis should be decided by the parents only after an appropriate genetic counseling and the considerations of benefits versus risks. Taking into account that no *SERPING1* mutations were detected in 8–10 % of cases [4] and that in up to 75 % of estrogen-associated HAE no *F12* mutations are found, preimplantation genetic diagnosis might be an option in families with C1-INH-HAE or FXII-HAE since it allows the selection of healthy embryos [131].

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