SUPPLEMENTARY MATERIAL

PATIENTS AND METHODS

Patients

We studied 9 patients with idiopathic angioedema (4 men and 5 women; age range: 32-83 years), during acute angioedema attacks. All patients presented to the emergency department of Ospedale Maggiore of Crema for angioedema reporting previous episodes of angioedema not responding to antihistamine, no family history or known causes of angioedema and no therapy with ACE inhibitors or non-steroidal anti-inflammatory drugs. Thus, they can be considered affected with idiopathic non-histaminergic acquired angioedema. Exclusion criteria were age less than 18 years and presence of severe comorbidities. The study was approved by Ethics Committee Valpadana of ASST Ospedale Maggiore Crema (No. 104, 22 march 2019) and was carried out in conformity with the 2013 revision of the Declaration of Helsinki and the code of Good Clinical Practice. All patients gave their written consent to participate in the study.

Blood was collected before any treatment from antecubital veins with minimal stasis using specific anticoagulant mixtures to avoid in vitro activation of the contact system as well as generation and degradation of vasoactive mediators [9-11]. Blood samples were immediately centrifuged and the plasma was frozen, as described below. Nine healthy subjects, sex- and age-matched with patients, served as controls. Control subjects were staff members of the hospital or their relatives, and their blood samples were obtained in the emergency department and processed with the same method used for patients.

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Methods

Measurement of cleaved HK

For cleaved HK measurement, blood was drawn into tubes containing an inhibitor cocktail (100 mM benzamidine, 400 g/ml hexadimethrine bromide, 2 mg/ml soybean trypsin inhibitor, 263 M leupeptin and 20 mM aminoethyl-benzenesulfonylfluoride) dissolved in acid-citrate-dextrose (100 mM trisodium citrate, 67 mM citric acid, and 2% dextrose, pH 4.5) to prevent in vitro activation of the contact system [10,11]. The tubes were centrifuged at 2,000g for 10 minutes at room temperature and the plasma aliquots were stored in polystyrene tubes at -80 °C until testing. The cleavage of HK was assessed by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in nonreducing conditions and immunoblotting analysis. After electrophoretic separation and the transfer of proteins from the gel to a polyvinylidene difluoride membrane (Immobilon; EMD Millipore Corp, Billerica, MA, USA), HK was identified using goat polyclonal anti-HK light chain (Nordic, Tilburg, The Netherlands) and visualized using a biotinylated rabbit anti-goat antibody (Sigma-Aldrich Co, St. Louis, MO, USA). The apparent molecular masses of the proteins were estimated by comparing them with the high-molecular-weight protein markers (Bio-Rad Laboratories, Hercules, CA, USA). Using this method, native HK appears as a single band with a relative molecular mass ($M_{r)}$ of 130,000, but upon maximal cleavage, it is progressively replaced by two bands with M_r values of 107,000 and 98,000. The density of the bands was measured using a Bio-Rad GS800 densitometer. Kaolin-incubated plasma was run on each electrophoresis as a control sample. The amount of cleaved HK (with M_r bands of 107,000 and 98,000) was expressed as a percentage of total HK (the sum of the three bands) [10,11]. Intra- and inter-assay coefficients of variation were less than 20%.

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Measurement of bradykinin

To measure bradykinin, venous blood was collected in pre-cooled syringes containing protease and peptidase inhibitors to obtain final concentrations of 21 μmol/L aprotinin, 73 μg/mL egg trypsin inhibitor chicken albumin, 305 μg/mL hexadimethrine bromide, 4.5 mmol/L 1.10-phenanthroline and 4.5 mmol/L edetic acid. Blood samples were immediately transferred into pre-cooled polypropylene tubes, centrifuged at 2,000g for 10 minutes at 4°C, and plasma aliquots were stored in polystyrene tubes at -80°C until the tests were performed [9]. Bradykinin was measured using a commercial enzyme immunoassay (RayBio® Bradykinin Enzyme Immunoassay (EIA) Kit, RayBiotech Life Inc. Peachtree Corners, GA, USA) following the manufacturer's instructions. In brief, a biotinylated bradykinin peptide was spiked into the samples and standards. The samples and standards were added to a microplate pre-coated with anti-IgG antibodies. The biotinylated bradykinin peptide competed with endogenous bradykinin for binding to an anti-bradykinin IgG that in turn is captured by the anti-IgG adsorbed to the plate. After washing, the captured biotinylated bradykinin interacted with horseradish peroxidase (HRP)-streptavidin, which catalysed the development of a colorimetric reaction. The absorbance at 450 nm was directly proportional to the amount of captured biotinylated bradykinin and inversely proportional to the amount of endogenous bradykinin in the samples. Intra- and inter-assay coefficients of variation were 10% and 15%, respectively.

Complement system analysis

For the complement evaluation, blood was drawn in silicone-coated Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing 0.13 mol/L of trisodium citrate. C1-INH and C1q antigens were measured by means of radial immunodiffusion (RID, NOR-Partigen, Siemens Healthcare Diagnostics, Munich, Germany). Intra- and inter-assay coefficients of variation were less than 13%. C1-INH function was assessed as the capacity of plasma to inhibit the esterase activity of exogenous C1s measured on a specific chromogenic substrate by means of a commercially available kit

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(Technoclone GmbH, Vienna, Austria). Intra- and inter-assay coefficients of variation were 4.5% and 6.6%, respectively.

Genetic analysis

Genes, the mutations of which are considered implicated in hereditary angioedema, were evaluated using the custom panel HaloPlex HS Target Enrichment System (Agilent, Santa Clara, CA, USA): *SERPING1* (NM_00006.2), *ANGPT1* (NM_001146.5), *PLG* (NM_000301.4), *MYOF* (NM_013451.4), *KNG1* (NM_001102416.3) and *F12* (NM_000505.3) [12]. Genomic DNA was extracted from the whole blood of proband using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The raw data were then processed according to the Genome Analysis Toolkit (GATK 1.6) and were analysed using the software BaseSpace Variant Interpreter Annotation Engine 3.15.0.0 (Illumina, San Diego, CA, USA). Variants were annotated according to the Human Genome Variation Society guidelines (HGVS), mapped to the human genome build GRCh37/UCSC hg19, and classified according to the criteria of the American College of Medical Genetics and Genomics [Richards S et al. Genet Med. 2015;17(5):405-24. doi: 10.1038/gim.2015.30]. Pathogenicity assessment for all rare genetic variants was performed according to ACMG2015 guidelines. To identify variants that were pathogenic, likely pathogenic or variant of unknown significance (VUS), we looked up the variants' minor allele frequency (MAF) in the Exome Aggregation Consortium (ExAC). We used 0.01 as an initial filtering criterion to limit the number of variants considered. In addition, further analysis was performed to identify variants associated with a given phenotype.

Statistical analysis

The sample size was calculated in order to obtain a statistical power of 80%, with an alpha error of 5%, based on our previous study [9]. Due to non-normal distribution, results were reported as medians and ranges (minimum - maximum), and nonparametric methods were used to assess statistical differences between groups. The significance level was set at p=0.05. The Spearman correlation

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coefficient was calculated to assess relationships between variables. The data were analysed using the SPSS PC statistical package, version 27 (IBM SPSS Inc., Chicago, IL, USA).

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