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A sequential variant prioritization to identify genetic causes of hereditary angioedema in affected families from Spain and Portugal

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A sequential variant prioritization to identify genetic causes of hereditary angioedema in affected families from Spain and Portugal

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Running title: Sequential WES for HAE in Spain and Portugal families

Abstract

Background: Hereditary angioedema (HAE) is a rare autosomal dominant disorder characterized by recurrent edema attacks mediated by bradykinin dysregulation. While *SERPING1* and *F12* account for most of cases worldwide, genetic causes in a significant proportion of patients are unknown, particularly in patients with normal C1 inhibitor plasmatic levels (HAE-nC1INH). **Methods:** We performed whole-exome sequencing (WES) on 102 families from insular Portugal and Spain and established a sequential variant prioritization strategy combining the HADA tool with a virtual panel of genes linked to the kinin-kallikrein (KKS) and complement pathways. **Results:** We identified 28 pathogenic/likely pathogenic variants, including seven novel *SERPING1* variants and two novel *F12* variants. In the cases lacking genetic alterations in established HAE genes, candidate variants were prioritized in KKS genes with the potential to explain the disease. Genetic testing of relatives beyond index cases improved diagnostic yield (88.3% in multiplex families vs. 21.1% in proband-only cases) despite functional studies are required to validate the effects of prioritized variants. **Conclusion**: Our study expands the mutational spectrum of HAE, proposes novel candidate variants in genes interacting with the classical bradykinin pathway and reinforces the need of genetic tests as first level diagnostic tool to reduce diagnostic delays and the precise management of HAE patients.

1 Introduction

Hereditary angioedema (HAE) is a rare genetic disease with dominant inheritance characterized by recurrent swellings (edema) affecting the skin, internal organs, mucosa, and/or the upper airways [1, 2]. HAE attacks can turn into a life-threatening episode if the edema develops in the laryngeal tract, which leads to the obstruction of the upper respiratory airways [3]. Pathogenesis is explained by the dysfunction of the C1 esterase inhibitor (C1-INH) or dysregulation of the kinin cascade, which led to bradykinin (BK) release. BK is a vasoactive peptide and the main activator of the B2R receptor located in endothelial cells [4]. Activation of B2R increases the vascular permeability, resulting in the swellings [5]. Mental stress [6], contraception hormones [7], infections [8], injury/surgery interventions [9], and weather changes [6] have been reported as triggering factors of HAE attacks.

HAE is typically classified into three main groups attending to C1-INH plasmatic levels. Type 1 accounts for approximately 85% of cases and is characterized by a deficiency of C1-INH, whereas type 2 results from dysfunctional C1-INH protein [11]. Both subtypes are explained by more than 890 pathogenic variants affecting *SERPING1*, the gene encoding C1-INH [10, 12-15]. These mutations can cause protein misfolding, alteration of glycosylation site, the accumulation of C1-INH protein aggregates within the endoplasmic reticulum or the activation of the nonsense-mediated mRNA decay (NMD) pathway [16, 17]. These leads to truncated gene expression or impaired protein secretion that prevents its inhibitory function within the complement cascade. However, 5-15% of clinically recognized HAE-C1INH type 1 and type 2 cases still lack an identified genetic cause [18]. Less than 5% of HAE patients present normal levels on functional C1-INH (HAE-nC1INH). Pathogenic variants have been identified in other genes involved in the BK-kinin pathway in these cases [10]. However, most cases remain without a molecular diagnosis [19].

The first gene identified affecting kinin pathway was *F12*, which encodes factor XII (FXII) protein and produces the activation of kinin pathway and increases BK accumulation, leading to increased vascular permeability [20]. Less than 20% of HAE-nC1INH affected families carry causal genetic variants in the *F12* gene [21]. Next generation sequencing (NGS) studies, particularly whole-exome sequencing (WES), have been used to improve the HAE-nC1INH genetic diagnosis. WES allowed to identify five more genes responsible for HAE attacks since 2018, doubling the number of known genetic factors since its first application. Genetic variants associated with HAE-nC1INH have been found in angiopoietin 1 (*ANGPT1*) [22], plasminogen (*PLG*) [23], kininogen 1 (*KNG1*) [24], heparan sulfate 3-O-sulfotransferase 6 (*HS3ST6*) [25], and myoferlin (*MYOF*) [26] genes. Although the exact mechanism

of action remains unclear for the latter two, the studies suggest that *MYOF* defects involve the VEGF-mediated signaling in HAE pathophysiology, while the incomplete synthesis of heparan sulfate increases the BK concentration. Recent efforts have also identified key factors involved in the pathogenesis, including the *DAB2IP* gene [27], as well as Tie-2, VEGFs, and C1s/C1INH [28]. Thus, many of underlying mechanisms of HAE remains unclear, especially for causal genes in patients without C1-INH protein deficiency.

HAE is poorly recognized due to its nonspecific manifestations and frequently misdiagnosed as histaminergic-mediated angioedema. Advances in genetic testing procedures have improved the diagnosis of HAE [29]. However, genetic testing is still not part of the HAE clinical diagnosis routine, despite recommended by the most recent management guidelines [10, 19, 30]. As result, delayed diagnosis and undertreatment remains common [31]. Because of that and the importance of establishing an accurate diagnosis to prescribe the optimal treatment, many countries have developed HAE patient registries [32], fostering the development of standards as clinical protocols for patient management and specialized facilities to assist in genetic diagnosis, while reducing the diagnosis delay.

Recent studies have reported a minimum estimated prevalence of 1.09:100,000 in Spain for HAE due to C1-INH deficiency [33], which is lower than that estimated for Portugal of 1.38:100,000 [34]. Strikingly, the estimated prevalence of HAE type 2 in Portugal is the highest reported for any studied country, with 40% of studied HAE-C1INH cases compared to 10-15% in patient series from other countries. Despite this, epidemiological studies have revealed significant regional variations within Spain, with certain regions reporting prevalence rates as high as 2:100,000 [32], exceeding the national average.

Herein, we aimed to characterize the underlying genetic causes of 102 HAE affected families from several regions of Spain and Portugal to further contribute to a comprehensive definition of the genetics of HAE.

2 Material and methods

2.1 Study setting and patient selection and recruitment

We studied familial and sporadic cases, with clinical suspicion of HAE from Spain and Portugal. Exclusion of acquired angioedema (AAE) was based on levels of C1q and anti-C1q autoantibodies in those patients with C1-INH deficiency and absence of family history, late onset, or association with

lymphoproliferative tumors [30]. Following the WAO/EAACI guidelines for clinical management of HAE, patients with prescribed medication that could trigger angioedema attacks (i.e., angiotensin-converting enzyme inhibitors) were treated as a hereditary form of the disease at first steps of diagnosis routine, avoiding the premature exclusion of borderline HAE patients. By contrast, other conditions that can potentially cause angioedema attacks (i.e., hepatitis, HIV infection, hepatic/renal disorders, immunological deficiencies, and infections due to *Helicobacter pylori*) were excluded. C4 and C1-INH plasmatic levels were determined by nephelometry (N Antisera to Human Coagulation Factors and C1 Inhibitor, BN II: Siemens Healthcare Diagnostics, Marburg, Germany), while the C1-INH activity determination was determined by functional chromogenic assay (Berichrom C1-inhibitor, Siemens Healthcare Diagnostics, Marburg, Germany). Clinical plasma determinations of C4 and C1-INH were performed by the laboratories in charge of the diagnosis from each participating hospital. DNA was extracted from 4 mL of peripheral blood with Illustra^{TM} blood genomicPrep kit (GE Healthcare; Chicago, IL). Alternatively, the OG-250 kit (DNA Genotek, Ontario, CA) was used according to the manufacturer's instructions for pediatric patients. The concentration of DNA was evaluated using the dsDNA BroadRange Assay Kit for Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The study was approved by the Ethics Committee of the Hospital Universitario Nuestra Señora de Candelaria (PI 57–17) and the Ethics Committee for Health and the Scientific Committee for Research of the Serviço Regional de Saúde da Região Autónoma da Madeira (SESARAM, EPERAM) (Approval n. 34/2019), and written informed consent was obtained from all patients or legal guardians.

2.2 Whole-exome sequencing and variant calling

DNA sequencing libraries were prepared using exome enrichment kits. Library sizes and concentrations were checked on a 4200 TapeStation (Agilent Technologies, Santa Clara, CA) and sequenced on Illumina instruments including 1% of PhiX (Illumina Inc.) as internal control. Sequencing was conducted at Instituto Tecnológico y de Energías Renovables (Tenerife, Spain). See **Supplementary Table 1** for futher details.

Sequencing reads were preprocessed with bcl2fastq v2.18 and mapped to hg19/GRCh37 with Burrows-Wheeler Aligner 0.7.15 [35], and BAM files were processed with Qualimap v2.2.1 [36], SAMtools v1.3 [35], BEDTools [37] and Picard v2.10.10 (http://broadinstitute.github.io/picard). Variant calling was performed using an in-house bioinformatics pipeline based on the Genome Analysis Toolkit (GATK) v. 3.8 [38]. The pipeline has been designed for the detection of nucleotide substitutions (SNVs) and small indels (<50 bp) following the GATK Best Practices [39] and its description is

publicly available (https://github.com/genomicsITER/benchmarking/tree/master/WES). Somalier v0.3.0 was used to verify sample identity and assess cryptic relatedness and cross-sample contamination. Analyses were conducted at the TeideHPC Supercomputing facility (http://teidehpc.iter.es/en).

2.3 Identification of mobile element insertions and splicing predictions

Given that at least 17 *Alu* elements in *SERPING1* have been identified to cause HAE and that these repetitive elements are estimated to account for ~15% of new HAE cases linked to C1-INH deficiency [40], we used a combination of the Mobile Element Locator Tool (MELT v2.2.2) for initial screening [41] and the Soft Clipped Read Alignment Mapper v1.0.1 (SCRAMble) [42] to improve detection accuracy of potential *Alu* elements affecting *SERPING1*.

To analyze candidate splicing variants and predict their effect, SQUIRLS v1.3.0 was used for prioritization based on its internal pathogenicity score [43]. Subsequently, the DECIPHER database was utilized to further validate the predicted splicing impact, as indicated by SQUIRLS, and to gather additional evidence on the variants through its integrated metrics and disease variant annotations [44].

2.4 Prioritization of previously reported causal variants and validation

The identification of the underlying genetic factors responsible for HAE was devised on the basis of a sequential variant prioritization strategy (**Figure 1**). The first level targeted established HAE genes (*SERPING1*, *F12*, *ANGPT1*, *PLG*, *MYOF*, *KNG1*, and *HS3ST6*) to identify causal variants explaining the patients' phenotypes. The second level was only conducted if the patient's genetics was not solved in the first level analysis. This was an exploratory level and involved a broader panel of genes from the kinin-kallikrein and complement pathways, with the specific purpose of identifying variants with potential biological relevance. However, the genes of this second level currently lack an established causal connection with HAE, and any prioritized variants from this stage should be interpreted as candidates requiring functional validation and further segregation studies to confirm their potential role in disease pathogenesis.

In the first level, we relied on the Hereditary Angioedema Database Annotation tool (HADA; http://hada.hpc.iter.es/), our in-house public variant prioritization and interpretation server, to identify known genetic alterations at seven known HAE genes (*SERPING1, F12, PLG, ANGPT1, KNG1, HS3ST6,* and *MYOF*), and variants that are known to affect the normal protein function [12]. HADA

also provides annotations from the literature and functional databases (allele frequency (AF), gene location, or functional consequences) and the pathogenic classification provided by ClinVar [45], according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) [46]. HADA was also used to extract novel genetic variants at previously known HAE genes, where no previously reported causal variants were found on a given patient.

In the second level, we relied on a virtual gene panel using Franklin (https://franklin.genoox.com) including genes from the first level and others involved in the BK-induced inflammation pathway and the kinin-kallikrein system (KKS): *DAB2IP*, *ACE*, *AGT*, *ANGPT2*, *BDKRB1*, *BDKRB2*, *C1R*, *C1S*, *C1QBP*, *C2*, *C3*, *C4A*, *C4B*, *C5*, *COL4A1*, *CPM*, *CPN1*, *DPP4*, *ELANE*, *F2*, *F11*, *GK*, *KLK3*, *KLKB1*, *KRT1*, *MPO*, *LP*, *MASP1*, *MASP2*, *MBL*, *MME*, *NOS3*, *NR3C1*, *PLAT*, *PLAU*, *PLAUR*, *PROC*, *PRCP*, *PTGIR*, *PTGIS*, *REN*, *SLC34A1*, *SLC30A2*, *SYTL2*, *TEK*, *TLR2*, *TLR4*, *TNFAIP3*, *VEGFA*, *KDR*, and *XPNPEP2*. This strategy aligns with recent evidence that expanding genetic screening beyond canonical genes enhances detection rates [25, 27].

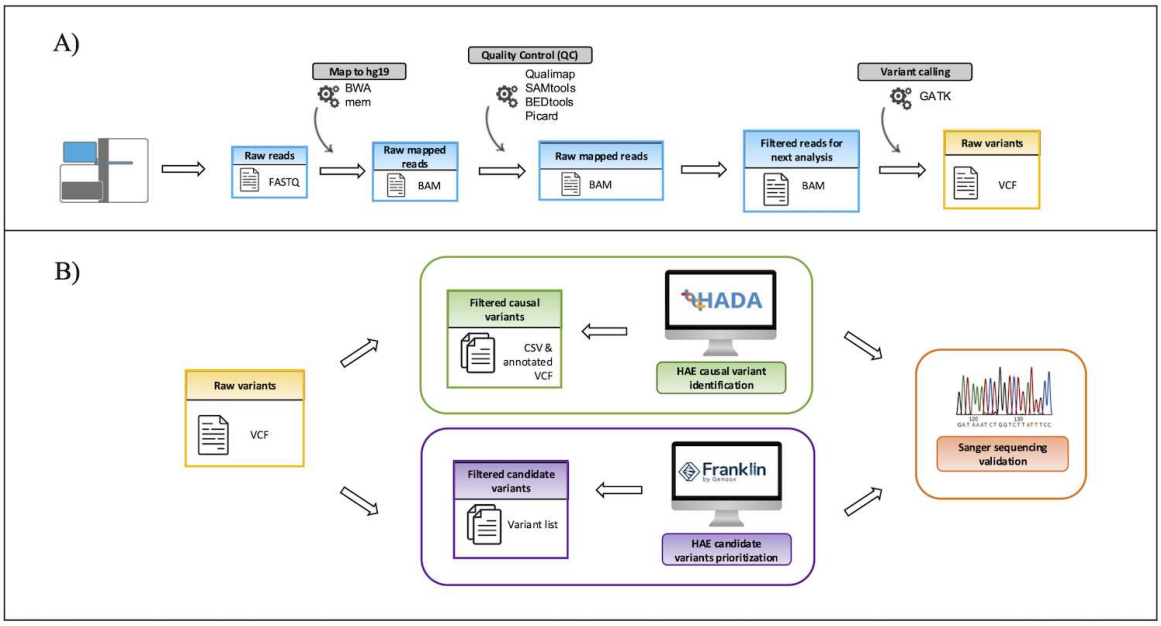


Figure 1. Schematic representation of the steps followed for patient genetic testing. Panel A: Algorithms are indicated in grey and the results files in blue. Panel B: Variants prioritized by HADA (first level) are shown in green, while those prioritized by Franklin (second level) are represented in purple. Finally, the Sanger validation step of candidate variants is shown in orange.

The population AF of the variants prioritized through Franklin was ascertained using two major public genomic databases: non-Finnish Europeans (NFE) from gnomAD v4.1.0 [47] and The 1000 Genomes Project [48]. These resources were employed to filter out common polymorphisms and to prioritize

214 variants with an AF<0.01, as recommended for autosomal dominant conditions in agreement with
215 current ACMG guidelines [46]. To assess the predicted pathogenicity of the prioritized variants, their
216 Combined Annotation Dependent Depletion (CADD) scores were evaluated against gene-specific
217 Mutational Significance Cutoff (MSC) thresholds [49]. For each gene included in the virtual gene
218 panel, a precomputed MSC value of >95% was set as a significance filter. Variants with a CADD score
219 exceeding their respective gene MSC threshold (MSC 95%) were considered to have a predicted
220 deleterious impact. Given that synonymous variants could impact in protein function by interfering
221 with splicing events and/or translation efficiency, all prioritized synonymous candidates were
222 rigorously evaluated using the usDSM (deleterious synonymous mutation prediction) algorithm [50].
223 Whenever possible, familial segregation analysis of the prioritized variant was conducted according to
224 the current ACMG classification guidelines (PP1 criteria).

225 Diagnostic yields were calculated with the observed data as proportions with 95% confidence intervals
226 (CIs) using the Wilson score method (http://vassarstats.net). No statistical comparisons between groups
227 were conducted, and family structure was not incorporated into these descriptive estimates.

228 Finally, all the prioritized variants that were previously unrelated to HAE in the first or second level of
229 the analyses and that could be reclassified as pathogenic (P) or likely pathogenic (LP) were subject to
230 validation by sequencing of direct PCR-amplified products using the BigDye Terminator v3.1 Cycle
231 Sequencing Kit (Thermo Fisher Scientific) as offered by Macrogen Spain Inc. using custom-designed
232 amplicons (**Supplementary Table 2**).

233 HGVS nomenclature was validated for novel variants using the tool LOVD HGVS syntax checker
234 (https://hgvs-nomenclature.org/stable/software/).

235 # 3 Results

236 ## 3.1 Characterization of participants and sequencing

237 A total of 177 subjects were recruited for this study from Spain (n=152) and insular Portugal (n=25)
238 (**Figure 2**). Seven of them did not meet the criteria to be included in our study (i.e., they had acquired
239 angioedema in autoimmune context or were induced by angiotensin-converting enzyme inhibitors or
240 by non-steroidal anti-inflammatory drugs). Thus, we finally included 150 patients with HAE and 20
241 asymptomatic family members from 102 independent families. Of them, 140 patients were of European
242 ancestry, three from Bolivia, two from Israel, two from India, one from Honduras, one from Venezuela,

and one from Argentina. There were 52 male patients (34.6%) and 98 females (65.4%) aged between 4 and 88 years (mean: 45.0 ± 19.7 years). Positive family history for HAE attacks was reported in 71 (47.3%) of them. Based on molecular analysis, all major types of HAE were present in this study. HAE-C1INH was the most abundant, with 65 (43.3%) patients being HAE-C1INH type 1 and 29 (19.3%) being HAE-C1INH type 2. The remaining 56 (37.4%) patients were diagnosed as HAE-nC1INH, of whom females were the majority (76.8%). Clinical data, C1-INH protein levels and activity, and C4 plasmatic levels for all subjects included in this study are provided in **Supplementary Table 3**. We extracted and quantified DNA from the 170 patient blood samples for further studies. WES yielded an average of 8.9 Gb per patient with an average of 99.7% of reads on-target offering a median depth of 118.3X and a mean transition/transversion ratio range of 3.0 to 3.3.

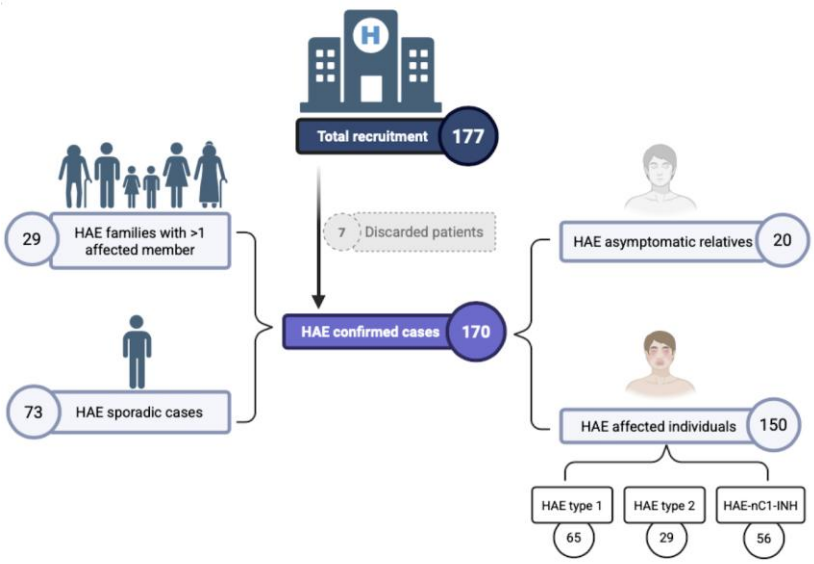


Figure 2. Patient recruitment and classification according to HAE clinical diagnosis.

3.2 Causal variant identification: first level studies

WES data from each patient were submitted to HADA to prioritize previously reported causal variants of HAE described in the scientific literature. In this first step, a total of 28 variants in HAE associated genes were detected in this cohort (**Table 1**, **Table 2**). Additional data regarding sequencing metrics, pathogenicity scores used for filtering and relevant information for prioritized variants is provided in **Supplementary Table 4**.

SERPING1 was the most frequently affected gene, harboring a total of 24 distinct variants with a pathogenic impact. Seven of these variants had not been previously linked to HAE-C1INH, and five

were small indels identified exclusively in the index cases and were absent in the asymptomatic relatives, whenever available (**Table 2**). The missense variant c.863T>A (p.Val288Asp) in exon 5 was detected in an affected father and his sibling from family 29. A novel splicing variant (*SERPING1*:c.-22-15_-22-3del) was identified in a member of family 5 and was initially classified as LP following ACMG guidelines. Additionally, the nonsense variants *SERPING1:*c.731dup (p.Tyr244Ter) and *SERPING1:*c.889+3A>C were prioritized by HADA in two unrelated affected families (families 35 and 38, respectively). The latter is predicted to trigger the Nonsense-Mediated Decay (NMD) pathway, leading to mRNA degradation and preventing the production of a truncated C1-INH protein. Another duplication event (*SERPING1*:c.1455_1459dup) was predicted to cause a frameshift and subsequent protein truncation, reducing the available plasma levels of C1-INH. This LP variant was only found in one affected member of family 37. A third duplication (*SERPING1*:c.336_337insC) was identified in the father and his son on family 59. This variant also predicts activation of the NMD pathway. HADA also prioritized a *SERPING1* intronic variant (c.551-48C>G) in a patient from Portugal (family 60), absent in population databases. However, due to the current limited knowledge, it was classified as a variant of uncertain significance (VUS). These novel variants categorized as VUS, LP, or P with potential clinical impact were reclassified as likely pathogenic based on ACMG guidelines (PP1 criteria; segregation) whenever possible.

The well-known causal variant of *F12* (c.983C>A; p.Thr328Lys) was detected in multiple members of the family 2, in three relatives of family 31, and the mother and his daughter of family 97, all having a clinical suspicion of HAE-nC1INH (**Table 1**). HADA also prioritized two novel variants in the *F12* gene in two unrelated families with affected members. The first variant, *F12*:c.230C>G, was detected in the proband of family 70 and was located in a moderately conserved a region of the gene. It was reported at low AF in population databases (PM2, moderate) and predicted to affect a region considered a mutational hotspot (PM1, supporting). The second variant, *F12*:c.530C>T, was identified in all four affected members of family 52 and predicted to impact the splicing of exon 7. The AF was estimated in 4.78×10^{-5} (PM2, moderate), and was also predicted by multiple *in silico* tools as damaging effect on splicing (PP3, moderate), and it showed co-segregation with the disease in the family (PP1, supporting). According to ACMG guidelines, these *F12* variants were classified as VUS and LP, respectively (**Table 2, Supplementary Table 4**).

HADA also allowed the identification of additional candidate variants with potential effects in other previously known HAE genes. All variants described below exceeded the MSC 95% based on their

CADD scores. Notably, the *MYOF* gene harbored the highest number of predicted functional variants (n=9), including four missense variants detected in affected family members while were absent in asymptomatic relatives (**Table 2**). HADA achieved a consensus among its integrated pathogenicity prediction algorithms, with >95% of predictors indicating a damaging effect on the protein. However, some variants showed conflicting interpretations. For example, the *MYOF*:c.4015G>A variant (family 14) was initially classified as likely benign (LB) following ACMG guidelines. Subsequent integration of additional evidence, as its presence at low AF in other population databases (PM2, moderate), supported its reclassification as a VUS. Two *MYOF* missense variants (c.508C>A;p.Val170Phe in family 63, and c.2177G>A in family 49) lacked sufficient evidence for reclassification and were considered as VUS. In contrast, other variants in this gene, such as *MYOF*:c.5697G>C, *MYOF*:c.5881G>A, and *MYOF*:c.5599-7delG in families 15, 73, and 74, respectively, were predicted to disrupt splicing by the SQUIRLs pathogenicity score [43] and DECIPHER data. These variants met the PM4 ACMG criteria, allowing the reclassification from LB to VUS. In family 83, HADA prioritized the missense variant *MYOF*:c.4208A>C in the index case. The available information supports its classification as VUS. Another variant, *MYOF*:c.3403A>G in family 91, was consistently predicted as deleterious or disease-causing by multiple tools. Finally, the *MYOF*:c.607G>A variant identified in two unrelated families (94 and 98) did not show a consensus among predictors albeit some provided strong evidence of pathogenicity, including a CADD (20.2) surpassing the MSC 95% (13.4).

HADA also prioritized candidate variants in *PLG* and *ANGPT1.* Two relevant variants were identified in *PLG*. The first, *PLG*:c.112A>G, detected in probands from families 76 and 77, has been reported by gnomAD at very low AF (0.00651) and was consistently predicted as deleterious, according to ClinVar classification as LP/P (**Supplementary Table 4**). The second variant, *PLG*:c.296A>T, detected in family 10, showed stronger *in silico* evidence, where splicing analysis algorithms predicted a splice site disruption, while protein modeling suggested that this missense change could induce a truncation caused by a frameshift event. However, the integration of ACMG criteria allowed its reclassification as VUS. Another splice site variant prioritized by HADA was identified in the *ANGPT1* gene (c.809-1G>T) in the index cases of two unrelated families 21 and 43. This variant was located in the canonical 5′ splice site of exon 5, and was predicted to cause haploinsufficiency of the angiopoietin protein, as indicated by DECIPHER.

Finally, a manual review of all candidate calls suggestive of potential inserted *Alu* elements in *SERPING1* revealed that all were likely false positives, resulting from low sequencing depth of coverage in the analyzed regions and lacking sufficient evidence to prompt validation experiments.

3.3 Candidate variants potentially affecting HAE: second level studies

To uncover novel HAE candidate variants in those families and proband-only cases where no variants were prioritized in first instance, we implemented a second level approach based on customized virtual gene panel targeting the KKS and complement cascade using Franklin platform (**Supplementary Figure 2**).

This approach yielded several notable findings. A striking finding was the identification of a *C5* splice-site variant (c.3151_3154+2del) in four unrelated sporadic HAE-nC1INH cases from the Canary Islands, Spain (**Table 3**). This variant was absent from population databases and was predicted to cause loss-of-function (PVS1 and PM2 ACMG criteria), supporting a LP classification. Additionally, a frameshift variant in *C9* (c.1042del) and canonical splice-site variants in *C3* (c.2583+1G>A) were prioritized, both predicted to trigger NMD.

A particularly interesting finding was a *TEK* missense variant (c.2404G>A) that showed complete co-segregation with the disease in family 50, being present in all affected individuals and in one asymptomatic relative, suggesting incomplete penetrance. This observation aligns with the established role of ANGPT-Tie2 signaling in HAE-nC1INH pathogenesis [22]. Additionally, other candidate variants were identified in *ANGPT2*, *KRT1*, *F11*, *VEGFA*, and *KDR*, all of which have plausible biological functions in vascular permeability and BK-mediated pathways. It is important to emphasize that all variants identified in this second-level analysis are candidates in biologically relevant genes, and that their causal relationship with HAE requires functional validation and segregation studies in larger cohorts. A complete list of all prioritized variants from this second-level study, their description and details including *in silico* prediction scores and ACMG classifications, is provided in **Supplementary Results 1** and **Supplementary Table 4**.

3.4 Genetic diagnosis yield

The described filtering strategies enabled the identification of novel causal variants in known genes and revealed a constellation of candidate variants in other disease-associated genes that may explain symptoms in affected individuals (**Figure 3**). The combined use of HADA for detecting previously

reported and novel variants in known genes and Franklin for prioritizing variants in KKS pathway genes provided a full concordance (28/28 variants prioritized by both tools). When considering only previously reported variants classified as LP/P in known genes among families with multiple affected members, we achieved a diagnostic yield of finding a genetic cause of 63.3% (95% confidence interval [CI]: 43.9-79.4%; 19/30 families). Specifically, for patients with laboratory-confirmed HAE-C1INH type 1 or type 2, the diagnostic yield for *SERPING1* was 88.3% (95% CI: 62.3-97.9%; 15/17 families) and 25.0% (95% CI: 13.7-40.6%; 11/44 proband-only). This yield decreased to 21.1% (95% CI: 13.2-32.0%; 15/71 cases) among proband-only. These findings strongly support the WAO/EAACI guidelines for HAE clinical management, which recommends the inclusion of unaffected family members for causal variant screening [30]. Furthermore, our findings highlight the diagnostic challenges in sporadic cases, consistent with recent reports of reduced detection rates in individuals without family history [51].

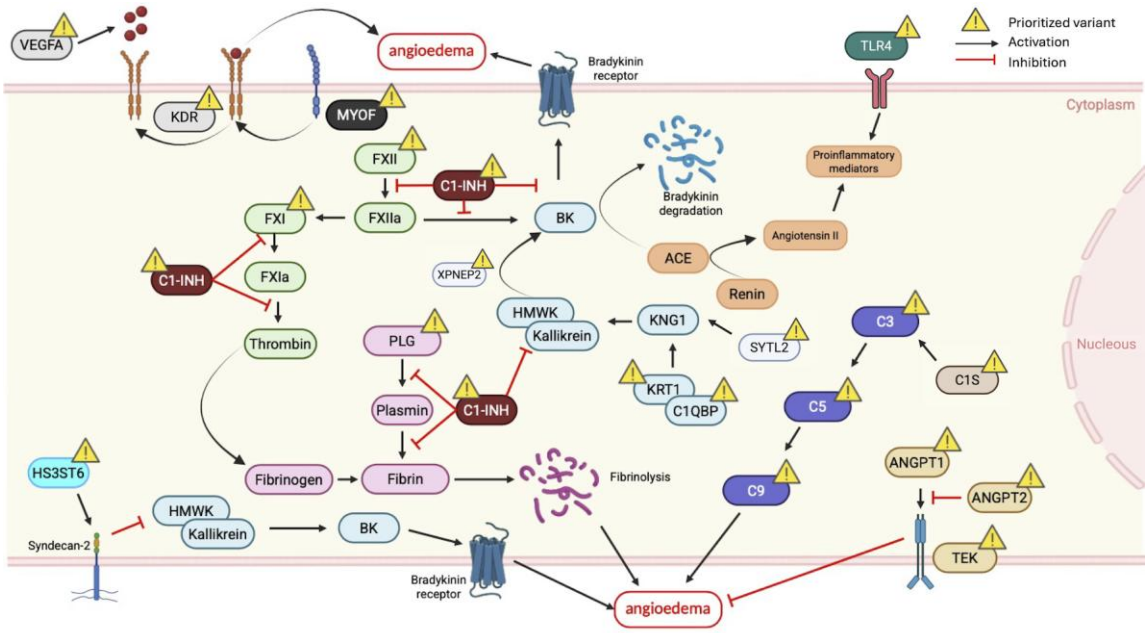


Figure 3. HAE-associated pathway map with prioritized candidate variants in genes of kinin-kallikrein system, the complement, and related gene networks. Yellow triangles denote genes containing the prioritized candidate genetic variants listed in Tables 2 and 3. Additional genes are shown to illustrate relevant functional interactions within the KKS network. Created in BioRender.

4 Discussion

This study represents the most comprehensive genetic characterization of HAE cases from Spain and insular Portugal to date, combining WES with a stepwise variant prioritization analysis pipeline aimed

at delineating the spectrum of potential genetic causes across patients, providing an unprecedented resolution of both known and candidate genetic factors, and presenting a framework for re-evaluating the true burden and molecular diversity of HAE. While previous epidemiological studies reported a C1-INH deficiency prevalence of 1.09:100,000 in Spain [33] versus 1.38:100,000 in Portugal [34], our data reveal striking regional disparities. We observed an unexpectedly high proportion (40%) of HAE-C1INH type 2 cases among Portuguese patients, compared to the 10-15% typically described globally. These findings are consistent with recent evidence suggesting widespread underdiagnosis of HAE, particularly in certain geographic regions [52, 53]. Persistent diagnostic delays, exemplified by a 50-year diagnosis delay in family 3, highlights the urgent need for improved diagnostic strategies aligned with the current management paradigm, requiring to precise HAE subtype identification for choosing an optimal therapy for patients. Remarkably, prior molecular studies of HAE cases from Spain and Portugal have been largely limited to targeted sequencing of *SERPING1* and *F12* or protein-level analyses, with the sole exception of one study that relied on a NGS panel targeting six HAE-nC1INH genes [54].

Our findings underscore the molecular heterogeneity of HAE, with pathogenic variants identified in *SERPING1*, *F12*, and in some of the emerging genes such as *MYOF*, *PLG*, and *ANGPT1*, reinforcing the pivotal role of BK-mediated pathways in the disease pathogenesis. It is important to emphasize that the genes included in our second level analysis represent biological candidates within the KKS and complement pathways, but they do not have yet a proven causal connection with HAE. Therefore, the variants prioritized from this exploratory level should be interpreted as potential contributors requiring rigorous functional validation and segregation studies in larger cohorts. The identification of causal genetic variants of HAE has become increasingly critical in recent years, as directly revealing new disease mechanisms [55]. However, a significant diagnostic gap remains, particularly for patients with normal C1-INH levels, where no pathogenic variants affecting function are detected. Also, the relatively low *SERPING1* detection rate of 25% observed in our proband-only HAE-C1INH cases warrants careful interpretation. Our findings highlight three crucial considerations when no causal *SERPING1* variants are identified in HAE-C1INH patients, especially for type 2 cases. First, while all patients met standard diagnostic criteria based on C1-INH levels and function at the time of diagnosis in accordance with WAO/EAACI clinical management guidelines, for a subset of historical patients recruited from multiple centers, the original laboratory reports containing specific biochemical values could not be retrieved from hospital archives, representing a limitation in data availability rather than a lack of diagnostic confirmation. Second, despite our dedicated bioinformatic screening for *Alu*-

mediated structural variants using MELT and SCRAMble, WES-based methods have inherent limitations for detecting certain types of structural rearrangements, which may account for some unresolved cases. Third, the genetic heterogeneity, with some patients harboring pathogenic variants in genes other than *SERPING1* that indirectly affect C1-INH levels or deep intronic or regulatory variants not captured by WES, might be missed by the sequencing approach. Complementary techniques such as MLPA and soft-clipping analysis could further enhance the detection of structural variants in *SERPING1*, particularly in patients with a precise clinical diagnosis where WES is negative [56].

Pre-analytical factors must be carefully evaluated, as inappropriate sample handling can significantly alter C1-INH plasma levels, functional activity, and complement protein measurements (particularly C4), potentially leading to misdiagnosis and delays in treatment initiation [57]. This diagnostic challenge is exemplified by family 97, in which an initial clinical suspicion of HAE-C1INH type 1 was revised to HAE-nC1INH due to the identification of the *F12*:c.983C>A variant, demonstrating how comprehensive genetic analysis can assist in diagnostic reclassification and inform the clinical management. The predominance of *SERPING1* variants in patients analyzed (43.3% HAE-C1INH type 1 and 19.3% HAE-C1INH type 2) aligns global estimates [58]. Importantly, the identification of seven novel pathogenic variants expands the mutational spectrum of HAE-C1INH. Particularly, the splicing variant *SERPING1*:c.889+3A>C predicts a triggering of NMD leading to haploinsufficiency, a recurrent pathogenesis mechanism in HAE [17]. Beyond the established genes, our second level analysis prioritized *C5*, *TEK*, and *KRT1* as genes of potential interest. The *C5*:c.3151_3154+2del variant may disrupt the complement-mediated inflammation pathway, a plausible contributor to vascular leakage [59]. Similarly, the segregation of *TEK* variant with the disease in the family 50 aligns with previously reported ANGPT-Tie2 signaling defects observed in HAE-nC1INH [60]. These findings advocate for inclusion of complement and angiogenesis-related genes in future diagnostic pipelines, particularly for unresolved cases. However, further studies will be needed, including validation through functional assays and segregation in independent cohorts, to establish if they are causal of HAE or not.

Our study has several limitations. First, it remains challenging to recruit family members for the genetic studies, significantly limiting our capability to assess segregation analyses. This limitation directly impacts the clinical interpretation of many of the identified variants, particularly of those in KKS genes with unconfirmed disease associations and significantly decreases the diagnosis yield. Second, the

study lacks functional assays to validate novel candidate variants and refine the pathogenic classifications, a common challenge in the field, as highlighted by others [27]. This challenge is particularly important for VUS in potentially novel disease genes, where the available evidence often meets only provisional levels of clinical validity according to ACMG/AMP guidelines. A third major limitation involves the lack to access to specialized protein assays for measuring C1-INH and C4 levels on site in Spanish and Portuguese hospitals, further affecting the diagnosis [61]. This gap is being solved by sample transportation to, sometimes distant, reference laboratories, introducing pre-analytical variables, such as sample stability and transport delays, that may compromise protein measurements, and potentially lead to misdiagnosis or delays in treatment initiation [62, 63]. Including a sample from an asymptomatic non-family member in the transport and analysis, as internal quality control, may allow monitoring and prevent erroneous diagnosis. Fourth, for a subset of individuals, particularly in historical or proband-only cases, we were unable to retrieve complete biochemical profiles (C1-INH levels and function) or samples from family members for segregation analysis. While we have compiled all available data, this absence of comprehensive clinical metadata for all participants represents a constraint for the definitive application of certain ACMG/AMP criteria and for establishing stronger genotype-phenotype correlations.

A key strength of our study was the systematic application of WES to elucidate novel genetic factors in HAE, successfully identifying multiple previously unreported pathogenic variants. This approach was particularly impactful in cases with borderline alterations in plasma protein levels, enabling direct molecular confirmation of clinical suspicions and assisting in establishing the correct HAE diagnosis [57]. In addition, we adopted a sequential prioritization strategy that allowed us to resolve clinical diagnoses with ambiguous serological profiles, as exemplified by family 97, in which HAE-C1INH was initially suspected based on clinical presentation. However, WES reclassified the diagnosis as HAE-nC1INH, reinforcing the role of NGS in supporting diagnosis when protein-level assays yield inconclusive results. This approach not only enhanced diagnostic yield but also revealed seven novel *SERPING1* variants absent from ClinVar, the Leiden Open Variant Database (LOVD) v3.0, and the Human Gene Mutation Database, further expanding the mutational spectrum of HAE. The approach also facilitated variant detection in pre-symptomatic family members, as in family 16, demonstrating its preventive potential through early diagnosis before symptoms onset. Finally, this variant prioritization framework also allowed to pinpoint potentially novel HAE associated variants.

Efforts should focus on fostering the recruitment of family members to better interpret the genetic findings, increase the implementation of WAO/EAACI diagnostic guidelines to enable early genetic testing in affected families, and resolve undiagnosed cases through first-degree relative sequencing. Such efforts would directly address WAO/EAACI recommendations 20 and 28 while helping reduce diagnostic odysseys. Furthermore, our results open new horizons for investigating how genetic variants influence treatment responses, which is critical given the current lack of genotype-guided therapy studies in HAE [64].

This study used a variant prioritization framework for the in-depth genetic investigations of HAE patients. Despite comprising the largest sequenced HAE cohort to date in Spain and Portugal, a significant proportion of patients still lack a definitive genetic diagnosis. The integration of WES with a customized KKS gene panel beyond known HAE genes has enabled the reclassification of ambiguous cases and uncovered previously unreported variants, including in *SERPING1* and *F12*. Our findings underscore the critical role of genetic testing in HAE diagnosis, particularly in reducing diagnostic delays and guiding personalized treatments strategies. By expanding the access to NGS-based diagnostics and establishing international HAE registries, one can improve early detection, refine genotype-phenotype correlations, and enhance therapeutic outcomes for patients worldwide. Further research is required to identify the underlying causes of disease in cases where current clinical and genetic evidence remains inconclusive, and to functionally validate the candidate variants and genes prioritized in this study.

5 Conflict of Interest

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

6 Author Contributions

C.F. conceived the study. A.M.-A., A.A.-G., D.B.-P., A.G.-D.R., A.M.-B., L.A.R.-R., D.J., Al.C., R.G.-M., and J.M.L.-S. designed the methodology. IMAGE coordinated data collection. Patient and sample recruitment was performed by L.A.-Q., V.C.-H., E.M.-F., K.B., S.C., R.R.-L., J.A.C.-G., I.M., A.R., A.M.V., E.P.-R., J.C.G.-R., and A.C. Sample preparation and DNA sequencing were carried out by A.M.-A., Al.C., A.A.-G., D.B.-P., A.G.-D.R., and R.G.-M. Formal analysis was performed by A.M.-A., A.M.-B., L.A.R.-R., and J.M.L.-S. A.M.-A. wrote the original draft, and all authors reviewed and edited the manuscript. Supervision was provided by C.F. and A.C. Funding acquisition was done

by A.C., J.C.G.-R., and C.F. All authors have read and agreed to the published version of the manuscript.

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8 Data Availability

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the restrictions established by the HUNSC Ethics Committee (PI 57–17).

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706 **Table 1.** Summary of causal *SERPING1* and *F12* causal variants in the HAE patients included in the
707 study.

Family ID	Individual ID	Gene	Chr	Position start-end	HGVS	Aminoacid change	HAE type	ACMG class
1	1, 2, 3	*SERPING1*	11	57381947-57381947	NM_001032295.2:c.1396C>T	p.Arg466His	HAE-C1INH type 2	Pathogenic
2 **31** **97** **101**	1, 2, 3, 7, 9 1, 2, 4 1 1	*F12*	5	176831232-176831232	NM_000505.4:c.983C>A	p.Thr328Lys	HAE-nC1INH	Likely pathogenic
3	1, 2, 3	*SERPING1*	11	57373471-57373471	NM_001032295.2:c.686-12A>G	None	HAE-C1INH type 1	Likely pathogenic
4	1, 2, 3	*SERPING1*	11	57373548-57373548	NM_001032295.2:c.751C>T	p.Leu251=	HAE-C1INH type 1	Benign
8	1	*SERPING1*	11	57369570-57369570	NM_001032295.2:c.613T>C	p.Cys205Arg	HAE-C1INH type 1	Likely pathogenic
9	1, 2	*SERPING1*	11	57367442-57367443	NM_001032295.2:c.143_144delCA	p.Thr48SerfsTer9	HAE-C1INH type 1	Likely pathogenic
16 **85**	1	*SERPING1*	11	57378700-57378700	NM_001032295.2:c.1100T>C	p.Leu367Pro	HAE-C1INH type 1	Likely pathogenic
26	1	*SERPING1*	11	57378700-57378700	NM_001032295.2:c.1367C>A	p.Ala456Glu	HAE-C1INH type 1	Pathogenic
27	1, 2	*SERPING1*	11	57373548-57373549	NM_001032295.2:c.106_107delAG	p.Ser36PhefsTer21	HAE-C1INH type 1	Pathogenic
28 **34**	1, 2 1	*SERPING1*	11	57382031-57382031	NM_001032295.2:c.1480C>T	p.Arg494Ter	HAE-C1INH type 1	Pathogenic
32	1	*SERPING1*	11	57367850-57367850	NM_001032295.2:c.550G>A	p.Gly184Arg	HAE-C1INH type 1	Pathogenic
33	1	*SERPING1*	11	57369619-57369619	NM_001032295.2:c.666_667del	p.Gln223AspfsTer33	HAE-C1INH type 1	Likely pathogenic
36	1	*SERPING1*	11	57379386-57379386	NM_001032295.2:c.1226T>C	p.Met409Thr	HAE-C1INH type 1	Likely pathogenic

39	1, 2	*SERPING1*	11	57373928-57373928	NM_001032295.2:c.937T>C	p.Phe313Leu	HAE-C1INH type 1	Pathogenic
44	1, 3, 4, 5, 6	*SERPING1*	11	57381947-57381947	NM_001032295.2:c.1396C>T	p.Arg466His	HAE-C1INH type 2	Pathogenic
45	1, 3, 4, 5, 6							
46	1, 2, 3	*SERPING1*	11	57367726-57367726	NM_001032295.2:c.430del	p.Asp144MetfsTer4	HAE-C1INH type 1	Pathogenic
53	1, 2	*SERPING1*	11	57379208-57379208	NM_001032295.2:c.1048T>C	p.Ser350Pro	HAE-C1INH type 1	Likely pathogenic
62	1	*SERPING1*	11	57373607-57373607	NM_001032295.2:c.816_818del	p.Asn272del	HAE-C1INH type 1	Pathogenic

Table 2. Novel variants prioritized in previously reported HAE genes.

Family ID	Individual ID	Gene	Chr	Position start-end	HGVS	Aminoacid change	HAE type	ACMG class
5	1	*SERPING1*	11	57365705-57365705	NM_001032295.2:c.-22-15_-22-3del	.	HAE-C1INH type 1	Likely pathogenic†
10	1	*PLG*	6	161132112-161132112	NM_000301.5:c.296A>T	p.Tyr99Phe	HAE-nC1INH	Uncertain significance†
14	1	*MYOF*	10	95109633-95109633	NM_013451.4:c.4015G>A	p.Glu1339Lys	HAE-nC1INH	Uncertain significance†
15	1	*MYOF*	10	95076472-95076472	NM_013451.4:c.5697G>C	p.Leu1899Phe	HAE-nC1INH	Uncertain significance†
21 **43**	1 1	*ANGPT1*	8	108315596-108315596	NM_001146.5:c.809-1G>T	.	HAE-nC1INH	Likely pathogenic†
29	1, 2	*SERPING1*	11	57373660-57373660	NM_001032295.2:c.863T>A	p.Val288Asp	HAE-C1INH type 1	Likely pathogenic†
35	1	*SERPING1*	11	57373528-57373528	NM_001032295.2:c.731dup	p.Tyr244Ter	HAE-C1INH type 1	Likely pathogenic†
37	1	*SERPING1*	11	57382004-57382004	NM_001032295.2:c.1455_1459dup	p.Lys487SerfsTer91	HAE-C1INH type 1	Likely pathogenic†
38	1	*SERPING1*	11	57373689-57373689	NM_001032295.2:c.889+3A>C	.	HAE-C1INH type 1	Likely pathogenic†
49	1, 2	*MYOF*	10	95134644-95134644	NM_013451.4:c.2177G>A	p.Arg726Gln	HAE-nC1INH	Uncertain significance†
52	1, 2, 3, 4	*F12*	5	176831915-176831915	NM_000505.4:c.530C>T	p.Ala177Val	HAE-nC1INH	Likely pathogenic†
59	1, 2	*SERPING1*	11	57367637-57367637	NM_001032295.2:c.336_337insC	p.Ser113LeufsTer20	HAE-C1INH type 1	Pathogenic†
60	1	*SERPING1*	11	57369460-57369460	NM_001032295.2:c.551-48C>G	.	HAE-C1INH type 1	Uncertain significance†
63	1	*MYOF*	10	95169422-95169422	NM_013451.4:c.508C>A	p.Val170Phe	HAE-nC1INH	Uncertain significance†
70	1	*F12*	5	176832792-176832792	NM_000505.4:c.230C>G	p.Pro77Arg	HAE-nC1INH	Uncertain significance†
73	1	*MYOF*	10	95072785-95072785	NM_013451.4:c.5881G>A	p.Val1961Ile	HAE-nC1INH	Likely pathogenic†

74	1	*MYOF*	10	95076577-95076577	NM_013451.4:c.5599-7delG	.	HAE-nC1INH	Uncertain significance†
76 **77**	1	*PLG*	6	161127501-161127501	NM_000301.5:c.112A>G	p.Lys38Glu	HAE-nC1INH	Pathogenic†
83	1	*MYOF*	10	95107415-95107415	NM_013451.4:c.4208A>C	p.Asp1403Ala	HAE-nC1INH	Uncertain significance†
91	1	*MYOF*	10	95115385-95115385	NM_013451.4:c.3403A>G	p.Arg1135Gly	HAE-nC1INH	Uncertain significance
94 **98**	1	*MYOF*	10	95168666-95168666	NM_013451.4:c.607G>A	p.Val203Ile	HAE-nC1INH	Uncertain significance

† Validated previously VUS reclassified according to publicly available genomic information.

Table 3. Candidate variants prioritized in the second level study.

Family ID	Individual ID	Gene	Chr	Position start-end	HGVS	Aminoacid change	HAE type	ACMG class
12 17 18 19	1	*C5*	9	123751844-123751844	NM_001735.3:c.3151_3154+2delGAAGGT	.	HAE-nC1INH	Likely pathogenic†
30	1, 2	*C9*	5	39311308-39311308	NM_001737.5:c.1042del	p.Ser348AlafsTer5	HAE-nC1INH	Likely pathogenic†
40	1	*TLR4*	9	120475248-120475248	NM_138554.5:c.842G>A	p.Cys281Tyr	HAE-nC1INH	Uncertain significance†
41	1	*C1S*	12	7175762-7175762	NM_001734.5:c.1198G>C	p.Glu400Gln	HAE-nC1INH	Conflicting interpretations of pathogenicity†
42	1	*XPNPEP2*	X	128881715-128881715	NM_003399.6:c.623A>G	p.Gln208Arg	HAE-nC1INH	Uncertain significance†
50	1, 3, 4	*TEK*	9	27206619-27206619	NM_001375476.1:c.2404G>A	p.Ala802Thr	HAE-nC1INH	Uncertain significance†
51	1, 2, 3	*KRT1*	12	53069047-53069047	NM_006121.4:c.1865G>T	p.Gly622Val	HAE-nC1INH	Uncertain significance†
54	1	*SYTL2*	11	85407399-85407399	NM_206927.4:c.6401G>T	p.Arg2134Leu	HAE-C1INH type 1	Uncertain significance†
57 69	1	*C1QBP*	17	5338269-5338269	NM_001212.4:c.401A>G	p.Asn134Ser	HAE-C1INH type 1	Uncertain significance †
67	1	*SYTL2*	11	85420487-85420487	NM_206927.4:c.5802A>C	p.Lys1934Asn	HAE-nC1INH	Uncertain significance†
66	1	*ANGPT2*	8	6360771-6360771	NM_024596.5:c.1342C>T	p.Asp448Asn	HAE-nC1INH	Uncertain significance†
68	1	*F11*	4	187209748-187209748	NM_000128.4:c.1858G>C	p.Glu620Gln	HAE-nC1INH	Uncertain significance†
75	1	*VEGFA*	6	43752340-43752340	NM_001317010.1:c.617C>T	p.Thr206Ile	HAE-nC1INH	Uncertain significance†
78	1	*KDR*	4	55984888-55984888	NM_002253.4:c.241G>A	p.Asp81Asn	HAE-nC1INH	Uncertain significance†
80	1	*C3*	19	6714253-6714253	NM_000064.4:c.606C>T	p.Gly202=	HAE-nC1INH	Uncertain significance†

95	1	*C3*	19	6697662-6697662	NM_000064.4:c.2583+1G>A	.	HAE-nC1INH	Likely pathogenic

† Validated previously VUS reclassified according to publicly available genomic information.

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