

Hereditary angioedema cosegregating with a novel kininogen 1 gene mutation changing the N-terminal cleavage site of bradykinin

To the Editor

Types of hereditary angioedema (HAE) comprise first, HAE due to C1 inhibitor (C1-INH) deficiency (HAE-C1-INH) and second, various types of HAE with normal activity of C1-INH (HAEnCI), including those with HAEnCI-specific variants in the factor XII gene (*F12*) (HAE-FXII), the plasminogen (*PLG*) gene (HAE-PLG), and the angiotensin-converting enzyme (*ACE*) gene (HAE-ACE)¹⁻⁴. In other HAEnCI families, the genetic background is still unknown (HAE-unknown).

Here, we report a large family with HAE in which all known HAE-specific variants were not present and aimed to identify a potentially disease-causing variant in symptomatic patients. By using next-generation sequencing of the whole exome sequencing (WES)⁵ of the two affected family members who were the first consented to donate blood, we identified the hitherto unknown variant c.1136T>A in exon 10 of the kininogen 1 (*KNG1*) gene. This *KNG1* variant causes an amino acid (AA) change of p.Met379 to p.Lys379 (p.Met379Lys; p.M379K) and is present in both high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK) isoproteins (Figure 1). The variant was present in all six affected and not present in eight asymptomatic relatives (Figure 2). Among the investigated 14 family members, there was no member who had HAE symptoms but did not carry the described variant. Pedigree analysis revealed that clinical symptoms of HAE occurred in three generations and cosegregated with the *KNG1* variant c.1136T>A in all analyzed patients. The variant was transmitted in a dominant inheritance pattern. The probability that a further patient will appear in this family who will not match to the cosegregation pattern of disease and variant is less than 0.001 (Fisher's exact test). The haplotypes linked with the mutant *KNG1* alleles were obtained from WES data. Haplotypes were determined to identify a common founder effect in case further families with the *KNG1* variant are found.

The variant c.1136T>A was absent from ExAC, 1000 Genomes, gnomAD databases, as well as 105 in-house controls. Furthermore, the variant was not present in any of the 38 index patients of HAEnCI families without HAEnCI-specific variants in the *F12*, *PLG*, or *ANGPT1* genes. In silico predictions regarding its effect on protein function were divergent. Whereas SIFT, CADD, PolyPhen-2, and MutationTaster classified and predicted the variant as benign, SNPs&GO predicted that p.Met379Lys is deleterious. Moreover, according to the ACMG 2015 guidelines, the pathogenicity potential of the described variant is "likely pathogenic." This is based on

the following: first, the strong segregation data (large family with affected members in three consecutive generations and a large number of investigated affected and unaffected family members); second, the significantly increased prevalence of the variant in affected individuals compared with controls; third, the absence of the variant from controls in ExAC, 1000 Genomes, and gnomAD as well as all WES analysis databases (see Appendix S1); and finally, the fact that the patients' phenotype combined with family history is highly specific for a disease with a single genetic etiology. Furthermore, the variant is located in a functional domain, the cleavage region for kinins including bradykinin, which is the presumed direct mediator for the symptoms of HAE.

Mean C1-INH activity and concentration, C4 concentration, FXI clotting activity, FXII clotting activity, and plasminogen activity for the six affected patients during attack-free intervals and controls were within the reference range. The mean FXII clotting activity was significantly increased ($P = 0.04$) in the patient group compared with the control group. The effect of the shortened APTT is tendentially significant ($P = 0.0518$), meaning the p -value lies between 0.05 and 0.10. However, due to the small sample size, the statistical power is low. Houlihan et al. found a modulation of APTT values by a polymorphism within the *KNG1* gene (rs710446)⁶. In the family's haplotype analysis, the polymorphism within the *KNG1* gene (rs710446, SNP c.1742 T/C) has been included (see Figure 2). One patient (II:10) has a c.1742T/T genotype, that is, 2 reference alleles, and another patient (IV:10) has a c.1742T/C genotype, that is, one reference allele and one rare allele. This shows that the functional variant c.1742C is not linked with the mutant *KNG1* allele in our family. Hence, we could rule out that all family members carry the c.1742C allele, which might influence the APTT level.

To rule out a significant influence of the *KNG1* variant c.1136T>A on the HMWK level, prekallikrein and HMWK activities and antigen levels were determined and found to be in the reference range.

Three AA cleavage sites for kinins are changed N-terminally before the bradykinin sequence by the *KNG1* variant c.1136T>A. This concerns the cleavage of bradykinin from HMWK, of Lys-bradykinin from LMWK, and of Met-Lys-bradykinin-Ser-Ser from HMWK⁷. Bradykinin is cleaved from HMWK by plasma kallikrein^{8,9}. The N-terminal cleavage site for bradykinin is between AA p.Lys380 and Arg 381. Lys-bradykinin is cleaved from LMWK by the serine protease tissue kallikrein. The N-terminal cleavage site is between AA p.Met379

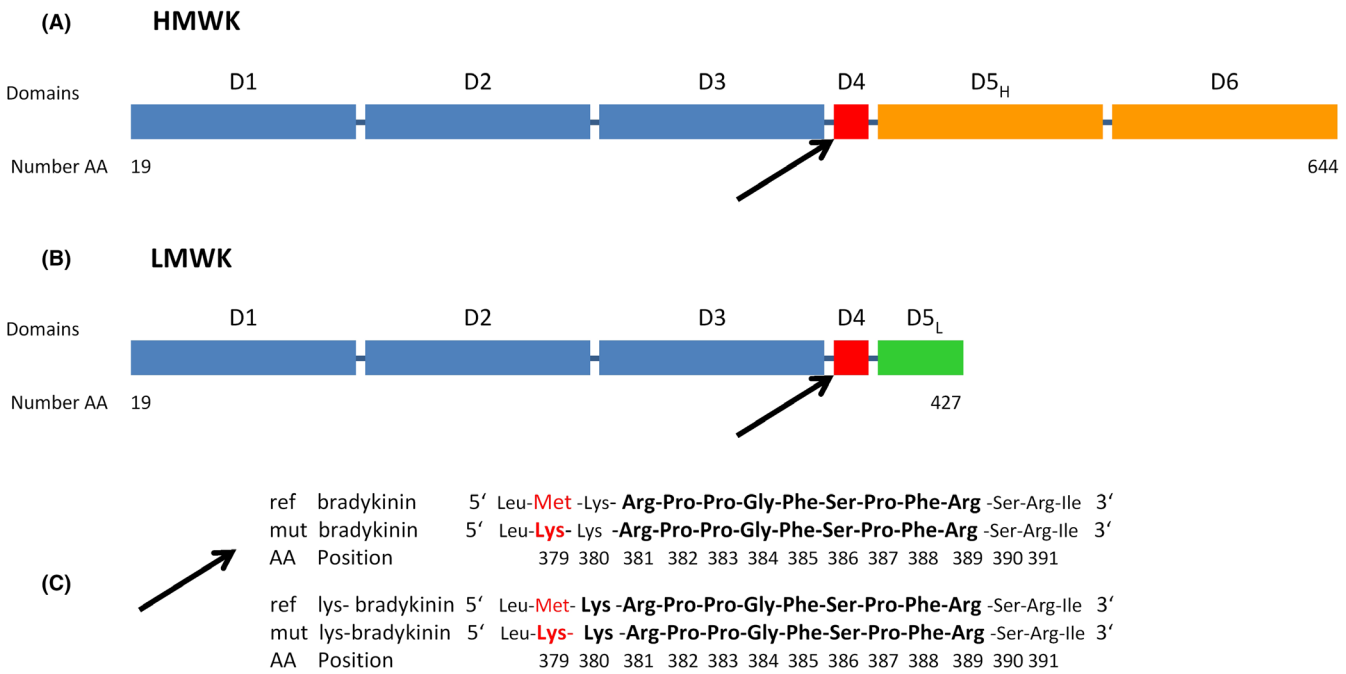


FIGURE 1 Domain structures (not to scale) of the isoproteins HMWK (A) and LMWK (B) from the *KNG1* gene and the localization of the described amino acid exchange in domain 4. D 1, 2, and 3: domains 1, 2, and 3 (cystatin kininogen domains 1 to 3), D4: bradykinin domain (contains bradykinin and Lys-bradykinin sequences); function of D5_L of LMWK is not known; D5_H of HMWK: surface binding, D6 of HMWK: prekallikrein and FXI binding. Black arrow: location of P.Met379Lys change. (C) Amino acid sequences of bradykinin and Lys-bradykinin (bold) and the flanking amino acids of reference and the allele with the P.Met379Lys variant. Methionine/Lysine change is marked in red. The C-terminal cleavage site of HMWK and LMWK is between Arg389 and Ser390; the N-terminal cleavage sites of HMWK are in Lys380/Arg381 (bradykinin) and of LMWK are in Met379/Lys380 (Lys-bradykinin). AA, amino acid; ref, reference AA sequence; mut, mutant AA sequence

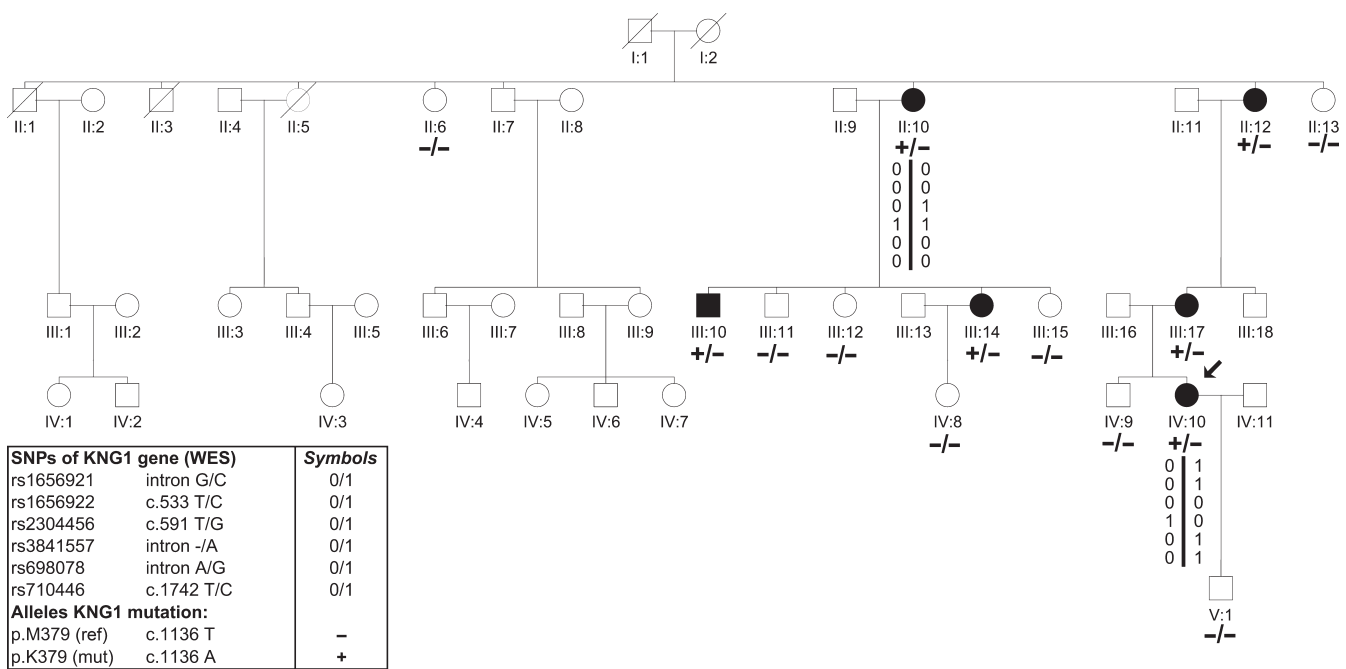


FIGURE 2 Pedigree of a family with the novel HAE-KNG1 variant c.1136T>A (p.Met379Lys). Results of whole exome sequencing and Sanger sequence analysis and haplotypes linked with the variant c.1136T>A are presented. Circle = female. Square = male. Symbols with a slash indicate deceased family members. Filled symbols = individuals affected by recurrent angioedema. Empty symbols = symptom-free subjects.

and Lys380. Met-Lys-bradykinin-Ser-Ser is cleaved from HMWK by the neutrophil proteinase-3 (PR3). The N-terminal cleavage site is between AA p.Leu378 and Met379. It is highly probable that the reported AA change modifies the bradykinin-forming process pointing toward a previously unreported pathomechanism of HAE.

We consider that the described *KNG1* variant changing the N-terminal cleavage site of bradykinin from HMWK and LMWK could lead to a functionally active but aberrant bradykinin in HAE-KNG1. An aberrant bradykinin could change the process of the inactivation by enzymes such as aminopeptidase 2, angiotensin-converting enzyme, and others, possibly resulting in a prolonged half-life time and thus a higher than normal activity of this mutant bradykinin.

Similar to bradykinin, Lys-bradykinin is a high-affinity agonist for the human B2 receptor. It can be cleaved to bradykinin by an arginine aminopeptidase (AAP) in plasma. We cannot rule out that the novel mutation also leads to a modified Lys-bradykinin. Another possibility could be that the mutant HMWK is more easily activated. We are planning to demonstrate the cleaved HMWK products.

The results, reported here, strongly support our assumption that the new variant in the *KNG1* gene leads to a novel type of HAE, HAE with normal C1-INH and a specific variant in the *KNG1* gene or HAE-KNG1.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supporting Information

Hereditary angioedema cosegregating with a novel kininogen1 gene mutation changing the N-terminal cleavage site of bradykinin

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METHODS

The study comprised analyses of whole exome sequencing (WES), Sanger sequencing and clinical and biochemical data of patients.

Probands

The probands were comprised of patients, symptom-free relatives of HAE_{NCI} patients, 38 unrelated index patients with HAE_{NCI} with unknown genetic basic defects, and controls. All probands came from the Angioedema Outpatient Service (AOS) Mainz. Patients were selected using the following criteria: (a) patients had recurrent swellings of the skin, abdominal pain attacks, tongue swellings or laryngeal attacks in the absence of urticaria; (b) patients had normal C1-INH function and concentration in plasma. All HAE_{NCI} patients were analyzed using WES and/or

Sanger sequencing methods and biochemical tests. Furthermore, 105 controls from the AOS Mainz (210 alleles), comprising 53 males and 52 females, were examined for the presence of the new KNG1 gene variant.

Collection of clinical data

Clinical data was collected from the patients' medical history and from clinical examinations as well as follow-up records. All subjects analyzed in this study gave written informed consent prior to participation. The local ethic committee approved the study.

Whole exome sequencing

EDTA blood was collected from all study participants. All samples were stored at -80°C. DNA was extracted from whole blood according to standard protocols (Qiagen, Hilden, Germany). Exome sequencing was performed as previously described.^{E1} Briefly, read alignment was performed with BWA (v. 0.5.8) and the human genome assembly hg19 (GRCh37) was used as reference. Single nucleotide variants and small insertions and deletions (indels) were detected using SAMtools (v 0.1.7) using default parameters. Furthermore, we required putative single nucleotide variants to fulfil the following criteria: (i) median base quality of the variant bases of at least 15; (ii) a minimum of 15% of reads showing the variant base; and (iii) the variant base is indicated by at least 5% of reads coming from different strands. We used annovar to annotate the single nucleotide variant.^{E2} Annotation was based on several databases provided by annovar such as UCSC known gene, dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), Exome Sequencing Project (<http://evs.gs.washington.edu/EVS/>), 1000 Genomes (<http://www.internationalgenome.org/>), and the Exome Aggregation Consortium (ExAC) (<http://exac.broadinstitute.org/>). In addition, we also annotated several functional prediction scores such as SIFT^{E3}, CADD^{E4}, PolyPhen-2^{E5} and MutationTaster.^{E6}

Haplotype analysis

Haplotype linked with the mutant KNG1 allele was determined in two patients in whom WES data was available (see box inserted in Figure 2). Six KNG1 gene single nucleotide polymorphisms (SNPs) flanking the described mutation were chosen for

haplotyping the mutant alleles. Five of the polymorphisms (rs 1656921; rs 1656922; rs 2304456; rs 3841557; rs 698078) were located in 5' and one of them in 3' direction of the new *KNG1* gene mutation (rs 710446). In the haplotypes the symbol 0 presented the reference and 1 the rare form of SNPs (Figure 2). For our analysis we used the classical indirect genomic diagnostics which have been used for diagnosing the carrier status and for prenatal diagnosis of numerous genetic diseases. All patients with the novel *KNG1* mutation in the family described here must have an identical combination of allele variants of SNPs flanking the *KNG1* mutation c.1136T>A because they are present in the same gene region. To show transmission of the SNP variants linked with the *KNG1* mutation informative allele constellations are required. All used SNPs were 5' and 3' flanking and closely linked with the novel *KNG1* mutation.

Sanger sequencing

Validation of the results of WES was performed by Sanger sequencing as described elsewhere.^{E7} Primers (Biomers, Ulm, Germany) that were used for validation of the novel *KNG1* gene mutations in exon 10 were: KN-10-1 (forward) 5'-TAA AAT CTG GCA AAC ACC TCC C-3' and KN-10-2 (reverse) 5'-CGA GTA TGC CCT TGT TCT TTT CC-3'.

The presence of the known HAE-specific mutations in the *F12*, *PLG* and *ANGPT1* genes were tested as described elsewhere.^{E7,E8,E9,E10}

Search for potentially disease-causing variants

As mentioned in the literature, the prevalence of HAE with normal C1-INH has been supposed to be less than 1:100,000. Therefore, we assumed that potentially HAE-causing gene variants must be rare in our population. Accordingly, we filtered the results of WES analysis of HAE patients on the rare gene variants with allele frequencies of 1% or lower. From the WES data of two HAE patients of the same family 121 different rare variants were identified. Forty of those variants in different genes were identified as non-synonymous. The next step was the search for mutations in risk genes for HAE (including *KNG1*) which might be involved in the pathomechanism. In the following step we investigated the presence of selected variants in HAE patients and unaffected relatives by Sanger sequencing. Then we calculated the statistical risk that the segregation of the proposed causal variant with

HAE could be only accidental and not disease-adherent. For this we determined the probability that a further patient appears in this family who develops the phenotype of HAE but does not carry the *KNG1* variant – i.e., does not match to the cosegregation pattern of disease and variant.

Biochemical analysis

Plasma from patients and controls was separated by means of centrifugation of freshly collected citrated blood at 1,500 g for 10 minutes at 4°C. All samples were divided in aliquots and stored at -80°C. Patient samples were collected during symptom-free intervals between attacks.

C1-INH activity in plasma was determined using the chromogenic substrate C₂H₅CO-Lys(ε-Cbo)-Gly-Arg-pNA (Technochrom C1-Inhibitor, Technoclone, Vienna, Austria).

Plasma levels of C1-INH antigen and C4 antigen were assayed by radial immunodiffusion (NOR Partigen C1-INH and C4, Siemens Healthcare Diagnostics, Marburg, Germany). For APTT determination, platelet poor plasma (PPP) was mixed and incubated with nonsettling colloidal silica and synthetic phospholipids [SynthASil[®], Instrumentation Laboratory (IL), Bedford, Mass., USA] for 180–220 s at 37°C. After this, the mixture was recalcified with 0.020 mol/l calcium chloride solution, and the clotting time measured. Determination of clotting activity of FXI and FXII was done by a modification of APTT using SynthASil[®] and FXI- and FXII-deficient (depleted) plasma (Instrumentation Laboratory (IL), Bedford, Mass., USA IL), respectively. Prothrombin time (PT) was determined by mixing PPP with a thromboplastin reagent containing recombinant tissue factor, phospholipids and calcium ions (RecombiPlasTin 2G kit, IL) and measuring the clotting time. INR was calculated with the formula: (patient's PT/mean of PT normal range)_{ISI}. APTT, FXI, FXII, and PT were determined using an automated coagulation analyzer (ACL TOP 700 CTS, IL). PLG activity was determined using a chromogenic assay. PLG is activated through reaction with an excess of streptokinase in the presence of fibrinogen. The PLG-streptokinase complex is determined by the rate of hydrolysis of the chromogenic substrate pyroGlu-Phe-Lys-pNA (HemosIL Plasminogen, Instrumentation Laboratory (IL), Bedford, Mass., USA). TAT was determined by ELISA technique (Enzygnost[®] TAT micro, Siemens Healthcare Diagnostics Product GmbH, Marburg, Germany).

Prekallikrein (PK) and HMWK clotting activities (PK:C and HMWK:C) were measured as described by Asmis et al., 2002 [Thrombosis Research] with minor modifications using reagents supplied by IL (Instrumentation Laboratory Company, Bedford, MA, USA; Factor Diluent, SynthASil, CaCl₂ Solution, ready to use), George King Bio-Medical Inc. (Overland Park, KS, USA; Prekallikrein Deficient Plasma) and CoaChrom Diagnostica GmbH (Maria Enzersdorf, Austria; Kininogen Deficient Plasma). Kininogen deficient plasma was immune depleted and supplemented with 0.40 U/mL PK. Prekallikrein deficient plasma was patient plasma. Clotting times were obtained by a KC4A Macro coagulation analyzer (SYCOMed e.K., Lemgo, Germany). Briefly, patients' plasma samples were diluted 1:10 in factor diluent. 25 µl of the diluted plasma was incubated at 37°C for 60 sec and then mixed with 25 µl prekallikrein deficient plasma or kininogen deficient plasma, respectively. After incubation at 37°C for 60 min 50 µl of pre-warmed SynthASil reagent were added; after a further 60 sec incubation step at 37°C the mixture was complemented by 50 µl pre-warmed CaCl₂ solution and clotting time was measured.

PK and HMWK antigen (PK:Ag and HK:Ag) were determined by ELISA using the matched-pair antibody set for ELISA of human prekallikrein and kininogen (Affinity Biologicals Inc., ON, Canada), respectively, following the manufacturer's instructions. The antibodies used in the HMWK EIA were polyclonal and are directed primarily to the light-chain of HMWK.

Statistical analysis

Results of the biochemical analysis of patients with the described *KNG1* variant were compared to a control group. For each parameter, a t-test was performed assuming unequal variances using STATA V14 (StataCorp, 2015). Significance was set at $\alpha \leq .05$.

RESULTS

We used 3 publicly available control samples: ExAC (<http://exac.broadinstitute.org>), 1000 Genomes (<http://www.internationalgenome.org>), and gnomAD (gnomad.broadinstitute.org) with more than 60,706, 2,504, and 15,708 individuals, respectively (accessed on February 19, 2019). The variant described here is absent

in these data sets. Moreover, in a fourth set of controls (105 healthy individuals from the area of Mainz) we could show that the variant was absent as well. Further evidence supporting the causality of the extremely rare variant in the *KNG1* gene was its distribution in the described family.

Haplotype analysis

The results of the haplotype analysis showed the SNP variants linked with the mutant *KNG1* allele. Results of patients II:10 and IV:10 are presented in the family's pedigree (Figure 2). Five of the 6 markers chosen for the analysis are very closely linked to the here reported *KNG1* mutation so that a crossing over can nearly be excluded. In patient II:10 the analysis showed that 5 of the 6 SNPs are equal in both alleles. The variant rs2304456 could not be ascribed initially. The haplotype constellation in the second patient (IV:10), however, showed that the SNP rs2304456 was 0/0 and revealed that in patient II:10 the SNP rs2304456 is transmitted with allele 0 (and not with 1). This confirms that the mutant allele is identical in both patients as could be expected.

Biochemical parameters of patients and controls

Mutations in the *KNG1* gene were reported to be associated with an HMWK deficiency. To rule out a pathogenic influence of the new *KNG1* variant on the HMWK level, we determined the HMWK and prekallikrein activities and antigen levels of all patients with the new *KNG1* variant outside of an attack and compared the data with a control group. The parameters' results were in the normal range and demonstrated no statistically significant differences between patients and controls (Table E1). The clotting activity [%] to antigen [%] ratios were 1.3 for HMWK in patients; 1.4 for HMWK in healthy persons; 1.2 for PK in patients; and 1.3 for PK in healthy persons. Hence, the new *KNG1* variant did not reduce the HMWK activity and did not cause HMWK deficiency. APTT was not prolonged (Table E2).

Table E2 shows that the mean C1-INH activity and concentration, C4 concentration, APTT, FXI clotting activity, FXII clotting activity, plasminogen activity, TAT for 6 patients and controls were within the reference range (RR) with the exception of patients' TAT. The reason for the elevated mean TAT-value in the patient group was a single elevated value.

Clinical data, estrogen impact and treatment

Age of onset of clinical symptoms and distribution of swelling sites in patients from the family with the new *KNG1* variant are summarized in Table E3. None of the tongue swellings required intubation, cricothyrotomy or ICU treatment. Laryngeal attacks and an erythema marginatum were not reported by any of the patients. Factors provoking swellings included pressure in two patients and stress in one patient. One patient had clinical symptoms solely during the period of oral contraceptives (OC) intake and in each of two pregnancies. In another patient, the intake of OC increased the number of swellings. No influence of OC was reported by three other women. Furthermore, hormonal replacement therapy by two women and a total of 12 pregnancies in 5 women reportedly had no influence on the clinical symptoms. Three patients had an arterial hypertension. One patient had three episodes of venous thromboses, after her 5th pregnancy. FXI and TAT were within RR.

Five patients received corticosteroids for a total of 6 lip/facial swellings and two tongue swellings and two patients received antihistamines for three facial attacks. All those treatments were ineffective. One patient responded to C1-INH concentrate, 1000 IU for two facial attacks, with symptom relief after 30 minutes.

Table E1. HMWK and prekallikrein (PK) in citrated plasma of 6 patients with hereditary angioedema (HAE) with p.Met379Lys variant during attack-free intervals and 6 controls

Pat. Nr	HMWK clotting activity [%]	HMWK antigen [%]	PK clotting activity [%]	PK antigen [%]
1	76.4	58.6	77.1	67.2
2	97.5	61.9	77.8	60.8
3	61.9	47.8	64.0	59.0
4	119.4	98.3	106.9	80.7
5	99.7	86.7	118.3	92.6
6	100.2	75.4	136.5	111.5
Mean	92.5	71.5	96.8	78.6
SD	20.3	18.9	28.2	20.6
controls				
Mean	96.8	63.8	110.5	88.3
SD	8.9	11.4	12.9	17.6
p	0.64	0.41	0.30	0.40
RR	70-130	53-153	50-150	42-133

RR; reference range

Table E2. Biochemical parameters of the kallikrein-kinin (contact) system and related systems in patients with HAE-KNG1 and controls

Pat. Nr	C1-INH activity [%]	C1-INH protein [mg/dl]	C4 [mg/dl]	APTT [sec]	INR	FXI clotting activity [%]	FXII clotting activity [%]	Plasminogen activity [%]	TAT [µg/l]
1	127	26.3	28.8	28.3	1	89	123	100	2.5
2	125	32.5	25.2	26.9	1	121	139	97	4.4
3	87	17.4	18.5	28.3	1	102	122	83	4.0
4	109	27.5	22.9	27.9	0.9	121	149	112	2.6
5	125	21.7	18.2	28.6	1.1	126	124	91	9.8
6	74	16.5	16.4	26.6	1	109	150	123	3.8
Mean	107.8	23.7	21.7	27.8	1.0	111.3	134.5	101.0	4.5
SD	22.5	6.2	4.8	0.8	0.1	14.1	13.2	14.5	2.7
controls									
Mean	105.5	20.1	23.4	30.7	1.0	111.5	108.8	95.0	3.2
SD	20.9	3.9	3.6	2.8	0.1	21.7	21.4	7.9	1.1
p	0.86	0.27	0.50	0.0518	0.57	0.99	0.04	0.40	0.31
RR	70-130	15.4-33.8	16.4-31.8	25-37	0.8-1.2	65-150	50-150	80-130	<4.1

RR; reference range

Table E3. Age of onset of clinical symptoms and distribution of swelling sites in 6 patients with HAE-KNG1

Pat. Nr	Patients marked in Figure 2 as:	Age at present [years]	Age at onset of symptoms [years]	Age at last symptoms	Number of symptomatic years	Number of lip/facial swellings	Number of hand swellings	Number of feet swellings	Number of tongue swellings	Number of abdominal attacks
1	II:10	81	55	64	3	3	0	0	0	0
2	II:12	79	37	69	32	35	0	0	0	0
3	III:10	59	51	59	8	1	28	3	9	0
4	III:14	55	17	48	31	2	0	3	0	51
5	III:17	57	33	39	2	0	0	0	0	2
6	IV:10	34	17	23	6	4	0	0	0	0

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