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DEPARTMENT OF IMMUNOLOGY AND HISTOCOMPATIBILITY

COMMUNAUTE UNIVERSITE GRENOBLE ALPES

ECOLE DOCTORALE CHIMIE ET SCIENCES DU VIVANT

GREPI LABORATOIRE

PROGNOSTIC AND PREDICTIVE BIOMARKERS OF THE SEVERITY OF BRADYKININ-MEDIATED ANGIOEDEMA

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A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in subject areas of Virology-Microbiology-Immunology (EDCSV, UGA) and Medicine-Life Sciences (UTH).

Larissa, 15/12/2020

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΕΡΓΑΣΤΗΡΙΟ ΑΝΟΣΟΛΟΓΙΑΣ ΚΑΙ ΙΣΤΟΣΥΜΒΑΤΟΤΗΤΑΣ

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Biomarqueurs pronostiques et prédictifs de la sévérité de l'angioedème héréditaire

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Publications

Articles

1. Loules G et al. Targeted next-generation sequencing for the molecular diagnosis of hereditary angioedema due to C1-inhibitor deficiency. *Gene*. 2018 Aug;667:76–82.
2 Vatsiou S et al. A novel deep intronic *SERPING1* variant as a cause of hereditary angioedema due to C1-inhibitor deficiency. *Allergol Int.* 2020 Jan 17;S1323-8930(20)30003-4.
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1. Loules G et al. *SERPING1* gene typing in the era of Next-Generation Sequencing (NGS). *Allergy, Asthma & Clinical Immunology* 13 (Suppl 2):18, 2017.
2. Zamanakou M et al. Genotyping unknown (U-HAE) and idiopathic non-histaminergic angioedema (InH-AAE): Preliminary evidence of pathogenic variants. *Allergy, Asthma & Clinical Immunology* 13 (Suppl 2):12, 2017.
3. Vincent D*, Parsopoulou F* et al. Families affected by Carboxypeptidase N deficiency, 37 years after the single description. *Molecular Immunology* 89:194-196, 2017.
4. Parsopoulou F et al. Plasminogen missense mutation p.Lys330Glu: altered plasminogen glycoforms type I & II and activation susceptibility. *Allergy, Asthma & Clinical Immunology* 15(Suppl 4):45, 2019.
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6. Vatsiou S et al. The silo effect in the annotation of *SERPING1* variation. *Allergy, Asthma & Clinical Immunology* 15(Suppl 4):45, 2019.

Conferences

1. Oral Presentation: Vincent D*, Parsopoulou F* et al. Carboxypeptidase N deficiency: variants in the *CPN1* gene and biological phenotype. 7th ISK2017, Tours, France, 2017.
2. Poster: Loules G et al. Frequency of *PLG* and *ANGPT1* pathogenic variants in U-HAE and InH-AAE. 5th Bradykinin Symposium, Berlin, Germany, 2018.
3. Poster: Parsopoulou F et al. Routine *SERPING1* genotyping by targeted NGS: New pathogenic variants. 69th HSBMB, Larissa, Greece, 2018.
4. Oral Presentation: Parsopoulou F et al. Frequency of pathogenic variants *PLG* K330E και *ANGPT1* A119S in patients with nl-C1INH-HAE. National Allergology Congress, State of the Art, Athens, Greece, 2019.

Preface

This Ph.D. thesis has been developed within the International Co-tutorship (Cotutelle) framework as laid out in both national regulations and the specific ones of the "Communauté Université Grenoble-Alpes (UGA)" at Grenoble, France and the "University of Thessaly (UTH)" at Larissa, Greece. The Ph.D. subject areas are "Virology-Microbiology-Immunology" in the "École Doctorale Chimie et Sciences du Vivant (EDCSV)" for the UGA and "Medicine and Life Sciences" in the Medical School for the UTH. The reception teams for the research were "GREPI Laboratory (Groupe d'Etude et de Recherche du Processus Inflammatoire) EA7408, UGA" and "Department of Immunology and Histocompatibility, Medical School, UTH", respectively. The follow-up committee of the doctorate program consists of Dr. David Laurin and Dr. Nicole Thielens. The PhD will be certified as "European doctorate".

The thesis is a result of a single scientific research project developed in collaboration between both universities. The research was performed in three parts:

PART I

This part took place in GREPI Laboratory, UGA from 1st December 2016 until 31st May 2018. The research was funded by APR2016 program. The main supervisor for this work was *Prof.* Christian Drouet and partially Dr. Arije Ghannam. During this time, the mandatory 120 hours of training were completed. More precisely, 135 hours of training in 4 different modules were followed, among which training in order to obtain the REI Label (Recherche, Entreprise et Innovation). The research performed in this part is presented in detail in Part I: Expression of the receptors of bradykinin (B1R, B2R).

PART II

During the first part of this PhD study, a six-month collaboration with KininX S.A.S was also achieved. KininX S.A.S. funded the project, Dr. Arije Ghannam was the supervisor of the research and the results that occured are presented in Part II: Plasminogen glycoforms alteration and susceptibility to activation associated with the missense variant p.Lys330Glu in HAE-PLG patients.

PART III

This part took place in the Department of Immunology and Histocompatibility of the Medical School of the UTH from 20th July 2018 until 30th November 2020. The research was funded by CeMIA S.A. The supervisor for this work was *Prof.* Anastasios Germenis. All patients' samples used in this part of the Ph.D. were accompanied by written informed consent from the subjects. The Ethics Committee of the University of Thessaly has approved the protocol of the study. The research performed in this part is presented in detail in Part III: Genetic biomarkers of the severity of C1-INH-HAE.

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I would like to thank Dr. Fotis Psarros for providing me samples from HAE patients and for being an advisor and friend during these years. For providing me materials to work with I am deeply grateful to *Prof*. Francois Marceau and Dr. Xavier Charest-Morin and for providing me patients' samples to *Prof*. Henriette Farkas, Dr. Gregor Porebski, Dr. Anna Valerieva, *Prof*. Markus Maurer, *Prof*. Markus Magerl. Finally, I am grateful to all these patients who accepted to contribute to this research project.

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Faidra Parsopoulou
University of Thessaly, Larissa
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Table of contents

Table of Abbreviations

Abbreviations	Definitions
A1AT	a1-antitrypsin
A1ATD	a1-antitrypsin deficiency
A2	Apple domain 2
A2M	a2-macroglobulin
AAE	Acquired angioedema
ACE	Angiotensin-I-convertin enzyme
ACEi-AAE	ACE-inhibitor-induced acquired angioedema
ACMG	American College of Medical Genetics
AMA	American Medical Association
AMP	Association of Molecular Pathology
ANGPT1	Angiopoietin-1
APP	Aminopeptidase P
AT	Antithrombin
AT1R	Angiotensin-II receptor
ATCC	American Type Culture Collection
B1R	B1 receptor of bradykinin
B2R	B2 receptor of bradykinin
BK	Bradykinin
BSA	Bovine serum albumin
C1-INH	C1-esterase inhibitor
C1-INH-HAE	Hereditary angioedema due to C1-inhibitor deficiency
CAS	Contact activation system
CK-1	Cytokeratin-1
COPD	Chronic obstructive pulmonary disease
CPH	Carboxypeptidase H
CPM	Carboxypeptidase M
CPN	Carboxypeptidase N
CVD	Cardiovascular disease
D	Domain
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleoside triphosphate
DPPIV	Dipeptidyl-peptidase 4
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ENFAF	European non-Finnish allele frequency
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FIX	Coagulation factor IX

FXI	Coagulation factor XI
FXII	Coagulation factor XII
FXIII	Coagulation factor XIII
gC1qR	Receptor for the globular heads of the C1q subcomponent of the first component of complement
GnomAD	Genome Aggregation database
GPCR	G-protein-coupled receptor
HC	Heavy chain
HGMD	Human Gene Mutation Database
HGVS	Human Genome Variation Society
HK	High molecular weight kininogen
HNF4α	Nuclear Factor 4α transcription factor
HRP	Horseradish peroxidase
HSP90	Heat-shock protein 90
HT	Hemorrhagic transformation
hUA-SMCs	human Umbilical Smooth Muscle Cells
IAB	Intitute for Advanced Biosciences
IF	Immunofluorescence
IGV	Integrative Genomics Viewer
IL	Interleukin
INFγ	Interferon γ
ISFET	Ion-sensitive field-effect transistor
KK	Plasma kallikrein
KKS	Kinin-kallikrein system
Km	Michaelis constant
KNG	Kininogen
KR	Kringle domain
LC	Light chain
LK	Low molecular weight kininogen
LOAD	Late-onset Alzheimer's disease
LOVD	Leiden Open Variation Database
LTP	Long-term prophylaxis
MAF	Global allele frequency
MAPK	Mitogen-activated protein kinase
MK	Maximakinin
MLPA	Multiplex ligation-dependent probe amplification
MPO	Myeloperoxidase
MyD	Myeloid differentiation primary response protein
nC1-INH-HAE	Hereditary angioedema with normal C1-inhibitor
NCBI	National Center for Biotechnology Information
NEP	Endopeptidase 24.11

NF-κB	Nuclear factor κB
NGS	Next-generation sequencing
NIH	National Institutes of Health
NO	Nitric oxide
NOX	NADPH oxidase
NSGC	National Society of Genetic Counselors
ORF	Open reading frame
P	Ligand peptide
PAI-1, -2	Plasminogen activator inhibitor-1, -2
PAp	Pan-apple domain
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PKC	Protein kinase C
pKK	Plasma prekallikrein
PLG	Plasminogen
PMA	Phorbol myristate acetate
pNA	para-nitroaniline
PRCP	Prolylcarboxypeptidase
RCL	Reactive center loop
RPMI	Roswell Park Memorial Institute growth medium
RT	Room temperature
S	Spacer peptide
serpin	Serine protease inhibitor
SK	Streptokinase
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
Sp	Signal peptide
SP	Substance P
SPase	Signal peptidase
TBS	Tris buffer saline
TLR	Toll-like receptor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
U-HAE	Angioedema with unknown cause
UK	Urokinase-type plasminogen activator
u-PAR	Urokinase plasminogen activator receptor
UTR	Untranslated region
VUS	Variant of uncertain significance
WB	Western Blot

Abstract (EN)

The heterogeneous clinical manifestations and the unpredictable nature of C1-INH-HAE require the identification of biomarkers and the development of accompanying bioassays. To contribute to this purpose this thesis focused on four topics:

I. The expression of bradykinin receptors. B1R and B2R are potential therapeutic targets. Molecular imaging agents enable the visualization and quantification of the receptors at a cellular level. Specific fluorescent ligands were prepared and used as imaging agents in order to examine the expression of the receptors on EA.hy926 and THP1 cell lines and subsequently, on the surface of patients' endothelial cells in resting conditions or during an attack. The detection of naturally expressed receptors was not successful due to low expression or due to low affinity of the ligands. Further investigation is required to develop a diagnostic tool and proceed in human blood samples.

II. Activation of PLG with p.Lys330Glu variant. The alteration of PLG glycosylation patterns was examined in heterozygous and homozygous carriers of PLG p.Lys330Glu variant, previously described as pathogenic for HAE-PLG. In the homozygous patient, a reversal of the glycosylation pattern was observed, while the heterozygous subjects presented the two glycoforms at the same level. A plasmin-specific chromogenic assay was developed in order to measure the PLG susceptibility to activation. Both homozygous and heterozygous carriers displayed a significantly high susceptibility to activation by streptokinase and urokinase. The qualitative *in vivo* impact of p.Lys330Glu on the protein may result in increased plasmin formation and excessive bradykinin production through kallikrein-kinin system activation.

III. Association of genetic variants with the severity of C1-INH-HAE. The concomitant carriage of variants on genes encoding for proteins involved in bradykinin metabolism and function may modify the clinical expression of C1-INH-HAE. Using NGS technology the study aimed to detect and classify rare variants (MAF≤1%) in 54 genes other than *SERPING1* and to associate the carriage of 18 selected functional SNPs (MAF≥1%) with C1-INH-HAE patients' age at disease onset, disease severity based on CALS score and need for LTP, regardless the *SERPING1* mutational status and separately in patients carrying a missense *SERPING1* variant. In the first group of patients, the presence of the C allele of *F12*-rs1801020 was significantly associated with an increase at disease severity; the presence of *SERPING1*-rs28362944 increased 2.5-fold the probability of LTP need; *SERPING1*-rs4926 was associated with later disease onset; *F13B*-rs6003, *SERPINA1*-rs28929474 and *PLAU*-rs2227564 were significantly associated with the severity of the disease. In carriers of a missense *SERPING1* mutation, the presence of the C allele of *F12*-rs1801020 was significantly associated with an increase at disease severity; the presence of *SERPING1*-rs28362944 increased 4.2-fold the probability of LTP need; *SERPINA1*-rs17580 and *SERPINE1*-rs6092 were significantly associated with earlier and later age at disease onset, respectively; *CPN1*-rs61751507 and

F2-rs1799963 were significantly associated with decrease of need for LTP and disease severity, respectively; *KLKB1*-rs3733402 and *KLK1*-rs5515 were associated with both the age at disease onset and the disease severity. Further analyses should be done in order to conclude on the contribution of the detected rare variants to the disease, their functional effects and their clinical validity.

IV. Global data sharing. In order for both researchers and physicians to assess the available genetic data, they need to be classified and shared in public, user-friendly, easily accessible databases. To this aim, we classified and submitted in ClinVar database 45 *SERPING1* variants previously detected in C1-INH-HAE patients of the Laboratory of Immunology and Histocompatibility of the UTH, accompanied by the supporting clinical evidence.

Key Words: C1-INH-HAE, Bradykinin, Biomarker, Receptors, Plasminogen, *SERPING1*

Abstract (FR)

L’expression clinique de l’angioedème héréditaire C1-INH-AOH est hétérogène. L'identification de biomarqueurs et la disponibilité de tests biologiques pourraient rendre compte de cette observation. Ainsi, notre travail a retenu quatre points:

I. L'expression des récepteurs de la bradykinine. Les récepteurs B1 et B2 sont des cibles thérapeutiques potentielles. Par les ligands fluorescents spécifiques pour B1 et B2, nous avons cherché à quantifier les récepteurs pour leur expression sur les lignées EA.hy926 et THP1, pour examiner l’expression sur les cellules endothéliales des patients en condition de repos ou en période symptomatique. L’expression spontanée des récepteurs n'a pu être quantifiée par faible expression ou faible affinité des ligands. Des recherches supplémentaires sont nécessaires pour développer un outil de diagnostic et procéder à des examens sur les échantillons humains.

II. Activation du variant p.Lys330Glu du plasminogène. Le variant a été précédemment décrit comme pathogène pour l’angioedème héréditaire HAE-PLG. Examiné chez des porteurs hétérozygotes et homozygotes, le variant p.Lys330Glu se présente avec une modification de la glycosylation du plasminogène, avec une inversion du modèle de glycosylation chez le porteur homozygote et deux bandes d'intensité égale pour les porteurs hétérozygotes. Le variant p.Lys330Glu présente une sensibilité significativement élevée à l'activation par la streptokinase et l’urokinase, par mesure enzymatique à l’aide d’un chromogène spécifique à la plasmine. L'impact du variant p.Lys330Glu provoque une augmentation de la transformation du plasminogène en plasmine, avec une production de BK par activation du système kallicréine-kinine.

III. Association de variants génétiques à la sévérité de l’angioedème C1-INH-AOH. La combinaison de variants sur des gènes codant pour des protéines impliquées dans le métabolisme et la fonction de la bradykinine peut modifier chez le porteur l'expression clinique de l’angioedème C1-INH-AOH. Par la technologie NGS, des variants rares (MAF≤1%) ont été recherchés dans 54 gènes pour être identifiés dans des combinaisons avec *SERPING1*. 18 polymorphismes fonctionnels ont été retenus (MAF≥1%) et s’associent à l'âge de l'apparition de la maladie, au score de sévérité et au besoin d’une prophylaxie, quel que soit le variant *SERPING1* combiné ou chez les porteurs d'un faux-sens de *SERPING1*. Concernant les variants fonctionnels communs et indépendamment du variant *SERPING1*, les porteurs de l’allèle C de *F12*-rs1801020 présentent une sévérité de la maladie augmentée; la présence du *SERPING1*-rs28362944 multiplient par 2,5 la probabilité d’un besoin de prophylaxie; *SERPING1*-rs4926 a été associé à un retard de l'âge de l'apparition des symptômes; *F13B*-rs6003, *SERPINA1*-rs28929474 et *PLAU*-rs2227564 ont été associés à la sévérité de C1-INH-AOH. Pour les porteurs d'un variant faux-sens de *SERPING1*, les porteurs de l’allèle C de *F12*-rs1801020 présentent de la sévérité augmentée; la présence du *SERPING1*-rs28362944 multiplient par 4,2 la probabilité d’un besoin de prophylaxie;

SERPINA1-rs17580 et *SERPINE1*-rs6092 ont été associés à l'âge de l'apparition des symptômes; l'hétérozygotie pour *CPN1*-rs61751507 est indépendamment associée à une diminution de 98% du besoin de prophylaxie; *F2*-rs1799963 a été associé à la sévérité de la maladie. Enfin, *KLKB1*-rs3733402 et *KLK1*-rs5515 ont été associés à l'âge de l'apparition des symptômes et à la sévérité. Ce premier examen des gènes devrait être poursuivi pour conclure sur la contribution à la maladie des variants rares détectés, leurs fonctions et leur validité clinique.

IV. Partage des données au niveau mondial. Nous avons classé et soumis dans la base de données ClinVar 45 variantes de *SERPING1* précédemment détectées chez des patients de C1-INH-AOH du Laboratoire d'immunologie et d'Histocompatibilité de l'UTH, accompagnées des preuves cliniques.

Mots clés: C1-INH-AOH, Bradykinine, Recepteurs, Plasminogène, *SERPING1*, Biomarqueur

Abstract (GR)

Η κλινική εικόνα των ασθενών με βραδυκινινεργικό αγγειοοίδημα χαρακτηρίζεται από μεγάλη ετερογένεια. Σκοπός της μελέτης αυτής ήταν η αναζήτηση γενετικών και βιοχημικών βιοδεικτών που θα καταστήσουν δυνατή την πρόβλεψη και την πρόγνωση της εμφάνισης ή της διαφορετικής έκφρασης της νόσου με σκοπό να επιτευχθεί η κατά το δυνατόν περισσότερο εξατομικευμένη θεραπεία των ασθενών. Η μελέτη πραγματοποιήθηκε σε τέσσερα μέρη.

Στο πρώτο μέρος, μελετήθηκε η έκφραση των υποδοχέων της βραδυκινίνης (B2R, B1R) σε κυτταρικές γραμμές ανθρώπινων ενδοθηλιακών κυττάρων (EA.hy926) και μονοκυττάρων (THP1), με τη βοήθεια ειδικών παραγόντων μοριακής απεικόνισης. Πρόκειται για φθορίζοντα πεπτίδια αποτελούμενα από πράσινη φθορίζουσα πρωτεΐνη (GFP) συνδεδεμένη με έναν αγωνιστή ή ανταγωνιστή του υποδοχέα-στόχου. Οι υποδοχείς της βραδυκινίνης αποτελούν πιθανό θεραπευτικό στόχο. Σκοπός της μελέτης ήταν να αναπτυχθεί ένα σύστημα μοριακής απεικόνισης που θα μπορεί να χρησιμοποιηθεί, απουσία αποτελεσματικών αντισωμάτων, ως διαγνωστικό εργαλείο σε απομονωμένα κύτταρα ασθενών με βραδυκινινεργικό αγγειοοίδημα, σε κατάσταση ηρεμίας ή κατά τη διάρκεια μίας κρίσης. Η φυσιολογική ή επαγόμενη έκφραση των υποδοχέων στις κυτταρικές γραμμές δεν επιτεύχθηκε, πιθανώς λόγω χαμηλής συγκέντρωσης των υποδοχέων στη μεμβράνη των κυττάρων ή λόγω χαμηλής συγγένειας των πεπτιδίων με τον υποδοχέα. Βελτίωση της ποιότητας των πεπτιδίων και συνέχεια της μελέτης απαιτείται για να αξιολογηθεί και να χρησιμοποιηθεί το σύστημα ως διαγνωστικό εργαλείο.

Στο δεύτερο μέρος, μελετήθηκε η αναστροφή των γλυκομορφών του πλασμινογόνου σε ετερόζυγους και μία ομόζυγη ασθενή για τη μετάλλαξη PLG p.Lys330Glu, η οποία έχει χαρακτηριστεί παθογονική για αγγειοοίδημα με μετάλλαξη στο πλασμινογόνο (HAE-PLG) και αναπτύχθηκε μία μεθοδολογία για τον έλεγχο της ενεργοποίησης του πλασμινογόνου από φυσικούς ενεργοποιητές (στρεπτοκινάση και ουροκινάση). Οι δύο γλυκομορφές εμφανίστηκαν στην ίδια ποσότητα σε ετερόζυγους ασθενείς ενώ στην ομόζυγο ασθενή παρατηρήθηκε αναστροφή των γλυκομορφών με 60% τύπου I και 40% τύπου II. Επιπλέον, τόσο οι ετερόζυγοι ασθενείς όσο και η ομόζυγη παρουσίασαν σημαντικά αυξημένη ευαισθησία στην ενεργοποίηση του πλασμινογόνου. Από τα αποτελέσματά μας, η p.Lys330Glu φαίνεται να προκαλεί αυξημένη ευαισθησία ενεργοποίησης του πλασμινογόνου σε πλασμίνη, γεγονός που μπορεί να οδηγήσει σε υπερπαραγωγή βραδυκινίνης, μέσω της ενεργοποίησης του συστήματος κινίνης-καλλικρεΐνης.

Στο τρίτο μέρος, σχεδιάστηκε και χρησιμοποιήθηκε πρωτόκολλο αλληλούχησης νέας γενιάς για τη γονοτύπηση του *SERPING1* και 54 γονιδίων που κωδικοποιούν πρωτεΐνες εμπλεκόμενες στο μεταβολισμό και τη δράση της βραδυκινίνης. Σκοπός ήταν η συσχέτιση 18 επιλεγμένων λειτουργικών πολυμορφισμών (συχνότητα αλληλίου στον παγκόσμιο πληθυσμό >1%) με το φαινότυπο ασθενών με αγγειοοίδημα λόγω ανεπάρκειας του C1-INH

(C1-INH-HAE). Τα φαινοτυπικά χαρακτηριστικά που μελετήθηκαν ήταν η ηλικία εμφάνισης της νόσου, η βαρύτητα υπολογισμένη με το σκορ CALS και η ανάγκη για μακροχρόνια προφυλακτική θεραπεία (ΜΠΘ). Η στατιστική ανάλυση πραγματοποιήθηκε σε 233 ασθενείς και ο γονότυπός τους για τους λειτουργικούς πολυμορφισμούς συσχετίστηκε με τα φαινοτυπικά χαρακτηριστικά ανεξαρτήτως του τύπου της *SERPING1* μετάλλαξης. Στη συνέχεια η ανάλυση επαναλήφθηκε στους ασθενείς που έφεραν μετάλλαξη αντικατάστασης στο *SERPING1*. Ανεξαρτήτως του τύπου της παθογονικής μετάλλαξης στο *SERPING1*, η παρουσία του C αλληλίου του *F12*-rs1801020 συσχετίστηκε σημαντικά με αυξημένη βαρύτητα της νόσου· η παρουσία του *SERPING1*-rs28362944 φάνηκε να αυξάνει κατά 2,5 φορές την ανάγκη για ΜΠΘ, ενώ ο πολυμορφισμός *SERPING1*-rs4926 συσχετίστηκε με καθυστέρηση εμφάνισης της νόσου· οι *F13B*-rs6003, *SERPINA1*-rs28929474 και *PLAU*-rs2227564 συσχετίστηκαν με τη βαρύτητα της νόσου. Σε ασθενείς που έφεραν μετάλλαξη αντικατάστασης στο *SERPING1,* η παρουσία του C αλληλίου του *F12*-rs1801020 συσχετίστηκε σημαντικά με αυξημένη βαρύτητα της νόσου· η παρουσία του *SERPING1*-rs28362944 φάνηκε να αυξάνει κατά 4,2 φορές την ανάγκη για ΜΠΘ· οι *SERPINA1*-rs17580 και *SERPINE1*-rs6092 συσχετίστηκαν σημαντικά με την ηλικία εμφάνισης της νόσου· η ετεροζυγωτία για τον *CPN1*-rs61751507 συσχετίστηκε ανεξάρτητα με 98% μείωση της ανάγκης για ΜΠΘ· η ετεροζυγωτία για τον *F2*-rs1799963 συσχετίστηκε με μειωμένη βαρύτητα της νόσου· οι *KLKB1*-rs3733402 και *KLK1*-rs5515 συσχετίστηκαν με τροποποιήσεις στην ηλικία εμφάνισης καθώς και στη βαρύτητα της νόσου.

Στο τέταρτο μέρος της μελέτης, πραγματοποιήθηκε χαρακτηρισμός και καταχώρηση στην παγκόσμια βάση δεδομένων ClinVar 45 *SERPING1* μεταλλάξεων που εντοπίστηκαν κατά τη διάρκεια της μελέτης σε ασθενείς με αγγειοοίδημα λόγω ανεπάρκειας του C1-INH συνοδευόμενων από κλινικά δεδομένα, σύμφωνα με τις οδηγίες της ACMG/AMP.

Λέξεις κλειδιά: Κληρονομικό αγγειοοίδημα, Βραδυκινίνη, Υποδοχείς, Πλασμινογόνο, *SERPING1,* Βιοδείκτης βαρύτητας

INTRODUCTION

I. HEREDITARY ANGIOEDEMA DUE TO C1-INH DEFICIENCY (C1-INH-HAE)

A. Clinical phenotype of C1-INH-HAE, the prototypical situation

Hereditary angioedema due to C1 inhibitor deficiency (C1-INH-HAE, OMIM*106100) is a relatively rare autosomal dominant bradykinin (BK)-mediated disorder. Its prevalence is estimated to be approximately 1:50,000 people worldwide, without known differences among ethnic groups[1]. It represents a chronic syndrome characterized by intermittent and unpredictable episodes of nonpruritic, nonpitting, subcutaneous or submucosal swelling (Greek: oedema) typically involving the arms, legs, hands, feet, bowels, genitalia, trunk, face, tongue or larynx[2]. The diagnosis of the disorder is particularly challenging because of its intermittent nature, its high heterogeneous clinical phenotype from asymptomatic to highly severe in patients of the same family, the common unfamiliarity among medical personnel and the lack of specialist centers[3,4].

In a typical episode, the oedema grows more severe for about 12-36 hours and then subsides after 2-5 days, but there are many exceptions to this rule. In general, the variability of this disorder is remarkable. Untreated patients with a severe HAE expression may have attacks every 7 to 14 days, with the frequency ranging from virtually never to every 3 days. In some patients attacks last less than 24 hours. The occasional patients' abdominal attacks, which are highly painful, last 4 days and rarely peripheral swelling attacks could last as long as 9 days[4,5,6].

Although attacks are sporadic and usually do not have a clear triggering cause, results of observational studies suggest that almost in one third of patients an attack is precipitated after trauma or pressure and in one third when they experience emotional stress. Many attacks also occur without an apparent trigger.

HAE attacks usually begin in childhood, become more severe at puberty for female patients, and persist throughout life, with unpredictable severity. However, they can begin anytime in the adulthood[6]. The autosomal dominant inheritance pattern would predict equal incidence and severity in men and women, but most physicians note a clear female predominance (60%). This might represent selection bias but could also reflect hormonal influences on disease severity. It is generally accepted that there are distinct patterns of HAE in women (oestrogen dependent, oestrogen sensitive and oestrogen-independent)[7]. Many women note that attack frequencies are highest at the time of menstruation and they decrease during pregnancy, more precisely in the second trimester.

Swelling is mainly associated with disfigurement and disability due to loss of tissue flexibility but not with pain. However, patients who present swelling of the wall of the bowel can suffer from severe abdominal pain. This pain is often spasmodic rather than steady, presumably increasing with each peristaltic wave. In many patients vomiting, constipation or diarrhea are regular features of the episode and this kind of attack can lead to surgery in the patient without a diagnosis because of the severity of the pain.

Swelling can be life threatening when it involves the airway, where it can lead to asphyxiation and death. Frank et al. have reported in their clinical studies that about one third of untreated relatives of family members with the disease had a history of asphyxiation.

In the last 15 years, new and effective acute therapies have become available, some of which have also provided short-term and long-term prophylaxis options, together with a better understanding of older prophylactic drugs[8].

B. Distinction between BK-mediated angioedema situations

1. History

Swelling has been recognized as a clinical condition since the 4th century BC, when Hippocrates in Prognosticon defined edema as a "soft, painless and pitting tumor of organs". In Graeco-Roman times a tumor included all abnormal swellings and it was explained on the basis of inflammation. In 1586, Marcello Donati described for the first time a swelling disorder with urticaria and in 1777, almost 200 years later, Franz Anton Mai referred in his book to a 62-year old man suffering from swelling illness, starting from eyelids and lips and finally attacking different organs, including the larynx. Further reference to the syndrome was made in 1843 and in 1876 by Robert James Graves and John Laws Milton respectively.

The first full medical report for angioedema is attributed to the German physician Heinrich Ireanus Quincke (1882) and his student, Eugen Dinkelacker, which explains the fact that "Quincke's edema" is still used as a term to define angioedema in many countries, particularly edema of the face and lips. This term was followed by "angioneurotic edema" in 1885, used by Paul Strubing, which reflected the belief that the process of edema formation was a consequence of neurosis. Sir William Osler enlightened the hereditary nature of the disease by recording five generations in a family with this syndrome and Osler's precise description holds true until today. During the first half of the twentieth century, only occasional case reports are found in the literature[9,10].

However, from 1961 until today, remarkable progress has been done in research which led to a better understanding of the pathogenesis of the disease. In 1961, Irwin Lepow discovered an inhibitor of the first component of the complement and named it C1-esterase inhibitor (C1-INH)[11]. At the same time, Landeman presented his observations on a group of HAE patients. More precisely, he described signs, symptoms, differential diagnosis of HAE and proposed criteria to differentiate an allergic from a non-allergic form and the hereditary from the non-hereditary type[12]. In 1963-1964, Donaldson and Evans defined a low circulating C1-INH as the main biochemical abnormality in angioedema patients (HAE Type I) and suggested a role of C1-INH in HAE[13,14], making the first key observation for the progress in understanding the syndrome. Subsequently, in 1965 Rosen et al. studied patients' plasma profile and proposed a distinction between HAE type I and type II, where HAE type II diagnostic is retained when an abnormal, nonfunctional protein is synthesized[15]. A few years

later, they presented evidence that HAE Type I and Type II are clinically indistinguishable[16] and many case reports followed these publications, suggesting that the syndrome was more common than originally considered[17,18,19]. At that time, Michael Frank and his colleagues had pointed out that both hereditary and acquired forms of angioedema were not histamine-mediated as it was the "allergic" form with urticaria[5].

In 1972, Caldwell et al. reported an acquired C1-INH deficiency in patients with lymphoproliferative disease[20], without giving definite answers concerning the mediators of these attacks. It took ten more years until the second key observation by Kaplan et al.[21] that swelling is predominantly a consequence of excessive generation of BK which was further supported by other research groups. More precisely, the team described the generation of plasma kinins due to the activation of kinin-kallikrein system (KKS).

In 1984-1985 two different types of acquired angioedema were presented, the angioedema in patients with lymphoproliferative disorders (AAE Type I) and the AAE characterized by the formation of anti-C1-INH autoantibodies (AAE Type II)[22,23].

Iatrogenic situations have next been reported, the first cases with ACE-induced acquired angioedema (ACEi-AAE) where patients were treated for hypertension with angiotensin-I-converting-enzyme inhibitors[24]. The mechanism was revealed by Israili and Hall[25] and by 1998, guidelines for management of hypertension were proposed in order to prevent these side effects[26]. Similar AE situations were reported for patients treated with angiotensin-II receptor (AT1R) antagonists though the risk was assessed as lower than with ACE inhibitors[27]. Moreover, Brown et al. (2009) reported that the treatment with dipeptidyl-peptidase-IV (DPP-IV) inhibitors may be associated with an increased risk of angioedema in diabetic patients who take an ACE or an AT1R inhibitor[28]. Hermanrud et al. (2017) reported a case of recurrent angioedema associated with pharmacological inhibition of DPP-IV[29]. Isolated iatrogenic AE cases have been reported concerning patients taking a 5-alpha reductase inhibitor. Finally, AE has been reported in patients with acute ischemic stroke treated with recombinant tissue plasminogen activator (rtPA) with a frequency of 1.9%.

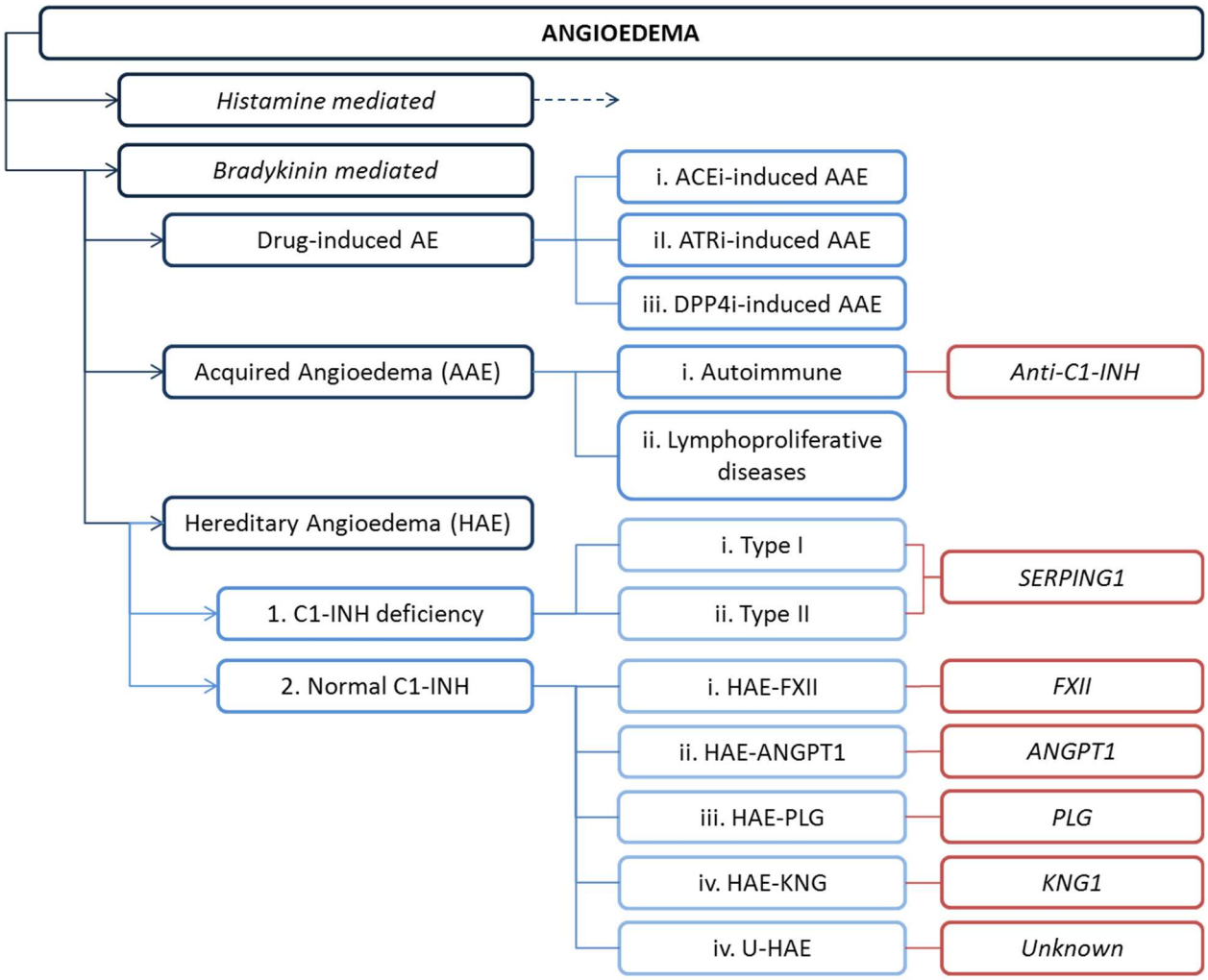
In 2000, a new form of HAE (type III HAE, nC1-INH-HAE) has been described simultaneously by Bork et al. and Binkley et al[30,31]. In this type of AE, the patients (predominantly females) exhibit the same clinical manifestations as in C1-INH-HAE, but function and antigenic C1-INH and antigenic C4 are normal, yet bradykinin overproduction is postulated. Between 2006 and 2013, four different mutations were identified in the *F12* gene coding for coagulation factor XII (FXII) with an increased susceptibility to activation and subsequent development of enzymatic function[32,33].

2. Distinction between HAE situations

Besides clinical and biochemical observations, genetics of angioedema has also been elucidated. During the last 30 years, over 700 mutations have been identified on *SERPING1* coding for C1-INH. Cases of carriers of *F12* mutations that have been considered as causative

for HAE type III have been more and more frequently reported. During the last two years, variants in genes coding for angiopoietin-1 (*ANGPT1*), plasminogen (*PLG*) and kininogen (*KNG1*)[34,35,36] have been found to segregate with angioedema symptoms in families with HAE and normal C1-INH, indicating that, in fact, angioedema represents a non-uniform entity with a complex pathophysiology and a variety of clinical manifestations. The current classification of BK-mediated angioedema is illustrated in Figure 1.

Figure 1. Illustration of BK-mediated AE types and causative factors for each type. Histamine-mediated AE is not discussed in this thesis. Drug-induced AE can be associated with treatment with ACE inhibitors, AT1R antagonists, DPP-4 inhibitors. Different types of AAE can be associated with lymphoproliferative disorders or characterized by the formation of anti-C1-INH autoantibodies. HAE is divided according to the presence or absence of C1-INH. Causal variants in *SERPING1* are responsible for C1-INH-HAE (Types I and II). HAE with normal C1-INH (nC1-INH-HAE) can be inherited due to variants in the *F12* (HAE-FXII), *PLG* (HAE-PLG), *ANGPT1* (HAE-ANGPT1), *KNG1* (HAE-KNG) genes or in yet unidentified loci (U-HAE).



II. HAE PATHOPHYSIOLOGY

A. Kinins, kinin-forming and kinin catabolism processes

1. Bradykinin (BK), the prototypical kinin of HAE

The progress in understanding HAE was based on the observation that accumulation of BK was the cause of swelling[37]. BK (from Greek brady=slow and kinin=indicating movement) is a nonapeptide generated by the cleavage of high molecular weight kininogen (HK) by plasma kallikrein (KK). BK and Lys-BK are the main kinin peptides in humans[38], potent vasodilators, components of the kinin-kallikrein system (KKS). Lys-BK represents a major ligand of B2 receptor of BK (B2R). Metabolites Lys-*des*Arg^9-BK and *des*Arg^9-BK represent ligands for B1 receptor of BK (B1R). Lys-*des*Arg^9-BK has high affinity for B1R, while *des*Arg^9-BK is an alternative ligand. Extreme variations in the kinin concentration in venous blood have been reported since the early 1970s and the values have come down from nanograms to picograms per ml of blood, even during attacks, with the improvement of the extraction procedures and the assays. These peptides should be considered as autacoids acting as autocrine/paracrine factors.

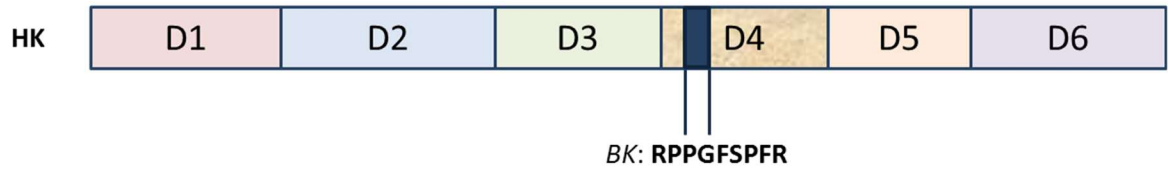
2. Kinin-forming processes

The kinins result from the hydrolysis of large circulating precursors called kininogens. In most mammalian species, there are two kininogens, called, based on their size, high molecular weight kininogen (HK; 628 amino-acid residues) and low molecular weight kininogen (LK; 409 amino-acid residues). HK exhibits a molecular mass of 88-115 kDa depending on the species origin and circulates in human plasma at a concentration of 90 μg/ml. The molecular mass of LK ranges from 50 to 68 kDa and the concentration in human plasma is 170 μg/ml. An N-terminal heavy chain of 50-60 kDa linked to a C-terminal light chain of variable length constitutes the basic structure of both kininogens.

The two forms of kininogens are encoded, by the same gene, *KNG1*, which is mapped to chromosome 3. *KNG1* extends over 27,000 bp and consists of 11 exons. The 9 exons in the 5'-terminal part of the gene encode for the common sequence. Exon 10 encodes for the domain D4, which contains the BK sequence and both D5 and D6 of HK. Exon 11 is located downstream from exon 10 and specifies the unique sequence for LK. The gene uses alternative splicing to generate the 2 different circulating proteins.

Hydrolysis of LK by tissue kallikrein type I releases Lys-BK. Lys-BK is identical to BK except for a Lys residue at its N-terminal part and has the same biological effects. However, it is not regulated by C1-INH and there is no evidence that this pathway contributes to AE. Two main mechanisms have been proposed to lead to the release of BK from HK: the activation of CAS and KKS and the activation of HK at the surface of endothelial cells.

Figure 2. Illustration of the 6 domains (D1-D6) of HK, including the nonapeptide BK.



The Contact Activation System (CAS) refers to a proteolytic system initiated by FXII also known as Hageman Factor (HF), which activates the proinflammatory KKS and the intrinsic coagulation pathway. The cascade is initiated by the autoactivation of FXII to FXIIa, once it is bound to negatively charged surfaces and results in the conversion of prekallikrein (pKK) to plasma KK. KK reciprocally activates FXII, creating a positive feedback loop of system activation and leading to FXI activation. FXI connects the CAS to the coagulation. FXII can be also activated by plasmin, while FXIIa and KK can also activate complement and fibrinolytic factors and mediate cross-talk between the contact, complement, and fibrinolytic systems[39].

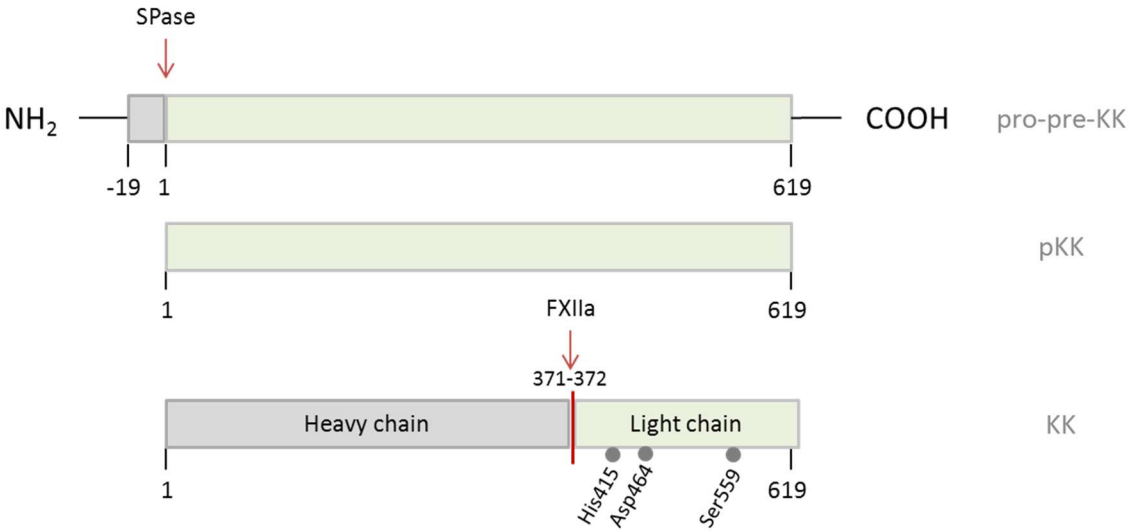
The KKS, also referred as kinin-kininogen-kallikrein system, is a complex network of proteins and peptides involved in several biological processes. It refers to active plasma KK cleaving HK at two positions within a disulfidic bond, generating BK. CAS and KKS are overlapping and interacting as KK is a part of both pathways[40]. However, KK in KKS can also be activated in a FXII-independent manner. More precisely, pKK can autoactivate when bound to HK and its autoactivation is accelerated by heat-shock protein 90 (HSP90)[39] and membrane-expressed enzyme prolylcarboxypeptidase (PRCP).

3. The proteins of CAS and KKS

a. Plasma kallikrein (KK)

Human plasma prekallikrein (pKK) is a glycoprotein that is predominantly synthesised in the liver. It is secreted into the circulation as a single chain zymogen that has an apparent MW of approximately 85-88 kDa. pKK has a concentration of approximately 50 μg/ml in the plasma of healthy individuals and circulates mainly as a complex with HK (75-90%). It is encoded by *KLKB1* gene, extending over 22,000 bp, and mapping to chromosome 4. *KLKB1* consists of 15 exons and 14 introns. Exon 1 codes for the 5'UTR of the mRNA and exon 2 for the signal peptide sequence. Exons 3 to 10 encode for the 4 homologous apple domains. Exons 9 to 15 encode for the catalytic domain of PK containing the catalytic triad (His^{415}, Asp^{464} and Ser^{559}). The only known member of the same family of *KLKB1*, is *F11*, which encodes for FXI, maps to the same chromosomal locus and has an identical exon-intron structure. This suggests that *KLKB1* and *F11* genes most likely arose by a gene duplication event.

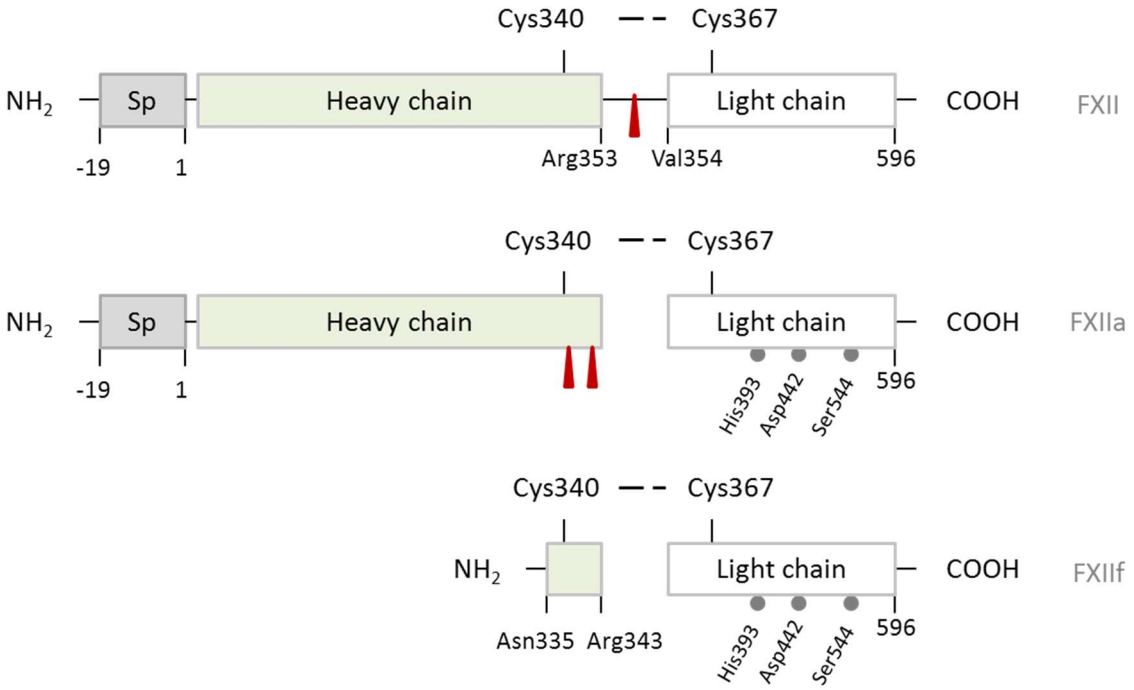
Figure 3. Processing of human pKK. The primary translation product pro-pre-KK consists of 638 residues. Signal peptidase (SPase) detaches the leader peptide (19 amino acid residues) to form pKK. FXIIa cleaves pKK at a single site to generate a heavy chain (HC) and a catalytic light chain (LC) connected. Grey circles illustrate the relative position of the residues of the catalytic triad (His^{415}, Asp^{464} and Ser^{559}).



b. Coagulation Factor XII (FXII)

FXII circulates as a single chain zymogen with no enzymatic activity. Its molecular weight is approximately 80 kDa on SDS gel electrophoresis. FXII is synthesized in the liver and circulates in human plasma at a concentration of 30-35 μg/ml. The autoactivation of FXII is a result of a conformational change that renders bound FXII a substrate for Factor XIIa. Further cleavage can occur at the C-terminal end of the heavy chain to produce a series of fragments the most prominent of which is a 30 kDa species termed Factor XIIf. These fragments lack the ability to initiate coagulation by converting FXI to FXIa. However, they continue to be potent activators of pKK. Thus formation of Factor XIIf allows BK production to continue in the fluid phase until the enzyme is inactivated and the reactions can therefore proceed at sites distant from the initiating surface.

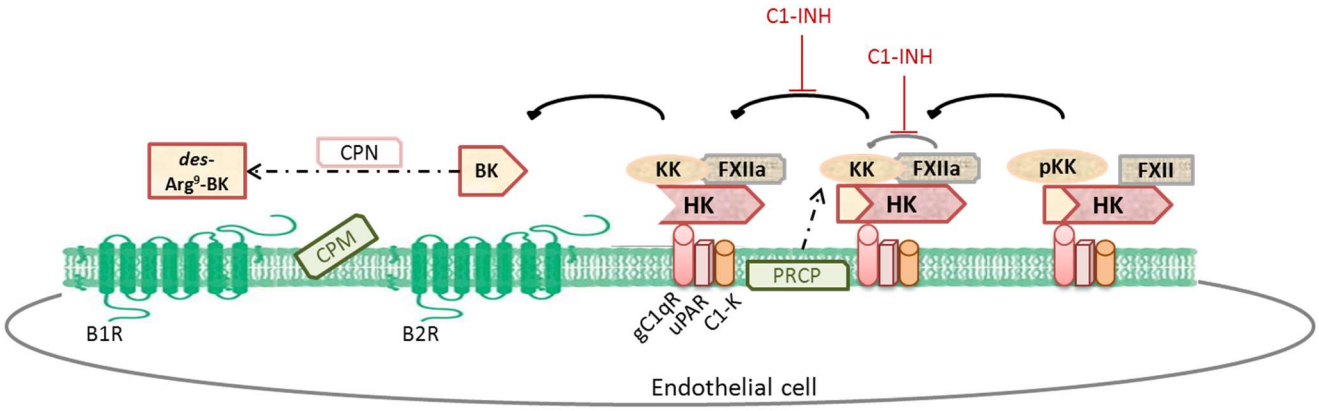
Figure 4. Proteolytic activation and processing of FXII. FXII is a single chain zymogen with a signal peptide (Sp) that mediates secretion from hepatocytes. Auto-activation and KK-mediated activation leads to cleavage of the Arg^{353}-Val^{354} peptide bond and formation of FXIIa. The active protease is composed of a heavy and light chain that is still connected via a disulfide bond (Cys^{340}-Cys^{367}). FXII light chain contains Ser^{544}, His^{393} and Asp^{442} that compose the catalytic triad. Further cleavage of FXIIa at the peptide bonds Arg^{343}-Leu^{344} and Arg^{334}-Asn^{335} by KK, results in the FXIIf (β-FXIIa)[41].



4. Role of the endothelium in the release of BK from HK

All the components of the BK-forming cascade have been demonstrated to bind to endothelial cells. Three endothelial cell binding sites for HK and FXII have been described so far: (a) the receptor for the globular heads of the C1q subcomponent of the first component of complement (gC1qR/p33)[42,43] (b) cytokeratin 1 (CK-1)[44,45] and (c) the urokinase plasminogen activator receptor (u-PAR)[46]. These components are displayed as complexes of either gC1qR-CK-1 or CK-1-uPAR, as well as uncomplexed gC1qR[47]. HK binds preferentially to gC1qR-CK-1 and free gC1qR, while FXII binds primarily to uPAR within the CK-1-uPAR complex. Binding of HK to endothelial cells leads to pKK activation in a FXII-dependent or a FXII-independent way. In any case, the activation of plasma pKK via both mechanisms respresents a critical step to the physiological release of BK by HK.

Figure 5. Strategic complement and KKS components in the angioedema episodes. FXII, pKK and HK interact with the endothelial cell membrane through a binding site formed by gC1qR/p33, uPAR and CK-1. The consequent activation of pKK by FXIIa and PRCP leads to the generation of BK that can stimulate the B2R. BK can be cleaved by carboxypeptidase M (CPM), on the cell membrane, or carboxypeptidase N (CPN), in the soluble phase, forming *des*Arg^9-BK, agonist for B1R.

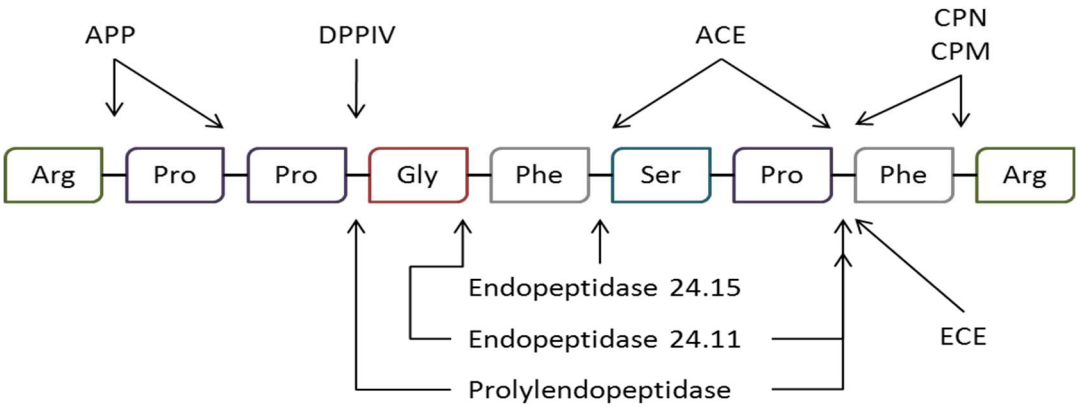


5. Kinin catabolism process

Five metallopeptidases are the main responsible enzymes for the catabolism of BK: angiotensin-I-converting enzyme (ACE), neutral endopeptidase 24.11 (NEP), carboxypeptidase N (CPN), carboxypeptidase M (CPM) and aminopeptidase P (APP). In order to hydrolase their substrates, they all require zinc in their catalytic site. Except for CPN, they are membrane-bound single-chain glycoproteins. However, they are also found in soluble form in biological fluids. Other peptidases can secondarily cleave BK; an important aminopeptidase is DPPIV, which will also be discussed below.

All the above referred enzymes rapidly cleave kinins in active or inactive metabolites. However, they hydrolyse several other oligopeptides, like anaphylatoxins, angiotensin peptides, substance P (SP) etc. The proteolytic actions of endopeptidases yield inactive fragments. The action of aminopeptidase N does not influence the biological activities of B2R agonists since BK and Lys-BK are equally active; however, deletion of N-terminal Lys causes a loss of activity on the human B1R because Lys-*des*Arg^9-BK is 100-fold more potent than *des*Arg^9-BK[48].

Figure 6. Amino acid sequence and catabolism enzymes of BK. CPN is known as kininase I and ACE as kininase II. CPM and CPN cleave residues from the C-terminus of the peptide. APP cleaves amino acid residues from the N-terminus of BK. ACE is a carboxy dipeptidase that produces sequential cleavage of dipeptides from the C-terminus of the peptide. DPPIV cleaves *des*Arg^{9}-BK.



a. Angiotensin-I-converting enzyme (ACE)

Angiotensin-I-converting enzyme (ACE, OMIM*106180) is a widely distributed enzyme, primarily found in abundance in the vascular endothelium. It is bound with a C-terminal sequence to cell membranes and its catalytic sites are exposed on the extracellular surface of the cell[49,50]. ACE has two well-recognized biological activities. It inactivates BK by hydrolysing two separate bonds on its C-terminal end. More specifically, it removes the dipeptide Phe^{8}-Arg^{9} and subsequently cleaves the Phe^{5}-Ser^{6} bond to generate the second dipeptide Ser^{6}-Pro^{7} [51,52]. Concerning its actions on kinins, ACE can degrade *des*Arg^{9}-BK by removing the C-terminal tripeptide Ser^{6}-Pro^{7}-Phe^{8} [53,54]. The second one is the activation of angiotensin II by the release of the C-terminal dipeptide His^{9}-Leu^{10} from angiotensin I[51,55]. Nevertheless, the Michaelis constant (Km) of ACE for BK (0.18 mM) is lower than for angiotensin I (16 mM); this means that ACE is mainly a kininase rather than an angiotensinase[56,57].

b. Neutral endopeptidase 24.11 (NEP)

Neutral endopeptidase 24.11 (NEP, OMIM*120520) is a membrane-bound enzyme encoded by *MME* gene, which is located in chromosome 3q25.2. *MME* exists in a single copy, extends over 80,000 bp and is composed of 24 exons. The gene is highly conserved among mammalian species[58]. NEP has a wide tissue distribution, but contrary to ACE, its expression in vascular endothelial cells is low[59]. NEP cleaves bonds on the N-terminal part of hydrophobic amino acid residues. It inactivates BK by cleaving the Pro^{7}-Phe^{8} bond to liberate the C-terminal Phe^{8}-Arg^{9} dipeptide. However, a prolonged incubation also results in the hydrolysis of the Gly^{4}-Phe^{5} bond[60]. Concerning *des*Arg^{9}-BK it is likely that, in contrast to ACE, NEP cleaves the Gly^{4}-Phe^{5} bond to generate two inactive peptides.

c. Carboxypeptidase N (CPN)

CPN (OMIM*603103) is synthesized in the liver and released into the plasma as a 280-kDa tetrameric complex consisting of 2 identical 83-kDa regulatory subunits and 2 identical 50-kDa catalytic subunits. CPN cleaves basic amino acid residues (-Arg or -Lys) from the C-terminus of peptides and proteins, generally -Lys faster than -Arg[61]. The enzyme plays a central role in regulating the biological activity of peptides such as kinins and anaphylatoxins, and therefore it is also known as kininase-I and anaphylatoxin inactivator. Skidgel et al. (1988)[62] and Gebhard et al. (1989)[63] each purified CPN and determined a partial sequence of the catalytic subunit. Concerning the cleavage of BK, it removes the C-terminal Arg residue of BK to generate the active B1R agonist (*des*Arg^9-BK).

Molecular cloning of the catalytic subunit has revealed sequence similarities to other metallocarboxypeptidases (14%-49%)[63] and 41-49% with CPM, and CPH[64]. The regulatory subunit showed identity neither with the catalytic subunit nor with other carboxypeptidases[65].

d. Carboxypeptidase M (CPM)

CPM (OMIM*114860) is a membrane-bound Arg/Lys carboxypeptidase found in many tissues and cultured cells. CPM is encoded by *CPM* gene, which is located in 12q15. The cloning and sequencing of the 2-kb cDNA from a human placental cDNA library contained an open reading frame (ORF) of 1,317 bp encoding for a 439-residue protein[64]. The deduced sequence of CPM is 41% identical with that of the catalytic subunit of human plasma CPN and 43% with human CPH[66].

Carboxypeptidases have important activities in many biological processes, including activation, inactivation or modulation of peptide hormone activity and alteration of physical properties of proteins and enzymes. CPM, identical to CPN, cleaves C-terminal Arg or Lys from a variety of synthetic and naturally occurring peptide substrates[61,67]. In contrast to CPN, it cleaves C-terminal Arg preferentially over Lys residue and generates the active B1R agonists *des*Arg^9-BK.

e. Aminopeptidase P (APP)

APP (X-prolyl aminopeptidase 2, OMIM* 300145) is a proline-specific metalloaminopeptidase that catalyses the removal of any unsubstituted N-terminal amino acid that is adjacent to a penultimate proline residue[68,69]. APP is a membrane-bound enzyme. The serum form of APP is probably released from the membrane of endothelial cells following phospholipase or proteinase action[70]. However, the soluble form in human leukocytes exhibits similar specificity and other characteristics to membrane-bound APP, but differs in size[71], suggesting that the two types represent separate gene products.

APP is encoded by *XPNPEP2* gene, located on chromosome X. APP cleaves BK by hydrolysing Arg[1]-Pro[2] bond. BK represents an excellent substrate for APP *in vitro*. However, the hydrolysis results in loss of biological activity.

f. Dipeptidyl peptidase IV (DPPIV)

DPPIV (OMIM*102720) is a multifunctional protein, member of the prolyl oligopeptidase family related proteins. It liberates a dipeptide from the N-terminal part of its substrates. Like other members of the family, it prefers -Pro and -Ala, although peptides with other residues (Gly, Ser, Val) at the same position can also be cleaved but more slowly[72]. DPPIV is encoded by *DPP4* gene, mapped on Chr2q24.2. It is a membrane protein expressed on cells throughout the body but also circulates as a soluble protein in the plasma. A large number of bioactive molecules can be cleaved *in vitro* by DPPIV but only a few of them have been demonstrated to be physiological substrates.

Apart from direct effect through B2R, BK stimulates substance P (SP) from sensory nerves which increases vascular permeability by acting on NK1 receptor[73]. ACE degrades SP and DPPIV sequentially degrades SP to SP5-11 which is susceptible to further degradation by APP. DPPIV contributes to the N-terminal degradation of both kinins and SP. However, BK and *des*Arg[9]-BK are inactivated by APP before cleavage by DPPIV. DPPIV contributes significantly to the inactivation of SP[74].

B. Bradykinin receptors

Kinins exert their physiological actions through two different cell surface receptors, called B1R and B2R. The receptors were pharmacologically defined in the late 1970s and this classification is currently supported by several molecular biology findings.

Both receptors belong to the rhodopsin-class of the G protein-coupled receptors (GPCR) superfamily which consist of a single polypeptide chain that spans the membrane seven times, with an extracellular N-terminal domain and an intracellular C-terminal domain, three extracellular loops and three intracellular loops[38]. B1R and B2R are encoded by *BDKRB1* and *BDKRB2* genes respectively, which are clustered on chromosome 14. The *BDKRB2* gene consists of three exons, two of which are non-coding, while the third contains the full-length coding region. The gene encodes for a protein of 364 amino acids. The human *BDKRB1* gene presents 36% homology with *BDKRB2*, but it contains an additional exon, which may originate from the insertion of an *Alu* repetitive sequence during evolution. Moreover, *BDKRB2* carries an alternatively spliced exon between exons 2 and 3[75].

BK and Lys-BK are natural B2R agonists, while their C-terminal *des*Arg metabolites, *des*Arg[9]-BK and Lys-*des*Arg[9]-BK (*des*Arg[10]-kallidin) are B1R agonists. In fact, the only subnanomolar agonist of the human B1R is Lys-*des*Arg[9]-BK. The metabolite *des*Arg[9]-BK is potent agonist of B1R in rodent species but it has a weak affinity for human B1R[76]. Therefore, B1R may be irrelevant to conditions associated with hyperactive plasma

kallikrein, such as HAE[77]. Active peptides *des*Arg^9-BK and Lys-*des*Arg^9-BK result from the cleavage of the respective kinin by peptidases belonging to kininase I group.

The activation of the receptors stimulates membrane phospholipid metabolism by activating phospholipase C, which causes intracellular calcium (Ca^{2+}) mobilization by inositol 1,4,5-triphosphate. Additionally, it promotes generation of diacylglycerol, which activates protein kinase C (PKC), release of nitric oxide (NO) and prostaglandins and activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways[78]. Their activation mediates multiple inflammatory kinin responses including vasodilatation, increased vascular permeability, hyperalgesia, and pain.

B2R is constitutively expressed on many cell types and it is considered an important receptor involved in HAE as it has high affinity for BK. B2R antagonist, icatibant, is approved for treatment of C1-INH-HAE. It is the only FDA-approved clinical application of a BK receptor ligand. The expression of B1R is strongly upregulated during inflammation in response to some cytokines, e.g. TNFα, IL-1β. In addition, endothelial cells can influence the expression of the receptors by secreting factors. Unlike B2R, which, in the presence of kinins, is desensitized, B1R is stabilized on the membrane. IL-1β, in a synergistic fashion with Lys-*des*Arg^9-BK upregulates B1R.

The role of B1R in HAE patients is unclear despite the fact that several observations suggest its involvement in the pathogenesis of HAE attacks. Levels of various cytokines are increased during HAE attacks as well as plasma C-reactive protein. Therefore, agonists of B1R are present in HAE attacks and B1R is possibly upregulated. However, B1R expression in patients with HAE is likely local[39]. Up to now, the detection of the intact receptors on the surface of the endothelial cells has not been achieved, but some clinical evidence suggest their expression as possible biomarkers of active inflammatory states[79].

C. Control of kinin-forming cascade and cross-talk between pathways

The role of C1-INH

Over the past fifty years, our knowledge has been significantly advanced concerning the structure and biological functions of C1-INH and its role in HAE and other conditions. The majority of research still focuses on the efficient treatments of C1-INH-HAE. However, the physiological activities of C1-INH are broader and it has also been considered as treatment for several other pathological conditions.

C1-INH is an important anti-inflammatory plasma protein with a wide range of inhibitory and non-inhibitory biological activities (Figure 7). C1-INH plays a unique role in the control of four major plasmatic cascade systems. It inactivates different proteases of the classical and lectin complement pathways (C1r, C1s, MASPs) and represents the primary regulator of the CAS and KKS by targeting FXIIa and KK. Furthermore, it participates in the regulation of the intrinsic coagulation pathway (FXI, thrombin) and the fibrinolytic system (tPA, plasmin)[80]. The inhibitory role of C1-INH in these pathways is illustrated in Figure 8. In addition to its

inhibitory potential, C1-INH also possesses a broad spectrum of non-inhibitory activities, including interactions with endogenous proteins, polyanions (glycosaminoglycans), various types of cells and infectious agents[81,82,83,84], which will not be discussed in this thesis.

Figure 7. Illustration of the currently known biological (inhibitory and non-inhibitory) activities of C1-INH.

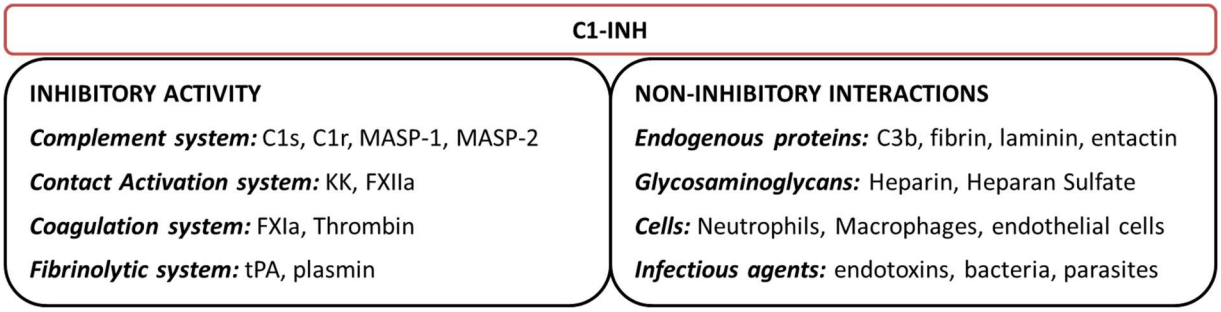


Figure 8. The role of C1-INH in the generation of BK by cleavage of HK in KKS and the inhibitory role in other pathways. FXII can be activated by blood contact with negative charged surfaces. The fibrinolytic system can also result to BK formation via activation of FXII by plasmin. Complement pathway is not further discussed in this thesis.

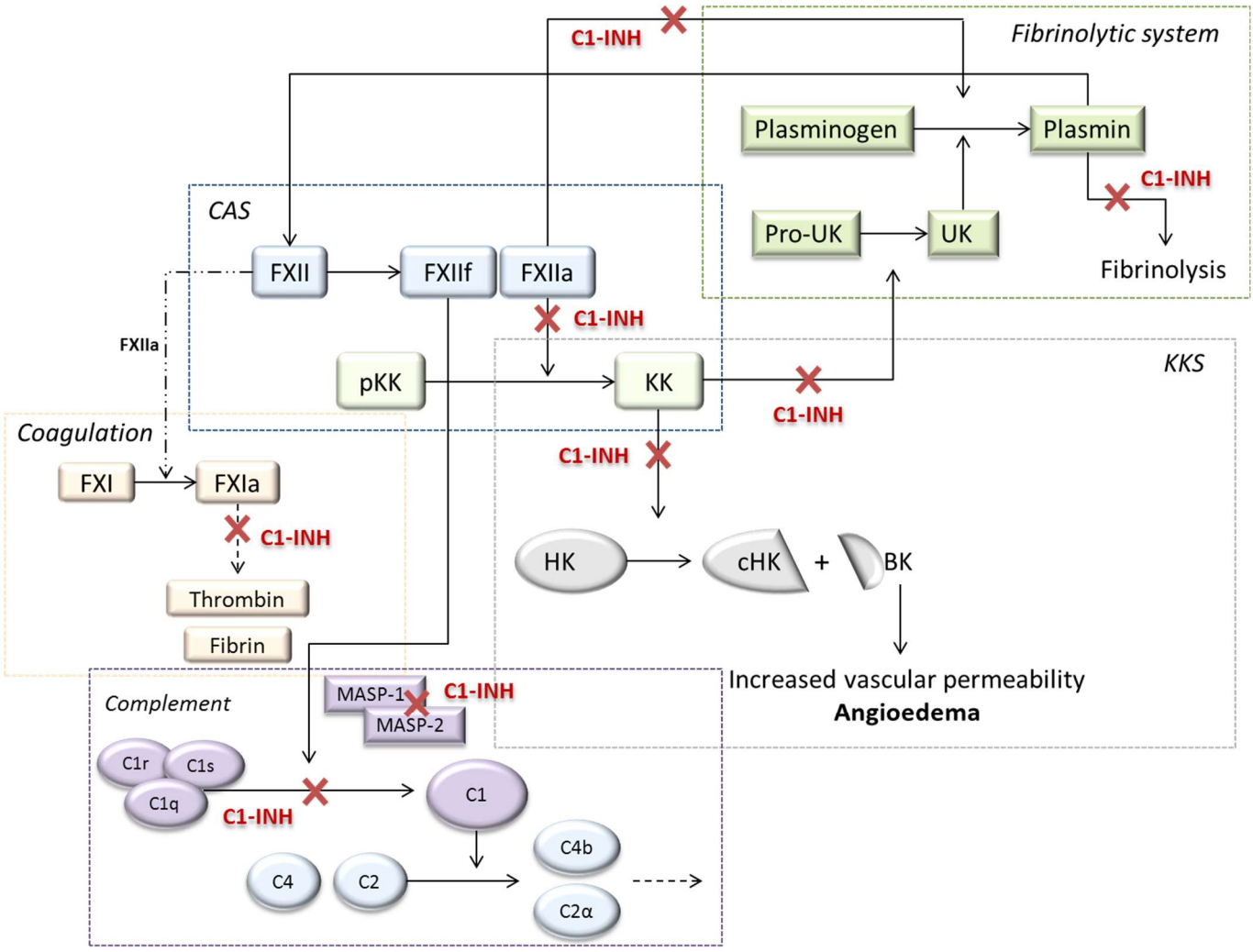


Table 1. Inhibitory activities of C1-INH and other significant inhibitors.

Pathway	Protease	C1-INH role	Other inhibitors
CAS	FXIIa	Major	-
KKS	pKK	Major	a2-macroglobulin (A2M), a2-antiplasmin (A2AP)
Complement	C1r C1s MASP-1, MASP-2	Major Major Major	- - -
Coagulation	FXI	50%	a2-antiplasmin, a1-antithrypsin (A1AT)
Fibrinolysis	plasmin	Minor	a2-antiplasmin
	tPA	Minor	PAI-1

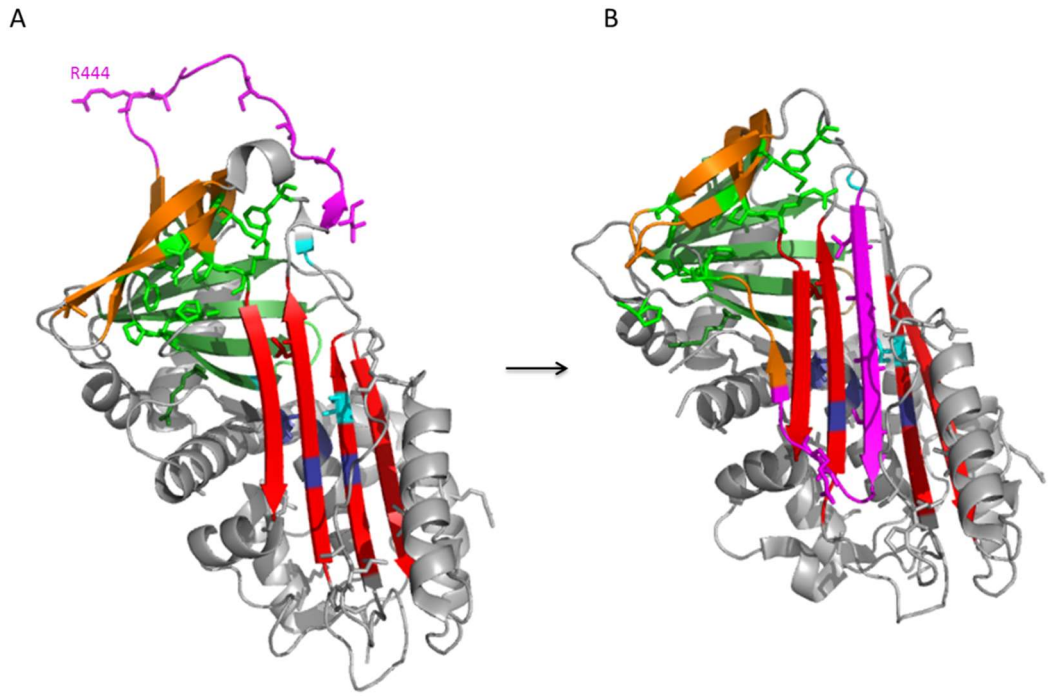
C1-INH is synthesized and secreted primarily by hepatocytes, but is also produced by monocytes, fibroblasts, macrophages, microglial cells, endothelial cells and other cells[85,86,87,88,89]. Biosynthesis of C1-INH can be stimulated by cytokines, particularly by interferon-γ (INFγ)[90,91]. It is a member of serine protease inhibitor (serpin) superfamily, the largest class of plasma protease inhibitors. Serpin superfamily includes inhibitory and non-inhibitory members among which antithrombin, α1-proteinase inhibitor and plasminogen activator inhibitor. Inhibitory proteases of the superfamily are characterized by a conserved tertiary structure to the C-terminal part (serpin domain), which contains 5 β-sheets, 8-9 α-helices (depending on the serpin) and a mobile reactive loop, essential for their activity.

C-terminal part of C1-INH (serpin domain) exhibits 27% homology with A1AT, the prototypical serpin[92] and a quite similar structural architecture with A1AT, A2AP or antithrombin-III. Its N-terminal part does not have homologies with other serpins. *O*-linked polysaccharides, but not *N*-linked polysaccharides, affect C1-INH function concerning the control of KKS but not the control of C1s, indicating the involvement of the N-terminal domain in C1-INH function[93]. Deglycosylation impact has not been demonstrated for another serpin canonical function, namely protease-inhibitor complex formation or the latent molecular species stability. Moreover, the functional role of the heavily glycosylated N-terminal domain is also probably essential for the protein's conformational stability, recognition and affinity to endotoxins and selectins. C1-INH contains two disulfide bridges, formed between Cys^{101} and Cys^{108} of the N-terminal domain and Cys^{406} and Cys^{183} of C-terminal domain[94] (Figure 9).

C1-INH, like other serpins, is notable for its mechanism of function, described as "molecular mousetrap"[95]. According to this model, the reactive center of the protein, in its native state, is exposed and accessible for interaction with the targeted proteases. The P1-P1' (Arg^{444}-Thr^{445}) bond behaves as a pseudo-substrate. Upon cleavage of this bond the active site of the protease forms a covalent bond with P1 and C1-INH undergoes a conformational rearrangement, acting like a mousetrap. Therefore, it swings the inactivated

protease from the upper to the lower pole with insertion of the reactive loop as an additional strand in the β-sheet. The conformational change is thermodynamically favorable and results in a complex with increased stability [37,96].

Figure 9. Overall structure of native (A) and latent (B) form of C1-INH serpin domain. The five crucial regions for the proper function of C1-INH are presented on the crystal structure generated using PyMOL Molecular Graphics System[97]. The reactive site loop (purple), which includes Arg^{444} (P1); the central β-sheet A (red) with the breach region (light blue) and the shutter domain (dark blue); the gate (highlighted with green sticks), including s3C and s4C of the β-sheet C; β-sheet B (green) and β-sheet C (orange).

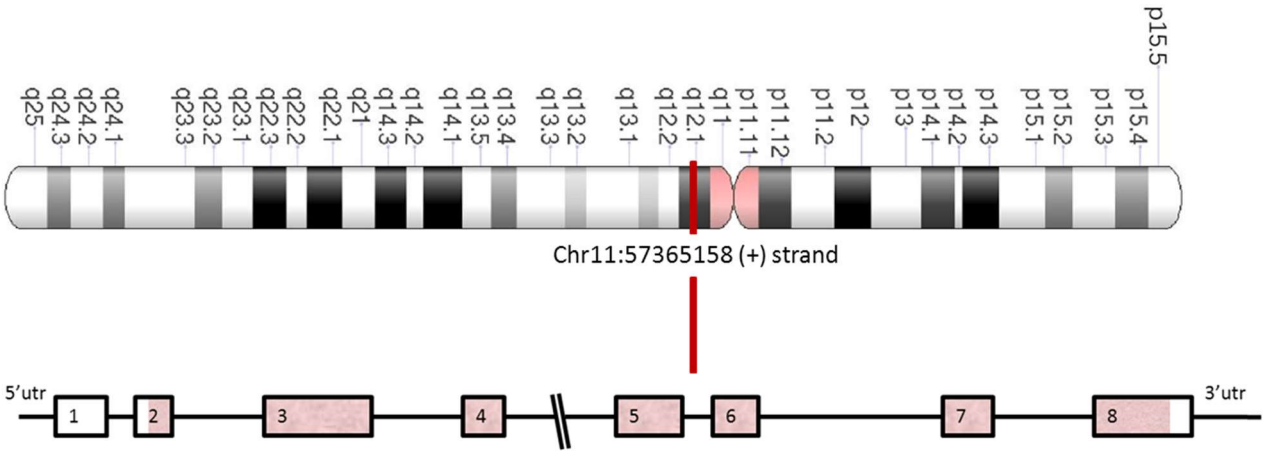


During the last years, several C1-INH products have been licensed in Europe for the treatment of C1-INH-deficient patients that improved the therapeutic options in the battle with this disorder. As a potent anti-inflammatory agent, C1-INH has also been considered for treatment of several other serious pathological conditions, including sepsis, acute myocardial infarction, vascular leakage syndromes, ischemia-reperfusion injury, brain ischemic injury and some other conditions[81-83,98].

III. C1-INH-HAE GENETICS

SERPING1 gene (serpin peptidase inhibitor, glade G, member 1) (OMIM*606860), extends over 17,159 bp on chr11q12.1. The gene consists of 8 exons, 7 introns and a promoter with TdT-like initiator and a polypurine-polypyrimidine tract rather than a TATA sequence. The product of gene transcription is an 1,827 bp mRNA[99]. The encoded protein, C1-INH, is a single-chain polypeptide composed by 478 amino acid residues. It consists of two domains, the 365 aa C-terminal domain and the 113 aa N-terminal domain.

Figure 10. The structure of *SERPING1* gene and the localization on the chromosome 11.[100]



Over 700 mutations related to C1-INH-HAE have been detected, located in all exons, exon/intron junctions and recently in intron 2[101]. A majority of these mutations are short deletions/duplications and missense mutations. Probably more than a half of the missense products are not functional and/or rapidly degraded. Mutations resulting in the production of misfolded proteins which are not efficiently secreted occur throughout the gene and are responsible for the common C1-INH-HAE type I. This type represents 80-85 % of all cases and results from failure to synthesize C1-INH. However, heterozygous patients for the deleterious mutations, on the contrary of what is expected, present antigenic C1-INH from 5 to 30% of the normal level. The clinically indistinguishable C1-INH-HAE type II (15-20% of cases) is caused by single amino acid substitutions, in particular in exon 8 that code for the reactive center. Some of these mutations affect the critical Arg^{444} residue and lead to the production of a dysfunctional C1-INH protein despite a normal or often elevated antigenic C1-INH. Another particular observation is that in a small proportion of patients with C1-INH-HAE, the analysis of the coding region does not reveal *SERPING1* alterations.

The fact that some C1-INH-HAE cases have no family history for the disease and present *de novo SERPING1* mutations with the same mutational spectrum as the familial cases, reflects the particular mutagenesis of *SERPING1*. This phenomenon may be explained by the localization of the gene on the chromosome. *SERPING1* is close to the centromere and presents high mutation rate, as it is expected for rapidly evolving centromeric regions. Moreover, *SERPING1* contains DNA repetitive elements (19 *Alu* repeats) which represent

hotspots for events that may cause partial deletions or duplications of the gene. Indeed, large INDELs related to *Alu* repeat sequences are common defects in C1-INH-HAE patients[102]. Finally, the gene contains CpG sites, which are potentially responsible for the higher than average mutation rates, as they may represent frequent targets for recurrent amino acid substitutions. A fine example of a CpG dinucleotide acting as hotspot is the CG dinucleotide in the codon 444 (CGC), encoding for the central Arg of the reactive center of C1-INH[103].

Another particular observation concerns the pathogenicity of missense mutations in *SERPING1* causing single amino acid substitutions. This type of mutations, without many exceptions, result in loss of the serpin function and consequently of the inhibitory activity of C1-INH. An evident explanation for that is that point substitutions result in oligomerization or polymerization of C1-INH. This results in an impaired secretion of C1-INH, a common molecular event in serpinopathies[104]. In fact, Madsen et al.[105] detected C1-INH polymers in plasma of C1-INH-HAE patients and Ponard et al. pointed out *SERPING1* variants with critical oligomerization capacity[106]. This serpin-specific functional property role must be more extensively investigated, particularly for a subsequent impaired control of the target proteases.

The molecular analysis of *SERPING1* starts with the prioritized PCR-amplification and direct sequencing of all exons and the exon/intron junctions. If no mutation is found, further analysis for the identification of large defects is performed, usually by two different techniques (long-range PCR and MLPA). This approach is fraught with pitfalls and it is highly dependent on user's experience and knowledge. For example, a novel missense mutation can be incorrectly characterized as the causal genetic defect because its detection during the early steps of the analysis would prevent the discovery of an INDEL leading to the production of a truncated protein. Moreover, deep intronic alterations which, in other conditions, have already been associated to pre-mRNA splicing and are possibly contributing to C1-INH-HAE phenotype, escape the conventional approach.

IV. HETEROGENEOUS CLINICAL MANIFESTATIONS OF C1-INH-HAE

The clinical expression of C1-INH-HAE is characterized by large heterogeneity. Even among family members, carriers of the same mutation, the features of the disease such as the age at disease onset, the frequency and triggers of the attacks, the localization of the symptoms, the severity and the need for long-term prophylaxis (LTP) vary broadly[2,107,108]. Some patients experience life-threatening attacks while others mild symptoms. About 14% of carriers of a causal *SERPING1* variant remain asymptomatic and about 5% of adults are identified only after their child is diagnosed, after presenting symptoms. Therefore, in order to optimize patients' management and avoid the potentially fatal manifestations, we need to define metrics for the disease severity and be able to predict the clinical course.

The large amount of *SERPING1* variants that have been identified over the recent decades has created a clear need for their functional characterization and their classification as disease-causing or benign. However, the low prevalence of the disease does not allow confirming strong correlation between the presence of a variant and a certain phenotypic trait. For such functional studies, very large patient cohort is required to include enough patients/carriers of the rare variants and achieve statistical significance.

The largest study ever realized in this field, included 265 C1-INH-HAE patients from 4 European countries. Speletas et al. (2015)[109] concluded that the probability of having an episode before the age of 10 is significantly lower in patients carrying missense mutations in *SERPING1* in comparison with other types of mutations (nonsense, regulatory, frameshift, INDELs, large defects). A similar correlation has been reported by Andrejević et al. (2015)[110] in Serbian patients. Taking into account that the early onset of the disease is usually associated with high severity, carriers of missense mutations may present a milder clinical course. Further studies have been realized in order to associate the presence or absence of common *SERPING1* SNPs with disease severity. As yet, no correlation was found between the p.Val480Met (c.1438G>A, rs4926) and C1-INH-HAE severity[111,112]. Concerning c.-21T>C (rs28362944), there are conflicting interpretations of the pathogenicity[113,114,115].

Apart from *SERPING1* variants, in order to achieve a precise diagnosis for AE patients, we need to take into account the underlying pathophysiology of the disease and the many different biological pathways implicated in the increase of vascular permeability and the generation of oedema. Despite the fact that, in the beginning, HAE was considered a disorder caused by a deficiency in the complement system, our understanding has been improved in the recent years. According to the definition proposed by Strimbu and Tavel[116], any measurable parameter reflecting biological, pathological processes or response to therapy would be a suitable biomarker. In angioedema the most suitable biomarkers could originate from the CAS, KKS, complement, fibrinolysis, coagulation or they could be endothelial-cell related or inflammatory factors. The biomarkers could, for example, include functional elements of the plasma cascade systems, receptors, cytokines, cell adhesion

molecules, tumor factors and stress proteins such as HSP90. Variants and common SNPs in the genes encoding for the above referred proteins could also represent genetic biomarkers, as they may affect the expression or activity of these proteins and they could be useful especially if these characteristics are changing during the attacks or the prodromes[117].

Setting up a C1-INH testing with highest sensitivity and specificity has been a challenge, because C1-INH is not the only serpin to control KKS activation, while C1r or C1s proteases, with 100% control by C1-INH, are not involved in the kinin-forming cascade. Only recently C1-INH testing on KKS activation has been achieved with optimum diagnostic cut-off values[118,119]. With considerable efforts, numerous studies have been performed in cohorts of AE patients in order to associate the levels and activity of other proteins with the severity of the disease or observe differences between episodes and episode-free periods in patients. For example, the levels of MASP-1 and MASP-2, serine proteases of the lectin pathway, have been reported to be lower in HAE patients[120,121] compared to healthy control individuals, while during attacks MASP-2 appears to be increased[122]. The same holds true for complexes of MASP-1 with C1-INH and antithrombin (AT)[120]. The stable complex of C1s/r with C1-INH is higher in HAE patients[123] and increases further during attacks[124]. The activated factor FXII, as well as KK, is measured at higher levels[125,126,127,128,129] and even more increased during attacks, while the FXIIa/C1-INH complex and the KK/C1-INH complex, respectively, are lower in patients compared to healthy subjects[125-130]. Almost similar observation has been made for FXIa which is higher in HAE patients[125], while the FXIa/C1-INH is decreased during attacks[127]. Zymogen to enzyme conversion, in relationship with kinin and C4a formation, has been successfully achieved in HAE plasma samples by Joseph et al.[126]. The most useful biomarker, already used by many laboratories in diagnosis, is cleaved HK species, the proteolytic product of HK after the release of BK, which appears elevated in HAE patients and increases further during attacks[131,132] From ROC analysis, cut-off values have been determined for women and men and the assay offers maximum specificity and sensitivity. In addition to its kinin-specificity adapted to HAE diagnostic, quantification of circulating molecular species improves patient follow-up in clinical trials or prophylactic management. Data obtained along the HAE attack suggests its use as a post-attack assay, with an advantage for HAE diagnostic[132]. Concerning the enzymes of catabolism of BK, APP and DPPIV have been studied mainly in patients with ACEi-AAE. However, APP and CPN have been studied once and their activity is inversely correlated with disease severity in C1-INH-HAE patients[133]. Lower than the normal values of a2-macroglobulin have also been detected in some HAE patients[134]. In the coagulation system, the activity of thrombin and its complex with antithrombin have been detected higher in HAE patients, while the complex further increases during HAE attacks[135]. In the fibrinolytic system, the levels of PAI-2 are higher in C1-INH-HAE patients compared to healthy controls and compared to FXII-HAE patients[134], while the PAI-1 levels are decreased only during episodes of AE[125]. On the contrary, the levels of the stable complex between plasmin and antiplasmin are measured high in an episode-free

period and they further increase during an attack[125,126,135,136]. Finally, neutrophil elastase and myeloperoxidase (MPO), a serine protease and an antibacterial enzyme from neutrophils respectively, as well as the proinflammatory cytokine TNF have been detected increased during HAE attacks in C1-INH-HAE patients[137].

Taking all the above into account, we understand why the clinical expression of HAE may be partially determined by genetic variants or common SNPs in genes other than *SERPING1* encoding for proteins involved in the kinin generation, catabolism and function. A fine example for this fact is the strong correlation of a common SNP in *F12* (c.-4T>C, rs1801020) with the age at disease onset. More specifically, Bors et al. (2013)[138] correlated the CC genotype of rs1801020 with an earlier expression of the symptoms of the disease. Speletas et al. (2015)[139] confirmed these results in a large cohort of 258 patients from 113 unrelated European families. In this study, the authors concluded that carrying the TT genotype of the *F12*-rs1801020 was significantly associated with a 7-year delay in the age of HAE onset independently of the type of *SERPING1* mutation. Moreover, homozygotes for the T allele did not need any long-term treatment. Interestingly, Rijavec et al.[140] investigated the influence of *F12*-rs1801020 in the penetrance of C1-INH-HAE. For this purpose, they examined the frequency of the variant in 88 well-characterized C1-INH-HAE European patients, including 9 asymptomatic adults from 42 unrelated families. They found out that the CC genotype and the C allele, separately, are highly over-represented in symptomatic C1-INH-HAE patients in comparison to asymptomatic ones.

Another example of a common functional variant affecting the age at disease onset and the need for LTP is the c.428G>A (p.Ser143Asn, rs3733402) in the *KLKB1* gene. The SNP causes a reduced plasma KK complex formation with HK and it has been associated with a significant delay of the disease onset. According to Gianni et al. (2017)[141] carriers of the G allele of the *KLKB1*-rs3733402 exhibit a significantly delayed disease onset by 4.1 years ($p<0.001$) depending on the zygosity status. Carrying both rs1801020 and rs3733402 was significantly associated with an 8.8-year delay in disease onset and a 64% lower probability of needing long-term prophylactic treatment.

Table 2. Potential genetic and biological biomarkers for C1-INH-HAE.

Gene	Potential Biomarker	Biological activity	Relevance
SERPING1	Antigenic, AgC1-INH	Serine protease inhibitor (CAS, KKS, complement, coagulation, fibrinolysis)	Serum level under 0.075 g/L predisposes attack
	C1-INH function		May predict attack frequency and C1-INH concentrate consumption
MASP1	Mannan-binding lectin serine protease 1	Serine protease of the lectin pathway	Lower level in HAE
MASP2	Mannan-binding lectin serine protease 2	Serine protease of the lectin pathway	Lower level in HAE Increases during attack
C1r/C1s	Complement components C1r, C1s	Stable complex of inactivated esterase with C1-INH	Higher level in C1-INH-HAE Increases further during attack May predict disease severity
F12	FXIIa	Serine protease of the CAS	Higher level in C1-INH-HAE Increases further during attack
KLKB1	KK KK/C1-INH	Serine protease of the KKS Stable complex of inactivated protease with C1-INH	Higher activity in HAE Increases further during attack
KNG1	Cleaved HK	Proteolytic product of HK after the release of BK	Higher level in HAE
XPNPEP2	Membrane APP	Peptidase involved in BK catabolism	Activity inversely correlated with disease in C1-INH-HAE patients not treated with danazol
CPN1	CPN	Peptidase involved in BK catabolism	Inversely correlated with disease in C1-INH-HAE patients not treated with danazol
A2M	a2-macroglobulin	Plasma serine protease inhibitor	Lower level in C1-INH-HAE
F11	FXIa	Serine protease of the coagulation system	Higher level in HAE
F2	Thrombin	Serine protease of the coagulation system	Higher activity in HAE Increases further during attack
SERPINC1	Thrombin/Antithrombin complex	Stable complex of thrombin/antithrombin	Higher level in C1-INH-HAE Increases further during attack
SERPINE1	PAI-1	Plasminogen activator inhibitor	Decreases during attacks
SERPINB2	PAI-2	Plasminogen activator inhibitor	Higher in C1-INH-HAE
PLG/ SERPINF2	Plasmin/ Antiplasmin (AP)	Stable complex of inactivated plasmin with AP	Higher level in HAE Increases further during attack
ELANE	Neutrophil elastase	Serine protease of neutrophils	Increases during attack
MPO	Myeloperoxidase	Enzyme from neutrophils	Increases during attack
TNF	Tumor Necrosis factor	Pro-inflammatory cytokine	Increases during attack

V. CLASSIFICATION OF VARIANTS AND REPORTING IN PUBLIC DATABASES

The identification of biomarkers and development of accompanying bioassays are required to accompany clinical trials and enable personalized medicine for HAE patients. As far as it concerns the biochemical biomarkers, once they are available as diagnostic tests, the result can be assessed by the physician. Concerning genomic data, defining the pathogenicity and the clinical validity of any detected variant is particularly challenging. The next step of detecting new variants and uncovering their association with the pathogenesis of HAE is expanding this information beyond research laboratories and use it about individuals as a part of their clinical care.

As sequencing technology is evolving rapidly and laboratories want to ensure quality results, professional standards and guidelines for the interpretation of pathogenicity have been developed by the American College of Medical Genetics (ACMG). In these guidelines, all criteria are initially weighed and then combined for a reliable curation. However, it is pointed out that pathogenicity itself should be independent of defining the cause of the disease in a given patient. These guidelines are used widely for variant classification by clinical testing laboratories. This kind of laboratories usually possess detailed phenotypic information by the physicians and have access to other approaches to delineate pathogenicity, such as sequencing family members, comparison to large number of controls and running functional studies. Yet, their data are not published, therefore not available by searching PubMed or Human Gene Mutation Database (HGMD) tables.

One criterion in ACMG standards and guidelines for variant classification is the report of the variant by reputable databases (criterion PP5 and BP6). The need for archiving evidence concerning variants originated from distinct sources and presenting their relationship to human health and disease is critical. The American Medical Association (AMA), the American College of Medical Genetics (ACMG) and the National Society of Genetic Counselors (NSGC) support ClinVar database to meet this need.

ClinVar was created by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) and provides a centralized, user-friendly, public open-access database which can help users in interpreting variants. Submitters to ClinVar include clinical testing laboratories, research entities, database curators, expert panels and practice guideline groups. Variants are collected from a variety of sources like clinical testing, literature-only evaluation, research, curation or other. Different entities can enrich each entry with additional evidence for the same variant. Even though a great number of pathogenic variants are reported in the literature and public databases, benign variants are rarely reported, leading to misinterpretation and discordances amongst laboratories. The ClinVar database allows for the deposition of all classified variants with clinical observations and assertions and with review status to enable a more transparent view of the quality of the curation. The aggregation of data for variants or variant-disease pairs allows ClinVar to

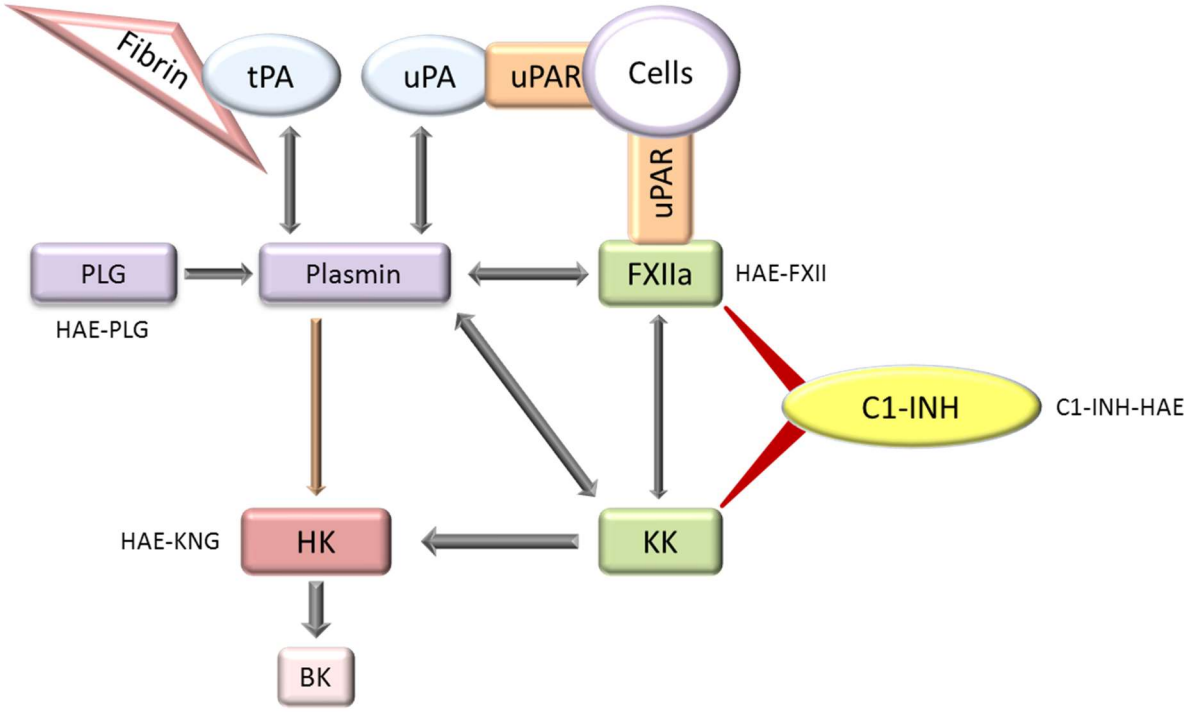
report when different submitters agree or disagree on the interpretation. In this way, it allows for variant conflict interrogation and resolution[142].

Other centralized databases of variation related to human disease existed before ClinVar; databases like HGMD and OMIM focused on variants found in the literature and COSMIC for somatic variation. Gene-specific databases also exist, like Leiden Open Variation Database (LOVD). In fact, in LOVD, 827 entries can be found about *SERPING1*, corresponding to 770 variants. However, ClinVar is evolving and since the first public release (April 2013) it has become a routine for many testing laboratories. As for November 2020, it contains more than 1,300,000 records for more than 32,000 genes, among which 1,134,618 are characterized using professional guidelines.

VI. PLASMINOGEN

Plasminogen (PLG) is the circulating zymogen form of plasmin, a serine protease known for its role in fibrinolysis and haemostasis. It is primarily synthesized in the liver[143] and its activation to plasmin is predominantly depending on urokinase-type (UK) and tissue type (tPA) activators, but in a less extent on FXIIa and pKK, proteases of the KKS. PLG activation is effectively amplified on the surface of fibrin. When bound onto cells or fibrin, both activators display an enhanced activity up to 10-fold; tPA receptors are found on endothelial cells and uPA is binding to the cell surface receptor of plasminogen activator receptor (uPAR). Both uPA and tPA are controlled by the plasminogen activator inhibitors -1 (PAI-1) and -2 (PAI-2). Under physiological conditions, the activation occurs through cleavage in the loop between Arg^{561} and Val^{562} [144]. Active plasmin targets numerous substrates, including FXII, fibrin, fibrinogen, complement components C3 and C5, vitronectin, osteocalcin, factors V, VIII and X, protease-activated receptor 1, injury-induced aggregated proteins and some collagenases. Plasmin also targets the key plasminogen activators (UK, tPA) and creates a positive feedback loop[144]. Therefore, it plays important physiological and pathological roles in pathways other than fibrinolysis and haemostasis, such as cell migration, tissue remodelling, wound healing, angiogenesis, embryogenesis, inflammation, tumor cell migration and immunity[144,145].

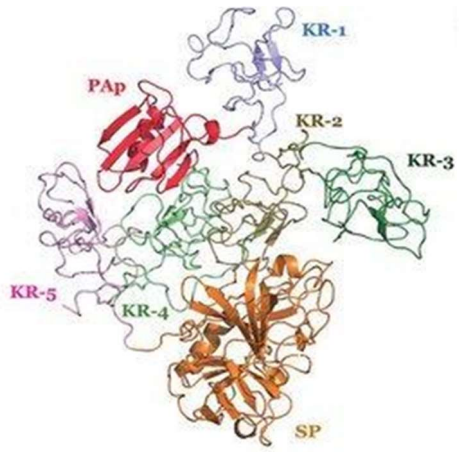
Figure 11. Links between PLG activation and components of fibrinolysis with CAS, KKS. On the figure are represented the forms of HAE related to gain-of function mutations in each factor.



Full-length plasminogen (Glu-PLG) comprises a 791-amino acid single chain with seven domains, an N-terminal Pan-apple domain (PAp), 5 kringle domains (KR-1-5) that mediate fibrin binding and the serine protease domain (SPD), homologous to trypsin-group proteases and supporting fibrinolysis and other proteolytic functions. (Figure 12). Glu-PLG circulates in a closed conformation that cannot be readily activated by tPA or uPA and adopts an open conformation when bound to the fibrin or the cell surface. The PAp domain is important for maintaining pre-activated PLG in a closed and globular conformation. Removal of the PAp domain by plasmin during pre-activation produces Lys-PLG. Recent crystal structures have been solved and led to understanding of the molecular actions that are essential for PLG to maintain the closed, activation resistant conformation. Of key importance are the five kringle domains, four of which are capable of binding to Lys residues. More precisely, the conformation of closed PLG is partially mediated by inter-domain interactions via lysine-binding sites[39,40].

There are two major glycoforms of plasma PLG: type I, (~33%), which is N-glycosylated at Asn^{289} and O-glycosylated at Thr^{346} and type II form (~67%), which is O-glycosylated at Thr^{346} (UniProtKB-P00747). These glycoforms have distinct biological activity, with glycoform I thought to be more prominent in intravascular fibrinolysis and glycoform II possessing greater affinity for the cell surface.

Figure 12. The PLG 3D structure and linear illustration with the distribution of PAp, kringle (KR) and serine protease (SPD) domains and the cleavage sites of Glu-PLG by plasmin and uPA/tPA.



VII. HAE-PLG

The HAE entity with normal C1-INH (nC1-INH-HAE), was first described in 2000 and associated with mutations in the F12 gene (HAE-FXII)[146]. Recently, three novel forms of HAE due to mutations in the *PLG* (HAE-PLG)[34], *ANGPT1* (HAE-ANGPT1)[36] and *KNG1* (HAE-KNG)[35] genes have been described. The remaining oestrogen-related HAE cases with normal C1-INH function and no genetic cause are referred to as unknown HAE forms (U-HAE).

HAE-PLG, with prevalence <1/1,000,000, is inherited in an autosomal dominant manner and is clinically characterized by a later disease onset (usually in adulthood) and by swellings affecting the face, the tongue and the larynx[34,147]. It has been hypothesized that the overproduction of BK in HAE-PLG could be related to an increased activation of the fibrinolytic system. From a mechanistic perspective, the implication of plasmin and the fibrinolytic system in edema formation is supported by the observations that (a) antifibrinolytic agents (for example tranexamic acid) are effectively used as LTP in C1-INH-HAE patients and (b) low levels of PAI-1 and PAI-2 are detected in HAE-FXII patients, indicating their consumption by PLG activators.

The *PLG* gene encoding for PLG (OMIM*173350) is located on chromosome 6q26 and consists of 19 exons. Up to now, more than 70 different mutations causing PLG deficiency have been reported in the HGMD, but only one specific missense *PLG* variant, an A>G substitution (NM_000301.4:c.988A>G, p.Lys330Glu) was observed in nC1-INH-HAE and interpreted as pathogenic for HAE-PLG[148]. The variant is located in exon 9 of the gene and affects Lys^{330} residue in the KR-3 domain of the protein. Bork et al.[34] first identified the mutation in 14 patients with nC1-INH-HAE belonging to 4 families. Up to date, 25 unrelated families have been recorded with HAE due to the PLG variant p.Lys330Glu (17 German [34,147,149,] , 1 Bulgarian, 1 Greek, 1 Spanish[150], 3 French[151] and 2 Japanese[152]) with an incomplete penetrance.

Although the pathophysiology of HAE-PLG is not entirely known, the pathologic nature of the variant is supported by co-segregation of HAE in carriers. Dewald[149] observed altered PLG patterns in two heterozygous patients for the p.Lys330Glu variant and suggested that the variant might have some quantitative as well as qualitative effects on PLG glycosylation.

OBJECTIVES OF THE STUDY

Angioedema, as a distinct clinical entity, can be challenging for physicians in everyday practice mainly because it can be life-threatening and also because established methods for laboratory diagnosis are only available for C1-INH-HAE and nC1-INH-HAE with genetic defects (HAE-FXII, HAE-PLG, HAE-ANGPT1 and HAE-KNG)[153]. Therefore, the clinicians dealing with HAE patients have to treat them based on their family history, clinical examination or using exclusion or non-validated therapeutic criteria[154]. The Precision Medicine approach for other rare and chronic diseases suggests that the combination of several biomarkers, when analyzed by robust statistical methods might lead to a specific diagnosis and individualized therapy.

The aim of this study was to investigate the factors that influence the heterogeneous clinical manifestations of C1-INH-HAE and assess their utility as prognostic or predictive biomarkers. Ideally, a biomarker should be detected in a sample that is easily accessible, such as blood/plasma sample and should be linked to the pathophysiological mechanisms of the disease. Finally, it should be measured via a sensitive, specific, rapid test and the results should be comparable across the different laboratories[155].

The first potential biomarker for HAE was the plasma levels of BK, when the peptide was observed and characterized as the mediator of oedema. However, critical observations have been raised against the measurement of BK plasma levels. Functional studies in HAE patients have been performed concerning the activity of other proteases implicated directly or indirectly in the generation, catabolism and function of BK like cytokines, growth factors, the presence of certain antibodies, the levels of D-dimers and hormones. Nowadays, the scientific community agrees that some of the studied proteins have been proved useful such as protease-serpin complexes and cleaved zymogen proteases.

The currently available biomarkers are the following:

1. C1-INH function and antigenic levels; the measurements are required for diagnosis of C1-INH-HAE and C1-INH-AAE. However, replacement therapy and treatment with danazol can influence both function and antigenic C1-INH.
2. C1-INH molecular species using an anti-C1-INH immunoblot assay; circulating C1-INH molecular species is recommended as a support to diagnosis of C1-INH-HAE and C1-AAE.
3. Antigenic C4 levels; an easily accessible parameter for C1-INH-HAE and C1-INH-AAE diagnosis. Antigenic C4 levels are easy to measure but they also vary broadly between healthy individuals. Moreover they are found within the normal range in 10% of *SERPING1* pathogenic variant carriers[128].
4. C1q levels and anti-C1-INH antibodies; they are used for the distinction between C1-INH-AAE, e.g. with anti-C1-INH autoimmunity.
5. pKK activity; the measurement of its spontaneous activity discriminates between histamine- and BK-mediated AE.

6. Cleaved HK molecular species; it discriminates C1-INH-HAE patients in resting conditions and during attacks.

Concerning the function and antigenic levels of C1-INH and the antigenic C4, they present some advantages in everyday practice but they do not reflect clinical course, prognosis or response to therapy for the patient.

In this thesis, the aim was to expand the research for potential biomarkers and for this purpose we investigated:

I. The expression of B1 and B2 receptors.

The activation of B1R and B2R mediates multiple inflammatory kinin responses. B2R is constitutively expressed on many cell types and it is considered an important receptor for HAE. B1R is induced by inflammatory cytokines like IL-1β or TNFα and its role is unclear despite the fact that several observations suggest its involvement in the pathogenesis of HAE attacks. As levels of various cytokines are increased and agonists of B1R are present during HAE attacks, it is possible that B1R is upregulated, even if its expression is likely local.

Up to now, the detection of the intact receptors on the surface of any type of cells has not been achieved, but some clinical evidence suggest their expression as possible biomarkers of active inflammatory states. The main objective of this part of the study is the detection of the receptors on endothelial cell lines, and subsequently, on the membrane of patients' endothelial cells in resting conditions and during an attack. The detection will be achieved using specific fluorescent ligands as molecular imaging agents in parallel with anti-B1R and anti-B2R antibodies. The fluorescent ligands can be used as a diagnostic tool and the direct detection of the receptors on the surface of different cells will enlighten their role in HAE pathogenesis. Finally, the expression of the receptors at a certain time, before or during an attack, can be associated with the phenotype of a HAE patient.

II. The susceptibility of PLG activation in presence of the p.Lys330Glu mutation.

The cross-talk of the CAS with fibrinolysis has been studied before and the implication of plasmin in the production of BK and the formation of oedema is supported by many observations. When we consider AE with normal C1-INH, p.Lys330Glu is characterized causal. Dewald (2018)[149] observed altered PLG patterns in two heterozygous patients for the p.Lys330Glu variant and suggested that the variant might have some quantitative as well as qualitative effect on PLG glycosylation. The aim of this part of the study is to investigate the biological consequences of the PLG p.Lys330Glu variant in heterozygous and homozygous patients and the PLG activation in presence of different activators using a plasmin-specific chromogenic assay.

As antifibrinolytic agents, such as tranexamic acid have been successfully used for LTP in C1-INH-HAE patients, we would expect that the PLG susceptibility to activation would point out a gain or loss of plasmin function. This is congruent with a recent observation where

p.Lys330Glu variant in the *PLG* gene alone or in co-existence with a *SERPING1* variant affects the clinical manifestation of the disease[156], and more precisely the localization of the attacks.

III. Variants on 54 genes other than *SERPING1* that can affect the phenotype of the disease in C1-INH-HAE patients.

C1-INH-HAE is inherited in an autosomal dominant manner due to deleterious mutations in *SERPING1* gene. Over 700 *SERPING1* alterations have been reported after systematic mutation analysis performed in different cohorts of HAE patients. However, the linkage between the presence of the mutations and the disease prevalence or the phenotypic traits is still under investigation. The functional impact of most of the variants is unknown and it remains elusive whether the presence of the mutations considered so far as pathogenic is the only responsible for the disease.

The clinical expression of HAE is heterogeneous with regards to disease severity, triggering factors and response to treatment. Numerous efforts have been made up to now with only few of them concluding in strong genotype-phenotype correlations. This part of the study examines if defects in genes other than *SERPING1* are able to modify the patients' clinical phenotype and/or the response to treatment. More precisely, in cases of C1-INH-HAE, we aimed to investigate if the severity of the disease, the phenotypic traits (triggering factors, frequency and localization of the attacks, age of disease onset) and the need for LTP can be attributed in the modifying effect of alterations in other genes, co-existing with the already characterized causal *SERPING1* defects.

IV. Reporting previously detected *SERPING1* variants in ClinVar database.

The next step of identifying genetic variants and establishing genotype-phenotype association is sharing the information among researchers, laboratories and physicians in reputable databases in order to be used in everyday clinical care. In order to participate to global data sharing, the aim of the last part of the study is the classification according to ACMG-AMP standards and guidelines of *SERPING1* variants previously detected in C1-INH-HAE patients of the Laboratory of Immunology and Histocompatibility at the UTH and reporting them in ClinVar database, accompanied by the supporting clinical evidence.

MATERIALS AND METHODS

PART I: EXPRESSION OF BRADYKININ RECEPTORS (B1R, B2R)

Materials

(a) The devices, pipets and plastic material used for the analysis of the expression of bradykinin receptors are presented in Appendix I (Table 1).

(b) The solutions, reagents and antibodies used for the analysis of the expression of bradykinin receptors are presented in Appendix I (Table 2).

Methods

I. Detection of B1R and B2R on endothelial cells using fluorescent recombinant proteins

The aim of this part of the study was to set up a detection system for BK receptors (B1R, B2R) in order to investigate their expression on endothelial cell lines and subsequently on the membrane of patients' endothelial cells under normal conditions and during an angioedema attack. This concept was based on the design and validation of selective fluorescent ligands that had been realized by Charest-Morin et al. at the Laval University of Quebec in Canada[79].

A. Production of Biotechnological ligands

The starting material for the production of fluorescent ligands was pcDNA 3.1(-) vectors which were kindly provided by F. Marceau. In these vectors, Ki9 and Ki10 sequences had been inserted, which encode for GFP proteins C-terminally extended. The extension is composed of 2 modular sequences designated S (spacer) and P (ligand peptide). The ligand peptide (P) sequence for both fluorescent ligands was *des*Arg^9-BK = P1. The spacer peptide used to design the B2R probe (S1) was derived from the amphibian peptide maximakinin (MK) (Figure 13). The plasmid map for the vector with Ki9 sequence is presented in Figure 14. The spacer peptide used to design the B1R probe (S4) was $(Asn\text{-}Gly)_{15}$. These vectors determine the production of non-secreted cytosolic GFP, C-terminally extended. The amino acid sequences of the extensions for both ligands are presented below:

Figure 13. Schematic representation of the design of fusion proteins prepared as molecular imaging agents for the detection of the receptors of BK.

Ligand for B2R (S1P1): DLPKINRKGP RPPGFSPF (Maximakinin-des-Arg)

Ligand for B1R (S4P1): $(NG)_{15}K$ RPPGFSPF

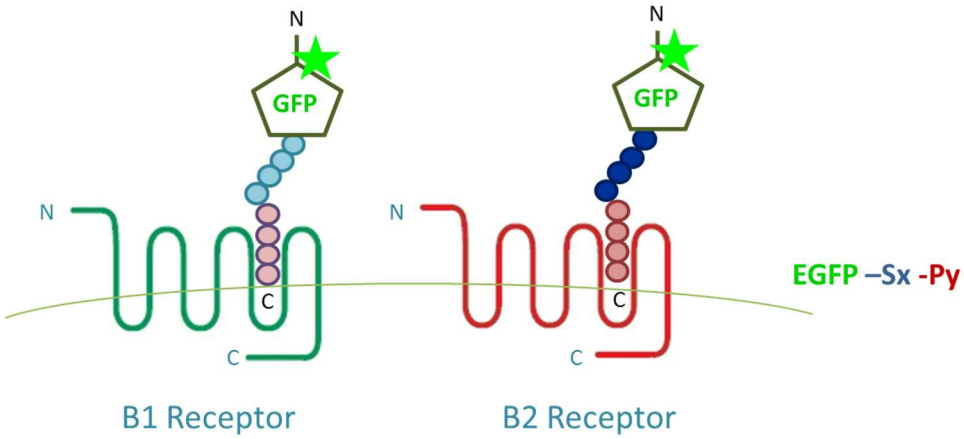
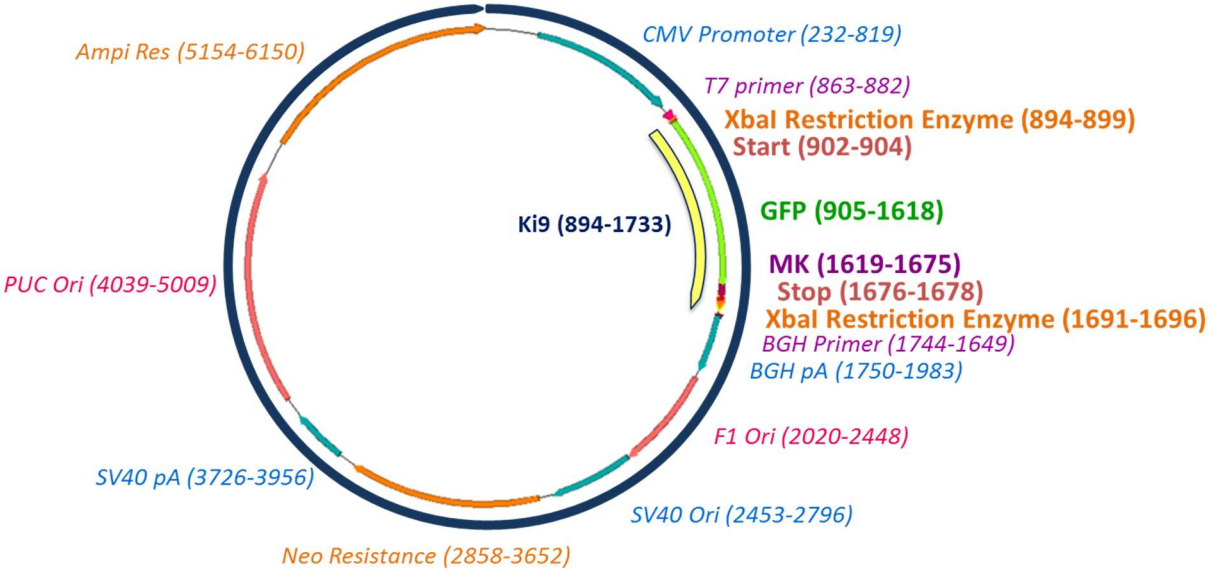


Figure 14. Plasmid map of vector pc3.1(-) including ki9 insert. ki9 contains EGFP protein C-terminally extended with a sequence that derives from the amphibian protein Maximakinin, start and stop codons as well as the restriction enzyme sequence used for the insertion.



1. Cell culture

HEK293 cells (ATCC® CRL-1573™) kindly provided by the University of Lyon and SV40 transformed kidney cells from African green monkey (COS-7, ATCC®CRL-1651™) were used for transfection with pcDNA 3.1(-) vectors containing different inserts. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose, pyruvate, Gibco™) containing 10% fetal bovine serum (Fetal Bovine Serum, qualified, E.U.-approved, South America origin, Gibco™), 1% penicillin/streptomycin (10,000 U/mL, Gibco™) and 1% L-glutamine (200 mM, Life technologies) at 37°C and 5% CO_2 atmosphere.

For subculturing, confluent adherent cells were treated with trypsin-EDTA (0.05%, phenol red, Gibco™), following the protocol and indications of ATCC. Briefly, cells were rinsed with phosphate buffer saline (PBS), pH=7.4, after removal of growth medium and subsequently treated with trypsin-EDTA for the cell layer to be dispersed. Two volumes of fresh growth medium were added, cells were aspirated gently and transferred to a centrifuge tube. The number of cells and their viability were determined using hematocytometer-like grid (KOVA™ Glasstic™ Slide 10 with Grids) and a 0.4% trypan blue in PBS. After centrifugation at 300 x g for 5 min, the supernatant was discarded and the cell pellet was resuspended into fresh medium. Cells were transferred in the recommended cell culture flasks.

2. Plasmid transformation and purification

Plasmid or vector transformation is the process by which exogenous DNA is transferred into the host cell. Transformation usually implies uptake of DNA into bacterial, yeast or plant cells, while transfection is a term usually reserved for mammalian cells[157]. The method for transformation which was followed was chemical transformation. pcDNA 3.1(-) vectors containing three different inserts encoding for EGFP, S4P1-EGFP and EGFP-MK were used. All vectors were previously sequenced in GREPI laboratory in order to validate their sequences. Plasmid transformation was performed using Library Efficiency DH5α competent cells (Invitrogen). Briefly, 1μl (1-10 ng DNA) was added to competent cells and they were incubated for 30 min on ice. Heat shock was applied to cells, by incubating them at 42°C water bath and subsequently for 2 min on ice. Transformants were incubated overnight at 37°C in Terrific Broth. One colony was selected and amplified overnight at 37°C. Plasmids were purified using HiSpeed Maxi Kit (Qiagen).

3. Cell transfection

Three different ways of cell transfection were performed in order to optimize the procedure. The aim of the optimization was to produce ligands with the lower possible cost and in high quantity to have a final solution of high concentration or stable clones that can continuously produce fluorescent ligands. Producer cells are highly fluorescent as these proteins are distributed in all the cellular water. Transgene expression was analysed 24 hours after transfection by observing cells under the microscope, using blue light (GFP emission at 460-500 nm).

a. Transient transfection using PEI

Polyethylenimine (PEI) is a stable cationic polymer that can be used on cells like HEK293 in order to introduce DNA into them. PEI condenses DNA into positively charged particles that bind to anionic cell surfaces. The DNA:PEI complex is endocytosed by the cells and the DNA is released into the cytoplasm[158]. All cells used for transfection were subcultured more than five times after thawed from liquid nitrogen. HEK293 cells were seeded in 24-well plates to a density of 10,000 - 50,000 cells per well, 18h-24h before transfection. Immediately before

transfection, they were rinsed and supplemented with fresh serum-free culture medium. pcDNA vectors were diluted in 150mM NaCl solution before the addition of PEI (c_i=100 mM). DNA-PEI complexes were incubated for 20-30 min at RT and added subsequently in the serum-free growth medium in a proportion 1:10. Cells were incubated 24 hours with complexes at 37°C humidified atmosphere and 5% CO_2. After incubation, cells were washed and incubated with fresh complete growth medium for another 24 hours.

b. Transient transfection using TurboFect™ Transfection Reagent

The TurboFect™ Transfection Reagent (Thermo Fischer Scientific) is a sterile solution of cationic polymers in water. For transient transfection using this reagent, HEK293 cells were seeded and transfected in 24-well plates according to manufacturer's protocol and indications. Briefly, cells were seeded in 1 ml DMEM to a density of 5,000 cells per well, 24 hours before transfection. 1 μg of pcDNA and 2 μL of transfection reagent per well were mixed in 100 μl serum-free DMEM and incubated 10-20 min at RT before being added to the wells. The plate was gently rocked to achieve even distribution of the complexes immediately after adding the transfection reagent and incubated at 37°C in a CO_2 incubator.

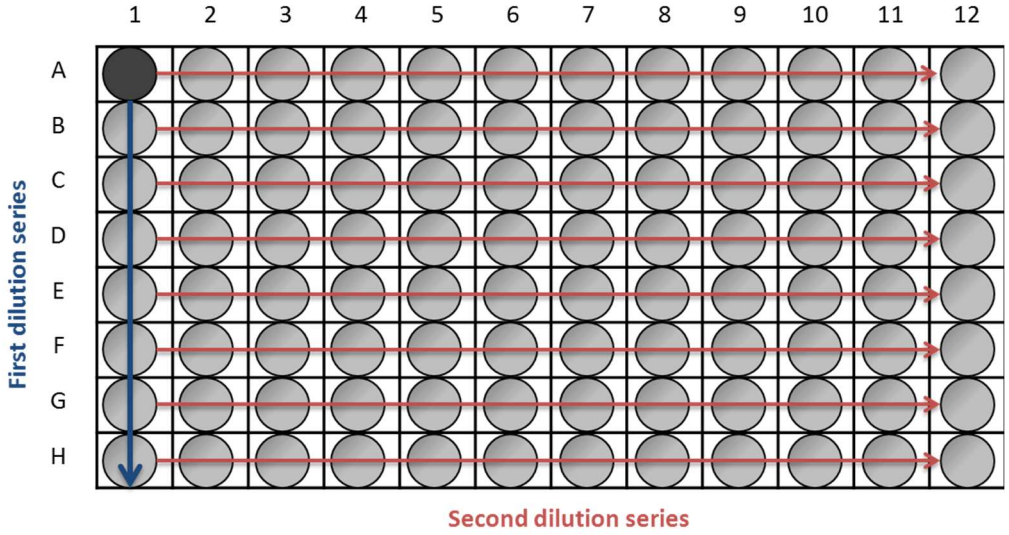
c. Stable transfection using Turbofect™ Transfection Reagent

For this procedure, COS-7 cells were seeded and transfected according to manufacturer's protocol and indications for Turbofect™ Transfection Reagent (Thermo Fischer Scientific). As the plasmids contain in their sequences a gene for resistance to geneticin (G418), after transfection they were grown in selective medium (G418 c_f=500 μg/ml) for 20 days. Subsequently, transfected cells were cloned by two different procedures, serial dilution and cell sorting in flow cytometer.

For cloning by serial dilution in 96-well plates, the Corning protocol "Single Cell Cloning by Serial Dilution" was followed. Briefly, 1,000 to 2,000 cells were added in well A1 of a 96-well plate. A serial dilution was done from well A1 to H1 (first column). Using a multi-channel pipet, a serial dilution was done from the first column until the last, having the same final volume to all wells. Plate was incubated undisturbed at 37°C in a humidified CO_2 incubator.

The cell sorting by FACS was performed at the Institute for Advanced Biosciences (I.A.B) in Grenoble. At day 20 after transfection and selection with G418, cells were harvested and resuspended in culture medium with 1% penicillin/streptomycin at approximately 5×10^6 viable cells/ml. The collected cells were then sorted on a FACS Aria, BD Biosciences. Living cells were gated based on forward/side scatter and events in the top 6% of GFP fluorescence were sorted and individually placed in 96-well plates.

Figure 15. Initial plate setup for serial dilution. After incubation, the cells with less than 2 cells were chosen and marked to follow-up (clones).



4. Cell lysis by freeze-thaw method

Cell lysis by freeze-thaw method was previously described by Charest-Morin et al.[79]. HEK293 transfected cells were rinsed with PBS pH=7.4, left without supernatant, frozen for 2 hours at -20°C, thawed and scraped. The resulting suspension was centrifuged (15,000 x g, 10 min, 4°C) in the presence of protease inhibitors and the supernatant (the lysate) was stored as a concentrated stock of fusion proteins at -80°C.

5. Protein analysis

The presence and concentration of cytosolic GFP proteins was verified by anti-GFP immunoblotting assay, using 15% SDS-polyacrylamide gels and transferring proteins on a nitrocellulose membrane. For protein detection, primary antibody, anti-GFP monoclonal antibody (Santa Cruz Biotechnology) was incubated overnight at 4°C, followed by labelled with horseradish peroxidase (HPR)-conjugated secondary antibody for 1 hour at RT. Protein signals were visualised by using Clarity Western ECL Substrate for detection of peroxidase activity. Band intensity was analysed densitometrically with the Molecular Imager ChemiDoc XRS™ and ImageLab™ software (Bio-Rad, USA).

B. Expression of Bradykinin Receptors

1. Cell Culture

The human endothelial cells (EA.hy926, ATCC® CRL-2922™) were grown in DMEM, high glucose, pyruvate (Gibco™) containing 10% FBS (qualified, E.U.-approved, South America origin, Gibco™), 1% penicillin/streptomycin (10,000 U/mL, Gibco™) and 1% L-glutamine (200 mM, Life technologies™) at 37°C and 5% CO_2 atmosphere. The generation time for all adherent cell lines (HEK293, COS-7 and EA.hy926) was calculated in order to define the rate of subculturing and the expected number of cells after seeding plates for different

experiments. Human peripheral blood monocytes, THP1 (ATCC® TIB-202™) were grown in RPMI-1640 medium (Gibco™) containing also FBS, penicillin/streptomycin and L-glutamine in the same concentration as DMEM medium. Falcon® 25 cm and 75 cm tissue culture flasks were used for growing and subculturing all cells.

For subculturing, confluent adherent cells (EA.hy926) were treated as described previously. Non-adherent cell cultures (THP1) were maintained either by the addition of fresh medium or replacement of medium. To maintain the indicated by ATCC concentration of cells, subculturing was sometimes performed by centrifugation with subsequent resuspension at 2-4 x 10^5 viable cells/ml. The cell concentration was not allowed to exceed 10^6 cells/ml.

2. Luminescence-based assay

Phagocytic cells, when activated, produce reactive oxygen species (ROS) which is essential for host defense against pathogens. One important enzyme involved in ROS production in non-leukocyte tissues is NADPH oxidase 1 (NOX1). As NOX2, NOX1 is responsible for the production of superoxide anion (O_2°-) which generates hydrogen peroxide (H_2O_2) in the presence of protons. H_2O_2 reacts with O2°- to form hydroxyl radical (OH°). In chemiluminescence, luminol is activated by an oxidant and emits a blue glow that can be measured by a luminometer. A solution containing hydrogen peroxide (H_2O_2) and hydroxide ions can be the activator.

In presence of phorbol myristate acetate (PMA), THP1 cells are differentiated into macrophages. Aim of this assay was to identify the activation of monocytes (THP1) in presence of PMA, in order to evaluate the performance of the cell line. In this assay, THP1 cells were washed with PBS and incubated with a solution containing 20 mM Glucose, 20 μM Luminol and 10 U/μl PBS enriched with $MgCl_2$ 0.8 mM and $CaCl_2$ 0.5 mM for 1 min at 37°C. Finally, the emission of the blue glow produced by luminol was measured in a luminometer or 1 hour (reading every 30 sec) in the presence of PMA 2 μg/ml.

3. Induction for the expression of B1R

B2R is constitutively expressed on different cells and cell lines. Based on literature[159], a mix of TNFα 10 ng/ml and INFγ 20 ng/ml was used to induce the expression of B1R. Cells were treated with the cocktail of cytokines for 24 hours before every analysis for the expression of B1R. For Immunoblot assays, based on previous results in GREPI Laboratory, the induction of B1R was done by applying only TNFα 10 ng/ml.

4. Immunofluorescence (IF) using biotechnological ligands

EA.hy926 cells were prepared by being seeded in the optimized cell density, fixed using Ethanol 70% and washed with PBS. The plate was saturated by using 1% bovine serum albumin (BSA) in PBS. The solution containing the ligand for B1R was added and incubated

for 1 hour at RT under agitation. IF analysis was performed by observing cells under the microscope, using blue light (GFP emission at 460-500 nm).

II. Detection of BK receptors using antibodies

A. Immunoblot

EA.hy926 cells were lysed as described previously for HEK293. Cell lysates containing the targeted proteins were migrated on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (BIORAD Transblot turbo). Membranes were blocked in 1% BSA in Tris Buffer Saline (TBS) solution containing 0.05% Tween20 for 1 hour. Western Blots were performed using primary antibodies anti-B1R (PsiScience™) and anti-B2R (Abcam™) produced in rabbits. Anti-rabbit IgG peroxidase labelled antibody was used as a secondary antibody. Peroxidase activity on membranes was visualized on a Molecular Imager ChemiDoc XRS™ and ImageLab™ software (Bio-Rad, USA) by using Clarity Western ECL™ Substrate. PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo scientific) was used to estimate the size of proteins.

B. FACS

Normal THP1 cells or treated with cytokines were washed with PBS, resuspended in a concentration of 100,000 cells/ml, centrifuged and left without supernatant. They were incubated for permeabilisation for 40 min at 4^{o}C with Fixation and permeabilization Solution (BD), washed and stained with different primary antibodies (anti-B1R, anti-B2R) in an antibody/Permwash solution overnight at 4^{o}C and with 1% secondary antibody in Permwash solution for 1 hour at RT. Finally, they were analyzed in FACS lysing solution (PFA 0.1%) using BD FACS Canto II /BD FACS Diva 6.1.3.

PART II: PLASMINOGEN GLYCOFORMS ALTERATION AND SUSCEPTIBILITY TO ACTIVATION ASSOCIATED WITH THE MISSENSE VARIANT p.Lys330Glu IN HAE-PLG PATIENTS

Materials

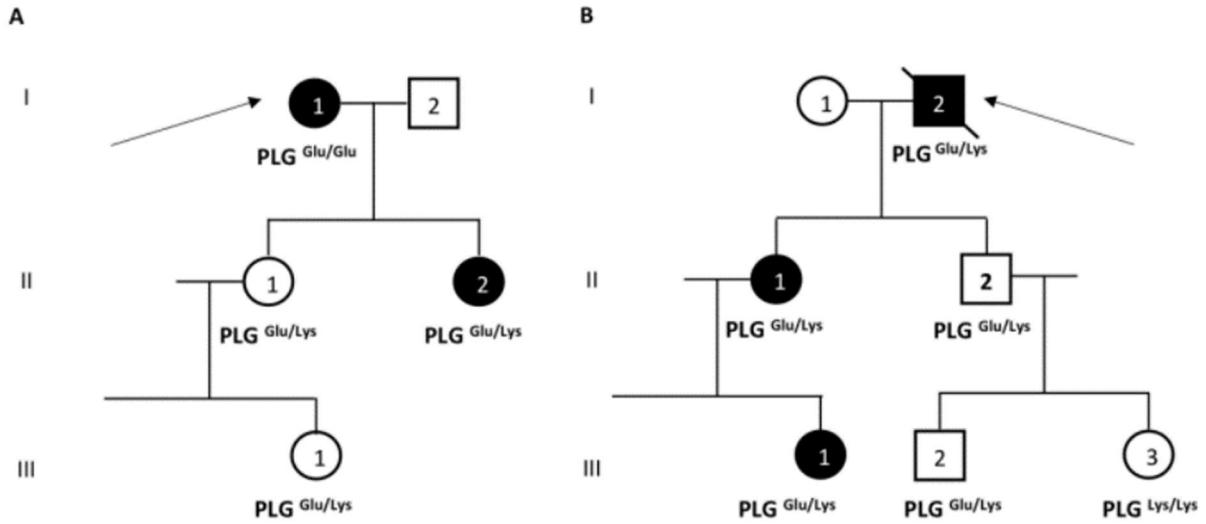
The materials used for the analysis of PLG activation are presented in Appendix I (Table 3).

Methods

I. Demographical, clinical and molecular data of patients

The aim of this part of the study was to investigate the consequences of the PLG p.Lys330Glu variant and the susceptibility to activation of PLG. For this purpose we included 3 symptomatic and 5 symptom-free individuals, members of two different families, one Greek and one Spanish. All individuals are carriers of the variant. The pedigrees are presented in Figure 16. The proband I.1 of family A is a homozygous carrier, a 75-year old woman with severe angioedema since the age of 40 years (18 attacks/year when out of prophylactic treatment). She does not have any family history of HAE and tranexamic acid is the only effective treatment. She has two daughters (subjects II.1 and II.2) and a granddaughter (subject III.1) who are heterozygous carriers. One daughter (II.2) developed first symptoms of angioedema at the same age as her mother, while the other members are asymptomatic. All members of family B (proband I.2, daughter II.1, son II.2, granddaughter III.1 and grandson III.2) were found heterozygous for the p.Lys330Glu variant. Nevertheless only the proband I.2, the daughter II.1 and the granddaughter III.1 developed angioedema symptoms. More specifically, the proband (subject I.2) died at the age of 94 years, suffered his first attack at the age of 75 after receiving treatment with ACE-inhibitor. The second attack appeared at the age of 83 after receiving dutasteride for benign prostatic hyperplasia. Thereafter, he suffered every 2-3 months, mild nondrug-induced attacks controlled by tranexamic acid. His 62-year-old daughter (subject II.1), firstly experienced an attack during pregnancy and afterwards she presented at least two moderate attacks of tongue swelling. Her daughter (subject III.1) suffers several severe oestrogen-induced attacks affecting tongue, lips, and larynx. Interestingly, two of them (proband I.2 and daughter II.2) were also found heterozygous carriers of a *PLG* variant (NM_000301.3:c.266G>A), while the granddaughter III.1 is carrying another *PLG* variant (NM_000301.3:c.1567C>T), both classified as likely benign with an allele frequency 0.6% and 0.7% respectively.

Figure 16. Pedigrees of the two families with PLG p.Lys330Glu. Panels A and B correspond to the Greek and Spanish families, respectively. Black symbols indicate symptomatic individuals; empty symbols indicate asymptomatic individuals and the arrows indicate the probands in each family. Incomplete penetrance has already been described for HAE-PLG. [Adapted by Parsopoulou et al. (2020)][160]



II. Samples

Citrated plasma samples were collected for biochemical investigation. The results from basic coagulation tests and clot lysis assay were within the normal range for all individuals. Furthermore, all subjects presented normal C1-INH function[118], normal KK activity[129] and kinin catabolism, except a low APP activity for the individuals of family A and BIII.2. HK cleavage pattern, as investigated by immunoblot[132], was comparable to control for all the heterozygous carriers at any stage of the disease. The homozygous patient also exhibited a HK cleavage comparable to healthy control during the resting situation, while more extended in samples collected a few hours after angioedema attack.

III. anti-PLG immunoblot

The glycosylation pattern of PLG was investigated by anti-PLG immunoblot. 60 ng of purified PLG and plasma sample from a healthy individual were used for control and quantification samples. Control samples and plasma samples from all 8 patients were migrated on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (BIORAD Transblot turbo). Membranes were saturated using 1% BSA in TBS solution containing 0.05% Tween 20 for 1 hour. Immunoblots were performed using primary antibodies anti-PLG (Goat, anti-human-PLG, Affinity Biological). Anti-goat IgG peroxidase labelled antibody was used as a secondary antibody. Peroxidase activity on membranes was visualized on Molecular Imager ChemiDoc XRS™ and ImageLab™ software (Bio-Rad, USA) by using Clarity Western ECL™ Substrate. PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo scientific) was used to estimate the size of proteins.

IV. Plasmin-specific chromogenic assay

As both PLG glycoforms are known to exhibit functional differences, including the susceptibility to activation, we investigated PLG activation in human citrated plasma, performing a plasmin-specific chromogenic assay using a -pNA substrate. A chromogenic substrate is composed of 3-5 amino acids that mimic the cleavage site of the natural protein substrate and has the chromophore 4-nitroaniline (pNA) attached to its end. When pNA is attached to the peptide chain it is colorless but when liberated by enzymatic cleavage the free pNA is yellow.

In this experiment, 10 µl of each plasma sample were added with 10 µl of one among three different activators [tPA, streptokinase (SK) and urokinase (UK)] in Tris-NaCl buffer 1X (50 mM, 150 mM, pH 7.8) in a 96-well plate (Corning Costar) and incubated for 10 min at 37°C. Then, 200 µl of Tris-NaCl buffer (pH 7.4) containing chromogenic substrate S-2403 (<Glu-Phe-Lys-pNA, Chromogenix) was added to each well, and absorbance at 405 nm was measured every 1 min for 30 min with a microplate reader (Sunrise, TECAN Austria GmbH, Grödig, Austria) at 37°C.

PART III: GENETIC BIOMARKERS OF THE SEVERITY OF C1-INH-HAE

Materials

(a) The devices, pipets and plastic material used for the detection of variants affecting the severity of the disease in C1-INH-HAE patients are presented in Appendix I (Table 4).

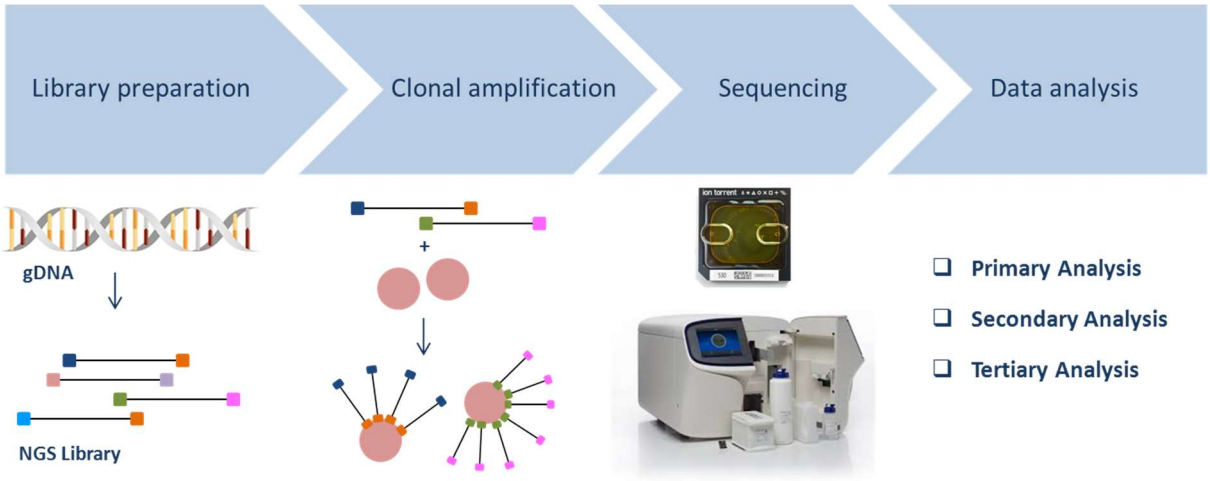
(b) The solutions, reagents and kits used for the detection of variants affecting the severity of the disease in C1-INH-HAE patients are presented in Appendix I (Table 5).

Methods

I. Next-Generation Sequencing

Next-generation sequencing (NGS), initially called “massively-parallel sequencing”, enables the sequencing of many DNA strands at the same time compared to Sanger sequencing by capillary electrophoresis. Regardless the instrument technology, the workflow for a NGS experiment includes similar steps, which are presented in Figure 17. At the first step, a library is created by performing PCR amplification of the targeted genetic regions and ligation of specific adaptor sequences, which include a unique molecular barcode, to the resulting DNA fragments. This allows for multiple samples to be mixed and sequenced in the same experiment. The DNA library is attached on a solid surface and clonally amplified in order to increase the final signal from each target during sequencing. Finally, all the libraries are sequenced at the same time. The method is called "sequencing by synthesis", where individual bases are read as they grow along a polymerized strand. In IonTorrent™ technology, the instruments use electrical detection to sense the pH change caused by the release of hydrogen ions, which naturally occurs when nucleotides are incorporated during DNA synthesis. Data analysis can be divided in three steps, primary, secondary and tertiary analysis. Primary analysis is automated and processes raw signals from instrument detectors into base calls. This step is mandatory as each NGS experiment generates large quantities of data which consist of short DNA reads. These raw data are collected during each sequencing cycle. The output of primary analysis is files containing base calls assembled into sequencing reads and their associated quality scores (Phred quality score). Secondary analysis involves filtering of the reads and trimming based on quality, followed by alignment of reads to a reference genome or assembly of reads for novel genomes, and finally by variant calling. The main output is a BAM file containing aligned reads. Tertiary analysis is the most challenging step, as it involves interpreting results and extracting meaningful information from the data[161].

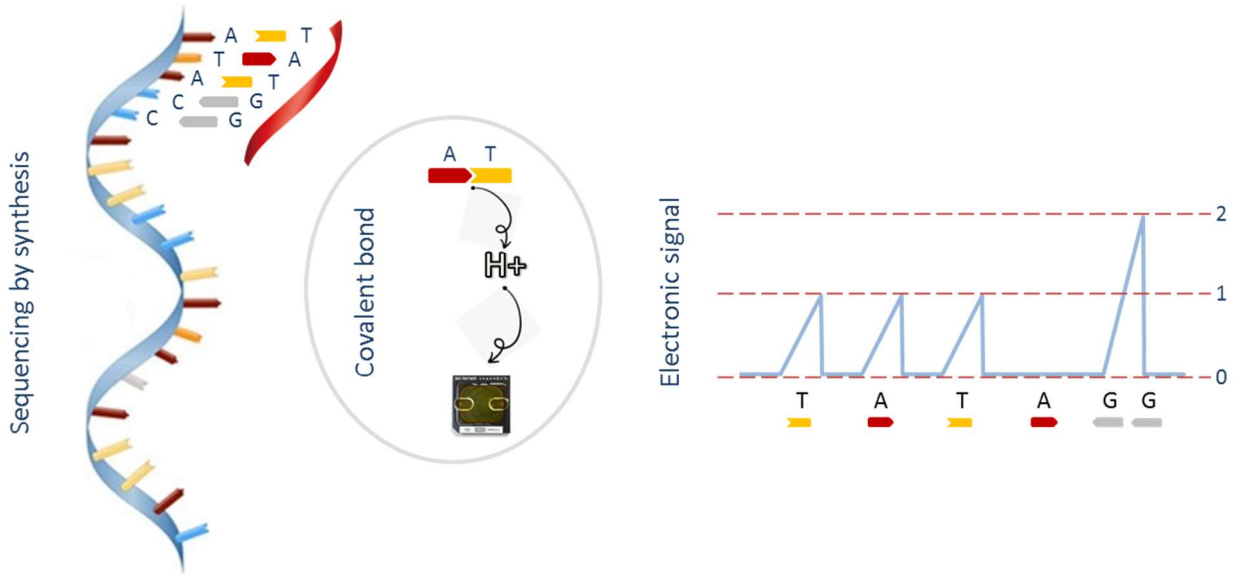
Figure 17. The four steps of an NGS experiment.



II. Ion Torrent Next-Generation Sequencing Technology

Ion Torrent™ technology connects chemical to digital information as it translates chemically encoded information (A, C, G, T) into digital information (0, 1) on a semiconductor chip. In nature, when a deoxyribonucleoside triphosphate (dNTP) is incorporated into a strand of DNA by a polymerase, a covalent bond is formed and a pyrophosphate and a positively charged hydrogen ion (H+) are released as by-products. In ion semiconductor sequencing, a microwell contains many copies of a unique single-stranded template DNA and DNA polymerase. This microwell is flooded in every cycle with a single species of dNTP. If the introduced dNTP is complementary to the template nucleotide, it is incorporated into the growing strand. This causes the release of a hydrogen ion. If the introduced dNTP is not complementary there is no incorporation and no biochemical reaction. The hydrogen ion that is released in the reaction changes the pH of the microwell, which is detected by an ion-sensitive field-effect transistor (ISFET). If homopolymer repeats are present in the template sequence, multiple dNTP molecules will be incorporated in a single cycle. This leads to a corresponding number of released hydrogens and a proportionally higher electronic signal. The unattached dNTP molecules are washed out before the next cycle when a different dNTP species is introduced[162]. In order to perform a NGS experiment with Ion technology, the workflow is the same as described previously. A library is created, the DNA library is attached on a solid surface and clonally amplified. The libraries are sequenced “by synthesis" and the instruments convert raw electrical signals from instrument detectors into digital information and subsequently to base calls.

Figure 18. Ion Torrent™ technology.



III. Rare variants affecting the severity of the disease in C1-INH HAE patients

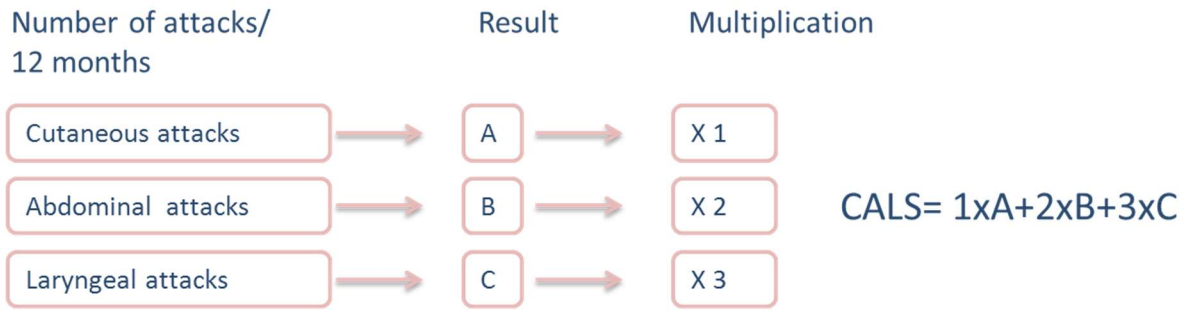
A custom NGS platform was designed using the Ion AmpliSeq Designer (www.ampliseq.com, Thermo Fisher Scientific), in order to analyze *SERPING1* in its full length [all exons, introns, promoter, 5' and 3' untranslated regions (UTRs)] and all coding regions and exon-intron splice junctions of 54 additional genes involved in angioedema pathogenesis and possibly affecting patients' clinical phenotype[163]. The gene list was compiled from literature data on angioedema and genetic predisposition, protein-protein interaction networks, and pathway analysis[153].

Targeted sequencing with the Ion Torrent System is able to identify single nucleotide variants (SNVs), small insertions and small deletions. This panel includes 825 amplicons in two primer pools among which 77 for *SERPING1*, and provides 99.61% coverage of all targeted regions (100% coverage of all translated *SERPING1* regions and UTRs with missing areas located only in intronic regions). The developed platform was validated against 135 C1-INH-HAE-associated variants along with 115 negative controls and 95 randomly selected DNA samples from affected family members of C1-INH-HAE index patients[163]. The specifications of the NGS panel and the missing areas are presented in Appendix II (Table 1-2).

For this part of the study, DNA samples from 144 unrelated C1-INH-HAE patients (43 Hungarian, 32 Polish, 30 Greek, 23 German and 16 Bulgarian) were analyzed in order to detect rare variants (≤1%) in the 54 additional genes encoding for proteins implicated in the metabolism and function of BK and investigate if the co-existence with a causal *SERPING1* variant could affect the severity of the disease. The severity of C1-INH-HAE was calculated with a score based on the number of oedematous attacks during the 12 months before evaluation and more precisely as it is presented in Figure 19.

Figure 19. Calculation of CALS score.

Example: The number of edematous attacks in the preceding 12-month period; Cutaneous=5, Abdominal=3, Laryngeal=1. CALS: (1×5)+(2×3)+(3×1)=14.



All patients were previously diagnosed clinically and by measuring C4, C1-INH levels and function. 134 patients were C1-INH-HAE Type I and 10 were C1-INH-HAE Type II. Their mean age at disease onset was 12.91 years. 38/144 patients were under LTP. 1/144 patient had never suffered any angioedema attack during his life up to the age of analysis. The clinical and demographical data is presented on Table 3.

10 ng of gDNA per primer pool were amplified in order to construct DNA libraries for each sample using the Ion AmpliSeq Library Kit 2.0 (Thermo Scientific) according to manufacturer's instructions (User Guide, Publication Number MAN0006735). Pooled amplicons were partially digested using FuPa reagent, ligated to a unique barcoded adapter (Ion Xpress barcode adapters kit, Thermo Scientific) and amplified for 5 cycles in order to obtain a sufficient quantity of barcoded library. Purification of barcoded libraries was performed using the Agencourt AMPure XP Beads (Beckman Coulter) and were quantified with Qubit 2.0 fluorometer (Thermo Scientific). Finally, the libraries were diluted to 100 pM and pooled in equimolar proportion. Template preparation, enrichment and chip loading were carried out on Ion Chef system (Thermo Scientific). Sequencing was performed on S5XL, on 520 and 530 chip, using Ion 510 & Ion 520 & Ion 530 Kit (Thermo Scientific). All procedures were performed according to the manufacturer's instructions (Application Guide MAN0016854, Thermo Fisher Scientific).

Base calling, demultiplexing and alignment to hg19 reference genome (GRCh37) of the sequencing raw data were performed on Torrent Suite 5.10 software (Thermo Scientific) using default parameters. Variant calling was performed by the VariantCaller v.5.8.0.19 plug-in and coverage analysis by the coverageAnalysis v.5.8.0.8 plug-in on Torrent Suite 5.10.

All variants detected after alignment to hg19 by the use of VariantCaller plug-in were annotated on Ion Reporter software v.5.6 (Thermo Scientific) with the gene name and for their possible presence in the Single Nucleotide Polymorphism Database (v135) (http://www.ncbi.nlm.nih.gov/projects/SNP/), the Genome Aggregation Database (GnomAD) (gnomad.broadinstitute.org), the 1000 Genomes project and the ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) according the recommendations of the Human Genome Variation Society (HGVS) (http://www.hgvs.org/mutnomen/). SIFT

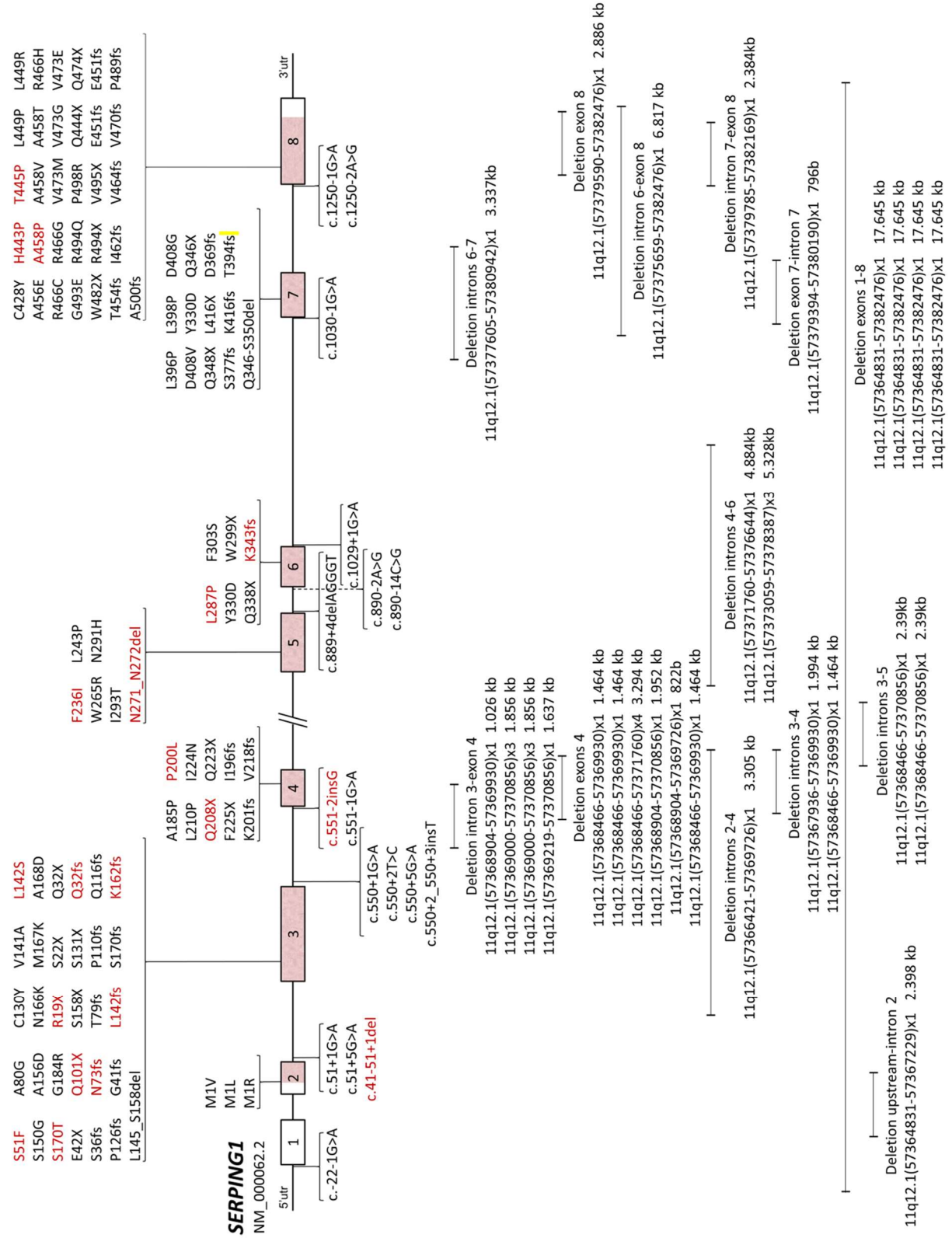
(http://sift.jcvi.org/) and PolyPhen version 2 (http://genetics.bwh.harvard.edu/pph2/) bioinformatics tools were used for the *in silico* pathogenicity prediction of the variants. Alignments and all obtained sequences were visually inspected using the Integrative Genomics Viewer (IGV) v2.2 (Broad Institute). Variants with worldwide frequency >1% (1000 Genomes Global Minor Allele Frequency, ExAC), polymorphisms (UCSC Common SNPs) for which no disease associations are reported in the ClinVar database as well as synonymous and intronic SNVs were excluded from further analysis.

We classified variants according to standards and guidelines from the American College of Medical Genetics and Genomics (ACMG) and Association of Molecular Pathology (AMP)[164]: pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB) and benign (B). We used the Varsome tool for ACMG classification in order to confirm our results[165].

Table 3: Demographic, clinical and molecular data of the C1-INH-HAE patients.

Clinical data	**Total**	**Greek**	**Polish**	**German**	**Hungarian**	**Bulgarian**
No of patients	144	30	32	23	43	16
Sex (male/female)	65/79	16/14	12/20	11/12	18/25	8/8
Age at analysis (median, range)	40.0 (2.5-85)	35.0 (2,5-67)	44.0 (25-85)	42.0 (13-81)	37 (12-70)	50 (8-81)
Age at disease onset (median, range)	13.0 (0.5-73)	10.0 (1-31)	17.0 (1-73)	10.0 (3-19)	12.0 (0.5-53)	12.5 (1-50)
HAE Type (I/II)	134/10	30/0	29/3	22/1	38/5	15/1
Longterm treatment (Yes/No/NA)	45/86/13	10/16/4	3/27/2	6/10/7	23/20/0	3/13/0
CALS Severity (median, range)	31.74 (0-238)	24.42 (4-69)	51.96 (0-238)	*Missing data*	8.15 (0-41)	39.38 (6-103)
***SERPING1* defects**						
Regulatory, n (%)	1 (0.69%)	0 (0.00%)	0 (0.00%)	1 (4.35%)	0 (0.00%)	0 (0.00%)
Missense mutations, n (%)	46 (31.94%)	6 (20%)	16 (50%)	8 (34.78%)	14 (32.56%)	2 (12.50%)
Nonsense mutations, n (%)	20 (13.90%)	5 (16.67%)	3 (9.375%)	2 (8.70%)	7 (16.28%)	3 (18.75%)
Splice defects, n (%)	15 (10.42%)	4 (13.33%)	1 (3.13%)	3 (13.04%)	5 (11.63%)	2 (12.50%)
Small deletions or insertions (frameshift alterations), n (%)	31 (21.53%)	11 (36.67%)	2 (6.25%)	6 (26.08%)	9 (20.93%)	3 (18.75%)
Large deletions or insertions, n (%)	14 (9.72%)	1 (3.33%)	3 (9.375%)	2 (8.70%)	6 (13.95%)	2 (12.50%)
Deep intronic, n (%)	1 (0.69%)	1 (3.33%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Unidentified defects, n (%)	16 (11.11%)	2 (6.67%)	7 (21.87%)	1 (4.35%)	2 (4.65%)	4 (25.00%)

Figure 20. The localization of 144 different C1-INH-HAE-associated *SERPING1* variants that were detected by the targeted NGS sequencing. Among them, 3 regulatory, 48 missense, 19 nonsense, 27 frameshift, 17 splice site mutations, 3 deletions and 27 large defects. The NGS platform presents an analytical sensitivity of 98.96%, a false negative rate of 1.05%, analytical specificity 100%, a false positive rate equal to zero, accuracy 99.35%, and repeatability 100%, based on the results for previously genotyped patients by the conventional method.



IV. SNPs affecting the phenotype of the disease in C1-INH HAE patients

Using the previously described NGS platform, we investigated the presence or absence of 18 common functional variants (Allele frequency≥1%) and associated them with three distinct phenotypic traits of C1-INH-HAE patients (the age at disease onset, the severity of the disease, based on the CALS score and the need for LTP). The SNPs were selected from the literature according to their effect on the protein activity, their frequency and the coverage that could be achieved by the platform (Table 5). 233 patients (113 Hungarian, 47 Polish, 31 Greek, 23 German, 19 Bulgarian) from 144 different families (43 Hungarian, 32 Polish, 30 Greek, 23 German, 16 Bulgarian) were enrolled at this study. More precisely, the study population included the 144 C1-INH-HAE unrelated patients from the previous part and 89 family members, carrying the same causal *SERPING1* variant and suffering from HAE attacks. 217/233 patients were C1-INH-HAE Type I and 16/233 were C1-INH-HAE Type II. Their mean age at disease onset was 12.5 years. 1/144 patient had never suffered any angioedema attacks during his life. 38/144 patients were on LTP. The clinical and demographical data is presented on Table 4.

Table 4. Demographic, clinical and molecular data of the C1-INH-HAE patients.

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

The library, template preparation, enrichment, chip loading, sequencing, primary and secondary analysis were performed as presented above. In the tertiary analysis, we searched for the presence or absence of the selected functional UCSC common SNPs.

Table 5. Selected common SNPs. Allele frequency according to GnomAD v2.1.1 (searched on 31/07/2020)

Gene	dbSNP	Nucl. change	aa change	MAF	EMAF
F12	rs1801020	c.-4T>C	NA (5'UTR)	65.20%	75.66%
F13A1	rs5985	c.103G>T	p.Val35Leu	20.46%	25.33%
F13B	rs6003	c.344G>A	p.Arg115His	88.22%	90.60%
F2	rs1799963	c.*97G>A	NA (3'UTR)	0.84%	1.25%
CPN1	rs61751507	c.533G>A	p.Gly178Asp	4.23%	4.92%
A2M	rs669	c.2998A>G	p.Ile1000Val	30.98%	34.14%
KLK1	rs5515	c.230G>A	p.Arg77His	3.12%	3.70%
KLKB1	rs3733402	c.428G>A	p.Ser143Asn	54.02%	50.59%
MASP2	rs72550870	c.359A>G	p.Asp120Gly	2.14%	3.23%
MPO	rs56378716	c.752T>C	p.Met251Thr	1.02%	1.35%
PLAU	rs2227564	c.422T>C	p.Leu141Pro	74.83%	76.39%
SERPINA1	rs17580	c.863A>T	p.Glu288Val	2.33%	3.65%
SERPINA1	rs28929474	c.1096G>A	p.Glu366Lys	1.11%	1.84%
SERPINA1	rs121912714	c.839A>T	p.Asp280Val	0.05%	0.05%
SERPINE1	rs6092	c.43G>A	p.Ala15Thr	9.52%	11.31%
TLR2	rs5743708	c.2258G>A	p.Arg753Gln	1.74%	2.81%
SERPING1	rs4926	c.1438G>A	p.Val480Met	22.20%	27.17%
SERPING1	rs28362944	c.-21T>C	NA (5'UTR)	2.91%	4.67%

rs1801020 (*F12*)

NM_000505.3:c.-4T>C, widely known as *F12*-46C/T is located in the promoter region of *F12* gene (OMIM*610619), four bases upstream from the ATG translation initiation codon. Among approximately 120,000 exomes of the GnomAD, the C allele has been detected in a frequency 65.20% in the global population and 75.66% in the European non-Finnish population. In ClinVar database, the SNP is characterized benign by different submitters, when associated with Factor XII deficiency or FXII-HAE.

Kanaji et al. (1998) first reported this SNP and investigated its relationship with plasma levels of FXII. They found different levels of the protein according to the genotype and despite the fact that both alleles were equally transcribed in hepatocytes of heterozygotes, the cDNA containing the T allele was producing less FXII in vitro than the one containing the C allele. Therefore, they concluded that the presence of the SNP affected the efficiency of translation, probably due to the creation of another ATG codon[166].

Endler et al. (2001) observed a 2.5-fold lower prevalence of the T genotype in patients with acute coronary syndrome, which indicated a protective effect on the development of

the syndrome in patients with preexisting stable coronary artery disease[167]. Homozygosity for the T allele was also suggested as a risk factor for ischemic stroke[168] and venous thrombosis[169,170,171,172].

Concerning C1-INH-HAE patients and the heterogeneous clinical manifestations of the disease, Speletas et al. (2015) provided evidence that the presence of the T allele of this variant is associated with a less severe clinical phenotype of C1-INH-HAE regardless of *SERPING1* mutational status. More precisely, carriers of the TT genotype exhibited a significantly delayed disease onset and did not need long-term treatment[139]. They attributed this fact to the effect that the polymorphism has on the synthesis of FXII, mainly in transcriptional level. Evidence provided by Rijavec et al. (2019)[140] indicates that the *F12* c.-4T>C variant influence the penetrance of C1-INH-HAE. The authors have shown that, among C1-INH-HAE individuals, carriers of the CC genotype compared to those of the TT genotype have a 25-fold greater risk of developing the disease.

rs5985 (*F13A1*)

NM_000129.3:c.103G>T (p.Val35Leu) is located in exon 2 of *F13A1* gene (OMIM*134570). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the T allele has been detected in frequency 20.46% in the global population and 25.33% in the European non-Finnish population. It is presented in a very low frequency in East-Asian population (0.09%). In NCBI, the Reference SNP number of this variant (rs5985) represents a multiallelic variant, as at the same position there is another substitution c.103G>A (p.Val35Met). The c.103G>A is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in exomes of 6/125,438 individuals of the GnomAD. In ClinVar database, there are conflicting interpretations of the pathogenicity of the SNP as it is characterized benign for FXIII Subunit A deficiency, protective for venous thrombosis and myocardial infraction by two different submitters, based on the literature and VUS by one submitter with no additional information concerning the condition or the carrier.

The variant was first detected in Finnish individuals by Mikkola et al (1994)[173], and characterized a common SNP as the frequency in controls was 23%. From 1998 until 2003, accumulated evidence[174,175,176,177,178] suggested strong protective effect of TT homozygosity against venous thrombosis and myocardial infraction. Up to 2007, the data for the association of the SNP with myocardial infraction is resumed and interpreted to a meta-analysis by Shafey et al.[179].

The mechanical strength and resistance to fibrinolysis are enhanced by the formation of covalent γ-glutamyl-γ-lysine bonds between fibrin monomers, reaction that is catalyzed by the activated factor XIII (FXIII)[180]. Arlens et al. (2000) reported the functional characterization of the SNP in the A subunit. More precisely, the substitution is located close to a thrombin

activation site and the nature of the amino acid at this position influences the activation rate of FXIII. The rate of proteolysis of the FXIII A subunit is faster and occur at lower thrombin concentrations when Val is substituted by Leu. Arlens et al. hypothesized that fibrin cross-linked by mutated FXIII may result in a finer meshwork, thinner fibers, and altered permeation characteristics[181]. Based on the same hypothesis and functional studies, González-Conejero et al. (2006) identified rs5985 as a marker for the efficacy of thrombolytic therapy and early mortality rates in patients with ischemic stroke[182].

rs6003 (*F13B*)

NM_001994.2:c.344G>A (p.Arg115His) is located in exon 3 of *F13B* gene (OMIM*134580). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 88.22% in the global population and 90.60% in the European non-Finnish population. In ClinVar database, it is characterized benign for FXIII Subunit B deficiency, but a risk factor for venous thrombosis, based on literature.

Komanasin et al. (2005) presented evidence that the presence of arginine in the position 115 resulted in faster dissociation between the A and B subunits of FXIII after activation by thrombin and was associated with moderately increased risk for venous thrombosis[183].

rs1799963 (*F2*)

NM_000506.5:c.*97G>A, which has historically been called prothrombin 20210G-A polymorphism is located in the 3'UTR region of *F2* gene (OMIM*176930). It has not been previously associated with C1-INH-HAE. Among approximately 15,000 genomes of the GnomAD, the A allele has been detected in frequency 0.84% in the global population and 1.25% in the European non-Finnish population. The variant is rare in Asian and African populations. In ClinVar database, there are conflicting interpretations of the pathogenicity of the SNP. It is characterized pathogenic for thrombophilia due to thrombin defect, congenital prothrombin deficiency and venous thrombosis, based on accumulated evidence from the literature and a risk factor for hereditary factor II deficiency, ischemic stroke, recurrent pregnancy loss and venous thromboembolism. However, one submitter has classified the variant VUS and another benign for congenital prothrombin deficiency.

While Rosendaal et al. (1998) studied the prevalence of this variant[184], Zivelin et al. performed studies to determine the origin and few years later the age of the variant[185,186]. Their data strongly supported a single origin and the age was estimated at 23,720 years. Gehring et al. investigated the mechanism by which the variant affects the generation of prothrombin. They demonstrated that the mutation does not affect the amount of pre-mRNA, the site of 3' end cleavage or the length of the poly(A) tail of the mature mRNA. They supported that the physiological F2 3' end cleavage signal is inefficient and that *F2* 20210 G-A

represents a gain-of-function mutation, causing increased cleavage site recognition, increased 3' end processing and increased mRNA accumulation and protein synthesis[187].

The variant was studied by different research teams in association with thrombophilia and ischemic stroke[188,189,190]. Moreover, it was associated with recurrent pregnancy loss, Budd-Chiari Syndrome and perception deafness, where it was characterized a risk factor[190].

rs61751507 (*CPN1*)

NM_001308.3:c.533G>A (p.Gly178Asp) is located in exon 3 of *CPN1* gene (OMIM*603103). This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 4.23% in the global population and 4.92% in the European non-Finnish population. In ClinVar database, the variant is characterized benign for anaphylatoxin inactivator deficiency but pathogenic for CPN deficiency, based on literature.

The variant has been previously associated with hereditary angioedema by Mathews et al. (1980)[191]. The 65-year-old patient described in that study was presenting a remarkably low CPN level with an 11-year history of episodic angioedema or chronic urticaria, as well as hay fever or asthma. However, plasma histamine was elevated during attacks while serotonin and kinin activity were not. The proband's sister had equally depressed CPN level and studies of other family members suggested an autosomal recessive inheritance of the enzyme deficiency.

Genotyping of the 65-year-old man by Cao and Hegele (2003)[192] revealed three *CPN1* variants. The affected individual was heterozygous for each variant. One of these variants, 385fsInsG was a rare frameshift variant that would be expected to have a profound effect on protein structure, with little or no active enzyme produced. The second variant, c.533G>A (p.Gly178Asp) altered the coding sequence, but was also seen in heterozygous state in normal control subjects (~1%). The Gly178 residue of CPN has been conserved in species as diverse as cow, rat and mouse and is also conserved among most members of the human carboxypeptidase family.

Applying the ACMG standards and guidelines, the variant is classified as benign. More precisely, (a) GnomAD exomes latino allele frequency = 0.0669 is greater than 0.05 threshold (Criterion BA1 Stand-alone), (b) ClinVar classifies this variant as benign, rated 2 stars, with 3 submissions, 1 publication and no conflicts. (Criterion BP6, using strength "Strong" because of the evidence presented by ClinVar).

However, the variant remains interesting as (a) UniProt protein CBPN_HUMAN region of interest 'Catalytic' has 1 non-VUS, non-synonymous, coding variant [(1 pathogenic and 0 benign), pathogenicity = 100.0% which is more than threshold 33.3%) (Criterion PM1 Moderate) and (b) UniProt classifies this variant as Pathogenic, associated with

Anaphylotoxin inactivator deficiency (PP5 Supporting) and (c) compound heterozygosity in *CPN1* may cause symptoms of HAE, due to CPN deficiency.

rs669 (*A2M*)

NM_000014.5:c.2998A>G (p.Ile1000Val) is located in exon 24 of the *A2M* gene (OMIM*103950). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the G allele has been detected in frequency 30.98% in the global population and 34.14% in the European non-Finnish population. In ClinVar database, the variant is characterized benign as an *A2M* common polymorphism, but as a risk factor for Alzheimer's disease.

Poller et al. (1992) located the SNP in the part of the gene encoding for the thiolester site of the protein, a functional domain, but they observed no difference of A2M serum levels for the 2 alleles[193]. The SNP has been mainly associated with increased risk of Alzheimer's[194] and Parkinson's disease[195]. In 2012, del Río-Espínola et al. performed a study in patients with acute ischemic stroke who were treated with t-PA in order to find genetic variations associated with hemorrhagic transformation (HT) and mortality rates after treatment and they present evidence for association of rs669 with HT[196].

rs5515 (*KLK1*)

NM_002257.4:c.230G>A (p.Arg77His) is located in exon 3 of *KLK1* gene (OMIM*147910). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 3.12% in the global population and 3.70% in the European non-Finnish population. It is remarkable that the frequency in African population is 10.2%. In ClinVar database, the variant is reported to decrease the urinary activity of kallikrein. The evidence is based on the literature, where Slim et al. (2002) revealed a major decrease in enzyme activity when arginine was replaced by histidine and their crystallographic data suggested that arginine is involved in substrate binding[197]. Azizi et al. (2005) observed 50 to 60% lower urinary kallikrein activity in heterozygous individuals in comparison with homozygous for arginine. Renal and hormonal adaptation to dietary changes in sodium and potassium were unaffected. However, in studies of brachial artery function, heterozygous individuals consistently exhibited an increase in wall shear stress and a paradoxical reduction in artery diameter and lumen. They concluded that the partial genetic deficiency in kallikrein activity is associated with an inward remodelling of the brachial artery, which is not adapted to a chronic increase in wall shear stress, indicating a new form of arterial dysfunction affecting 5-7% of white people[198].

rs3733402 (*KLKB1*)

NM_000892.5:c.428G>A (p.Ser143Asn) is located in exon 5 of *KLKB1* gene (OMIM*229000). This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 54.02% in the global population and 50.59% in the European non-Finnish population. In ClinVar database, there are conflicting interpretations of pathogenicity for this variant. It is characterized once pathogenic and by another submitter benign for prekallikrein deficiency.

The apple domains of KK bind to the C-terminal domain of HK. The exon 5 of the gene encodes for part of the apple domain 2 (A2) of the heavy chain. Katsuda et al. reported three Japanese patients with plasma pKK deficiency carrying rs3733402 in compound heterozygous state with rs121964952, both in exon 5 and they concluded that the binding capacity of A2 on HK was reduced[199].

Biswas et al. (2016) measured active plasma renin in 1,180 individuals and performed the genotyping of these subjects which revealed that the carriers of the functional variants of *KLKB1* (rs3733402) and *F12* (rs1801020) had a significant association with reduced levels of active plasma renin because they disrupted the cascade of enzymatic events, influencing the conversion of zymogen prorenin to renin[200]. The study suggests that the mutated factor XIIa had reduced ability to activate mutated pKK to KK. As a result KK had reduced efficacy in converting prorenin to renin. Gittleman et al. (2016) studied two *PRCP* and two *KLKB1* SNPs in 2,243 individuals with cardiovascular disease (CVD). They found that the G allele of *KLKB1* rs3733402 was associated with reduced history of CVD[201].

Concerning C1-INH-HAE patients, Gianni et al. (2017) studied 249 C1-INH-HAE patients from 114 European families in order to explore possible associations of carrying the variant rs3733402 with C1-INH-HAE clinical features, combined or not with carrying the *F12* rs1801020. Carriers of the G allele of the *KLKB1*-rs3733402 exhibited a significantly delayed disease onset, depending on the zygocity status, while carriers of both the *KLKB1* and the *F12* polymorphisms displayed an 8.8-year delay in disease onset and a 64% lower probability of needing LTP.

rs72550870 (*MASP2*)

NM_006610.4:c.359A>G (p.Asp120Gly) is located in exon 2 of *MASP2* gene (OMIM*605102). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the G allele has been detected in frequency 2.14% in the global population and 3,23% in the European non-Finnish population. In ClinVar database, there are conflicting interpretations of pathogenicity of this variant. It is characterized pathogenic and likely pathogenic for MASP2 deficiency and benign with no additional information concerning the condition or the carrier.

Stengaard-Pedersen et al. (2003) studied a patient presenting frequent infections and chronic inflammatory disease and they identified MASP2 deficiency. The deficiency was caused by homozygosity for p.Asp120Gly (rs72550870), while members of his family were heterozygous and they were presenting only partial deficiency[202]. However, García-Laorden et al (2006) suggested that MASP2 deficiency presented low clinical penetrance as they have also detected homozygosity for p.Asp120Gly in a patient who had no similar symptoms of chronic inflammatory disease[203]. Totally, according to Sokolowska et al., up to 2015, homozygosity for the p.Asp120Gly mutation had been found in eight patients suffering from various diseases and six healthy controls[204] and Fu et al (2016) in a meta-analysis found no association between the functional polymorphism rs72550870 and infectious diseases[205].

St Swierzko et al. (2009) concluded that heterozygosity for this allele significantly influenced the protein concentration, but not the lectin pathway of complement activity (MBL-MASP-2 complex activity)[206]. Despite the fact that different studies found no clear disease association with low levels of MASP2, they all agree to the fact that carrying this allele significantly reduces MASP2 concentration.

rs56378716 (*MPO*)

NM_000250.2:c.752T>C (p.Met251Thr) is located in exon 3 of *MPO* gene (OMIM*606989). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious according to SIFT algorithm, but as benign according to PolyPhen. Among approximately 120,000 exomes of the GnomAD, the C allele has been detected in frequency 1.02% in the global population and 1.35% in the European non-Finnish population.

In ClinVar database, the SNP is evaluated as pathogenic for myeloperoxidase deficiency, based on a publication by Romano et al. (1997)[207]. However, the article describes a 5-year old girl and her father presented with MPO deficiency caused by a 14-bp deletion within exon 9. Although the father is compound heterozygote for p.Met251Thr and the deletion and the two individuals appear with a different mRNA splicing, Romano et al. does not conclude that there is an effect of the SNP to the splicing. In fact, they refer to another regulatory mutation in mother's DNA. The latest evaluations in ClinVar suggest that the variant is of uncertain significance in favor of likely pathogenic, because the majority patients with myeloperoxidase deficiency are clinically asymptomatic except if they are also diabetic and based on Romano et al (2015)[207] and on Marchetti et al. who identified an individual with myeloperoxidase deficiency, harboring a compound heterozygote for c.752T>C and c.1705C>T[208].

rs2227564 (*PLAU*)

NM_002658.6:c.422T>C (p.Leu141Pro) is located in exon 4 of *PLAU* gene (OMIM*191840). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the C allele has been detected in frequency

74.83% in the global population and 76.39% in the European non-Finnish population. In ClinVar database, the SNP is characterized by one submitter benign with no additional information concerning the condition or the carrier, but is also evaluated as a risk factor for late-onset Alzheimer's disease (LOAD), based on literature. More precisely, Finckh et al. (2003) suggested that the C allele (Pro141) was a recessive risk allele for LOAD while the T allele (Leu141) was conferring protection[209]. On the contrary, Ertekin-Taner et al. (2005) found that the CT and TT genotypes were associated with LOAD[210].

As a consequence of contradictory results, the association between rs2227564 and Alzheimer's disease (AD) risk has been widely reported across different ethnic populations. The meta-analysis by Wu et al. (2013) showed that T allele of rs2227564 polymorphism in PLAU gene could increase the effects on risk of AD[211].

rs28929474, rs17580, rs121912714 (*SERPINA1*)

Deficiency of alpha-1-antitrypsin (A1AT, OMIM*613490) is primarily associated with the risk of emphysema and liver disease. The A1AT locus is pleiomorphic, with approximately 75 alleles identified. The A1AT alleles can be conveniently classified as "normal" (alleles coding for A1AT proteins present in normal amount and with normal function) and "at risk" (alleles that place the individual at risk for disease). Crystal (1989) listed by sequencing 10 "normal" A1AT alleles and divided "at risk" alleles into "deficiency" alleles and "null" alleles. He stated that except for the rare Pittsburgh allele, which is associated with a bleeding disorder, only those phenotypes comprising 2 "at risk" alleles place the individual at risk for development of disease[212].

rs28929474: NM_001127700.2:c.1096G>A (p.Glu366Lys), historically reported as p.Glu342Lys and commonly known as the Z allele or PI*Z represents the most frequent allele leading to a high risk of emphysema (and liver disease) in homozygous state. It is widely reported in the literature, but it has not been previously associated, to our knowledge, with C1-INH-HAE. This variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of GnomAD, the A allele has been detected in frequency 1.11% in the global population and 1.84% in the European non-Finnish population. The allele reaches polymorphic frequencies in Caucasians and is rare or absent in Asian and African population. It is present in homozygous state in 0.015% (19/125,680). In ClinVar database the variant is characterized by multiple submitters with no conflicts pathogenic or risk factor for A1AT deficiency.

Functional studies demonstrate that this variant is five times less effective than the normal M allele as an inhibitor of neutrophil elastase, it forms polymers in the lung that can be chemoattractants for neutrophils, thereby increasing inflammation[213,214,215], and alters the global structural dynamics of A1AT[216]. When found in homozygous state, the Z allele is responsible for 95% of all clinical cases of A1AT deficiency and in compound heterozygosity with the S allele it is associated with 20-50% risk for emphysema[217,218].

rs17580: NM_001127700.2:c.863A>T (p.Glu288Val), historically reported as p.Glu264Val and commonly known as the S allele or PI*S allele, is a common variant associated with partial A1AT deficiency. It has not been previously associated with C1-INH-HAE. This variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the T allele has been detected in frequency 2.33% in the global population and 3.65% in the European non-Finnish population. It is present in homozygous state in 0.1% (131/125,740). In ClinVar database the variant is characterized by multiple submitters pathogenic or risk factor for A1AT deficiency and once as VUS for chronic obstructive pulmonary disease (COPD).

Functional studies by Curiel et al. (1989) has shown that the S allele cause reduced cellular secretion of A1AT because the newly synthesized S-type A1AT protein is degraded intracellularly before secretion[219]. The variant is not usually associated with a high risk for liver disease, but it is reported to confer a risk for lung disease (emphysema, COPD) when it is present in compound heterozygosity with the Z allele [217,218,220,221,222]. Because of the high frequency of the S allele such compound heterozygotes are relatively frequent. Individuals who are homozygous for the S variant do not appear to be at risk for lung disease[223].

rs121912714: NM_001127700.2:c.839A>T (p.Asp280Val), also known as p.Asp256Val, PI*Lowell allele, PI*Duarte allele, or PI*Null Cardiff allele has been associated in the literature with A1AT deficiency. It has not been previously associated with C1-INH-HAE. This rare variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the T allele has been detected in frequency 0.047% in the global population and 0.055% in the European non-Finnish population. No homozygous individuals have been detected and the variant is absent in Asian and African population.

Experimental studies have shown that this missense change disrupts protein folding and reduces secretion of A1AT due to intracellular degradation[224,225,226]. It has been observed to segregate with A1AT deficiency in families[224,227] and has been observed in individuals affected with A1AT deficiency (A1ATD)[228,229,230,231,232,233].

rs6092 (*SERPINE1*)

NM_000602.4:c.43G>A (p.Ala15Thr) is located in exon 2 of *SERPINE1* gene (OMIM*173360). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 9.52% in the global population and 11.31% in the European non-Finnish population. In ClinVar database, the SNP is characterized likely benign for plasminogen activator inhibitor type 1 (PAI-1) deficiency.

Complete PAI-1 deficiency is inherited in an autosomal recessive manner. However, in a Chinese patient with PAI-1 deficiency, Zhang et al. (2005)[234] identified this variant in the

signal peptide and performed family segregation and functional studies. More precisely, the patient had bleeding episodes and decreased activity and antigen levels of PAI-1 (about 10%). His father was heterozygous for the p.Ala15Thr mutation; he had moderately reduced PAI-1 antigen and activity, but no bleeding history. The functional studies showed PAI-1 activity at about 70%. The authors suggested that the change from a hydrophobic non-polar amino acid (Ala) to a hydrophilic polar amino acid (Thr) in the hydrophobic core region (h-region) of the signal peptide of the protein may perturb its function.

rs5743708 (*TLR2*)

NM_003264.5:c.2258G>A (p.Arg753Gln) is located in exon 3 of *TLR2* gene (OMIM*603028). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 1.74% in the global population and 2.81% in the European non-Finnish population. In ClinVar database, the SNP is characterized a risk factor for mycobacterium tuberculosis based on different references from the literature.

Xiong et al. (2012) studied the impact of the p.Arg753Gln polymorphism on TLR2 expression, hetero-dimerization with TLR6, tyrosine phosphorylation, and recruitment of myeloid differentiation primary response protein (MyD) 88 and MyD88 adapter-like (Mal). They concluded that the polymorphism renders TLR2 signalling incompetent by impairing its tyrosine phosphorylation, dimerization with TLR6, and recruitment of Mal and MyD88[235].

rs28362944 (*SERPING1*)

NM_000062.3:c.-21T>C is located near the splice site before exon 2 of *SERPING1.* Among approximately 74,000 exomes of the GnomAD, the C allele has been detected in frequency 2.91% in the global population and 4.67% in the European non-Finnish population. In ClinVar database, the SNP is characterized benign for HAE type I.

After its first description by Verpy et al. (1996)[236], other research teams suggested that this variant in heterozygous state had probably no effect on the translation of the protein[111,237] and that the presence of the SNP has no correlation with the biochemical values of C1-INH function and the clinical severity score [114]. Functional studies by Duponchel et al. proposed c.-21T>C as a modifier of disease severity as they found that the variant yields low but significant levels of exon 2 skipping in transfected cells. Therefore, this allele may contribute, at the RNA level, to more severe forms of angioedema[238].

Later, the variant was reported twice in homozygous state. The first case was a 27-year-old female patient with recurrent abdominal edema of unknown etiology. She presented a C1-INH deficiency associated with a normal C1q level which suggested HAE, but C4 level was not reduced. Subsequently, C1-INH level normalized and disease symptoms did not recur during the 2-year-long follow-up. DNA analysis revealed homozygous g.566T>C (c.-21T>C). The authors suggested that the variant might become pathogenic in homozygous state[239]. In

the second case report, the variant was detected in a patient with clinical symptoms compatible with HAE, relatively late symptom onset (40 years), and reduced C1-INH activity. He carried no mutation responsible for the disease, except the homozygous variant g.566T>C (c.-21T>C). This patient was receiving LTP (danazol), and the frequency of edema attacks had been diminished with therapy. She had been also supplied with C1-INH concentrate and had responded well to the administration during angioedema attacks. The authors concluded that homozygosity for c.-21T>C in the *SERPING1* gene might be responsible for the disease and even if the frequency of heterozygous c.-21T>C was previously reported to be similar in patients with HAE and in healthy individuals, the variant could be associated with a more severe HAE form[240].

rs4926 (*SERPING1*)

NM_000062.3:c.1438G>A (p.Val480Met), historically reported as Val458Met or Val451Met, is located in exon 8 of *SERPING1*. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 22.20% in the global population and 27.17% in the European non-Finnish population. In ClinVar database, the SNP is characterized by multiple submitters benign for C1-INH-HAE type I. Gösswein et al. (2008) initially characterized pathogenic a rare missense mutation detected in C1-INH-HAE patient which resulted in the same amino acid substitution (c.1439T>G, p.Val480Gly)[241]. Functional studies by Cumming et al. (2003) found no detectable effect on protein stability, plasma levels or HAE disease expression. The function of mutated C1-INH was studied and shown to be unaffected. The authors did not exclude a consequence of the variant on other functions of C1-INH as a modulator of the coagulation and kinin release pathways[113].

Statistical analysis

Categorical variables were analyzed with Fisher's exact test. Normality of continuous variables was assessed with Kolmogorov-Smirnov test. Normally distributed data were analyzed with Student's t-test and one-way ANOVA as appropriate. Skewed data were analyzed with nonparametric methods (Mann-Whitney test or Kruskal-Wallis test as appropriate). Given the fact that our patient population consisted of correlated subjects (members of individual families), we implemented generalized estimating equations (GEE), an extension of the generalized linear model that accounts for the within-subject correlation. GEE was used to model the association of response variables (age at disease onset, need for long-term treatment, CALS severity) with explanatory variables (polymorphisms, sex e.t.c.). Age at disease onset and CALS severity score were modeled as continuous variables in linear GEE models and need for long-term treatment was entered as a binary variable in logistic GEE models. In all GEE models an unstructured correlation structure was used and the Quasi Likelihood Information Criterion (QIC) was used for model

selection. Data analysis was performed with SPSS 17.0 (IBM Corporation, NY, 2008). For all analyses, alpha was set at 0.05 (two-tailed).

V. ClinVar

This part of the study focused on the report and interpretation of *SERPING1* variants in public databases and in the literature. We searched the latest mutation update regarding *SERPING1* variants. More precisely, Ponard et al. (2019)[106] reported a list of 528 *SERPING1* variants complied from the literature and 120 novel variants. Subsequently, we searched the number of these mutations that have already been reported in ClinVar database and HAEdb (http://hae.enzim.hu/), a specific database used for many years by the HAE scientific community. Finally, we submitted the variants that we have detected in total in our laboratory in ClinVar database.

ClinVar was created at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) and provides a centralized, user-friendly, public open-access database which can help users in interpreting variants. Submitters to ClinVar include clinical testing laboratories, research entities, database curators, expert panels and practice guideline groups. Variants are collected from a variety of sources like clinical testing, literature-only evaluation, research, curation, or other. Different entities can enrich each entry with additional evidence for the same variant. The aggregation of data for variants or variant-disease pairs allows ClinVar to report when different submitters agree or disagree on the interpretation. In this way, it allows for variant conflict interrogation and resolution.

Other centralized databases of variation related to human disease existed before Clin-Var; databases like the Human Gene Mutation Database and OMIM focused on variants found in the literature and COSMIC for somatic variation. Gene-specific databases also exist, like Leiden Open Variation Database (LOVD). However, ClinVar is evolving and since the first public release (April 2013) has become a routine for many testing laboratories. As for November 2020, it contains more than 1,300,000 records for more than 32,000 genes, among which 1,134,618 are characterized using professional guidelines.

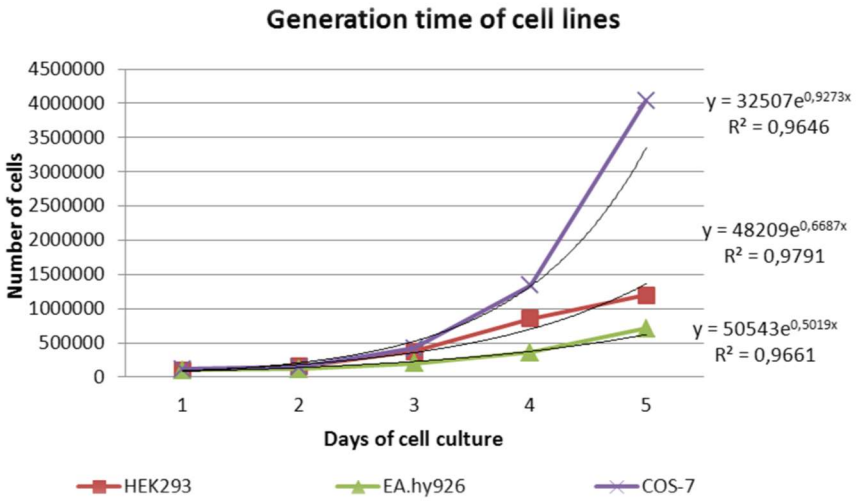
RESULTS

PART I: EXPRESSION OF BRADYKININ RECEPTORS (B1R, B2R)

I. Detection of B1R and B2R on endothelial cells using fluorescent recombinant proteins

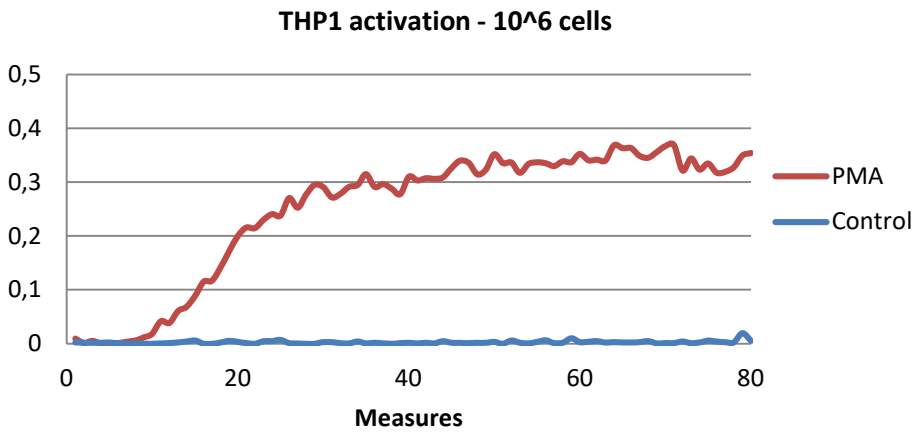
Before starting any experiments, the generation time of all cell lines was calculated in order to record the growth characteristics of the cells, control at any moment their manipulation and observe any changes to their life. The generation time or doubling time is the interval between the consecutive divisions of a cell, that is the time that it takes for a population of single-celled organisms to double its cell number. The exponential generation of cell lines is presented on Figure 21.

Figure 21. Exponential growing of (a) EA.hy926 cells (b) HEK293 cells and (c) COS-7 cells.



Moreover, a luminescence-based assay was performed for THP1 cells in order to evaluate the performance of the cell line. The cell line was activated normally in presence of PMA and the results are presented in Figure 22.

Figure 22. Activation of THP1 cells after incubation with 2 μg/ml PMA.



A. Production of Biotechnological ligands

The plasmid concentration after transformation and purification with HiSpeed Maxi Kit (Qiagen) was:

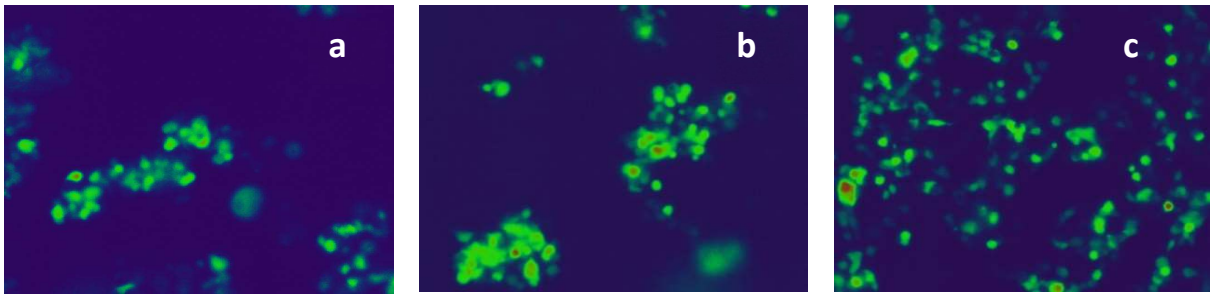
- EGFP- S4-P1 [EGFP- $(NG)_{15}$KRPPGFSPF] = 1,502 ng/µl (R 260/280= 2.00)
- EGFP- MK = 1,541 ng/µl (R 260/280= 2.01)

1. Cell transfection

a. Transient transfection of HEK293 cells

Transient transfection using PEI complexes was not successful. On the contrary, producer HEK293 cells transfected with TurboFect Transfection reagent (Invitrogen) when expressing the ligands for BK receptors were highly fluorescent. EGFP proteins are distributed in the cellular water. Before performing cell lysis, transgene expression was analysed by observing cells under the microscope, using blue light (GFP emission at 460-500 nm) (Figure 23).

Figure 23. Epifluorescence of HEK293 cells after being transfected by plasmids encoding for fluorescent ligands of BK receptors. (a) EGFP (b) EGFP-MK (c) EGFP-S4-P1.

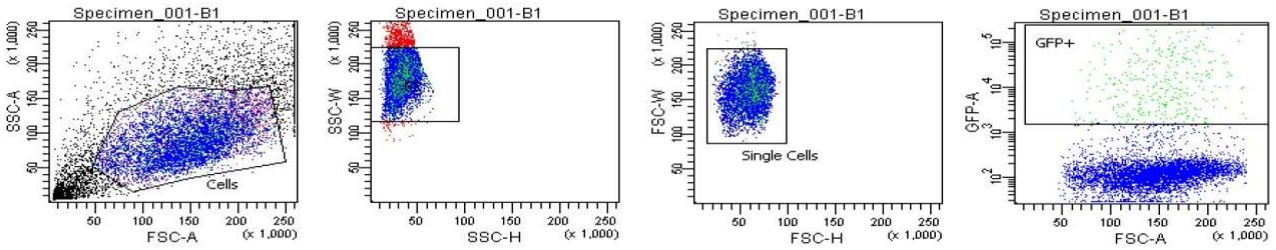


b. Stable transfection using TurboFect Transfection Reagent

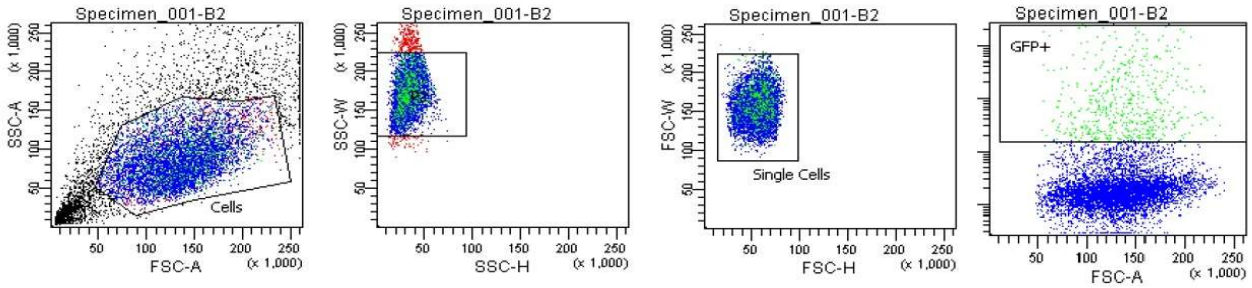
The most important point of this strategy was the selection of a parental cell line with high expression of the reporter gene. Cells with the highest expression of EGFP ligands (top 3-6%) were sorted by FACS on day 30 after transfection and selection in growth media containing geneticin (Figure 24). To select an EGFP high-expression parental cell line, the cells from the FACS were individually seeded in 96-well plates and subcultured when in confluence. The FACS sorted cells and the cells selected after cloning by serial dilution were observed under the microscope using blue light for the emission of GFP protein. The resulting clones were not homogenous as the cells were losing their ability to express the fluorescent ligands after a certain number of divisions.

Figure 24. Cell sorting by FACS. Cells with the highest expression of fluorescent ligands were gated and individually separated in plates. (a) EGFP-S4-P1, 3.5% of total single cells. (b) EGFP-MK, 5.5% of total single cells.

(a)



(b)



2. Protein analysis

The homogeneity and molecular masses of the ligands have been estimated by anti-GFP immunoblotting of the cell lysates with the monoclonal antibody JL8 (Figure 25). The concentration of the proteins in the cell lysates were determined by ImageLab BIORAD (semi-quantitative method) and are presented on Table 6.

Figure 25. Western Blot anti-GFP for B1R and B2R ligands. Control for semi- quantification: purified GFP protein. Positive control cell lysate from cells transfected with plasmid coding for EGFP non terminally extended.

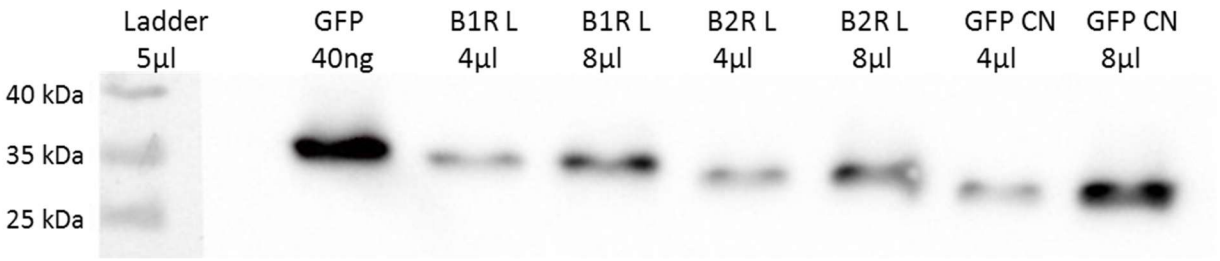


Table 6. Calculation of ligands' concentration in cell lysates, after semi-quantification using as control GFP purified protein.

Protein	Quantity on electrophoresis	Intensity	Concentration (ng/μl)	Average
purified GFP	40 ng	$14.4 * 10^6$	-	-
B1R Ligand EGFP-S4P1	4 μl	$2.33 * 10^6$	1.62	1.45 ng/μl
	8 μl	$5.38 * 10^6$	1.87	
B2R Ligand EGFP-MK	4 μl	$2.25 * 10^6$	1.56	1.71 ng/μl
	8 μl	$5.35 * 10^6$	1.86	
EGFP in cell lysate	4 μl	$2.027 * 10^6$	1.41	2.39 ng/μl
	8 μl	$9.68 * 10^6$	3.36	

B. Expression of bradykinin receptors

1. Immunofluorescence using fluorescent ligands

Charest Morin et al.[79] applied the lysate of producer cells to other HEK293a recipient cells transiently expressing hB1R-FLAG in order to test the most competent ligand. EGFP-S4-P1 (final concentration 32-74 nM) produced the brightest labeling of B1R-FLAG-expressing cells. Moreover, he applied the ligands on human Umbilical Artery Smooth Muscle Cells (hUA-SMCs) but epifluorescence was difficult to detect because of the low concentration of the targeted receptor on the cell membrane. EGFP-S4-P1 did not support cell membrane B1R imaging in cytokine-treated hUA-SMCs and evidence based on [3H]Lys-*des*Arg^9-BK binding shows that the latter cells express approximately 2.4 fmol of B1Rs per cm^2 of culture surface, whereas recombinant B1R-FLAG is expressed at approximately 9.5 fmol/cm^2 in transfected HEK293a cells[79]. In this experiment, the lysate containing EGFP-S4-P1 ligand was applied to EA.hy926 cells treated with cytokines to naturally express B1R. EGFP-S4-P1 ligand failed to label the intact receptors.

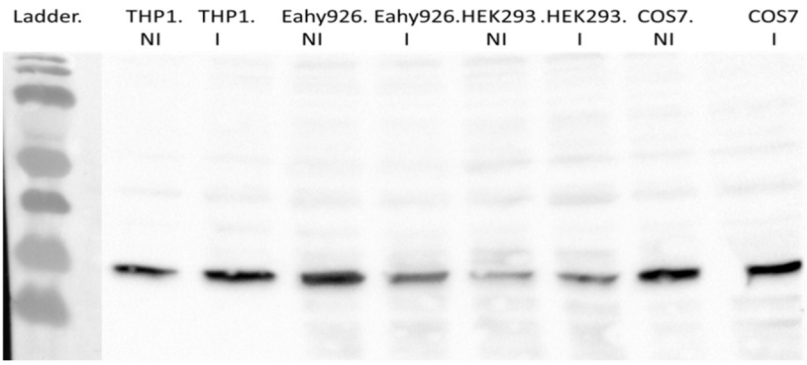
II. Detection of BK receptors using antibodies

A. Analysis of BK receptor expression by anti-B1R and anti-B2R Immunoblots

The expression of B2R and B1R was tested on the cell lysates of EA.hy926, HEK293, COS-7, THP1 by performing Western Blots (WBs), using anti-B1R and anti-B2R produced in rabbits and anti-rabbit IgG peroxidase labeled antibodies. The results of immunoblots and the quantification of the bands are presented on Figure 26. EA.hy926 were expected to express constitutively B2R and after induction with TNFα, B1R. HEK293 and COS-7 cells were expected to express neither B2R nor B1R, while for THP1 it was interesting to investigate the presence of the receptors. For B1R, the size of the protein acquired (~30 kDa) does not correspond at the expected size of the receptor (54 kDa). For B2R, the size of the protein indicated by the manufacturer is about 78 kDa, although the size of the receptor is also 55 kDa.

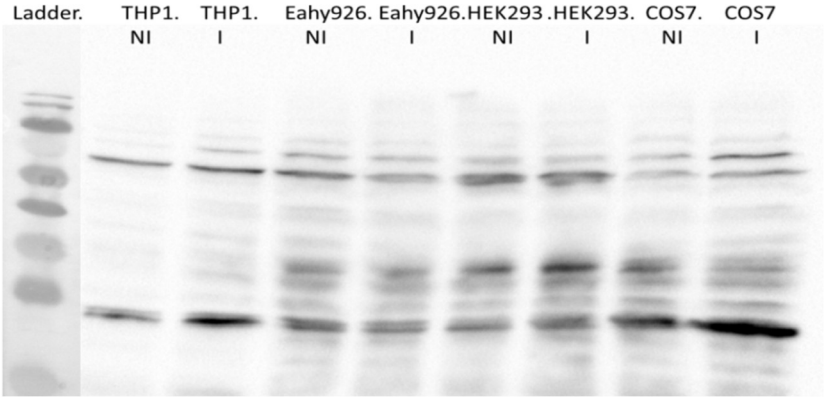
Figure 26. Western Blots and band quantification of (a) anti-B1R, anti-rabbit IgG-HRP and (b) anti-B2R, anti-rabbit IgG-HRP.

(a)



B1R L	Mol. Wt. (KDa)	Rf	Volume (Int)	Band %	Lane %
Lane 1- THP1	~35	0.50	11 917 269	100	75.007831
Lane 2- THP1+TNFα	~35	0.52	18 703 944	100	64.64758
Lane 3- EA.hy926	~35	0.53	19 832 832	100	64.197152
Lane 4- EA.hy926 +TNFα	~35	0.52	10 291 072	100	46.164933
Lane 5- HEK293	~35	0.52	5 319 296	100	33.878035
Lane 6- HEK293+TNFα	~35	0.53	8 559 872	100	47.345933
Lane 7- COS-7	~35	0.51	1 9865 088	100	70.41465
Lane 8- COS-7+TNFα	~35	0.50	18 289 600	100	63.111738

(b)



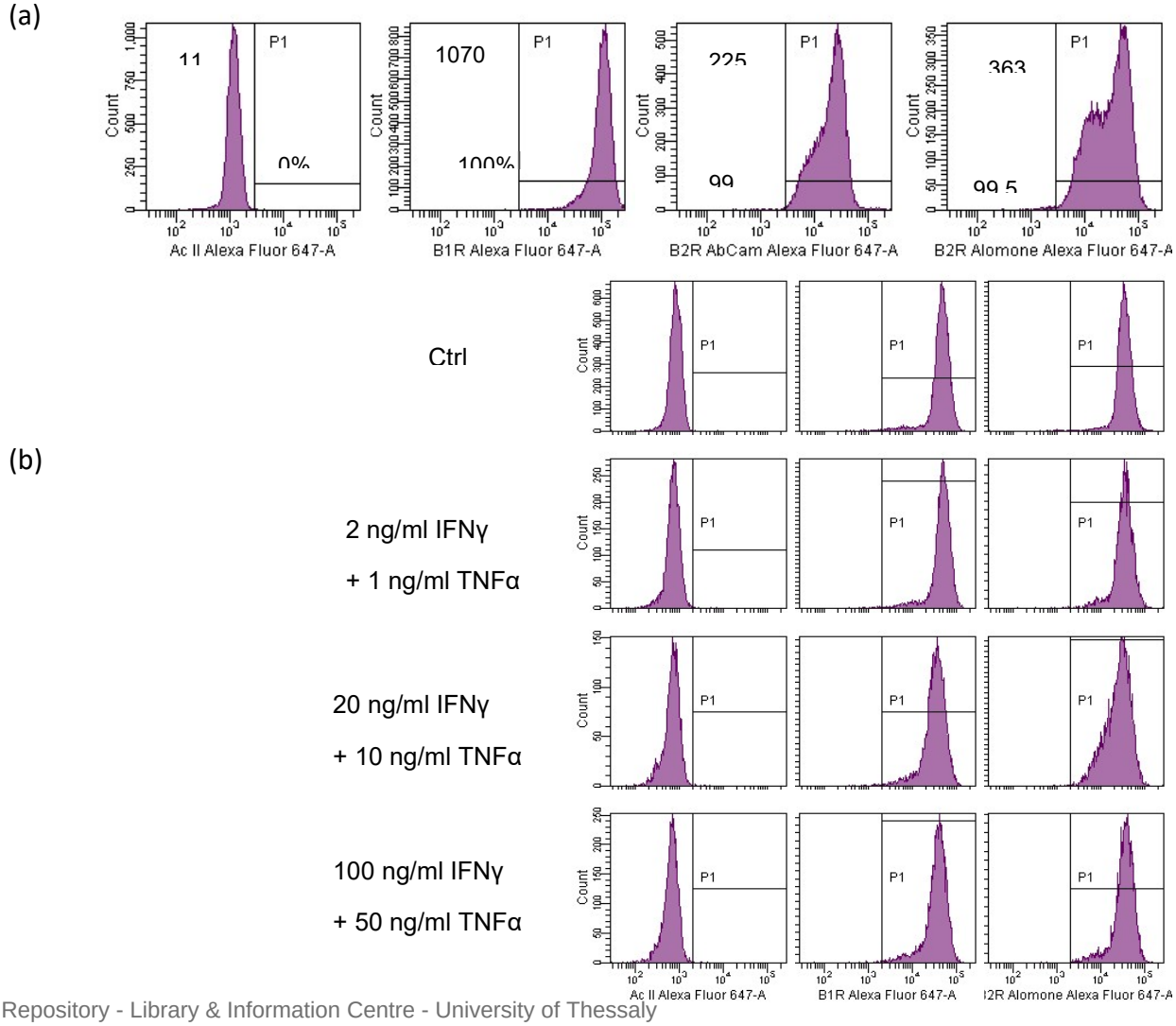
B2R L	Mol. Wt. (KDa)	Rf	Volume (Int)	Band %	Lane %
Lane 1- THP1	~75	0.13	5 258 316	38.25	25.34
Lane 2- THP1+TNFα	~75	0.15	7 152 982	32.01	20.18
Lane 3- EA.hy926	~75	0.16	7 972 260	33.61	20.13
Lane 4- EA.hy926 +TNFα	~75	0.17	5 200 635	30.06	16.00
Lane 5- HEK293	~75	0.17	9 633 227	41.44	24.30
Lane 6- HEK293+TNFα	~75	0.16	9 814 237	32.86	20.18
Lane 7- COS-7	~75	0.16	3 068 647	11.52	6.69
Lane 8- COS-7+TNFα	~75	0.16	5 997 388	11.03	6.96

B. Analysis of kinin receptors by flow cytometry

In the first analysis [Figure 27 (a)], difference between staining with monoclonal or polyclonal anti-B2R antibodies and competence of anti-B1R antibody were tested, while the constitutive expression of both receptors was also investigated. Both anti-B2R antibodies are at the same level competent. Nearly 100% of human monocytes from THP1 cell line are expressing B1R and B2R. However, as the epitopes for all antibodies are intracellular and the cell membrane is treated to be permeable, we cannot conclude if the receptors are present on the cell membrane or stocked in the cell.

In the second analysis [Figure 27 (b)], a standard curve was produced using different concentrations of cytokines in order to test the induction of B1R. We cannot detect any differences between samples. This could be a result of (a) non-specific fixation of the antibody on a constitutively expressed protein, (b) fixation of the antibody on the molecules of receptor which are stocked in different particles in the cells (c) need for different conditions in order to induce B1R on THP1 cells. The results of flow cytometry verify the results of Western Blots, as the antibodies used for both techniques are the same.

Figure 27. Flow cytometry analysis of (a) B1R and B2R expression by using different antibodies (b) B1R expression, after induction of the expression by treating with different concentrations of cytokines.



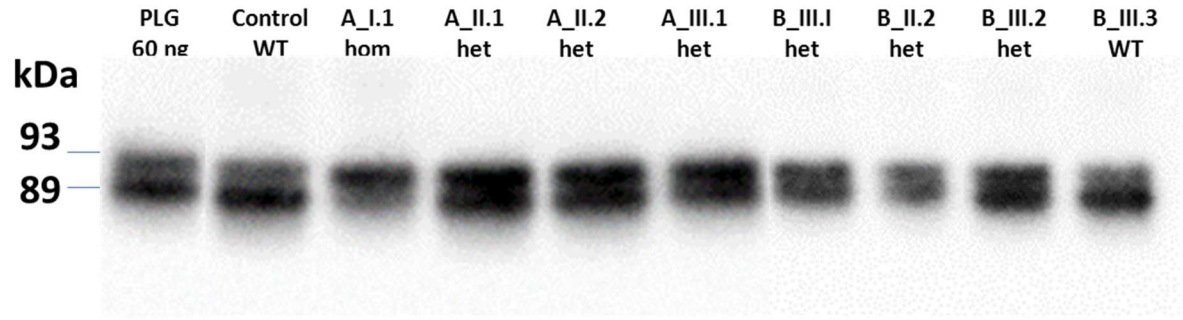
PART II: PLASMINOGEN GLYCOFORMS ALTERATION AND SUSCEPTIBILITY TO ACTIVATION ASSOCIATED WITH THE MISSENSE VARIANT p.Lys330Glu IN HAE-PLG PATIENTS

Dewald (2018)[149] observed altered PLG patterns in two heterozygous patients for the p.Lys330Glu variant and suggested that the variant might have some quantitative as well as qualitative effect on PLG glycosylation. In our experiments, we detected a reversal of the glycosylation pattern in the homozygous patient with ~60% of PLG type I glycoform and around 40% of PLG type II form, while the heterozygous subjects present two bands of approximately equal intensity. Non carrier individuals display around 40% of PLG type I glycoform, as observed for the control sample. In addition, we confirm that PLG type I displays a slight molecular weight decrease from 93 KDa to 92 KDa. The results are presented in Figure 28.

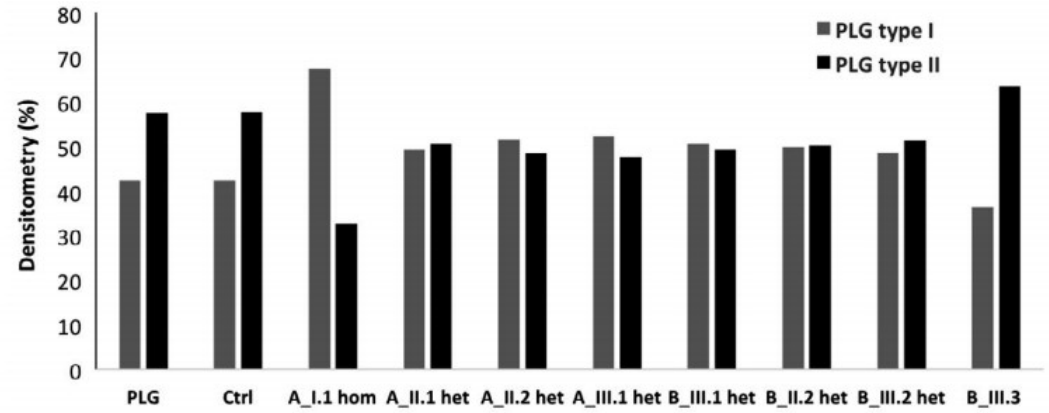
As both PLG glycoforms are known to exhibit functional differences, including the susceptibility to activation, we investigated PLG activation using a chromogenic substrate (S-2403, Chromogenix) and three different activators, tPA, streptokinase (SK) and urokinase (UK). Homozygous (n=1) or heterozygous (n=8) carriers of the PLG p.Lys330Glu display a significantly high susceptibility to activation by SK and UK ($p<0.002$ and $p<0.002$), compared to healthy controls (n=6, Mann Whitney test for non-parametric values and $\alpha<0.05$; Figure 28B).

Figure 28. A. Anti-plasminogen immunoblots. Plasma samples from homozygous and heterozygous patients (symptomatic AI.1, AII.2 and BIII.2), asymptomatic (AII.1, AIII.1, BIII.1, BII.2 and BIII.3) carriers, purified PLG (lane 1) and healthy control (lane 2) have been submitted to SDS-PAGE under nonreducing conditions followed by anti-plasminogen immunoblot (Goat anti-human-PLG, Affinity Biological). Lanes identification is linked to Figure 16. Molecular weights are indicated. B PLG signal is assessed using densitometric data. C. Plasminogen activation of plasma samples in the presence of three activators (tPA, SK and UK). tPA, tissue-plasminogen activator; SK, streptokinase; **p<0.01, ***p<0.001. ([Adapted by Parsopoulou et al. (2020)[160]]

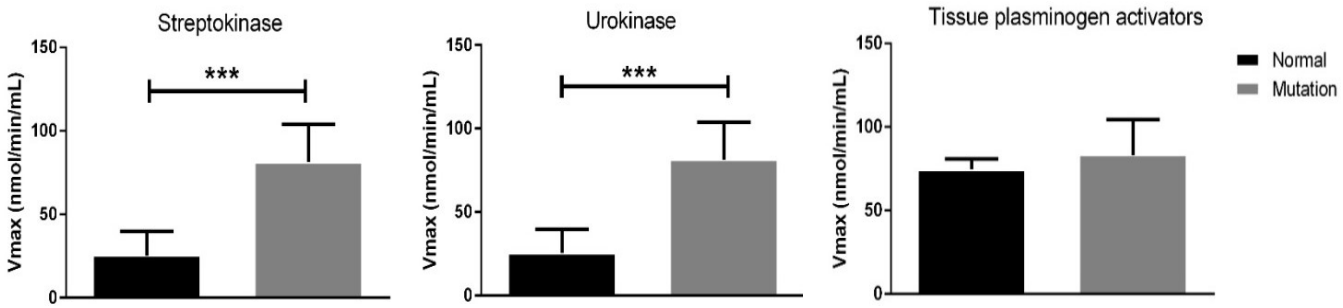
(A)



(B)



(C)



PART III: GENETIC BIOMARKERS OF THE SEVERITY OF C1-INH-HAE

I. Rare variants affecting the severity of the disease in C1-INH HAE patients

187 rare variants (MAF ≤1%) in 52 out of 54 genes included in our NGS platform were detected, among which 19 novel that are presented in the table below.

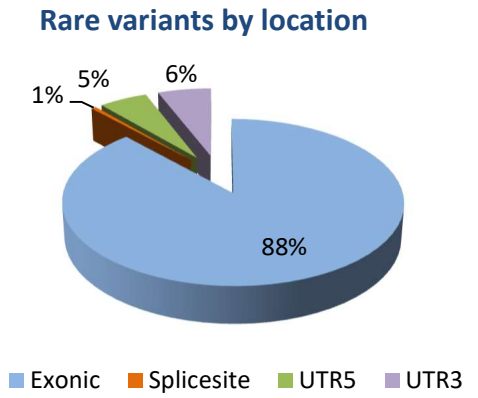
Table 7. Novel rare variants detected in C1-INH-HAE patients.

Gene	Transcript	Location	Nucleotide change	Amino acid change
ACE	NM_000789.3	exonic	c.2677G>A	p.Asp893Asn
XPNPEP2	NM_003399.5	exonic	c.604C>G	p.Gln202Glu
DPP4	NM_001935.3	exonic	c.1393G>T	p.Ala465Ser
DPP4	NM_001935.3	exonic	c.942G>T	p.Gln314His
DPP4	NM_001935.3	exonic	c.248A>G	p.Tyr83Cys
BDKRB2	NM_000623.3	exonic	c.1006G>A	p.Gly336Ser
KNG1	NM_001102416.2	3'UTR	c.*35T>C	N/A
KLKB1	NM_000892.3	exonic	c.7T>A	p.Leu3Ile
KLK3	NM_001648.2	exonic	c.515A>G	p.Gln172Arg
PLAT	NM_000930.3	exonic	c.769C>A	p.Gln257Lys
TPSG1	NM_012467.3	exonic	c.312C>A	p.Phe104Leu
TAC1	NM_003182.2	exonic	c.8T>G	p.Ile3Ser
A2M	NM_000014.4	exonic	c.781C>A	p.Pro261Thr
C1QBP	NM_001212.3	exonic	c.41C>G	p.Ser14Cys
TLR2	NM_003264.3	exonic	c.2341G>T	p.Ala781Ser
HSP90AA1	NM_001017963.2	exonic	c.1930A>G	p.Ile644Val
KRT1	NM_006121.3	3'UTR	c.*17T>G	N/A
KRT1	NM_006121.3	exonic	c.173C>G	p.Ala58Gly
PROC	NM_000312.3	exonic	c.554G>A	p.Arg185Lys

We detected no variants in *XPNPEP1* and *KLK1*. 165 out of 187 variants were located in exonic regions, 1 was splice site and 21 were located in UTRs (10 5'UTR, 11 3'UTR). Moreover, 161/165 exonic variants were missense, 2 nonsense and 2 frameshift variants. The automated ACMG criteria applied by Varsome.com were used in order to classify these variants. Among 187 variants, 32 were benign, 94 likely benign, 4 likely pathogenic, 3 pathogenic and 54 VUS. The classification of these variants according to their location, their effect on the protein and their pathogenicity are presented in Figure 29. The detailed information about all 187 variants is presented in Appendix III. Information about the 3 pathogenic and 4 likely pathogenic variants is presented below. In addition, information about *F12* rs369991760 and *F13B* rs145637157 are exceptionally presented in this section.

Figure 29. Classification of all detected rare variants according to their location (A), their effect on the protein (B) and their pathogenicity according to ACMG/AMP Standards and Guidelines (C).

A.



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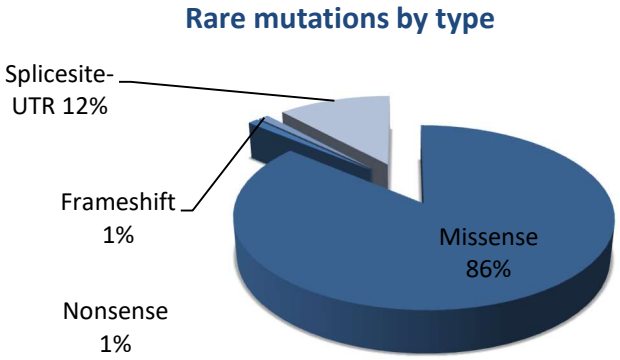
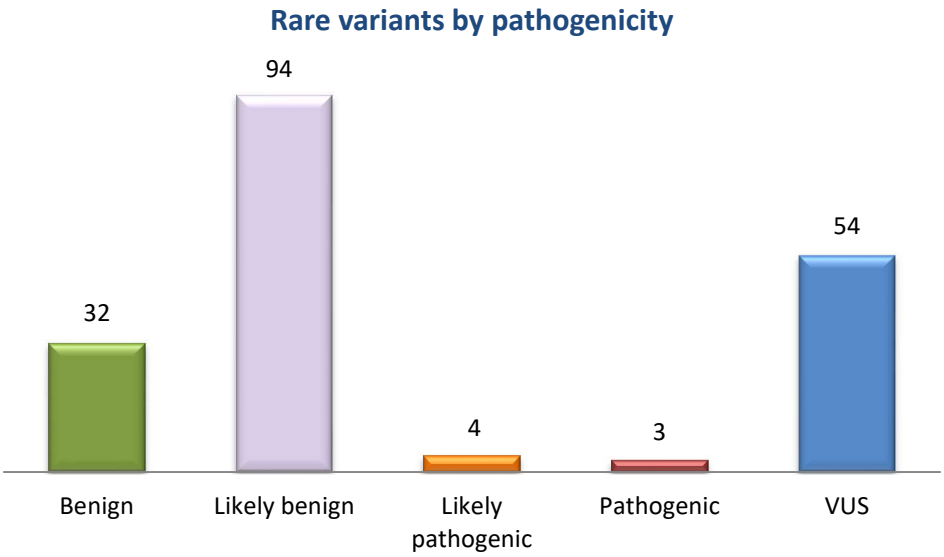


C.



rs369991760 (*F12*)

The NM_000505.3:c.-8C>T has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected, according to GnomAD, in low frequency in the worldwide (0.043%) and European non-Finnish (0.078%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Bulgarian male patient with C1-INH-HAE type I. In ClinVar database, the variant is characterized by one submitter likely benign for FXII-deficiency and for HAE.

The variant is located in a putative binding site for the Hepatocyte Nuclear Factor 4-α transcription factor (HNF4α). HNF4α is a liver-enriched transcription factor that has a crucial role in the expression of blood coagulation factors. Sabater-Lleal et al. (2011)[242] suggested

that this mutation, as well as c.-13C>T, cause a significant reduction in expression levels of FXII as they modify the transcription binding site and as a consequence, they alter the binding affinity of HNF4a. Moreover, Sabater-Lleal et al. indicate that c.-8C>T mutation in cis-combination with 46C/T polymorphism (rs1801020) lead to a FXII-deficient phenotype (<1%) due to a synergistic effect and increase the risk of thrombosis.

rs145637157 (*F13B*)

The NM_001994.2:c.709G>A (p.Val237Ile), historically known as Val217Ile, is located in exon 5 of *F13B* gene (OMIM*134580) encoding for FXIII B subunit. The variant has been detected in low frequency in the global (0.023%) and European non-Finnish (0.04%) population. It is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively and it has been previously investigated in the literature in association with FXIII deficiency. In our laboratory, it was detected in 1 Bulgarian male patient with C1-INH-HAE Type I. However, this patient carries another mutation on *F13B* (NM_001994.2:c.1318G>A, p.Glu440Lys). Mutations in homozygous or compound heterozygous state in *F13B* have already been described as causative for FXIII deficiency. In ClinVar Database, there are no clinical assertions for this variant.

The variant is located in the fourth Sushi domain which is essential for FXIII-B homodimer assembly. Ivaskevicius et al. (2010) reported that p.Val237Ile does not reflect a significant change in FXIII size, polarity or charge and conclude that there is a high possibility that Ile237 might represent a polymorphism[243]. Thomas et al (2015), after investigating the molecular pathological mechanism as well as the heterozygous expression phenotype for this variant, they agree that p.Val237Ile is pathologically neutral in terms of secretion, structural and functional stability of FXIII. They observed no effect on heterotetramer assembly, while low degree of sequence and structural conservation further supports the neutral status of this mutation. They also characterize the variant as a functional polymorphism rather than true causative mutation[244], which comes to an agreement with Ivaskevicius et al.

Table 8. Pathogenic and likely pathogenic variants, according to ACMG Standards and Guidelines.

Gene	Location	Nucleotide change	Amino acid change	Rs	ACMG classification and criteria
F11	Exonic	c.1556G>A	p.Trp519Ter	rs201007090	Pathogenic Criteria: PVS1, PM2, PP3, PP5
PLG	Exonic	c.2251G>A	p.Gly751Arg	rs121918033	Pathogenic Criteria: PS1, PM1, PM2, PP3, PP5, BP1
MPO	Splicesite	c.2031-2A>C		rs35897051	Pathogenic Criteria: PVS1, PM2, PP3, PP5
F11	Exonic	c.281C>T	p.Ala94Val	rs367856671	Likely pathogenic Criteria: PM1, PM2, PP2, PP3
F2	Exonic	c.1628G>A	p.Arg543His	rs143064939	Likely pathogenic Criteria: PM1, PM2, PP2, PP3
MME	Exonic	c.1390G>A	p.Glu464Lys	rs200864897	Likely pathogenic Criteria: PM1, PM2, PP2, PP3
MME	Exonic	c.335A>G	p.Asp112Gly	rs1409620468	Likely pathogenic Criteria: PM1, PM2, PP2, PP3

rs201007090 (*F11*)

The NM_000128.3:c.1556G>A (p.Trp519Ter) has not been previously associated with C1-INH-HAE. This nonsense variant is located in exon 13 of *F11* gene (OMIM*264900) and reduces the protein length (FXI) by the formation of a termination codon in the position 519. The predicted truncated protein is missing 107aa. Among approximately 120,000 exomes of the GnomAD the variant has been detected in 0.0032% frequency (8/251,434 alleles) in the global population. In the European non-Finnish population the variant has been detected once in one Bulgarian male patient. Among 233 C1-INH-HAE patients of this study, it has been detected in one Bulgarian male patient with C1-INH-HAE type I. In ClinVar Database, the variant is characterized by 2 different submitters pathogenic/likely pathogenic for hereditary factor XI deficiency and pathogenic for abnormal bleeding.

Factor XI (FXI) is a haemostatic plasma glycoprotein circulating as a zymogen of the serine protease FXIa in non-covalent interaction with cofactor HK. FXIa is required for the activation of factor IX (FIX) to maintain thrombin production during the coagulation process. FXI deficiency shows a high variability in clinical phenotype. To date, many allele variants have

been shown to cause this bleeding disorder. Homozygotes or compound heterozygotes typically show severe FXI deficiency, whereas heterozygotes show partial or mild deficiency. Iijima et al. (2000) first reported a 65-year old man, carrier of this mutation with severe FXI deficiency[245], but in a later publication Berber et al. (2010) detected compound heterozygosity (p.Trp519* and p.Gly217Ser) in this individual[246]. Tiscia et al. (2017), by investigating 2 different families, confirmed that this mutation causes severe FXI deficiency only when found in compound heterozygosity with another missense mutation in *F11*[247].

rs121918033 (*PLG*)

The NM_000301.3:c.2251G>A (p.Gly751Arg) is located in exon 18 of *PLG* gene (OMIM* 173350). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms. Among approximately 120,000 exomes of the GnomAD, the A allele has been detected in frequency 0.0016% (4/251,440 alleles) in the global population and 0.0026% in the European non-Finnish population (3/113,726 alleles). Among 233 C1-INH-HAE patients of this study, it has been detected in 2 female patients, members of a Hungarian family, with C1-INH-HAE type I. In ClinVar Database, the variant is characterized pathogenic for dysplasminogenemia, based on evidence by Higuchi et al. (1998). More specifically, Higuchi et al. described a male carrier of this variant with plasminogen Kanagawa-I, a dysplasminogen. He did not have any thrombotic episodes but his plasma plasminogen activity was 51.4%. His father and grandfather were carriers of the same mutation in heterozygous state, with his grandfather carrying another mutation on *PLG*. Compound heterozygosity was resulting in activity 7.7% (reference interval 70-130%)[248].

rs35897051 (*MPO*)

The NM_000250.1:c.2031-2A>C in *MPO* gene (OMIM*606989) has not been previously associated with C1-INH-HAE. Among approximately 120,000 exomes of the GnomAD, the G allele has been detected in frequency 0.44% in the global population and 0.71% in the European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek male patient with C1-INH-HAE type I. In ClinVar Database, the variant is characterized by multiple submitters pathogenic for myeloperoxidase deficiency. More precisely, the variant has been reported in association with myeloperoxidase deficiency, when it is present in homozygous state or when in trans with another disease-causing variant. Functional studies by Marchetti et al. showed that it destroys the canonical splice acceptor site in intron 11, and causes the activation of a cryptic splice site located 109 nucleotides upstream of the authentic splice site. The 109 nucleotide insertion causes a shift in the reading frame that is predicted to lead to the generation of an abnormal MPO precursor lacking enzymatic activity[208].

rs367856671 (*F11*)

The NM_000128.3:c.281C>T (p.Ala94Val) is located in exon 4 of *F11* gene (OMIM* 264900). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms. Among approximately 120,000 exomes of the GnomAD, the T allele has been detected in frequency 0.0052% (13/251,264 alleles) in the global population and 0.0044% (5/113,564 alleles) in the European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Greek male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar Database. In NCBI and in ExAC database, the Reference SNP number of this mutation (rs367856671) presents a multiallelic variant, as at the same position there is another SNV c.281C>A (p.Ala94Glu) which is predicted as deleterious and probably damaging by the upper referred bioinformatic tools, but it has been detected only once among the 120,000 individuals of ExAC database and no other information are available in NCBI, GnomAD, ClinVar databases concerning its pathogenicity.

The variant is considered likely pathogenic after automated application of ACMG criteria as (a) UniProt protein "FA11_HUMAN domain Apple 1" has 23 non-VUS, non-synonymous, coding variants (23 pathogenic and 0 benign), pathogenicity = 100.0%, (b) the variant is detected in very low frequency in GnomAD, (c) 132 out of 137 non-VUS missense variants in gene *F11* are pathogenic = 96.4% which is more than threshold of 51.0%, and 201 out of 297 clinically reported variants in gene *F11* are pathogenic = 67.7% which is more than threshold of 12.0% and (d) there are multiple bioinformatics tools predicting the variant damaging/pathogenic.

rs143064939 (*F2*)

The NM_000506.4:c.1628G>A (p.Arg543His) is located in exon 12 of *F2* gene (OMIM*176930). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms. Among approximately 120,000 exomes of GnomAD, the A allele has been detected in frequency 0.0052% (13/251,144 alleles) in the global population and 0.0062% (7/113,536 alleles) in the European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar Database. However, in NCBI and in ExAC database, the Reference SNP number of this mutation (rs143064939) represents a multiallelic variant, as at the same position there is another SNV c.1628G>T (p.Arg543Leu) which is a common polymorphism for Latino individuals with allele frequency 3.45%. The c.1628G>T (p.Arg543Leu) is predicted as tolerated and benign by the upper referred bioinformatic tools, but it has never been detected in European non-Finnish individuals of the ExAC database (0/128,952 alleles). In ClinVar database, there are conflicting

interpretations of the pathogenicity of the c.1628G>T (p.Arg543Leu) variant. It is characterized VUS for venous thrombosis and benign for congenital prothrombin deficiency.

rs200864897 (*MME*)

NM_007288.2:c.1390G>A (p.Glu464Lys) is located in exon 14 of *MME* gene (OMIM*120520). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms. Among approximately 120,000 exomes of GnomAD, the A allele has been detected in frequency 0.0012% (3/250,540 alleles) in the global population and 0.00% (0/113,050 alleles) in the European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 male patients with C1-INH-HAE Type I, members of a Hungarian family. There are no clinical assertions for this variant in ClinVar Database.

rs1409620468 (*MME*)

The NM_007288.2:c.335A>G (p.Asp112Gly) is located in exon 4 of *MME* gene (OMIM*120520). It has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms. Among approximately 120,000 exomes of GnomAD, the G allele has been detected in frequency 0.0008% (2/251,404 alleles) in the global population and 0.0009% (1/ 113,710 alleles) in the European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in one Greek male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar Database.

II. SNPs affecting the severity of the disease in C1-INH HAE patients

The allele frequency of the 18 selected common functional variants in the 233 C1-INH-HAE patients of this study was compared with the global allele frequency (MAF) and European non-Finnish allele frequency (ENFAF), as it is recorded in GnomAD (Table 9). The prevalence of the variants for patients from different countries was also calculated (Table 10).

Moreover, we performed univariable analyses to associate the presence of the common functional variants with each phenotypic trait (age at disease onset, need for long-term prophylaxis and disease severity, based on CALS score). As univariable analysis can yield misleading results in cases in which multivariate analysis is more appropriate, we continued with a multivariable analysis with dependent variable the age at disease onset, the LTP or the CALS, and with those of the variants presenting significant associations in univariable analysis fitted as independent variables. This type of analysis was performed for two groups of C1-INH-HAE patients (a) independently of the *SERPING1* variant and (b) for patients carrying a missense variant in *SERPING1*. The results are summarized on table 11.

Table 9. Allele frequency of the 18 selected common SNPs in our cohort, compared to GMAF and EMAF.

SNP	Gene	Reference Allele	Homozygote Patients, %	Heterozygote Patients, %	Wild type Patients, %	Allele Frequency	GMAF	EMAF
rs1801020	***F12***	T	153/ 65.67%	73/ 31.33%	7/ 3.00%	81.33%	65.20%	75.66%
rs5985	***F13A1***	G	12/ 5.15%	77/ 33.05%	144/ 61.80%	21.67%	20.46%	25.33%
rs6003	***F13B***	G	173/ 74.25%	56/ 24.03%	4/ 1.72%	86.27%	88.22%	90.60%
rs1799963	***F2***	G	0/ 0.00%	12/ 5.15%	221/ 98.85%	2.58%	0.84%	1.25%
rs61751507	***CPN1***	G	0/ 0.00%	27/ 11.59%	206/ 88.41%	5.79%	4.23%	4.92%
rs669	***A2M***	A	27/ 11.59%	98/ 42.06%	108/ 46.35%	32.62%	30.98%	34.14%
rs5515	***KLK1***	G	0/ 0.00%	8/ 3.43%	225/ 96.57%	1.72%	3.12%	3.70%
rs3733402	***KLKB1***	G	71/ 30.47%	117/50.21%	45/ 19.31%	55.58%	54.02%	50.59%
rs72550870	***MASP2***	A	0/ 0.00%	26/ 11.16%	207/ 88.84%	5.58%	2.14%	3.23%
rs56378716	***MPO***	T	0/ 0.00%	4/ 1.72%	229/ 98.28%	0.86%	1.02%	1.35%
rs2227564	***PLAU***	T	138/ 59.23%	78/ 33.48%	17/ 7.30%	75.97%	74.83%	76.39%
rs17580	***SERPINA1***	A	0/ 0.00%	9/ 3.86%	224/ 96.14%	1.93%	2.33%	3.65%
rs28929474	***SERPINA1***	G	0/ 0.00%	3/ 1.29%	230/ 98.71%	0.64%	1.11%	1.84%
rs121912714	***SERPINA1***	A	0/ 0.00%	7/ 3.00%	226/ 97.00%	1.50%	0.05%	0.05%
rs6092	***SERPINE1***	G	1/ 0.43%	51/ 21.89%	181/ 77.68%	11.37%	9.52%	11.31%
rs5743708	***TLR2***	G	0/ 0.00%	12/ 5.15%	221/ 94.85%	2.58%	1.74%	2.81%
rs4926	***SERPING1***	G	13/ 5.58%	78/ 33.48%	142/ 60.94%	22.32%	22.20%	27.17%
rs28362944	***SERPING1***	T	2/ 0.86%	39/ 16.74%	192/ 82.40%	9.23%	2.91%	4.67%

Table 10. Allele frequency of the 18 selected SNPs in C1-INH-HAE patients according to their nationality, compared to GMAF and EMAF.

SNP	Gene	Allele Frequency						GMAF	EMAF
		ALL (n=233)	BUL (n=19)	GR (n=31)	GER (n=23)	HUN (n=113)	POL (n=47)		
rs1801020	***F12***	81.33%	81.58%	87.10%	80.43%	79.65%	81.91%	65.20%	75.66%
rs5985	***F13A1***	21.67%	26.32%	14.52%	26.09%	21.68%	22.34%	20.46%	25.33%
rs6003	***F13B***	86.27%	84.21%	88.71%	89.13%	87.61%	80.85%	88.22%	90.60%
rs1799963	***F2***	2.58%	2.63%	4.84%	0.00%	3.54%	0.00%	0.84%	1.25%
rs61751507	***CPN1***	5.79%	2.63%	8.06%	2.17%	6.19%	6.38%	4.23%	4.92%
rs669	***A2M***	32.62%	39.47%	16.13%	32.61%	32.30%	41.49%	30.98%	34.14%
rs5515	***KLK1***	1.72%	5.26%	4.84%	2.17%	0.00%	2.13%	3.12%	3.70%
rs3733402	***KLKB1***	55.58%	47.37%	43.55%	47.83%	57.96%	64.89%	54.02%	50.59%
rs72550870	***MASP2***	5.58%	2.63%	4.84%	4.35%	6.19%	6.38%	2.14%	3.23%
rs56378716	***MPO***	0.86%	2.63%	1.61%	0.00%	0.88%	0.00%	1.02%	1.35%
rs2227564	***PLAU***	75.97%	78.95%	91.94%	78.26%	70.80%	75.53%	74.83%	76.39%
rs17580	***SERPINA1***	1.93%	0.00%	0.00%	0.00%	3.54%	1.06%	2.33%	3.65%
rs28929474	***SERPINA1***	0.64%	0.00%	0.00%	2.17%	0.00%	2.13%	1.11%	1.84%
rs121912714	***SERPINA1***	1.50%	2.63%	0.00%	0.00%	2.65%	0.00%	0.05%	0.05%
rs6092	***SERPINE1***	11.37%	13.16%	6.45%	8.70%	12.83%	11.70%	9.52%	11.31%
rs5743708	***TLR2***	2.58%	0.00%	0.00%	2.17%	3.54%	3.19%	1.74%	2.81%
rs4926	***SERPING1***	22.32%	21.05%	19.35%	23.91%	19.91%	29.79%	22.20%	27.17%
rs28362944	***SERPING1***	9.23%	5.26%	4.84%	2.17%	13.72%	6.38%	2.91%	4.67%

A. Independently of the *SERPING1* variation

1. Age at disease onset

a. Univariable analysis

SERPING1-rs4926, *F12*-rs1801020 and *SERPINA1*-rs17580 were significantly associated with lower or higher age at disease onset. More precisely, the mean age at disease onset was by 7.2 years higher in *SERPING1*-rs4926 homozygotes (AA) compared with patients without the polymorphism (GG) (p=0.03), while heterozygotes (AG) displayed a difference of 3.2 years later onset (borderline significance, p=0.05). In *F12*-rs1801020 homozygotes (CC), the mean age at disease onset was by 5.0 years lower compared with heterozygotes (CT) (p<0.001). However, age at disease onset did not significantly differ in carriers of the polymorphism in either form compared with patients lacking the polymorphism. Finally, the mean age at disease onset was by 6.0 years lower in *SERPINA1*-rs17580 heterozygotes (TA) compared with patients without the polymorphism (AA) (p=0.002).

b. Multivariable analysis

In a multivariable model with *SERPING1*-rs4926, *F12*-rs1801020 and *SERPINA1*-rs17580 fitted as independent variables with dependent variable the age at disease onset, it was found that only *SERPING1*-rs4926 was independently associated with age at disease onset. Heterozygotes (AG) displayed a 3.6-year delay (p=0.018) and homozygotes (AA) a trend towards a 6.3-year delay (p=0.058) of disease onset compared with patients lacking the polymorphism (GG). Heterozygotes for *SERPINA1*-rs17580 (TA) displayed a trend towards earlier disease onset by 4.2 years (p=0.56). *F12*-rs1801020 was a confounder.

2. Long-term prophylaxis

a. Univariable analysis

The presence of the C allele of *SERPING1*-rs28362944, treated as a binary variable (presence vs. absence) was significantly associated with a 2.8-fold increase in the probability of LTP need (OR=2.81, 95% confidence interval 1.40 – 5.61, p=0.004). *A2M*-rs669 homozygotes (GG) had a significantly higher probability of LTP need compared with patients lacking the polymorphism (AA). However, neither an association nor a trend was found in the group of heterozygotes.

b. Multivariable analysis

In a model with *SERPING1*-rs28362944 (as a binary variable) and *A2M*-rs669 as independent variables, the adjusted estimates of the effect remained practically unchanged. Therefore, from this type of analysis we concluded that the presence of the C allele of *SERPING1*-rs28362944 increases 2.5-fold the probability of LTP need (p=0.012). A trend towards 2.2 increase of the above probability was found for *A2M*-rs669 homozygotes (GG)

compared with patients lacking the polymorphism (AA). Models adjusted for age at onset and sex did not yield significantly different estimates.

3. Disease severity (CALS)

Only patients with no long-term treatment were included in the analyses below.

a. Univariable analysis

Presence of *SERPING1*-rs28362944 was associated with 17.72 higher disease severity (CALS) compared with absence (borderline significance p=0.052). Heterozygosity (TC) and homozygosity (CC) for *F12*-rs1801020 were associated with 19.32 (p<0.001) and 28.21 (p<0.001) higher disease severity (CALS), respectively, compared with absence of the C allele. Heterozygosity for *F13B*-rs6003 (GA) was associated with 14.35 lower disease severity (CALS) compared with absence of the A allele (GG) (p<0.001). Presence of the A allele of *F2*-rs1799963 was associated with 13.93 lower disease severity (CALS) compared with absence (p=0.011). Presence of *SERPINA1*-rs28929474 was associated with 87.99 higher disease severity (CALS) compared with absence (p<0.001). Finally, homozygosity for *PLAU*-rs2227564 (TT) was associated with 15.31 lower disease severity (CALS) compared with absence (CC) (p<0.001).

b. Multivariable analysis

SERPINA1-rs28929474 and *F12*-rs1801020 were the most significant predictors of disease severity. In fact, *F12*-rs1801020 displays a robust linear trend among the ordinal categories (homozygosity-heterozygosity-absence), a result that agrees with the univariable analysis. More precisely, heterozygosity (TC) and homozygosity (CC) for *F12*-rs1801020 were associated with 18.69 (p=0.002) and 28.21 (p<0.001) higher disease severity (CALS) compared with absence of the C allele. Presence of *SERPINA1*-rs28929474 was associated with 80.16 higher disease severity (CALS) compared with absence (p=0.003).

In addition, heterozygosity (GA) for *F13B*-rs6003 was associated with 11.84 lower disease severity (CALS) compared with lack of the variant (p=0.024) and homozygosity for *PLAU*-rs2227564 (TT) was associated with 13.67 lower disease severity (CALS) compared with absence (CC) (p=0.004). Two more complex models, including common variants with borderline significance (*SERPING1*-rs28362944, *CPN1*-rs61751507, *SERPINA1*-rs17580, and *KLKB1*-rs3733402) or adjustment for sex were not more informative.

B. Carriers of a missense *SERPING1* variant

Sixty eight patients carried a missense mutation in *SERPING1*. The common functional variants *SERPINA1*-rs121912714, *SERPINA1*-rs28929474 and *MPO*-rs56378716 were not detected in any patient of the missense group.

1. Age at disease onset

Linear GEE models were fitted with age at onset disease as the dependent variable, family as a within-subject variable and common functional variants as independent variables.

a. Univariable analysis

F12-rs1801020, *SERPINA1*-rs17580, *KLKB1*-rs3733402, *KLK1*-rs5515 and *SERPINE1*-rs6092 were significantly associated with higher or lower age at disease onset. A trend was found regarding *SERPING1*-rs28362944, *SERPING1*-rs4926 and *A2M*-rs669.

F12-rs1801020 in the missense group was significantly associated with a delay in the age at disease onset. Mean age at disease onset was by 19.0 years higher in heterozygotes (CT) and by 12.6 years higher in homozygotes (CC) compared with patients lacking the polymorphism (TT) ($p<0.001$). Mean age at disease onset in heterozygotes (TA) for *SERPINA1*-rs17580 was by 8.1 years lower compared with patients without the polymorphism (AA) ($p=0.001$). *KLKB1*-rs3733402 was also significantly associated with lower age at disease onset ($p=0.019$). Mean age at disease onset is by 5.7 years lower in heterozygotes (AG) (borderline significance $p=0.057$) and by 8.1 years lower in homozygotes (AA) compared with patients lacking the polymorphism ($p=0.005$). On the contrary, in heterozygotes (AG) for *KLK1*-rs5515 the mean age at disease onset is by 12.3 years higher compared with patients lacking the polymorphism (GG) ($p=0.023$). Finally, *SERPINE1*-rs6092 was significantly associated with higher age at disease onset ($p=0.002$). More specifically, mean age at disease onset was by 3.4 years higher in heterozygotes (AG) and by 4.6 years higher in homozygotes (GG) compared with patients lacking the variant (AA) ($p=0.001$). Heterozygotes did not display significant difference compared with wild-type patients.

b. Multivariable analysis

In a multivariable model with *F12*-rs1801020, *SERPINA1*-rs17580, *KLKB1*-rs3733402, *KLK1*-rs5515 and *SERPINE1*-rs6092 fitted as independent variables and with dependent variable the age at disease onset, it was found that *F12*-rs1801020 and *SERPINA1*-rs17580 were the strongest predictors of the age at disease onset. Only homozygosity for *KLKB1*-rs3733402 and *SERPINE1*-rs6092 were significantly associated with age at disease onset. *KLK1*-rs5515 displayed an association of borderline significance.

Mean age at disease onset was by 18.0 years higher in *F12*-rs1801020 heterozygotes (TC) and by 13.4 years higher in homozygotes (CC) compared with patients lacking the polymorphism (TT) ($p<0.001$). Mean age at disease onset was by 8.0 years lower in

SERPINA1-rs17580 heterozygotes (TA) (p<0.001) and 7.0 years lower in *KLKB1*-rs3733402 homozygotes (AA) (p=0.029) compared with patients without the variants. Mean age at disease onset was by 8.95 years higher in *KLK1*-rs5515 heterozygotes (AG) (borderline significance p=0.05) and by 8.4 years higher in *SERPINE1*-rs6092 homozygotes (AA) (p=0.009) compared with patients lacking the polymorphism.

A second multivariable model including as independent variables variants with a trend of association in univariable analysis i.e. *SERPING1*-rs28362944 (p=0.089), *SERPING1*-rs4926 (p=0.102) and *A2M*-rs669 (p=0.124) was not more informative. The estimates of the significantly associated variants did not notably change, except for *KLK1*-rs5515, which lost significance.

2. Long-term prophylaxis

Logistic regression GEE models were fitted with the need for long-term treatment as a binary dependent variable, family as a within subject variable and polymorphisms as independent variables.

a. Univariable analysis

In the missense group there were no *SERPING1*-rs28362944 homozygotes. Heterozygosity (TC) was significantly associated with a 5.2-fold increase in probability of need for LTP (OR=5.19, 95% confidence interval 1.67 – 16.1, p=0.004). *F13A1*-rs5985 homozygosity (TT) and *CPN1*-rs61751507 heterozygosity (GA) displayed a trend towards a 5-fold increase (p=0.080) and an 85% decrease (p=0.080), respectively, in the probability for LTP need.

b. Multivariable analysis

By studying three models of multivariable analysis we concluded that *SERPING1*-rs28362944 heterozygosity (TC) increases 4.2-fold the probability of LTP need compared with absence of polymorphism (p=0.02). A trend towards 8.8 increase of the above probability was found for *F13A1*-rs5985 homozygotes (TT) compared with patients non-carriers of the T allele. Heterozygosity (GA) for the *CPN1*-rs61751507 was independently associated with 98% decrease of the probability of long-term prophylaxis need (p=0.017).

The first model included all three common variants which displayed a significant association or a trend of association with the need for LTP, i.e. the *SERPING1*-rs28362944, *F13A1*-rs5985 and *CPN1*-rs61751507. The second and third model included *SERPING1*-rs28362944/*CPN1*-rs61751507 and *SERPING1*-rs28362944/*F13A1*-rs5985 as independent variables, respectively. Models adjusted for age at disease onset and sex did not yield significantly different estimates.

3. Disease severity (CALS)

Only patients out of prophylactic therapy were included in the analyses below (n=44/68). CALS severity was modeled as dependent variable in linear GEE models with family as within-

subject variable, and polymorphisms as dependent variables. CALS change represents mean change in CALS severity between comparator (homozygote or heterozygote) and reference category (absence of polymorphism).

a. Univariable analysis

F12-rs1801020, *F2*-rs1799963, *KLKB1*-rs3733402, *KLK1*-rs5515, *PLAU*-rs2227564 and *TLR2*-rs5743708 were significantly associated with higher or lower disease severity (CALS).

Heterozygosity (TC) and homozygosity (CC) for *F12*-rs1801020 were associated with 15.70 (p=0.002) and 30.27 (p<0.001) higher disease severity (CALS), respectively, compared with absence. Presence of A allele for *F2*-rs1799963 as well as *KLK1*-rs5515 were associated with 22.27 (p<0.001) and 25.15 (p<0.001) lower disease severity, respectively, compared with absence of the variants. On the contrary, presence of the A allele for *TLR2*-rs5743708 was associated with 48.22 (p=0.001) higher disease severity (CALS). Homozygosity for *KLKB1*-rs3733402 was associated with 36.36 higher disease severity (CALS) (p<0.001), while homozygosity for *PLAU*-rs2227564 with 14.04 lower disease severity (CALS) (p=0.014).

b. Multivariable analysis

The first model of multivariable analysis included the most strongly associated variants from the above univariable analyses and concluded that all variants but *PLAU*-rs2227564 are independently associated with higher or lower disease severity (CALS). From the second model, simplified by excluding *PLAU*-rs2227564, we concluded that in *F12*-rs1801020 homozygotes (CC), mean disease severity is by 25.48 higher compared with CALS in patients lacking the polymorphism (p=0.002) and in *F12*-rs1801020 heterozygotes (TC), mean CALS severity is by 13.88 higher compared with CALS in patients lacking the polymorphism (p=0.003). In *F2*-rs1799963 heterozygotes (GA), mean disease severity is by 25.97 lower compared with CALS in patients lacking the polymorphism (p=0.017). Finally, in *KLKB1*-rs3733402 homozygotes (AA) mean CALS severity is by 30.45 higher (p<0.001) and in *KLK1*-rs5515 heterozygotes it is by 16.79 lower (p=0.029) compared with CALS in patients lacking the polymorphisms. A model adjusted for age at disease onset and a model adjusted for sex were not more informative.

Table 11. Summary of the associations of common functional variants with patients' phenotype (age at disease onset, need for LTP, CALS).

	SNP (*Gene*)	Nucl. change	Genotype	Age at disease onset		Need for LTP		CALS	
All *SERPING1* variants	*SERPING1*-rs4926	c.1438G>A	GA AA	+3.6 years +6.3 years	p=0.018 p=0.058	- -		- -	
	SERPING1-rs28362944	c.-21T>C	TC and CC	-		2.5-fold	p=0.012	-	
	A2M -rs669	c.2998A>G	GG	-		2.2-fold (trend)		-	
	F12-rs1801020	c.-4T>C	CT CC	- -		- -		+18.69 +28.21	p=0.002 p<0.001
	F13B -rs6003	c.344G>A	GA	-		-		-11.84	p=0.024
	SERPINA1-rs28929474	c.1096G>A	GA and AA	-		-		+80.16	p=0.003
	PLAU -rs2227564	c.422T>C	CC	-		-		-13.67	p=0.004
Missense *SERPING1* variants	*F13A1*-rs5985	c.103G>T	TT	-	-	8.8-fold (trend)	-	-	
	SERPINA1-rs17580	c.863A>T	AT	-8 years	p<0.001	-		-	
	KLKB1-rs3733402	c.428G>A	AA	-7 years	p=0.029	-		+30.45	p=0.001
	KLK1-rs5515	c.230G>A	GA	+8.95	p=0.05	-		-16.79	p=0.029
	SERPINE1-rs6092	c.43G>A	AA	+8.4	p=0.009	-		-	
	SERPING1-rs28362944	c.-21T>C	TC	-		4.2-fold	p=0.02	-	
	CPN1-rs61751507	c.533G>A	GA	-		98% decrease	p=0.017	-	
	F12-rs1801020	c.-4T>C	CT CC	+18 years +13.4 years	p<0.001 p<0.001	- -	- -	+13.88 +25.48	p=0.003 p=0.002
	F2-rs1799963	c.*97G>A	GA	-		-		-25.97	p=0.017

III. ClinVar

We submitted through the standard ClinVar protocol 45 *SERPING1* variants associated with HAE Type I or II. The document for submission is presented in Appendix IV. For the classification of variants we used Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines (2015), verifying our assessment with the variant interpretation tool from University of Maryland (https://www.medschool.umaryland.edu/Genetic_Variant_Interpretation_Tool1.html/). All the variants were submitted with supporting evidence, which included clinical information for the patients, such as number and frequency of angioedema attacks, family history and segregation of the mutation in the family, sex of family members etc. We performed no functional studies, but all the patients were previously diagnosed with C1-INH-HAE. Some of the submitted variants were already classified by other submitters using guidelines or by independent method.

DISCUSSION

PART I: EXPRESSION OF BRADYKININ RECEPTORS (B1R, B2R)

B1R and B2R are implicated in a series of responses, including the regulation of blood vessel tonus, inflammatory endothelial permeability and pain processing mechanisms. Some clinical evidence suggests their expression as possible biomarker of active inflammatory states. The B2R is considered as the main intermediate responsible for HAE attacks and has been largely studied. Endothelial cells constitutively express B2R and treatment with the B2R antagonist, icatibant, successfully controls attacks in patients. From a pharmacological point of view, once activated by kinins, B2R is rapidly desensitized, a fact that is not consistent with the long course of swelling. Moreover, despite the immediate relief obtained by the use of icatibant, complete resolution of symptoms requires several hours, supporting a hypothesis that other ligands/receptors should be considered responsible for the maintenance of HAE. The high severity risk of HAE patients with low APP activity provides an indirect evidence for the involvement of B1R ligands in the pathological process. A prolonged signal from B1R could sustain the swelling during an episode. However, among its ligands, *des*Arg^{9}-BK, has been reported to display low affinity and no evidence has been provided that the potent ligand Lys-*des*Arg^{9}-BK is generated during attacks, suggesting that B1R may not play an important role in the generation of the swelling.

Conversely to B2R, B1R displays a low physiological expression, being upregulated under stressful stimuli, e.g. inflammatory agents (TNFα, IL-1β). The contribution of B1R in endothelial permeability of HAE processes has been poorly investigated. *In vitro* BSA leakage using Transwell® system and *in vivo* models have shown that B2R antagonist and either of the two B1R antagonists partially reduced endothelial permeability, whereas combined treatment with B1R and B2R antagonists completely inhibited the leakage[249]. Up to now, it has been postulated that B1R is locally upregulated during attacks, but the detection of B1R on the surface of the patients' endothelial cells during attacks has not been achieved.

Charest-Morin et al., in an effort to define novel diagnostic and analytical tools alternative to antibodies, reported the design of fluorescent fusion proteins as ligands of B2R and B1R. The ligands are composed of an EGFP prolonged at its N-terminus by a spacer peptide linked to a peptide agonist or antagonist. These ligands could detect the intact receptor at the cell surface and give a rapid, direct fluorescent signal. In this thesis, the fusion proteins EGFP-S4-P1 and EGFP-S1-P1 (EGFP-MK) were successfully produced by transient transfection of HEK293a cells with pcDNA vectors, kindly offered to our laboratory by *Prof*. F. Marceau. Efforts to generate stable clones of cells that can constantly produce the ligands were not successful.

Charest-Morin et al.[79] had applied the ligands in cytokine-treated human umbilical artery smooth muscle cells (hUA-SMCs). In the experiments performed for this thesis, the ligands were used *in vitro* on cytokine-treated endothelial cells (EA.hy926) and monocytes (THP1) cells, in parallel with anti-B1R and anti-B2R immunoblots and flow cytometry.

Immunofluorescence using the fluorescent ligands failed to detect the receptors, possibly due to low expression of the naturally expressed receptors on the cell surface. Moreover, B2R ligand presented unexpected low affinity for the human receptor. Concerning the antibodies, it was not possible to conclude, neither on their specificity nor on the localization of the receptors.

Protein ligands may be intrinsically superior to peptides and they can be used to detect the signal originating from the intact receptor expressed on the cell surface. However, optimization of the spacer sequence and additional assays, possibly in combination with gene expression study, should be performed in order to achieve the desired outcome and proceed in human blood samples. B2R and B1R transcripts can be investigated, as *Prof*. F. Marceau also suggested. A strategy for receptor quantification using RT-PCR on peripheral neutrophils prepared from asthmatic individuals has been successfully developed by Bertram et al. (2007)[250]. No difference has been observed between asthmatic and non-asthmatic subjects. Similar results have been obtained using peripheral eosinophils or monocytes. However, B1R mRNA expression has been found by neutrophils at significantly lower amounts in asthmatic subjects under inhaled corticosteroid. No investigation has been yet reported with successful observations concerning patients presenting symptoms of angioedema. Tissue injury, such as myocardial ischemia and inflammation, can cause the expression of B1 receptors on cell surfaces, e.g. with subsequent hypotensive state seen during septic shock. But among important areas that remain poorly understood concerning kinin angioedema, the role of the B1R must be included.

PART II: PLASMINOGEN GLYCOFORMS ALTERATION AND SUSCEPTIBILITY TO ACTIVATION ASSOCIATED WITH THE MISSENSE VARIANT p.Lys330Glu IN HAE-PLG PATIENTS

HAE-PLG due to p.Lys330Glu variant, which is located in exon 9 of the gene and affects Lys^{330} residue in the KR-3 domain of the protein, has been associated with swelling of the lips and tongue (nearly 78% of cases). The variant carriers have been successfully treated by icatibant or plasma-derived C1-INH, demonstrating that HAE-PLG could be mediated by bradykinin. Scarce data is currently available concerning the prevalence and penetrance of this variant. Dewald (2018) predicted the structural and functional impact of the mutation and observed altered PLG patterns in two heterozygous patients for the p.Lys330Glu variant. He strongly supported that the substitution is a disease-causing mutation and it defines a new type of HAE. He also implied that, apart from the quantitative impact on PLG it may also have a qualitative effect.

In the experiments of the present study, the altered glycosylation pattern in heterozygous subjects, observed by Dewald, has been confirmed. The additional examination of a homozygous patient revealed a reversed glycosylation pattern. The patients presented ~60%

of PLG type I glycoform and around 40% of PLG type II form. Takada et al. (1983)[251] have already observed that PLG type I is more susceptible to activation by UK or SK than PLG type II. A plasmin-specific chromogenic assay was developed to investigate the susceptibility of PLG activation by natural activators in samples from our patients. Both homozygous and heterozygous carriers display a significantly high susceptibility to activation of PLG by SK ($p<0.002$) and UK ($p<0.002$) compared to healthy controls.

The enhanced susceptibility to proenzyme activation may be related to reversed glycosylation patterns. It could be hypothesized that p.Lys330Glu may develop a higher binding ability of the mutant protein with lysine residues of protein ligands. Concerning HAE-PLG, the PLG p.Lys330Glu seems to promote a qualitative impact on the protein and may result in increased plasmin formation. Plasmin and tissue plasminogen activator, as well, can also act as FXII activators, with transformation of zymogen FXII into protease FXIIa. Plasmin can also act as a direct and reciprocal prekallikrein activator[252]. This results in KKS activation and subsequent excessive BK production.

Finally, some patients in our cohort were also carriers of *PLG* variants c.266G>A, (p.Arg89Lys) and c.1567C>T (p.Arg523Trp), which are predicted to be likely benign according to bioinformatics tools. The developed plasmin-specific assay used in our study can be further developed to define a possible contribution of these variants on the PLG susceptibility to activation.

PART III: GENETIC BIOMARKERS OF THE SEVERITY OF C1-INH-HAE

C1-INH-HAE has profound and lifelong effects on individuals and families in a personal, psychological and economic level. Several studies have attempted to quantify the burden of HAE on the individual. The results showed significant quality of life impairment in all physical and mental components. Newly developed tools concerning the measurement of angioedema-specific quality of life (QoL) already provide specific data on outcomes. Tools that measure the disease activity have also been introduced into the clinical care.

The clinical expression of C1-INH-HAE is characterized by large heterogeneity with regards to severity, triggering factors and response to treatment. The clinical heterogeneity is rather unpredictable and efforts have been made to uncover strong genotype-phenotype correlations. Even among family members, carriers of the same *SERPING1* variant, the features of the disease such as the age at disease onset, the frequency and triggers of the attacks, the localization of the symptoms, the severity and need for LTP vary broadly. This part of the study aimed to associate genetic variants with the severity of C1-INH-HAE and to assess their utility as genetic biomarkers, based on the hypothesis that they can act as modifiers of the clinical phenotype. Uncovering biological parameters that modify patients' phenotype and possibly impact the effectiveness of the various treatment modalities might result in the optimization of HAE management. A metrics of disease severity (CALS)

calculating the number of cutaneous, abdominal and laryngeal oedematous attacks that a patient experienced during the 12 months before evaluation has been proposed by *Prof*. H. Farkas and used in this thesis for the enrolled patients.

A. Common functional variants

In order to investigate the association of the 18 selected common functional variants (Allele frequency≥1%) with three distinct phenotypic traits (age at disease onset, need for LTP and disease severity, based on CALS score) we performed univariable analyses, which provided an initial summary for all the variables. In this way, it was possible to choose for every phenotypic trait the variants which presented significant associations and continue with the more appropriate multivariable analysis where the variants were fitted as independent variables and the phenotypic traits as dependent variables. Given the fact that our patient population consisted of correlated subjects (family members), we fitted the family factor as a within-subject variable. This type of analysis was performed for 233 C1-INH-HAE patients, independently of the *SERPING1* mutational status. Then, we repeated the analysis for 68 patients carrying a missense *SERPING1* variant.

Two common functional variants (*F12*-rs1801020 and *SERPING1*-rs28362944) were found in association with at least one phenotypic trait in both groups. Heterozygosity (TC) and homozygosity (CC) for *F12*-rs1801020 were significantly associated with an increase at disease severity in the first and the second group of patients. Our results agree with evidence provided by Bors et al. (2013)[138] who suggested that the carriage of the T allele of *F12*-rs1801020 variant is independently associated with a less severe C1-INH-HAE clinical phenotype. Moreover, they agree with evidence provided by Rijavec et al. (2019)[140] who investigated the influence of *F12*-rs1801020 in the penetrance of C1-INH-HAE. More specifically, the authors have shown that the C allele and CC genotype were represented more in symptomatic patients, compared to asymptomatic and they suggested that carriers of the CC genotype have a 25-fold greater risk of developing the disease compared to those carrying the TT genotype. They characterized the T allele as possibly protective for C1-INH-HAE. In a previous study, Endler et al. (2001)[167] had observed a 2.5-fold lower prevalence of the T genotype in patients with acute coronary syndrome, which also indicated a protective effect on the development of the syndrome in patients with preexisting stable coronary artery disease. The protective effect of the T allele seems to appear in our results, as *F12*-rs1801020 displays a robust linear trend among the ordinal categories (homozygosity-heterozygosity-absence). Therefore, we observe that the disease severity is influenced by the presence of the C allele when it is found in heterozygous state, but when found in homozygous state it clearly affects more the calculated score and with statistically increased significance. This effect could be explained based on functional studies by Kanaji et al. (1998)[166], who investigated the levels of FXII in plasma. The authors observed different levels of FXII in plasma, depending on the genotype and despite the fact that both alleles were

equally transcribed in hepatocytes of heterozygotes, the cDNA containing the T allele was producing less FXII *in vitro* than the one containing the C allele. Therefore, the presence of the variant is affecting the efficiency of translation. Based on this observation and on the fact that C1-INH-HAE patients with early disease onset usually display a more severe phenotype, we would also expect to observe a delay at disease onset in T carriers. Speletas et al. (2015)[139] supported this hypothesis when they observed that the age at disease onset was significantly affected by the carriage of the variant in C1-INH-HAE patients regardless of *SERPING1* mutational status and they characterized *F12*-rs1801020 an independent modifier of C1-INH-HAE severity. More precisely, patients carrying the TT genotype presented a 7-year delay at disease onset. During the univariable analysis that we performed in the first group of patients of the present study, we detected a significant delay at disease onset in heterozygous patients (TC) comparing to homozygous (CC) patients ($p<0.001$), which agrees with the results by Speletas et al. (2015). However, this result is not reported after the multivariable analysis, because *F12*-rs1801020 was a confounder and it should not be described in terms of associations.

SERPING1-rs28362944 (c.-21T>C) was studied as a binary variable, calculating the presence (TC and CC) compared to the absence (TT) of the C allele in the first group of patients-carriers of any type of *SERPING1* variant. In the second group, there were no homozygotes (CC). Therefore, the heterozygosity and the presence of the C allele coincided. Regardless the *SERPING1* variation, the presence of the C allele for *SERPING1*-rs28362944 increased 2.5-fold the probability of need for LTP. In carriers of a missense *SERPING1* variant, heterozygosity (TC) increased 4.2-fold the probability of need for LTP. Previous studies suggested that *SERPING1*-rs28362944 in heterozygous state had probably no effect on the translation of the protein, no correlation with the biochemical values of C1-INH function or the clinical severity score. Functional studies by Duponchel et al.[238] proposed the variant as a modifier of disease severity as they found that the variant yields low but significant levels of exon 2 skipping in transfected cells. Therefore, this allele may contribute, at the RNA level, to more severe forms of AE. When reported for the first time in homozygous state, the variant was characterized likely pathogenic and in the second case report, the homozygous carrier with symptoms of HAE was receiving LTP (danazol). The frequency of edema attacks had been diminished with therapy. The authors suggested that the homozygous form of *SERPING1*-rs28362944 might be pathogenic for the disease and even if the frequency of heterozygotes was reported to be similar in patients with HAE compared to healthy controls, the variant could be associated with a more severe HAE form.

We observed that the common variants which affect the phenotype of the patients of the first group are different compared to the variants affecting the phenotypic traits of the patients belonging to the second group. However, with a closer look and considering the facts that three variants were not detected in any patient of the missense group and that the

first group included about 3 times more patients than the second, the groups did not extremely differ (Table 12). The results are elaborately discussed below.

Table 12. Significant associations of common functional variants with the phenotype between the two patients groups of this study. A: Significant association, *Trend*: borderline significance in multivariable analysis. A: Significant association, *Trend*: borderline significance in multivariable analysis.

Variant	**Independently of the *SERPING1* mutation**			**Missense *SERPING1* mutation**		
	Disease onset	Need for LTP	Disease severity	Disease onset	Need for LTP	Disease severity
rs28362944	A				A	
rs1801020	*In univariable analysis*		A	A		A
rs4926	A			*Trend*		
rs28929474			A			*No patients with this variant*
rs2227564			A			*In univariable analysis*
rs6003			A			*No association*
rs17580	*In univariable analysis and trend*			A		
rs3733402	*No association*		*Borderline significance in univariable analysis*	A		A
rs1799963			*In univariable analysis*			A
rs5515	*No association*			A		
rs6092	*No association*			A		
rs61751507	*No association*				A	

Independently of the *SERPING1* mutation

SERPING1-rs4926 was significantly associated with later disease onset, while *F13B*-rs6003, *SERPINA1*-rs28929474 and *PLAU*-rs2227564 were associated with disease severity. More precisely, for *SERPING1*-rs4926, despite the fact that a trend was found for carriers of a missense *SERPING1* variant, only in the first group of patients we detected a significant delay at the age of disease onset (3.6 years and a trend towards 6.3 years respectively) in heterozygotes (GA) and homozygotes (AA). This common variant (p.Val480Met), historically reported as Val458Met or Val451Met, is predicted as deleterious and possibly damaging according to bioinformatic tools, because the highly conserved amino acid (Val) is important for the folding of the C1-INH protein into its native conformation[95]. The valine-methionine

and/or valine-glycine amino acid changes had been initially reported in this position as disease causing mutations by Verpy et al. (1996)[236], Blanch et al. (2002)[253] and Gösswein et al. (2008)[241]. However, it is a well-documented common variant and characterized by different groups as benign in public databases. Functional studies by Cumming et al. (2003)[113] found no detectable effect on C1-INH structure, stability, plasma levels or HAE disease expression. The function of mutated C1-INH was studied and shown to be unaffected. However, the authors did not exclude a consequence of the variant on other functions of C1-INH as a modulator of the coagulation and kinin release pathways.

Heterozygosity (GA) for *F13B*-rs6003 (c.344G>A) was significantly associated with decreased disease severity. The variant is rather benign for FXIII Subunit B deficiency, but it has been characterized a risk factor for venous thrombosis. Komanasin et al. (2005)[183] presented evidence that the presence of arginine in the position 115 (p.Arg115His), corresponding to the carriage of the G allele, resulted in faster dissociation between the A and B subunits of FXIII after activation by thrombin and was associated with moderately increased risk for venous thrombosis.

Presence of the A allele of *SERPINA1*-rs28929474 was associated with a significant increase of the disease severity, compared with absence. *SERPINA1*-rs28929474 commonly known as the Z allele of A1AT is responsible for 95% of all clinical cases of A1AT deficiency, when found in homozygous state. A1AT deficiency is primarily associated with the risk of emphysema and liver disease. Functional studies demonstrate that this common variant alters the global structural dynamics of A1AT and renders it five times less effective as an inhibitor of neutrophil elastase than the normal allele. Moreover, it results in the formation of polymers in the lung which can be chemoattractants for neutrophils and increase the inflammation. When the Z allele is found in compound heterozygosity with the S allele (*SERPINA1*-rs17580) it is associated with 20-50% risk for emphysema. We detected no such cases in our cohort and no clinical data are available regarding A1AT deficiency phenotype. There were no patients carrying this variant in the second subgroup of patients.

Homozygous carriers (CC) of *PLAU*-rs2227564 presented a significant decrease in the disease severity, regardless the *SERPING1* variation. The same result appeared after the univariable analysis for *PLAU*-rs2227564 in patients of the missense group, but the association lost its significance in the following multivariable analysis. Urokinase, encoded by *PLAU*, is a plasminogen activator and there is accumulating information regarding the role of plasminogen and plasmin in the activation of the kinin-kallikrein system and the generation of BK. The amino acid change (p.Leu141Pro) caused by this variant is located within the kringle domain of urokinsase at the junction between two β-pleated sheets[254,255]. The presence of the T allele does not appear to affect the activity of urokinase, but according to Yoshimoto et al.[254], the zymogen containing Pro^{141} binds fibrin aggregates less efficiently

than the one containing Leu[141], suggesting the possibility of altered extracellular UK localization or stability.

Carriers of a missense *SERPING1* variant

In the second group including patients-carriers of a missense *SERPING1* variant, *SERPINA1*-rs17580 and *SERPINE1*-rs6092 were significantly associated with the age at disease onset, while *CPN1*-rs61751507 and *F2*-rs1799963 were significantly associated with need for LTP and disease severity, respectively. *KLKB1*-rs3733402 and *KLK1*-rs5515 were associated with both the age at disease onset and the disease severity. More precisely, homozygosity (AA) for *KLKB1*-rs3733402 was significantly associated with 7 years earlier disease onset and increased disease severity compared with GG carriers. Similar observation, regarding the age at disease onset of C1-INH-HAE patients had been previously made by Gianni et al. (2017)[141] who also investigated the contribution of this functional variant to the disease phenotype. According to Gianni et al. (2017), carriers of the G allele of the *KLKB1* c.428G>A exhibited a significantly delayed disease onset by 4.1 years ($p<0.001$) depending on the zygocity status. Gianni et al. (2017) reported no significant association between the type of *SERPING1* mutation or C1-INH-HAE type and the age at disease onset. No data are available, concerning the percentage of carriers of a missense variant in the cohort of the study by Gianni et al. However, our study confirms the results only when the patients carried a missense *SERPING1* mutation.

On the contrary, heterozygous carriers (GA) of the *KLK1*-rs5515 displayed a significant higher mean age at disease onset by 8.95 years and a decrease in the disease severity compared to GG carriers. According to functional studies, heterozygous carriers of the *KLK1*-rs5515 have 50-60% lower urinary KK activity, while in studies of branchial artery function, they have been reported to exhibit an arterial dysfunction (increase in wall shear stress and reduction in artery diameter and lumen)[200].

Heterozygosity (AT) for *SERPINA1*-rs17580 was significantly associated with earlier age at disease onset by 8 years, compared to AA carriers. *SERPINA1*-rs17580 is commonly known as the S allele of A1AT. Functional studies by Curiel et al. (1989)[219] have shown that the S allele cause reduced cellular secretion of A1AT because the newly synthesized protein is degraded intracellularly before secretion. The S allele is not disease causing and even homozygous carriers are not presented with the common expressions of A1AT deficiency. It represents a risk factor when it is found in compound heterozygosity with the Z allele, and because of its high frequency such compound heterozygotes are relatively frequent. As it is previously referred, we detected no such cases among our patients.

Homozygous carriers (AA) of *SERPINE1*-rs6092 displayed a significantly higher mean age at disease onset by 8.4 years. The variant has been previously characterized likely benign for PAI-1 deficiency, but functional studies in a heterozygous patient showed activity at about 70% and Zhang et al. (2005)[234] suggested that the change from a hydrophobic non-polar

amino acid (Ala) to a hydrophilic polar amino acid (Thr) in the hydrophobic core region (h-region) of the signal peptide of the protein may disturb its function.

Heterozygosity for *CPN1*-rs61751507 was independently associated with 98% decrease of the probability of LTP. This variant has been previously associated with HAE by Mathews et al. (1980)[191] and Cao and Hegele (2003)[192], when found in compound heterozygosity with another *CPN1* variant. The frequency measured by Cao and Hegele in heterozygous state in normal control subjects was about 1%. Today, the global allele frequency is, according to GnomAD, 4,23%. The Gly178 residue of CPN has been conserved in diverse species and is also conserved among most members of the human carboxypeptidase family. The plasma levels of CPN can be easily measured and concurrent biological test in patients carriers of the SNP can give us further information about the functional impact of the variant and the association with the disease severity or the various phenotypic traits of the carriers.

Finally, heterozygous carriers (GA) of *F2*-rs1799963 had a significant decrease in the disease severity, compared to GG carriers. Gehring et al.[187] supported that *F2*-rs1799963 represents a gain-of-function mutation, affecting the generation of prothrombin.

B. Rare variants

The importance of rare variants (MAF<1%) is increasing while their role in the genetics of the diseases with a complex pathophysiology is not easy to define. They have distinctive features, like a higher impact on gene expression and function and larger population specificity. High-throughput technology, like Whole Genome Sequencing (WGS) or targeted NGS platforms have offered the possibility to detect a great number of rare variants and have elucidated novel biological pathways involved in the target diseases.

In this study, we used a targeted NGS platform including genes coding for proteins implicated in the metabolism and function of BK. We aimed to analyze the contribution of rare variants in the heterogeneous clinical manifestations of C1-INH-HAE. We presumed that, in cases of C1-INH-HAE, the number of rare variants co-existing with a causal *SERPING1* variant or the carriage of an additional variant classified as pathogenic in a gene important for BK function could modify the clinical phenotype and/or the response to treatment. If the severity of the disease in such patients is attributed in the modifying effect of other genes alterations, the determination of optimal therapy should be based on individualization according to patients' history and genomic data. For example, replacement therapy with plasma-derived C1-INH might be less effective than the administration of medications directly blocking the function of BK.

In 144 unrelated C1-INH-HAE patients from 5 different European countries, 187 rare variants (MAF≤1%) in the 52/54 genes were detected, among which 19 (10,16%) novel. Further research is required concerning the contribution of rare variants in the phenotype of our patients, taking into account evidence like the number of co-existing variants in an individual and the role of each protein in the pathogenicity of HAE (gene priority) and

limitations of this type of analysis, such as the size and genetic background of the reference population and the usually unknown functional impact of each variant, rendering complicated a population genotype-phenotype association.

Incidental findings

During this research, 3 incidental findings have occurred. More precisely, 3 pathogenic variants classified according to ACMG/AMP Standards and Guidelines. All three patients carrying pathogenic variants in heterozygous state presented a very low age at disease onset (1-4 years old). The age at disease onset of the Bulgarian patient carrying the *F11* c.1556G>A (p.Trp519Ter) was 2 years old. The patient was a carrier of a splicesite variant in *SERPING1* and a compound heterozygote for 2 more benign rare variants in *ACE*. FXI is circulating as a zymogen in non-covalent interaction with HK and is required to maintain thrombin production during the coagulation process. Heterozygotes for the *F11* variant show partial or mild FXI deficiency and bleeding disorder. We have no clinical data indicating a co-existing phenotype of abnormal bleeding and we cannot conclude about the contribution of this variant to the HAE phenotype.

The age at disease onset of the Hungarian patient carrying *PLG* c.2251G>A (p.Gly751Arg) was 3 years old. The patient carried a frameshift variant (deletion) in *SERPING1* and another rare missense variant in *DPP4*, characterized likely benign according to ACMG/AMP standards and guidelines. The patient, except for the early age at disease onset, was suffering from laryngeal attacks rather than attacks in the gastrointestinal tract, characteristic for HAE-PLG patients and he was receiving LTP. However, a member of his family with the same *SERPING1* and *PLG* variants presented frequent abdominal attacks and no laryngeal attacks. Bork et al. (2020)[156] suggested a strong genotype/phenotype correlation in patients carrying separately a pathogenic for HAE-PLG variant or a pathogenic for C1-INH-HAE variant, but observed no change in C1-INH-HAE symptoms in patients carrying both pathogenic variants. We cannot conclude about the contribution of the co-existing *PLG* variant in the localization of the attacks of our C1-INH-HAE patient. However, the *PLG* c.2251G>A is characterized pathogenic for dysplasminogemia and the PLG activity of the heterozygous carrier described by Higuchi et al. (1998)[248] was lower (51,4%), compared to reference (70-130%). Further functional investigation should be performed (PLG glycosylation patterns and susceptibility to activation) in the carriers of this mutation. The same holds true for other rare *PLG* variants. More precisely, among 144 patients unrelated patients of this study, 7 patients carriers of different *SERPING1* variants were compound heterozygous with 6 different PLG variants. Among them, 5 patients suffer from laryngeal attacks. Three out of five present additional abdominal swelling, while 4 receive LTP.

Finally, the patient carrying a missense *SERPING1* variant and a pathogenic splicesite variant in MPO (c.2031-2A>C) was 4 years old when he presented the first HAE symptoms and he was carrying no other rare variants in the examined genes. However, the variant is

characterized pathogenic for MPO deficiency when detected in homozygous state or in trans with another disease-causing variant. The patient's MPO may lack enzymatic activity, but we cannot conclude about a possible contribution of the variant in the C1-INH-HAE phenotype.

C. ClinVar

The rapid evolution of sequencing technology has led to the identification of large number of genetic variants associated with many clinical phenotypes. Defining the clinical validity and the pathogenicity of detected variants is a particularly challenging task. The low frequency of most variants makes population genotype-phenotype associations impractical. A better understanding of their functions should facilitate the implementation of many more variants in genomic-driven medicine. For genes with known functions and clinical utility, these variants can be used to assess risk and guide treatment based on their effect on the gene and gene product.

In order for clinical laboratories to ensure quality results regarding the interpretation of pathogenicity of the detected variants, the American College of Medical Genetics (ACMG) has developed professional standards and guidelines, where all criteria are initially weighed and then combined for a reliable curation. One criterion in ACMG standards and guidelines for variant classification is the report of the variant by reputable databases (criterion PP5 and BP6). Concerning *SERPING1* variants detected by different research entities, they are stored mainly in local databases, siloed between laboratories or shared only through publications in journals. After publication, they are sometimes submitted and maintained by their users in the gene specific HAE database (http://hae.enzim.hu/).

The final part of the present study aimed to participate to the effort for data sharing among entities and physicians dealing with HAE patients or research on HAE pathophysiology and genotype/phenotype correlations. ACMG along with the American Medical Association (AMA) and the National Society of Genetic Counselors (NSGC) propose and support ClinVar database. We classified, by application of ACMG standards and guidelines, 45 *SERPING1* variants previously detected in C1-INH-HAE patients of the Laboratory of Immunology and Histocompatibility of the UTH and reported the variants in ClinVar database, accompanied by the supporting clinical evidence. We aim to continue with submission of the benign *SERPING1* variants and the rare novel variants detected in our cohort.

GENERAL CONCLUSIONS

Studies included in the current PhD thesis led to the following conclusions:

I. The development of molecular imaging probes targeting B1R and B2R is limited to preclinical settings. The *Prof*. F. Marceau group, who kindly provided us the material for the production of fluorescent ligands, is one of the early research groups to use fluorescently tagged peptides to study bradykinin receptors. Protein ligands may be intrinsically superior to peptides and they can be used, when antibodies are not available, in order to detect the intact BK receptors expressed on the cell surface. However, optimization of the fluorescent ligands and additional assays should be performed in order for this diagnostic tool to be validated for human blood samples.

II. Altered glycosylation patterns are detected in heterozygous carriers of PLG p.Lys330Glu with PLG Type I and II appearing to be at the same level after quantification of the bands on the anti-PLG immunoblot. An inversion is detected in the homozygous patient with ~60% of PLG type I glycoform and around 40% of PLG type II. Both homozygous and heterozygous carriers of the variant display a significantly high susceptibility to PLG activation by streptokinase and urokinase compared to healthy controls. The developed plasmin-specific chromogenic assay can also be used to measure the effect of other pathogenic PLG variants on the susceptibility to PLG activation.

III. Common functional variants affecting proteins implicated in BK metabolism and function seem to have modifying effects on the clinical phenotype of C1-INH-HAE patients. Three distinct phenotypic traits were studied (age at disease onset, need for LTP and disease severity, calculated by CALS score). Among 18 selected functional SNPs, the presence of the C allele of *F12*-rs1801020 was significantly associated with an increase at disease severity. Presence of *SERPING1*-rs28362944 increased 2.5-fold the probability of LTP need. *SERPING1*-rs4926 was associated with later disease onset. *F13B*-rs6003, *SERPINA1*-rs28929474 and *PLAU*-rs2227564 were associated with increase or decrease of disease severity. In carriers of a missense *SERPING1* mutation, the presence of the C allele of *F12*-rs1801020 was significantly associated with an increase at disease severity. The presence of *SERPING1*-rs28362944 increased 4.2-fold the probability of LTP need. *SERPINA1*-rs17580 and *SERPINE1*-rs6092 were significantly associated with the age at disease onset, while *CPN1*-rs61751507 and *F2*-rs1799963 were significantly associated with need for LTP and disease severity, respectively. *KLKB1*-rs3733402 and *KLK1*-rs5515 were associated with both the age at disease onset and the disease severity.

IV. The role of rare variants (MAF<1%) in the genetics of complex diseases like C1-INH-HAE is not easy to define and further investigation is required. However, as for *SERPING1,* where functional studies are not always an option, submission of genetic data and sharing among laboratories and physicians should facilitate the interpretation of their pathogenicity and the implementation in genomic-driven medicine.

APPENDIX I

Table 1. Devices, pipets and plastic material used for the analytical investigation of the expression of BK receptors.

Material	Manufacturer-Provider	Serial No/REF/ Cat #
Device		
NanoDrop 2000 Spectrophotometer	Thermo Scientific	
GeneQuant Pro	Amersham BioSciences	95804
pHmeter pH700 EUTECH Instruments	Fischer Scientific	678819
TransBlot Turbo	BIORAD	690BR019417
Molecular Imager ChemiDoc XRS+ Model No Universal Hood II	BIORAD	721BR02750
Consort Bioblock E455 Microcomputer Electrophoresis Power Supply	BIOBLOCK SCIENTIFIC	
BD FACS Canto II	Becton Dickinson	338960
FACS Tri		
Beckman Coulter Allegra X30R	Beckman Coulter	ALZ14H017
CR422	Thermo Scientific	29212423/1175330
MiniSpin plus	Eppendorf	5453 06616
Microscope Terex		
AxioVert 135M	Zeiss Germany	
OHAUS Traveller TA302		B327533901
Luminoscan Ascent	Labsystems	
iCycler	BIORAD	582BR 001740
Environmental Incubator Shaker	New Brunswick Scientific Co, Inc.	
KOVA Glasstic Slide 10 with Grids	KOVA	87144
Waterbath	BIOBLOCK SCIENTIFIC	33194
Waterbath	BIOBLOCK SCIENTIFIC	86611
Vortex Genie 2 G-560E	Scientific Industries	#2.86805
Magnetic Agitator	RSLab	81121050142
Mini See-saw rocker agitator SSM4	Stuart	R000100606
Pipet Aid Portable XP	Drummond	4-000-201
Pipets		
Single chain pipet 0.5-10μl	ErgoOne	57100-0510
Single chain pipet 2-20μl	ErgoOne	57100-0220
Single chain pipet 10-100μl	ErgoOne	57100-1100
Single chain pipet 20-200μl	ErgoOne	57100-2200
Single chain pipet 100-1000μl	ErgoOne	57110-1000
25ml FALCON	Corning	357525
10ml FALCON	Corning	357530
5ml FALCON	Corning	357529
10ml Nunc	Thermo Scientific	170361
5ml Nunc	Thermo Scientific	170360
1ml FALCON	Corning	357521
Flasks and Petri		
25	Corning	353082
75	Corning	353136

175	Thermo Scientific	178883
Petri Dishes Falcon	Corning	353003
96-well plate	Corning Falcon	353072
24-well plate	Costar	3526
6-well plate	Costar	3516
Tubes		
Falcon 50ml	Corning	352070
Falcon 15ml	Corning	352096
Falcon 5ml	Corning	352054
Microtubes 0.65ml multinatural		
Microtubes 1.5 ml clearline Natural		
Cryovial Tubes	Thermo Scientific	377267
Syringes and filters		
Plastipak 60ml	Becton Dickinson	300866
Plastipak 10ml	Becton Dickinson	302188
Plastipak 5ml	Becton Dickinson	302187
Plastipak 1ml	Becton Dickinson	303172
Filtre Millipore 0.22 Express PES membrane	Millipore	SLGP033RS
Tips		
1-10µl Filter tips	Clearline	130340CL
1-330µl One touch	Sorenson Bioscience	10330
100-1000µl Safety Space Filter Tips	Satorius	791001F
Other Materials		
Gloves Polysem Medical NR Latex Free	Polysem Medical	171013040169
Cell scraper 24cm	TPP	99002
Amersham Protran 0.45µm NC 300mm x 4m 1 roll/PK	Dominique Dutscher	10600002
Whatman Paper 3MM CHR	Dominique Dutscher	036347
Filter Paper	Dominique Dutscher	074021

Table 2. Solutions, reagents and antibodies used for the analytical investigation of the expression of BK receptors.

Material	Manufacturer	Serial No/REF/ Cat #
Solutions and Reagents		
DMEM	Gibco, life technologies	41966-029
RPMI	Gibco, life technologies	31870-025
FBS	Gibco, life technologies	10270-106
P/S	Gibco, life technologies	15140-163
B-mercaptoethanol	Gibco, life technologies	31350-010
L-glutamine	Gibco, life technologies	25030-032
Trypsin 0.05%-EDTA	Thermo Scientific	25300-054
Trypan Blue 0.5% solution in 0.85% saline	Flow Laboratories	1691049 (7-0700)
Bromophenol Blue-Xylene	Sigma Aldrich	B3269-5ml
Glycine (aminoacetic acid)	EUROMEDEX	26-128-6405-C
Tween 20	Prolabo	28829296
Tris Base	EUROMEDEX	200923A
Sodium dodecyl Sulfate	vWR BDH Prolabo	27.926.295
Ammonium peroxodisulfate	vWR BDH Prolabo	21300.293
NaCl	EUROMEDEX	1112-A
D(+)-Saccharose	EUROMEDEX	200-301B
EDTA Disodium Salt	Sigma Chemical Company	ED255
Bovine Serum Albumin	Sigma Aldrich	A7906-100G
Na_2HPO_4 (2H2O)	EUROMEDEX	1309
KCl	Merck	1.04936.0500
KH_2PO_4	Prolabo	26.927.292
Deoxycholic Acid	Sigma Chemical Company	D6780
Triton X 100	Sigma Aldrich	T8787-100ml
Polyethylenimine, branched	Sigma Aldrich	408727
Turbofect Transfection Reagent	Thermo Scientific	R0531
G418 50mg/ml Sulfate Salt	Euromedex	EU0601
Luria Broth's Base	Invitrogen	12795-027
Agar	Invitrogen	30391-023
BactoYeast Extract	Becton Dickinson	212750
Bacto Tryptone	Becton Dickinson	211705
Library Efficiency DH5a Competent Cells	Invitrogen	18263-012
HiSpeed Plasmi Maxi Kit	Qiagen	
Acrylamide/bis-acrylamide 40% solution	Sigma Aldrich	A7168-100ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Sigma Aldrich	T9281-25ml
TNFα	Sigma Aldrich	T0157-10UG
INFγ Human	BioVendor Laboratory Medicine	RP17620630020
TRIZOL Reagent	Invitrogen	15596026/15596018
Ecotainer Aqua B.Braun	B.Braun	75/12604016/0410
Clarity Western ECL Substrate	BIORAD	170-5061
Superscript III First Strand Synthesis	Invitrogen	

Supermix for qRT-PCR		
RapidOut DNA Removal Kit	Thermo Scientific	K2981
PageRuler Prestained Protein Ladder, 10 to 180 kDa	Thermo Scientific	26616
Perm Wash Buffer	Becton Dickinson	554723
Fixation and permeabilization Solution	Becton Dickinson	554722
Ethanol absolute	vWR Chemicals	20821.296 1L
Antibodies and Serums		
anti-BDK1 Antibody	ProSciΨ	PSI-63-618
anti-B2 Bradykinin Receptor	Alomone Labs	ABR012
anti-BDKRB2 [EPR5646]	AbCam	Ab134118
ECL Anti rabbit IgG, Horseradish Peroxidase-linked, whole antibody	GE Healthcare UK	NA934
anti-GFP monoclonal antibody (clone B-2) Alexa Fluor 488 100μg/2ml	Santa Cruz Biotechnology, Inc.	SC9996 AF488
AlexaFluor 647 conjugated AffiniPure F(ab)2 Fragment Donkey anti-rabbit IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc.	711-606-152
Normal Donkey Serum	Jackson ImmunoResearch Laboratories, Inc.	017-000-121
anti-VE cadherin (BV9)	Santa Cruz Biotechnology, Inc.	sc-52751
anti-mouse IgG-HRP	GE Healthcare UK	NA931

Table 3. Materials used for the analysis of plasminogen activation*.

**Table 3 includes materials which have not been previously mentioned in tables 1 and 2.*

Material	**Manufacturer**	**Serial No/REF/Cat #**
Device		
Multiskan GO 1.00.40	Thermo Scientific	1510-02417
Microplate reader	TECAN Austria, GmbH	
Vortex Genie 2 G-560E	Scientific Industries	#2.86805
pHmeter pH700 EUTECH Instruments	Fischer Scientific	678819
Solutions and Reagents		
Human Plasmin	Enzyme Research Laboratories	HPlas
Human Plasminogen	Enzyme Research Laboratories	
tPA, Alteplase™	Boehringer Ingelheim	Actilyse®
uPA, Urokinase 100.000 I.U	EuMedica Pharmaceuticals	Actosolv®
Streptokinase	Sigma Aldrich	S3134-10KU
Fibrinogen	Sigma Aldrich	F3879-250mg
S-2403™	Chromogenix	

Table 4. Devices, pipets and plastic material used for the detection of variants in the genetic investigation of C1-INH-HAE patients.

Material	Manufacturer	Serial No/REF/ Cat #
Device		
DNA extractor, iprep	Thermo Scientific	0809D0094
Spectrophotometer Genova Plus	Jenway	51856
Thermal Cycler Veriti R 96-well	Life Technologies	2990224332
-20°C FREEZER FFGL2330V22	Thermo Scientific	0116762001120412
+4°C FREEZER Medica lux 700	FIOCCHETTI	26944
Vortex GVLab	GILSON	01.438505
Centrifuge CL40	Thermo Scientific	41011433
Qubit Fluorometer 2.0	Life Technologies	2286613946
Genetic Analyser ABI3730xl	Life Technologies	1414-002
Ion Reporter server system	Thermo Scientific	743V8N2
Ion Chef 4484178	Life Technologies	242470092
Ion S5 XL SYSTEM	Life Technologies	2772817010125
Pipets		
Single chain pipet 0.5-10µl	Gilson	F144802
Single chain pipet 2-20µl	Gilson	F123600
Single chain pipet 10-100µl	Gilson	F123615
Single chain pipet 20-200µl	Gilson	F123601
Single chain pipet 100-1000µl	Gilson	F123602
8-chain pipet x20M	Gilson	F81005
8-chain pipet x300M	Gilson	F81011
Tubes and plates		
Eppendorf Tubes Conical 5ml	Eppendorf	15188344
50ml FALCON	Thermo Scientific	
5ml FALCON	Thermo Scientific	
Microcentrifuge Tube 1.7ml	Axygen	MCT-175-C
PCR TUBES 0.5 ml thin wall, clear	Axygen	PCR-05-C
0,2 ml tubes RNAse DNase free	Costar	6571
MicroAmp Optical 96-Well Reaction Plate no Barcode	Applied Biosystems	N8010560
FG-OPTICAL CAP (8 CAPS/STRIPS) EACH	Applied Biosystems	4323032
Tips		
TIPS D200	Gilson	F161930
TIPS DL10	Gilson	F161450
TIPS DF1200ST	Gilson	F171803
TIPS DF1000ST	Gilson	F171703
TIPS DF300ST	Gilson	F171603
TIPS D200ST	Gilson	F171301
TIPS D100ST	Gilson	F171403
TIPS DFL10ST	Gilson	F171203
Other Materials		
Latex examination Gloves S	Mediworld	NLGDS
FG, 7700 Compression Pad Kit	Thermo Scientific	4312639
96well magnetic separator	Invitrogen	CS15096
MagnaRack	Invitrogen	CS15000

Table 5. Solutions, reagents and kits used for the detection of variants in the genetic investigation of C1-INH-HAE patients.

Material	Manufacturer	Serial No/REF/ Cat #
Solutions and Reagents		
Ethanol, absolute	Scharlau	ET00162500
10x TAE Buffer, Ultra Pure	Gibco	15558-042
Agarose	Invitrogen	16500-500
SYBR® Safe DNA Gel Stain (400μl)	Invitrogen	S33102
iPrep Pure Link DNA blood kit	Invitrogen	IS-10005
Ethyenediaminetetraacetic Acid,EDTA	Scharlau	AC09630250
Sodium acetate, anhydrous	Scharlau	SO00360500
Photometer Cuvette UV 70-550 Ml	BRAND	759235 (#10386712)
Hi-Di Formamide	Applied Biosystems	4311320
100 bp DNA Ladder (50μg)	Invitrogen	15628-019
DreamTaq DNA Polymerase EA	Thermo Fisher	EP0702
Long Amp Taq DNA Polymerase		M0323S
BTL, 5X SEQ BUFFER MEDIUM EACH	Thermo Fisher	4336699
3730 Buffer 10x with EDTA	Applied Biosystems	4335613
50mM Magnesium Chloride	SIGMA-ALDRICH	B0300-1VL
POP-7 Polymer for 3730/3730xl DNA Analyzers	Applied Biosystems	4335615
Ion Ampliseq Library kit 2.0-96LV	Life Technlogies	4480441
Ion Xpress Barcode Adapters 1-96 kit	Thermo Fisher	4474517
Qubit® dsDNA HS Assay Kits	Life Technologies	Q32854
Agencourt® AMPure® XP	BECKMAN COULTER	A63881
NGS primers HAE-SERPING1	Ion Torrent	IAD92366_241
Ion S5 Chef Solutions	Thermo Fisher	A27754
Ion S5 Sequencing Solutions	Thermo Fisher	A27767
Ion S5 Sequencing Reagents	Thermo Fisher	INS10122841
ION 520 / 530 EXT KΓ-CHEF EA	Thermo Fisher	A30670
ION 520 CHIP KIT EACH	Thermo Fisher	A27762
ION 530 CHIP KIT EACH	Thermo Fisher	A27764
Ion Chip Storage Box	Thermo Fisher	A27141
Ion PGM Hi-Q Wash 2 Bottle Kit	Life Technologies	A25591
ION PGM 2.5 L WASTE BOTTLE	Life Technologies	4482565
Ion PGM™ Calibration Standard	Life Technologies	A27832
Ion PGM™ Enrichment Beads	Life Technologies	4478525
Ion 318 Chip Kit V2 BC-4-PACK	Life Technologies	4488146
Ion 316 sequencing chips	Life Technologies	4466616
Ion 316 Chip Kit V2 BC-4 Pack	Life Technologies	4488145
Ion PGM™ Hi-Q™ OT2 Kit	Life Technologies	A27739
Ion PGM™ Hi-Q™ Sequencing Kit	Life Technologies	A25592
Ion PGM™ Hi-Q™ View Sequencing Kit	Life Technologies	A30044
Ion 314 Chip Kit V2 BC-8-PACK	Thermo Fisher	4488144
ION PGM TEMPLATE IA 500 KIT EA	Thermo Fisher	A24622

APPENDIX II

Table 1. Specifications of the custom NGS platform.

Encoded protein	Function	Gene	Chr. Position	Reference	Coverage %	Exons	OMIM*
Alpha-2-Macroglobulin	*Serine protease inhibitor*	***A2M***	12p13.31	NM_000014.4	99.7	36	103950
Angiotensin I-Converting Enzyme	*Enzyme involved in bradykinin breakdown*	***ACE***	17q23.3	NM_000789.3	97.4	26	106180
Androgen Receptor	*Endothelial cell receptor*	***AR***	Xq12	NM_000044.3	92.3	9	313700
Bradykinin Receptor B1	*Endothelial cell receptor*	***BDKRB1***	14q32.2	NM_000710.3	100	1	603337
Bradykinin Receptor B2	*Endothelial cell receptor*	***BDKRB2***	14q32.2	NM_000623.3	100	2	113503
Complement component C1q-binding protein	*Endothelial cell receptor*	***C1QBP***	17p13.2	NM_001212.3	100	6	601269
Complement component C1r	*Serine protease*	***C1R***	12p13.31	NM_001733.4	100	9	608974
Complement component C1s	*Serine protease*	***C1S***	12p13.31	NM_201442.2	100	11	120580
Carboxypeptidase M	*Enzyme involved in bradykinin breakdown*	***CPM***	12q15	NM_001005502.2	100	8	114860
Carboxypeptidase N	*Enzyme involved in bradykinin breakdown*	***CPN1***	10q24.2	NM_001308.2	92.3	9	603103
Dipeptidyl peptidase IV	*Enzyme involved in bradykinin breakdown*	***DPP4***	2q24.2	NM_001935.3	100	26	102720
Neutrophil-expressed elastase	*Serine protease*	***ELANE***	19p13.3	NM_001972.2	89.7	5	130130
Oestrogen-related receptor alpha	*Endothelial cell receptor*	***ESRRA***	11q13.1	NM_004451.4	94.0	7	601998
Coagulation factor XI	*Other*	***F11***	4q35.2	NM_000128.3	100	14	264900
Coagulation factor XII	*Serine protease*	***F12***	5q35.3	NM_000505.3	96.8	14	610619
Factor XIII, A1 Subunit	*Other*	***F13A1***	6p25.1	NM_000129.3	100	14	134570
Factor XIII, B Subunit	*Other*	***F13B***	1q31.3	NM_001994.2	100	12	134580
Coagulation Factor II	*Serine protease*	***F2***	11p11.2	NM_000506.3	95.7	14	176930
G protein-coupled oestrogen receptor	*Endothelial cell receptor*	***GPER1***	7p22.3	NM_001098201.1	100	1	601805

Histamine receptor H1	*Endothelial cell receptor*	***HRH1***	3p25.3	NM_001098212.1	100	1	600167
Heat-shock protein, 90KD, alpha, class A, member 1	*Other*	***HSP90AA1***	14q32.31	NM_001017963.2	99.5	12	140571
Eosinophil differentiation factor	*Other*	***IL5***	5q31.1	NM_000879.2	100	4	147850
Kallikrein 1	*Serine protease*	***KLK1***	19q13.33	NM_002257.3	100	5	147910
Kallikrein-related peptidase 2	*Serine protease*	***KLK2***	19q13.33	NM_005551.4	100	8	147960
Kallikrein-related peptidase 3	*Serine protease*	***KLK3***	19q13.33	NM_001648.2	98.9	7	176820
Plasma Kallikrein B (Prekallikrein)	*Serine protease*	***KLKB1***	4q35.2	NM_000892.3	100	14	229000
Kininogen 1	*Other*	***KNG1***	3q27.3	NM_001102416.2	99.2	12	612358
Keratin 1, Type II	*Endothelial cell receptor*	***KRT1***	12q13.13	NM_006121.3	100	9	139350
Mannan-binding lectin serine protease 1	*Serine protease*	***MASP1***	3q27.3	NM_139125.3	100	18	600521
Mannan-binding lectin serine protease 2	*Serine protease*	***MASP2***	1q36.22	NM_006610.3	94.8	12	605102
Membrane metalloendopeptidase (neutral endopeptidase, enkephalinase, neprilysin, CALLA)	*Enzyme involved in bradykinin breakdown*	***MME***	3q25.2	NM_007288.2	100	22	120520
Myeloperoxidase	*Other*	***MPO***	17q22	NM_000250.1	97.4	12	606989
Tissue plasminogen activator (tPA)	*Serine protease*	***PLAT***	8p11.21	NM_000930.3	100	13	173370
Urinary plasminogen activator (urokinase, plasminogen activator)	*Serine protease*	***PLAU***	10q22.2	NM_001001791.2	100	11	191840
Plasminogen activator receptor, Urokinase Type (uPAR)	*Endothelial cell receptor*	***PLAUR***	19q13.31	NM_002659.3	93.8	8	173391
Plasminogen	*Serine protease*	***PLG***	6q26	NM_000301.3	95.9	20	173350
Prolylcarboxypeptidase (Lysosomal Pro-X carboxypeptidase)	*Enzyme involved in des*Arg^{9}*-BK breakdown*	***PRCP***	11q14.1	NM_199418.2	100	10	176785
Protein C	*Serine protease*	***PROC***	2q14.3	NM_000312.3	97.1	8	612283

Serine protease inhibitor, clade a, member 1 (a1-antitrypsin)	*Serine protease inhibitor*	***SERPINA1***	14q32.13	NM_001127700.1	100	4	107400
Serine protease inhibitor, clade b, member 2, Plasminogen activator inhibitor 2 (PAI-2)	*Serine protease inhibitor*	***SERPINB2***	18q21.3-q22.1	NM_002575.2	100	7	173390
Serine protease inhibitor, clade c, member 1 (Antithrombin III)	*Serine protease inhibitor*	***SERPINC1***	1q25.1	NM_000488.3	100	7	107300
Serine protease inhibitor, clade e, member 1 (Nexin, PAI-1)	*Serine protease inhibitor*	***SERPINE1***	7q22.1	NM_000602.4	100	8	173360
Serine protease inhibitor, clade f, member 1 (a2-antiplasmin)	*Serine protease inhibitor*	***SERPINF1***	17p13.3	NM_002615.5	97.0	7	172860
Serine protease inhibitor, clade g, (C1-INH)	*Serine protease inhibitor*	***SERPING1***	11q12.1	NM_000062.2	100	8	608860
Preprotachykinin 1	*Other, Precursor of Substance P*	***TAC1***	7q21.3	NM_003182.2	100	6	162320
Toll-like receptor 2	*Endothelial cell receptor*	***TLR2***	4q31.3	NM_003264.3	100	1	603028
Toll-like receptor 4	*Endothelial cell receptor*	***TLR4***	9q33.1	NM_138554.4	100	5	603030
Toll-like receptor 9	*Endothelial cell receptor*	***TLR9***	3p21.2	NM_017442.3	97.7	2	605474
Tumor necrosis factor	*Other*	***TNF***	6p21.33	NM_000594.3	100	4	191160
Tryptase alpha/beta 1	*Other*	***TPSAB1***	16p13.3	NM_003294.3	66.9	5	191080
Tryptase delta 1	*Other*	***TPSD1***	16p13.3	NM_012217.2	98.1	5	609272
Tryptase gamma 1	*Other*	***TPSG1***	16p13.3	NM_012467.3	100	6	609341
XProlyl aminopeptidase 1	*Enzyme involved in tachykinin breakdown*	***XPNPEP1***	10q25.1	NM_020383.3	100	21	602443
XProlyl aminopeptidase 2	*Enzyme involved in des*Arg^{9}*-BK breakdown*	***XPNPEP2***	Xq26.1	NM_003399.5	91.2	21	300145
XProlyl aminopeptidase 3	*Enzyme involved in des*Arg^{9}*-BK breakdown*	***XPNPEP3***	22q13.2	NM_022098.3	98.6	11	613553

Table 2. Overall coverage of the genes and missed bases.

Name	Chr	No of Amplicons	Total Bases	Covered Bases	Missed Bases	Overall Coverage
A2M	chr12	36	6225	6204	21	99.7%
ACE	chr17	34	5420	5379	41	99.2%
AR	chrX	21	3233	3225	8	99.8%
BDKRB1	chr14	6	1112	1112	0	100%
BDKRB2	chr14	6	1276	1276	0	100%
C1QBP	chr17	8	1149	1149	0	100%
C1R	chr12	15	2336	2336	0	100%
C1S	chr12	14	2617	2617	0	100%
CPM	chr12	9	1732	1732	0	100%
CPN1	chr10	11	1827	1825	2	99.9%
DPP4	chr2	27	3601	3601	0	100%
ELANE	chr19	8	1054	1041	13	98.8%
ESRRA	chr11	11	1572	1499	73	95.4%
F11	chr4	16	2578	2578	0	100%
F12	chr5	16	2548	2544	4	99.8%
F13A1	chr6	16	2899	2899	0	100%
F13B	chr1	18	2586	2586	0	100%
F2	chr11	17	2569	2569	0	100%
GPER1	chr7	6	1178	1178	0	100%
HRH1	chr3	8	1514	1514	0	100%
HSP90AA1	chr14	20	3165	3149	16	99.5%
IL5	chr5	4	605	605	0	100%
KLK1	chr19	8	1039	1039	0	100%
KLK2	chr19	6	1073	1073	0	100%
KLK3	chr19	7	1173	1160	13	98.9%
KLKB1	chr4	14	2617	2617	0	100%
KNG1	chr3	15	2566	2546	20	99.2%
KRT1	chr12	14	2385	2385	0	100%
MASP1	chr3	22	3937	3937	0	100%
MASP2	chr1	19	2675	2675	0	100%
MME	chr3	25	3353	3353	0	100%
MPO	chr17	16	2838	2803	35	98.8%
PLAT	chr8	14	2339	2339	0	100%
PLAU	chr10	13	1880	1880	0	100%
PLAUR	chr19	9	1500	1500	0	100%
PLG	chr6	23	3387	3387	0	100%
PRCP	chr11	14	2054	2054	0	100%
PROC	chr2	12	1786	1735	51	97.1%
SERPINA1	chr14	9	1457	1457	0	100%
SERPINB2	chr18	9	1598	1598	0	100%
SERPINC1	chr1	11	1745	1745	0	100%
SERPINE1	chr7	10	1609	1609	0	100%
SERPINF1	chr17	10	1607	1558	49	97%
SERPING1	chr11	199	14692	14692	0	100%
TAC1	chr7	6	690	690	0	100%

TLR2	chr4	13	2405	2405	0	100%
TLR4	chr9	16	2670	2670	0	100%
TLR9	chr3	19	3199	3125	74	97.7%
TNF	chr6	5	902	902	0	100%
TPSAB1	chr16	7	1078	1033	45	95.8%
TPSD1	chr16	8	979	960	19	98.1%
TPSG1	chr16	8	1266	1266	0	100%
XPNPEP1	chr10	21	3051	3051	0	100%
XPNPEP2	chrX	23	3075	3075	0	100%
XPNPEP3	chr22	15	2178	2147	31	98.6%

APPENDIX III

Table 1. List of rare (MAF<1%) benign, likely benign and VUS variants detected in C1-INH-HAE patients. The variants which were previously presented in "Results:Part III" were excluded from this table.

Families	Patients	Genes	Transcript	Location	Coding	aa change	dbSNP	Varsome ACMG	exon	Locus	Variant Effect	Genotype	Ref
1	1	*F12*	NM_000505.3	exonic	c.41T>C	p.Leu14Ser	rs143809932	VUS	1	chr5:176836488	missense	A/G	A
2	2	*F12*	NM_000505.3	exonic	c.1025C>T	p.Pro342Leu	rs2230939	Benign	10	chr5:176831085	missense	G/A	G
1	2	*F12*	NM_000505.3	exonic	c.1553T>C	p.Phe518Ser	rs752701059	VUS	13	chr5:176829678	missense	A/G	A
1	1	*F12*	NM_000505.3	exonic	c.418C>G	p.Leu140Val	rs35515200	Benign	6	chr5:176832166	missense	G/C	G
1	2	*F13A1*	NM_000129.3	exonic	c.1552A>C	p.Asn518His	rs771866520	Likely Benign	12	chr6:6175008	missense	T/G	T
1	1	*F13A1*	NM_000129.3	exonic	c.1225C>T	p.Arg409Trp	rs752786253	VUS	10	chr6:6196110	missense	G/A	G
3	3	*F13A1*	NM_000129.3	exonic	c.1832C>G	p.Ala611Gly	rs777280256	VUS	13	chr6:6167767	missense	G/C	G
1	3	*F13A1*	NM_000129.3	exonic	c.1730C>T	p.Thr577Met	rs143711562	Likely Benign	12	chr6:6174830	missense	G/A	G
2	3	*F13A1*	NM_000129.3	exonic	c.1766T>A	p.Leu589Gln	rs5983	Likely Benign	13	chr6:6167833	missense	A/T	A
1	1	*F13B*	NM_001994.2	exonic	c.1318G>A	p.Glu440Lys	rs766408940	Likely Benign	8	chr1:197024881	missense	C/T	C
1	1	*F13B*	NM_001994.2	exonic	c.1730T>G	p.Leu577Trp	rs764924610	Likely Benign	10	chr1:197019835	missense	A/C	A
4	4	*F13B*	NM_001994.2	exonic	c.1025T>C	p.Ile342Thr	rs17514281	Benign	7	chr1:197026289	missense	A/G	A
1	1	*F13B*	NM_001994.2	exonic	c.1163A>T	p.Glu388Val	rs5991	Likely Benign	7	chr1:197026151	missense	T/A	T
1	1	*F2*	NM_000506.4	exonic	c.1547G>C	p.Gly516Ala	rs144885724	VUS	12	chr11:46751004	missense	G/C	G
1	1	*F2*	NM_000506.4	utr_5	c.-29T>C	NA	rs757072408	VUS	1	chr11:46740757		T/C	T
1	1	*F2*	NM_000506.4	exonic	c.1542C>A	p.Asn514Lys	rs199772906	Likely Benign	12	chr11:46750999	missense	C/A	C
1	1	*F2*	NM_000506.4	exonic	c.284C>T	p.Thr95Met	rs189436668	VUS	4	chr11:46742358	missense	C/T	C
1	1	*ACE*	NM_000789.3	exonic	c.731A>G	p.Tyr244Cys	rs3730025	Benign	5	chr17:61557773	missense	A/G	A
1	1	*ACE*	NM_000789.3	exonic	c.1681C>T	p.Arg561Trp	rs4314	Likely Benign	11	chr17:61561304	missense	C/T	C
3	3	*ACE*	NM_000789.3	exonic	c.1060G>A	p.Gly354Arg	rs56394458	Benign	7	chr17:61559041	missense	G/A	G
1	2	*ACE*	NM_000789.3	exonic	c.2749C>G	p.Pro917Ala	rs748317639	Likely Benign	19	chr17:61568579	missense	C/G	C
1	1	*ACE*	NM_000789.3	exonic	c.2677G>A	p.Asp893Asn	Novel	Likely Benign	18	chr17:61568350	missense	G/A	G
1	2	*ACE*	NM_000789.3	exonic	c.3521delG	p.Gly1174fs	rs754265941	VUS	24	chr17:61574173	frameshift	TG/T	TG

3	3	*ACE*	NM_000789.3	exonic	c.3836G>A	p.Arg1279Gln	rs4980	Benign	25	chr17:61574642	missense	G/A	G
3	3	*ACE*	NM_000789.3	exonic	c.3108C>A	p.Asn1036Lys	rs142947404	Likely Benign	20	chr17:61570992	missense	C/A	C
1	1	*XPNPEP2*	NM_003399.5	exonic	c.1177G>A	p.Asp393Asn	rs199809120	Benign	12	chrX:128888517	missense	G/A	G
1	3	*XPNPEP2*	NM_003399.5	exonic	c.1567G>A	p.Gly523Ser	rs1388103985	VUS	17	chrX:128895216	missense	G/A	G
1	1	*XPNPEP2*	NM_003399.5	utr_5	c.-166C>T	NA	rs187905432	Benign	1	chrX:128873024		C/T	C
2	2	*XPNPEP2*	NM_003399.5	exonic	c.644C>T	p.Thr215Ile	rs138365897	Benign	8	chrX:128884450	missense	C/T	C
1	1	*XPNPEP2*	NM_003399.5	utr_3	c.*31C>T	NA	rs754445186	VUS	21	chrX:128902492		C/G	C
1	1	*XPNPEP2*	NM_003399.5	exonic	c.604C>G	p.Gln202Glu	Novel	VUS	7	chrX:128881696	missense	G/G	C
1	1	*XPNPEP3*	NM_022098.3	exonic	c.1244G>A	p.Arg415Gln	rs79385822	Likely Benign	9	chr22:41320373	missense	G/A	G
1	1	*DPP4*	NM_001935.3	exonic	c.740A>C	p.Gln247Pro	rs201266487	VUS	9	chr2:162891708	missense	T/G	T
1	1	*DPP4*	NM_001935.3	exonic	c.1393G>T	p.Ala465Ser	Novel	Likely Benign	16	chr2:162875266	missense	C/A	C
1	1	*DPP4*	NM_001935.3	exonic	c.1228C>G	p.Leu410Val	rs138404587	Likely Benign	14	chr2:162876755	missense	G/C	G
1	1	*DPP4*	NM_001935.3	exonic	c.942G>T	p.Gln314His	Novel	VUS	11	chr2:162881395	missense	C/A	C
1	1	*DPP4*	NM_001935.3	exonic	c.1582A>G	p.Met528Val	rs200348396	Likely Benign	19	chr2:162870961	missense	T/C	T
1	1	*DPP4*	NM_001935.3	utr_3	c.*81A>C	NA	rs201341836	VUS	26	chr2:162849712		T/G	T
1	2	*DPP4*	NM_001935.3	exonic	c.248A>G	p.Tyr83Cys	Novel	Likely Benign	4	chr2:162903462	missense	T/C	T
1	1	*DPP4*	NM_001935.3	exonic	c.796G>A	p.Val266Ile	rs56179129	Likely Benign	10	chr2:162890142	missense	C/T	C
1	1	*CPM*	NM_001005502.2	exonic	c.472G>A	p.Asp158Asn	rs747472128	VUS	5	chr12:69264139	missense	C/T	C
1	2	*CPM*	NM_001005502.2	exonic	c.432G>C	p.Arg144Ser	rs752185553	VUS	5	chr12:69264179	missense	C/G	C
3	3	*CPM*	NM_001005502.2	exonic	c.440A>G	p.Tyr147Cys	rs140860259	Likely Benign	5	chr12:69264171	missense	T/C	T
3	3	*CPN1*	NM_001308.2	utr_3	c.*89A>G	NA	rs45546135	Benign	9	chr10:101802095		T/C	T
1	1	*PRCP*	NM_199418.3	exonic	c.253A>G	p.Thr85Ala	rs140677570	Likely Benign	3	chr11:82571138	missense	T/C	T
1	1	*MME*	NM_007288.2	exonic	c.2067C>A	p.Asn689Lys	rs146536523	VUS	21	chr3:154889992	missense	C/A	C
3	7	*MME*	NM_007288.2	exonic	c.674G>C	p.Gly225Ala	rs147564881	Likely Benign	8	chr3:154836554	missense	G/C	G
1	2	*MME*	NM_007288.2	exonic	c.1946T>G	p.Ile649Ser	rs184666602	VUS	20	chr3:154886552	missense	T/G	T
1	1	*SERPINA1*	NM_001127700.1	exonic	c.1177C>T	p.Pro393Ser	rs61761869	VUS	5	chr14:94844866	missense	G/A	G
1	1	*SERPINA1*	NM_001127700.1	exonic	c.514G>T	p.Gly172Trp	rs112030253	Likely Benign	2	chr14:94849061	missense	C/A	C
1	1	*SERPINC1*	NM_000488.3	exonic	c.731T>A	p.Val244Asp	rs769820027	VUS	4	chr1:173879923	missense	A/T	A
2	3	*BDKRB1*	NM_000710.3	exonic	c.844C>T	p.Arg282Ter	rs145322761	Likely Benign	3	chr14:96730863	nonsense	C/T	C

5	6	*BDKRB1*	NM_000710.3	exonic	c.721G>A	p.Gly241Arg	rs45528332	Likely Benign	3	chr14:96730740	missense	G/A	G
2	4	*BDKRB1*	NM_000710.3	exonic	c.342C>G	p.Asn114Lys	rs33997210	Likely Benign	3	chr14:96730361	missense	G/G	C
1	1	*BDKRB1*	NM_000710.3	exonic	c.1054C>T	p.Arg352Trp	rs140613086	Likely Benign	3	chr14:96731073	missense	C/T	C
1	1	*BDKRB2*	NM_000623.3	utr_5	c.-20A>G	NA	rs1413929175	VUS	2	chr14:96703425		A/G	A
2	2	*BDKRB2*	NM_000623.3	exonic	c.1010A>G	p.Lys337Arg	rs144659195	Likely Benign	3	chr14:96707675	missense	A/G	A
1	1	*BDKRB2*	NM_000623.3	exonic	c.1006G>A	p.Gly336Ser	Novel	VUS	3	chr14:96707671	missense	G/A	G
1	1	*BDKRB2*	NM_000623.3	exonic	c.607G>A	p.Asp203Asn	rs772874206	VUS	3	chr14:96707272	missense	G/A	G
1	1	*KNG1*	NM_001102416.2	utr_5	c.-46T>A	NA	rs374559724	Benign	1	chr3:186435286		T/A	T
1	1	*KNG1*	NM_001102416.2	utr_3	c.*35T>C	NA	Novel	VUS	10	chr3:186460155		T/C	T
1	1	*KLKB1*	NM_000892.3	exonic	c.7T>A	p.Leu3Ile	Novel	Likely Benign	2	chr4:187149356	missense	T/A	T
1	1	*KLKB1*	NM_000892.3	exonic	c.1325A>C	p.Gln442Pro	rs4253316	Benign	12	chr4:187175753	missense	A/C	A
4	8	*KLK2*	NM_005551.4	exonic	c.155G>A	p.Gly52Asp	rs139063242	VUS	2	chr19:51378085	missense	G/A	G
1	1	*KLK2*	NM_005551.4	exonic	c.227G>A	p.Gly76Asp	rs142081380	VUS	3	chr19:51379748	missense	G/A	G
1	1	*KLK2*	NM_005551.4	exonic	c.460G>A	p.Ala154Thr	rs572630957	Likely Benign	3	chr19:51379981	missense	G/A	G
1	1	*KLK3*	NM_001648.2	exonic	c.629C>T	p.Ser210Leu	rs61729813	Likely Benign	4	chr19:51361850	missense	C/T	C
8	9	*KLK3*	NM_001648.2	exonic	c.629C>G	p.Ser210Trp	rs61729813	Likely Benign	4	chr19:51361850	missense	C/G	C
1	1	*KLK3*	NM_001648.2	exonic	c.515A>G	p.Gln172Arg	Novel	VUS	4	chr19:51361736	missense	A/G	A
1	1	*KLK3*	NM_001648.2	exonic	c.170C>T	p.Pro57Leu	rs545098001	VUS	2	chr19:51359619	missense	C/T	C
3	5	*PLG*	NM_000301.3	exonic	c.1567C>T	p.Arg523Trp	rs4252129	Benign	12	chr6:161152905	missense	C/T	C
1	1	*PLG*	NM_000301.3	exonic	c.1380T>A	p.Ser460Arg	rs116573785	Benign	11	chr6:161152206	missense	T/A	T
1	2	*PLG*	NM_000301.3	exonic	c.598A>G	p.Thr200Ala	rs149145958	VUS	6	chr6:161135876	missense	A/G	A
2	3	*PLG*	NM_000301.3	exonic	c.782G>A	p.Arg261His	rs4252187	Likely Benign	7	chr6:161137790	missense	G/A	G
1	1	*PLG*	NM_000301.3	exonic	c.266G>A	p.Arg89Lys	rs143079629	Benign	3	chr6:161128812	missense	G/A	G
1	1	*PLG*	NM_000301.3	exonic	c.1481C>T	p.Ala494Val	rs4252128	Benign	12	chr6:161152819	missense	C/T	C
1	1	*SERPINF1*	NM_002615.6	exonic	c.808G>A	p.Gly270Arg	rs369314029	VUS	7	chr17:1679847	missense	G/A	G
1	1	*PLAT*	NM_000930.3	exonic	c.1384C>T	p.Arg462Trp	rs545287158	Likely Benign	13	chr8:42036561	missense	G/A	G
1	1	*PLAT*	NM_000930.3	exonic	c.928C>T	p.Arg310Cys	rs151006962	Likely Benign	10	chr8:42038165	missense	G/A	G
1	1	*PLAT*	NM_000930.3	exonic	c.193C>T	p.Arg65Trp	rs776401170	VUS	4	chr8:42046512	missense	G/A	G
1	1	*PLAT*	NM_000930.3	exonic	c.860C>T	p.Thr287Met	rs114878147	Likely Benign	9	chr8:42039484	missense	G/A	G

1	1	*PLAT*	NM_000930.3	exonic	c.769C>A	p.Gln257Lys	Novel	Likely Benign	8	chr8:42040271	missense	G/T	G
9	15	*SERPINE1*	NM_000602.4	utr_3	c.*180C>T	NA	rs41334349	Likely Benign		chr7:100780903		C/T	C
2	3	*SERPINB2*	NM_002575.2	exonic	c.738A>G	p.Ile246Met	rs138446596	Likely Benign	7	chr18:61569697	missense	A/G	A
4	5	*SERPINB2*	NM_002575.2	exonic	c.686G>A	p.Arg229His	rs6100	Likely Benign	7	chr18:61569645	missense	G/A	G
1	1	*SERPINB2*	NM_002575.2	exonic	c.653G>C	p.Gly218Ala	rs143202684	Likely Benign	6	chr18:61569091	missense	G/C	G
1	1	*SERPINB2*	NM_002575.2	exonic	c.561A>T	p.Glu187Asp	rs978515084	Likely Benign	6	chr18:61568999	missense	A/T	A
1	1	*SERPINB2*	NM_002575.2	exonic	c.1076T>C	p.Met359Thr	rs144131011	Likely Benign	8	chr18:61570367	missense	T/C	T
1	1	*PLAU*	NM_002658.3	utr_5	c.-25C>T	NA	rs2227579	Benign		chr10:75671289		C/T	C
2	2	*PLAU*	NM_002658.3	exonic	c.43G>T	p.Val15Leu	rs2227580	Benign	2	chr10:75671356	missense	G/T	G
3	3	*PLAU*	NM_002658.3	exonic	c.172G>A	p.Gly58Arg	rs55744193	Benign	4	chr10:75672059	missense	G/A	G
1	1	*PLAU*	NM_002658.3	exonic	c.236G>A	p.Arg79Gln	rs201299522	Benign	5	chr10:75672724	missense	G/A	G
1	1	*PLAU*	NM_002658.3	exonic	c.1048T>C	p.Tyr350His	rs72816325	Benign	10	chr10:75675086	missense	T/C	T
1	1	*PLAU*	NM_002658.3	exonic	c.539T>C	p.Ile180Thr	rs771200321	Likely Benign	7	chr10:75673375	missense	T/C	T
2	3	*PLAUR*	NM_002659.3	utr_5	c.-87C>T	NA	rs147665588	Likely Benign	1	chr19:44174359		G/A	G
1	1	*PLAUR*	NM_002659.3	exonic	c.238C>T	p.Arg80Trp	rs752525518	Likely Benign	3	chr19:44169540	missense	G/A	G
1	1	*PLAUR*	NM_002659.3	exonic	c.476G>A	p.Arg159His	rs140011964	Likely Benign	5	chr19:44159722	missense	C/T	C
1	4	*PLAUR*	NM_002659.3	exonic	c.802A>G	p.Met268Val	rs138492321	Likely Benign	7	chr19:44153248	missense	T/C	T
1	4	*PLAUR*	NM_002659.3	exonic	c.508G>A	p.Gly170Ser	rs146166394	VUS	5	chr19:44159690	missense	C/T	C
1	1	*PLAUR*	NM_002659.3	exonic	c.922C>T	p.Pro308Ser	rs779002802	Likely Benign	7	chr19:44153128	missense	G/A	G
1	1	*PLAUR*	NM_002659.3	exonic	c.391C>G	p.Arg131Gly	rs772718099	Likely Benign	4	chr19:44160712	missense	G/C	G
1	1	*MPO*	NM_000250.1	exonic	c.1924A>C	p.Ile642Leu	rs112737382	Likely Benign	11	chr17:56349122	missense	T/G	T
7	14	*MPO*	NM_000250.1	exonic	c.995C>T	p.Ala332Val	rs28730837	Benign	7	chr17:56355397	missense	G/A	G
1	1	*MPO*	NM_000250.1	exonic	c.1705C>T	p.Arg569Trp	rs119468010	VUS	10	chr17:56350196	missense	G/A	G
1	1	*MPO*	NM_000250.1	exonic	c.1873A>C	p.Lys625Gln	rs749013683	Likely Benign	11	chr17:56349173	missense	T/G	T
3	3	*TPSAB1*	NM_003294.3	exonic	c.407A>G	p.His136Arg	rs1064780	Likely Benign	4	chr16:1291608	missense	A/G	A
1	1	*TPSAB1*	NM_003294.3	utr_3	c.*3C>G	NA	rs768747120	VUS	6	chr16:1292244		C/G	C
1	1	*TPSD1*	NM_012217.2	exonic	c.173G>A	p.Arg58His	rs113256300	Benign	2	chr16:1306607	missense	G/A	G
1	2	*TPSD1*	NM_012217.2	exonic	c.685G>A	p.Gly229Ser	rs142998820	Likely Benign	5	chr16:1308333	missense	G/A	G
1	1	*TPSD1*	NM_012217.2	exonic	c.376G>A	p.Gly126Arg	rs200563281	Likely Benign	3	chr16:1306919	missense	G/A	G

2	2	*TPSG1*	NM_012467.3	exonic	c.106G>A	p.Gly36Ser	rs201921574	Likely Benign	3	chr16:1273562	missense	C/T	C
2	4	*TPSG1*	NM_012467.3	exonic	c.508G>A	p.Gly170Arg	rs117769620	Likely Benign	4	chr16:1272655	missense	C/T	C
2	2	*TPSG1*	NM_012467.3	exonic	c.844C>A	p.Leu282Ile	rs117001332	Likely Benign	6	chr16:1271910	missense	G/T	G
1	1	*TPSG1*	NM_012467.3	exonic	c.110G>A	p.Arg37Gln	rs201153431	Likely Benign	3	chr16:1273558	missense	C/T	C
1	1	*TPSG1*	NM_012467.3	exonic	c.128C>T	p.Ala43Val	rs369071915	Likely Benign	3	chr16:1273540	missense	G/A	G
1	1	*TPSG1*	NM_012467.3	exonic	c.312C>A	p.Phe104Leu	Novel	Likely Benign	4	chr16:1272851	missense	G/T	G
1	1	*TAC1*	NM_003182.2	exonic	c.8T>G	p.Ile3Ser	Novel	VUS	2	chr7:97361932	missense	T/G	T
1	1	*TAC1*	NM_003182.2	utr_3	c.*196C>T	NA	rs113735261	Likely Benign	7	chr7:97369428		C/T	C
1	1	*A2M*	NM_000014.4	exonic	c.3554G>A	p.Arg1185His	rs777433561	Likely Benign	29	chr12:9227358	missense	C/T	C
1	1	*A2M*	NM_000014.4	exonic	c.661G>A	p.Val221Met	rs578188069	Likely Benign	6	chr12:9262475	missense	C/T	C
1	2	*A2M*	NM_000014.4	exonic	c.3889C>T	p.Arg1297Cys	rs376258518	Benign	30	chr12:9225335	missense	G/A	G
1	1	*A2M*	NM_000014.4	exonic	c.781C>A	p.Pro261Thr	Novel	Likely Benign	8	chr12:9260218	missense	G/T	G
1	1	*C1R*	NM_001733.4	exonic	c.336G>C	p.Met112Ile	rs139531404	Benign	3	chr12:7242740	missense	C/G	C
1	1	*C1R*	NM_001733.4	exonic	c.1033A>C	p.Ile345Leu	rs117402032	Benign	7	chr12:7241211	missense	T/G	T
1	1	*C1S*	NM_201442.2	exonic	c.979G>C	p.Val327Leu	rs2239170	Likely Benign	8	chr12:7173929	missense	G/C	G
3	3	*C1S*	NM_201442.2	exonic	c.943G>A	p.Asp315Asn	rs117907409	Benign	8	chr12:7173893	missense	G/A	G
3	5	*MASP1*	NM_139125.3	exonic	c.1528A>G	p.Ser510Gly	rs28945070	Likely Benign		chr3:186944222	missense	T/C	T
1	2	*MASP1*	NM_139125.3	exonic	c.910C>A	p.Leu304Ile	rs145057248	Likely Benign	7	chr3:186969523	missense	G/T	G
1	4	*MASP1*	NM_139125.3	exonic	c.1931C>T	p.Thr644Met	rs146714674	Likely Benign	11	chr3:186953728	missense	G/A	G
1	1	*MASP1*	NM_139125.3	exonic	c.1520C>T	p.Thr507Met	rs200778904	Likely Benign	11	chr3:186954139	missense	G/A	G
2	2	*MASP1*	NM_139125.3	exonic	c.1507C>T	p.Arg503Cys	rs201025468	Likely Benign	11	chr3:186954152	missense	G/A	G
1	1	*MASP1*	NM_139125.3	utr_5	c.-22C>T	NA	rs375911990	VUS	1	chr3:187009442		G/A	G
1	1	*MASP2*	NM_006610.3	exonic	c.296G>A	p.Arg99Gln	rs61735600	Benign	3	chr1:11106729	missense	C/T	C
1	1	*MASP2*	NM_006610.3	exonic	c.1304G>A	p.Gly435Glu	rs915010179	VUS	11	chr1:11087699	missense	C/T	C
3	3	*MASP2*	NM_006610.3	exonic	c.881C>T	p.Thr294Met	rs139962539	Benign	6	chr1:11102940	missense	G/A	G
1	1	*MASP2*	NM_006610.3	exonic	c.1731A>C	p.Gln577His	rs144471433	Benign	11	chr1:11087272	missense	T/G	T
2	2	*MASP2*	NM_006610.3	exonic	c.1882A>G	p.Ser628Gly	rs148079537	VUS	11	chr1:11087121	missense	T/C	T
3	5	*MASP2*	NM_006610.3	exonic	c.467G>A	p.Cys156Tyr	rs41307788	Likely Benign	4	chr1:11105542	missense	C/T	C
1	1	*MASP2*	NM_006610.3	exonic	c.193G>A	p.Asp65Asn	rs372294851	Likely Benign	2	chr1:11106989	missense	C/T	C

1	1	*C1QBP*	NM_001212.3	exonic	c.41C>G	p.Ser14Cys	Novel	VUS	1	chr17:5342353	missense	G/C	G
1	1	*TLR2*	NM_003264.3	exonic	c.1216G>A	p.Gly406Arg	rs770658906	Likely Benign	3	chr4:154625275	missense	G/A	G
2	2	*TLR2*	NM_003264.3	exonic	c.798G>C	p.Leu266Phe	rs150388453	Likely Benign	3	chr4:154624857	missense	G/C	G
1	1	*TLR2*	NM_003264.4	utr_3	c.*105G>A	NA	rs543157250	VUS	3	chr4:154626519		G/A	G
2	2	*TLR2*	NM_003264.3	exonic	c.650T>C	p.Phe217Ser	rs139227237	Likely Benign	3	chr4:154624709	missense	T/C	T
1	1	*TLR2*	NM_003264.3	exonic	c.619A>G	p.Met207Val	rs1560751270	Likely Benign	3	chr4:154624678	missense	A/G	A
1	1	*TLR2*	NM_003264.4	exonic	c.337T>C	p.Ser113Pro	rs768229096	Likely Benign	3	chr4:154624396	missense	T/C	T
1	1	*TLR2*	NM_003264.3	exonic	c.2341G>T	p.Ala781Ser	Novel	VUS		chr4:154626400	missense	G/T	G
1	2	*TLR4*	NM_138554.4	exonic	c.2207T>C	p.Val736Ala	rs754642038	VUS	3	chr9:120476613	missense	T/C	T
2	2	*TLR4*	NM_138554.4	exonic	c.842G>A	p.Cys281Tyr	rs137853920	Likely Benign	3	chr9:120475248	missense	G/A	G
1	2	*TLR4*	NM_138554.4	exonic	c.2411G>A	p.Arg804Gln	rs149989546	Likely Benign	3	chr9:120476817	missense	G/A	G
2	2	*TLR4*	NM_138554.4	exonic	c.2500C>A	p.Gln834Lys	rs5030724	Likely Benign	3	chr9:120476906	missense	C/A	C
1	1	*TLR4*	NM_138554.4	exonic	c.43A>T	p.Met15Leu	rs202028525	Likely Benign	1	chr9:120466793	missense	A/T	A
2	2	*TLR9*	NM_017442.3	utr_3	c.*18G>C	NA	rs5743848	Likely Benign	2	chr3:52255215		C/G	C
4	4	*TLR9*	NM_017442.3	exonic	c.2588G>A	p.Arg863Gln	rs5743845	Likely Benign	2	chr3:52255744	missense	C/T	C
3	3	*HRH1*	NM_001098212.1	exonic	c.57G>C	p.Lys19Asn	rs2067466	Likely Benign	2	chr3:11300780	missense	G/C	G
1	1	*IL5*	NM_000879.2	exonic	c.329G>A	p.Arg110Gln	rs775344061	VUS	4	chr5:131877579	missense	C/T	C
1	1	*TNF*	NM_000594.3	exonic	c.251C>T	p.Pro84Leu	rs4645843	Likely Benign	3	chr6:31544562	missense	C/T	C
1	1	*HSP90AA1*	NM_001017963.2	exonic	c.302delA	p.Gln101fs	rs764790477	VUS	2	chr14:102568275	frameshift Deletion	CT/C	CT
1	1	*HSP90AA1*	NM_001017963.2	exonic	c.1102G>A	p.Glu368Lys	rs3208445	VUS	6	chr14:102551263	missense	C/T	C
1	1	*HSP90AA1*	NM_001017963.2	exonic	c.1930A>G	p.Ile644Val	Novel	VUS	10	chr14:102549562	missense	T/C	T
1	1	*HSP90AA1*	NM_001017963.2	utr_5	c.-3C>G	NA	rs747975238	Likely Benign	1	chr14:102605744		G/A	G
1	1	*HSP90AA1*	NM_001017963.2	exonic	c.37C>T	p.Pro13Ser	rs201793224	Likely Benign	1	chr14:102605705	missense	G/A	G
9	9	*KRT1*	NM_006121.3	exonic	c.1669A>G	p.Ser557Gly	rs77846840	Benign	9	chr12:53069243	missense	T/C	T
1	1	*KRT1*	NM_006121.3	utr_3	c.*17T>G	NA	Novel	VUS	9	chr12:53068960		A/C	A
1	1	*KRT1*	NM_006121.3	exonic	c.281A>C	p.Tyr94Ser	rs2741152	VUS	1	chr12:53073852	missense	T/G	T
1	1	*KRT1*	NM_006121.3	exonic	c.173C>G	p.Ala58Gly	Novel	VUS	1	chr12:53073960	missense	G/C	G
1	1	*PROC*	NM_000312.3	exonic	c.554G>A	p.Arg185Lys	Novel	VUS	7	chr2:128183679	missense	G/A	G

1	1	*ESRRA*	NM_004451.4	utr_3	c.*150G>A	NA	rs1250712844	VUS	7	chr11:64083588		G/A	G
4	4	*GPER1*	NM_001098201.1	exonic	c.14C>T	p.Ser5Phe	rs117290655	Likely Benign	2	chr7:1131378	missense	C/T	C
1	1	*GPER1*	NM_001098201.1	exonic	c.808G>A	p.Val270Ile	rs376978032	Likely Benign	2	chr7:1132172	missense	G/A	G
1	1	*GPER1*	NM_001098201.1	exonic	c.100T>A	p.Ser34Thr	rs201711034	Likely Benign	2	chr7:1131464	missense	T/A	T
3	3	*GPER1*	NM_001098201.1	exonic	c.1119T>G	p.Ser373Arg	rs138657567	Likely Benign	2	chr7:1132483	missense	T/G	T
1	1	*AR*	NM_000044.3	exonic	c.1288G>T	p.Ala430Ser	rs755088348	VUS	1	chrX:66766276	missense	G/T	G
1	3	*AR*	NM_000044.3	exonic	c.2395C>G	p.Gln799Glu	rs137852591	VUS	6	chrX:66941751	missense	C/G	C
1	3	*AR*	NM_000044.3	utr_5	c.-207C>A	NA	rs189146053	Likely Benign	1	chrX:66764782		C/A	C
1	1	*AR*	NM_000044.3	exonic	c.1174C>T	p.Pro392Ser	rs201934623	VUS	1	chrX:66766162	missense	C/T	C
2	2	*AR*	NM_000044.3	exonic	c.814C>T	p.Leu272Phe	rs148972137	Likely Benign	1	chrX:66765802	missense	C/T	C
1	1	*ELANE*	NM_001972.2	exonic	c.770C>T	p.Pro257Leu	rs17216663	Benign	5	chr19:856130	missense	C/T	C

The information concerning the following variants has been collected by GnomAD v2.1.1, dbSNP (NCBI), ClinVar, Varsome, SIFT and PolyPhen. The correct nomenclature, according to HGVS[256] has been confirmed using Mutalyzer[257].

The ***F12*** **c.41T>C (p.Leu14Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.017%), mainly in Ashkenazi Jewish and Latino individuals. It has been detected in one European non-Finnish male individual (0.0009%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Bulgarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***F12*** **c.1025C>T (p.Pro342Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.08%) and European non-Finnish (0.15%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 female patients (1 Polish and 1 Hungarian) with C1-INH-HAE Type I. In ClinVar database, it is characterised by one submitter likely benign for FXII-deficiency and for HAE and by another submitter likely benign with no additional information concerning the condition or the carrier. In NCBI and GnomAD, the reference SNP number (rs2230939) represents a multiallelic variant, as at the same position there are two other substitutions, c.1025C>G (p.Pro342Arg) and c.1025C>A (p.Pro342Gln). The other two variants have lower frequency than c.1025C>T, but they are also predicted as tolerated and benign by the upper referred bioinformatic tools.

The ***F12*** **c.1553T>C (p.Phe518Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0012%) and European non-Finnish (0.0027%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 twin patients, both females, members of a Hungarian family with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F12*** **c.418C>G (p.Leu140Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and Polyphen algorithms, respectively. It has been detected in low frequency in the worldwide (0.22%) and European non-Finnish (0.34%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 German patient with C1-INH-HAE Type I. In ClinVar database, it is characterized likely benign for FXII-deficiency and for HAE.

The ***F13A1*** **c.1552A>C (p.Asn518His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0008%) and European non-Finnish (0.0009%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 male patients, members of a Bulgarian family with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F13A1*** **c.1225C>T (p.Arg409Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0008%) and European non-Finnish (0.0018%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F13A1*** **c.1832C>G (p.Ala611Gly)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0044%) and European non-Finnish (0.0088%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 unrelated female patients (2 Hungarian, 1 Greek) with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F13A1*** **c.1730C>T (p.Thr577Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.14%) and European non-Finnish (0.25%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 male patients, members of a Hungarian family with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F13A1*** **c.1766T>A (p.Leu589Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.38%) and European non-Finnish (0.58%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 patients (1 male, 1 female), members of a Hungarian family and in 1 German patient, all with C1-INH-HAE Type I. In ClinVar database, there are conflicting interpretations of the pathogenicity of this variant as it is characterised by two submitter VUS, once for FXIII subunit A deficiency and by one submitter benign with no additional information concerning the condition or the carrier.

The ***F13B*** **c.1318G>A (p.Glu440Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0012%) and European non-Finnish (0.0018%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Bulgarian male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F13B*** **c.1730T>G (p.Leu577Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0028%) and European non-Finnish (0.0062%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Bulgarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F13B*** **c.1025T>C (p.Ile342Thr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen

algorithms, respectively. It has been detected in low frequency in the worldwide (0.728%) and European non-Finnish (1.058%) population. In terms of allele frequency, the variant could be characterized a common SNP for European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in 4 unrelated Hungarian male patients with C1-INH-HAE Type I (Allele frequency 0.86%). There are conflicting interpretations of the pathogenicity of this variant in ClinVar database. It is characterized VUS for FXIII Subunit B deficiency but benign with no additional information concerning the condition or the carrier.

The ***F13B*** **c.1163A>T (p.Glu388Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.16%) and European non-Finnish (0.27%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 German patient with C1-INH-HAE Type II. In ClinVar database, the variant is characterized VUS for Factor XIII Subunit B Deficiency.

The ***F2*** **c.1547G>C (p.Gly516Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected only once among 120,000 individuals of the GnomAD, in a European non-Finnish female individual. Among 233 C1-INH-HAE patients of this study, it has been detected in a Polish female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database. In NCBI and GnomAD, the reference SNP number (rs144885724) represents a multiallelic variant, as at the same position there is another SNV c.1547G>T (p.Gly516Val) which is also predicted as tolerated and benign by the upper referred bioinformatic tools, but it has been detected 10 times among approximately 120,000 individuals of GnomAD, all European non-Finnish and 4 times more in male than in female individuals. No other information is available in NCBI, GnomAD, ClinVar databases concerning the pathogenicity of the rs144885724.

The ***F2*** **c.-29T>C** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide (0.013%) and European non-Finnish (0.0099%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***F2*** **c.1542C>A (p.Asn514Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms respectively. It has been detected in low frequency in the worldwide (0.029%) and European non-Finnish (0.023%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Greek male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database. In NCBI and GnomAD, the reference SNP number (rs199772906) represents a multiallelic variant as at the same position there is another SNV c.1542C>T (p.=) which is a synonymous variant and it has been detected to 3 individuals among 120,000 of GnomAD.

The ***F2*** **c.284C>T (p.Thr95Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.006%) and European non-

Finnish (0.0026%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 German patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***ACE*** **c.731A>G (p.Tyr244Cys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively, but in terms of allele frequency it is a common polymorphism. More precisely it has been detected in 1.068% of the global population and 1,542% of the European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Bulgarian male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***ACE*** **c.1681C>T (p.Arg561Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.078%) and European non-Finnish (0.0044%) population. On the contrary, for the African population the variant is a common SNP as it is detected in a frequency of 1.037%. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Polish male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***ACE*** **c.1060G>A (p.Gly354Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.78%). For the European non-Finnish population the variant is a common SNP as it is detected in a frequency of 1.021%. Among 233 C1-INH-HAE patients of this study, it has been detected in two unrelated Polish female patients and one German patient, all with C1-INH-HAE Type I. In ClinVar database, the variant is characterized once benign and once likely benign for renal tubular dysgenesis.

The ***ACE*** **c.2749C>G (p.Pro917Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected only once among approximately 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in two female patients with C1-INH-HAE Type I, members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database.

The ***ACE*** **c.2677G>A (p.Asp893Asn)** is a novel missense variant in *ACE* exon 18. It is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***ACE*** **c.3521delG (p.Gly1174fs)** has not been previously associated with C1-INH-HAE. This INDEL variant causes interruption of the reading frame by the formation of a termination codon (*12aa) which results in a truncated protein. It has been detected in low frequency in the worldwide (0.047%) and European non-Finnish (0.025%) population. Among 233 C1-INH-HAE patients of this

study, it has been detected in two female patients with C1-INH-HAE Type I, members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database.

The ***ACE*** **c.3836G>A (p.Arg1279Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.41%) and European non-Finnish (0.658%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in three unrelated patients with C1-INH-HAE Type I (1 Greek, 1 Bulgarian, 1 German). In ClinVar database the variant is characterized by two different submitters benign.

The ***ACE*** **c.3108C>A (p.Asn1036Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.10%) and European non-Finnish (0.08%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 unrelated patients with C1-INH-HAE Type I (1 Greek, 2 German). In ClinVar database, the variant is characterized VUS for renal tubular dysgenesis.

The ***XPNPEP2*** **c.1177G>A (p.Asp393Asn)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.053%) and European non-Finnish (0.081%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Polish female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***XPNPEP2*** **c.1567G>A (p.Gly523Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected only once among approximately 120,000 of the GnomAD, in a European non-Finnish male individual. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 patients (2 female, 1 male) with C1-INH-HAE Type I, members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database.

The ***XPNPEP2*** **c.-166C>T** has not been previously associated with C1-INH-HAE. This 5'UTR prime variant has been detected, according to GnomAD, in low frequency (GMAF=0.15%, EMAF=0,21%) among approximately 10,000 individuals. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***XPNPEP2*** **c.644C>T (p.Thr215Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.251%) and European non-Finnish (0.399%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 unrelated Greek female patients with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***XPNPEP2*** **c.*31C>T** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected only once in an African female individual among approximately 50,000 of

GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Greek female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***XPNPEP2*** **c.604C>G (p.Gln202Glu)** is a novel missense variant in *XPNPEP2* exon 7. It is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a German patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***XPNPEP3*** **c.1244G>A (p.Arg415Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.075%) and European non-Finnish population (0.12%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian male patient with C1-INH-HAE Type I. In ClinVar database the variant is characterized VUS for Nephronophthisis-like nephropathy 1.

The ***DPP4*** **c.740A>C (p.Gln247Pro)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.014%) and European non-Finnish population (0.0017%). Among approximately 120,000 individuals of GnomAD, it is mainly detected in South Asian population (32 out of 35 individuals, Allele frequency 0.11%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Bulgarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.1393G>T (p.Ala465Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected in 120,000 individuals of the GnomAD. However, at the same position, a different mutation has been detected once (NM_001935.3:c.1393G>A, rs1285035755), resulting in the substitution of the alanine by a threonine. Among 233 C1-INH-HAE patients of this study, c.1393G>T has been detected in a Polish female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.1228C>G (p.Leu410Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.0036%) but most of the individuals are European-non-Finnish (8 out of 9, Allele frequency 0.007%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.942G>T (p.Gln314His)** is a novel missense variant in *DPP4* exon 11. It is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of the GnomAD. However, at the same position a synonymous mutation has been detected once (NM_001935.3:c.942G>A, rs751885146). Among 233 C1-INH-HAE patients of this study, c.942G>T was detected in a Hungarian female patient with C1-INH-HAE Type II. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.1582A>G (p.Met528Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It is detected in low frequency in the worldwide (0.013%) and European non-Finnish (0.0071%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.*81A>C** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected once in a European non-Finnish female individual and once in a Finnish female individual among approximately 15,000 of GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in one Greek male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.248A>G (p.Tyr83Cys)** is a novel variant in *DPP4* exon 4. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected in 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in two C1-INH-HAE Type I patients (1 male, 1 female), members of a Greek family. There are no clinical assertions for this variant in ClinVar database.

The ***DPP4*** **c.796G>A (p.Val266Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.33%) and European non-Finnish (0.55%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek female patient with C1-INH-HAE Type I. In ClinVar database the variant is characterized likely benign.

The ***CPM*** **c.472G>A (p.Asp158Asn)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of the GnomAD, 1 North-Western European female and 1 South-Asian female individual. Among 233 C1-INH-HAE patients of this study, it has been detected in one Polish male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***CPM*** **c.432G>C (p.Arg144Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected once among approximately 120,000 individuals of GnomAD (1 European non-Finnish male individual). Among 233 C1-INH-HAE patients of this study, it has been detected in two patients (1 male, 1 female) with C1-INH-HAE Type I, members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database.

The ***CPM*** **c.440A>G (p.Tyr147Cys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.15%), mainly in European non-Finnish individuals (0.25%). Among 233 C1-INH-HAE patients of this study, it has been detected in 3 unrelated patients (1 Bulgarian male, 1 Hungarian male, 1 Greek female) with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***CPN1*** **c.*89A>G** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected in low frequency in the worldwide (0.23%) and European non-Finnish (0.25%) population and in a higher frequency in Ashkenazi Jewish population (7.25%). Among 233 C1-INH-HAE patients of this study, it has been detected in 3 unrelated patients (1 Bulgarian female, 1 Hungarian female, 1 Greek female) with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***PRCP*** **c.253A>G (p.Thr85Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.27%), mainly in Ashkenazi Jewish individuals (2.69%). The allele frequency In European non-Finnish population is close to the global (0.24%). Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Bulgarian male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***MME*** **c.2067C>A (p.Asn689Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.02%) and European non-Finnish (0.013%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Bulgarian male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***MME*** **c.674G>C (p.Gly225Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.16%) and European (0.2%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 5 patients with C1-INH-HAE Type I, members of a Hungarian family and two additional unrelated patients. In ClinVar database, the variant is characterized likely benign. In NCBI and GnomAD, the reference SNP number (rs147564881) represents a multiallelic variant, as at the same position there is another SNV c.674G>A (p.Gly225Asp) which is predicted as deleterious and probably damaging by the upper referred bioinformatic tools, but it has been detected only twice among the 120,000 individuals of GnomAD and no other information is available in NCBI and ClinVar databases concerning its pathogenicity.

The ***MME*** **c.1946T>G (p.Ile649Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.12%) and European (0.02%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 patients (1 male, 1 female) with C1-INH-HAE Type I, members of a Hungarian family. In ClinVar database, the variant is characterized VUS. In NCBI and GnomAD, the reference SNP number (rs184666602) represents a multiallelic variant, as at the same position there is another SNV c.1946T>C (p.Ile649Thr) which is also predicted as deleterious and probably damaging by the upper referred bioinformatic tools and it has been detected 9 times among approximately 120,000 individuals of ExAC database. In ClinVar database c.1946T>C (p.Ile649Thr) is also characterized VUS.

The ***SERPINA1* c.1177C>T (p.Pro393Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.03%) and European non-Finnish (0.05%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Greek female patient with C1-INH-HAE Type I. In ClinVar database, there are conflicting interpretations of the pathogenicity of this variant for A1ATD. It is characterized by two submitters likely pathogenic, by three submitters pathogenic and by one submitter VUS. In NCBI, the reference SNP number (rs61761869) represents a multiallelic variant, as at the same position there is another SNV, c.1177C>A (p.Pro393Thr), which is also predicted as deleterious and probably damaging by the upper referred bioinformatic tools but it has never been detected among approximately 120,000 individuals of GnomAD. In ClinVar database, there are also conflicting interpretations of pathogenicity concerning c.1177C>A (p.Pro393Thr), as the variant has been associated with A1ATD (pathogenic), but it is also submitted once as VUS with no additional information concerning the condition or the carrier. The p.Pro393Ser variant in *SERPINA1* (M Wurzburg allele) has been reported in the literature in at least 5 individuals with *SERPINA1*-related phenotypes, including alpha-1-antitrypsin deficiency (A1ATD), asthma, elevated transaminases, and intrahepatic inclusions[229,258,259,260,261]. Three of these individuals were heterozygous, while 2 individuals with liver phenotypes were compound heterozygous. The variant segregated in at least 1 family member with low levels of A1AT and 2 members of a family with asthma. Functional studies provide evidence that the p.Pro393Ser may impact the protein function; however, these types of assays may not accurately represent biological function. A different variant at the same position (p.Pro393Leu) has been also reported by Poller et al. and also classified in ClinVar database as pathogenic by one submitter, supporting that a change at this position may not be tolerated.

The ***SERPINA1* c.514G>T (p.Gly172Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.09%) and European non-Finnish (0.13%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Greek male patient with C1-INH-HAE Type I. In ClinVar database, there are conflicting interpretations of the pathogenicity of this variant as it is characterized by one submitter likely benign and by two submitters VUS. A different variant at the same position, c.514G>A (p.Gly172Arg), has been classified once as likely benign and three times as VUS for A1ATD.

The ***SERPINC1* c.731T>A (p.Val244Asp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.005%) and European non-Finnish (0.0035%) population. More specifically, it has been detected in 12 among approximately 120,000 individuals of GnomAD, 4 European non-Finnish. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Hungarian male patient with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB1* c.844C>T (p.Arg282Ter)** has not been previously associated with C1-INH-HAE. This nonsense variant reduces the protein length of B1R by the formation of a termination codon in the position 282 of the protein. The truncated protein is missing 72 aa. The variant has been detected in

low frequency in the worldwide (0.29%) and European non-Finnish (0.32%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 2 female patients, members of a Hungarian family and an unrelated Hungarian male patient, all with C1-INH-HAE Type I. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB1*** **c.721G>A (p.Gly241Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. Among 233 C1-INH-HAE patients of this study, it has been detected in 6 patients of five different families with C1-INH-HAE. There are no clinical assertions for this variant in ClinVar database. In NCBI and in GnomAD, the reference SNP number (rs45528332) represents a multiallelic variant, as at the same position there is another SNV (c.721G>T) resulting in the same substitution in B1R and which has been detected once among approximately 120,000 individuals of GnomAD in a European non-Finnish individual.

The ***BDKRB1*** **c.342C>G (p.Asn114Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.015%) and European non-Finnish (0.019%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 4 patients from 2 different families. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB1*** **c.1054C>T (p.Arg352Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms respectively. It has been detected in low frequency in the worldwide (0.08%) and European non-Finnish (0.05%) population. The allele frequency is higher to the Latino population (0.35%). Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Hungarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB2*** **c.-20A>G** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected once among approximately 120,000 individuals of GnomAD in an Estonian female individual. Among 233 C1-INH-HAE patients of this study, it has been detected in a Polish female patient with C1-INH-HAE type II. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB2*** **c.1010A>G (p.Lys337Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.18%) and European non-Finnish (0.56%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Hungarian and 1 German patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB2*** **c.1006G>A (p.Gly336Ser)** is a novel variant in *BDKRB2* exon 3. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 of GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***BDKRB2*** **c.607G>A (p.Asp203Asn)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of the GnomAD, in 1 male and 1 female Bulgarian individuals. Among 233 C1-INH-HAE patients of this study, it has been detected in 1 Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KNG1*** **c.-46T>A** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide (0.19%) and European non-Finnish (0.07%) population. The allele frequency is higher in the South-Asian population (1.1%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I.

The ***KNG1*** **c.*35T>C** has not been previously associated with C1-INH-HAE. This 3'UTR variant has never been detected among approximately 120,000 of the GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KLKB1*** **c.7T>A (p.Leu3Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms respectively. The variant has never been detected among approximately 120,000 of GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KLKB1*** **c.1325A>C (p.Gln442Pro)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and Polyphen algorithms, respectively. It has been detected in low frequency in the worldwide (0.27%) and European non-Finnish (0.47%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a German patient with C1-INH-HAE type I. In NCBI and GnomAD, the reference SNP number (rs4253316) represents a multiallelic variant, as at the same position there is another substitution, c.1325A>G (p.Gln442Arg). This substitution has never been detected among 120,000 individuals of GnomAD. There are no clinical assertions for these two variants in ClinVar database.

The ***KLK2*** **c.155G>A (p.Gly52Asp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.2%) and European non-Finnish (0.3%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 8 patients from 4 different families with C1-INH-HAE. In NCBI and GnomAD, the reference SNP number (rs139063242) represents a multiallelic variant, as at the same position there is another substitution, c.155G>C (p.Gly52Ala). This substitution has been detected once among 120,000 individuals of GnomAD in a European non-Finnish male individual and is predicted as tolerated but probably damaging according to SIFT and PolyPhen respectively. There are no clinical assertions for these two variants in ClinVar database.

The ***KLK2*** **c.227G>A (p.Gly76Asp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen

algorithms, respectively. It has been detected only 3 times among approximately 120,000 individuals of GnomAD (European non-Finnish individuals). Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KLK2*** **c.460G>A (p.Ala154Thr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.01%) and European non-Finnish (0.025%) population according to GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KLK3*** **c.629C>T (p.Ser210Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.012%) and European non-Finnish (0.014%) population. In NCBI and GnomAD, the reference SNP number (rs61729813) represents a multiallelic variant as in the same position there is another substitution, c.629C>G (p.Ser210Trp). The c.629C>G is predicted as deleterious but benign by the upper referred bioinformatic tools and is detected in higher frequency than c.629C>T (0.75%) in the global population. In European non-Finnish population, this substitution is a common SNP with allele frequency 1.22%. Among 233 C1-INH-HAE patients of this study, it has been detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KLK3*** **c.629C>G (p.Ser210Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms respectively. It has been detected in low frequency in the worldwide population (0.75%), but it is a common polymorphism in European non-Finnish population with allele frequency 1.22%. In NCBI and GnomAD, the reference SNP number (rs61729813) represents a multiallelic variant as in the same position there is another substitution, c.629C>T (p.Ser210Leu). The c.629C>T is predicted as tolerated and benign by the upper referred bioinformatic tools and is detected in lower frequency than c.629C>G. Among 233 C1-INH-HAE patients of this study, c.629C>G was detected in 9 patients from 8 different families with C1-INH-HAE. There are no clinical assertions for this variant in ClinVar database.

The ***KLK3*** **c.515A>G (p.Gln172Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE type I. There is no information regarding its pathogenicity in Ensembl and NCBI.

The ***KLK3*** **c.170C>T (p.Pro57Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in 3 Southern European and 3 Latino individuals among approximately 120,000 in GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in

a German patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***PLG*** **c.1567C>T (p.Arg523Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.66%), but in higher frequency in the European non-Finnish population (1.24%). Among 233 C1-INH-HAE patients of this study, it was detected in 5 patients from 3 different families with C1-INH-HAE. In ClinVar database, the variant is classified as benign by one submitter with no additional information concerning the condition or the carrier.

The ***PLG*** **c.1380T>A (p.Ser460Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.48%) and European non-Finnish (0.24%) population, but for the African population is a common polymorphism with an allele frequency 4.17%. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian male patient with C1-INH-HAE type I. In ClinVar database, the variant is classified as benign by one submitter with no additional information concerning the condition or the carrier.

The ***PLG*** **c.598A>G (p.Thr200Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.07%) and European non-Finnish (0.15%) population. Among 233 C1-INH-HAE patients of this study, it was detected in two female patients, members of a Hungarian family. In NCBI and GnomAD, the reference SNP number (rs149145958) represents a multiallelic variant, as at the same position there is another substitution, c.598A>T (p.Thr200Ser). This substitution has been detected once among approximately 120,000 individuals of GnomAD, in a North-Western European individual and is predicted as deleterious and possibly damaging by the upper referred bioinformatic tools. There are no clinical assertions for these two variants in ClinVar database.

The ***PLG*** **c.782G>A (p.Arg261His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.25%) and European non-Finnish (0.43%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 patients from 2 different families with C1-INH-HAE. In NCBI and GnomAD, the reference SNP number (rs4252187) represents a multiallelic variant, as at the same position there is another substitution, c.782G>C (p.Arg261Pro), which is predicted as tolerated and benign by the upper referred bioinformatic tools and it has been detected only once among approximately 120,000 individuals of GnomAD. In ClinVar database, c.782G>A is characterised once benign with no additional information on the condition or the carrier and once VUS for susceptibility to otitis media. Arg^{261} is reported as a highly conserved amino acid residue and c.781C>T which also leads to its substitution (p.Arg261Cys) has already been associated with plasminogen deficiency.

The ***PLG*** **c.266G>A (p.Arg89Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms,

respectively. It has been detected in low frequency in the worldwide population (0.62%) but in a higher frequency in European non-Finnish population (1.17%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type II. In ClinVar database, the variant is characterized by one submitter benign with no additional information concerning the condition or the carrier. In NCBI, the reference SNP number of this variant (rs143079629) represents a multiallelic variant as at the same position there is another substitution (c.266G>C, p.Arg89Thr). The c.266G>C is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of the GnomAD in two male individuals (1 Latino, 1 European non-Finnish).

The ***PLG*** **c.1481C>T (p.Ala494Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.98%) and European non-Finnish (0.42%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian male patient with C1-INH-HAE type I. In ClinVar database, the variant is characterized by one submitter benign with no additional information concerning the condition or the carrier and by another submitter VUS for susceptibility to otitis media. Osborne et al.[262] detected the variant in a patient with atypical haemolytic syndrome and characterized it likely benign. In NCBI, the reference SNP number (rs4252128) represents a multiallelic variant, as at the same position there is another substitution (c.1481C>A, p.Ala494Glu). The c.1481C>A is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.007%) and European non-Finnish (0.002%) population. According to GnomAD, it is mainly detected in Ashkenazi Jewish individuals (0.2%).

The ***SERPINF1*** **c.808G>A (p.Gly270Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0096%) and European non-Finnish (0.018%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Bulgarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs369314029) represents a multiallelic variant, as at the same position there is another substitution (c.808G>T, p.Gly270Ter). The c.808G>T reduces the protein length (PEDF) by the formation of a termination codon in the position 270. The predicted truncated protein is missing 149aa. The nonsense variant has never been detected among approximately 120,000 individuals of GnomAD.

The ***PLAT*** **c.1384C>T (p.Arg462Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0052%) and European non-Finnish (0.0046%) population. The allele frequency, according to GnomAD is higher in South Asian (0.026%) and Ashkenazi Jewish (0.03%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number of this variant (rs545287158) represents a multiallelic variant, as at the same position there are two additional substitutions c.1384C>G (p.Arg462Gly) and c.1384C>A (p=). The c.1384C>G missense

variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen and it has been detected once among approximately 120,000 individuals of GnomAD in an East-Asian individual. On the contrary, c.1384C>A is a synonymous variant and has never been reported in GnomAD.

The ***PLAT* c.928C>T (p.Arg310Cys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0076%) and European non-Finnish (0.13%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***PLAT* c.193C>T (p.Arg65Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected once among approximately 120,000 individuals of GnomAD in a Finnish male individual. Among 233 C1-INH-HAE patients of this study, it has been detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***PLAT* c.860C>T (p.Thr287Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.17%) and European non-Finnish (0.27%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. In ClinVar database, there are conflicting interpretations of the pathogenicity for the variant, as it is characterized by one submitter likely benign and by another submitter VUS with no additional information concerning the condition or the carriers.

The ***PLAT* c.769C>A (p.Gln257Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. A missense variant changing the same amino acid (c.771G>C, p.Gln257His, rs746988345) has been detected once among approximately 120,000 individuals of the GnomAD in a Southern European individual and it is also predicted as tolerated and benign.

The ***SERPINE1* c.*180C>T** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected in low frequency in the worldwide population (0.66%) but in higher frequency in European non-Finnish population (1.01%). Among 233 C1-INH-HAE patients of this study, it was detected in 15 patients from 9 different families. In ClinVar database, the variant is characterized likely benign for congenital plasminogen activator inhibitor type 1 deficiency.

The ***SERPINB2* c.738A>G (p.Ile246Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.14%) and

European non-Finnish (0.2%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 patients from 2 different families. There are no clinical assertions for this variant in ClinVar database.

The ***SERPINB2*** **c.686G>A (p.Arg229His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.14%) and European non-Finnish (0.23%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 5 patients from 4 different families. In ClinVar database, the variant is characterized by one submitter likely benign with no additional information concerning the condition or the carrier.

The ***SERPINB2*** **c.653G>C (p.Gly218Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.29%) and European non-Finnish (0.18%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***SERPINB2*** **c.561A>T (p.Glu187Asp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of GnomAD, in 2 European non-Finnish individuals (1 male, 1 female). Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***SERPINB2*** c**.1076T>C (p.Met359Thr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.04%) and European non-Finnish (0.022%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE. There are no clinical assertions for this variant in ClinVar database.

The ***PLAU*** **c.-25C>T** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide population (0.6%) but in higher frequency in European non-Finnish population (0.95%). Among 233 C1-INH-HAE patients of this study, it was detected in 1 Polish male patient with C1-INH-HAE type I. In ClinVar database, the variant is characterized likely benign for Quebec platelet disorder by one submitter. In NCBI, the reference SNP number of this variant (rs2227579) represents a multiallelic variant, as at the same position there is another substitution c.-25C>A, which has been detected only 3 times among approximately 120,000 individuals of GnomAD.

The ***PLAU*** **c.43G>T (p.Val15Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It is a common polymorphism for the worldwide (1.05%) and European non-Finnish (1.05%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 unrelated patient with C1-INH-HAE type I (1 Polish male, 1 Hungarian female). In ClinVar database, the variant is characterised benign for Quebec platelet disorder by one submitter. In NCBI, the reference SNP

number of this variant (rs2227580) represents a multiallelic variant, as at the same position there are two more substitutions c.43G>C and c.43G>A. The c.43G>C results in the same amino acid change on the protein (p.Val15Leu) and it is also predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected once among approximately 120,000 individuals of the GnomAD in a Southern European non-Finnish male individual. The c.43G>A (p.Val15Met) is predicted as deleterious and probably damaging and it has been detected once among the individuals examined and included in GnomAD.

The ***PLAU* c.172G>A (p.Gly58Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.47%) and European non-Finnish (0.82%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 unrelated patients with C1-INH-HAE. In ClinVar database, the variant is characterized by one submitter VUS for Hirschsprung disease 1. Moreover, it is characterized by two different submitters likely benign, once for Quebec platelet disorder and once with no additional information concerning the condition or the carrier.

The ***PLAU* c.236G>A (p.Arg79Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.024%) and European non-Finnish (0.033%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish female patient with C1-INH-HAE type I. In ClinVar database, the variant is characterised by one submitter likely benign for Quebec platelet disorder. In NCBI, the reference SNP number (rs201299522) represents a multiallelic variant, as at the same position there is another substitution c.236G>C (p.Arg79Pro). The c.236G>C is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.0048%) and only in South-Asian individuals.

The ***PLAU* c.1048T>C (p.Tyr350His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.39%) and European non-Finnish (0.57%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs72816325) represents a multiallelic variant, as at the same position there is another substitution c.1048T>A (p.Tyr350Asn). The c.1048T>A is also predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of the GnomAD, in 2 African male individuals.

The ***PLAU* c.539T>C (p.Ile180Thr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected 3 times among approximately 120,000 exomes of the GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs771200321) represents a multiallelic variant, as at the same position there is another

substitution c.539T>G (p.Ile180Ser). The c.539T>G is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected once among approximately 120,000 individuals of GnomAD in a European non-Finnish female individual.

The ***PLAUR* c.-87C>T** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide (0.29%) and European non-Finnish (0.43%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 male patients with C1-INH-HAE type I, members of two different families (2 Hungarians, one Greek). There are no clinical assertions for this variant in ClinVar database.

The ***PLAUR* c.238C>T (p.Arg80Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.0032%) but only in European non-Finnish individuals (8 individuals, EMAF=0.007%). Among 233 C1-INH-HAE patients of this study, it was detected in a Polish male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***PLAUR* c.476G>A (p.Arg159His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.14%) but mainly in European non-Finnish individuals (0.27%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish male patient with C1-INH-HAE type I. In ClinVar database, the variant is characterized by one submitter likely benign with no additional information concerning the condition or the carrier.

The ***PLAUR* c.802A>G (p.Met268Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.36%) and European non-Finnish (0.48%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 4 patients, members of a Hungarian family, with C1-INH-HAE type I. In ClinVar database, the variant is characterized by one submitter likely benign with no additional information concerning the condition or the carrier.

The ***PLAUR* c.508G>A (p.Gly170Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected in the worldwide population. Among 233 C1-INH-HAE patients of this study, it was detected in 4 patients, members of a Hungarian family, with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. Another variant in the same codon (c.509G>A, p.Gly170Asp) has been detected in 2 Latino individuals among approximately 120,000 individuals of the GnomAD which is also predicted as deleterious and probably damaging.

The ***PLAUR* c.922C>T (p.Pro308Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (3 individuals, 0.0012%) and European non-Finnish (2 individuals, 0.0018%) population. Among 233 C1-INH-HAE

patients of this study, it was detected in a Hungarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***PLAUR*** **c.391C>G (p.Arg131Gly)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in 7 Latino individuals among approximately 120,000 of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs772718099) represents a multiallelic variant, as at the same position there is another substitution c.391C>T (p.Arg131Trp). The c.391C>T is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected 3 times among approximately 120,000 individuals of GnomAD.

The ***MPO*** **c.1924A>C (p.Ile642Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.26%) and European non-Finnish (0.4%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***MPO*** **c.995C>T (p.Ala332Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It is a common polymorphism in the worldwide (1,24%) and European non-Finnish (1.81%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 14 patients from 7 different families. Among 141 unrelated C1-INH-HAE patients, it was detected in 4.96% (7/141). In ClinVar database, the variant is characterised VUS. More specifically, it is characterized once as pathogenic for MPO deficiency and by two other submitters VUS. As it is explained by the latest submitter (2020, Reproductive Health Research and Development, BGI Genomics) mutations in the *MPO* lead to hereditary MPO deficiency in an autosomal recessive manner. Although a number of 45 homozygous occurrences is observed in GnomAD, the majority of patients with MPO deficiency are clinically asymptomatic except if they are also diabetic. Marchetti et al.[208] identified an individual with a partial MPO deficiency, compound heterozygous for this variant and c.1715T>G. Therefore, the evidence is not enough for the classification as pathogenic. The variant is considered VUS according to ACMG/AMP criteria (PM3, PP4, BS1).

The ***MPO*** **c.1705C>T (p.Arg569Trp)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.15%) and European non-Finnish (0.29%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian male patient with C1-INH-HAE type I. In ClinVar database, the variant is characterised by multiple submitters pathogenic for myeloperoxidase deficiency.

The ***MPO*** **c.1873A>C (p.Lys625Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0087%) and European non-

Finnish (0.0044%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TPSAB1*** **c.407A>G (p.His136Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.04%) and European non-Finnish (0.06%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 unrelated female patients with C1-INH-HAE type I (2 Polish, 1 Hungarian). There are no clinical assertions for this variant in ClinVar database.

The ***TPSAB1*** **c.*3C>G** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected 2 times among approximately 120,000 individuals of GnomAD, 1 European non-Finnish. Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs768747120) represents a multiallelic variant, as at the same position there is another substitution c.*3C>A. The c.*3C>A has been detected also twice among approximately 120,000 individuals of GnomAD, in 2 Latino individuals.

The ***TPSD1*** **c.173G>A (p.Arg58His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.72%) and European non-Finnish (0.56%) population but it is a common SNP for African population (5.6%). Among 233 C1-INH-HAE patients of this study, it was detected in a Polish male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TPSD1*** **c.685G>A (p.Gly229Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.8%) but in a higher frequency in the European non-Finnish (1.05%). Among 233 C1-INH-HAE patients of this study, it was detected in two female patients with C1-INH-HAE type II, members of a Hungarian family. In ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier.

The ***TPSD1*** **c.376G>A (p.Gly126Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0096%) and European non-Finnish (0.0062%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs200563281) represents a multiallelic variant, as at the same position there is another substitution c.376G>C which results in the same amino acid change (p.Gly126Arg). The c.376G>C has been detected once among approximately 120,000 individuals of GnomAD, in an African male individual.

The ***TPSG1*** **c.106G>A (p.Gly36Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms,

respectively. It has been detected in low frequency in the worldwide (0.14%) and European non-Finnish (0.24%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 unrelated patients with C1-INH-HAE type I (1 Polish male, 1 Hungarian female). There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs201921574) represents a multiallelic variant, as at the same position there is another substitution c.106G>C (p.Gly36Arg). The c.106G>C is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively, but It has never been detected among approximately 120,000 individuals of GnomAD.

The ***TPSG1*** **c.508G>A (p.Gly170Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.57%) and European non-Finnish (0.73%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 4 Polish patients with C1-INH-HAE type I, members of two different families. There are no clinical assertions for this variant in ClinVar database.

The ***TPSG1*** **c.844C>A (p.Leu282Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.25%) and European non-Finnish (0.47%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 patients with C1-INH-HAE type I (1 Polish male, 1 Greek female). In ClinVar database, the variant is characterized by one submitter likely benign with no additional information concerning the condition or the carrier.

The ***TPSG1*** **c.110G>A (p.Arg37Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.13%) and European non-Finnish (0.16%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs201153431) represents a multiallelic variant, as at the same position there is another substitution c.110G>T (p.Arg37Leu). The c.110G>T is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively but it has never been detected among approximately 120,000 individuals of GnomAD.

The ***TPSG1*** **c.128C>T (p.Ala43Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0083%) and European non-Finnish (0.0071%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TPSG1*** **c.312C>A (p.Phe104Leu)** is a novel variant which has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately

120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE type I. There are no clinical assertions for this variant in dbSNP and ClinVar databases.

The ***TAC1*** **c.8T>G (p.Ile3Ser)** is a novel variant which is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Bulgarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in dbSNP and ClinVar databases.

The ***TAC1*** **c.*196C>T** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected in low frequency in the worldwide population (0.66%) but in higher frequency in the European non-Finnish population (1.08%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***A2M*** **c.3554G>A (p.Arg1185His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (4 individuals, 0.0016%) and European non-Finnish (1 individual, 0.0009%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***A2M*** **c.661G>A (p.Val221Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0087%) and European non-Finnish (0.015%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***A2M*** **c.3889C>T (p.Arg1297Cys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0076%) and European non-Finnish (0.012%) population. Among 233 C1-INH-HAE patients of this study, it was detected in two female patients with C1-INH-HAE type I, members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs376258518) represents a multiallelic variant, as at the same position there is another substitution c.3889C>A (p.Arg1297Ser). The c.3889C>A is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD.

The ***A2M*** **c.781C>A (p.Pro261Thr)** is a novel variant which is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. However, at the same position, another variant has been detected (c.781C>T,

p.Pro261Leu, rs182493380). The c.781C>T is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.75%). The variant is a common polymorphism for Latino individuals (5.3%) but in European non-Finnish population it has been detected once in a North-western European female individual. In ClinVar database, it is characterized by one submitter benign with no additional information concerning the condition or the carrier.

The ***C1R*** **c.336G>C (p.Met112Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.28%) and European non-Finnish (0.47%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish male patient with C1-INH-HAE type II. There are conflicting interpretations of pathogenicity of this variant in ClinVar database as it is characterised by one submitter VUS for Ehlers-Danlos syndrome type 8, but also likely benign by another submitter with no additional information concerning the condition or the carrier.

The ***C1R*** **c.1033A>C (p.Ile345Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It is a common polymorphism worldwide (2.5%), while the highest frequency is detected in Latino population (13.5%). However, in European non-Finnish population it is rarely detected (0.023%). Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***C1S*** **c.979G>C (p.Val327Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.02%), mainly in South-Asian individuals while in European non-Finnish population the frequency is about 0.008%. Among 233 C1-INH-HAE patients of this study, it was detected in a Bulgarian male patient with C1-INH-HAE type I. In ClinVar database the variant is characterised likely benign by one submitter with no additional information concerning the condition or the carrier. In NCBI, the reference SNP number (rs2239170) represents a multiallelic variant, as at the same position there is another substitution c.979G>A (p.Val327Ile). The c.979G>A is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected once among approximately 120,000 individuals of GnomAD, in an East-Asian individual. Moreover, at the same codon, an INDEL variant has been detected (c.980_981dupTT, p.Val328LeufsTer26, rs1565622421), which does not affect Val327 but changes the reading frame by the formation of a termination codon (*26aa) which results in a truncated protein. The variant has been detected once among approximately 120,000 individuals of GnomAD in an African individual.

The ***C1S*** **c.943G>A (p.Asp315Asn)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.35%) and European non-Finnish (0.55%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 unrelated C1-INH-HAE patients (1 Polish male type II, 2 Hungarians type I, 1 male, 1

female). There are conflicting interpretations of pathogenicity for this variant in ClinVar database, as it is characterised by two submitters VUS for complement component C1s deficiency and by another two submitters likely benign with no additional information concerning the condition or the carrier.

The ***MASP1*** **c.1528A>G (p.Ser510Gly)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.18%) and European non-Finnish (0.26%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 5 C1-INH-HAE patients from 3 different families. There are no clinical assertions for this variant in ClinVar database.

The ***MASP1*** **c.910C>A (p.Leu304Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.025%), mainly in European non-Finnish individuals (0.053%). Among 233 C1-INH-HAE patients of this study, it was detected in 2 Hungarian female twins with C1-INH-HAE type I. In ClinVar database, the variant is characterised by three different submitters VUS with no additional information concerning the condition or the carrier. In NCBI, the reference SNP number (rs145057248) represents a multiallelic variant, as at the same position there is another substitution c.910C>G (p.Leu304Val). The c.910C>G is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of GnomAD, in European non-Finnish male individuals.

The ***MASP1*** **c.1931C>T (p.Thr644Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.033%) and European non-Finnish (0.048%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 4 C1-INH-HAE type II patients (1 male, 3 female), members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database.

The ***MASP1*** **c.1520C>T (p.Thr507Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.028%) and European non-Finnish (0.048%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. In ClinVar database, the variant is characterised by one submitter as VUS with no additional information concerning the condition or the carrier.

The ***MASP1*** **c.1507C>T (p.Arg503Cys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.045%) and European non-Finnish (0.03%) population. Among 233 C1-INH-HAE patients of this study, it was detected in two unrelated C1-INH-HAE type I patients (1 Hungarian, 1 German). In ClinVar database, the variant is characterised by one submitter VUS with no additional information concerning the condition or the carrier.

The ***MASP1*** **c.-22C>T** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide (0.014%) and European non-Finnish (0.023%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***MASP2*** **c.296G>A (p.Arg99Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.5%) and European non-Finnish (0.034%) population. However, for the African population it is a common polymorphism (6.9%). Among 233 C1-INH-HAE patients of this study, it was detected in a Bulgarian male patient with C1-INH-HAE type I. In ClinVar database, the variant is characterised by one submitter likely benign for MASP2 deficiency and by another submitter benign with no additional information concerning the condition or the carrier.

The ***MASP2*** **c.1304G>A (p.Gly435Glu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Bulgarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. Another variant changing the same amino acid (c.1303G>A, p.Gly435Arg, rs1341653478) has been detected twice among approximately 120,000 individuals of GnomAD, in two South Asian female individuals. c.1303G>A is predicted as deleterious and probably damaging by SIFT and PolyPhen algorithms, respectively.

The ***MASP2*** **c.881C>T (p.Thr294Met)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.68%) and European non-Finnish (0.48%) population. However, it is a common polymorphism for Finnish (2.7%) and South Asian (1.5%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 unrelated C1-INH-HAE type I patients (1 Polish male, 1 Greek male, 1 Bulgarian female). In ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier.

The ***MASP2*** **c.1731A>C (p.Gln577His)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.4%) and European non-Finnish (0.0079%) population. The variant is a common polymorphism for East Asian population (4.1%). Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I. In ClinVar database the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier and by another submitter likely benign for MASP2 deficiency.

The ***MASP2*** **c.1882A>G (p.Ser628Gly)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.01%) and

European non-Finnish (0.02%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 unrelated C1-INH-HAE patients (1 Hungarian male and 1 Greek female). There are no clinical assertions for this variant in ClinVar database.

The ***MASP2*** **c.467G>A (p.Cys156Tyr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.6%) and European non-Finnish (0.9%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 5 patients from 3 different families with C1-INH-HAE type I. In ClinVar database, there are conflicting interpretations of the pathogenicity of this variant. It is characterised twice benign and likely benign with no additional information concerning the condition or the carrier. However, it is characterised by one submitter VUS for MASP2 deficiency. At the same codon, a frameshift variant has been detected once in a South Asian male individual, c.466dupT (p.Cys156LeufsTer21, rs1557677609).

The ***MASP2*** **c.193G>A (p.Asp65Asn)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.0016%) and European non-Finnish (0.0013%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***C1QBP*** **c.41C>G (p.Ser14Cys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TLR2*** **c.1216G>A (p.Gly406Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.003%), mainly in South Asian individuals. It has never been detected in European non-Finnish population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TLR2*** **c.798G>C (p.Leu266Phe)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.096%) and European non-Finnish (0.18%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 unrelated C1-INH-HAE patients (1 Hungarian male, 1 Greek male). There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs150388453) represents a multiallelic variant, as at the same position there is another substitution c.798G>C (p.=), a synonymous variant which has been detected once among approximately 120,000 individuals of GnomAD, in an East-Asian female individual.

The ***TLR2*** **c.*105G>A** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected once among approximately 120,000 individuals of the GnomAD in an Ashkenazi Jewish male individual. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TLR2*** **c.650T>C (p.Phe217Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.41%) and European non-Finnish (0.52%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 unrelated patients (1 Greek, 1 German) with C1-INH-HAE type I. In ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier.

The ***TLR2*** **c.619A>G (p.Met207Val)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it has been detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. Another missense variant, changing the same amino acid (c.620T>C, p.Met207Thr, rs901988067) has been detected once in an African female individual among approximately 120,000 individuals of GnomAD. The c.620T>C is predicted as tolerated but possibly damaging by SIFT and PolyPhen algorithms, respectively.

The ***TLR2*** **c.337T>C (p.Ser113Pro)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.001%). More specifically, it has been detected in 3 male European non-Finnish individuals (0.003%). Among 233 C1-INH-HAE patients of this study, it was detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TLR2*** **c.2341G>T (p.Ala781Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of the GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***TLR4*** **c.2207T>C (p.Val736Ala)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.002%), only in European individuals, 4 European non-Finnish (0.0036%), 1 Finnish (0.0046%). Among 233 C1-INH-HAE patients of this study, it was detected in 2 C1-INH-HAE type I patients (1 male, 1 female), members of a Polish family. There are no clinical assertions for this variant in ClinVar database. Another variant changing the same amino acid (c.2206G>T, p.Val736Leu, rs762970720) has

been detected in a Finnish male individual. The c.2206G>T is predicted as deleterious and probably damaging by SIFT and PolyPhen algorithms, respectively.

The ***TLR4*** **c.842G>A (p.Cys281Tyr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and probably damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.23%) and European non-Finnish (0.32%) population. Among 233 C1-INH-HAE patients of this study, it was detected in two C1-INH-HAE type II female patients, members of a Hungarian family. In ClinVar database, there is a submission with no interpretation of pathogenicity and no additional information concerning the condition or the carrier.

The ***TLR4*** **c.2411G>A (p.Arg804Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.07%) and European non-Finnish (0.12%) population. Among 233 C1-INH-HAE patients of this study, it was detected in two C1-INH-HAE type I patients (1 male, 1 female), members of a Hungarian family. There are no clinical assertions for this variant in ClinVar database.

The ***TLR4*** **c.2500C>A (p.Gln834Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.08%) and European non-Finnish (0.11%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in two unrelated Greek patients with C1-INH-HAE type I (1 male, 1 female). There are no clinical assertions for this variant in ClinVar database.

The ***TLR4*** **c.43A>T (p.Met15Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide population (0.0028%), only in European non-Finnish individuals (0.006%). Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs202028525) represents a multiallelic variant, as at the same position there is another substitution c.43A>G (p.Met15Val). The c.43A>G is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. However, it has never been detected among approximately 120,000 individuals of GnomAD.

The ***TLR9*** **c.*18G>C** has not been previously associated with C1-INH-HAE. This 3'UTR variant has been detected in low frequency in the worldwide (0.62%) and European non-Finnish (0.91%) population. Among 233 C1-INH-HAE patients of this study, it was detected in two unrelated patients, 1 Bulgarian, 1 German. There are no clinical assertions for this variant in ClinVar database.

The ***TLR9*** **c.2588G>A (p.Arg863Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.83%) and European non-Finnish (0.44%) population. However, it is a common polymorphism for South-Asian (2.6%) and African (3.4%) populations. Among 233 C1-INH-HAE patients of this study, it was detected in 4 unrelated patients (2 Hungarian male, 1 Greek female, 1 Polish male) with C1-INH-HAE type I. In

ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier. Two studies[263,264] investigated the association of this variant with susceptibility to lung disease and Vogt-Koyanagi-Harada (VKH) disease respectively, with no significant results.

The ***HRH1*** **c.57G>C (p.Lys19Asn)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.41%) and European non-Finnish (0.58%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 3 unrelated C1-INH-HAE patients (1 Hungarian, 1 Greek, 1 German). In ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier.

The ***IL5*** **c.329G>A (p.Arg110Gln)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected twice among approximately 120,000 individuals of the GnomAD, in 2 South Asian male individuals. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs775344061) represents a multiallelic variant, as at the same position there is another substitution c.329G>T (p.Arg110Leu). The c.329G>T is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. However, it has never been detected among approximately 120,000 individuals of GnomAD. Another variant changing the same amino acid (c.328C>T, p.Arg110Trp, rs201223432) has been detected in low frequency in the worldwide (0.0024%) and European non-Finnish (0.0018%) population. The c.328C>T is predicted as tolerated and benign by SIFT and PolyPhen algorithms, respectively.

The ***TNF*** **c.251C>T (p.Pro84Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.25%) and European non-Finnish (0.24%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I. In ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier. In NCBI, the reference SNP number (rs4645843) represents a multiallelic variant, as at the same position there is another substitution c.251C>G (p.Pro84Arg). The c.251C>G is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. However, it has never been detected among approximately 120,000 individuals of GnomAD.

The ***HSP90AA1*** **c.302delA (p.Gln101ArgfsTer22)** has not been previously associated with C1-INH-HAE. This deletion causes interruption of the reading frame by the formation of a termination codon (*22aa) which results in a truncated protein. It has been detected in low frequency in the worldwide population (0.002%) and only in European non-Finnish individuals (0.004%). Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***HSP90AA1*** **c.1102G>A (p.Glu368Lys)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated according to SIFT algorithm. It has been detected twice among approximately 120,000 individuals of the GnomAD, in 2 South Asian individuals. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. An INDEL variant has been detected in the same position (c.1101_1102insAAG, p.Lys367dup, rs1364962160) in 2 European non-Finnish and 1 Latino individual of the GnomAD.

The ***HSP90AA1*** **c.1930A>G (p.Ile644Val)** is a novel variant which is predicted as tolerated according to SIFT algorithm. It has never been detected in the worldwide population according to GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***HSP90AA1*** **c.-3C>G** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide population (0.008%) but never in European non-Finnish individuals according to GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Hungarian female patient with C1-INH-HAE type II. There are no clinical assertions for this variant in ClinVar database.

The ***HSP90AA1*** **c.37C>T (p.Pro13Ser)** has not been previously associated with C1-INH-HAE. This missense variant has been detected in low frequency in the worldwide (0.2%) and European non-Finnish (0.35%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KRT1*** **c.1669A>G (p.Ser557Gly)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated according to SIFT algorithm. It has been detected in low frequency in the worldwide (0.03%) and European non-Finnish (0.026%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 9 unrelated C1-INH-HAE patients. In ClinVar database, the variant is characterised by one submitter likely benign for epidermolytic hyperkeratosis and nonepidermolytic palmoplantar hyperkeratosis.

The ***KRT1*** **c.*17T>G** has not been previously associated with C1-INH-HAE. This 3'UTR variant has never been detected in the worldwide population according to GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Bulgarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KRT1*** **c.281A>C (p.Tyr94Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***KRT1*** **c.173C>G (p.Ala58Gly)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected in the worldwide population according to GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a German patient with C1-INH-HAE type I.

There are no clinical assertions for this variant in ClinVar database. At the same position, an INDEL variant (NM_006121.3:c.155_172del, p.Gly52_Gly57del, rs771524812) has been reported once in GnomAD in a Southern European male patient.

The ***PROC*** **c.554G>A (p.Arg185Lys)** is a novel missense variant which is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected in the worldwide population according to GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Greek male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***ESRRA*** **c.*150G>A** has not been previously associated with C1-INH-HAE. This novel 3'UTR variant has never been detected in the worldwide population according to GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***GPER1*** **c.14C>T (p.Ser5Phe)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.92%) and European non-Finnish (0.44%) population. However, it is a common polymorphism for Latino (3.6%) and East Asian (2.3%) populations. Among 233 C1-INH-HAE patients of this study, it was detected in 4 unrelated patients. In ClinVar database, the variant is characterised by one submitter benign with no additional information concerning the condition or the carrier.

The ***GPER1*** **c.808G>A (p.Val270Ile)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.018%) and European non-Finnish (0.037%) population. Among 233 C1-INH-HAE patients of this study, it was detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs376978032) represents a multiallelic variant, as at the same position there is another substitution c.808G>T (p.Val270Phe). The c.808G>T is predicted as tolerated but possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has never been detected among approximately 120,000 individuals of GnomAD.

The ***GPER1*** **c.100T>A (p.Ser34Thr)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.19%) and European non-Finnish (0.067%) population. However, it is a common polymorphism in Finnish population (1.8%). Among 233 C1-INH-HAE patients of this study, it was detected in a Polish female patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***GPER1*** **c.1119T>G (p.Ser373Arg)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.4%) and European non-Finnish (0.65%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 unrelated patients. There are no clinical assertions for this variant in ClinVar database.

The ***AR* c.1288G>T (p.Ala430Ser)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has never been detected in approximately 120,000 individuals of GnomAD. Among 233 C1-INH-HAE patients of this study, it was detected in a Bulgarian male patient with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database. In NCBI, the reference SNP number (rs755088348) represents a multiallelic variant, as at the same position there is another substitution c.1288G>A (p.Ala430Thr). The c.1288G>A is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected once among approximately 120,000 individuals of GnomAD, in an Estonian female individual.

The ***AR* c.2395C>G (p.Gln799Glu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0,14%) and European non-Finnish (0.24%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 patients with C1-INH-HAE type I, members of a Polish family. In ClinVar database, the variant is characterised by one submitter pathogenic for partial androgen insensitivity and by another submitter as likely pathogenic with no further information concerning the condition or the carrier.

The ***AR* c.-207C>A** has not been previously associated with C1-INH-HAE. This 5'UTR variant has been detected in low frequency in the worldwide (0.28%) and European non-Finnish (0.47%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in 3 patients (2 female, 1 male) with C1-INH-HAE type I. There are no clinical assertions for this variant in ClinVar database.

The ***AR* c.1174C>T** (p.Pro392Ser) has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and benign according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.45%) and European non-Finnish (0.27%) population. However, it is a common polymorphism for South Asian population (2.48%) Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I. In ClinVar database, there are conflicting interpretations of pathogenicity. It is characterised by two different submitters VUS for androgen resistance syndrome and X-linked hypospadias 1, respectively. Moreover, it is characterised once pathogenic for reifenstein syndrome and finally, benign with no additional information concerning the condition or the carrier.

The ***AR* c.814C>T (p.Leu272Phe)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as tolerated and possibly damaging according to SIFT and PolyPhen algorithms, respectively. It has been detected in low frequency in the worldwide (0.013%) and European non-Finnish (0.0086%) population. Among 233 C1-INH-HAE patients of this study, it was detected in 2 unrelated patients with C1-INH-HAE type I (1 Hungarian female, 1 Greek male). There are no clinical assertions for this variant in ClinVar database.

The ***ELANE* c.770C>T (p.Pro257Leu)** has not been previously associated with C1-INH-HAE. This missense variant is predicted as deleterious but benign according to SIFT and PolyPhen algorithms,

respectively. It has been detected in low frequency in the worldwide (0.64%) and European non-Finnish (0.85%) population. Among 233 C1-INH-HAE patients of this study, it has been detected in a Hungarian female patient with C1-INH-HAE type I. In ClinVar database, the variant is characterised by 4 different submitters benign/likely benign, with no additional information on the condition or the carrier and once as benign for severe congenital neutropenia.

APPENDIX IV

Figure 1. ClinVar TemplateLite

#Version L1.5, lite spreadsheet template for submitting data to ClinVar. This spreadsheet does not support all the data elements in ClinVar; if you wish to submit more detailed information, please review our full submission spreadsheet template.

Release date 8/21/2018

Complete instructions available here: www.ncbi.nlm.nih.gov/clinvar/docs/submit/
If you have any questions about this form, please contact us at clinvar@ncbi.nlm.nih.gov

Please register in the ClinVar Submission Portal before you submit:
https://submit.ncbi.nlm.nih.gov/clinvar/
After your registration is approved, you can upload your spreadsheet in the Submission Portal.

Please note this workbook consists of five sheets, including this one. **TheVariant and ExpEvidence worksheets are required for all submission files.**

Sheet name	Description	Comment
READ_ME	This sheet. Overview of the template and general instructions.	
SubmissionInfo (hidden)	To register information about the submitter, and information that pertains to all data in the submission file.	Only used by submitters who drop their submission files on the ftp site. Submitters who use the Submission Portal provide the information from this worksheet directly in the Submission Portal and should not use this worksheet at all.
Variant	To register summary-level information about variant interpretations, including the variant, condition, and clinical significance.	REQUIRED
ExpEvidence	To register aggregate-level evidence for the interpretations reported on the Variant worksheet.	REQUIRED
Deletes	To delete previously submitted interpretations.	REQUIRED for deleted interpretations. This worksheet is used only when you are requesting to delete one or more of your previously submitted records.

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Methodological paper

Targeted next-generation sequencing for the molecular diagnosis of hereditary angioedema due to C1-inhibitor deficiency

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ABSTRACT

SERPING1 genotyping of subjects suspicious for hereditary angioedema due to C1-INH deficiency (C1-INH-HAE) is important for clinical practice as well as for research reasons. Conventional approaches towards the detection of C1-INH-HAE-associated *SERPING1* variants are cumbersome and time-demanding with many pitfalls. To take advantage of the benefits of next-generation sequencing (NGS) technology, we developed and validated a custom NGS platform that, by targeting the entire *SERPING1* gene, facilitates genetic testing of C1-INH-HAE patients in clinical practice. In total, 135 different C1-INH-HAE-associated *SERPING1* variants, out of the approximately 450 reported, along with 115 negative controls and 95 randomly selected DNA samples from affected family members of C1-INH-HAE index patients, were included in the forward and reverse validation processes of this platform. Our platform's performance, i.e. analytical sensitivity of 98.96%, a false negative rate of 1.05%, analytical specificity 100%, a false positive rate equal to zero, accuracy of 99.35%, and repeatability of 100% recommends its implementation as a first line approach for the genetic testing of C1-INH-HAE patients or as a confirmatory method. A noteworthy advantage of our platform is the concomitant detection of single nucleotide variants and copy number variations throughout the whole length of the *SERPING1* gene, moreover providing information about the size and the localization of the latter. During our study, 15 novel C1-INH-HAE-related *SERPING1* variants were detected.

1. Introduction

Hereditary angioedema is a relatively rare, disabling and potentially life-threatening disorder characterized by recurrent attacks of subcutaneous or submucosal edemas, which develop as a result of a transient and localized increase of bradykinin and increased permeability of

Abbreviations: C1-INH, C1-inhibitor; C1-INH-HAE, hereditary angioedema due to C1-INH deficiency; CI, confidence interval; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; CNV, copy number variation; SNV, single-nucleotide variant; *SERPING1*-NGS platform, the custom NGS platform developed for *SERPING1* genotyping; U-HAE, unknown hereditary angioedema

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blood vessels. C1-INH-HAE is the most common and best-characterized form of hereditary angioedema (Cicardi et al., 2014). It is attributed to genetic alterations in the *SERPING1* gene encoding C1-INH, a regulatory protein of the contact system. *SERPING1* gene extends over a 17,159 bp genomic region located on chromosome 11q12-q13.1. Its pronounced allelic heterogeneity as well as the fact that approximately 20–25% of all unrelated patients with C1-INH-HAE represent sporadic cases carrying de novo *SERPING1* mutations with the same mutational spectrum as the familial ones, makes *SERPING1* a prime example of mutagenic lability. *SERPING1*'s proximity to the centromere and the high incidence of Alu repeat sequences and CpG sites explain its higher than average mutation rates (Pappalardo et al., 2002; Cicardi et al., 2005). Over 450 different *SERPING1* mutations related with C1-INH-HAE have been detected up to now, scattered over the eight exons and their flanking intronic sequences (Germenis & Speletas, 2016). The majority of C1-INH-HAE-related *SERPING1* mutations are SNVs (34% missense mutations, 10% splice-site defects, 7% nonsense mutations, 1% regulatory mutations) followed by CNVs (31% frameshift alterations and small indels, 17% large gene rearrangements) (Speletas et al., 2015a). In a small proportion of patients with C1-INH-HAE (approximately 5–10%) no *SERPING1* alterations can be detected even after meticulous analysis of the coding region (Speletas et al., 2015a; Pappalardo et al., 2008; Csuka et al., 2015) implying that the defect that modifies C1-INH expression may be located in an intronic or an untranslated region of the gene, like those recently reported (Maas et al., 2008; Kang et al., 2006; Colobran et al., 2014).

The diagnosis of C1-INH-HAE is based on its clinical appearance and is confirmed by the detection of low plasma levels of antigenic and/or functional C1-INH. However, with every passing day, *SERPING1* genotyping of patients who suffer from hereditary angioedema becomes more indispensable in the clinical practice (Farkas et al., 2017) as well as for research reasons. The conventional molecular analysis of *SERPING1* relies on 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, further analyses for the identification of large gene rearrangements are performed, usually by two different techniques, namely the long-range PCR and the MLPA. This cumbersome and time-consuming approach is fraught with pitfalls as it is highly dependent on users' experience and knowledge. Sometimes, for example, a novel missense mutation is incorrectly held to be the causal genetic defect because its detection during the early steps of the analysis has prevented the discovery of an indel leading to premature truncation of protein synthesis. Moreover, deep intronic alterations that may affect pre-mRNA splicing and possibly contributing to C1-INH-HAE phenotype, as it is true for many other diseases (Vaz-Drago et al., 2017), escape the conventional approach.

NGS overcomes many of shortcomings of traditional approaches. Here, we report the development and the validation of a custom NGS platform that targets the entire 11q12-q13.1 locus, including the promoter, coding, intron-exon boundary as well as intronic regions of the *SERPING1* gene, following the recently published professional standards and guidelines (Rehm et al., 2013; Weiss et al., 2013).

2. Material and methods

2.1. DNA samples

102 DNA samples from already diagnosed and genotyped C1-INH-HAE index patients (99 type I, 3 type II; 31 Hungarian, 33 Greek, 17 German, 11 Romanian, 10 Spanish) carrying different *SERPING1* variants were used for the forward validation of the newly developed NGS platform for *SERPING1* genotyping. The previously reported C1-INH-HAE associated SNVs and indels (76), and CNVs (22) are shown in Tables 1 and 2, respectively. Four additional samples in which the detection of a causal *SERPING1* defect had not been achieved were also included in the analysis. As negative controls, 115 DNA samples from patients diagnosed with U-HAE (normal antigenic and functional C1-INH plasma levels) previously genotyped and free of *SERPING1* mutations were used.

Another cohort of 91 not genotyped patients (4 Greek, 30 Bulgarian, 36 Polish, 11 German, and 10 Romanian) already diagnosed with C1-INH-HAE (88 type I, 3 type II) belonging to 55 different families was used for the blind reverse validation of the developed *SERPING1*-NGS platform.

In order to test the repeatability of the *SERPING1*-NGS platform, 95 randomly selected DNA samples from C1-INH-HAE-affected family members of the above index patients were analyzed.

2.2. Next-generation sequencing

A NGS custom panel was designed using the Ion AmpliSeq Thermo Fisher Scientific Designer, in order to analyze *SERPING1* in its full length (all exons, introns, promoter, 5′- and 3′-untranslated regions –UTRs). 77 amplicons in two primer pools provide 100% coverage of all translated regions and UTRs with missing areas located only in intronic regions (overall coverage ≈83%). Additional amplicons (300 in total) for different genes in all chromosomes, selected among those encoding for proteins involved in bradykinin metabolism, were included in the panel, in order to achieve a more robust CNVs analysis.

DNA libraries for each sample were constructed using the Ion AmpliSeq Library Kit 2.0 (Thermo Scientific). Briefly, 10 ng of gDNA per primer pool was amplified and indexed with a unique adapter using Ion Xpress barcode adapters kit (Thermo Scientific). Equal volumes of the two pools were combined and barcoded libraries were purified using the Agencourt AMPure XP Beads (Beckman Coulter), quantified with Qubit 2.0 fluorometer (Thermo Scientific), diluted to 100 pM and pooled in equimolar proportion. Template preparation was carried out on OneTouch2 system using the Ion OT2 HI-Q Template kit (Thermo Scientific) and the enrichment of Ion Sphere Particles (ISPs) on Ion One Touch ES (Thermo Scientific). Sequencing was performed on PGM using Ion PGM Hi-Q sequencing kit (Thermo Scientific), on 318 chip. All procedures were performed according to the manufacturer's instructions.

Sequencing raw data were analyzed for base calling, demultiplexing, alignment to hg19 reference genome (GRCh37), coverage analysis and variant calling on Torrent Suite 5.2 software using default parameters. More specifically, variant calling was performed by the VariantCaller v.5.2 plug-in and coverage analysis by the coverageAnalysis v.5.2.1.2 plug-in. Annotation of variants and CNVs analysis was performed on Ion Reporter software v.5.2 (Thermo Scientific). Detected variants were annotated with the gene name and for their possible presence in the dbSNP database according the recommendations of the Human Genome Variation Society (HGVS) (http://www.hgvs.org/mutnomen/). In silico pathogenicity prediction of the variants was performed by the usage of SIFT (http://sift.jcvi.org/) and PolyPhen version 2 (http://genetics.bwh.harvard.edu/pph2/) bioinformatics tools. CNVs were detected by Hidden Markov Model algorithm on Ion Reporter software v.5.2 using paired CNVs analysis and default parameters. CNV analysis workflow uses depth of coverage and is limited by the number and extend of amplicons that cover the affected regions. Alignments and all obtained *SERPING1* sequences were visually inspected using the Integrative Genomics Viewer (IGV) v2.2 (Broad Institute).

2.3. Variants verification

All identified variants were verified by Sanger sequencing as previously described (Speletas et al., 2015a). Emerged CNVs were verified by a commercial MLPA kit (MRC-Holland for *SERPING1*) and by long-range PCR. In MLPA, 60 ng of gDNA from each sample were analyzed. Three reference samples (without CNVs), one positive and one negative control were included. All procedures were performed according to

Table 1
The SNVs carried by the previously genotyped patients used in the forward validation of the *SERPING1*-NGS platform.

cDNA	Protein	Location	Type of mutation	Reference
c.1A > G	M1V	Exon 2	Regulatory	(Gösswein et al., 2008)
c.1A > C[a]	M1L	Exon 2	Regulatory	(Speletas et al., 2015b)
c.2 T > G	M1R	Exon 2	Regulatory	(Speletas et al., 2015b)
c.51 + 1G > A		Intron 2	Splice defect	(Gösswein et al., 2008)
c.51 + 5G > A		Intron 2	Splice defect	(Verpy et al., 1996)
c.65C > G	S22X	Exon 3	Nonsense	(Speletas et al., 2015a)
c.94C > T	Q32X	Exon 3	Nonsense	(Kalmár et al., 2003)
c.121delG	G41 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.124G > T	E42X	Exon 3	Nonsense	(Speletas et al., 2015b)
c.235 delA	T79 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.239C > G	A80G	Exon 3	Missense	(Speletas et al., 2015b)
c.325_337delCTCCCAACAGATT	T111 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.347delA	Q116fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.357_358insGGGTCCTTCTGCCCAGGACC	V127 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.389G > A	C130Y	Exon 3	Missense	(Kalmár et al., 2003)
c.392_393delCT	S131X	Exon 3	Nonsense	(Speletas et al., 2015a)
c.435_476delTTTGGTAGATTTCTCCCTGAAGCTCTACCACGCCTTCTCAGC	L145_S158del	Exon 3	Deletion	(Kalmár et al., 2003)
c.448 T > C	S150P	Exon 3	Missense	(Speletas et al., 2015b)
c.467C > A	A156D	Exon 3	Missense	(Gösswein et al., 2008)
c.498C > A	N166 K	Exon 3	Missense	(Cagini et al., 2016)
c.500T > A	M167 K	Exon 3	Missense	(Speletas et al., 2015b)
c.508delT	S170 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.550G > A	G184R	Exon 3	Missense	(Verpy et al., 1996)
c.550 + 2_550 + 3insT		Exon 3	Frameshift	(Kalmár et al., 2003)
c.550 + 1G > A		Intron 3	Splice defect	(Kalmár et al., 2003)
c.550 + 2 T > C		Intron 3	Splice defect	(Speletas et al., 2015b)
c.550 + 5G > A		Intron 3	Splice defect	(Roche et al., 2005)
c.551-1G > A		Intron 3	Splice defect	(Speletas et al., 2015b)
c.553G > C	A185P	Exon 4	Missense	(Speletas et al., 2015a)
c.586-589DelATCC	I195fs	Exon 4	Frameshift	(Speletas et al., 2015b)
c.600insC	K201 fs	Exon 4	Frameshift	(Speletas et al., 2015b)
c.650_651delGT	V218 fs	Exon 4	Frameshift	(Speletas et al., 2015b)
c.675–76TC > AA	F225X	Exon 4	Nonsense	(Speletas et al., 2015b)
c.667C > T	Q223X	Exon 4	Nonsense	(Kalmár et al., 2003)
c.671 T > A	I224N	Exon 4	Missense	(Gösswein et al., 2008)
c.728 T > C	L243P	Exon 5	Missense	(Speletas et al., 2015a)
c.871A > C	N291H	Exon 5	Missense	(Gösswein et al., 2008)
c.878 T > C	I293T	Exon 5	Missense	(Pappalardo et al., 2000)
c.889 + 4delAGGGT		Intron 5	Splice defect	(Speletas et al., 2015b)
c.890-14C > G		Intron 5	Splice defect	(Speletas et al., 2015b)
c.896G > A[b]	W299X	Exon 6	Nonsense	(Speletas et al., 2015b)
c.908 T > C	F303S	Exon 6	Missense	(Gösswein et al., 2008)
c.1029 + 1G > A		Intron 6	Splice defect	(Gösswein et al., 2008)
c.1030-1G > A		Intron 6	Splice defect	(Speletas et al., 2015b)
c.988 T > G	Y330D	Exon 7	Missense	(Speletas et al., 2015a)
c.1036C > T	Q346X	Exon 7	Nonsense	(Verpy et al., 1996)
c.1038_1052delGCTGCAGCTCTCCCA	Q346_S350del	Exon 7	Deletion	(Speletas et al., 2015b)
c.1106delA	D369fs	Exon 7	Frameshift	(Speletas et al., 2015b)
c.1127_1128insC	S377 fs	Exon 7	Frameshift	(Speletas et al., 2015a)
c.1180delA	T394 fs	Exon 7	Frameshift	(Speletas et al., 2015b)
c.1187 T > C	L396P	Exon 7	Missense	(Gösswein et al., 2008)
c.1193 T > C	L398P	Exon 7	Missense	(Gösswein et al., 2008)
c.1223A > T	D408V	Exon 7	Missense	(Kalmár et al., 2003)
c.1243delA	K416 fs	Exon 7	Frameshift	(Gösswein et al., 2008)
c.1250-2A > G		Intron 7	Splice defect	(Gösswein et al., 2008)
c.1341_1342insGAACTGAC_dupl	E451fs	Exon 8	Frameshift	(Speletas et al., 2015b)
c.1346 T > G	L449R	Exon 8	Missense	(Speletas et al., 2015b)
c.1353_1354delGA	E451fs	Exon 8	Frameshift	(Gösswein et al., 2008)
c.1356_1357delTG	T454 fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1367C > A	A456Q	Exon 8	Missense	(Skriver et al., 1991)
c.1373C > T	A458V	Exon8	Missense	(Siddique et al., 1992)
c.1382_1383insGGGGTGGAGGCGGCTGCAGCCTCCGC	I462fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1391_1392delTG	V464 fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1396C > T	R466C	Exon 8	Missense	(Skriver et al., 1991)
c.1396C > G	R466G	Exon 8	Missense	(Gösswein et al., 2008)
c.1397G > A	R466H	Exon 8	Missense	(Faiyaz-Ul-Haque et al., 2010; Rijavec et al., 2013)
c.1409insG	V470 fs	Exon 8	Frameshift	(Speletas et al., 2015b)
c.1417G > A	V473 M	Exon 8	Missense	(Verpy et al., 1996)
c.1418 T > G	V473G	Exon 8	Missense	(Blanch et al., 2002)
c.1418 T > A	V473E	Exon 8	Missense	(Kalmár et al., 2003)
c.1420C > T	Q474X	Exon 8	Nonsense	(Speletas et al., 2015b)
c.1446G > A	W482X	Exon 8	Nonsense	(Speletas et al., 2009)
c.1466delC	P489fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1478G > A	G493E	Exon 8	Missense	(Blanch et al., 2002)

(*continued on next page*)

Table 1 (*continued*)

cDNA	Protein	Location	Type of mutation	Reference
c.1480C > T	R494X	Exon 8	Nonsense	(Verpy et al., 1996)
c.1483-7delGTATA	V495X	Exon 8	Nonsense	(Speletas et al., 2015b)
c.1493C > G	P498R	Exon 8	Missense	(Speletas et al., 2015a)
c.1499_1505delCCTGAGA	A500fs	Exon 8	Frameshift	(Speletas et al., 2015b)

[a] Previously reported by conventional genotyping as large insertion in exon 5.
[b] Previously reported by conventional genotyping as large deletion in exon 6.

manufacturers' instructions. Fragment analysis was carried out on ABI 3730xl (Applied Biosystems) and MLPA data analysis in Coffalyser.Net software (MRC-Holland). Long-range PCR was performed as previously described (Pappalardo et al., 2000).

3. Results

3.1. Validation

Our NGS panel generates 300 amplicons of 204 bp on average, covering all translating regions, exon-intron boundaries, UTRs and most of the intronic regions of *SERPING1* gene, with overlapping amplicons across the gene. In the 102 DNA samples from conventionally genotyped C1-INH-HAE patients analyzed (48 SNVs, 28 indels, 22 large defects, and 4 without variants of *SERPING1*), a mean of 313,270 reads was produced per sample. On average, 91.5% of these reads mapped to the targeted regions, with a mean depth of coverage of 270× and 90% uniformity. For variant calling, a minimum 20× coverage was set as threshold.

Forward analysis of DNA samples with our *SERPING1*-NGS platform was concordant with conventional *SERPING1* typing in 97/102 cases, i.e. 47/48 SNVs, 26/28 indels, 20/22 large defects and 4/4 with no defect. Out of the five observed discrepancies, the conventional approach had miss-assigned two substitutions as large defects, i.e. a large insertion located in exon 5 and a large deletion located in exon 6. NGS analysis identified in the first of these samples a SNP altering the start codon of *SERPING1* (c.2 T > G, M1R) and in the second a mutation resulting in an early termination codon (c.896G > A, W299X). The presence of both of these mutations was confirmed by Sanger sequencing, whereas large defects were not detected by MLPA and long-range PCR. CNV analysis by our *SERPING1*-NGS platform did not uncover large defects in these samples.

The other three discrepancies were a 4 bp deletion (c.586-589DelATCC, I196fs), a missense mutation (c.553G > C, A185P) and a deletion of a single base (c.347delA, Q116fs). The first one was identified by VariantCaller as c.585C > G. However, by visually reviewing the IGV file, we found a complex variation with a 4 bp deletion and a single nucleotide substitution (c.585_589delCATCCinsG) assigned as S195 fs that thereafter was confirmed by Sanger sequencing. After adjustment of the VariantCaller by setting the allow_complex parameter to 1 instead of 0 and re-analysis, this complex variation was correctly recognized. Similarly, VariantCaller did not detect the other two variants (c.553G > C and c.347delA) that were again recognized after visually reviewing the IGV files. In fact, VariantCaller had highlighted these positions but as high background that was filtered out due to strand bias (< 1% in + strand). Adjustment of VariantCaller strand bias settings and re-analysis uncovered the presence of these mutations; however, this adjustment resulted in a high proportion of false positive variants which precluded its permanent use.

It has to be noted that all the 20 real CNVs of the initial cohort used for the forward validation of our *SERPING1*-NGS platform were correctly detected regardless of their type (deletion, duplication) and location. Moreover, information about their size and CytoBand localization was provided (Table 2). Similarly, in the 4 samples initially reported as not carrying any defect, the *SERPING1*-NGS platform also did not detect a defect.

Reverse validation of our *SERPING1*-NGS platform by blindly genotyping 91 C1-INH-HAE patients from 55 unrelated families detected *SERPING1* variants or their absence in all cases that were a posteriori confirmed by appropriate methods when required. Forty-four different

Table 2
The CNVs carried by the previously genotyped patients used in the forward validation of the *SERPING1*-NGS platform. (Two samples initially reported as CNVs by the conventional genotyping but not finally confirmed are not shown. See footnotes of Table 1).

CytoBand[a]	Length[a]	Location[a]	MLPA or long range PCR	Reference
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Roche et al., 2005)
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Roche et al., 2005)
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Speletas et al., 2015a)
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Speletas et al., 2015a)
11q12.1(57364831-57367229)x1	2.398 kb	upstream-intron 2	deletion exons 1–2	(Roche et al., 2005)
11q12.1(57366421-57369726)x1	3.305 kb	intron 2-intron 4	deletion exons 3–4	(Speletas et al., 2015a)
11q12.1(57367936-57369930)x1	1.994 kb	intron 3-intron 4	deletion exon 4	(Speletas et al., 2015a)
11q12.1(57368466-57369930)x1	1.464 kb	intron 3-intron 4	deletion exon4	(Speletas et al., 2015a)
11q12.1(57368466-57370856)x1	2.39 kb	intron 3-intron5	deletion exon 4	(Roche et al., 2005)
11q12.1(57368466-57370856)x1	2.39 kb	intron 3-intron 5	deletion exon 4	(Roche et al., 2005)
11q12.1(57368904-57369930)x1	1.026 kb	intron 3-exon 4	deletion exon 4	(Speletas et al., 2015a)
11q12.1(57369000-57370856)x3	1.856 kb	intron 3-intron 4	duplication exon 4	(Speletas et al., 2015a)
11q12.1(57369000-57370856)x3	1.856 kb	intron 3-intron 4	duplication exon 4	(Roche et al., 2005)
11q12.1(57369219-57370856)x1	1.637 kb	intron 3-intron 4	deletion exon 4	(Speletas et al., 2015a)
11q12.1(57371760-57376644)x1	4.884 kb	intron 4-intron 6	deletion exons 5–6	(Roche et al., 2005)
11q12.1(57373059-57378387)x3	5.328 kb	intron 4-intron 6	duplication exon 5–6	(Roche et al., 2005)
11q12.1(57375659-57382476)x1	6.817 kb	intron 6-exon 8	deletion exon 7–8	(Speletas et al., 2015a)
11q12.1(57377605-57380942)x1	3.337 kb	intron 6-intron 7	deletion exon 7	(Roche et al., 2005)
11q12.1(57379394-57380190)x1	796b	exon 7-intron 7	deletion exon 7	(Speletas et al., 2015a)
11q12.1(57379785-57382169)x1	2.384 kb	intron 7-exon 8	deletion exon 8	(Roche et al., 2005)

[a] Final information obtained by CNV analysis using the *SERPING1*-NGS platform.

Table 3
The different *SERPING1* variants detected during the reverse validation of *SERPING1*-NGS platform.

Origin	cDNA	Protein	Location	Type	Reference
PL	c.550G > A	G184R	Exon 3	Missense	(Verpy et al., 1996)[a]
BL	c.550 + 1G > A		Intron 3	Splice defect	(Roche et al., 2005)[a]
BL	c.600_601insC	K201 fs	Exon 4	Frameshift	(Speletas et al., 2015b)[a]
ROM	c.988 T > G	Y330D	Exon 6	Missense	(Speletas et al., 2015a)[a]
PL	c.1396C > T	R466C	Exon 8	Missense	(Skriver et al., 1991)[a]
GER	c.1396C > G	R466G	Exon 8	Missense	(Gösswein et al., 2008)[a]
GR	c.1480C > T	R494X	Exon 8	Nonsense	(Verpy et al., 1996)[a]
BL	c.-22-1G > A		Intron 1	Splice defect	(Verpy et al., 1996)
GR	c.55A > T	R19X	Exon 3	Nonsense	Novel
PL	c.94delC	Q32fs	Exon 3	Frameshift	Novel
PL	c.152C > T	S51F	Exon 3	Missense	Novel
BL	c.218delA	N73 fs	Exon 3	Frameshift	Novel
PL	c.301C > T	Q101X	Exon 3	Nonsense	Novel
ROM	c.424_425insGT	L142 fs	Exon 3	Frameshift	Novel
PL	c.425 T > C	L142S	Exon 3	Missense	Novel
BL	c.473C > G	S158X	Exon 3	Nonsense	(López-Lera et al., 2011)
PL	c.485_498delAGGTGGAGACCAACinsTGCTGAGA	K162 fs	Exon 3	Frameshift	Novel
ROM	c.503C > A	A168D	Exon 3	Missense	(Suffritti et al., 2014)
GER	c.508 T > A	S170 T	Exon 3	Missense	Novel
PL	c.551-2insG		Intron 3	Splice defect	Novel
PL	c.622C > T	Q208X	Exon 4	Nonsense	Novel
PL	c.793 T > C	T265R	Exon 5	Missense	(Bafunno et al., 2014)
BL	c.813_818delCAACAA	N271_N272del	Exon 5	Nonsense	Novel
GER	c.890-2A > G		Intron 5	Splice defect	(Bowen et al., 2001)
ROM	c.860 T > C	L287P	Exon 6	Missense	Novel
BL	c.897G > A	W299X	Exon 6	Nonsense	(Gösswein et al., 2008)
GR	c.1012C > T	Q338X	Exon 6	Nonsense	(Iwamoto et al., 2012)
GER	c.1027insC	K343 fs	Exon 6	Frameshift	Novel
ROM	c.1042C > T	Q348X	Exon 7	Nonsense	(Gösswein et al., 2008)
ROM/PL	c.1223A > G	D408G	Exon 7	Missense	(Martinho et al., 2013)
GR	c.1247 T > A	L416X	Exon 7	Nonsense	(Pappalardo et al., 2008)
GER	c.1250-1G > A		Intron 7	Splice defect	(Gösswein et al., 2008)
BL	c.1283G > A	C428Y	Exon 8	Missense	(López-Lera et al., 2011)
BL	c.1330C > T	Q444X	Exon 8	Nonsense	(Uyguner & Kesim, 2008)
PL	c.1333A > C	T445P	Exon 8	Missense	Novel
GER	c.1346 T > C	L449P	Exon 8	Missense	(Pappalardo et al., 2008)
PL	c.1372G > A	A458T	Exon 8	Missense	(Levy et al., 1990)
GER	11q12.1(57368466-57369930)x1	1.464 kb	Exon 4	Large defect	[b]
ROM	11q12.1(57368466-57369930)x1	1.464 kb	Exon 4	Large defect	[b]
ROM	11q12.1(57368466-57371760)x4	3.294 kb	Exon 4	Large defect	[b]
BL	11q12.1(57368904-57370856)x1	1.952 kb	Exon 4	Large defect	[b]
PL	11q12.1(57368904-57369726)x1	822b	Exon 4	Large defect	[b]
PL	11q12.1(57368466-57369930)x1	1.464 kb	Exon 4	Large defect	[b]
PL	11q12.1(57379590-57382476)x1	2.886 kb	Exon 8	Large defect	[b]

[a] Detected in forward validation.
[b] Large defects in the same region (exons 4 and 8) have been reported. However, data on their exact extend and localization are not available; therefore, it is unknown whether these are exactly the same defects.

variants were detected of which 15 (34.1%) were missense, 11 (25%) nonsense, 7 (15.9%) large defects, 6 (13.6%) frameshift mutations and 5 (11.4%) splice-site defects (Table 3). No *SERPING1* alteration was detected in 8 out of the 55 families (14.5%), neither by the *SERPING1*-NGS platform nor the confirmatory conventional genotyping. Twenty two out of the 37 SNVs detected during reverse validation had been already reported in the literature, while 15/37 were not yet reported alterations (Table 3). Interestingly, one large defect reported by the *SERPING1*-NGS platform as a large duplication of 3.3 kb [11q12.1(57368466–57371760) × 4, including exon 4] was confirmed by long-range PCR, but not by MLPA. Possibly, the observed duplication consists only of intronic regions and, as a result, the CNVs analysis of *SERPING1*-NGS platform led to reporting a more extended defect.

In total, 135 different C1-INH-HAE-associated *SERPING1* variants were included in the overall validation process of our *SERPING1*-NGS platform and the platform failed to detect only two variants. Further, analysis of negative controls did not reveal any C1-INH-HAE-associated variants, although there were some polymorphisms of the *SERPING1* gene (e.g. V480 M). As such, the *SERPING1*-NGS platform's analytical sensitivity was 98.96% (95% CI 96.31–99.87%) with a false negative rate 1.05%, its analytical specificity was 100% (95% CI 96.84–100.00%) with a false positive rate equal to zero, and its accuracy was 99.35% (95% CI 97.67–99.92%). The repeatability of the *SERPING1*-NGS platform was found to be 100%.

3.2. Novel SERPING1 mutations

Out of the 15 novel mutations detected during the reverse validation, 5 (33.3%) were missense, 4 (26.6%) nonsense, 5 (33.3%) frameshift mutations and 1 (6.6%) splice-site defect. All novel missense mutations were predicted to be deleterious by SIFT and PolyPhen prediction tools; however, none had been detected in > 65,000 Europeans of the ExAC Database (except for S51F, with an allele frequency of < 0.001%). Pathogenicity of eight of the novel mutations was supported by family segregation studies. Family segregation also proved that three of nonsense variants represented de novo mutations. The fact that one novel missense mutation (c.508 T > A) occurred at amino acid residue where a different missense change has been previously observed in association with C1-INH-HAE [c.508 T > C (Pappalardo et al., 2000)] is further evidence of that mutation's pathogenicity (Richards et al., 2015). Similarly, the novel mutation c.551-2insG occurs at the −2 position of the acceptor site of exon 4, where a

substitution (c.551-2A > G) was previously reported in four patients from three C1-INH-HAE families (Gösswein et al., 2008). Finally, the observed c.301C > T (Q101X) has been previously detected in three affected members of a Serbian family (Andrejević et al., 2015), but it was reported as c.300C > T (Q101X).

4. Discussion

In the vast majority of patients, the laboratory diagnosis of C1-INH-HAE is based on the measurement of antigenic and/or functional C1-INH plasma levels. However, genetic testing is often helpful or required. For example, patients younger than 1 year-of-age present with C1-INH levels lower than in adults (Craig et al., 2012; Nielsen et al., 1994), making diagnosis by traditional means difficult. Similarly, genetic testing is useful in the workflows for prenatal and preimplantation diagnostics (Farkas et al., 2017), as well as for confirmation of causal *SERPING1* variants carried by apparently healthy individuals whose relative(s) have been diagnosed with C1-INH-HAE (Kasami et al., 2018). Further, peculiarities of the C1-INH-HAE-associated *SERPING1* variants frequently make the conventional approach towards their detection quite laborious and time-demanding. Confirmation of the results obtained by some widely used methods, like MLPA for the detection of large defects, is usually recommended. The NGS platform we developed and validated takes advantages of the benefits of NGS technology, targets the entire *SERPING1* gene, and facilitates genetic testing of C1-INH-HAE patients in clinical practice.

The developed *SERPING1*-NGS platform was validated against 135 C1-INH-HAE-associated variants of all types (SNVs and CNVs) representing about 1/3 of the up to now reported variants (≈450). Its performance and estimated analytical parameters combined with its time and cost saving advantages justify its use in the clinical practice as a first line approach for the genetic testing of C1-INH-HAE patients. A significant advantage of our platform is that it screens the whole length of the *SERPING1* gene, simultaneously detecting SNVs and CNVs, while also providing information about the size and the localization of CNVs. According to our findings, confirmation of the results obtained by the *SERPING1*-NGS platform would be required mainly in cases of negative results in patients suspected for C1-INH-HAE. It is true that large defects remain a demanding issue, but no single method can cover the requirements for the detection of all types of large defects. Finally, the *SERPING1*-NGS platform provides automated assignment of variants in a user-friendly manner, thereby minimizing the possibility of nomenclature errors that commonly occur with traditional methods for cases of multiple nucleotide variants.

The fact that we did not use a standard conventional procedure for *SERPING1* analysis to validate the performance of our platform might represent a limitation of our study. However, such a “gold standard” has not yet been proposed by the community with different researchers following various workflows using classical techniques. Thus, we decided to compare the performance of a high-throughput approach against the overall bias presented by conventional methodologies.

Overall coverage of our *SERPING1*-NGS platform is ≈83%; however, all of the missing regions are in deep intronic regions. Given that deep intronic variants have not yet been associated with C1-INH-HAE, we did not made any further effort to improve the coverage. Besides, the existing coverage of intronic regions already gives our platform a significant advantage in accurate detection of the extent and localization of CNVs.

During the forward validation of the *SERPING1*-NGS platform, we discovered that up to 2.94% (3/102) of variants are miss-assigned by conventional genotyping. Clearly, even the results of the conventional approaches require confirmation, which can be provided by our platform. Alternatively, the *SERPING1*-NGS platform could be the primary genotyping approach for C1-INH-HAE. After all, our study detected a large number of novel *SERPING1* mutations and analysis by our *SERPING1*-NGS platform provided substantial information on *SERPING1* polymorphisms, including within intronic regions and synonymous variants (data not presented).

5. Conclusion

We present a novel and validated NGS platform that targets the entire *SERPING1* gene, facilitating genetic testing of C1-INH-HAE patients in clinical practice. The platform screens the entire *SERPING1* gene, simultaneously detecting SNVs and CNVs and providing information about the size and the localization of CNVs. Obviously, potential users must address the special demands and quality prerequisites of NGS technology (in general and with respect to the specific platform), especially if employing it as the primary approach for genetic testing. Nevertheless, the *SERPING1*-NGS platform offers a powerful approach for clinical analysis of patients with respect to C1-INH-HAE. Fifteen novel C1-INH-HAE-related *SERPING1* variants were detected during the validation process of the platform.

Author contributions

G.L., M.Z.: Designed the experiments, performed experiments, analyzed the data, writing of the manuscript; F.P., S.V.: Performed experiments; C.D., A.L.-L., M.L.-T., M.S. H.F.: Provided conventionally genotyped samples; F.P., G.P., K.O., A.V., M.S., D.M., M.M., M.M.: Provided non-genotyped samples; A.E.G. Conceived and coordinated the study, analyzed the data, writing of the manuscript. All authors reviewed the manuscript.

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Conflict of interest statement

Authors have no conflicts of interest to declare.

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LETTER TO THE EDITOR

Plasminogen glycoforms alteration and activation susceptibility associated with the missense variant p.Lys330Glu in HAE-PLG patients

To the Editor

Hereditary angio-oedema (HAE) represents a heterogeneous disease with clinical nondistinguishable phenotypes but more complex underlying genotypes than originally considered. HAE may be associated with severe clinical course and sometimes can be life-threatening. Since the first description of HAE type III (OMIM:#610618), four individual forms of angio-oedema with normal C1 inhibitor have been described.

The rare missense variant NM_000301.3:c.988A > G;(p.Lys330Glu) in the plasminogen (*PLG*) gene was characterized as pathogenic for HAE-PLG.[1,2] The variant is located in exon 9 of the gene and affects Lys^{330} residue in the kringle 3 domain of the protein. Kringle domains likely function as recognition units for interaction of plasminogen with its ligands and substrates. Up to date, 26 unrelated families have been recorded with HAE-PLGp.Lys330Glu in Germany, Greece, Bulgaria, Spain, France, Japan and the United States, with an incomplete penetrance. One hundred and fifty-nine symptomatic individuals have been described.

Plasminogen (PLG) is the circulating zymogen of plasmin, a serine protease of the fibrinolytic pathway. After plasminogen activation to plasmin, fibrin is fragmented within blood clots. The activation is predominantly depending on urokinase-type (UK) and tissue type (tPA) plasminogen activators, but in a less extent by activated factor XII (FXIIa) and prekallikrein PK, proteases of the Kallikrein-Kinin System (KKS). Besides fibrin, plasmin, also targets fibrinogen, FXII, complement C3 and C5, factors V, VIII, von Willebrand and X and plasminogen activators tPA and UK. Therefore, PLG and plasmin play important physiological roles in fibrinolysis and haemostasis, while there are strong arguments for an involvement of plasmin beyond fibrinolysis.[3]

The question arises about the biological consequences of the PLG p.Lys330Glu variant. There are two major glycoforms of plasma plasminogen: type I (~33%), which is *N*-glycosylated at Asn^{289} and *O*-glycosylated at Thr^{346} and type II form (~67%), which is *O*-glycosylated at Thr^{346} (UniProtKB-P00747). Dewald[4] observed altered PLG patterns in two heterozygous patients for the p.Lys330Glu variant and suggested that the variant might have some quantitative as well as qualitative effect on plasminogen glycosylation.

We investigated plasma samples from four patients, without prophylaxis and without oestrogen intake, and four symptom-free subjects belonging to two families A and B, from Greece and Spain respectively, both carrying the p.Lys330Glu variant. All individuals have signed the consent for genetic investigations. First observations of families A and B have been described by Germenis et al,[2] with pedigrees illustrated in Figure 1. The proband I.1 of family A is the first homozygous carrier, a 75-year old woman with severe angio-oedema since the age of 40 years. She has two daughters II.1 and II.2 and a granddaughter III.1 who are heterozygous carriers. Daughter II.2 was asymptomatic, and she developed first symptoms of HAE sometime; thereafter, the other members are asymptomatic. All members of family B (proband I.2, daughter II.1, son II.2, granddaughter III.1 and grandson III.2) were found heterozygous for the p.Lys330Glu variant, nevertheless only the proband I.2, the daughter II.1 and the granddaughter III.1 developed angio-oedema symptoms. Interestingly, two of them (proband I.2 and daughter II.2) were also found heterozygous carriers of a rare *PLG* polymorphism (NM_000301.3:c.266G > A), while the granddaughter III.1 is carrying another rare polymorphism (NM_000301.3:c.1567C > T), both classified as likely benign with an allele frequency of 0.01.

Plasma citrate samples from eight individuals were collected for biochemical investigation. Data from basic coagulation tests and clot lysis assay were within the normal range. All subjects presented normal C1 inhibitor function,[5] normal kallikrein activity[6] and kinin catabolism except a low aminopeptidase P (APP) activity for the individuals of family A and the individual III.2 of family B. High molecular weight kininogen (HK) cleavage pattern, as investigated on plasma samples by anti-HK L-chain immunoblot,[7] was comparable to control. The homozygous patient also exhibited a HK cleavage comparable to healthy control during the resting situation, while more extended in samples collected a few hours after angio-oedema attack. Clinically, all patients were presenting attacks localized to the face, tongue, larynx and typical manifestations of HAE-PLG, and the treatment with tranexamic acid was effective.

The PLG glycosylation pattern PLG was investigated by anti-plasminogen immunoblot. Figure 2A illustrates a reversal of the glycosylation pattern in the homozygous patient with ~60% of PLG type I and ~40% of PLG type II, while the heterozygous subjects present

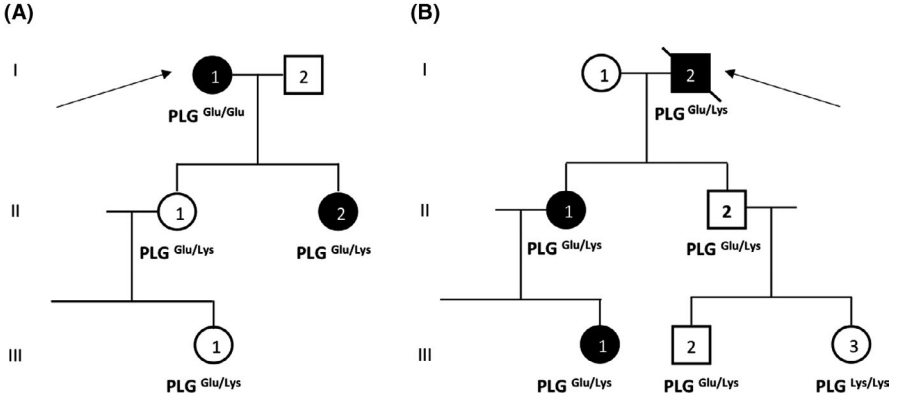

FIGURE 1 Pedigrees carrying the PLG p.Lys330Glu variant. Panels A and B correspond to the Greek and Spanish families, respectively. Black symbols indicate symptomatic individuals; empty symbols indicate asymptomatic individuals, and the arrows indicate the probands in each family. Incomplete penetrance has already been described for HAE-PLG

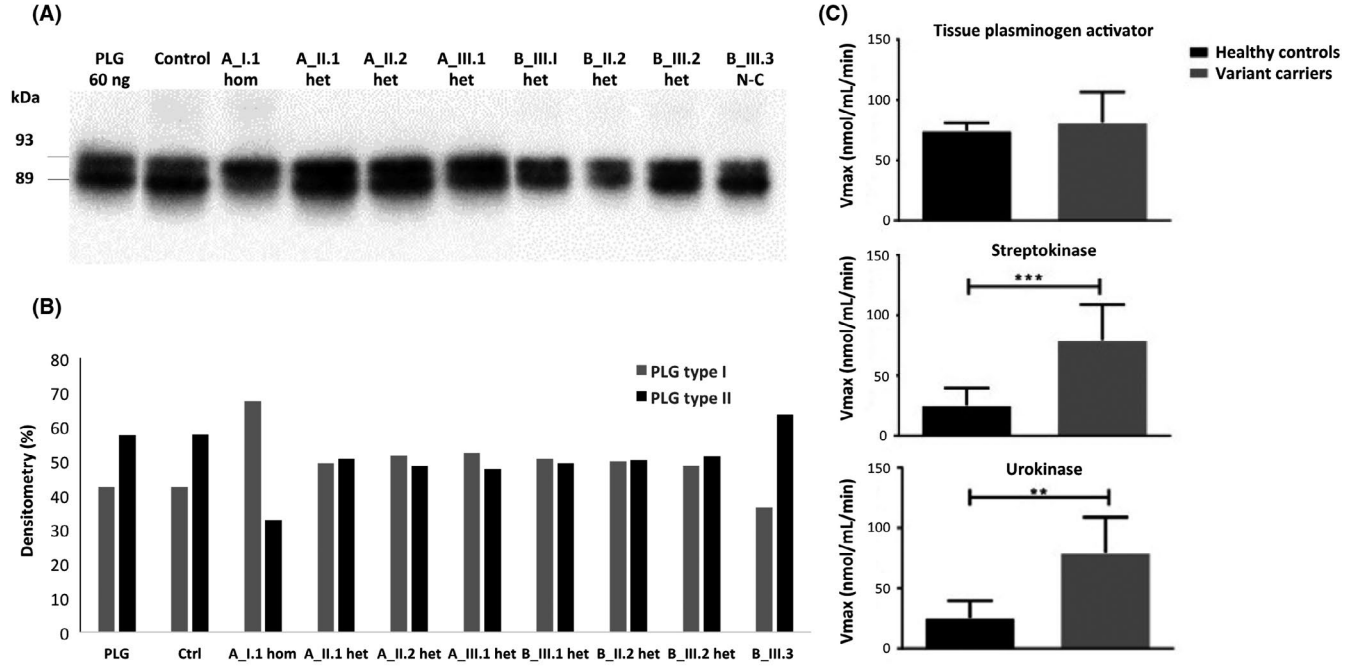

FIGURE 2 A, Anti-plasminogen immunoblots. Plasma samples from homozygous and heterozygous patients (symptomatic AI.1, AII.2 and BIII.2), asymptomatic (AII.1, AIII.1, BIII.1, BII.2 and BIII.3) carriers, purified PLG (lane 1) and healthy control (lane 2) have been submitted to SDS-PAGE under nonreducing conditions followed by anti-plasminogen immunoblot (Goat anti-human-PLG, Affinity Biological). Lanes identification is linked to Figure 1. Molecular weights are indicated. B, PLG signal is assessed by using densitometric data. C, Plasminogen activation of plasma samples in the presence of three activators (tPA, SK and UK). tPA, tissue-plasminogen activator; SK, streptokinase; UK, urokinase; hom, homozygous; het, heterozygous, N-C, noncarrier; $^{**}P < .01$, $^{***}P < .001$

with two bands of approximately equal intensity. Noncarrier individuals display around 40% of PLG type I, as observed for the control sample (Figure 2B). In addition, we observe that PLG type I displays a slight molecular weight decrease from 93 KDa to 92 KDa.

As both PLG glycoforms are known to exhibit functional differences, including a susceptibility to activation, we investigated plasminogen activation using a chromogenic substrate (S-2403, Chromogenix) and three different activators, tPA, streptokinase (SK) and urokinase (UK). Homozygous or heterozygous (n = 8) carriers of p.Lys330Glu display a significantly high susceptibility to the PLG activation by SK and UK ($P < .001$ and $P < .01$), compared with healthy controls (n = 6, Mann-Whitney test for nonparametric values and $\alpha < .05$; Figure 2C). However, a participation of cell components cannot be excluded in in vivo plasminogen activation, where S100A10 receptor within a S100A10-Annexin A2 tetramer develops plasminogen conformational change and plasmin generation.

In conclusion, the altered glycosylation patterns in heterozygous individuals and the reversed glycosylation patterns in the homozygous patient suggest that p.Lys330Glu has a qualitative impact on PLG function. The enhanced susceptibility of PLG to proenzyme activation may be related to reversed patterns, in agreement with the observation of Takada and Takada.[8] showing that type I is more susceptible to activation by UK or SK than type II. These data add to ACMG criteria for its classification as pathogenic. Moreover, p.Lys330Glu may develop a higher binding ability to interact with lysine residues of protein ligands, for example tissue factor TF. This

hypothesis adds to an increased TF binding capacity of type I,[9] the major species displayed by homozygous proband. As a result, the p.Glu330Lys variant may promote a qualitative impact on PLG with subsequent high plasmin forming, KKS activation and sustained BK production.

KEYWORDS

angioedema, biologics, biomarkers, genetics

CONFLICTS OF INTEREST

Disclosure of potential conflict of interest: FP is currently employed at CeMIA SA. DC, MT and AG are employed at KininX SAS. AEG received funds from Shire. CM has a patent WO 2018/154044 A1 issued. The other authors declare no competing financial interests.

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Original Article

A novel deep intronic *SERPING1* variant as a cause of hereditary angioedema due to C1-inhibitor deficiency

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ACMG, American College of Medical Genetics and Genomics; ASSP, Alternative Splice Site Predictor; AMP, Association for Molecular Pathology; C1-INH, C1-inhibitor; C1-INH-HAE, hereditary angioedema due to C1-INH deficiency; CADD, Combined Annotation Dependent Depletion; GERP, Genomic Evolutionary Rate Profiling; HSF, Human Splicing Finder; ISS, intronic splicing silencer; NGS, next-generation sequencing

ABSTRACT

Background: In about 5% of patients with hereditary angioedema due to C1-inhibitor deficiency (C1-INH-HAE) no mutation in the *SERPING1* gene is detected.
Methods: C1-INH-HAE cases with no mutation in the coding region of *SERPING1* after conventional genotyping were examined for defects in the intronic or untranslated regions of the gene. Using a next-generation sequencing (NGS) platform targeting the entire *SERPING1*, 14 unrelated C1-INH-HAE patients with no detectable mutations in the coding region of the gene were sequenced. Detected variants with a global minor allele frequency lower than the frequency of C1-INH-HAE (0.002%), were submitted to *in silico* analysis using ten different bioinformatics tools. Pedigree analysis and examination of their pathogenic effect on the RNA level were performed for filtered in variants.
Results: In two unrelated patients, the novel mutation c.-22-155G > T was detected in intron 1 of the *SERPING1* gene by the use NGS and confirmed by Sanger sequencing. All bioinformatics tools predicted that the variant causes a deleterious effect on the gene and pedigree analysis showed its co-segregation with the disease. Degradation of the mutated allele was demonstrated by the loss of heterozygosity on the cDNA level. According to the American College of Medical Genetics and Genomics 2015 guidelines the c.-22-155G > T was curated as pathogenic.
Conclusions: For the first time, a deep intronic mutation that was detected by NGS in the *SERPING1* gene, was proven pathogenic for C1-INH-HAE. Therefore, advanced DNA sequencing methods should be performed in cases of C1-INH-HAE where standard approaches fail to uncover the genetic alteration.

Introduction

C1-INH-HAE (OMIM#106100) is a potentially fatal autosomal dominant disease that manifests clinically with episodes of non-pruritic and nonpitting swelling of the deeper layers of the skin or mucosa.[1] The disease is caused by mutations of the *SERPING1* gene, which encodes for C1-INH. *SERPING1* is located on chromosome 11 and consists of 8 exons.[2] Currently, 748 different disease-causing *SERPING1* variants have been published.[3] However, in

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approximately 5% of C1-INH-HAE patients no causal mutation is identified by standard mutational screening which is ordinarily restricted to the coding exons and exon-intron boundaries of the *SERPING1* gene.[4,5]

Intronic sequences were initially assumed to be largely non-functional and mutations located deep within introns (i.e., more than 100 base pairs away from exon-intron boundaries) were ignored as possible causes of human disease. However, genomic approaches in clinically oriented studies have identified many deep intronic variants with significant association to diseases, and intron functionality is supported by several independent lines of evidence.[6,7] Recently, Vaz-Drago *et al.*[7] have reviewed, between 1983 and 2016, 185 deep intronic mutations across 77 different disease-associated genes.

Hitherto, conventional methods for genotyping of C1-INH-HAE patients did not allow the analysis of *SERPING1* intronic regions. Even when some introns are sequenced along with their upstream and downstream exons, possible detection of deep intronic variants is disregarded. Thus, no deep intronic variant has been reported in association with C1-INH-HAE. To overcome some of the shortcomings of traditional approaches we recently developed a custom next-generation sequencing (NGS) platform that allows analyzing *SERPING1* in its full length.[8] Here, we use this platform for genotyping C1-INH-HAE patients with no detectable mutations in the coding region of *SERPING1*, and we describe our results and their relevance.

Methods

Study population

Fourteen patients (3 Greek, 2 Hungarian, 4 Polish, 3 Bulgarian, 2 German; 5 male, mean age 43 ± 17 years) diagnosed with type I C1-INH-HAE, according to the criteria of the Hereditary Angioedema International Working Group,[9] but without *SERPING1* mutations after conventional molecular analysis, were initially enrolled in the study. Pedigree analysis became feasible in one case. Three healthy first degree relatives of the patient and three first degree relatives with C1-INH-HAE were examined. Informed consent was obtained from all patients and family members investigated, and the study has been approved by the Ethics Committee of the Faculty of Medicine, University of Thessaly.

Genetic analysis

Genomic DNA was extracted from peripheral blood with iPrep PureLink DNA blood kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Conventional genotyping had been performed by sequencing all *SERPING1* translated regions and intron-exon boundaries, long-range PCR and multiplex ligation-dependent probe amplification (MLPA), as previously described.[4]

A custom NGS panel was designed by the Ion AmpliSeq Thermo Fisher Scientific Designer, as previously described,[8] covering the whole *SERPING1* gene (NM_000062.2) including its complete 5′ and 3′ untranslated regions, exonic and intronic regions (Chr11: 57,364,831–57,382,476; GRCh37). Coverage of 99.9% and 79.9% of exonic and intronic regions of *SERPING1* was achieved, respectively. Missing intronic regions were mainly those of introns 3, 4, and 6 (41,4%, 42,2% and 22,5%, respectively). Briefly, amplicon libraries were prepared using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) and Ion Xpress™ Barcode Adapter 1–96 Kit (Thermo Fisher Scientific). Pooled, barcoded libraries were clonally amplified using the Ion OneTouch™ system and Ion OT2 HI-Q Template kit (Thermo Fisher Scientific). Ion sphere particles (ISPs) were enriched with the Dynabeads® MyOne™ Streptavidin C1 Beads (Thermo Fisher Scientific) and washed with the Ion OneTouch Wash Solution included in the kit using the Ion One Touch ES system (Thermo Fisher Scientific). NGS was performed on the Personal Genome Machine (PGM) using the Ion PGM Hi-Q sequencing kit (Thermo Fisher Scientific) resulting in a mean depth of coverage 20x. All procedures were performed according to the manufacturer's instructions.

Torrent Suite 5.2 software was used for the analysis of NGS data. Raw data were aligned to the complete *SERPING1* gene of the human reference sequence hg19 (GRCh37), variant calling was performed by the VariantCaller v.5.2 plug-in and coverage analysis by the coverageAnalysis v.5.2.1.2 plug-in. Annotation of variants was performed on Ion Reporter software v.5.2 (Thermo Fisher Scientific). The annotated variants were evaluated and visualized via integrative genome viewer (IGV).

Variants that were present in the reference population data sets [Genome Aggregation Database (gnomAD), Exome Aggregation Consortium[10] release 0.3 (ExAC Browser), Database of Single Nucleotide Polymorphisms[11] build 141 GRCh37.p13 (dbSNP)] at a global minor allele frequency greater than that the frequency C1-INH-HAE (<0.002%) were filtered out from the analysis.

Sanger sequencing was performed for the confirmation of novel variants and family segregation study. The intron 1 of the *SERPING1* gene was amplified by using the primers 5′-CTGCACC-CAAGCTTCCCCGTTCAC-3′ and 5′-CCCCGTCCCCCATCCCACAAG-3'. The PCR was initiated at 94 °C for 2 min, followed by denaturation at 94 °C for 45 s, annealing at 65 °C for 45 s, and extension at 72 °C for 1 min for 30 cycles. The amplified products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed in ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA). Data were evaluated by the Sequencing Analyses software 5.2 (Applied Biosystems, Foster City, California, USA).[12]

In silico analyses

The putative effect of filtered-in intronic mutations was evaluated by using ten different bioinformatics analysis tools. Sequence segments with and without corresponding changes in their sequences were submitted to five bioinformatics tools examining potential splice effects: NNSPLICE,[13] Netgene2,[14] ASSP,[15] FSPLICE and HSF.[16] All of them provide probability scores for the use of potential donor and acceptor splice sites. Further assessment of the pathogenicity was attempted by the use of 5 additional tools. The CADD provides prediction of the functional impact of the observed variants by integrating different functional aspects and diverse annotations into a single outcome, namely the C-score[17]; the Transcript-inferred Pathogenicity score (Trap score) evaluates a single nucleotide variant's ability to cause disease by damaging the final transcript[18]; DANN is a pathogenicity scoring methodology based on deep neural networks[19]; GERP is a conservation score calculated by quantifying substitution deficits across multiple alignments of orthologues using the genomes of 35 mammals[20]; finally, SpliceAid 2 bioinformatics foresee the splicing pattern alteration and guide the identification of the molecular effect due to the mutations.[21]

Transcriptional analysis

In order to further evaluate the function effect of the variant c.-22-155G > T, whole blood was collected from the proband and his three suffering family members carrying the mutation and being heterozygous for the rs4926 (c.1438G > A) polymorphism. Mononuclear cells were separated by a Ficoll gradient, and total RNA was extracted with phenol-chloroform, ethanol precipitated, and purified by guanidinium thiocyanate dissociation and isopropanol

precipitation.[22,23] First-Strand cDNA was synthesized from 1 μg RNA in 25 μl reactions with 25 μM Primer random p[dN]6 (Roche), 10 mM dNTPs (Invitrogen, Carlsbad, CA, USA) and 200 U/μL M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) employed in 72 °C for 2 min, 42 °C for 75 min and 65 °C for 10 min.

In the produced cDNA, a fragment from exons 6–8 was amplified, sequenced and compared to the gDNA sequence. The forward 5′AACTCAGTTATAAAAGTGCCCATGATGAAT3′[24] and the reverse 5′CCCTTTTGGTGGATAGCG-3′[25] primers were used. The amplification of the cDNA was performed using 10 pmol of each primer and 35 cycles in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, USA), comprising of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. The amplified products were sequenced and analyzed, as described above.

Variant curation

The interpretation of sequence variants was based on the criteria established by the ACMG and the AMP.[26] Data from our previous study[8] in regard with the presence of variants in healthy individuals were used to this aim.

Results

Apart from the known polymorphisms rs28362944 and rs4926, NGS genotyping of our C1-INH-HAE patients confirmed the absence of disease-causing *SERPING1* exonic mutations. However, 35 different intronic mutations were revealed in these patients, 29 of which were filtered out as their global minor allele frequency was greater than

Table 1
Filtered out intronic mutations presenting in the Genome Aggregation Database (gnomAD) with a global minor allele frequency greater than 0.002%.

Locus	Genotype	Coding	dbSNP	Intron	Frequency (%)
chr11:57365895	G/A	c.51 + 101G > A	rs28362945	2	12.56
chr11:57366405	C/CGT	c.51 + 625_51 + 626dupTG	rs3054018	2	38.99
chr11:57366656	C/T	c.52−696C > T	rs1005511	2	39.51
chr11:57367222	C/T	c.52−130C > T	rs1005510	2	38.72
chr11:57369008	C/G	c.551-500C > G	rs28362947	3	22.03
chr11:57369013	A/C	c.551-495A > C	rs28362948	3	22.03
chr11:57369353	A/G	c.551-155A > G	rs2936694	3	36.84
chr11:57369730	G/A	c.685 + 88G > A	rs11229063	4	22.24
chr11:57370742	C/T	c.685 + 1100C > T	rs78364821	4	22.52
chr11:57371911	G/T	c.686-1572G > T	rs28362949	4	22.08
chr11:57371918	G/A	c.686-1565G > A	rs28362950	4	22.42
chr11:57372526	A/G	c.686−957A > G	rs28362951	4	22.19
chr11:57373304	A/G	c.686-179A > G	rs189335964	4	0.1068
chr11:57374280	G/A	c.1029 + 260G > A	rs191053716	6	0.1356
chr11:57374332	T/C	c.1029 + 312T > C	rs11603020	6	22.79
chr11:57374871	C/G	c.1029 + 851C > G	rs11229066	6	22.79
chr11:57374946	G/T	c.1029 + 926G > T	rs11229067	6	22.79
chr11:57375463	G/C	c.1029 + 1443G > C	rs78624400	6	22.82
chr11:57375517	A/G	c.1029 + 1497A > G	rs17661117	6	0.6972
chr11:57376130	T/C	c.1029 + 2110T > C	rs2454659	6	61.20
chr11:57376131	A/G	c.1029 + 2111G > A	rs138770460	6	22.7
chr11:57377215	G/C	c.1030-1975G > C	rs151035150	6	1.1044
chr11:57377676	CT/C	c.1030-1513delT	rs1184255008	6	0.003227
chr11:57377968	A/G	c.1030−1222A > G	rs3824988	6	22.78
chr11:57377992	G/T	c.1030-1198G > T	rs2508443	6	32.92
chr11:57378325	C/T	c.1030-865C > T	rs2511989	6	38.14
chr11:57379170	A/G	c.1030-20A > G	rs2511988	6	61.09
chr11:57381263	T/C	c.1250-538T > C	rs10896631	7	22.76
chr11:57381519	T/C	c.1250-282T > C	rs1557522	7	30.64

Table 2
Bioinformatic analysis of the six filtered-in intronic mutations.

Variant	NNSPLICE[†]	Netgene2[‡]	ASSP[§]	FSPLICE[¶]	HSF[‖]	CADD[#]	Trap score[††]	DANN[‡‡]	GERP RS[§§]
c.-22-155G > T	D:0.96	D:0.79	D:0.46	D:10.16	New intronic cryptic donor site	21.2	0.686	0.9507	1
c.551-156A > G	NI	NI	NI	NI	Creation of an ISE	1.427	0.103	0.3543	NP
c.686−1488_686-1487insT	NI	NI	NI	NI	Creation of an ISE	NP	NP	NP	0.0435
c.686−1335T > A	NI	NI	NI	NI	Alteration of an ISS/Creation of an ISE	0.131	0.078	0.1134	−1.335
c.686−1333A > T	NI	NI	NI	NI	NI	0.969	0.012	0.1669	−1.875
c.1250-154C > G[¶¶]	NI	NI	NI	NI	Creation of an ISE	3.911	0.122	0.5833	0.3333

NI, No impact; NP, Not provided; D, new donor; ISS, Intronic splicing silencer; ISE, Intronic splicing enhancer.
[†] Range 0–1, with minimum score for 5′ and 3′ splice site 0.4 (www.fruitfly.org/seq_tools/splice.html).
[‡] Confidence range 0.5–0.95 (www.cbs.dtu.dk/services/NetGene2/).
[§] Confidence range 0–1, false splice site cutoff for acceptor sites: 2.2, false splice site cutoff for donor sites: 4.5 (Alternative Splice Site Predictor: wangcomputing.com/assp/).
[¶] Acceptor site threshold: 4.175, donor site threshold: 6.099 (www.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind).
[‖] www.umd.be/HSF/HSF.shtml.
[#] Range 10–30 (cadd.gs.washington.edu).
[††] Range 0–1 (http://trap-score.org/Search?version=v2).
[‡‡] Range 0–1.
[§§] −12.3 to 6.17.
[¶¶] Observed in 2 patients (trans with the c.-22-155G > T variant) and one healthy member of the studied family.

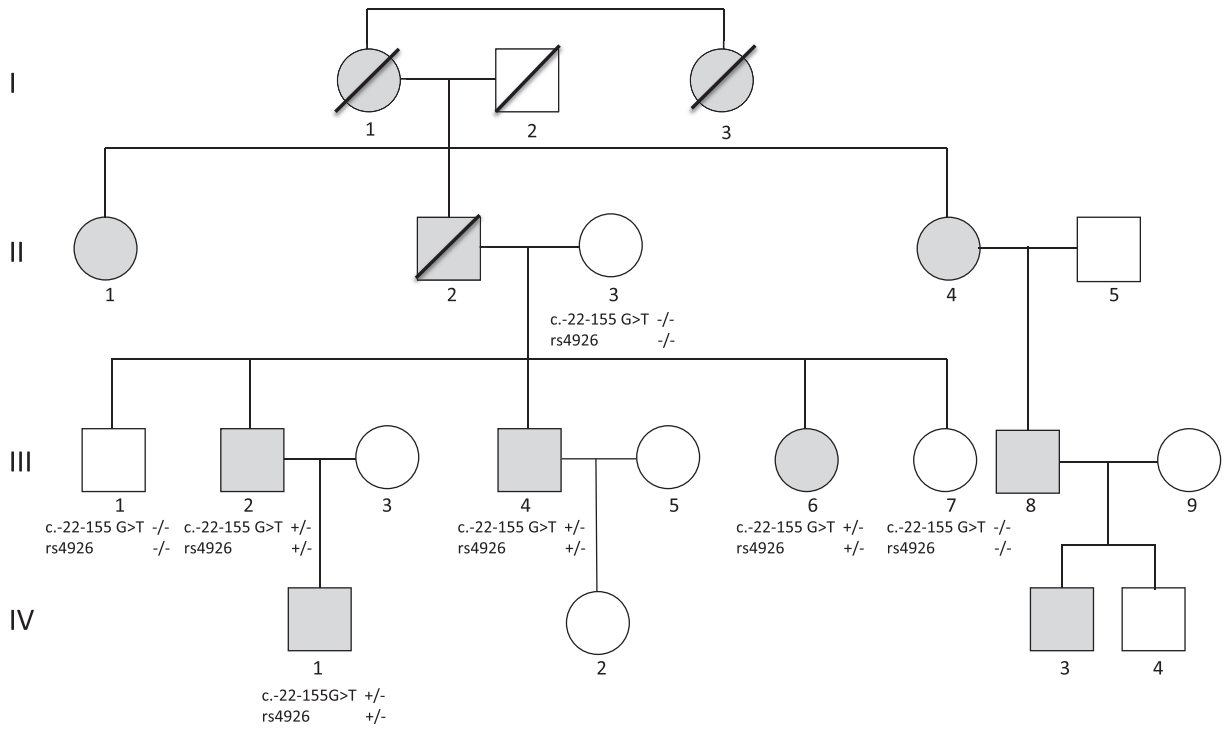

Fig. 1. Pedigree analysis of a C1-INH-HAE family carrying the deep intronic c.-22-155G > T variant. All examined suffering members of the family (III.2, III.4, III.6, IV.1) —carriers of the c.-22-155G > T— were heterozygous for the rs4926 (c.1438G > A) polymorphism, while all examined unaffected members (II.3, III.1, III.7) were homozygous for the wild type of this polymorphism.

0.002% (Table 1). Four out of the remaining 6 variants were unreported (novel), and 2 were reported once in global databases.

Only one of the six filtered-in variants, the novel variant c.-22-155G > T located in intron 1 of the *SERPING1* gene (chr11:57,365,567), was predicted as pathogenic by all used bioinformatics tools (Table 2). Moreover, this variant was also the only one that was detected in two unrelated C1-INH-HAE Greek patients, i.e. with a frequency 14.3% among the cohort of our C1-INH-HAE patients without any mutation in the exonic region of the gene, while it was also undetectable in our healthy control group.[8] Assuming a 5% frequency of C1-INH-HAE patients with no causal mutation identified in the coding exons and exon-intron boundaries of the *SERPING1* gene,[4,5] the estimated frequency of this variant among C1-INH-HAE patients is 0.7%. Thus, the c.-22-155G > T variant was the only one that was further studied.

Pedigree analysis revealed that the c.-22-155G > T variant co-segregated with C1-INH-HAE in all of the 4 analyzed patients belonging to two generations, while it was absent from all of the 3 healthy family members who were also analyzed (Fig. 1). Examined patients were of 8, 40, 44 and 45 (the proband, III.2) years-of-age with an age at disease onset of 8, 7, 24 and 13 years, and a mean frequency of attacks (mainly cutaneous) 2, 5, 14 and 5 per year, respectively. Their antigenic C1-INH concentration at diagnosis was varying from 4% to 40% of the reference. Information about the other suffering members of the family was unobtainable since they are immigrants in Australia. The fifth, unrelated patient examined was a man of 45 years-of-age without family history of angioedema presenting with rare cutaneous attacks since the age of 25 years. No other member of his family was available for analysis.

Further bioinformatic analysis with the use of SpliceAid 2 tool showed that the c.-22-155G > T variant alters the transcriptional motif recognized by hnRNP H1, hnRNP H2, hnRNP F and hnRNP H3 transcriptional factors preventing their binding in the corresponding gene region.

Transcript analysis supported the above indications of the deleterious effect of the c.-22-155G > T variant. By this approach loss of heterozygosity was demonstrated for the exon 8 rs4926 (c.1438G > A) polymorphism located in the same allele with the c.-22-155G > T variant. More specifically, as it is shown in Figure 1, in the gDNA, all

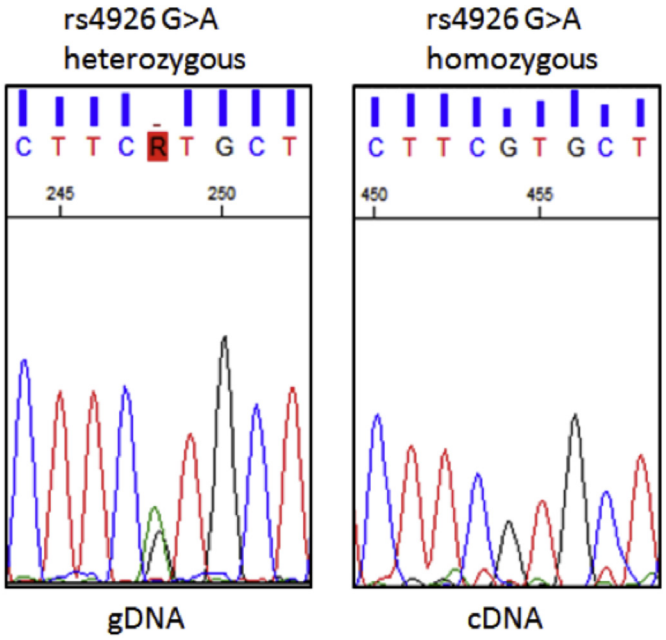

Fig. 2. cDNA and gDNA sequencing of one of the patients (III.6) carrying the c.-22-155 G > T variant and the rs4926 G > A polymorphism. Sequencing of the amplified cDNA demonstrate that while in the gDNA the two alleles (G/A) of the rs4926 are equally represented, in the cDNA only one allele (G) is present.

Table 3
The 49 reported intronic mutations in *SERPING1* gene associated with C1-INH-HAE. The majority of them are located in the donor and acceptor site or a few (up to 5) nucleotides from these regions. Only the c.1029 + 84G > A mutation is located 84 nucleotides from the acceptor site.

Mutation	Intron	Chromosome position	dbSNP	References
c.-22-2A > C	1	chr11:57365720		Aabom *et al.*, 2017[33]
c.-22-2A > G	1	chr11:57365720		Bygum *et al.*, 2011[34]; Xiong *et al.*, 2015[6]
c.-22-1G > A	1	chr11:57365721		Verpy *et al.*, 1996[35]; Gösswein *et al.*, 2008[36]; Xiong *et al.*, 2015[6]; Duponchel *et al.*, 2006[37]; Andrejević *et al.*, 2015[38]
c.51+6T > G	2	chr11:57365800		López-Lera *et al.*, 2011[39]
c.51+5G > A	2	chr11:57365799		Verpy *et al.*, 1996[35]; Duponchel *et al.*, 2006[37]; Xiong *et al.*, 2015[6]
c.51 + 1G > T	2	chr11:57365795		Yamamoto *et al.*, 2012[40]; Cagini *et al.*, 2016[41]
c.51 + 1G > A	2	chr11:57365795	rs1470120365	Gösswein *et al.*, 2008[36]; Pappalardo *et al.*, 2008[4]; Kalmár *et al.*, 2003[42]; Xiong *et al.*, 2015[6]
c.51 + 2T > C	2	chr11:57365796		Cagini *et al.*, 2016[41]
c.51 + 3A > G	2	chr11:57365797		Roche *et al.*, 2005[43]; Pappalardo *et al.*, 2008[4]; Duponchel *et al.*, 2006[37]
c.52−2A > G	2	chr11:57367350		Pappalardo *et al.*, 2008[4]; Xiong *et al.*, 2015[6]
c.52−1 G > A	2	chr11:57367351	rs886041353	Gösswein *et al.*, 2008[36]; Xiong *et al.*, 2015[6]
c.550+1G > A	3	chr11:57367851		Kalmár *et al.*, 2003[42]; Xiong *et al.*, 2015[6]
c.550+2T > C	3	chr11:57367852		Roche *et al.*, 2005;[43]
c.550 + 5G > C	3	chr11:57367855		Roche *et al.*, 2005[43]; Xiong *et al.*, 2015[6]
c.550 + 5G > A	3	chr11:57367855	rs1314284778	Roche *et al.*, 2005[43]; Xiong *et al.*, 2015[6]
c.551-2delA	3	chr11:57369506		Roche *et al.*, 2005;[43]
c.551-1G > A	3	chr11:57369507		Speletas *et al.*, 2015[5]
c.551−2A > G	3	chr11:57369506		Gösswein *et al.*, 2008[36]; Grodecká *et al.*, 2017[44]
c.551−3 C > G	3	chr11:57369505		Gösswein *et al.*, 2008[36]
c.551−5 T > A	3	chr11:57369503		Pappalardo *et al.*, 2008[4]
c.685 + 31G > A	4	chr11: 57369673	rs751335805	Suffritti *et al.*, 2014[45]
c.685 + 1G > T	4	chr11:57369643		Pappalardo *et al.*, 2008[4]; Xiong *et al.*, 2015[6]
c.685 + 1 G > A	4	chr11:57369643	rs113263597	Gösswein *et al.*, 2008[36]; Xiong *et al.*, 2015[6]
c.685 + 2T > A	4	chr11:57369644		Colobran *et al.*, 2014[46]
c.685 + 2T > G	4	chr11:57369644		Pappalardo *et al.*, 2008[4]; Xiong *et al.*, 2015[6]
c.686−12 A > G	4	chr11: 57373471		Pappalardo *et al.*, 2008[4]; Grodecká *et al.*, 2017[44]; Andrejević *et al.*, 2015[38]
c.686-3C > G	4	chr11:57373480		Roche *et al.*, 2005[43]; Xiong *et al.*, 2015[6]
c.889 + 1 G > T	5	chr11:57373687		Gösswein *et al.*, 2008[36]; Xiong *et al.*, 2015[6]
c.889 + 2T > C	5	chr11:57373688		Roche *et al.*, 2005[43]; Xiong *et al.*, 2015[6]
c.889 + 3A > T	5	chr11:57373689		Johnsrud *et al.*, 2015[47]
c.890-14C > G	5	chr11: 57373867		Speletas *et al.*, 2015[5]
c.890-2A > G	5	chr11: 57373879		Bowen *et al.*, 2001[12]; Xiong *et al.*, 2015[6]
c.890-1G > A	5	chr11: 57373880		Sekijima *et al.*, 2004[48]; Xiong *et al.*, 2015[6]
c.1029 + 1 G > T	6	chr11:57374021		Siddique *et al.*, 1991[49]; Xiong *et al.*, 2015[6]
c.1029 + 1 G > A	6	chr11:57374021		Gösswein *et al.*, 2008[36]; Xiong *et al.*, 2015[6]
c.1029 + 84G > A	6	chr11:57374104	rs118132731	Pappalardo *et al.*, 2008[4]
c.1029 + 3_ c.1029 + 6 del4bp	6	chr11:57374023		Gösswein *et al.*, 2008[36]
c.1029 + 4 delA	6	chr11:57374024		Bygum *et al.*, 2011[34]
c.1030−1 G > A	6	chr11:57379189		Speletas *et al.*, 2015[5]
c.1030−1 G > C	6	chr11:57379189		Gösswein *et al.*, 2008[36]; Verpy *et al.*, 1996[35]; Xiong *et al.*, 2015[6]
c.1249 + 1G > C	7	Chr11: 57379410		López-Lera *et al.*, 2011[39]
c.1249 + 1G > A	7	Chr11: 57379410	rs112565881	Gösswein *et al.*, 2008[36]; Xiong *et al.*, 2015[6]
c.1249 + 2T > A	7	Chr11: 57379411		Kawachi *et al.*, 1998[50]; Xiong *et al.*, 2015[6]
c.1249 + 2delT	7	Chr11: 57379411		Roche *et al.*, 2005;[43]
c.1249 + 5G > T	7	Chr11: 57379414		Pappalardo *et al.*, 2008[4]; Grodecká *et al.*, 2017[44]
c.1249 + 5G > A	7	Chr11: 57379414		Colobran *et al.*, 2017[25]; Grodecká *et al.*, 2017[44]
c.1250−13 G > A	7	chr11: 57381788		Gösswein *et al.*, 2008[36]; Kesim *et al.*, 2011[51]
c.1250-1G > A	7	chr11: 57381800		Aabom *et al.*, 2017[33]
c.1250−2 A > G	7	chr11: 57381799		Gösswein *et al.*, 2008[36]

patients of the examined family —carriers of the c.-22-155G > T— were heterozygous for the rs4926 (c.1438G > A) polymorphism, while all unaffected members were homozygous for the wild type. Interestingly, in the cDNA, all patients were found homozygous for the wild type —only one allele (G) is present— indicating that the mRNA from the mutated allele is probably degraded (Fig. 2).

According to the ACMG-AMP 2015 guidelines[26] the pathogenicity potential of the c.-22-155G > T variant is “pathogenic” based on: (a) The strong segregation data. The variant co-segregates with disease in multiple affected family members in a gene definitively known to cause the disease (PP1 criterion used as strong evidence). (b) The absence of the variant from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium (PM2). (c) The observation of the variant in two unrelated probands with the same phenotype, and its absence in controls (PS4). (d) Prediction by multiple bioinformatics tools that the variant causes a deleterious effect on the gene (PP3). (e) The patient's phenotype and family history is highly specific for the disease (PP4). (f) *In vitro* functional studies support a damaging effect on the gene (PS3). Based on the available criteria (BS2, BS4, BP4, BP5) the remaining intronic variants detected in our C1-INH-HAE patients were classified as “benign” or “likely benign”.

Discussion

To identify missing noncoding variants in C1-INH-HAE cases, we sequenced the *SERPING1* locus in 14 patients. In two of them, we detected a novel deep intronic variant (c.-22-155G > T) which was classified as pathogenic, according to ACMG-AMP 2015 guidelines.[26] In recent years, increasing numbers of deep intronic variants located at least 100 bp from the nearest canonical splice site, have been reported and their role in human diseases has been

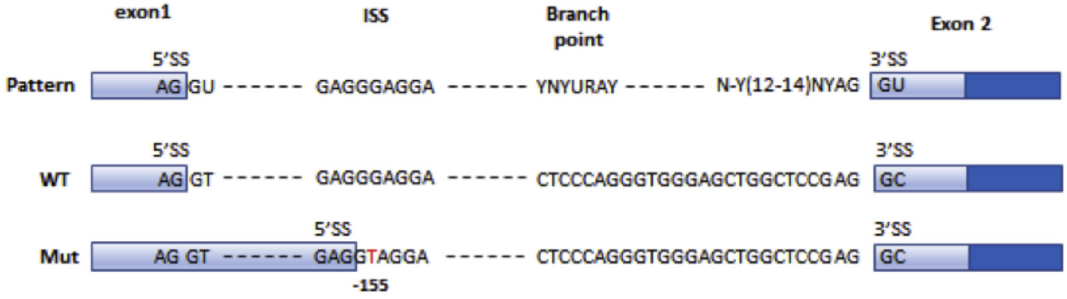

Fig. 3. Graphical presentation of the impact of the c.-22-155G > T variant according to bioinformatics analysis. The variant is predicted to form an alternative donor site leading to a modified mutant RNA longer (>372 bp) than the wild type.

largely demonstrated. To the best of our knowledge, this is the first deep intronic variant of *SERPING1* gene associated with C1-INH-HAE that has been reported in the literature. Heretofore, 49 different intronic mutations of *SERPING1* gene have been associated with C1-INH-HAE, all of them located in the donor and acceptor site or a few nucleotides from these regions[4–6,12,25,33–51] (Table 3).

Deleterious deep intronic variants most commonly lead to pseudo-exon inclusion due to activation of non-canonical splice sites or changes in splicing regulatory elements, or they can disrupt transcription regulatory motifs and non-coding RNA genes.[7] As far as the c.-22-155G > T variant is regarded, the most probable scenario indicated by the findings of the *in silico* and the transcriptional analysis, is that it affects splicing and alters the transcribed RNA. The mutant mRNA is susceptible to degradation by mRNA surveillance pathways. Consequently, only the wild type allele is translated, and this results in C1-INH deficiency.

In more detail, by the use of the NNSPLICE, NetGene2 and FSPLICE, ASSP bioinformatics tools, it has been shown that the c.-22-155G > T variant introduces in the genome a new donor site stronger than the wild type leading to a larger exon 1 in the RNA level (Fig. 3). This was confirmed by HSF indicating, in parallel, that the variant disrupts an ISS recognized by a number of transcriptional factors and, as a result, stops the suppression of the intronic cryptic donor site. Additionally, by analyzing both the wild type and the mutated sequence, SpliceAid 2 concluded that different splicing motifs are destroyed, including the splicing motifs for the transcriptional factors hnRNP H1, hnRNP H2, hnRNP F, hnRNP H3 which act as silencers in the wild type sequence. Transcriptional analysis confirmed that the mutant mRNA is susceptible to degradation. To this aim, an informative exonic SNP, i.e. the polymorphism rs4926 (c.1438G > A) carried by the patients in heterozygous state, was used. On the RNA level, this polymorphism was found in homozygous state, which indicates that the mutated allele is degraded, obviously through mRNA surveillance pathways. Further studies are required in order to define which of the three translation-associated mRNA surveillance pathways (nonsense-mediated mRNA decay, no-go decay, nonstop decay) that target mRNAs for degradation is involved.[7]

In favor of the pathogenicity of the c.-22-155G > T variant is its location in a chromosomal region of intron 1 which, by the use of GERP, a statistically rigorous and biologically transparent framework for constrained element identification,[27] was found highly conserved. Generally, it is considered that the first intron is highly conserved and that its conservation is related to its enrichment with regulatory elements[28] and a specific pattern of chromatin organization.[29] However, the first intron of *SERPING1* is not the longest among all other downstream introns within the gene as it happens in most species.[30]

In conclusion, our study verified the earlier hypothesis that intronic alterations could be the cause of the disease in cases of C1-INH-HAE where standard genotyping approaches cannot uncover any DNA damage, highlighting one more advantage of NGS[8] in the molecular analysis of these patients.[31] Therefore, advanced DNA sequencing methods should be performed in cases of C1-INH-HAE where standard approaches fail to uncover the genetic alteration. Finally, despite that one fifth of *SERPING1* intronic length is escaping the analysis by our NGS panel, the possibility of intronic alterations to be the invisible damage whenever standard genotyping cannot detect the cause of C1-INH-HAE, is minimized. The regulatory mechanisms of gene expression comprise diverse molecular circuits involving multiple dedicated components. Thus mechanisms intervening to the expression of *SERPING1,* other than alterations in non-coding regions, should be considered. As Dirk A. Kleinjan and Veronica van Heyningen have noted[32]: "… the laborious identification of the disease loci and regulatory mechanisms involved in currently 'unsolved' human disorders remains a huge but rewarding task".

Conflict of interest

MZ, GL and FPa are employed by CeMIA SA. AEG is stock owner of CeMIA SA. The rest of the authors have no conflict of interest.

Authors' contributions

SV participated in the study design, carried out the basic experimental analysis and data interpretation and drafted the manuscript. MZ and GL conceived and participated in the design of the study, carried out the data interpretation and performed critical revision of the manuscript. FPa involved in some of the basic experimental analyses. FPs, DC, AV, MS, GP, KO, MMag, MMau, MS and HF were responsible for collection of blood samples and assembly of clinical data. AEG conceived and coordinated the study, participated in the study design, and critically revised of the article for important intellectual content. All authors have read and approved the final version of the manuscript.

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Deciphering the Genetics of Primary Angioedema with Normal Levels of C1 Inhibitor

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Abstract: The genetic alteration underlying the great majority of primary angioedema with normal C1 inhibitor (nl-C1-INH-HAE) cases remains unknown. To search for variants associated with nl-C1-INH-HAE, we genotyped 133 unrelated nl-C1-INH-HAE patients using a custom next-generation sequencing platform targeting 55 genes possibly involved in angioedema pathogenesis. Patients already diagnosed with *F12* alterations as well as those with histaminergic acquired angioedema were excluded. A variant pathogenicity curation strategy was followed, including a comparison of the results with those of genotyping 169 patients with hereditary angioedema due to C1-inhibitor deficiency (C1-INH-HAE), and only filtered-in variants were studied further. Among the examined nl-C1-INH-HAE patients, carriers of neither the *ANGPT1* p.Ala119Ser nor the *KNG1* p.Met379Lys variant were found, whereas the *PLG* p.Lys330Glu was detected in four (3%) unrelated probands (one homozygote). In total, 182 different variants were curated, 21 of which represented novel mutations. Although the frequency of variants per gene was comparable between nl-C1-INH-HAE and C1-INH-HAE, variants of the *KNG1* and *XPNPEP1* genes were detected only in nl-C1-INH-HAE patients (six and three, respectively). Twenty-seven filtered variants in 23 different genes were detected in nl-C1-INH-HAE more than once, whereas 69/133 nl-C1-INH-HAE patients had compound heterozygotes of filtered variants located in the same or different genes. Pedigree analysis was performed where feasible. Our results indicate the role that alterations in some genes,

like *KNG1*, may play in disease pathogenesis, the complex trait that is possibly underlying in some cases, and the existence of hitherto unrecognized disease endotypes.

Keywords: next-generation sequencing; pedigree analysis; primary angioedema; primary angioedema with normal C1 inhibitor

1. Introduction

Primary angioedema is defined as localized and self-limiting edema of the subcutaneous and submucosal tissue occurring in the absence of wheals and of a causative factor. According to the criteria of the Hereditary Angioedema International Working Group [1], all hereditary forms as well as the two idiopathic forms of acquired angioedema (histaminergic and non-histaminergic) can be considered as primary angioedema. Hereditary angioedema due to C1-INH deficiency (C1-INH-HAE), the prototype of primary angioedema, is an autosomal dominant disease caused by deleterious mutations in the *SERPING1* gene, leading to quantitative and/or functional C1 inhibitor (C1-INH) deficiency [2]. Normal C1-INH levels and function characterize all other forms of primary angioedema, which clinically present with individual attacks indistinguishable from C1-INH-HAE attacks, despite differing from C1-INH-HAE in many aspects [3]. Until recently, the only genetic defects known to be associated with the hereditary forms of primary angioedema with normal C1-INH levels were mutations in the *F12* gene [4,5]. All other familial cases of angioedema with normal C1-INH levels were characterized as unknown angioedema. In 2019, next-generation sequencing technologies provided new insights into the genetics of primary angioedema with normal C1 inhibitor (nl-C1-INH-HAE). Two new missense mutations in *ANGPT1* (c.807G>T, p.Ala119Ser) and *PLG* (c.988A>G, p.Lys330Glu) genes were detected in association with the disease, whereas family segregation and meticulous functional studies have proved their pathogenicity [6–8]. Recently, Bork et al. [9] reported a hitherto unknown variant in exon 10 of the *KNG1* gene (c.1136T>A, p.Met379Lys) co-segregated with clinical symptoms of hereditary angioedema (HAE) with normal C1-INH levels in three generations of a large German family.

Interestingly, the recently discovered pathogenic variants expanded our concept of nl-C1-INH-HAE pathophysiology beyond the contact system indicating new disease endotypes [6,10,11]. Moreover, a series of patients misdiagnosed as idiopathic non-histaminergic acquired angioedema (InH-AAE) have been reported in the literature, who, after genotyping, were proved to be suffering nl-C1-INH-HAE associated with *F12, PLG*, or *ANGPT1* mutations [12]. Thus, further uncovering the genetic basis of nl-C1-INH-HAE is expected not only to facilitate a better understanding of disease pathophysiology that could drive the discovery of new therapeutic targets but also to provide useful indicators for the clinical management of the disease. To this aim, here, we applied a custom next-generation sequencing (NGS) platform targeting a series of genes entangled in the metabolism and function of bradykinin to detect candidate genes involved in the pathogenesis of nl-C1-INH-HAE.

2. Experimental Section

2.1. Patients

Patients fulfilling the diagnostic criteria of primary angioedema according to the Hereditary Angioedema International Working Group [1] and presenting with normal C1-INH plasma levels were included in the study. Beyond those diagnosed with hereditary angioedema with normal C1-INH, patients with idiopathic non-histaminergic acquired angioedema were included in this group, since they represent a temporary exclusion diagnosis that does not rule out either the appearance of angioedema in the next generation or the presence of a yet unidentified genetic background [12]. Patients already

diagnosed with hereditary angioedema with normal C1-INH and factor XII mutation (FXII-HAE), as well as those with histaminergic acquired angioedema, were excluded.

In total, 133 unrelated patients (53 Hungarian, 32 Italian, 27 Spanish, 12 Greek, and 9 Polish) (35 male; age 40.8 ± 17.4 years) were enrolled in the study. Their mean (±SD) age at disease onset was 27.0 ± 16.4 years (median: 24 years). One in 133 patients had suffered only one angioedema attack during their life; the other 132/133 had a mean frequency of angioedema attacks of 7.8 per year (median: 5 per year). Of the 133 patients, 104 presented with a family history of angioedema, whereas 31/133 patients were on long-term prophylaxis with tranexamic acid. A further 169 patients with C1-INH-HAE were genotyped as controls to search for the presence of variants common in the two forms of angioedema that possibly affect the clinical expression of the disease.

The study was carried out according to the principles of good clinical practice and adhered to the ethical standards of the Declaration of Helsinki with written informed consent from all subjects. The Ethics Committee of the University of Thessaly approved the protocol of the study.

2.2. Genotyping

A custom NGS panel was designed using the Ion AmpliSeq Thermo Fisher Scientific Designer (Thermo Scientific, Waltham, Massachusetts, US) to analyze 55 genes (all coding regions and exon–intron splice junctions) (Supporting Information, Table S1) possibly involved in angioedema pathogenesis and/or the clinical phenotype. The gene list was compiled from literature data on angioedema and genetic predisposition, protein–protein interaction networks, and pathway analysis. In total, 825 amplicons in two primer pools provide 99.61% coverage of all targeted regions.

To construct DNA libraries for each sample using the Ion AmpliSeq Library Kit 2.0 (Thermo Scientific, Waltham, MA, USA), 10 ng of gDNA per primer pool was used. The produced libraries were indexed with a unique adapter using the Ion Xpress barcode adapter kit (Thermo Scientific, Waltham, MA, USA). Barcoded libraries were purified using the Agencourt AMPure XP Beads (Beckman Coulter, Brea, CA, USA), quantified with a Qubit 2.0 fluorometer (Thermo Scientific, Waltham, MA, USA), diluted to 100 pM and pooled in equimolar proportion. Template preparation, enrichment, and chip loading were carried out on the Ion Chef system (Thermo Scientific, Waltham, MA, USA). Sequencing was performed on S5XL on 520 and 530 chips, using the Ion 510, Ion 520, and Ion 530 Kit - Chef (Thermo Scientific, Waltham, MA, USA). All procedures were performed according to the manufacturer's instructions.

Base calling, demultiplexing, and alignment to the hg19 reference genome (GRCh37) of the raw sequencing data were performed in Torrent Suite 5.10 software (Thermo Scientific, Waltham, MA, USA) using the default parameters. Variant calling was performed by the VariantCaller v.5.8.0.19 plug-in and coverage analysis by the CoverageAnalysis v.5.8.0.8 plug-in in Torrent Suite 5.10.

Confirmatory Sanger sequencing was appropriately performed where necessary. Since the causative *ANGPT1* variant (c.807G>T) had not been described at the time of the design of our NGS panel, this gene was not included among those analyzed by this method. Thus, *ANGPT1* genotyping was performed by Sanger sequencing as previously described [6].

2.3. Variant Pathogenicity Curation

All variants detected after alignment to the hg19 genome using the VariantCaller plug-in were annotated in Ion Reporter software v.5.6 (Thermo Scientific, Waltham, MA, USA) with the gene name and for their possible presence in the Single Nucleotide Polymorphism Database (v135) [13], the Exome Aggregation Consortium (ExAC) [14], the 1000 Genomes project [15], and the ClinVar [16] according to the recommendations of the Human Genome Variation Society (HGVS) [17]. SIFT [18] and PolyPhen version 2 [19] bioinformatics tools were used for in silico pathogenicity prediction of the variants. Alignments and all obtained sequences were visually inspected using the Integrative Genomics Viewer (IGV) v.2.2 (Broad Institute, Cambridge, MA, USA).

Variants with a worldwide frequency of >1% (1000 Genomes Global Minor Allele Frequency, ExAC) and polymorphisms for which no disease associations are reported in the ClinVar database, as well as synonymous and intronic single-nucleotide variants (SNVs), were excluded from further analysis.

3. Results and Discussion

Among the nl-C1-INH-HAE patients, no carriers of the *ANGPT1* p.Ala119Ser variant were found, indicating that at least this mutation of the *ANGPT1* gene represents very rare causative genetic damage. However, the *PLG* p.Lys330Glu variant was detected in four (3%) unrelated probands (one homozygote), which have already been described in detail elsewhere [20]. Pedigree analysis of these cases confirmed the incomplete penetrance of this alteration. Including our cases, more than 100 patients with nl-C1-INH-HAE due to this mutation have been reported in the literature since its first description [21].

Among the variants identified in the 55 analyzed genes, 182 different variants were filtered in and included in further analysis. Twelve alterations occurred in the 5′ untranslated region (UTR) (6.6%) and 18 in the 3′-UTR (10%). Missense mutations corresponded to 76.6% of the total, followed by small insertions/deletions leading to frameshift (1%), non-sense (3.3%), splice site (0.5%), stop-loss (0.5%), and non-frameshift insertions/deletions (1.5%). A list containing all variants is found in Table S2 (Supporting Information). Of the 182 mutations (indicated in black in Table S2), 21 were not previously reported in population databases (novel mutations). The frequency of variants per gene was not significantly different between nl-C1-INH-HAE and C1-INH-HAE patients, with the exception of *KNG1* and *XPNPEP1* genes, where six and three variants were detected, respectively, in the nl-C1-INH-HAE group but none in the C1-INH-HAE group.

A series of 27 filtered variants in 23 different genes was detected in our material more than once. As shown in Table 1, in a proportion of these variants, their allele frequencies among nl-C1-INH-HAE patients were significantly different from those in the European population or even in our C1-INH-HAE cohort. According to the guidelines of the American College of Medical Genetics and Genomics [22], this is a criterion in favor of the pathogenicity of the variants. The exact contribution of each one of these variants in the pathogenesis or in the clinical phenotype of the disease is difficult to envisage. However, a finding that merits particular attention is the frequency of filtered androgen receptor gene (*AR*) variants, which, among nl-C1-INH-HAE patients, is significantly higher than that in both the European population and in the cohort of C1-INH-HAE controls. Further studies are worth undertaking to investigate the possible correlation of these variants with the estrogen-dependence of the disease's clinical phenotype.

Sixty-nine of the examined nl-C1-INH-HAE patients were heterozygous for more than one and up to nine filtered variants located in the same or different genes (compound heterozygotes). No correlation was found between the number of heterozygous variants carried by patients and their age at disease onset or the frequency of attacks.

Family segregation studies were performed when feasible and provided useful information. Firstly, the variants p.Leu140Val and p.Ala177Val of the *F12* gene proved to be non-pathogenic. However, the novel *PLG* p.Val728Glu (c.2183T>A) variant was found to co-segregate with angioedema symptoms in a Greek patient (male, 15 years old) and their suffering father (52 years old) but not in his unaffected mother. His 10-year-old sister also carries the variant but she has not demonstrated disease symptoms as yet. The p.Val728Glu variant is located inside the plasmin serine protease domain (residues 562–791), which is an active serine protease with a wide substrate specificity [23]. Thus, the p.Val728Glu substitution could eventually affect functional interrelationships between the plasminogen/plasmin system and the kinin pathway, leading to an alteration in vasopermeability.

Table 1. Variants with a worldwide allelic frequency of <1% that were detected in our material more than once. EMAF: European minor allele frequency; ExAC ENFAF: ExAC European non-Finnish allele frequency; nl-C1-INH-HAE AF: allele frequency among nl-C1-INH-HAE patients; C1-INH-HAE AF: allele frequency among C1-INH-HAE patients, P1: EMAF vs. nl-C1-INH-HAE AF, P2: EMAF vs. C1-INH-HAE AF, P3: ni-C1-INH-HAE AF vs. C1-INH-HAE AF.

Genes	Coding	Amino Acid Change	dbSNP	SIFT	PolyPhen	EMAF	ExAC ENFAF	nl-C1-INH-HAE AF	C1-INH-HAE AF	P1	P2	P3
BDKRB1	c.721G>A	p.Gly241Arg	rs45528332	tolerated	probably damaging	0.0037	0.0052	0.0113	0.0148	0.1500	0.0350	0.7000
MME	c.674G>C	p.Gly225Ala	rs147564881	tolerated	probably damaging	0.0023	0.0033	0.0113	0.0030	0.0310	0.7400	0.2100
PLAUR	c.802A>G	p.Met268Val	rs138492321	tolerated	possibly damaging	0.0062	0.0045	0.0188	0.0000	0.0440	0.1500	0.0110
C1S	c.943G>A	p.Asp315Asn	rs117907409	deleterious	probably damaging	0.0053	0.0052	0.0113	0.0059	0.2400	0.8300	0.4700
F13B	c.1025T>C	p.Ile342Thr	rs17514281	deleterious	possibly damaging	0.0097	0.0098	0.0263	0.0059	0.0380	0.4900	0.0390
F2	c.*97G>A		rs1799963			0.0080		0.0263	0.0148	0.0130	0.2600	0.3100
TLR4	c.842G>A	p.Cys281Tyr	rs137853920	deleterious	probably damaging	0.0044	0.0027	0.0150	0.0030	0.0420	0.7900	0.1000
KRT1	c.1669A>G	p.Ser557Gly	rs77846840	tolerated	benign		0.0019	0.0263	0.0296			0.8000
SERPINE1	c.*180C>T		rs41334349			0.0110		0.0226	0.0266	0.1400	0.0400	0.7500
AR	c.-207C>A		rs189146053			0.0000		0.0188	0.0030	<0.0001	0.0844	0.0500
AR	c.1174C>T	p.Pro392Ser	rs201934623	tolerated	benign	0.0000	0.0041	0.0113	0.0000	0.0007		0.0500
TPSAB1	c.407A>G	p.His136Arg	rs201820654	tolerated	benign		0.0034	0.0113	0.0089			0.7600
TPSG1	c.508G>A	p.Gly170Arg	rs117769620	tolerated	benign	0.0065	0.0073	0.0188	0.0118	0.0757	0.3893	0.4832
ELANE	c.770C>T	p.Pro257Leu	rs17216663	tolerated	benign	0.0108	0.0080	0.0188	0.0030	0.3062	0.1775	0.0530
F12	c.418C>G	p.Leu140Val	rs35515200	tolerated	possibly damaging	0.0042	0.0033	0.0075	0.0030	0.4533	0.7904	0.4287
F12	c.530C>T	p.Ala177Val	rs144821595	tolerated	benign	0.0002	0.0001	0.0075	0.0000	<0.0001	0.7948	0.1103
ACE	c.1453C>G	p.Pro485Ala	rs202178737	deleterious	benign	0.0000	0.0001	0.0075	0.0000	0.0059		0.1103
BDKRB1	c.844C>T	p.Arg282Ter	rs145322761			0.0035	0.0038	0.0075	0.0030	0.4533	0.7904	0.4287
PLG	c.266G>A	p.Arg89Lys	rs143079629	tolerated	benign	0.0100	0.0108	0.0075	0.0030	0.7164	0.2177	0.4287
KLK3	c.629C>G	p.Ser210Trp	rs61729813	deleterious	probably damaging	0.0110	0.0109	0.0075	0.0178	0.6223	0.3319	0.2748
DPP4	c.796G>A	p.Val266Ile	rs56179129	tolerated	benign	0.0060	0.0045	0.0075	0.0000	0.7755	0.1547	0.1103
PLAU	c.1048T>C	p.Tyr350His	rs72816325	deleterious	probably damaging	0.0058	0.0059	0.0075	0.0000	0.7755	0.1547	0.1103
PLAUR	c.-87C>T		rs147665588			0.0060		0.0075	0.0089	0.7755	0.5702	0.8550
F13A1	c.1730C>T	p.Thr577Met	rs143711562	tolerated	benign	0.0029	0.0020	0.0075	0.0000	0.2930	0.3149	0.1103
TNF	c.251C>T	p.Pro84Leu	rs4645843	tolerated	benign	0.0030	0.0028	0.0075	0.0030	0.2930	0.9945	0.4287
GPER1	c.14C>T	p.Ser5Phe	rs117290655	tolerated	benign	0.0048	0.0045	0.0075	0.0089	0.6173	0.4193	0.8550
MPO	c.2031-2A>C		rs35897051			0.0072	0.0071	0.0075	0.0059	0.9227	0.8391	0.8096

The recently reported *KNG1* p.Met379Lys variant [9] was not detected in any of our nl-C1-INH-HAE patients. However, the *KNG1* p.Pro574Ala (c.1720C>G) variant was detected in three affected members (two brothers and their father) of an Italian family but not in three asymptomatic relatives. Two of the patients suffered typical disease with repeated angioedema attacks, whereas the third had only experienced one attack during his life following a viral infection. Interestingly, the two patients who suffered repeated attacks were also carriers of the *ACE* p.Arg487Cys (c.1459C>T) variant. The same variant, despite it being predicted as deleterious by bioinformatics tools, was also detected in one of the three analyzed asymptomatic relatives (Figure 1). It seems that the *KNG1* p.Pro574Ala variant presents with incomplete penetrance or that its possible pathogenicity depends upon its compound heterozygosity with the *ACE* p.Arg487Cys variant. In conjunction with the abovementioned high frequency of filtered *KNG1* variants observed among nl-C1-INH-HAE patients, these findings indicate that variations in the *KNG1* gene could contribute to the pathogenesis of the disease; thus, they deserve further consideration.

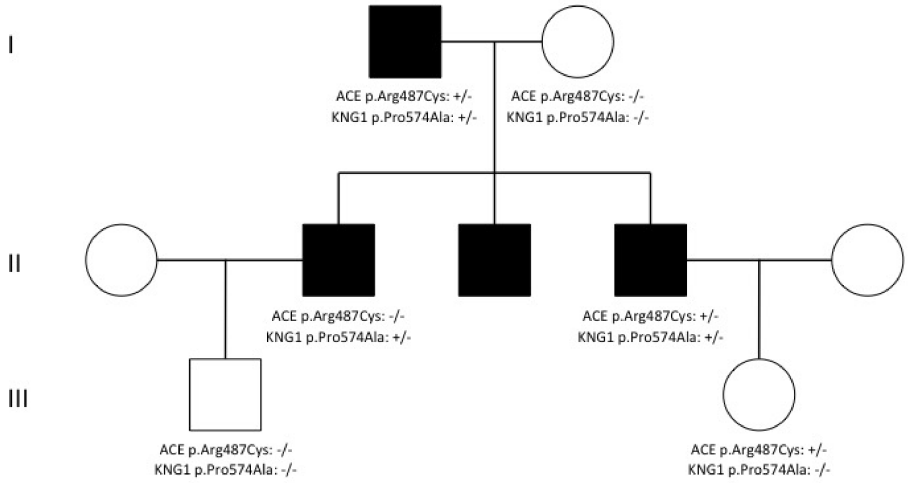

Figure 1. Pedigree demonstrating co-segregation of the missense mutation of the *KNG1* p.Pro574Ala (c.1720C>G) and the *ACE* p.Arg487Cys (c.1459C>T) variants with nl-C1-INH-HAE.

The genes encoding for tryptases (*TPSAB1, TPSD1*, and *TPSG1*) were included in the panel of analyzed genes because raised serum tryptase has been occasionally observed in cases of acquired angioedema [24,25]. The variant p.Arg158Gln (c.473G>A) of the *TPSG1* gene was detected in all three affected women in three generations and in one of the three examined asymptomatic first-degree relatives of an Italian family. Should this finding be confirmed by further studies, it would implicate new pathways or cells (e.g., mastocytes) in the pathogenesis of nl-C1-INH-HAE.

A final remarkable finding was that the two suffering members (a mother and her daughter) of a Hungarian family were carriers of the same series of novel or rare variants in different genes: *BDKRB1* p.Arg282Ter, *CPN1* p.Glu407Lys, *SERPING1* c.*57C>G (3'UTR), *PLAUR* p.Met268Val, *MASP1* p.Val680Ala, *TLR4* p.Cys281Tyr, and MPO p.Arg524His. This observation suggests that, at least in certain cases, nl-C1-INH-HAE could be the result of the cumulative effect of multiple gene variations.

Taken together, the above observations clearly demonstrate that, genetically, nl-C1-INH-HAE is an extremely complex disorder. The relatively small size of the examined cohort of patients and the non-availability of families for pedigree analysis represent the main limitations of the study, which is true for all rare disease studies. Thus, strong evidence of the causative effect of certain variants has not been provided. Nevertheless, the results of the study helpfully highlight the role that alterations in some genes, like *KNG1*, may play in the pathogenesis of the disease, the complex trait that is possibly underlying some cases, and the existence of hitherto unrecognized disease endotypes. Finally, it must be underlined that every day, contemporary genomic approaches discover new genes associated with the disease, indicating the involvement of new pathways in its pathogenesis (e.g., the *ANGPT1* [6]

and the *MYOF* [26] genes). Therefore, beyond the genes examined in this study, there are many other candidate disease genes remaining to be examined, like many endothelium-associated ones.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/11/3402/s1: Table S1: The genes analyzed by our custom NGS panel and their coverages; Table S2: The filtered-in variants detected among nl-C1-INH-HAE patients. Variants not previously reported in population databases (novel mutations) are indicated in bold.

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