LUANA SELLA MOTTA MAIA

**Correlações genótipo/fenótipo em pacientes brasileiros com angioedema
hereditário por deficiência do inibidor de C1**

Tese apresentada à Faculdade de Medicina de
Ribeirão Preto da Universidade de São Paulo para
obtenção do título de Doutor em Ciências Médicas
Área de concentração: Clínica Médica –
Investigação Biomédica

Orientadora: Prof. Dra. Luisa Karla de Paula Arruda
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FOLHA DE APROVAÇÃO

Luana Sella Motta Maia

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*“A verdadeira coragem é ir atrás de seu sonho
mesmo quando todos dizem que ele é impossível.”*

Cora Coralina

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RESUMO

MAIA, L.S.M. **Correlações genótipo/fenótipo em pacientes brasileiros com angioedema hereditário por deficiência do inibidor de C1** 2019. 138f. Tese (Doutorado)- Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2019.

Resumo: O angioedema hereditário devido à deficiência de inibidor de C1 (AEH-C1-INH) é caracterizado por episódios recorrentes de angioedema do tecido subcutâneo e do trato gastrointestinal e respiratório. Nosso objetivo foi identificar mutações no gene *SERPING1*, que codifica o C1-INH, e investigar o impacto do tipo de mutação nas características clínicas de pacientes com AEH-C1-INH. Além disso, investigamos a ancestralidade em pacientes com AEH com C1-INH normal com mutações no gene que codifica o fator XII da coagulação, designado como AEH-FXII. **Métodos:** A análise de *SERPING1* foi realizada em 205 indivíduos de 16 famílias de pacientes-índice com AEH-C1-INH. Os dados clínicos foram coletados durante entrevistas médicas. DNA genômico foi isolado do sangue total e PCR foi realizada com *primers* específicos. A análise funcional prevista das mutações foi realizada usando as ferramentas de bioinformática SIFT, PolyPhen2 e MutationTaster. A gravidade da doença foi avaliada por dois escores independentes. Os pacientes foram classificados como portadores de deleções, inserção, duplicação, mutação *nonssense* e mutações que afetam Arg466 no centro reativo (grupo 1, n = 48); e mutações *missense*, exceto as de Arg466 (grupo 2, n = 12). Análise de ancestralidade da mutação dominante encontrada em pacientes com AEH-FXII, c.983C>A (p.Thr328Lys), foi realizada em 6 pacientes com AEH-FXII e seus pais (sendo um portador da mutação) avaliando hapótipos previamente descritos em pacientes europeus. **Resultados:** Sessenta pacientes (33F: 27M), de 10 meses a 63 anos de idade, apresentaram mutações em *SERPING1*. Cinquenta e oito apresentaram AEH-1 e dois tinham AEH-2. As mutações de *SERPING1* incluíram 10 mutações *missense* e 6 não-*missense* (mutações estruturais que levam a *frameshifts* ou mutações *nonssense*), seis das quais eram novas. Não foram observadas diferenças significantes entre os pacientes dos grupos 1 e 2 em relação à idade, sexo, sensibilidade ao estrogênio, idade de início dos sintomas, locais de apresentação do angioedema, necessidade de profilaxia a longo prazo e escores de gravidade clínica. **Conclusões:** Os presentes resultados mostraram que uma variedade de mutações leva a manifestações clínicas heterogêneas de AEH-C1-INH e que a gravidade da doença pode não estar estritamente relacionada ao tipo de mutação. Foi encontrado nos pacientes com AEH-FXII o mesmo padrão de hapótipos de pacientes europeus, sugerindo que esta mutação tenha o mesmo fundador comum europeu.

Palavras-chave: Angioedema hereditário, Inibidor de C1, Bradicina, *SERPING1*.

ABSTRACT

MAIA, L.S.M. **Genotype-phenotype correlations in Brazilian patients with hereditary angioedema due to C1 inhibitor deficiency** 2019. 138p. Thesis (Doctorate)- Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, 2019.

Background Hereditary angioedema due to C1 inhibitor deficiency (C1-INH-HAE) is characterized by recurrent episodes of angioedema of the subcutaneous tissue and the gastrointestinal and respiratory tract. Our objective was to identify mutations in the *SERPING1* gene, which codes for C1-INH, and to investigate the impact of the mutation type on the clinical characteristics of patients with A C1-INH-HAE. In addition, we investigated the ancestry in patients with normal C1-INH-HAE with mutations in the gene encoding coagulation factor XII, designated as AEH-FXII. **Methods:** *SERPING1* analysis was performed on 205 individuals from 16 families of C1-INH-HAE index patients. Clinical data were collected during medical interviews. Genomic DNA was isolated from whole blood and PCR was performed with specific primers. Predicted functional analysis of mutations was performed using the bioinformatics tools SIFT, PolyPhen2 and MutationTaster. Disease severity was assessed by two independent scores. Patients were classified as having deletions, insertion, duplication, nonsense mutation and mutations affecting Arg466 in the reactive center (group 1, n = 48); and missense mutations, except those of Arg466 (group 2, n = 12). Ancestry analysis of the dominant mutation found in patients with AEH-FXII, c.983C> A (p.Thr328Lys), was performed on 6 patients with AEH-FXII and their parents (being a carrier of the mutation) evaluating previously described multiple subjects Europeans. **Results:** Sixty patients (33F: 27M), aged 10 months to 63 years, presented mutations in *SERPING1*. Fifty-eight had HAE-1 and two had HAE-2. *SERPING1* mutations included 10 missense and 6 non-missense mutations (structural mutations leading to frameshifts or nonsense mutations), six of which were new. No significant differences were observed between patients in groups 1 and 2 regarding age, gender, estrogen sensitivity, age at onset of symptoms, angioedema presentation sites, need for long-term prophylaxis, and clinical severity scores. **Conclusions:** The present results showed that a variety of mutations lead to heterogeneous clinical manifestations of C1-INH-HAE and that the severity of the disease may not be closely related to the type of mutation. AEH-FXII patients were found to have the same pattern of Europeans' multiple subjects, suggesting that this mutation has the same common European founder.

Keywords: Hereditary angioedema, C1 inhibitor, Bradykinin, *SERPING1*.

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Introdução

1 INTRODUÇÃO

Angioedema hereditário (AEH) é uma doença rara, com herança autossômica dominante, que afeta 1:50.000 indivíduos (MAURER et al., 2018). Pacientes com AEH apresentam episódios recorrentes de edema subcutâneo e de submucosa, afetando principalmente a pele, o trato gastrointestinal e as vias aéreas superiores. Angioedema de vias aéreas superiores pode levar a asfíxia, com risco à vida (MAURER et al., 2018c). Na maioria dos casos, a doença resulta da deficiência do inibidor do C1 (AEH-C1-INH), causada por mutações em *SERPING1*, o gene que codifica a proteína C1-INH (MAURER et al., 2018c). A diminuição da atividade de C1-INH pode causar impacto em diversas vias metabólicas; contudo, há evidências substanciais que a elevação na bradicinina tem papel central na patogênese do edema tecidual em pacientes com AEH-C1-INH, como resultado da ativação incontrollada do sistema cinina-caliceína (KAPLAN; JOSEPH, 2017). Nos últimos anos, AEH com inibidor de C1 normal também foi identificado, associado a mutações nos genes que codificam o fator XII da coagulação (*F12*), angiopoietina-1 (*ANGPT1*), plasminogênio (*PLG*), e cininogênio 1 (*KNG1*), e acredita-se que outras mutações poderão vir a ser descobertas no futuro (DEWALD; BORK, 2006; BAFUNNO et al., 2018; BORK et al., 2018, 2019; ZURAW, 2018).

Figura 1 - Regulação pelo inibidor de C1. O inibidor de C1 atua na ativação do fator XII (via da coagulação), na pré-caliceína e caliceínas e consequente liberação da bradicinina pelo cininogênio de alto peso molecular (via das cininas). Adaptado de Kaplan e Joseph Immunol Allergy Clin N Am 2017.

As manifestações clínicas podem surgir em qualquer idade, porém geralmente iniciam na infância, em torno dos 10 a 12 anos de idade. Os sintomas incluem edema subcutâneo, que pode se localizar em face, lábios, pescoço, abdome, genitais e membros superiores e inferiores; edema de vias aéreas superiores, podendo levar a óbito por asfixia (BORK et al., 2013); e episódios recorrentes de dor abdominal, que pode ser intensa e acompanhada de náuseas, vômitos e diarreia, podendo simular um quadro de abdome agudo levando a cirurgias desnecessárias (BORK; FISCHER; DEWALD, 2003; CICARDI et al., 2014; GIAVINA-BIANCHI et al., 2017). Fatores desencadeantes das crises podem ser físicos como trauma ou pressão; stress emocional; exposição a hormônios estrogênicos pelo uso de contraceptivo oral ou terapia de reposição hormonal contendo estrógeno, ou gravidez; medicamentos, particularmente os inibidores da enzima conversora da angiotensina, iECA; ou infecções (CICARDI et al., 2014; FERRARO et al., 2014; GIAVINA-BIANCHI et al., 2017b).

O diagnóstico de AEH-C1-INH é baseado em sintomas clínicos sugestivos, e pode ser dificultado pela existência de angioedema por outras causas, como o angioedema alérgico mediado por IgE ou outros mecanismos de ativação de mastócitos; angioedema adquirido, associado a uso de iECA e outras drogas que atuam no metabolismo da bradicinina; o angioedema autoimune e o idiopático (FARKAS et al., 2016), contudo a história familiar positiva, juntamente com baixos níveis quantitativos e qualitativos de C1-INH e baixo nível de C4 (MAURER et al., 2018b) podem auxiliar no diagnóstico. Nível baixo de C1q pode ser útil para diferenciar o angioedema adquirido (AAE-C1-INH) do AEH-C1-INH (BOVA et al., 2018; MAURER et al., 2018c). Episódios de dor abdominal podem também ser comuns a várias outras doenças. Entretanto, a história familiar pode estar ausente em até 25% dos pacientes com AEH-C1-INH porque a doença pode ser causada por mutações *de novo* (BOVA et al., 2018; MAURER et al., 2018c). No AEH-C1-INH, níveis quantitativos e qualitativos de C1-INH estão em geral abaixo de 50% dos valores de referência. Entretanto, valores limítrofe ou valores inconsistentes podem ser encontrados devido a problemas técnicos (WAGENAAR-BOS et al., 2008; LI et al., 2014). Níveis baixos de C4 tem sido propostos como triagem, entretanto podem estar normais, especialmente se não forem medidos durante a crise de AEH. Níveis de C1q estão baixos em 70% dos pacientes com AAE-C1-INH, e normais entre pacientes com AEH-C1-INH (BOVA et al., 2018). A análise genética é recomendada para elucidar casos inconclusivos (BOVA et al., 2018; MAURER et al., 2018c).

Figura 2 - Classificação do Angioedema hereditário. Modificado de Cicardi et al. Allergy 2014; Bork et al. Allergy 2017, 2019; Bafuno et al. J Allergy Clin Immunol 2017; Giavina-Bianchi et al. Arq Asma Alerg Imunol 2017.

1.1 Aspectos genéticos do angioedema hereditário por deficiência do inibidor de C1

O inibidor de C1 (C1-INH) é responsável pela regulação de várias vias metabólicas, incluindo ativação do sistema complemento (C1r, C1s, *mannose-binding lectin-associated serine protease* MASP 1 e MASP 2), via da coagulação (plasmina e plasminogênio tecidual) (KAPLAN; JOSEPH, 2016) e via da produção de cininas ou via de contato (calicreína plasmática e Fator XIIa). No AEH- C1-INH, a redução da concentração e/ou da atividade funcional desta proteína resulta na ativação excessiva da via cininogênio-cinina, levando a produção aumentada de bradicinina com vasodilatação e aumento da permeabilidade vascular, sendo o principal mecanismo de desenvolvimento do angioedema. Foram encontradas mais de 500 diferentes mutações no gene *SERPING1*, que codifica a proteína C1-INH, em pacientes com AEH (KALMÁR et al., 2005). Mutações *de novo* podem ser encontradas no gene *SERPING1* em até 25% dos pacientes com AEH, portanto a ausência de história familiar não exclui o diagnóstico de AEH (FARKAS et al., 2016; MAURER et al., 2018b)(GERMENIS; SPELETAS, 2016).

O gene *SERPING1* compõe-se de 17 kb e está localizado no cromossomo 11, consistindo de oito exons, dos quais sete codificam o inibidor de C1 (NM_000062.2). As

regiões intrônicas do gene *SERPING1* contem uma alta concentração de Alu-elementos repetitivos, tornando o gene susceptível a deleções e duplicações (BOCK et al., 1986).

No AEH por deficiência quantitativa do C1-INH (tipo 1), a concentração da proteína produzida é baixa devido à expressão deficiente do gene, a atividade funcional da proteína é diminuída, e a concentração de C4 é baixa na maior parte dos pacientes; ocorre em cerca de 85% dos casos descritos. Já no AEH por deficiência qualitativa do C1-INH (tipo 2), a concentração de C1-INH é normal, porém sua atividade funcional é baixa, e a concentração de C4 é baixa na maior parte dos pacientes; está presente em 15% dos casos (AGOSTONI et al., 2004). Mutações do gene *SERPING1* em pacientes com AEH do tipo 1 ocorrem por todo o gene e resultam em proteínas truncadas que não são eficientemente secretadas, levando a níveis antigênicos e funcionais do C1-INH diminuídos, enquanto que mutações em pacientes com AEH do tipo 2 normalmente envolvem o exon 8, e causam alterações no sítio ativo da molécula ou perto dele, resultando na secreção de uma proteína mutante que é disfuncional (MAURER et al., 2018b). Níveis de C4, e dosagem quantitativa e qualitativa (funcional) do C1-INH são utilizados para o diagnóstico de C1-INH-AEH (FARKAS et al., 2016). No AEH os níveis de C1q estão normais, enquanto que no angioedema por deficiência adquirida do C1-INH, estes níveis estão diminuídos (FARKAS et al., 2016; MAURER et al., 2018b). Nosso grupo é um dos pioneiros no estudo genético de pacientes com AEH em nosso meio, e descreveu em 2011 pela primeira vez uma nova mutação, a deleção c.351delC, em uma família de pacientes com AEH-C1-INH da cidade de Carmo do Rio Claro, MG, sendo uma das famílias com maior número de pacientes já relatada (FERRARO et al., 2011).

Embora existam algoritmos bem estabelecidos para a investigação de pacientes com suspeita clínica de AEH (GIAVINA-BIANCHI et al., 2017a), há retardo significativo no diagnóstico da doença. O tempo desde o início dos sintomas até o diagnóstico do AEH é em média de 13 a 15 anos, no Brasil e em outros países do mundo (ZURAW; CHRISTIANSEN, 2010; FERRARO et al., 2011). Razões para atraso diagnóstico no AEH incluem: limitações no conhecimento da doença por médicos, outros profissionais de saúde e população; sintomas comuns a outras doenças, dificultando o diagnóstico diferencial; disponibilidade limitada de exames para confirmação diagnóstica; limitações técnicas na realização de exames do sistema complemento, incluindo labilidade dos componentes e instabilidade na temperatura de acondicionamento e transporte do material para laboratórios de referência, gerando inconsistência nos resultados.

1.2 Angioedema hereditário com inibidor de C1 normal

Mais recentemente foram descritas famílias de pacientes com AEH que apresentavam concentração e atividade funcional de C1-INH normais. Este tipo de angioedema, previamente designado como AEH tipo III, é atualmente designado como AEH com C1-INH normal (CICARDI et al., 2014). Mutações no gene que codifica o fator XII da coagulação foram identificadas inicialmente em subgrupos de pacientes com AEH com C1-INH normal (AEH-FXII) (DEWALD; BORK, 2006). Em seguida mutações em outros genes associadas a AEH com C1-INH normal foram descritas. Entretanto, ainda existem pacientes com AEH com C1-INH normal e história familiar de AEH, nos quais variantes genéticas não foram identificadas, como causa da doença; estes pacientes são designados como pacientes com AEH de causa desconhecida (*unknown*, AEH-U)(MAURER et al., 2018c).

1.2.1 Angioedema hereditário por mutação no gene F12

Em meados dos anos 2000, foi descrito pela primeira vez, o AEH com inibidor de C1 normal, com concentração e atividade funcional de C1-INH, e os níveis de C4 normais associado a mutações no gene *F12* que codifica o Fator XII da coagulação (FXII)(BORK, 2006; CICHON et al., 2006).

Em estudos em diferentes partes do mundo, a mutação c.983C>A (p.Thr328Lys) foi identificada como dominante, estando presente em 519/551 (94,2%) em pacientes pertencentes 155/167 (92,8%) famílias. (DIAS et al., 2018). Mutação *missense* na mesma localização, c.983C>G (p.Thr328Arg), foi encontrada em número bem menor de famílias. (DIAS et al., 2018). Deleção de 72 pares de bases c.971_1018 + 24del 72, foi relatada em uma família de origem turca e em uma família brasileira (BORK et al., 2011; VERONEZ et al., 2018). Duplicação de 18 pares de bases c.890-909dup (p.298_303dup) foi descrita em um paciente caucasiano (KISS et al., 2013). Até o presente momento, as mutações associadas a AEH-FXII descritas estão localizadas no exon 9 do gene *F12*, que corresponde a uma região codificadora da proteína rica em prolina.

Em estudo conduzido por de Maat e cols. os autores desenvolveram variantes da proteína FXII mutadas, e compararam a FXII normal. As proteínas com mutações patogênicas Thr328Lys e Thr328Arg, e com a deleção c.971_1018+24del48, que alteram a glicosilação; bem como as proteínas com as mutações não patogênicas Thr328Ala e c.971_del48, que não alteram a glicosilação, foram produzidas como proteínas recombinantes. Os resultados

demonstraram que as proteínas FXII com mutações patogênicas que alteram a glicosilação tiveram atividade enzimática aumentada, nível mais elevado de FXII pós-clivagem, resposta maior à quebra por plasmina, maior produção de FXIIa e consumo mais elevado de cininogênio de alto peso molecular. Já as proteínas FXII normal e com mutações não patogênicas e que não alteram glicosilação tiveram valores diminuídos destes parâmetros. As mutações patogênicas levaram ao aumento da sensibilidade do FXII à ativação por plasmina, superando a modulação pelo C1-INH, e culminando em elevada produção de bradicinina. De forma interessante, análogos de lisina como o ácido tranexâmico, atenuaram a ativação de FXII e regularam a liberação de bradicinina (DE MAAT et al., 2015). Portanto, ficou demonstrado que a plasmina é um gatilho natural para edema em pacientes com AEH-FXII. Mutações causadoras de AEH-FXII introduzem novos sítios de glicosilação em FXII, tornando a molécula mais susceptível a clivagem enzimática por plasmina, assim resultando em rápida ativação de FXII, com escape da inibição por C1-INH. Esses achados estão de acordo com a observação clínica de que os análogos de lisina, incluindo o ácido tranexâmico, tem eficácia terapêutica particularmente em pacientes com AEH-FXII, por atenuarem o mecanismo descrito acima (DE MAAT et al., 2015).

1.2.2 Angioedema hereditário por mutações nos genes *PLG*, *ANGPT1*, e *KNG1*

A identificação de mutações nos genes *PLG*, *ANGPT1* e *KNG1* associadas a AEH é recente, e o mecanismo pelo qual estas mutações levam a AEH ainda não está completamente elucidado.

A mutação descrita em *PLG* está localizada no domínio *kringle 3*. Os domínios *kringle* facilitam a ligação de plasminogênio a grandes superfícies e têm influência na estrutura e função da proteína. O plasminogênio é um precursor enzimático inativo que é convertido em plasmina por clivagem de ligação peptídica. A plasmina é a principal enzima da fibrinólise. Bork et al. 2018 investigaram a atividade de PLG, determinada por ensaio cromogênico através de reação em excesso de estreptoquinase, na presença de fibrinogênio. A atividade de PLG foi normal em 3 pacientes sintomáticos com AEH-PLG, indicando que a mutação no gene *PLG* não parece ter influência sobre a atividade de PLG no plasma sanguíneo. Entretanto, os autores sugerem que a mutação em *PLG* poderia levar a uma mudança estrutural do plasminogênio, induzindo a aumento da ativação do sistema fibrinolítico com posterior formação de plasmina, ativação do sistema calicreína-cinina e aumento da bradicinina (BORK et al., 2018).

Bafunno e cols. 2018 propõem que a mutação descrita em *ANGPT1* altere a permeabilidade endotelial gerando angioedema mediado por bradicinina, pois a angiopoietina 1 tem função de reduzir a ação vasodilatadora induzida por bradicinina, manter a barreira endotelial e regular a integridade vascular. A proteína mutada é produzida com perda de função que afeta a capacidade de formar multímeros, reduzindo sua habilidade a ligar-se ao receptor TIE2 (BAFUNNO et al., 2018).

Em 2019, Bork e cols. descreveram uma nova variante c.1136T> A no exon 10 do gene do cininogênio 1 (*KNIG1*) associada a AEH com C1-INH normal. Esta variante no gene *KNIG1*, p.Met379Lys, está presente em ambas as isoproteínas, de cininogênio de alto peso molecular (HMWK) e de cininogênio baixo peso molecular (LMWK). Considerando que a variante *KNIG1* possa mudar o local de clivagem do N-terminal da bradicinina de HMWK e LMWK, isso poderia levar a uma bradicinina funcionalmente ativa, porém irregular no AEH-*KNIG1*, que poderia alterar o processo de inativação por enzimas como ECA, aminopeptidase 2, entre outras, possivelmente resultando em um tempo de meia-vida prolongado e, portanto, em uma atividade mais alta que o normal da bradicinina mutante (BORK, K. , WULFF, K. , ROSSMANN, H. , STEINMÜLLER-MAGIN, L. , BRÆNNE, I. , WITZKE, G. AND HARDT, 2019).

1.3 Correlação genótipo-fenótipo no angioedema hereditário e ancestralidade

Estudos prévios sugerem que o tipo de mutação no gene *SERPING1* poderia influenciar a apresentação clínica e gravidade da doença entre os pacientes com AEH-C1-INH. Foi descrito que pacientes com mutações *missense* apresentaram sintomas mais tardiamente, cursaram doença clinicamente menos grave, e tiveram menor necessidade de medicação profilática quando comparados a pacientes com outros tipos de mutações que causam mudanças mais profundas na molécula (BORS et al., 2013; SPELETAS et al., 2014; ANDREJEVIĆ et al., 2015; GRIVČEVA-PANOVSKA et al., 2018). Assim sendo, para valor diagnóstico, a caracterização do tipo de mutação do gene *SERPING1* poderia ser útil na predição clínica dos fenótipos da doença.

O AEH-C1-INH ocorre devido a mutações *de novo* em 25% dos pacientes, sendo que deleções ou inserções, e rearranjos genômicos são encontrados em até 15% dos casos. Esses fenômenos são atribuídos à presença de elementos Alu distribuídos pelo locus. Elementos Alu são sequências repetitivas de aproximadamente 300 pares de bases, que representam os elementos transponíveis mais abundantes no genoma humano. Devido à sua orientação em

tandem e com natureza repetitiva, promovem eventos de recombinação não homólogos que resultam em rearranjos genômicos (STOPPA-LYONNET et al., 1987).

No AEH-FXII, a mutação prevalente c.983C>A (p.Thr328Lys) ocorre no domínio rico em prolina. Em 2006, Cichon e colaboradores analisaram se havia um fundador em comum nas amostras europeias do sudeste europeu (Alemanha e França) ou se a mutação era proveniente de um evento mutante aleatório. Esta variante foi originada na população europeia por volta do século XI e é frequentemente associada ao AEH com C1-INH normal no sul da Europa (CICHON et al., 2006). Nagy e cols. também encontraram esse mesmo haplótipo em uma família inglesa de origem italiana (NAGY et al., 2009). A investigação da ancestralidade em pacientes com AEH não foi relatada até o momento em pacientes brasileiros.

No presente estudo, identificamos e caracterizamos mutações no gene *SERPING1* em pacientes brasileiros com AEH-C1-INH, por deficiência quantitativa (AEH-1) ou qualitativa (AEH-2) de C1-INH. As características clínicas dos pacientes AEH-C1-INH com mutação *missense* foram comparadas com aquelas presentes em pacientes que tinham outros tipos de mutação (deleção, duplicação, inserção), incluindo mutações que afetam a região Arg466 que é o centro reativo da proteína. A ancestralidade foi investigada em famílias de pacientes brasileiros com AEH- FXII, com análise de haplótipos encontrados em populações europeias.

Hipótesis

2 HIPÓTESES

Alterações genéticas específicas no gene *SERPING1* poderiam influenciar nos fenótipos clínicos de angioedema hereditário entre pacientes brasileiros com a doença.

Pacientes brasileiros com angioedema hereditário com mutação no Fator XII da coagulação teriam um fundador comum europeu, devido à grande imigração europeia para o Brasil a partir do século XVI.

Objetivos

3 OBJETIVOS

3.1 Objetivo geral

Analisar os aspectos clínicos de pacientes brasileiros com angioedema hereditário por deficiência do inibidor de C1 em relação ao tipo de mutação encontrada e investigar a ancestralidade dos pacientes com angioedema hereditário com inibidor de C1 normal com mutação no gene *F12*.

3.2 Objetivos específicos

- Identificar e caracterizar mutações no gene *SERPING1* em pacientes com AEH tipos 1 e 2 e seus familiares.
- Comparar a gravidade dos sintomas (idade do início dos sintomas, localização do edema, sensibilidade ao estrógeno, necessidade de profilaxia de longo prazo) em relação ao tipo de mutação encontrada.
- Investigar a ancestralidade dos pacientes com angioedema hereditário com inibidor de C1 normal com mutação no gene *F12* (AEH-FXII).

Sujeitos e Métodos

4 SUJEITOS E MÉTODOS

4.1 Sujeitos

Sessenta pacientes diagnosticados com AEH-C1-INH, pertencentes a dezesseis famílias distintas, foram investigados para a presença e caracterização de mutações no gene *SERPING1*. Cinquenta e cinco dos pacientes relatavam episódios recorrentes de angioedema e/ou dor abdominal, e cinco eram assintomáticos.

Os pacientes foram selecionados dentre aqueles atendidos no Serviço de Alergia e Imunologia do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto (HC-FMRP-USP) ou tiveram amostras enviadas para nossa Instituição para análise genética por seus médicos, ou através de colaboração com a Associação Nacional de Pacientes com Angioedema Hereditário (ABRANGHE). O diagnóstico de AEH-C1-INH foi estabelecido de acordo com critérios de consenso internacional, que incluem dosagem do inibidor de C1 quantitativo e qualitativo (funcional) e de C4 e/ou presença de mutação no gene *SERPING1* (MAURER et al., 2018).

Para estudo de ancestralidade, foram selecionadas 6 famílias de pacientes diagnosticados com AEH-FXII, atendidas no Serviço de Alergia e Imunologia do HC-FMRP-USP, que participaram de estudo prévio do nosso grupo (VERONEZ et al., 2017). Trios foram constituídos com o paciente índice e seus pais.

Heredogramas foram construídos a partir de informações sobre pelo menos um familiar além do caso índice, utilizando o programa Genopro 2016. Previamente, nosso grupo descreveu uma das famílias incluídas no presente estudo (Família 7), sendo uma das maiores famílias descritas na literatura de pacientes com AEH por deficiência quantitativa de C1-INH (AEH-1) (FERRARO et al., 2011b). No presente estudo, adicionamos informações do acompanhamento clínico dos pacientes desta família, incluindo gravidade e duração da doença. Cinco pacientes adicionais foram diagnosticados com AEH-1, totalizando 33 pacientes nesta família.

Para avaliar a gravidade da doença e suas características clínicas, os sessenta pacientes foram divididos em dois grupos, de acordo com Andrejevic et al. and Grivčeva-Panovska et al. (ANDREJEVIĆ et al., 2015; GRIVČEVA-PANOVSKA et al., 2018). O Grupo 1 compreendeu pacientes que apresentaram deleções, inserções, duplicações, mutações *nonsense* e mutações afetando a região enzimática da proteína Arg466, no exon 8 (n=48). O Grupo 2 incluiu pacientes com mutações *missense*, com exceção daquelas em Arg466 (n=12). As características clínicas analisadas foram idade atual, gênero, idade do início dos sintomas, idade do diagnóstico (apenas para a Família 7), sensibilidade ao estrógeno, uso de profilaxia e relatos de crises de edema subcutâneo, edema de face, dor abdominal, e edema de vias aéreas superiores.

O estudo foi aprovado pelo Comitê de Ética em Pesquisa do HC-FMRP-USP (protocolos HCRP 14000521/2012 e HCRP 15036/2015) e todos os pacientes ou seus responsáveis legais assinaram o Termo de Consentimento Livre e Esclarecido para participação no estudo.

4.2 Quantificação dos níveis de C1-INH e C4

Os níveis de C1-INH e C4 foram medidos por nefelometria (Dade Behring, Newark, Del., USA), e a atividade de C1-INH foi determinada usando método cromogênico (Technoclone GmbH, Vienna, Austria).

4.3 Amplificação dos exons do gene SERPING1 por Polymerase Chain Reaction (PCR)

DNA genômico foi extraído a partir de sangue periférico total usando o kit de purificação DNA Wizard Genomic (Promega, Madison, WI, USA), e para as crianças abaixo de 2 anos, o DNA foi extraído a partir de material de mucosa oral obtido com *swab* (Jiangsu Rogue Technology, China). PCR foi realizada utilizando *primers* específicos para o gene *SERPING1*, para amplificar os 8 exons e regiões intrônicas adjacentes. As sequências dos *primers* utilizados estão descritas na Tabela 1.

Tabela 1 - *Primers* utilizados para amplificação dos 8 exons do gene *SERPING1*

Exon	<i>Primers</i>	Sequência (5' – 3')	Tamanho pb
1	<i>Forward</i>	TTCCCCGTTACCCCCACCTA	415
	<i>Reverse</i>	TAGGAGGAGGCAGAAGTCCCAGGT	
2	<i>Forward</i>	GGTGGGAGCTGGCTCCGAGCGT	175
	<i>Reverse</i>	CCGCACCCCGCACCATCCCG	
3	<i>Forward</i>	CACACCTTCTCTTCCTGCTTTGAG	652
	<i>Reverse</i>	GTCTGGATTGGTGACTCTTATGGG	
4	<i>Forward</i>	CCCGACTCATCC TGC AAG TAT C	329
	<i>Reverse</i>	CTGTAGCTCCAACATTCCCTCTGT	
5	<i>Forward</i>	TTGCCGAGCCATAGTCACATAGC	569
	<i>Reverse</i>	GCAGGGACATTGAGATCCGATTG	
6	<i>Forward</i>	AAGGCTTTTAGCTCCTCTTCATCC	302
	<i>Reverse</i>	AGCATGTACCCCAAATGATGG	
7	<i>Forward</i>	GAGTTGGATCAGCACTGTTCCACC	557
	<i>Reverse</i>	AATAGCACCTACCCCTCTAACTTGC	
8	<i>Forward</i>	CAAGGGAAGAGGAAGAAGAGAGCACTG	428
	<i>Reverse</i>	TGAGAGCTGAGGCTGGAGAGGTAG	

Reações de PCR foram realizadas com volume final de 25 uL contendo: 1X tampão de PCR (2 mM Tris-HCl (pH 8,0), 4 mM NaCl, 0,2 mM Fosfato de Sódio, 0,01 mM EDTA, 0,1 mM DTT, estabilizadores, 50% (v/v) glicerol), 0,2 mM de cada de desoxinucleotídeo trifosfato (dNTP); 0,5 mM of MgCl₂; 1,0 U de DNA Polimerase (Invitrogen Platinum, Carlsbad, CA 92008 USA) e 100 ng de DNA genômico. As condições de ciclagem foram: 94°C por 8 min, seguido por 30 ciclos de 94°C por 30 segundos, 60°C por 30 segundos e 72°C por 2 minutos; e a extensão final sendo de 5 min a 72°C para todos os exons com exceção do exon 2, que requer uma temperatura de anelamento de 64°C por 30 segundos e 32 ciclos de amplificação. Os produtos de PCR foram analisados em gel de agarose 1%, purificados por ExoSAP-IT™ (Affymetrix, Cleveland, Ohio, USA), e sequenciados no ABI Prism 3100 Genetic Analyzer com Big Dye Terminator™ V3.1 Cycle Sequencing reagentes (Applied Biosystems, Foster City, CA, USA).

4.4 Análise das sequências de nucleotídeos e aminoácidos

As sequências de DNA foram analisadas usando o Seqman *software* (Lasergene; DNA Star, Inc., Madison, WIS), com a sequência do gene *SERPING1* obtida a partir do GenBank como referência. Variações na sequência foram numeradas e identificadas usando a sequência de cDNA do gene (GenBank número de acesso NM_000062.2, X54486), de acordo com diretrizes da *Human Genomic Variation Society* (HGVS), (DEN DUNNEN; ANTONARAKIS, 2000).

Com relação a análise de aminoácidos, a sequência da proteína madura, incluindo o peptídeo sinal (22 resíduos de aminoácidos N-terminais) foi utilizada. As bases de dados utilizadas foram HAEdb, UniProt, HGMD, e PubMed.

4.5 Predição da patogenicidade das mutações

Classificação das mutações em relação à patogenicidade foi realizada de acordo com os padrões e diretrizes do *American College of Medical Genetics and Genomics* (ACMG)(ACMG BOARD OF DIRECTORS, 2019).

Além disso, todas as mutações *missense* identificadas no estudo foram analisadas usando as ferramentas de bioinformática: SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html)(NG; HENIKOFF, 2003), PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>) (ADZHUBEI et al., 2010), e MutationTaster

(<http://www.mutationtaster.org/>) (SCHWARZ et al., 2010), que também avalia mutações *nonsense*. Para prever a patogenidade, SIFT classifica as mutações como *Damaging* ou *Tolerated*; Polyphen2 classifica as mutações como *Probably Damaging* ou *Possibly Damaging*; e MutationTaster como *Disease-Causing* ou *Polymorphism*.

4.6 Avaliação da gravidade do angioedema hereditário

Dois escores clínicos previamente publicados foram aplicados para classificar a gravidade da doença entre os pacientes com AEH-C1-INH (BYGUM et al., 2011; FERRARO et al., 2011b). Os parâmetros usados para calcular os escores de gravidade estão descritos nas Tabelas 2 e 3. De acordo com o escore proposto por Ferraro et al., a gravidade da doença é classificada baseada na frequência e intensidade dos sintomas, sendo considerada grave (≥ 7 pontos), moderada (5 ou 6 pontos), leve (≤ 4 pontos), e paciente assintomático (0 ponto), com máximo de 8 pontos (FERRARO et al., 2011b). No escore desenvolvido por Bygum et al., os seguintes parâmetros são usados: idade de início dos sintomas, manifestações clínicas, e necessidade de profilaxia em longo prazo, com valores variando de 0 a 10 (BYGUM et al., 2011).

Tabela 2 - *Score* de gravidade por Ferraro et al. 2011 (máximo 8 pontos).

Frequência	Pontos
Mais de um episódio por mês	3
Entre 6 e 12 episódios por ano	2
Menos de 6 episódios por ano	1
Sem sintomas de angioedema	0
Intensidade	Pontos
Desconforto, sem prejuízo das atividades diárias	2
Desconforto que afeta as atividades diárias	4
Crise grave com necessidade de hospitalização	5
Score	
Grave	≥ 7
Moderado	5 or 6
Leve	≤ 4
Assintomático	0

Tabela 3 - Score de gravidade por Bygum et al. 2011 (de 0 a 10 pontos).

Parâmetro	Pontos
Idade de início 0-5 anos	3 pontos
Idade de início 6-10 anos	2 pontos
Idade de início 11-20 anos	1 ponto
Idade de início > 20 anos	0 ponto
Edema subcutâneo	1 ponto
Edema abdominal doloroso	2 pontos
Edema laríngeo	2 pontos
Outras manifestações clínicas	1 ponto
Profilaxia a longo prazo	1 ponto

4.7 Análise da ancestralidade em famílias com angioedema hereditário e mutação no gene do Fator XII da coagulação AEH-FXII

Foram analisadas 6 diferentes famílias, com trios constituídos por pai, mãe e paciente afetado. A análise da ancestralidade foi realizada utilizando o DNA genômico e *primers* específicos, que foram desenhados a partir de sequências na região 5q35.2-q35.5 do cromossomo 5. Marcadores genéticos específicos pré-selecionados (CICHON et al., 2006) foram utilizados para desenhar os 10 haplótipos diferentes, incluindo a mutação patogênica c.983C>A (p.Thr328Lys) no gene *F12* (Tabelas 4 e 5).

Tabela 4 - Descrição dos haplótipos utilizados para análise da ancestralidade.

Haplótipos	Posição cromossômica	rs	Aminoácidos
HAP01	177410517	2731676	A
HAP02	177410503	2252316	T
HAP03	177410489	2252317	C
HAP04	177409531	1801020	C
HAP05	177406414	4976649	T
HAP06	177406305	4976692	T
HAP07	177406302	4976648	A
HAP08	177404231	118204456	A
HAP09	1774504118	17876031	C
HAP10	177403626	17876032	T

Tabela 5 - Descrição dos *primers* e seus respectivos haplótipos.

Haplótipos	<i>Primers</i>	Sequência (3' - 5')	Tamanho pb
HAP01/02/03	1		
	<i>Forward</i>	CACCACGCCCAGCTAATTTT	181
	<i>Reverse</i>	AATAAGGAGAATGGCAGGGC	
HAP04	2		
	<i>Forward</i>	CCACAGCACTCACCGAAAG	200
	<i>Reverse</i>	GCTTTCCACAAACAGCCTGT	
HAP05/06/07	3		
	<i>Forward</i>	ATCACGAGGTCAGGAGATCA	188
	<i>Reverse</i>	CAGGTTGTGGAGTCAGGAGT	
HAP08	4		
	<i>Forward</i>	CCACTTCCTAACCTCCCGG	237
	<i>Reverse</i>	GCTACAGGAACCCGGACAA	
HAP10	5		
	<i>Forward</i>	CACCACCGTCAGATCCTCG	190
	<i>Reverse</i>	AACCCGTGCCTACCTTCTC	
HAP10	6		
	<i>Forward</i>	CACCACCGTCAGATCCTCG	203
	<i>Reverse</i>	AACCCGTGCCTACCTTCTC	

As reações de PCR foram realizadas com volume final de 25 uL contendo: 10X tampão de PCR (KAPA Taq Buffers A e B), 10 M de desoxinucleotídeo trifosfato (dNTP); 25 mM of MgCl₂; 1,0 U de DNA Polimerase (KAPA Taq PCR Kit, Kapa Biosystems Manufacturing, R&D Cape Town, South Africa) e 25 ng de DNA genômico. As condições de ciclagem foram: 95°C por 5 min, seguido por 15 ciclos de 95°C por 30 segundos, 66°C por 20 segundos e 72°C por 20 segundos; seguido por 22 ciclos de 95°C por 30 segundos, 64°C por 20 segundos e 72°C por 30 segundos e extensão final de 7 min a 72°C para os *primers* 1 e 6. Para os *primers* 2 as condições foram: 95°C por 5 min, seguido por 15 ciclos de 95°C por 30 segundos, 60°C por 20 segundos e 72°C por 20 segundos; seguido por 22 ciclos de 95°C por 30 segundos, 58°C por 20 segundos e 72°C por 30 segundos; e extensão final de 7 min a 72°C. Para o *primer* 3, as condições foram: 95°C por 5 min, seguido por 15 ciclos de 95°C por 30 segundos, 64°C por 20 segundos e 72°C por 20 segundos; seguido por 22 ciclos de 95°C por 30 segundos, 62°C por 20 segundos e 72°C por 30 segundos; e extensão final de 7 min a 72°C. Para os *primers* 4 e 5, os parâmetros foram: 95°C por 5 min, seguido por 15

ciclos de 95°C por 30 segundos, 61°C por 20 segundos e 72°C por 20 segundos; seguido por 22 ciclos de 95°C por 30 segundos, 59°C por 20 segundos e 72°C por 30 segundos; e extensão final de 7 min a 72°C. Todos os produtos foram sequenciados no ABI Prism 3100 Genetic Analyzer com Big Dye Terminator™ V3.1 Cycle Sequencing reagentes (Applied Biosystems, Foster City, CA, USA).

4.8 Análise estatística

Um modelo de regressão log-binomial de efeito misto foi usado para estimar riscos relativos com intervalos de confiança de 95% para associar características clínicas incluindo gênero, locais de angioedema, sensibilidade ao estrógeno e a necessidade do uso profilaxia de longo prazo entre os grupos 1 e 2. O mesmo modelo foi usado para comparar os grupos de acordo com a gravidade da doença por Ferraro e colaboradores (FERRARO et al., 2011b). O modelo de efeito aleatório linear foi utilizado para comparar a idade de início dos sintomas entre os pacientes de ambos os grupos. Um modelo Bayesiano de regressão beta-binomial de efeitos mistos foi ajustado para comparar grupos de acordo com os escores de Bygum e colaboradores (BYGUM et al., 2011). Para todos os modelos de regressão, um efeito aleatório foi introduzido para considerar a variabilidade dos indivíduos de uma mesma família. O teste não paramétrico de Wilcoxon foi usado para comparar o tempo desde o início dos sintomas até o diagnóstico entre os pacientes da Família 7, dentro do subgrupo de pacientes que receberam cuidados antes e depois do teste genético. As análises foram conduzidas no software SAS versão 9.3 (SAS Institute, Cary, NC) e OpenBUGS 3.2.3.

4.9 Aspectos éticos

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, processo número 15036/2015. Todos os pacientes ou seus responsáveis legais assinaram o Termo de Consentimento Livre e Esclarecido antes da inclusão no estudo.

Resultados

5 RESULTADOS

5.1 Heredogramas das famílias

Dos 16 pacientes índice que participaram do estudo, informações sobre os familiares estavam disponíveis para 14 famílias sendo possível serem construídos os heredogramas (Figura 3). Para os outros 2 casos índice, informações de familiares não estavam disponíveis.

5.2 Diagnóstico de AEH-C1-INH

Sessenta pacientes, sendo 33 do sexo feminino (F) e 27 do sexo masculino (M) (33F:27M), com idades entre 10 meses e 63 anos, foram diagnosticados com AEH-C1-INH; 55 eram sintomáticos. Os 5 pacientes assintomáticos eram 4 crianças: uma de 10 meses de idade (F), um de 3 anos (M), uma de 8 anos (F), e outra de quatorze anos (F); e uma mulher com 51 anos de idade. Cinquenta e oito pacientes apresentaram deficiência quantitativa do inibidor de C1 (AEH-1), enquanto 2 apresentaram deficiência qualitativa do inibidor de C1 (AEH-2).

Dos 60 pacientes com AEH-C1-INH, 57 tinham informações clínicas obtidas através de entrevistas durante consultas médicas de acompanhamento, e apresentavam mutação no gene *SERPING1*. Três pacientes pertencentes a família número 30 tiveram dados obtidos de prontuários médicos, dos quais duas (números III.3 e IV.5, Figura 3) eram carreadoras obrigatórias da mutação c.995delT no gene *SERPING1*, de acordo com o heredograma da família. Revisão dos prontuários médicos revelou o diagnóstico de AEH em um terceiro membro desta família, que apresentava episódios de angioedema e baixos níveis de componentes do sistema complemento; a paciente faleceu aos 15 anos de idade (número IV.4, Figura 1).

Características clínicas, e dados demográficos e laboratoriais de todos os 60 pacientes estão mostrados na Tabela 6. O tempo entre o início dos sintomas e o diagnóstico de AEH foi analisado na Família 7, compreendendo 33 pacientes com AEH-1 com a mutação c.351delC (Figura 3). O tempo médio de diagnóstico foi de 15,7 anos (DP 13,3 anos) e 1,06 anos (DP 7,03 anos) nos grupos de pacientes que receberam cuidado antes e depois do teste genético estar disponível, respectivamente ($p < 0,01$, Wilcoxon test). A análise genética possibilitou o diagnóstico antes do desenvolvimento dos sintomas de vários pacientes (IV-2, V-7, IV-13, IV-11, V-8, V-5, V-12).

Figura 3 - Heredograma de 14 famílias com pacientes índice que apresentam AEH-C1-INH. Para a família 7, descendentes dos indivíduos I-1 e I-2 estão mostrados nos heredogramas 7B e 7C, respectivamente.

Figura 4 - Tempo em anos entre a idade do início dos sintomas e a idade do diagnóstico entre pacientes com AEH-C1-INH (Família 7) em relação às gerações afetadas. O tempo médio para o diagnóstico foi de 15,7 anos (DP 13,3 anos) e 1,06 anos (SD 7,03 anos) nos grupos de pacientes que receberam cuidados antes e depois da disponibilidade de testes genéticos, respectivamente ($p < 0,01$, teste de Wilcoxon). A análise genética permitiu que o diagnóstico fosse estabelecido antes do desenvolvimento dos sintomas em vários pacientes (IV-2, V-7, IV-13, IV-11, V-8, V-5, V-12).

Tabela 6 - Características clínicas, escores de gravidade e mutações no SERPING1 em pacientes com AEH-C1-INH.

Paciente id#	Gênero	Idade (anos)	Idade do inícios dos sintomas (anos)	Crises abdominais	Crises de vias aéreas superiores	Edema subcutâneo	Sensibilidade ao Estrógeno	Profilaxia de longo termo	Escore de gravidade por Ferraro et al. ⁸	Escore de gravidade por Bygum et al. ⁹	AEH -C1-INH- tipo	C4 (g/L)	C1-INH (g/L)	C1-INH funcional (%) [§]	Mutação no gene <i>SERPING1</i>
7.1	F	34	0.2	Sim	Sim	Sim	Sim	Sim	Grave	9	Tipo 1	0.05	0.05	22%	c.351delC
7.2	M	37	13	Sim	Não	Sim	NA	Sim	Grave	5	Tipo 1	0.07	<0.03	10%	c.351delC
7.3	F	35	16	Sim	Não	Sim	Sim	Sim	Grave	5	Tipo 1	0.06	0.05	16%	c.351delC
7.4	M	36	30	Sim	Não	Sim	NA	Sim	Grave	4	Tipo 1	0.17	0.1	33%	c.351delC
7.5	M	7	4	Sim	Não	Sim	NA	Não	Moderado	7	Tipo 1	0.07	0.08	ND	c.351delC
7.6	F	Falecido	6	Sim	Sim	Sim	Sim	Sim	Grave	8	Tipo 1	0.1	0.06	20%	c.351delC
7.7	M	58	23	Sim	Sim	Sim	NA	Não	Moderado	6	Tipo 1	0.11	0.1	13%	c.351delC
7.8	M	25	18	Sim	Não	Sim	NA	Não	Leve	4	Tipo 1	0.07	0.08	23%	c.351delC
7.9	F	12	11	Sim	Não	Não	Sim	Sim	Grave	4	Tipo 1	0.19	0.1	ND	c.351delC
7.10	F	14	Assintomático	Sim	Não	NA	Não	NA	Assintomático	0	Tipo 1	0.12	0.1	16%	c.351delC
7.11	M	6	5	Sim	Não	Sim	NA	NA	Grave	7	Tipo 1	0.05	0.07	ND	c.351delC
7.12	F	16	16	Sim	Não	No	Sim	NA	Moderado	4	Tipo 1	<0.02	<0.03	10%	c.351delC
7.13	M	44	3	Sim	Sim	Sim	NA	Não	Grave	9	Tipo 1	0.11	0.09	30%	c.351delC
7.14	F	8	Assintomático	NA	NA	NA	NA	NA	Assintomático	0	Tipo 1	0.13	0.09	ND	c.351delC
7.15	M	60	38	Não	Não	Sim	NA	Não	Leve	1	Tipo 1	0.21	<0.03	10%	c.351delC
7.16	M	50	9	Sim	Sim	Sim	NA	Sim	Grave	9	Tipo 1	0.06	0.08	26%	c.351delC
7.17	F	24	10	Sim	Não	Sim	Não	Não	Moderado	6	Tipo 1	0.05	0.09	26%	c.351delC
7.18	M	40	8	Sim	Não	Sim	NA	Sim	Moderado	6	Tipo 1	0.07	0.05	13%	c.351delC
7.19	F	63	30	Sim	Não	Sim	Sim	Sim	Grave	6	Tipo 1	0.09	0.09	30%	c.351delC
7.20	F	79	40	Sim	Não	Sim	Sim	Não	Leve	4	Tipo 1	0.07	0.07	20%	c.351delC
7.21	F	14	10	Sim	Não	Não	Sim	Sim	Grave	5	Tipo 1	0.08	0.1	30%	c.351delC
7.22	F	53	19	Sim	Sim	Sim	No	Sim	Grave	7	Tipo 1	0.07	0.07	16%	c.351delC
7.23	F	25	19	Sim	Sim	Sim	Sim	Sim	Grave	7	Tipo 1	0.04	0.04	13%	c.351delC
7.24	M	8	7	Sim	Não	Não	NA	Sim	Grave	4	Tipo 1	0.09	0.09	30%	c.351delC
7.25	M	12	4	Sim	Sim	Sim	NA	Sim	Grave	9	Tipo 1	0.12	0.08	23%	c.351delC
7.26	M	62	46	Sim	Não	Sim	NA	Sim	Grave	5	Tipo 1	0.04	0.05	9%	c.351delC
7.27	F	31	5	Sim	Sim	Sim	Sim	Sim	Grave	9	Tipo 1	0.02	0.06	20%	c.351delC
7.28	F	52	10	Sim	Sim	Sim	Sim	Sim	Grave	8	Tipo 1	0.07	0.07	23%	c.351delC
7.29	F	17	2	Sim	Sim	Sim	Não	Sim	Grave	10	Tipo 1	0.06	0.07	23%	c.351delC
7.30	F	36	19	Sim	Sim	Sim	Sim	Sim	Grave	8	Tipo 1	0.07	0.08	31%	c.351delC
7.31	M	21	0.5	Sim	Sim	Sim	NA	Sim	Grave	10	Tipo 1	0.04	0.04	20%	c.351delC
7.32	M	33	29	Sim	Sim	Sim	NA	Sim	Grave	7	Tipo 1	0.07	0.08	26%	c.351delC
7.33	F	1	Assintomático	NA	NA	NA	NA	NA	Assintomático	0	Tipo 1	ND	ND	ND	c.351delC

Paciente id#	Gênero	Idade (anos)	Idade do inícios dos sintomas (anos)	Crises abdominais	Crises de vias aéreas superiores	Edema subcutâneo	Sensibilidade ao Estrógeno	Profilaxia de longo termo	Escore de gravidade por Ferraro et al. ⁸	Escore de gravidade por Bygum et al. ⁹	AEH -C1-INH- tipo	C4 (g/L)	C1-INH (g/L)	C1-INH funcional (%) [§]	Mutação no gene <i>SERPING1</i>
17.1*	F	43	1	Sim	Sim	Sim	Não	Sim	Grave	10	Tipo 1	0.05	0.06	ND	c.939T>G
17.2	M	3	Assintomático	Não	Não	Não	NA	NA	Assintomático	0	Tipo 1	0.24	0.08	46%	c.939T>G
18.1	M	28	15	Sim	Sim	Sim	NA	Sim	Grave	7	Tipo 2	0.02	0.98	27%	c.1396C>T
25.1	F	27	13	Sim	Sim	Sim	Sim	Sim	Grave	8	Tipo 1	0.04	0.12	17%	c.871A>T
25.3	M	52	18	Não	Não	Sim	NA	Não	Leve	2	Tipo 1	0.09	0.06	ND	c.871A>T
28.1*	M	22	10	Sim	Sim	Sim	NA	Sim	Grave	8	Tipo 1	0.02	0.04	45%	c.679T>C
30.1	M	50	18	Sim	Sim	Sim	NA	Sim	Grave	7	Tipo 1	0.05	0.08	ND	c.995delT
30.4	M	16	8	Não	Não	Sim	NA	Não	Leve	3	Tipo 1	0.04	0.04	23%	c.995delT
30.5	M	15	7	Não	Não	Sim	NA	Não	Leve	3	Tipo 1	0.04	0.05	23%	c.995delT
30.6	F	Falecido	27	Sim	Não	Sim	Desconhecido	Não	Leve	3	Tipo 1	0.03	0.03	ND	Carreador obrigatório - c.995delT
30.7	F	Falecido	1	Sim	Sim	Sim	Desconhecid	Não	Grave	8	Tipo 1	0.06	0.14	ND	ND
30.8	F	32	15	Sim	Sim	Sim	Sim	Sim	Grave	7	Tipo 1	0.02	ND	ND	Carreador obrigatório - c.995delT
30.9	F	54	32	Sim	Sim	Sim	Não	Sim	Grave	6	Tipo 1	0.06	0.04	28%	c.995delT
36.1	F	33	2	Sim	Não	Sim	Não	Sim	Grave	9	Tipo 1	0.09	0.12	ND	c.1480C>T
38.1	F	32	19	Sim	Não	Sim	Não	Sim	Grave	6	Tipo 1	0.07	0.08	ND	c.730T>G
40.1	F	32	5	Sim	Sim	Sim	Sim	Não	Grave	9	Tipo 1	0.04	0.09	ND	c.1431C>G
57.1	F	35	23	Sim	Não	Sim	Sim	Sim	Grave	4	Tipo 1	0.09	0.07	34%	c.1334insA
60.1	F	32	15	Sim	Não	Sim	Sim	Sim	Moderado	6	Tipo 2	0.02	0.27	<10%	c.1397G>A
65.1	M	23	4	Sim	Sim	Sim	NA	Sim	Grave	9	Tipo 1	0.05	0.06	ND	c.550G>A
69.1	F	44	19	Sim	Sim	Sim	Sim	Sim	Grave	7	Tipo 1	0.03	0.09	ND	c.728T>C
70.1	M	44	19	Sim	Sim	Sim	NA	Sim	Moderado	7	Tipo 1	0.11	0.11	ND	c.314_317dup
70.2	M	20	7	Sim	Sim	Sim	NA	Sim	Leve	5	Tipo 1	0.06	0.04	ND	c.314_317dup
71.1	F	31	14	Sim	Sim	Sim	Não	Sim	Moderado	7	Tipo 1	0.02	<0.02	ND	c. 249delT
71.2	M	5	17	Sim	Sim	Sim	NA	Sim	Grave	7	Tipo 1	0.06	0.05	ND	c. 249delT
72.1	F	28	14	Sim	Sim	Sim	Sim	Sim	Grave	7	Tipo 1	0.07	0.05	ND	c.1010A>G
72.2	F	51	Assintomático	Não	Não	NA	Não	NA	Assintomático	0	Tipo 1	0.08	0.08	ND	c.1010A>G

Valores de referencia foram: C4 0.1-0.45 g/L; C1-INH quantitativo 0.14-0.39 g/L; C1-INH qualitativo 70%-100%. Níveis de C1-INH e de C4 foram determinados por nefelometria (Dade Behring, Newark, USA) e atividade funcional de C1-INH foi medida usando método cromogênico (Technoclone GmbH, Vienna, Austria).

Código do paciente foi desenvolvido com o primeiro número representando o código da família e o segundo número indicando o número individual de cada paciente.

* Sem história familiar de AEH e pais negativos para a mutação no gene *SERPING1*.

§ Atividade funcional de C1-INH não foi realizada para alguns pacientes. O diagnóstico foi realizado através da presença de sintomas clínicos, história familiar, nível baixo de C4, nível baixo de C1-INH e teste genético. NA- não aplicável; ND - não determinado (a).

Tabela 7 - Características das mutações no gene *SERPING1* identificadas nos pacientes com AEH-C1-INH.

Localização (exon)	Numeração cDNA (CDS)	Efeito na proteína (HUGO)	Tipo de mutação	Classificação de acordo com ACMS [†]	Referência
3	c. 249delT	p.Asp84Metfs*64	Deleção	Patogênica	Cimbollek S and Garcia-Lozano R – HAEdbase
3	c.314_317dupCAAC	p.Thr107Asnfs*27	Duplicação	Patogênica	Nova
3	c.351delC	p. Pro117fsx	Deleção	Patogênica	Ferraro et al. 2011 ⁸
3	c.550G>A	p.Gly184Arg	<i>Missense</i>	Patogênica	Verpy et al. 1996
5	c.689T>C	p.Leu230Pro	<i>Missense</i>	Patogênica	Xu et al. 2012
5	c.728T>C	p.Leu243Pro	<i>Missense</i>	Patogênica	Pappalardo et al. 2008
5	c.730T>G	p.Tyr244Asp	<i>Missense</i>	Provavelmente patogênica	Nova
5	c.871A>T	p.Asn291Tyr	<i>Missense</i>	Patogênica	Nova
6	c.939T>G	p.Phe313Leu	<i>Missense</i>	Patogênica	Nova, descrita a nível de proteína por Siddique et al. 1995
6	c.995delT	p.Val332Glyfs*9	Deleção	Patogênica	Nova
6	c.1010A>G	p.Asp337Gly	<i>Missense</i>	Patogênica	Pappalardo et al. 2008
8	c.1334_1335insA	p.Val446Serfs*27	Inserção	Patogênica	Nova
8	c.1396C>T	p.Arg466Cys	<i>Missense</i> [‡]	Patogênica	Skriver et al. 1989
8	c.1397G>A	p.Arg466His	<i>Missense</i> [‡]	Patogênica	Skriver et al. 1989
8	c.1431C>G	p.Phe477Leu	<i>Missense</i>	Patogênica	Guarino et. al.
8	c.1480C>T	p.Arg494*	<i>Nonsense</i>	Patogênica	Verpy et al. 1995

Classificação foi realizada de acordo com o *American College of Medical Genetics and Genomics* (ACMG) guia e padrões.

[‡] Mutações *missense* na região Arg466 foram incluídas com o Grupo 1 juntamente com deleção, inserção, duplicação e mutação *nonsense* para a análise de correlação genótipo-fenótipo.

Mutações novas estão marcadas em negrito. Mutações *de novo* foram c. 939T>G (Família 17) e c.689T>C (Família 28), identificadas entre os pacientes sem história familiar de AEH e confirmadas por ausência de mutação por sequenciamento do DNA dos pais.

5.3 Análise genética

Análise genética foi realizada em duzentos e cinco indivíduos (110F:95M). Dezesesseis diferentes mutações patogênicas foram encontradas no presente estudo. Dez foram mutações *missense*, dentre elas três novas mutações, incluindo a mutação c.939T>G, previamente descrita a nível de proteína por Siddique et al.(SIDDIQUE; MCPHADEN; WHALEY, 1995); sete mutações já haviam sido descritas na literatura em famílias com AEH-C1-INH (SKRIVER et al., 1989; SIDDIQUE; MCPHADEN; WHALEY, 1995; VERPY et al., 1996; PAPPALARDO et al., 2008; XU et al., 2012).

Sobre as seis mutações não-*missense*, uma era uma mutação *nonsense*, previamente descrita por Verpy et al.(VERPY et al., 1995); as outras foram mutações estruturais, incluindo uma duplicação (nova), uma inserção (nova), e três deleções, compreendendo uma nova e duas previamente descritas por Cimbollek e Garcia-Lozano(STEFAN CIMBOLLEK, base de dados HAEdb), e Ferraro et al.(FERRARO et al., 2011b) (Tabela 2). Todas as mutações estruturais resultam em *frameshift*, levando ao surgimento de *stop codons* prematuros. Além disso, três variantes não-patogênicas (c.25A>C, c.1030-20A>G, e c.1438G>A) foram identificadas. Das 16 mutações descritas, duas são mutações *de novo*, identificadas entre os pacientes índice sem história familiar de AEH e confirmadas pela ausência da mutação nos pais (c. 939T>G, Família 17; e c.689T>C, Família 28) (Figura 3 e Tabela 7).

5.4 Análise *in silico*

A análise das mutações *missense* foi também realizada através de três ferramentas de bioinformática, incluindo a ferramenta Mutation Taster, que analisa mutações *missense* e *nonsense*, mostrando concordância na maioria dos resultados. Quando o escore SIFT foi utilizado, 8/9 das mutações foram caracterizados como mutações *Damaging*; pela análise por PolyPhen-2, 6/9 das mutações foram consideradas *Probably Damaging*, e pelo Mutation Taster, 9/10 das mutações foram caracterizadas como *Disease-Causing*. De forma geral, a análise sugeriu que todas as mutações *missense* eram deletérias, afetando a estrutura e atividade funcional da proteína C1-INH.

Tabela 8 - Avaliação das mutações de acordo com a probabilidade de ser patogênica.

Substituição de aminoácidos	<i>SIFT (Probability score)</i>	<i>PolyPhen-2 (score, sensitivity, specificity)</i>	<i>MutationTaster (probability)</i>
Leu230Pro	<i>Damaging</i> 0	<i>Probably damaging</i> (1.000, 0.00, 1.00)	<i>Disease causing</i> -0.999
Leu243Pro	<i>Damaging</i> 0	<i>Probably damaging</i> (0.987, 0.73, 0.96)	<i>Disease causing</i> -0.999
Tyr244Asp	<i>Damaging</i> 0	<i>Probably damaging</i> (1.000, 0.00, 1.00)	<i>Disease causing</i> -0.999
Asn291Tyr	<i>Damaging</i> 0	<i>Probably damaging</i> (1.000, 0.00, 1.00)	<i>Disease causing</i> -0.999
Phe313Leu	<i>Damaging</i> 0	<i>Probably damaging</i> (1.000, 0.00, 1.00)	<i>Polymorphism</i> -0.904
Asp337Gly	<i>Tolerated</i> -0.08	<i>Possibly damaging</i> (0.516, 0.88, 0.90)	<i>Disease causing</i> -0.999
Phe477Leu	<i>Damaging</i> 0	<i>Probably damaging</i> (1.000, 0.00, 1.00)	<i>Disease causing</i> -0.999
Arg466Cys	<i>Damaging</i> -0.03	<i>Possibly Damaging</i> (0.921, 0.81, 0.94)	<i>Disease causing</i> -0.999
Arg466His	<i>Damaging</i> -0.03	<i>Possibly Damaging</i> (0.921, 0.81, 0.94)	<i>Disease causing</i> -0.999
Arg472Stop	—	—	<i>Disease causing</i> -0.999

5.5 Avaliação da gravidade do angioedema hereditário entre os pacientes com diferentes tipos de mutação

Dos sessenta pacientes que eram carreadores das mutações *SERPING1*, 48 pacientes pertencentes a 6 famílias apresentavam mutações não-*missense* (Grupo 1) enquanto 12 pacientes, pertencentes a 10 famílias, apresentavam mutações *missense* (Grupo 2). Três pacientes eram carreadores assintomáticos de mutações não-*missense*, e dois de mutações *missense*.

Os parâmetros clínicos não foram significativamente diferentes entre o Grupo 1 e o Grupo 2. Não houve diferença significativa na gravidade da doença entre os Grupos 1 e 2. De acordo com o escore descrito por Ferraro et al. (FERRARO et al., 2011), doença grave esteve

presente em 30/48 (62,5%) e 8/12 (66,6%) dos indivíduos nos Grupos 1 e 2, respectivamente (Figura 5A). Da mesma forma, os valores médios para os escores de gravidade, como definidos por Bygum et al. (BYGUM et al., 2011), foram 5,9 para o grupo 1 e 6,0 para o grupo 2, sem diferenças significantes (Figura 3B).

Figura 5 - A gravidade da doença entre os pacientes com AEH-C1-INH que possuem mutações nonsense, ou seja, deleções, inserções, duplicações ou afetando o centro reativo Arg466 no exon 8 (Grupo 1); e as mutações missense excluindo as Arg466 (Grupo 2). A: *Score* de gravidade segundo Ferraro et al. ; B: *Score* de gravidade segundo Bygum et al. .

Tabela 9 - Associação de genótipo e fenótipo entre os pacientes com angioedema hereditário por deficiência do inibidor de C1 de acordo com o tipo de mutação.

		Grupo 1 [†] n = 48	Grupo 2 [‡] n = 12
Idade mediana, intervalo (anos)		33,0 (1-79)	30,5 (3-52)
Idade do início dos sintomas mediana, intervalo (anos) [§]		14,0 (0.2-46)	13,5 (1-19)
Gênero	F	26/48 (54,2%)	7/12 (58,3%)
	M	22/48 (45,8%)	5/12 (41,7%)
Sensibilidade ao estrógeno [¶]	Sim	15/24 (62,5%)	4/7 (57,1%)
	Não	9/24 (37,5 %)	3/7 (42,9%)
Profilaxia	Sim	37/45 (82,2%)	8/10 (80%)
	Não	8/45 (17,8%)	2/10 (20%)
Locais do angioedema			
Subcutâneo		41/45 (91,1%)	10/10 (100%)
Dor abdominal		42/45 (93,3%)	9/10 (90%)
Edema de face		34/45 (75,5%)	8/10 (80%)
Edema de vias aéreas superiores		24/45 (53,3%)	6/10 (60%)
Score de gravidade por Ferraro et al. [#]	Assintomático	3/48 (6,2%)	2/12 (16,6%)
	Leve	7/48 (14,5%)	1/12 (8,3%)
	Moderado	8/48 (16,6%)	1/12 (8,3%)
	Grave	30/48 (62,5%)	8/12 (66,6%)
Score de gravidade por Bygum et al., mediana [#]		6,0	7,0

[†]Grupo 1: Deleção, inserção, duplicação, ou mutação *nonsense* e mutação que afetam o centro reativo Arg466 no exon 8.

[‡]Grupo 2: Todas as mutações missense com exceção da Arg466.

[§] Quarenta e cinco pacientes nos grupos 1 e 2, respectivamente. Cinco AEH- C1-INH pacientes foram assintomáticos.

[¶] Sensibilidade de estrógeno foi definida como desencadeadora e/ou que piora os sintomas com associação a exposição ao estrógeno, sendo com o uso de contraceptivo oral contendo estrógeno ou terapia de reposição hormonal, ou durante a gravidez, entre as pacientes mulheres.

[#]No *score* de Ferraro et al.(FERRARO et al., 2011b), a gravidade da doença é classificada baseada na frequência e intensidade dos sintomas. No *score* de Bygum et al., os parametros usados são idade do início dos sintomas, manifestações clínicas, e necessidade de profilaxia de longo prazo, com valores abrangendo de 0 a 10, sendo o ultimo o mais grave em apresentação clínica.

5.6 Ancestralidade genética em famílias de pacientes com AEH-FXII

Nossos resultados sugerem a presença de alelo mutado ancestral comum entre os indivíduos afetados do Brasil e os encontrados da Europa Ocidental, pois possuem exatamente os mesmos 10 haplótipos, com a mesma sequência (Fig 6).

Figura 6 - Análise de haplótipos nos trios de pais e filhos de seis famílias brasileiras, mostrando a mutação p.Thr328Lys, com o uso de 10 SNPs cobrindo a região genômica completa do gene F12, em vermelho o SNP (rs118204456) causador da doença.

Discussão

6 DISCUSSÃO

No presente estudo, foram identificados seis novas mutações, assim como dez mutações previamente descritas, no gene *SERPING1* como causa do angioedema hereditário por deficiência do inibidor de C1 (AEH-C1-INH) em dezesseis famílias brasileiras. A análise das características clínicas dos pacientes de acordo com o tipo de mutação revelou que gênero, idade de início dos sintomas, sensibilidade a estrógeno, uso de profilaxia de longo prazo, e os locais afetados pelo angioedema não foram significativamente diferentes entre os indivíduos que apresentaram deleção, inserção, duplicação, mutação *nonsense*, e mutações que afetam o centro reativo Arg466 que causa diminuição da função de C1-INH, quando comparados com pacientes que apresentaram mutação *missense*. Da mesma forma, a gravidade da doença, avaliada por 2 escores independentes, não mostrou diferença significativa entre os dois grupos. A razão para a comparação dos dois grupos de pacientes resultou da hipótese de que as mutações que causam alterações mais profundas na estrutura da proteína poderiam causar doença mais grave, ao contrário das mutações *missense*, que estariam associadas a um curso mais leve da doença, por causarem modificações menos marcantes na estrutura e função da proteína C1-INH (ANDREJEVIĆ et al., 2015; GRIVČEVA-PANOVSKA et al., 2018). Contudo, nossos resultados sugeriram que alterações genéticas específicas no gene *SERPING1* não foram suficientes para determinar fenótipos clínicos de AEH.

Pacientes com AEH-C1-INH do presente estudo apresentaram características clínicas semelhantes às relatadas entre pacientes de outras partes do mundo. A média da idade do início dos sintomas foi de 14,3 anos em pacientes do presente estudo, estando de acordo com estudos prévios, com variação de 10 a 14 anos de idade (KAPLAN, 2010; CICARDI et al., 2014; ANDREJEVIĆ et al., 2015; FARKAS et al., 2017; GRIVČEVA-PANOVSKA et al., 2018). A maioria das famílias apresentou AEH-1; 12,5% apresentaram AEH-2 (ZURAW, 2008; FARKAS et al., 2017).

Pacientes em sua maioria (90%) eram sintomáticos, e precisaram de tratamento profilático para controle dos sintomas, além de tratamento sob demanda para as crises agudas, como previamente descrito (ANDREJEVIĆ et al., 2015). No presente estudo, somente um adulto era assintomático, enquanto que os outros pacientes assintomáticos compreenderam em um bebe e três crianças. Os sintomas mais comuns foram angioedema subcutâneo e de submucosa, com frequente envolvimento de face e lábios, assim como dor abdominal, em concordância com estudos já publicados (BORK et al., 2006). A frequência de mutações *de*

novo foi verificada por sequenciamento do DNA de ambos os pais dos casos-índice, e foi um pouco menor no presente estudo (12,5 %) quando comparada à frequência relatada por Pappalardo et al., que foi de pelo menos 25% (PAPPALARDO et al., 2000). Estes resultados indicam que os pacientes brasileiros com AEH-C1-INH apresentaram características comuns àqueles de outras áreas, permitindo comparações de genótipo-fenótipo.

Um dos aspectos mais frequentemente debatidos sobre o diagnóstico de AEH-C1-INH é se a análise genética deve ser indicada. As principais razões para preocupação são o custo do teste genético e o fato de que, em raras ocasiões, uma mutação no gene *SERPING1* pode não ser encontrada (MAURER et al., 2018c). Consensos recomendam que ensaios do complemento devem ser repetidas vezes, para afastar a possibilidade de que flutuações normais do complemento sejam confundidas com concentrações baixas por deficiência do inibidor de C1 causada por mutações no gene *SERPING1* (BOVA et al., 2018; MAURER et al., 2018c). Aspectos metodológicos também podem dificultar a confiabilidade nos resultados de testes de complemento. Por exemplo, o método preferido para determinar o C1-INH qualitativo é o ensaio cromogênico, que pode não estar disponível nos laboratórios de rotina. Deve-se ter um cuidado particular com o estoque e transporte do soro sendo crucial o controle da temperatura, recomendada abaixo de -20°C em todos os passos para que os resultados sejam mais precisos. Alternativamente, a genotipagem para duas mutações p.Arg466Cys e p.Arg466His, localizadas no exon 8, que afetam o centro reativo responsável pela atividade catalítica de C1-INH, pode ser útil para prover um diagnóstico definitivo de AEH-C1-INH qualitativo. Essas mutações foram previamente relatadas como causa de AEH-2 em pacientes de diversos países, incluindo Hungria (KALMÁR et al., 2003), Itália (BAFUNNO et al., 2014; ZANICHELLI et al., 2015), Alemanha (GÖSSWEIN et al., 2008), Espanha (LÓPEZ-LERA et al., 2011), China (XU et al., 2012), Eslovênia (RIJAVEC et al., 2013), Portugal (MARTINHO et al., 2013), Dinamarca (AABOM et al., 2017), e Brasil (CAGINI et al., 2016).

Na prática clínica, famílias afetadas com AEH-C1-INH têm ansiedade para verificar se uma criança herdou uma mutação, que poderia representar um alto risco para desenvolver os sintomas da doença. Nossa experiência com o seguimento de mais de 20 anos de uma grande família com AEH-C1-INH revelou que o teste genético foi muito útil na diminuição do tempo de diagnóstico, possibilitando um diagnóstico preciso, que foi a chave para lidar com preocupações e fornecer orientação. Em bebês, o método de coleta de DNA menos invasivo é por *swab* da mucosa oral, que se mostrou adequado para obtenção de DNA para análise genética, diminuindo o desconforto. Para mutações conhecidas dentro de uma mesma família,

o teste genético específico para a mutação pode ter custo comparável a repetidos testes do complemento, recomendados em consensos internacional e nacional. Contudo, devida a grande variabilidade dentro dos países, benefícios e limitações tem que pesados. Além disso, recentes trabalhos que aplicam tecnologia de sequenciamento de nova geração apontam para estratégias inovadoras para o diagnóstico de AEH-C1-INH na prática clínica (URS C. STEINER, MELANIE KELLER, PIRMIN SCHMID, SVEN CICHON, 2017; LOULES et al., 2018). Nossa abordagem diagnóstica é manter a recomendação do atual consenso de testar crianças de famílias afetadas com AEH-C1-INH assim que possível, sendo o ideal antes do início das manifestações clínicas (MAURER et al., 2018c).

A variabilidade da apresentação clínica do AEH, mesmo entre pacientes que compartilham a mesma mutação na mesma família, tem levado pesquisadores à busca por biomarcadores para prever o desenvolvimento dos sintomas a gravidade da doença, dentro dos conceitos de medicina de precisão (KAPLAN; MAAS, 2017). Os níveis quantitativos e qualitativos de C1-INH e C4 usados para diagnóstico não mostram correlação com a gravidade da doença (FERRARO et al., 2011b; BOVA et al., 2018). Níveis sanguíneos de bradicinina ou do pentapeptídeo Arg-Pro-Pro-Gly-Phe derivado da degradação bradicinina podem não ser adequados como biomarcadores de gravidade da doença por problemas técnicos em quantificar estes mediadores (KAPLAN; MAAS, 2017). A clivagem do cininogênio de alto peso molecular (HMWK) foi aumentada em pacientes com AEH, especialmente durante as crises agudas, quando comparados a controles saudáveis (SUFFRITTI et al., 2014; HOFMAN et al., 2017). Em testes clínicos, a diminuição da ativação do HMWK foi observada de forma dose-dependente após tratamento com lanadelumabe, um anticorpo monoclonal totalmente humanizado anti-caliceína, utilizado no tratamento de AEH, corroborando o papel deste biomarcador para avaliar a resposta aos agentes terapêuticos para AEH (BANERJI et al., 2017). Estudos futuros, incluindo aqueles com foco no papel das modificações epigenéticas, serão necessários para correlacionar fenótipos clínicos com alterações genéticas, no sentido de melhorar o manejo clínico dos pacientes com AEH.

Em conclusão, nossos resultados estão em concordância com estudos prévios que evidenciaram elevada heterogeneidade de mutações no gene *SERPING1* associadas ao AEH-C1-INH. Além disso, os resultados mostraram que as características clínicas da doença, incluindo a gravidade da doença, podem não estar restritas ao tipo de mutação, sugerindo que outros fatores podem estar envolvidos, incluindo elementos epigenéticos, e interações gene-gene ou gene-ambiente. Apesar do presente estudo ser limitado devido ao pequeno número de

famílias participantes, a importância dele se deve ao fato de que os pacientes foram muito bem caracterizados clinicamente e seguidos ao longo do tempo por médicos experientes com o diagnóstico e o manejo de AEH, permitindo uma avaliação precisa da gravidade. Além disso, nossos resultados contribuíram com a caracterização de aspectos clínicos e genéticos do AEH-C1-INH entre pacientes da América Latina, aonde há ainda poucos estudos (FERRARO et al., 2011b; GRUMACH et al., 2013; FABIANI et al., 2014; CAGINI et al., 2016; VERONEZ et al., 2016; RODRÍGUEZ; NARVÁEZ, 2018). Nossa experiência clínica com famílias com AEH-C1-INH nos permite sugerir que a análise genética deveria ser considerada como uma ferramenta valiosa para aprimorar a precisão diagnóstica, e para prover um cuidado de excelência aos pacientes com AEH.

Conclusões

7 CONCLUSÕES

- Foram identificadas novas mutações, bem como mutações previamente descritas no gene *SERPING1* como causa de angioedema hereditário por deficiência do inibidor de C1 (tipos 1 e 2) em pacientes brasileiros.
- Manifestações clínicas e gravidade da doença foram heterogêneas e não apresentaram correlação com o tipo de mutação no gene *SERPING1*.
- Investigação da ancestralidade da mutação c.983C>A (p.Thr328Lys) no gene *F12*, realizada pela primeira vez em pacientes brasileiros com AEH-FXII, revelou o mesmo padrão de haplótipos demonstrado em 2 grupos europeus, sugerindo fortemente que a mutação veio de um ancestral comum europeu.

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Anexos

ANEXOS

ANEXO 1

LETTERS TO THE EDITOR

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SUPPORTING INFORMATION

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

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Genotype-phenotype correlations in Brazilian patients with hereditary angioedema due to C1 inhibitor deficiency

To the Editor,

Hereditary angioedema (HAE) is a rare disease with autosomal dominant inheritance that affects 1 in every 50 000 individuals.¹ Patients with HAE present recurrent episodes of edema of subcutaneous tissue and submucosa that mainly affects the skin, gastrointestinal tract, and upper airways. In most cases, the disease results from the deficiency of C1 inhibitor (C1-INH) owing to mutations in *SERPING1*, the gene encoding C1-INH protein. The decrease in C1-INH activity leads to uncontrolled activation of the kallikrein-kinin system and increased formation of bradykinin, resulting in angioedema. HAE with normal C1-INH has also been characterized.² Diagnosis of C1-INH-HAE is based on clinical symptoms, positive family history, low levels and/or functional activity of C1-INH, and decreased C4.¹ Whether genetic analysis should be performed in routine clinical practice is yet debated. Variability in clinical presentation of HAE has prompted researchers to look for novel biomarkers. Kaplan and Maas have recently discussed the role of potential biomarkers, including blood levels of bradykinin or the pentapeptide Arg-Pro-Gly-Phe derived from bradykinin degradation and cleaved high-molecular-weight kininogen, in assessing HAE severity and response to therapeutic agents.³ The type of mutation in *SERPING1* could account for clinical phenotypes. Patients with missense mutations have been shown to present symptoms at later ages, milder clinical course, and lesser need for prophylactic medications than those with mutations that cause more profound changes in the molecule.^{4,5} Grouping patients with missense mutations in *SERPING1* affecting Arg466 at the reactive center with those carrying mutations leading to more significant changes in C1-INH molecule revealed association with more severe disease.^{6,7} In the present study, we aimed to identify

and characterize mutations in *SERPING1* in Brazilian patients with C1-INH-HAE and investigate the impact of the type of mutation on clinical features of the disease.

Sixty patients with C1-INH-HAE from 16 distinct families were characterized based on mutations in *SERPING1*. Diagnosis was established according to consensus criteria (Data S1).¹ One of the families (Family 7) has been previously reported by our group; we provide follow-up information, including five new members with HAE-1.⁸ Patients were divided into the following groups: group 1 comprising patients with deletion, insertion, duplication, or nonsense mutation and mutations affecting Arg466 at the reactive center ($n = 48$); and group 2 comprising patients with missense mutations, with the exception of mutations at Arg466 ($n = 12$). The rationale for dividing patients into these groups was the fact that mutations causing more profound alterations in the structure of the protein could lead to more severe disease, as previously reported.^{6,7}

Genomic DNA was extracted from whole blood or oral mucosa material using the DNA Wizard Genomic DNA Purification Kit (Promega, Madison, WI). PCR was conducted using *SERPING1* primers (Data S2, Table S1), and DNA sequences were analyzed using the SeqMan software™ (Lasergene; DNA Star, Inc., Madison, WI). Sequence variations were identified by comparison with GenBank accession number NM_000062.2, X54486. Protein effect was defined using the mature protein sequence, including signal peptide. Criteria of the American College of Medical Genetics and Genomics (ACMG) were applied for classifying sequence variants. Predicted functional analysis of missense mutations was performed using the bioinformatics tools SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>), and MutationTaster,

which could also assess nonsense mutations (<http://www.mutationtaster.org/>). Two proposed scores, though nonvalidated, were applied to classify the disease severity.^{8,9} Statistical analysis is described in Data S3. For regression models, a random effect was introduced to consider variability in the individuals from the same family.

Among the 16 index patients, information on family members was available for 14 families (Figure S1). Sixty patients (33F:27M), from 10 months to 79 years of age, were diagnosed with C1-INH-HAE; of these, 55 were symptomatic. Fifty-eight patients presented HAE-1, whereas two had HAE-2 (Table S2). Time from onset of symptoms to diagnosis of HAE was examined in Family 7, comprising 33 patients carrying the c.351delC mutation (Figure S2). Mean time for diagnosis was 15.7 years (SD 13.3 years) and 1.06 years (SD 7.03 years) in the groups of patients receiving care before and after genetic testing became available, respectively ($P < 0.01$).

A total of 205 individuals (110F:95M) underwent genetic analysis. *SERPING1* mutations included 10 missense and 6 nonmissense mutations (structural mutations leading to frameshift or nonsense mutations). Six mutations were novel and two were de novo mutations (Table 1). According to ACMG guidelines, 15/16 variants were classified as pathogenic and 1/16 as likely pathogenic. With SIFT, 9/10 missense mutations were characterized as damaging, while PolyPhen-2 characterized 7/10 missense mutations as probably damaging. MutationTaster deemed 10/11 missense/nonsense mutations as disease-causing (data not shown). Clinical parameters and severity of

disease were not significantly different among patients from groups 1 and 2 (Table 2).

Patients with C1-INH-HAE from our cohort presented similar clinical features as well as some common genetic alterations previously reported in patients from other areas. Although Brazilian population is a mixture of native, European, and African ancestry, European ancestry is predominant, ranging from 60.6% to 77.7% (Pena et al. 2011, Supporting Information). The mean age of 14.3 years at onset of symptoms was in agreement with previous reports.¹ The majority of families presented HAE-1: only 12.5% presented HAE-2. The majority of our patients (91.6%) were symptomatic, and 81% required prophylactic treatment at a rate higher than that previously reported,⁶ owing to the limited availability of the specific on-demand HAE medications in Brazil. Common symptoms were angioedema of subcutaneous and submucosal tissues, with the frequent involvement of face and lips, and abdominal pain attacks, in accordance with published studies.¹ Frequency of de novo mutations was slightly lower in the present study (12.5%) than that previously reported (approximately 25%). These results indicate that Brazilian patients with C1-INH-HAE share common features with those from other areas, allowing for genotype-phenotype comparisons.

Our results showed that clinical severity of C1-INH-HAE may not be strictly related to the type of mutation. On the other hand, genetic testing was helpful in substantially decreasing the time for the diagnosis to allow precise diagnosis, which was key to dealing

TABLE 1 Characteristics of *SERPING1* mutations identified in patients with C1-INH-HAE

Location (exon)	cDNA numbering (CDS)	Protein effect (HUGO)	Type of mutation	Classification according to ACMG ^a	Reference
3	c. 249delT	p.Asp84Metfs*64	Deletion	Pathogenic	Cimbollek S and Garcia-Lozano R – HAEdbase
3	c.314_317dupCAAC	p.Thr107Asnfs*27	Duplication	Pathogenic	Novel
3	c.351delC	p. Pro117fsx	Deletion	Pathogenic	Ferraro et al. (2011) ⁸
3	c.550G>A	p.Gly184Arg	Missense	Pathogenic	Verpy et al. (1996)
5	c.689T>C	p.Leu230Pro	Missense	Pathogenic	Xu et al. (2012)
5	c.728T>C	p.Leu243Pro	Missense	Pathogenic	Pappalardo et al. (2008)
5	c.730T>G	p.Tyr244Asp	Missense	Likely pathogenic	Novel
5	c.871A>T	p.Asn291Tyr	Missense	Pathogenic	Novel
6	c.939T>G	p.Phe313Leu	Missense	Pathogenic	Novel, described at protein level Siddique et al. (1995)
6	c.995delT	p.Val332Glyfs*9	Deletion	Pathogenic	Novel
6	c.1010A>G	p.Asp337Gly	Missense	Pathogenic	Pappalardo et al. (2008)
8	c.1334_1335insA	p.Val446Serfs*27	Insertion	Pathogenic	Novel
8	c.1396C>T	p.Arg466Cys	Missense ^b	Pathogenic	Skriver et al. (1989)
8	c.1397G>A	p.Arg466His	Missense ^b	Pathogenic	Skriver et al. (1989)
8	c.1431C>G	p.Phe477Leu	Missense	Pathogenic	Guarino et al.
8	c.1480C>T	p.Arg494*	Nonsense	Pathogenic	Verpy et al. (1995)

Novel mutations are marked in bold. De novo mutations were c. 939T>G (Family 17) and c.689T>C (Family 28), identified among patients without family history of HAE and confirmed by the absence of mutation upon sequencing of the parental DNA.

Full references are presented as Supporting Information.

^aClassification was performed according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.

^bMissense mutations at Arg466 were included within group 1 together with deletion, insertion, duplication, and nonsense mutation for the analysis of genotype-phenotype correlations.

TABLE 2 Genotype-phenotype associations among patients with C1-INH-HAE according to the type of mutations

	Group 1 ^a n = 48	Group 2 ^b n = 12
Age median, range (years)	33.0 (1-79)	30.5 (3-52)
Age at onset of symptoms median, range (years) ^c	14.0 (0.2-46)	13.5 (1-19)
Gender		
F	26/48 (54.2%)	7/12 (58.3%)
M	22/48 (45.8%)	5/12 (41.7%)
Estrogen sensitivity ^d		
Yes	15/24 (62.5%)	4/7 (57.1%)
No	9/24 (37.5%)	3/7 (42.9%)
Prophylaxis		
Yes	37/45 (82.2%)	8/10 (80%)
No	8/45 (17.8%)	2/10 (20%)
Angioedema sites		
Subcutaneous edema	41/45 (91.1%)	10/10 (100%)
Abdominal attacks	42/45 (93.3%)	9/10 (90%)
Facial swelling	34/45 (75.5%)	8/10 (80%)
Laryngeal attacks	24/45 (53.3%)	6/10 (60%)
Severity score by Ferraro et al. ^{8,e}		
Asymptomatic	3/48 (6.2%)	2/12 (16.6%)
Mild	7/48 (14.5%)	1/12 (8.3%)
Moderate	8/48 (16.6%)	1/12 (8.3%)
Severe	30/48 (62.5%)	8/12 (66.6%)
Severity score by Bygum et al. ⁹ , median ^e	6.0	7.0

^aGroup 1: Deletion, insertion, duplication, or nonsense mutation and mutations affecting Arg466 at the reactive center in exon 8.

^bGroup 2: All missense mutations with the exception of those at Arg466.

^cForty-five and ten symptomatic patients in groups 1 and 2, respectively. Five C1-INH-HAE patients were asymptomatic.

^dEstrogen sensitivity was defined as triggering and/or worsening of symptoms in association with estrogen exposure owing to the use of an estrogen-containing oral contraceptive or a hormone replacement therapy, or during pregnancy, among female patients only.

^eIn the score by Ferraro et al.⁸, disease severity is classified based on symptom frequency and intensity. In the score by Bygum et al.⁹, the parameters used are age at disease onset, clinical manifestations, and need for long-term prophylaxis, with values ranging from 0 to 10, the latter being the most severe clinical presentation.

with concerns and providing guidance. Moreover, recent reports using next-generation sequencing technologies for C1-INH-HAE diagnosis point to a breakthrough strategy in clinical practice. Our approach is in keeping with the current consensus recommendation of testing children from C1-INH-HAE-affected families as soon as possible, ideally before the onset of clinical manifestations¹.

In conclusion, our results suggest that factors including epigenetic elements and gene-gene or gene-environment interactions may play a role in shaping the clinical course of C1-INH-HAE. Although our study may be limited by the small number of participating families, it has the strength that patients were well characterized

clinically and followed over time by physicians experienced with diagnosis and management of HAE, allowing for accurate evaluation of severity. Furthermore, our data add contributions on genetic and clinical aspects of C1-INH-HAE to the few reports in patients from Latin America. Our clinical experience with families with C1-INH-HAE prompts us to suggest that genetic testing should be considered a valuable tool for improving diagnostic accuracy and, ultimately, excellence of care for patients with HAE.

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CONFLICT OF INTERESTS

LSM Maia is a recipient of a Doctoral scholarship from the São Paulo Research Foundation (FAPESP) (grant numbers 2014/26693-5 and 2017/18669-5). She also received research support from the Coordination of Superior Level Staff Improvement (CAPES) and the Brazilian National Council for Scientific and Technological Development (CNPq), in addition to receiving travel support from Shire. AS Moreno is a recipient of postdoctoral fellowships from the Coordination of Superior Level Staff Improvement (CAPES) and São Paulo Research Foundation (FAPESP grant number 2011/23439-2). She also received travel support from Shire. MPL Ferriani has received lecture fees from Shire. FL Nunes has received lecture fees from Shire and is a recipient of an Investigator-Initiated Research Grant from Shire. P Roxo-Junior has received lecture fees from Shire and CSL Behring. SOR Valle is on the board for Novartis, Sanofi, and CSL Behring, and has received lecture fees from Novartis and Takeda. FS Serpa has received travel support and lecture fees from Shire, Novartis, and AstraZeneca. LK Arruda is a recipient of a Brazilian National Council for Scientific and Technological Development (CNPq) Research Productivity Grant, and has received travel support and lecture fees from Shire, Novartis, and Sanofi. MF Ferraro, MM Dias, FC Dias, S Levy, MLO Alonso, AT França, AA Motta, WA Silva Jr., W Sarti, DC Aragon, FGM Maia, S Cichon, and K Bork have no relevant conflict of interests to declare.

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AUTHOR CONTRIBUTIONS

LMS Maia participated in acquisition of patients' data, conducted the sequencing experiments and analysis, and prepared the data for the manuscript. LK Arruda and AS Moreno designed the study, analyzed the data, prepared the first draft of the manuscript, and revised the manuscript to its final version. MPL Ferriani, FL Nunes, MF Ferraro, P Roxo Jr, SOR Valle, S Levy, MLO Alonso, A França, FS Serpa, AA Motta, and W Sarti contributed with recruitment of patients and acquisition of patients' data. MPL Ferriani prepared the complete set of clinical data for analysis and critically revised the manuscript. MM Dias, FC Dias, and FGM Maia assisted with acquisition and analysis of sequencing data. WA Silva Jr. supervised the final analysis of sequencing data and critically revised the manuscript. DC Aragon conducted the statistical analysis. S Cichon and K Bork contributed to the interpretation of the data and critically revised the manuscript. All authors contributed to the interpretation of the data, revised the manuscript, and approved the final version.

Keywords

bradykinin, C1 Inhibitor, complement, hereditary angioedema, SERPING1

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SUPPORTING INFORMATION

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

ANEXO 2

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Clinical Communications

A cost-effective algorithm for diagnosis of hereditary angioedema with normal C1 inhibitor: Applying molecular approach to clinical practice

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Clinical Implications

- Diagnosis of hereditary angioedema with normal C1 inhibitor is often a challenge to the clinician. The c.983C>A mutation in the gene encoding coagulation factor XII (*F12*) has been predominant as a cause of hereditary angioedema with normal C1-inhibitor among patients worldwide. A cost-effective algorithm may facilitate a definitive diagnosis by genetic analysis.

TO THE EDITOR:

Hereditary angioedema (HAE) is an autosomal-dominant disease characterized by recurrent edema of subcutaneous tissue, gastrointestinal tract, and upper airways.¹ Over a decade ago, HAE with normal C1 inhibitor (C1-INH) has been described, associated with mutations in *F12*, the gene encoding coagulation factor XII (FXII).² Subsequently, mutations in Plasminogen (*PLG*), Angiotensinogen 1 (*ANGPT1*), and Kininogen 1 (*KNG1*) genes have been reported as cause of HAE with normal C1-INH.³⁻⁵ The newly described *KNG1* mutation could lead to a functionally active but aberrant bradykinin, which would undergo modified inactivation resulting in prolonged half-life.⁵ Four mutations in *F12*, all in exon 9, have been identified in patients with FXII-HAE: missense mutations c.983C>A (p.Thr328Lys) and c.983C>G (p.Thr328Arg), deletion c.971_1018+24del72, and duplication c.892_909dup.⁶ In a cohort of European patients with HAE with normal C1-INH, 26% of 265 individuals were carriers of *F12* mutations.⁶ A study from Brazil reported *F12* mutations in 134 of 195 patients with HAE with normal C1-INH, belonging to 42 families.⁷ In studies from different parts of the world, the c.983C>A (p.Thr328Lys) mutation has been identified in 519 of 551 (94.2%) patients belonging to 155 of 167 (92.8%) families (see Table E1 in this article's Online Repository at www.jaci-inpractice.org). We aimed to develop a cost-effective algorithm for the diagnosis of patients with HAE with normal C1-INH, using allelic discrimination to genotype the c.983C>A mutation as the initial step.

Patients with clinical suspicion of HAE with normal C1-INH, defined as those with symptoms suggestive of HAE, levels of C4 and quantitative and functional C1-INH within normal limits, and/or absence of mutations in *SERPINC1*, were enrolled. A subgroup of patients participated in previous studies, comprising 15 families and 68 patients.^{7,8} Patients' selection, ethical aspects and methods for allelic discrimination, Sanger sequencing, and

calculation of costs are described in this article's Online Repository at www.jaci-inpractice.org.

One hundred eighty-four individuals, belonging to 51 families with index patients, underwent DNA analysis. Individuals were aged 2 months to 91 years, with a median age of 35 years, and 136 of 184 (73.9%) were female. Allelic discrimination was positive for the c.983C>A *F12* mutation in 24 of 51 (47%) index patients. On detection of the mutation in the index patient, respective family members underwent genetic analysis. Overall, 96 patients belonging to 24 families were positive for the c.983C>A *F12* mutation by allelic discrimination; all of them presented the c.983C>A *F12* mutation by Sanger sequencing (see Figure E1 in this article's Online Repository at www.jaci-inpractice.org). Twenty-seven index patients who were negative for the c.983C>A mutation as well as 61 individuals belonging to the 24 families described above who were negative on allelic discrimination were also negative for mutations in *F12* by Sanger sequencing. Kappa index was 1.00, indicating perfect concordance. Among the 96 patients bearing the c.983C>A mutation, 70 (72.9%) were symptomatic. The proportion of symptomatic male carriers (56%) was higher as compared with that reported among European patients (4%-37.5%).^{7,8}

None of the 27 index patients negative for the c.983C>A mutation presented mutations in *PLG* or *ANGPT1* genes. Patients with family history and no causative genetic variants identified were characterized as HAE with normal C1-INH presenting as unknown HAE (*n* = 11); those with angioedema and normal C1-INH without family history were diagnosed with idiopathic nonhistaminergic acquired angioedema (*n* = 16).

Costs and time for performing allelic discrimination were US \$91.08 and US \$194.37, and 6 and 7.5 hours, for 1 and 10 samples, respectively. Values for DNA sequencing by the Sanger method were US \$496.69 and US \$776.28, and 38.5 and 43.5 hours, for 1 and 10 samples, respectively (Figure 1, see Table E2 in this article's Online Repository at www.jaci-inpractice.org).

We identified the c.983C>A mutation in 100% of our patients with FXII-HAE. Allelic discrimination was more cost-effective, with reduction of 82% in costs and decrease of 84% in performance time, compared with Sanger sequencing. Additional cost reduction and gain in time could be obtained by testing multiple samples. Reasons for these differences include fewer steps, fewer reagents used, and shorter labor time. Therefore, we propose that allelic discrimination for the c.983C>A mutation in *F12* could be used as an initial diagnostic test for HAE with normal C1-INH. No other mutations in *F12* and no previously reported mutations in *PLG* and *ANGPT1* were identified in patients negative for the c.983C>A mutation. These results led us to suggest an algorithm for the diagnosis of HAE with normal C1-INH, which could be useful in areas where the c.983C>A mutation in *F12* is predominant (Figure 2).

Genetic analysis revealed the diagnosis of FXII-HAE in 96 individuals belonging to 24 families with index patients with clinical suspicion of the disease, including 26 asymptomatic patients who otherwise would remain undiagnosed. Medications for acute attacks licensed for patients with C1-INH-HAE including icatibant, ecallantide, and plasma-derived C1-INH concentrate have been successfully used in patients with HAE with normal C1-INH.⁹ In addition, patients with FXII-HAE

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FIGURE 1. Comparison of (A) total costs and (B) time, including blood collection and DNA preparation, for performing Sanger sequencing and allelic discrimination for the c.983C>A mutation in *F12*, for 1 and 10 samples.

FIGURE 2. Algorithm to investigate HAE with normal C1-INH. *ANGPT1*-HAE, HAE with mutation in the gene encoding Angiotensinogen-converting enzyme 1 (*ANGPT1*); *FXII*-HAE, HAE with mutation in the gene encoding coagulation factor XII (*F12*); *InH*-AAE, idiopathic nonhistaminergic acquired angioedema; *PLG*-HAE, HAE with mutation in the gene encoding Plasminogen (*PLG*); *U*-HAE, unknown HAE. HAE with normal C1-INH types are circled in red.

show a good response to tranexamic acid for prophylaxis of HAE attacks.⁹ Therefore, patients diagnosed early would benefit from prompt treatment and preventive strategies and would be spared of diagnostic uncertainties.

In conclusion, the allelic discrimination method was developed for the detection of c.983C>A mutation in *F12*. Sanger

sequencing including sequence of exon 9 and intronic adjacent regions of *F12* would be indicated as the next step in patients with negative allelic discrimination results for the c.983C>A mutation. Allelic discrimination could be developed for simultaneous detection of missense mutations c.983C>G in *F12*,² c.988A>G in *PLG*,³ c.807G>T in *ANGPT1*,⁴ and c.1136T>A in *KNG1*,⁵ in a multiplex assay format. Patients negative to known mutations in *F12*, *PLG*, *ANGPT1*, and *KNG1* genes and angioedema with normal C1-INH could benefit from further investigation of pathogenic genetic variants by next-generation sequencing. Alternatively, in areas where *F12* mutations are rare, whole-exome sequence could be considered as the diagnostic method of choice. Uncertain diagnosis often leads to patients' anxiety, because of the bothersome and sometimes life-threatening nature of the attacks. Therefore, despite higher costs of next-generation sequencing approaches (see Table E2), precise diagnosis would be worth pursuing, providing grounds for adequate treatment and genetic counseling. Further studies in other areas need to be conducted to establish whether allelic discrimination would have a lower cost and provide faster results.

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METHODS

Patients' selection and ethical aspects

Patients attended the Allergy Clinics of the Clinical Hospital of Ribeirão Preto Medical School or had biological material sent to our laboratory for genetic investigation. The study was approved by the Ethics Committee of our institution (protocol no. 2344/2017), and all participants or their legal guardians signed written informed consent.

Allelic discrimination and Sanger sequencing

Genomic DNA was extracted from whole blood or oral mucosa cells using the DNA Wizard Genomic DNA Purification Kit (Promega, Madison, Wis). Allelic discriminations were carried out by real-time PCR using chromophore probes VIC (554 nm) designed for the wild-type allele and FAM (518 nm) designed for the allele with the c.983C>A mutation. Primers were F 5'-CTAGGCTTCATGTCCCACTCAT-3' and R 5'-CCCCCAGCTTCCTAACCTC-3', and reporters were Reporter 1 VIC TCAGCCCAAGACCCG and Reporter 2 FAM CAGCCCAAGACCCG. Real-time PCR was performed in 10.5 µL volume containing 15 ng genomic DNA, TaqMan Genotyping Master Mix, and TaqMan Probe (Thermo Fisher Scientific, Waltham, Mass). Samples were processed in the Applied Biosystems 7500 Real Time Thermal Cycler (Applied Biosystems, Foster City, Calif) using the following steps: 1 minute at 60°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C followed by 1 minute at 60°C, and 60° for 1 minute. Results were analyzed by ABI 7500 SDS software (Thermo Fisher Scientific, Waltham, MA).

Sequencing of exon 9 of *F12* gene by Sanger technique was performed using Applied Biosystems 3500 Genetic Analyzer sequencer (Applied Biosystems). PCR was performed to generate a 395 base-pair fragment, comprising a region of exon 9 and adjacent intronic regions of *F12*, which allows for the detection of all previously described mutations in *F12* associated with FXII-HAE.^{E1} PCR product was purified by ExoSAP-IT (Thermo Fisher Scientific) and sequenced.

To investigate previously described mutations in *PLG* and *ANGPT1* genes, the following primers were used: *PLG* primers F 5'-CTT AGT TTT AGT TAC TGT AGG AAC GCA GG-3' and R 5'-CAG GCT TTC TGA CCA CAA TAG C-3' to amplify a 494 base-pair fragment^{E2}; *ANGPT1* primers F 5'-GTTGACAACTGGATTCTGTGTG-3' and R 5'-GTTGACAACTGGATTCTGTGTG-3' to amplify a 446 base-pair fragment. *ANGPT1* primers were designed using the Gene Runner Software v.3.05 (Hastings Software, Inc, Hastings, NY). PCR was carried out in 25 µL volume containing 100 ng of genomic DNA, 0.5 µL of each primer 10 mM (IDT, Coralville, Iowa), DNA polymerase Taq Platinum (Thermo Fisher Scientific) using 1.0 µL of MgCl₂ for *PLG* and 0.5 µL of MgCl₂ for *ANGPT1*, and 1.0 µL dNTP 5 mM (Thermo Fisher Scientific). Samples were processed in Eppendorf Mastercycle Pro (Eppendorf, Hamburg, Germany) using the following steps: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes. PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific) and sequenced by Sanger technique using Applied Biosystems 3500 Genetic Analyzer sequencer (Applied Biosystems).

Evaluation of costs

Direct costs included costs of reagents and consumables, direct labor, sequencing service, energy, and maintenance of equipment directly involved in performing the tests, which were calculated on the basis of Brazilian guidelines.^{E3} Calculation was based on Brazilian values in Reais and converted to US dollars at the rate of US \$ 1.00 = R\$ 3.15. Costs for the collection of blood were provided by the Clinical Hospital of Ribeirão Preto Medical School financial management office. Attempts to evaluate indirect costs, including building maintenance and security, water, telephone, internet, warehouse materials, administrative services, and equipment not directly involved in the tests, were hampered by the fact that it was not possible to measure them accurately at each process.

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TABLE E1. Description of mutations in the *F12* gene reported among patients with HAE with normal C1-INH

Reference	Origin	Type of mutation in the <i>F12</i> gene	No. of families with mutation	No. of patients with mutation*
Veronez et al, ^{E1} 2017	Brazil	c.983C>A (p.Thr328Lys)	41 of 42 (97.6%)	132
		c.971_1018+24del 72	1 of 42 (2.4%)	2
Bouillet et al, ^{E4} 2017	France	c.983C>A (p.Thr328Lys)	1	2
Gallais Sérézal et al, ^{E5} 2016	Sweden	c.983C>A (p.Thr328Lys)	3	3
Piñero-Saavedra et al, ^{E6} 2016	Spain	c.983C>A (p.Thr328Lys)	9 of 9 (100%)	35
Bork et al, ^{E7} 2015	Germany/Europe	c.983C>A (p.Thr328Lys)	19 of 23 (82.6%)	92
		c.983C>G (p.Thr328Arg)	2 of 23 (8.7%)	5
		c.971_1018+24del 72	2 of 23 (8.7%)	7
Charignon et al, ^{E8} 2014	France, Spain, Algeria, Germany, Switzerland	c.983C>A (p.Thr328Lys)	40 of 40 (100%)	118
Bork et al, ^{E9} 2014	Turkey	c.971_1018+24del 72	2	7
Kiss et al, ^{E10} 2013	White	c.892_909dup	1	5
Marcos et al, ^{E11} 2012	Spain	c.983C>A (p.Thr328Lys)	13 of 13 (100%)	29
Bacza et al, ^{E12} 2011	Morocco/Arab	c.983C>A (p.Thr328Lys)	1	4
Vitrat-Hincky et al, ^{E13} 2010	France	Mutation not reported	3	6
Picone et al, ^{E14} 2010	France	c.983C>A (p.Thr328Lys)	2	3
Bork et al, ^{E15} 2009	Germany	c.983C>A (p.Thr328Lys)	11 of 13 (85%)	32
		c.983C>G (p.Thr328Arg)	2 of 13 (15%)	3
Hentges et al, ^{E16} 2009	Luxemburg	c.983C>A (p.Thr328Lys)	1	1
Nagy et al, ^{E17} 2009	UK/Italy	c.983C>A (p.Thr328Lys)	1	5
Duan et al, ^{E18} 2009	Italy	c.983C>A (p.Thr328Lys)	1	9
Prieto et al, ^{E19} 2009	Spain	c.983C>A (p.Thr328Lys)	1	4
Bell et al, ^{E20} 2008	Australia	c.983C>A (p.Thr328Lys)	1	1
Martin et al, ^{E21} 2007	France	c.983C>A (p.Thr328Lys)	1	7
Bouillet et al, ^{E22} 2007	France	c.983C>A (p.Thr328Lys)	1	2
Cichon et al, ^{E23} 2006	Germany/France	c.983C>A (p.Thr328Lys)	1	6
Dewald and Bork, ^{E24} 2006	Germany/Europe	c.983C>A (p.Thr328Lys)	5 of 6 (83.3%)	27
		c.983C>G (p.Thr328Arg)	1 of 6 (16.7%)	4
Combined studies		c.983C>A (p.Thr328Lys)	155 of 167 (92.8%)	519 of 551 (94.2%)

*May include asymptomatic carriers.

TABLE E2. Costs and time for performing Sanger sequencing and allelic discrimination

Parameter	Sanger sequencing*		Allelic discrimination†	
	1 sample	10 samples	1 sample	10 samples
Blood collection	1.19	11.90	1.19	11.90
Preparation of DNA‡	34.58	89.22	34.58	89.22
Reagents and consumables	10.00	47.96	9.28	32.13
Technical workforce	213.94	237.71	41.60	47.54
Equipment cost§	0.23	2.34	3.40	11.03
Equipment maintenance	0.05	0.47	0.68	2.20
Power consumption	1.48	1.48	0.35	0.35
Sequencing	4.76	47.62	NA	NA
Repeated analysis	230.46	337.58	NA	NA
Total (US \$)¶	496.69	776.28	91.08	194.37
Time (h)	38.5	43.5	6.0	7.5

NA, Not applicable.

*Sanger sequencing steps were conventional PCR, electrophoresis, purification of PCR products, sequencing reactions, precipitation, sequencing, and analysis of results.

†Allelic discrimination steps were real-time PCR and analysis of results by VIC and FAM fluorescence.

‡Preparations of DNA steps were extraction, quantification, and dilution of DNA.

§Equipment cost was estimated on the basis of actual cost and durability of the equipment, and the predicted time for performing experiments.

||Repeated analysis was performed when chromatograms did not fulfill quality requirements to allow accurate readings, requiring that all sequencing steps be repeated. In addition, the presence of mutation by Sanger sequencing was confirmed by repeating sequencing steps. For the purpose of calculation of costs, it was assumed that all sequencing reactions were performed in duplicate.

¶For next-generation sequencing approaches, estimates in US dollars ranged from \$555 to \$5,169 for a single whole-exome sequence (WES) test and from \$1,906 to \$24,810 for a single whole-genome sequence (WGS) test, according to a recent systematic review of the literature, based on published data from different parts of the world: the United States, the United Kingdom, Canada, Australia, the Netherlands, and France, in various genetic conditions, most commonly neurological or neurodevelopmental disorders.^[25] Similar figures were encountered in Brazil, with \$1,894 for the WES test and \$3,834-\$5,225 for the WGS test.

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FIGURE E1. Genotyping of variant c.983C>A in exon 9 of *F12* gene. (A) Representative TaqMan assay for genotyping the c.983C>A variant (heterozygous) in 7 individuals, highlighting 1 patient with the c.983C>A variant (*green*) and 1 individual with wild-type allele (*red*), with experiments performed in duplicate. Sequencing by the Sanger method in (B) a patient with the c.983C>A mutation (*arrow*) and in (C) a normal individual.

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ANEXO 3

Clinical Allergy – Research Article

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Serum Baseline Tryptase Level as a Marker for the Severity of Anaphylaxis

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Keywords

Anaphylaxis · Tryptase · Allergy · IgE · Epinephrine

Abstract

Background: Anaphylaxis is a severe and potentially fatal allergic disease or hypersensitivity reaction with variable clinical presentation. Biomarkers in anaphylaxis could be useful to improve diagnosis, to allow endotyping of patients, and to predict risk. **Objective:** To investigate the role of serum basal tryptase (sBT) levels in the management of patients with anaphylaxis. **Methods:** Patients with at least 1 episode of anaphylaxis were selected among those who attended the Allergy Clinics of the Clinical Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil, upon evaluation by allergy/immunology specialists of our medical staff. Demographic and clinical data were obtained using a structured questionnaire. sBT levels were determined using the ImmunoCAP Tryptase immunoassay. **Results:** 57 patients (56.1% female) with a median age of 35 years (range 7–87 years) participated in the study. sBT levels ranged from 2.57 to 21.19 ng/mL (mean 5.17 ng/mL), with no significant differences in patients with anaphylaxis due to different triggers. Mean levels were 4.93; 5.2; 5.41, and 5.24 ng/mL for

patients who had anaphylaxis due to *Hymenoptera* venom ($n = 17$), foods ($n = 13$), drugs ($n = 13$), and idiopathic disease ($n = 14$), respectively. Significantly higher sBT levels were observed in patients with severe anaphylaxis (grade IV) than in patients with mild-moderate disease (grades II/III) (mean levels 6.61 vs. 4.71 ng/mL, respectively). **Conclusion:** High sBT levels may help to identify patients at increased risk of more severe anaphylaxis, prompting physicians to initiate immediate therapy to avoid further acute episodes.

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Introduction

Anaphylaxis is a severe and potentially fatal allergic disease or hypersensitivity reaction with variable clinical presentation, which usually occurs suddenly after contact with a triggering antigen. IgE-mediated reactions frequently underlie the development of anaphylaxis; however, other pathways have also been described [1]. Due to the lack of universally accepted clinical criteria for the diagnosis of

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anaphylaxis, this condition is often underrecognized, underdiagnosed, and, consequently, undertreated [2–5]. Prevalence of anaphylaxis is increasing, with estimates of 0.3–5.1% depending on the definition used [6].

Currently, anaphylaxis phenotypes have been defined as type-I-like reactions, when classical allergic symptoms due to the release of inflammatory mediators from mast cells and basophils are present, including flushing, pruritus, urticaria, angioedema, dyspnea, wheezing, nausea, vomiting, diarrhea, hypotension, and cardiovascular collapse; cytokine storm-like reactions and mixed reactions, which are frequently provoked by chemotherapy or monoclonal antibodies and induce atypical symptoms such as chills, fever, or pain, in addition to classical symptoms, and reactions mediated by complement, which can be caused by contrast dyes or dialysis membranes, and often induce hypotension and oxygen desaturation [1, 7, 8]. Endotypes underlying these clinical presentations include IgE and non-IgE-mediated mechanisms, cytokine release, direct mast cell and basophil activation, complement activation with generation of anaphylatoxins C3a and C5a, contact system activation with bradykinin release, IgG-mediated cell activation which is still debatable in humans, and mixed reactions [1, 7, 8].

In keeping with precision medicine concepts, biomarkers in anaphylaxis would be very useful to improve diagnosis, particularly if point-of-care diagnostic biomarkers could be developed, to allow endotyping of patients, to propose new therapies based on the stratification of severity, and to predict risk [7, 8]. To date, serum tryptase levels are the best-characterized biomarker of anaphylaxis and have been included as part of institutional protocols [9–15]. Tryptase is a serine protease produced and released in large amounts by mast cells and to a lesser extent by basophils. Baseline tryptase levels have been shown to increase after mast cell activation in anaphylaxis [9, 10], but specificity may be limited by the fact that it can be increased in other conditions, particularly mast cell disorders such as systemic mastocytosis [9]. Serum tryptase concentrations >11.4 ng/mL indicate mast cell/basophil activation, and during anaphylaxis, levels correlate with severity. In patients with low baseline tryptase levels, levels may increase during anaphylaxis but still remain in the normal range; therefore, levels of 2 ng/mL plus 1.2 times baseline have been considered significantly increased [1]. Measurements of the histamine metabolite methylhistamine; prostaglandin D₂ and its metabolite 9α-11β prostaglandin F₂; and leukotrienes E₄ and C₄ have also been investigated; however, difficulties in obtaining 24-h urine samples in the scenario of ana-

Table 1. Signs and symptoms (*n*) reported by patients (*n* = 57) during their first episode of anaphylaxis

Cutaneous/mucosal signs/symptoms	
Skin rash	44 (77.2%)
Itching	44 (77.2%)
Angioedema	42 (73.7%)
Urticaria	33 (57.9%)
Respiratory signs/symptoms	
Dyspnea	51 (89.5%)
Glottis edema	45 (78.9%)
Dysphagia	34 (59.6%)
Nasal pruritus	30 (52.6%)
Cough	26 (45.6%)
Wheezing	26 (45.6%)
Stridor	22 (38.6%)
Hoarseness	22 (38.6%)
Cyanosis	20 (35.1%)
Gastrointestinal signs/symptoms	
Nausea	26 (45.6%)
Vomiting	16 (28.1%)
Abdominal pain	11 (19.3%)
Diarrhea	7 (12.3%)
Fecal incontinence	2 (3.5%)
Cardiovascular signs/symptoms	
Sweating	34 (59.6%)
Tachycardia	34 (59.6%)
Palpitation	32 (56.1%)
Hypotension	31 (54.4%)
Pallor	31 (54.4%)
Presyncope	20 (35.1%)
Chest pain	16 (28.1%)
Arrhythmia	9 (15.8%)
Shock	5 (8.8%)
Cardiopulmonary arrest	3 (5.3%)
Bradycardia	2 (3.5%)
Neurological signs/symptoms	
Dizziness	25 (43.9%)
Fatigue	22 (38.6%)
Mental confusion	21 (36.8%)
Headache	19 (33.3%)
Behavior changes	12 (21.1%)
Seizure	2 (3.5%)
Genitourinary signs/symptoms	
Uterine cramping	6 (10.5%)
Urinary incontinence	5 (8.8%)

Reported symptoms were in keeping with those described in the comprehensive review article on anaphylaxis by Castells [1].

phylaxis may hamper their practical use [7, 8]. Elevated IL-6 levels have been proposed as a marker of cytokine storm-type anaphylaxis [1].

In the present study, we aimed to investigate the role of serum basal tryptase (sBT) in the management of patients

with anaphylaxis and to establish its possible use in daily routine care by evaluating associations of sBT levels with the severity of anaphylactic episodes in a way that the findings could be used to predict a greater risk of new and potentially worse events. We also wanted to know whether sBT levels would vary among patients with anaphylaxis triggered by different factors, and whether sBT would be useful to screen our patients who had anaphylaxis for systemic mastocytosis and other mast cell disorders.

Patients and Methods

Patients

Patients participating in the present study were selected among those who attended the Allergy Clinics of the Clinical Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil (HCFMRP-USP). Patients were evaluated by allergy/immunology specialists of our medical staff and included in the study if they fulfilled the criterion of having had at least 1 episode of anaphylaxis based on their medical interview and analysis of medical records. Although it would be desirable that all patients who had an acute anaphylactic reaction would be promptly transferred to an allergy consultation from the emergency department, in our public system patients need a referral from their primary care doctors to allow for consultation at our specialty clinic. However, we issue high priority allergy consultation to all patients referred to us for evaluation of anaphylaxis.

Anaphylaxis was defined as a serious systemic allergic or hypersensitivity reaction following exposure to a known or unknown trigger involving 2 or more organs, or associated with hypotension/upper respiratory symptoms, that can be life-threatening or fatal [16–18]. Patients were divided into 4 groups according to the trigger factor responsible for their first episode of anaphylaxis: foods, drugs, venom of *Hymenoptera* insects, and idiopathic disease when the trigger could not be identified.

All patients or patients' caregivers agreed to participate in the present study and signed a written informed consent. Ethical approval was obtained from the HCFMRP-USP Ethics Committee and from Brazil's Platform of Ethics (protocol No. 3773/2016).

Questionnaire

Each patient completed a questionnaire addressing clinical and epidemiological aspects focusing on signs and symptoms during their first episode of anaphylaxis, treatment received, treatment beyond the anaphylactic reaction, including having or not self-injectable epinephrine, and simultaneous comorbidities. Signs and symptoms assessed in our questionnaire are outlined in Table 1. After data collection, the severity of the first anaphylaxis episode was defined according to Ring and Messmer [19]: grade I, only cutaneous symptoms; grade II, mild symptoms; grade III, moderate-severe symptoms; and grade IV, cardiac and/or respiratory arrest.

Serum Baseline Tryptase Levels

sBT levels were determined using the ImmunoCAP Tryptase immunoassay (Thermo Fisher Scientific, Uppsala, Sweden). Levels >11.4 ng/mL were considered elevated [20], and levels >20 ng/mL led to further investigation for systemic mastocytosis [21]. Tryptase

Table 2. Demographic and clinical characteristics of the patients with anaphylaxis ($n=57$) enrolled in the study

Median age (range), years	35 (7–87)
At the first episode	29 (1–82)
Gender, males/females	25/32
Mean tryptase levels (range), ng/mL	5.17 (2.57–21.19)
Severity of the first episode, n (%)	
Grade II	4 (7.0%)
Grade III	40 (70.2%)
Grade IV	13 (22.8%)
Trigger factors, n (%)	
<i>Hymenoptera</i> venom	17 (29.8%)
Food	13 (22.8%)
Drugs	13 (22.8%)
Idiopathic disease	14 (24.6%)
Anaphylactic episodes, n (%)	
1–5 episodes	41 (71.9%)
6–10 episodes	9 (15.8%)
11–20 episodes	6 (10.6%)
>20 episodes	1 (1.7%)

Anaphylaxis grades were defined using the criteria proposed by Ring and Messmer [19]: grade I, cutaneous symptoms; grade II, mild symptoms; grade III, moderate-severe symptoms; grade IV, cardiac and/or respiratory arrest.

Table 3. Types of acute and preventive therapies reported by patients with anaphylaxis

Therapy modality	Patients, n
<i>Acute treatment</i>	
Epinephrine	36 (65.5%)
Antihistamines	43 (78.2%)
Corticosteroids	42 (75.0%)
β_2 -Agonists	18 (31.6%)
Oxygen	24 (42.1%)
Intubation	5 (8.8%)
Cardiopulmonary resuscitation	1 (1.7%)
<i>Preventive therapy</i>	
Allergen-specific immunotherapy	15 (26.3%)
Exclusion diet	13 (22.8%)
Desensitization	2 (3.5%)
Anti-IgE	6 (10.5%)
Epinephrine auto-injector	16 (28.0%)
None	21 (36.9%)

Although all patients had prescriptions for epinephrine auto-injectors, only 16 were successful in fulfilling the prescription due to lack of availability and/or financial constraints. We provided epinephrine ampules to 3 of the patients who could not obtain epinephrine auto-injectors after weighing risks and benefits.

tase levels during the acute event(s) of anaphylaxis were not available in the present study. Most patients received care for their acute events in other hospitals or health care facilities before being referred to our specialty clinic at a reference tertiary hospital for investigation, so we could not obtain information on tryptase measurement accurately from the patients or from medical records. In addition, tryptase measurement is usually not part of the routine protocol in emergency departments in our country, in most cases due to financial constraints.

Statistical Analysis

Means, medians, standard deviations, and quartiles were calculated for each group, and Bayesian linear regression models were fitted to estimate differences in means, with respective 95% credible intervals (95% CrI), using the software OpenBugs.

Results

Clinical and Epidemiological Characterization

A total of 57 patients participated in our study, and 32 (56.1%) were female. Their median age was 35 years (ranging from 7 to 87 years), including 10 children (7–16 years old). Seven of the 57 patients (12.2%) had >10 episodes.

The age at the first anaphylaxis episode varied from 1 to 82 years, with a median of 29 years. Triggers of the first anaphylaxis episode were *Hymenoptera* venom in 17 patients; foods in 13 patients; and drugs in 13 patients. Fourteen patients were characterized as having idiopathic anaphylaxis. The most relevant triggers in the *Hymenoptera* venom group were wasp/hornet, bee, and fire ant, in decreasing order of frequency. Among foods, shrimp, fish, peanut, and milk/dairy foods were the most frequent triggers. Within the drug group, antibiotics (cefazolin and sulfadiazine) and anti-inflammatory nonsteroidal drugs (ibuprofen, parecoxib, and dipyrrone) were the dominant triggers. Moderate reactions (grade III in 40 patients) were most commonly found, followed by severe reactions (grade IV in 13 patients), and mild reactions (grade II in 4 patients). These data are summarized in Table 2.

Regardless of the severity of anaphylaxis episodes, the majority of patients had cutaneous/mucosal and respiratory symptoms simultaneously (55/57 patients, 96.4%). Next in frequency, patients presented cardiovascular (52/57 patients, 91.2%), neurological (44/57 patients, 77.1%), gastrointestinal (27/57 patients, 47.3%), and genitourinary symptoms (9/57 patients, 15.7%). Some patients reported unusual symptoms, including hypothermia, metallic taste, neck pain, peeling skin on hands and feet, sore throat, fluorescent vision, mouth ulcers, leg pain, paresthesia, and anasarca.

Table 4. Concurrent conditions and medications reported by patients with anaphylaxis

Asthma	21 (36.8%)
Rhinitis	34 (59.6%)
Atopic dermatitis	5 (8.8%)
Urticaria	16 (28.1%)
Hypothyroidism	5 (8.8%)
Use of β -blockers	1 (1.7%)
ACE inhibitor use ¹	1 (1.7%)
Food allergy (not the primary trigger of anaphylaxis)	28 (49.1%)
Eosinophilic gastrointestinal disease	2 (3.5%)

¹The same patient used a β -blocker and angiotensin-converting enzyme (ACE) inhibitor due to multiple comorbidities, including high blood pressure, diabetes mellitus type II, dyslipidemia, chronic renal disease, and previous stroke. The patient also had a pacemaker for atrioventricular blockade. There were no alternative pharmacological options suitable for his conditions.

Most patients were treated in the hospital/emergency room (50/57 patients, 87.7%), including 2 patients who needed treatment in the intensive care unit. However, 3 patients received care at a primary care unit (3/57, 5.3%), 3 patients self-administered medication at home (3/57, 5.3%), and 1 patient received treatment at a physician's office (1/57, 1.7%).

Regarding treatment received during this first episode of anaphylaxis, patients reported the use of antihistamines (75.4%) and corticosteroids (73.6%) most often. Epinephrine and β_2 -agonists were used in 63.1 and 31.5% of the patients, respectively. Twenty-four patients (42.1%) reported need of oxygen supplementation by nasal cannula or face mask, and 5 patients (8.8%) were intubated. One patient had cardiac arrest and was resuscitated by cardiopulmonary resuscitation protocol (Table 3).

Beyond the acute treatment, preventive treatment included subcutaneous allergen-specific immunotherapy in 15/17 (88.2%) patients with *Hymenoptera* venom-induced anaphylaxis; elimination diets for all patients with food-induced anaphylaxis; and desensitization for 2 patients with drug-induced anaphylaxis. In addition, 6 patients received anti-IgE therapy with the monoclonal antibody omalizumab, whereas 21 patients did not receive any kind of preventive treatment. Only 16/57 (28%) patients had epinephrine auto-injectors for reasons including financial constraints and previous advice to not use the medication. Three patients were given epinephrine ampules (Table 3).

Fig. 1. Serum baseline tryptase (sBT) levels in patients with anaphylaxis due to different triggers. Median values and 25th and 75th percentiles are represented. Median values are expressed above the boxes, as well as 1 outlier (21.19); this patient underwent further investigation of mastocytosis. Mean values of 4.93, 5.20, 5.41, and 5.24 ng/mL were found in patients who had anaphylaxis due to *Hymenoptera* venom, foods, drugs, and idiopathic disease, respectively.

Concurrent conditions and medications reported included asthma, rhinitis, atopic dermatitis, urticaria, hypothyroidism, and use of angiotensin-converting enzyme inhibitors and β -blockers. Allergy to foods not identified as the trigger of anaphylaxis was reported by 28 patients, and eosinophilic gastrointestinal disease was reported by 2 patients (Table 4).

Serum Baseline Tryptase Levels

sBT levels ranged from 2.57 to 21.19 ng/mL (mean 5.17 ng/mL), with no significant differences among patients who had anaphylaxis due to different triggers. Mean levels of 4.93, 5.2, 5.41, and 5.24 ng/mL were found in patients who had anaphylaxis due to *Hymenoptera* venom, foods, drugs, and idiopathic disease, respectively (Fig. 1). In the idiopathic group, there was 1 patient with an sBT level of 21.19 ng/mL (Fig. 1). At her initial evaluation for the study in July 2017, at the age of 40 years, this patient had had 8 episodes of anaphylaxis with no identifiable triggers, starting at the age of 23 years. She also presented with chronic urticaria refractory to antihistamines, leading to treatment with 300 mg omalizumab every 4 weeks. After the start of omalizumab therapy, her chronic urticaria was controlled, and the frequency and severity of anaphylactic episodes showed a marked decrease. Levels of sBT obtained in August 2017 (1 week after omalizumab injections) and repeated in September 2017 (4 weeks after omalizumab injections) were 21.17 and 13.25 ng/mL, respectively. She was promptly investigated for systemic mastocytosis at the Hematology Clinics in our

Hospital and underwent bone marrow biopsy, which was negative for mast cell disease.

Regarding the severity of anaphylaxis, mean sBT levels were 5.06, 4.67, and 6.61 ng/mL for patients with grade II, III, and IV anaphylaxis, respectively. Significantly higher sBT levels were observed in patients with severe anaphylaxis (grade IV) than in patients with more moderate disease (grades II/III), with mean levels of 6.61 and 4.71 ng/mL, respectively (mean difference = -1.92; 95% CrI -3.55 to -0.37) (Fig. 2).

Discussion

The use of tryptase measurement for the diagnosis of anaphylaxis is well established [13, 15, 22]. However, one single acute measurement of the tryptase level may not be decisive to establish the correct diagnosis of anaphylaxis. Baseline measurements are useful for allowing comparison to acute measurements, therefore enhancing diagnostic capacity. Although anaphylactic reactions to intravenous drugs have been shown to elicit greater increases in tryptase levels than oral triggers such as foods [13], our observational study has not identified significant differences in sBT levels and the types of triggers involved, but sBT levels were significantly higher among patients with more severe anaphylaxis. Our results are in keeping with those of Sahiner et al. [23], who showed the relevance of sBT levels in predicting the risk of anaphylaxis in food allergy: a cutoff of 5.7 ng/mL was associated with a 50%

probability of moderate-severe anaphylaxis. The work by Dua et al. [24] reinforced the importance of sBT levels as a tool for the comparison with acute measurements in food-allergic reactions, and showed a correlation of sBT levels with the severity of symptoms in food allergy. Yavuz et al. [25] reported similar findings in children with insect venom allergy, demonstrating that sBT levels ≥ 4.8 ng/mL were a significant risk factor for severe systemic reactions, supporting the role of sBT measurements as an instrument to help in deciding when to indicate preventive treatment with venom immunotherapy.

sBT measurements play also an important role in the investigation of mast cell-related diseases, particularly systemic mastocytosis [26, 27]. In the present study, only 1 patient presenting with multiple severe idiopathic anaphylactic episodes had sBT levels which prompted us to investigate mastocytosis. Although systemic mastocytosis was unlikely after bone marrow biopsy showing no abnormalities in mast cells, we could not exclude a mast cell activation syndrome (MCAS) as the underlying cause of symptoms in this patient. According to WHO criteria, additional findings including mast cells expressing CD2 and/or CD25 and presence of the *KIT* D816V mutation would help in the diagnosis of clonal MCAS [7, 28]. Nonclonal MCAS could also be suspected; however, we could not find common features of this condition in our patient, including IgE-mediated allergy, inflammatory and autoimmune diseases, neoplasms, Ehlers-Danlos syndrome, postural orthostatic tachycardia syndrome, bone abnormalities, or osteomuscular and central nervous symptoms, as described [7]. On the other hand, the elevated sBT level in conjunction with the clinical presentation of this patient was fundamental to indicate treatment with omalizumab, leading to clinical improvement, as previously described [28–31]. In addition, sBT levels have also been shown to be helpful to assess the risk for anaphylaxis in patients with confirmed systemic mastocytosis [32].

Recognition of the acute reaction and prompt epinephrine administration remain crucial issues in anaphylaxis. Results of our study indicate that there is room for improving anaphylaxis management. Epinephrine is recommended as first-line treatment for anaphylaxis, and its no-use is related to a greater occurrence of fatal outcomes [33–35]. There are strong reasons for that recommendation, since epinephrine reaches its maximal pharmacological effect within 10 min of intramuscular administration into the thigh, while antihistamines and corticosteroids have a delayed peak of action, and may not prevent respiratory and/or cardiovascular arrest [33]. In our study, the most common treatment modalities reported

Fig. 2. Serum baseline tryptase (sBT) levels according to the severity of anaphylaxis. sBT levels were significantly higher in patients with severe anaphylaxis (grade IV reactions, $n = 13$) than in those who had mild-moderate anaphylaxis (grades II and III reactions: $n = 4$ and $n = 40$, respectively). Mean levels are represented above the bars (mean difference = -1.92 ; 95% CrI -3.55 to -0.37).

by our patients were antihistamines and corticosteroids; epinephrine was used in only 65.5% of the patients during the acute episode of anaphylaxis. This observation is in accordance with other studies in the literature [33–35]. Most of our patients failed to fulfill one of the fundamental recommendations for anaphylaxis follow-up: to carry an epinephrine auto-injector all the time. There are many barriers for appropriate use of epinephrine auto-injectors [36], but in our country, the main factors are availability only through importation and financial barriers.

Our study has some limitations, including the small number of patients evaluated, and possibly the fact that more severe cases could have been selected, as a result of being a reference public hospital in the field. However, we tried to minimize these effects by choosing a diverse group of patients, comprising male and female subjects, as well as children and adults, and different triggers of anaphylaxis implicated, and our findings were in accordance with other studies. In addition, it was not possible to investigate the *KIT* mutation D816V in the peripheral blood of our patients, particularly in those with idiopathic anaphylaxis and *Hymenoptera* venom-induced anaphylaxis with normal sBT level in order to recommend a bone marrow biopsy appropriately [37].

In conclusion, our study showed that sBT levels were significantly higher among patients with more severe anaphylaxis, independent of the timing between sBT level measurement and anaphylaxis occurrence. Although the measurement of tryptase levels in the emergency setting has previously been identified as a suitable biomarker for anaphylaxis, high sBT levels may identify patients at increased risk of more severe anaphylaxis. Therefore, sBT should be routinely measured in all patients with a previous anaphylactic reaction. This is not usually performed, and it would be helpful to evaluate future risks.

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Statement of Ethics

All subjects or their parents or guardians have given their written informed consent.

Approval for the study protocol was obtained by the HCFMRP-USP Ethics Committee and Brazil's Platform of Ethics (protocol No. 3773/2016).

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

V.A. applied the questionnaires, collected clinical data, and prepared the first draft of the manuscript; M.M.D. carried out the tryptase measurements in the laboratory; J.M.L.M. selected patients and collected clinical data; O.T.-N. collected clinical data; D.C.A. helped with the design of the study and performed statistical analysis; L.S.M.M. collect blood samples and helped with tryptase measurements; A.S.M. supervised all the experiments in the laboratory and revised the manuscript; L.K.A. designed the study, supervised collection of clinical data, participated in the analysis of the data, and revised the manuscript to its final version. All authors approved of the final version of the manuscript and agreed with its submission.

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ANEXO 4

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Research paper

Gene mapping strategy for *Alu* elements rearrangements: Detection of new large deletions in the *SERPING1* gene causing hereditary angioedema in Brazilian families

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ABSTRACT

Background: Hereditary angioedema (HAE) is a rare genetic disorder mainly caused by mutations in the *SERPING1* gene, determining a deficit of C1 inhibitor (C1-INH). In approximately 10% of the cases, HAE with C1-INH deficiency (C1-INH-HAE) is caused by large gene rearrangements, which are not detected by Sanger sequencing. Here we present the exon quantification technique (EQT), a molecular diagnostic test for the detection of large genetic rearrangements in *SERPING1*, mapping the exact size and location of the deletion caused by the recombination of *Alu* elements. EQT analysis was performed on total DNA extracted from blood of patients belonging to two Brazilian families with a medical history of HAE, low plasma levels of C4 and C1-INH and no pathogenic alteration in *SERPING1* analyzed by Sanger sequencing.

Results: Two large deletions were found, one of 1356 pb and one of 1804 pb, which resulted from recombination of two *Alu* elements present in introns 3 and 4 of the gene.

Conclusion: These results showed that the EQT could be used as a simple, rapid, and efficient diagnosis test for analysis of large deletions and insertions involving *SERPING1*, otherwise not detected by Sanger sequencing, serving as a support technique for molecular diagnosis of HAE.

1. Introduction

Hereditary angioedema (HAE) (OMIM#106100) is a rare autosomal dominant genetic disease, with an estimated prevalence of 1 affected in every 50,000 people (ranging from 1: 10,000 to 1: 150,000 worldwide) (Roche et al., 2005a; Bygum, 2009; Lumry, 2013). In most cases, HAE is caused by mutations in the gene encoding C1 inhibitor (C1-INH), *SERPING1* (OMIM # 606860; GenBank NM_000062.2), leading to C1-INH deficiency (Cicardi et al., 2014; Drouet et al., 2018; Maurer et al., 2018). HAE with C1-INH deficiency (C1-INH-HAE) can be classified into two types, including HAE type 1, due to quantitative C1-INH

deficiency comprising 80–85% of cases, and HAE type 2, due to dysfunction of the protein, representing 15–20% of the cases. HAE-1 is caused by mutations distributed in all regions of the gene, resulting in defects of synthesis or secretion of the protein; therefore, quantitative levels of C1-INH are low. HAE-2 is mainly due to mutations at the final portion of exon 8, which codes for the active site of the molecule and presents with normal or even high quantitative levels of C1-INH, but reduced functional activity (Cicardi et al., 2014; Maurer et al., 2018; Germenis and Speletas, 2016). Approximately 20–25% of cases of HAE are caused by *de novo* mutations (Lumry, 2013; Germenis and Speletas, 2016).

Abbreviations: C1s, Complement C1s; C1r, Complement C1r; C1q, Complement C1q; C1-INH, C1 inhibitor; DNA, deoxyribonucleic acid; EQT, Exon Quantitation Technique; FECH, Ferrochelatase; HAE, Hereditary angioedema; MASP-2, Mannan Binding Lectin Serine Peptidase 2; MLPA, Multiplex ligation probe amplification; PCR, polymerase chain reaction; QMPSF, Quantitative Multiplex PCR Short Fluorescent Fragment; RFU, relative fluorescence unit

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C1-INH is an inhibitor of serine proteases belonging to the serpin family, and it is able to inactivate proteases of the complement system (C1r, C1s, MASP-2), contact system (factor XII, plasma kallikrein), and fibrinolytic pathway (plasmin, tissue plasminogen activator) (Davis et al., 2008). Decreased activity of C1-INH leads to the uncontrolled production of the pro-inflammatory mediator bradykinin, resulting in vasodilation, increased vascular permeability, and leakage of body fluids from vessels to the subcutaneous and submucosal tissues. This process results in recurrent episodes of non-pruritic edema, most often located on the face and extremities, gastrointestinal tract and upper airways, causing life-threatening edema (Cicardi et al., 2014; Maurer et al., 2018).

Diagnosis of HAE is based on clinical symptoms, family history and laboratory tests, including C4, quantitative levels and functional activity of C1-INH and C1q. Genetic testing is currently not performed routinely, however, due to the incomplete penetrance of the disease, it is of fundamental importance in children under one year old when antigenic and functional levels of C1-INH and complement measurements are inconclusive. In addition, its use is paramount in order to detect early asymptomatic patients, as well as to genetic counseling (Farkas et al., 2017) and for differentiation of HAE from acquired forms of angioedema. Early diagnosis is fundamental to decrease morbidity and mortality, and failure to diagnose HAE has been linked to increase in deaths due to laryngeal edema (Bork et al., 2012).

More than 500 mutations in *SERPING1* related to HAE have been described in the Human Gene Mutation Database (HGMD*) (Stenson et al., 2014). Missense alterations and small indels are responsible for approximately 65% of C1-INH-HAE, whereas large gene rearrangements are found in 10–20% of the cases (Germenis and Speletas, 2016). *SERPING1* intronic regions have 17 sequences of repeating elements (*Alu*), which may represent hotspots for non-homologous recombination events, making the gene prone to deletions, insertions and duplications (López-Lera et al., 2011; Germenis and Speletas, 2016).

Most of the disease-causing mutations in *SERPING1* can be detected by Sanger sequencing (Germenis and Speletas, 2016). However, to investigate large alterations, number-quantification techniques based on PCR-Multiplex, Multiplex Ligation Probe Amplification (MLPA) and Quantitative Multiplex PCR Short Fluorescent Fragment (QMPSF) are required (Schouten et al., 2002; Rouzier et al., 2014). In the present study, we have developed an Exon Quantitation Technique (EQT) for detection of large rearrangements of *SERPING1*, which was efficient as an additional method for molecular diagnosis for HAE in patients with clinical diagnosis of HAE-1/2 and no mutations in *SERPING1* identified by Sanger sequencing.

2. Methods

2.1. Ethical considerations

Informed consent was obtained from all individuals included in the study. This protocol was approved by the Research Ethics Committees of the Federal University of São Paulo (CAAE: 04032912.6.0000.5505) and the Clinical Hospital of Ribeirão Preto Medical School (Protocol No. 14521/2012).

2.2. Study subjects

Two index patients from Brazilian families presenting with C1-INH-HAE, with low plasma levels of C4 and C1-INH, had the eight exons of *SERPING1* sequenced by the Sanger technique as previously described (Cagini et al., 2016); however, no alterations were found. Clinical characteristics of these HAE patients and complement levels are shown on Table 1.

2.3. Levels of C1-INH and C4

C1-INH and C4 levels were measured by nephelometry (Dade Behring, Newark, Del., USA) and C1-INH activity was assessed using a chromogenic substrate method (Technoclone GmbH, Vienna, Austria). Reference values for tests were those provided by the manufacturers.

2.4. DNA extraction and quantification

Genomic DNAs were extracted from whole blood collected in BD Vacutainer® Blood Collection Tubes containing EDTA (Becton, Dickinson and Company) using the equipment QIAcube (QIAamp® DNA Blood Mini Kit Qiagen). Samples were quantified using NanoDrop® ND-1000 Spectrophotometer (Uniscience™), and DNA concentration was adjusted to 45 ng/μL when the purity of the genomic DNA ranged from 1.76 to 1.90.

2.5. Primer design

Geneious® Program Basic 6.1 (Biomatters) was used to design primers for the exons 2 to 8 of *SERPING1* (NG_009625.1) and produced fragments ranging from 200 bp to 488 bp (Table 2). All sense primers were labeled with a FAM fluorophore for subsequent analysis of the generated fragments by capillary electrophoresis. A pair of primers to amplify the exon 6 of ferrochelatase gene (*FECH* - NG_008175) present in chromosome 18 was used as internal control (Dobrovolsky et al., 2011). The primers were tested in different concentrations to obtain similar levels of fluorescence. The concentrations of the primers varied between 4.0 μM and 15.5 μM (Table 2). All primers were purchased from Exxtend Biotechnology Company (Campinas, Brazil).

2.6. Exon quantitation technique (EQT)

2.6.1. Multiplex PCR

Two multiplex PCRs were performed using four pairs of primers in each reaction to amplify the fragments/exons of interest. Multiplex PCR Mix-1 contained primers to amplify exons 2, 5/6 and 8 of *SERPING1* and multiplex PCR Mix-2 contained primers to amplify exons 3, 4 and 7. Both multiplex PCR-Mixes contained primers to amplify the exon 6 of *FECH*. The two multiplex PCRs were performed using GoTaq® kit Colorless 2 × Master Mix (Promega Corporation), 45 ng/μL of genomic DNA, primers (Table 2), and nuclease-free water to make up 20 μL of each reaction. Reactions were performed in Veriti® thermocycler (Applied Biosystems®) with an initial denaturation at 95 °C for 5 min, followed by 25 cycles of [95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s], and a final extension at 72 °C for 60 min.

2.6.2. Separation and quantification of fragments by capillary electrophoresis

Denaturation of 0.5 μL of multiplex PCRs was performed with 9.3 μL of formamide Hi-Di™ (Applied Biosystems®) and 0.2 μL of Standard size (GeneScan™ - 600 LIZ™ size Standard - Applied Biosystems®) for 3 min. at 95 °C, followed by –20 °C for 30 s. The fragments were separated and quantified in an ABI automated sequencer 3500xL Genetic Analyzer (Applied Biosystems®) using the protocol predetermined by the equipment manufacturer (FragmentAnalysis50_POP7). The amount of each fragment/amplified exon was captured and converted in relative fluorescence unit (RFU) using the GeneMapper software v.4.1 (Applied Biosystems®). Intrasample normalization was performed dividing the RFU of each *SERPING1* amplified fragment by the RFU of the exon 6 amplified fragment of *FECH*. Intersample normalization was performed by dividing the result of the first normalization by the mean of the control group. A reference interval was performed with the mean values obtained from the control group multiplied by the 95% confidence interval, then multiplied by the standard deviation. This reference interval was calculated for each fragment of *SERPING1* in

Table 1
Clinical symptoms and laboratory evaluation of HAE patients.

Patient	Age (years)	Gender	Age at onset of symptoms (years)	Duration of attacks (days)	Location of symptoms				
					Face	Lips	Extremities	Abdominal	Other locations
1	55	Female	20	3	Yes	No	Yes	Yes	Genitals
2	30	Female	25	3	Yes	Yes	Yes	Yes	Ear, elbow, knee

Patient	Parameters (normal range) ^a		
	C4 (0.01 to 0.04 g/L)	Quantitative C1-INH (0.021 to 0.039 g/L)	Functional C1-INH (80–120%)
1	0.06	0.09	54%
2	0.04	0.09	53%

^a C1-INH and C4 levels were measured by nephelometry and C1-INH functional activity was assessed using a chromogenic substrate method. References values for tests were those provided by the manufacturers.

comparison to the control group in triplicates.

2.7. Multiplex ligation probe amplification – MLPA

MLPA was performed using the Salsa MLPA Probemix P243-A3 *SERPING1* according to manufacturer's protocol (MRC-Holland, Amsterdam, Netherlands). In each MLPA reaction, four samples of control DNA were used in parallel with the tested sample and negative control (no DNA). Data were analyzed by Coffalyser software 9.4 (SALSA MLPA probemix P243-A3 *SERPING1* kit, MRC-Holland, Amsterdam, The Netherlands).

2.8. Long-range PCR and restriction enzyme analysis

A fragment of 6361 bp comprising part of intron 3, the whole exon 4 and part of intron 4 of the *SERPING1* gene was amplified from genomic DNA of control and HAE patients using the forward primer (5'-TCTCC TACCCAGCCCACTACT-3') and the reverse primer (5'-TGAAATGGGCC ACAGGTAC-3'). PCR was carried out using 12.5 µL of GoTaq® Long PCR Master Mix (Promega), 10 µM of each primer, 0.25 µg of genomic DNA and nuclease-free water to 25 µL, by incubating at 94.5 °C for 2 min, followed by 35 cycles of 93 °C for 20 s, 68 °C for 6 min and 30 s, followed by 72 °C for 10 min. Generated fragments were separated in agarose gel (1.5%), purified using QIAquick Gel Extraction Protocol Kit (Qiagen) and 300 ng of the purified fragments were subsequently digested with restriction enzymes *SmaI* (Invitrogen™), *SacI* (Invitrogen™), *NdeI* (Thermo Scientific) and *BglI* (Promega) in 20 µL reactions at 37 °C for 4 h.

Table 2
Primers for fluorescent multiplex PCR.

Primer	Sequence	Concentration (µM)	Size (bp)
C1INH_2FFAM	5' – GGGGAAAACAAAACAGAGGGA – 3'	4.0	331
C1INH_2R	5' – TTAATCTCAGCCACCGC – 3'		
C1INH_3FFAM	5' – TCTCTACCCAGCCCACTACT – 3'	3.5	352
C1INH_3R	5' – CCTCTACCCAGCCCACTACT – 3'		
C1INH_4FFAM	5' – CCAACCCCTCATTCCAA – 3'	4.0	265
C1INH_4R	5' – CCTTCAGAGTCTCTCAGGA – 3'		
C1INH_5.6FFAM	5' – CAACAGTGACGCCAACTGG – 3'	5.0	442
C1INH_5.6R	5' – TGAAATGGGCCACAGGGTAC – 3'		
C1INH_7FFAM	5' – CTGACTGATGCTTGTGAAA – 3'	15.5	488
C1INH_7R	5' – GCACGTACCCCTCTAACT – 3'		
C1INH_8FFAM	5' – ACCTTAACCTGTGTGGGCTG – 3'	4.0	200
C1INH_8R	5' – GACAGGGAACCTGTGTGCTC – 3'		
FECH6_FFAM	5' – TGCATCTACTAAGTGGTCTC – 3'	8.0 (1)	150
FECH6_R	5' – CACTGGCTTACCTGGATGAG – 3'	6.0 (2)	

FAM primers are fluorescence labeled primers for detection using ABI sequencer. The pair of FECH primers is used as a quantitative internal standard. (1) primer concentration used in Mix-1 (2, 5/6, 8 and FECH6) and (2) primer concentration used in Mix-2 (3, 4, 7 and FECH6).

2.9. Sequence of the deleted fragment

In order to define precisely the missing region of the mutated alleles, a smaller band was amplified using the forward primer (5'-TAT TGGGGGTGGGGTAGAGG-3') and reverse primer (5'-GAGTGCACTGG CGTCTATCTCG-3') and sequenced. The reaction was performed using 10.0 µL GoTaq 2× Master Mix (Promega), 0.5 µM of each primer, 45.0 ng/µL genomic DNA (patients 1 and 2, two control samples and a negative control) and water nuclease-free to complete the volume to 20 µL. Incubation was performed by an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 63 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 7 min. After electrophoresis on agarose gel at 1.5% (Invitrogen™), purification of the amplicon was performed using the Qiagen - QIAquick Gel Extraction Kit Protocol. The sequencing reaction was performed using BigDye™ Terminator Ready sequencing kit (Invitrogen™) in the ABI automated sequencer 3500xL Genetic Analyzer (Applied Biosystems®). The sequences were aligned with the reference sequence of the coding region of the *SERPING1* gene (NG_009625.1).

3. Results

3.1. EQT and MLPA analysis

Two female patients from Brazilian families with a clinical diagnosis of C1-INH-HAE were analyzed by EQT. Both patients present edemas located in some extremities of the body, crises frequency with a duration of three days, low plasma levels of C4 and C1-INH (Table 1). Sanger sequencing detected no mutations in the coding exons of

Fig. 1. Fragment analysis obtained by capillary electrophoresis. Electropherograms of PCR-Multiplex Mix 2 by EQT from (A) a healthy subject and (B) patient 1, which detected amplification of a single allele of exon 4 (black arrow).

Table 3

Interval of reference values observed for EQT fragment analysis or MLPA in accordance with the normalization for each *SERPING1* gene fragment/exon.

EQT	Exon 2	Exon 3	Exon 4	Exon 5,6	Exon 7	Exon 8
IR	[0.92; 1.08]	[0.89; 1.11]	[0.90; 1.10]	[0.95; 1.05]	[0.81; 1.19]	[0.82; 1.18]
1	0.94	1.06	0.50	0.99	1.01	1.01
2	1.05	1.09	0.50	0.90	0.93	1.15

MLPA	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7
1	1.00	1.01	0.55	0.99	0.99	0.99
2	0.99	1.03	0.58	1.14	0.99	1.12

IR = interval of reference; MLPA – IR [0.70; 1.30].

The IR of the MLPA is the same for all exons performed by the coffalyser 9.4 software. According to the manufacturer's standardization and instructions (MRC-Holland, Amsterdam, Netherlands).

SERPING1 of both patients. EQT revealed a 50% reduction in the fluorescence intensity of the fragments containing exon 4 as compared to the control, indicating amplification of only one of the alleles in this region (Fig. 1A and B). This deletion was also confirmed by the MLPA technique (data not shown). All the fragments containing the other exons were within the reference range by both techniques (Table 3).

3.2. Mapping the deletion in the *SERPING1*

Deletion of exon 4 in the *SERPING1* gene was also confirmed by long-range PCR technique. A 6361 bp fragment corresponding to the normal allele and a shorter fragment (4557 bp) related to the deleted allele were obtained (Fig. 2A). Both the long and short fragments were digested with restriction enzymes *SmaI*, *ScaI*, *NdeI* and *BglI* (data not shown). Based on the restriction pattern, the deleted regions in the alleles of both patients were determined. A PCR flanking for the deleted region was performed and Sanger sequencing of the generated

fragments (Fig. 2B) revealed that a recombination event between *Alu* elements was responsible for the deletion (Fig. 3). From these results, it was possible to identify the exact length (1804 bp for patient 1 and 1356 bp for patient 2) and the position of the deletion within the gene (c.550 + 428del1804 and c.550 + 695del1356 respectively for patient 1 and 2).

4. Discussion

Hereditary Angioedema is a disease that can be elicited, among other causes, by a quantitative and/or qualitative deficiency in C1-INH encoded by *SERPING1*. Presence of this gene in chromosome 11 within a region near the centromere may be one of the causes for high mutation rates in this gene (Germenis and Speletas, 2016). Fifty large deletions and six large insertions/duplications in *SERPING1* have been described in patients with HAE, listed in The Human Gene Mutation Database (HGMD*) (Stenson et al., 2014). In the present study, we have

Fig. 2. Large deletion in the *SERPING1* gene. (A) Amplification of DNA fragments of the *SERPING1* gene from a healthy volunteer (C) and patient 1 (P-1) by long-range PCR, showing the 6361-bp fragment and lacking a 4557-bp fragment of DNA, within the region of exon 4. The diagram below describes the two alleles in the patient, amplified with primers EQT-3F and EQT-5/6R, comprising one fragment with similar length to the normal allele, and one fragment corresponding to the deleted allele (heterozygosity). (B) Fragments amplified by PCR with primers flanking the deleted regions (424 bp for patient 1, and 872 bp for patient 2).

Fig. 3. Location and size of the deleted allele in the *SERPING1* gene from both HAE patients. The two repetitive *Alu* sequences (1 and 4, 2 and 3) and their position in the *SERPING1* gene are represented in (A) and (B), respectively. (A) Recombination of *Alu* 1 and 4 led to the deletion of 1804 bp in patient 1. (B) Recombination of *Alu* 2 and 3 led to the deletion of 1356 bp in patient 2.

successfully used the EQT, adapted from the Quantitative Multiplex PCR Short Fluorescent Fragment method (QMPSF) described by Duponchel et al. (2001) and Roche et al. (2005b), to search for large deletions in two patients with clinical diagnosis of C1-INH-HAE and no mutation detected by Sanger sequencing. Our *in-house* technique was efficient and enabled rapid detection of large deletions and insertions/duplications in *SERPING1*. This approach could be used for molecular diagnosis of HAE and other genetic diseases. Currently, Loules et al. (2018) used next-generation sequencing (NGS) technology to develop and validate a custom NGS platform to sequence the whole *SERPING1* gene aiming to facilitate genetic testing of C1-INH-HAE patients in the clinical practice. Using this NGS approach, the authors were able to

identify and localize simultaneously single-nucleotide variants and copy number variations (Loules et al., 2018) in the whole *SERPING1* gene. Despite these important advantages, this approach still requires laborious work and steps of validation of careful pipelines for bioinformatic analysis (Roy et al., 2018). On the other hand, the mapping of rearrangements using EQT assay is a cheaper, faster and more direct technique, requiring no extensive computational resources and presenting a sensitive and precise result.

Using EQT, we were able to detect large deletions including the whole exon 4 in patients from two different families. According to our results, these large deletions were caused by recombination promoted by two different *Alu* elements present in the exons 3 and 4 of *SERPING1*.

Alu elements are mobile repetitive sequences frequently involved in genomic rearrangements in the human genome due to their abundance and sequence identity. It is the most abundant transposable element in the human genome, one of the short interspersed elements (SINES), composing ~10% of the human genome and is related to approximately 0.1% of genetic diseases (Kim et al., 2016). Seventeen repetitive sequences have been detected within the seven introns of *SERPING1*, and most of these repeats are found in introns 4 and 6 (Germenis and Speletas, 2016). In the present study, we have detected two *Alu* recombinations not yet described in the literature. These *Alu* elements are at the beginning of intron 3 and in the middle of intron 4, causing recombination and deletion of 1804-bp fragments and 1356-bp fragments in patients 1 and 2 respectively, including exon 4. In addition to the size, it was possible to identify the exact position of the deletion in heterozygosity in both patients evaluated. According to studies conducted by Verpy et al. (1993), the protein formed by the allele with deletion of exon 4 would not be secreted, being retained in the endoplasmic reticulum due to the loss of 45 amino acids encoded by exon 4. This abnormality likely induces an abnormal bending of the protein, preventing it from being secreted. The deletion in exon 4 was confirmed by MLPA, which is the reference technique for detection of large deletions and insertions (Kozłowski et al., 2008; Johnsrud et al., 2015). However, MLPA is time-consuming and of high cost, hampering its utilization in many countries including Brazil. Therefore, this less expensive alternative technique described in this study could be very helpful for the molecular diagnosis of HAE in situations where Sanger sequencing is not appropriate to perform. In addition, it is noteworthy to emphasize that EQT, besides being a less expensive technique, could be very helpful to uncover mutations in families with large indels and to solve unsuccessful identification of variants associated with C1-INH-HAE.

5. Conclusion

In summary, in the present study, we developed an alternative method to detect large deletions and/or insertions in *SERPING1*, which has a high recombination rate from *Alu* elements. EQT, associated with Sanger sequencing and long range PCR comprises a precise method of mapping the size and exact location of the deletion promoted by the recombination of the *Alu* elements. Considering that Sanger sequencing does not detect these types of mutations, this mapping method is essential for identifying and diagnosing symptomatic patients and conducting family investigations of asymptomatic C1-INH-HAE carriers. Thus, we suggest that this internal, efficient and rapid method of the mapping strategy could be implemented in routine laboratories as one more tool for the precise molecular diagnosis of HAE.

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Conflict of interest

None declared.

Author contributions

Priscila Nicolicht designed and performed all experiments, performed data analysis, drafted the manuscript and approved the final version.

Douglas O. S. Faria helped in the design of the initial experiments; Luana S. M. Maia; Antônio A. Motta and Anete S. Grumach contributed with the initial data bank.

Douglas O. S. Faria; Leonardo Martins-Silva; Luana S. M. Maia; Adriana S. Moreno; L. Karla Arruda; Antônio A. Motta; Anete S. Grumach revised the manuscript and approved the final version.

João B. Pesquero designed the study, revised the manuscript and approved the final version. Corresponding author and guarantor for the article.

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ANEXO 5

Original Article

Hereditary Angioedema with Normal C1 Inhibitor and F12 Mutations in 42 Brazilian Families

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What is already known about this topic? Hereditary angioedema (HAE) with mutations in the *F12* gene (FXII-HAE) is a rare genetic disease with characteristics similar to those of angioedema with C1 inhibitor deficiency, which mainly affects female patients, increasing the expression and severity of symptoms.

What does this article add to our knowledge? The higher frequency of symptomatic male patients in the Brazilian population suggests that the gender penetrance previously described for FXII-HAE should be reconsidered. We report the highest number of families with FXII-HAE outside the European continent.

How does this study impact current management guidelines? Our results demonstrate the usefulness of genetic testing to diagnose patients presenting with clinical features of HAE with normal C1 inhibitor, including male patients, even in the absence of a family history or estrogen influence.

BACKGROUND: Hereditary angioedema (HAE) with normal C1 inhibitor (C1-INH) is a rare condition with clinical features similar to those of HAE with C1-INH deficiency. Mutations in

the *F12* gene have been identified in subsets of patients with HAE with normal C1-INH, mostly within families of European descent.

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Abbreviations used

ACEI-Angiotensin-converting enzyme inhibitor
 C1-INH- C1 inhibitor
 C1-INH-HAE-Hereditary angioedema with C1 inhibitor deficiency
 eOC-Estrogen-containing oral contraceptive
 FXII-Coagulation factor XII
 FXII-HAE-Hereditary angioedema with F12 gene mutations
 HAE-Hereditary angioedema
 HRT-Hormonal replacement therapy
 U-HAE-HAE with normal C1-INH of unknown cause

OBJECTIVES: Our aim was to describe clinical characteristics observed in Brazilians from 42 families with HAE and *F12* gene mutations (FXII-HAE), and to compare these findings with those from other populations.

METHODS: We evaluated a group of 195 individuals, which included 102 patients clinically diagnosed with FXII-HAE and their 93 asymptomatic relatives.

RESULTS: Genetic analysis revealed that of the 195 subjects, 134 individuals (77.6% females) carried a pathogenic mutation in *F12*. The T328K substitution was found in 132 individuals, and the c.971_1018+24del72 deletion was found in 2 patients. The mean age at onset of symptoms in patients with FXII-HAE was 21.1 years. The most common symptoms were subcutaneous edema (85.8% of patients), abdominal pain attacks (69.7%), and upper airway edema (32.3%). Of male individuals carrying *F12* mutations, 53.3% (16 of 30) were symptomatic. Compared with reports from Europe, fewer female patients (68.6%) reported an influence of estrogen on symptoms.

CONCLUSIONS: Our study included a large number of patients with FXII-HAE, and, as the first such study conducted in a South American population, it highlighted significant differences between this and other study populations. The high number of symptomatic males and patients with estrogen-independent FXII-HAE found here suggests that male sex and the absence of a hormonal influence should not discourage clinicians from searching for *F12* mutations in cases of HAE with normal C1-INH. © 2017 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2018;6:1209-16)

Key words: Hereditary angioedema; Normal C1 inhibitor; *F12* mutations; Estrogen; Brazilian population

Hereditary angioedema (HAE) is a rare condition characterized by uncontrolled activation of the contact system with excessive bradykinin production and recurrent episodes of angioedema.¹ In most patients, HAE is associated with mutations in *SERPING1*, the gene encoding C1 inhibitor (C1-INH). C1-INH deficiency renders the contact system prone to activation, resulting in increased production of bradykinin. HAE with normal C1-INH was first described in 2000^{2,3} and, in subsets of patients,⁴⁻⁶ it has subsequently been associated with mutations in *F12*, the gene encoding coagulation factor XII (FXII-HAE).⁷⁻¹⁰ Patients are designated as cases of unknown HAE when they display clinical features of HAE and belong to families with no identified mutation in *SERPING1* or the *F12* gene.¹

Patients with HAE and normal C1-INH present clinical features that are similar to those of HAE caused by C1 inhibitor

deficiency (C1-INH-HAE), including subcutaneous, gastrointestinal, and laryngeal edema. However, different characteristics are also apparent in patients with FXII-HAE, including a lower frequency of abdominal pain attacks and a higher frequency of facial and tongue swelling, a higher number of symptomatic female patients, marked estrogen sensitivity, and delayed onset of disease.^{11,12} In addition, hemorrhagic lesions at the sites of angioedema, which develop 1 to 2 days after the onset of swelling, have been reported in HAE with normal C1-INH, but they have not yet been described in patients with C1-INH-HAE.^{11,13}

To date, all mutations described in patients with FXII-HAE are located in exon 9 of the *F12* gene, and affect a proline-rich region of coagulation factor XII. The missense mutations T328K and T328R are associated with a defect in O-linked glycosylation of FXII, which has been shown to increase contact activation of zymogen FXII. This loss of glycosylation ultimately increases activation of the kallikrein-kinin pathway, leading to higher levels of bradykinin, without affecting C1-INH binding to FXII.^{14,15} New pathways leading to excessive bradykinin formation in patients with HAE that involve plasmin activation of factor XII have been reported recently, which could have implications for diagnosis and treatment of the disease,^{16,17} although further studies are needed to confirm these mechanisms.

More than 60 families with FXII-HAE have been described worldwide; however, the prevalence of FXII-HAE in a South American population has not been established. A recent study estimated the prevalence of FXII-HAE to be 1:400,000.⁶ In the Brazilian population, few families with HAE with normal C1-INH have been reported. Those reported include 2 unrelated FXII-HAE families with members carrying a homozygous T328K mutation.¹⁸⁻²² In the present study, we describe the clinical characteristics of 134 Brazilian individuals with FXII-HAE, who are from 42 unrelated families, and compare their clinical features to those reported in patients from other countries.

METHODS**Subjects**

After genetic testing, 102 Brazilian patients with recurrent episodes of subcutaneous edema without wheals, normal quantitative and/or functional C1-INH plasma levels, and no clinical response to high doses of antihistamines according to the criteria established by Cicardi et al¹ were included in this study. All patients who showed mutations in the *F12* gene known to be related to HAE were characterized as having FXII-HAE, a criterion for entering the study. Family history was a relevant factor for inclusion in the study; however, its absence was not an exclusion criterion. Clinical data regarding angioedema sites, triggering factors, and therapy were recorded using an established protocol (see Figure E1 in this article's Online Repository at www.jaci-inpractice.org). Symptomatic and asymptomatic family members were also evaluated. Patients were referred to this study from 9 different medical centers in Brazil. Approval for this study was obtained from the Ethics Committee of the Federal University de São Paulo (CAAE: 04032912.6.0000.5505), the Clinical Hospital of Ribeirão Preto Medical School (HCRP 14000521/2012), and the School of Medicine of ABC (CAAE: 51896015.0.1001.0082). All participants provided informed consent.

FIGURE 1. Distribution of FXII-HAE in Brazilian families. Of the 195 individuals analyzed from 42 families, 134 were found to have mutations in the *F12* gene. One hundred two *F12* mutation carriers were symptomatic and 32 were asymptomatic. The predominant mutation was T328K. F, Female; M, male.

Quantitative and functional C1-INH

Quantitative and functional C1-INH levels were estimated by nephelometry or radial immunodiffusion using a Human C1 Inactivator Bindarid Radial Immunodiffusion Kit (The Binding Site Group Ltd, Birmingham, UK) and colorimetric enzyme assay using a Technochrom C1-INH kit (Technoclone GmbH, Vienna, Austria), respectively.

Genetics

Blood samples were collected in EDTA, and DNA was purified using an Illustra blood genomicPrep Mini Spin Kit (GE Healthcare UK Ltd, Buckinghamshire, UK). Exon 9 of the *F12* gene (NG_007568) was amplified by PCR using primers (forward, 5'-CTA CCG GAA CGT GAC T-3'; reverse, 5'-CAT CGA AGA CAG ACT CT-3') that covered its exon-intron boundaries. PCR amplification was performed using GoTaq Green Master Mix (Promega, Madison, Wis) and the following cycle parameters: denaturation at 95°C for 5 minutes and 35 amplification cycles at 95°C for 30 seconds, 54°C for 30 seconds and 45 seconds, and 72°C for 10 minutes. PCR fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using an ABI Prism 3500xl Genetic Analyzer sequencer and BigDye Terminator v3.1 reagent (Life Technologies, Foster City, Calif). Chromatograms were analyzed in Geneious Basic version 8.1.7 (<http://www.geneious.com>).²³

The polymorphism *F12-46C/T*, present in the promoter region of *F12* gene, was analyzed by sequencing with the following primers: forward (5'-GTG GGT ATT GTT GTA AGA TGC-3') and reverse (5'-CCA AGC TCA CCA GCA GGA AC-3').

Frequency of symptoms among females and males carrying *F12* mutations

Of the 42 unrelated families studied, further analyses were performed on a subgroup of 20 families. Families selected for the subgroup analysis were those with at least 2 members with *F12* gene mutation, and with family pedigree data available. This analysis was performed to determine the frequency of symptomatic females and males and did not include 14 of 42 families with an index case only, and 8 of 42 families lacking accurate data on family pedigrees, including uncertainties in relatedness and clinical status. GenoPro

software version 2.5.3.9 (2011) was used to construct family pedigrees.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6.04 for Windows, GraphPad Software, Inc, La Jolla, Calif; www.graphpad.com). The percentage of mutation-carrying patients was compared by sex using the Fisher exact test. Differences in the age of onset of disease according to sex, hormonal influence, and *F12-46C/T* genotype were compared by Mann-Whitney testing.

RESULTS

Genetic analyses

We performed genetic analyses of 195 individuals belonging to 42 unrelated families. One hundred two were symptomatic patients (86 females, 16 males) who presented pathogenic mutations in the *F12* gene that were previously associated with HAE, and 32 were asymptomatic relatives (18 females, 14 males) who were carriers of *F12* mutations. One hundred thirty-two patients (103 females, 29 males), distributed in 41 families, presented the pathogenic T328K mutation in the *F12* gene (Figure 1). Two siblings presented the deletion c.971_1018+24del72 (1 female, 1 male), and the homozygous T328K mutation was found in 2 unrelated patients (1 female, 1 male), as previously published.^{21,22}

The presence of the polymorphism *F12-46C/T* was analyzed in 38 patients with symptomatic T328K FXII-HAE, and 9 patients were identified as having the CT genotype.

Characteristics of the patients with FXII-HAE

Overall, we had information confirmed by family members on 450 individuals. Clinical features of symptomatic patients are presented and compared with those reported in other large FXII-HAE studied populations in Table I.^{6,13,24,25} For the genetic screening in the present study, exon 9 of *F12* was Sanger sequenced using methods from the studies presented in Table I. This approach allowed the detection of all 4 currently described mutations, including missense mutations, a deletion, and a duplication. Interestingly, 5 patients (12% of the 42 families) reported no family history.

Age at onset of symptoms

The mean age ($\pm$ SEM) at onset of symptoms was 21.1 years ($\pm$ 11.7), ranging from 2 to 68 years, for the 97 patients who could identify the age at which they experienced their first angioedema episode. The onset of clinical symptoms occurred before the age of 12 years in 10 of 97 (10.3%) patients, between the ages of 12 and 25 years in 70 of 97 (72.2%) patients, and after the age of 25 years in 17 of 97 (17.5%) patients (Figure 2, A). The mean age at the onset of first symptoms was 20.1 and 26.3 years for female and male patients, respectively (Figure 2, B). There were no differences in mean ages at onset of disease among patients with the promoter region genotype CC compared with those with genotype CT (18.2 and 21.3 years, respectively).

Clinical symptoms

Most of the symptomatic patients presented subcutaneous swellings (85.8%) that affected the face (55.5%), lips (48.5%), periorbital area (15.1%), extremities (47.5%), genitals (11.1%), and other areas of the body (neck, ears) (7%). Attacks of abdominal pain were reported by 69 patients (69.7%), edema of upper airways affected 32 patients (32.3%), tongue swelling

TABLE I. Clinical and molecular characteristics of Brazilian patients with FXII-HAE compared with those in other populations

Characteristic	FXII-HAE Brazil (present study)	FXII-HAE France ²⁴	FXII-HAE Germany ⁶	FXII-HAE Spain ²⁵	FXII-HAE Spain ¹³
<i>F12</i> gene mutation carriers, n	134	57	104	35	35
Sex, n (%)					
Female	104 (77.6)	46 (81)	79 (76)	27 (77)	28 (80)
Male	30 (22.4)	11 (19)	25 (24)	8 (23)	7 (20)
Symptomatic, n (%)	102 (76.1)	19 (33)	35 (34)	6 (17)	13 of 35 (37)
Female	86 of 104 (82.7)	36 of 46 (78.3)	68 of 79 (86.1)	26 of 27 (96.3)	20 of 28 (71.4)
Male	16 of 30 (53.3)	2 of 11 (18.2)	1 of 25 (4)	3 of 8 (37.5)	2 of 7 (28.6)
Families, n	42	24	23	13	9
Family history, n (%)	37 (88)	16 (67)	22 (96)	12 (92)	6 (67)
Age at disease onset (y)	n = 97	n = 38	n = 61	n = 29	n = 16 F; n = 2 M
Mean $\pm$ SD	21.1 $\pm$ 11.7	21	20.3 $\pm$ 9.2	23.9 $\pm$ 8.9	19.9 F; 61.5 M
Median (range)	18 (2-68)	22 (8-40)	NA (1-65)	21 (14-55)	NA (14-28 F; 55-68 M)
Mutation (carriers/families)					
T328K	132/41	57/24	92/19	35/13	35/9
T328R	—	—	5/2	—	—
c.971_1018+24delT2	2/1	—	7/2	—	—
Location of swelling, n (%)	n = 99	n = 38	n = 61	n = 29	n = 16*
Subcutaneous	85 (85.8)	35 (92)	61 (100)	NA	NA
Extremities	47 (47.5)	(87)	30 (50)	NA	10 (62.5)
Face	55 (55.5)	(91)	61 (100)	NA	12 (75)
Lips	48 (48.5)	NA	NA	NA	14 (87.5)
Eyes	15 (15.1)	NA	NA	NA	10 (62.5)
Genitals	11 (11.1)	NA	9 (15)	NA	5 (31.2)
Tongue	12 (12.1)	NA	20 (33)	NA	7 (43.8)
Abdominal	69 (69.7)	(80)	40 (66)	10 (35)	14 (87.5)
Larynx	32 (32.3)	NA	15 (25)	5 (17)	14 (87.5)
Death from asphyxiation, n/total of symptomatic	1/102	0	2/69	NA	NA
Triggering factors, n (%)					
Unknown	20 (20.2)	NA	NA	NA	NA
Stress	48 (48.5)	NA	NA	NA	9 (56.3)
Trauma	47 (47.5)	NA	NA	8 (28)	9 (56.3)
Hormones	59 (68.6) [†]	34 (94)	48 of 58 (83)	25 (96)	13 (81.3)
Pregnancy	12 (20.3) [‡]	NA	43 of 92 (47)	NA	8 (61.5)
Estrogen-containing oral contraceptives	49 (83) [‡]	NA	43 of 48 (90)	NA	12 (92.3)
Surgery	6 (6)	NA	NA	NA	NA
Dental intervention	8 (8)	NA	NA	NA	3 (18.7)
Pressure	8 (8)	NA	NA	NA	9 (56.3)
Weather changes	2 (2)	NA	NA	NA	NA

NA, Not available.

*Analysis of 16 symptomatic females, excluding 4 paucisymptomatic females.¹⁴[†]Percentage of hormone-related events in relation to the total number of symptomatic female patients (86 Brazilian, 36 French,²⁴ 58 German,⁶ 26 Spanish,²⁵ and 20 Spanish¹³).[‡]Pregnancy occurrence and estrogen-containing oral contraceptive use (percentage calculated from the total number of patients who indicated a hormonal influence on their symptoms, n = 56).

was reported by 12 patients (12.1%), and uvula swelling was experienced by 2 patients (2%). Three patients presented edema of extremities only, and 9 presented edema affecting the lips and/or tongue only. Eight patients reported abdominal pain attacks only, and 1 patient reported laryngeal edema and no other symptoms. One patient reported spontaneous bruising preceding attacks of abdominal pain that disappeared just after edema was established. These lesions are not similar to hemorrhagic lesions or ecchymoses limited to the sites of the angioedema, which were described in some patients with HAE with normal C1-INH by Bork et al¹¹ and Piñero-Saavedra

et al.¹³ Coagulopathy was not identified or manifested in any of our patients with FXII-HAE.

Triggering factors

Stress and trauma were associated with swelling episodes in 48.5% and 47.5% of the patients, respectively (Table I). A hormonal influence was suspected in 59 of 86 symptomatic female patients (68.6%), with estrogen-containing oral contraceptives (eOCs) being the presumed hormonal trigger in most cases (49 of 59 [83%]) and exclusively in 43 of the 59 (72.8%) female patients (Table I). In 2 patients, the attacks were reported to worsen during menses, and, in

FIGURE 2. Age at disease onset in patients with FXII-HAE. Age (mean $\pm$ SEM) at disease onset in Brazilian patients with FXII-HAE was 21.1 years ($\pm$ 11.7) years, ranging from 2 to 68 years (A). The age (mean $\pm$ SEM) at onset of symptoms did not differ between male and female patients (B). Error bars in (Figure 2, B) indicate the SD.

1 patient, estrogen-containing hormonal replacement therapy (HRT) triggered the onset of HAE symptoms.

In 1 patient, symptoms began at the age of 68 years, after anti-hypertensive therapy with the angiotensin-converting enzyme inhibitor (ACEI) enalapril was initiated. He presented with recurrent episodes of angioedema of the face, lips, tongue, and genitals, which did not resolve with the withdrawal of ACEI and lasted for 5 years. A thorough clinical investigation included a molecular analysis of the *F12* gene, which revealed the presence of the c.971_1018+24del72 mutation. This mutation was also identified in the patient's asymptomatic sister.²² Treatment with tranexamic acid resulted in a marked reduction in the frequency and severity of angioedema episodes. Twenty symptomatic patients (20.2%) could not identify the factors precipitating angioedema.

HAE TREATMENT

Management of patients with FXII-HAE focused on the discontinuation of potential trigger factors, including eOCs,

HRT, or ACEI, and providing drug therapy, as recently reported by Bork et al.²⁶ eOC was discontinued in 44 female patients who reported worsening of symptoms with these medications, and 27 switched to progestin as a contraceptive medication and/or were prescribed progestin as a treatment. In 1 patient who had symptoms that began after HRT was initiated, withdrawal of this medication led to little improvement, and the patient required danazol for control of her symptoms. One male patient had persistent and frequent angioedema episodes, despite withdrawal of ACEI treatment; he was subsequently treated with tranexamic acid. Nine patients with acute attacks were treated with icatibant, and 4 patients were treated with plasma-derived C1-INH. All 13 patients showed a good response, with a marked reduction in the duration and intensity of attacks. Treatment modalities and clinical outcomes in our patients with FXII-HAE are presented in Table II.

Analysis of the frequency of symptoms according to sex within family pedigrees

Overall, relevant information was provided for 450 individuals by family members. A subgroup of 20 families, comprising 419 individuals (230 females, 189 males) with known clinical statuses, was selected for a pedigree analysis (see Figure E2 in this article's Online Repository at www.jaci-inpractice.org). Genetic screening was performed on 195 of 450 individuals in the cohort and on 164 of 419 individuals in the selected subgroup. Of the 164 individuals in the selected subgroup who were genetically analyzed, 118 were females and 46 were males, indicating a female:male ratio of 2.5:1. Females and males showed similar percentages of *F12* gene mutations, with 77 of 118 (65%) and 29 of 46 (63%) for females and males, respectively. Among females with *F12* gene mutations in the selected subgroup, 62 of 77 (80.5%) were symptomatic, whereas 15 of 29 (51.7%) males presented HAE symptoms. The frequency of symptomatic female and male patients within the overall group was 86 of 104 (82.7) and 16 of 30 (53.3%), respectively, similar to that of the 20-family subgroup. An example of a family pedigree is shown in Figure 3.

DISCUSSION

In the present study, we described the clinical features of Brazilian patients with FXII-HAE from 42 families, who were evaluated at 9 HAE reference centers coordinated by the Brazilian Study Group on Hereditary Angioedema (GEBRAEH). Since the first report of this disease in Germany, several families presenting FXII-HAE have been identified in France, Spain, Italy, the United Kingdom, Luxembourg, Turkey, Australia, and Morocco,²⁷⁻³⁸ with T328K as the most commonly associated mutation. In South America, few Brazilian families presenting FXII-HAE have been reported previously.¹⁸⁻²² Our results from Brazilian patients were compared with those from other large FXII-HAE cohorts in France, Germany, and Spain,^{6,13,24,25} and they suggested a higher proportion of symptomatic male patients and less dependence on estrogen influence in our patients with FXII-HAE.

The reasons for these significant differences within the studied populations are unclear. There are data to support the hypothesis that mutation p.Thr328Lys originated from a common founder.⁸ In keeping with this, we did not identify any FXII *de novo* mutations in our patients, and, to our knowledge, there are no reports of FXII *de novo* mutations leading to FXII-HAE in

TABLE II. Treatment modalities and outcomes of patients with FXII-HAE

Treatment	Procedure/Drug	Female (n)	Male (n)	Outcome
Discontinuation of triggering factors	Withdrawal of eOC	44	NA	Complete control or reduction in the number/duration of attacks
	Withdrawal of HRT	1	NA	No effect; required danazol
	Withdrawal of ACEI	0	1	No effect; required tranexamic acid
Long-term prophylaxis	Progestin	27	NA	Complete control or reduction in number/duration of attacks
	Tranexamic acid	30	2	
	EACA	1	1	
	Danazol	7	0	
	Oxandrolone	4	0	
Acute therapy	Icatibant	9	0	Marked reduction in the duration and intensity of attacks
	pdC1-INH	4	0	

EACA, Epsilon amino caproic acid; NA, not applicable; pdC1-INH, plasma-derived C1 inhibitor concentrate.

FIGURE 3. Pedigree of a large family with FXII-HAE. The index case (III-2) first developed symptoms at age 46 years after starting HRT. Her diagnosis prompted the genetic analysis of 45 of 96 family members, leading to the identification of a total of 19 family members with the T328K mutation. Of these, 9 (7 females, 2 males) were symptomatic. II.3 and IV.5 are obligatory carriers in the family pedigree.

families from other countries. The Brazilian population has a mixture of native, European, and African ancestry; however, a previous study analyzing 934 individuals from the 4 most populous regions of Brazil revealed that European ancestry was predominant, ranging from 60.6% to 77.7%.³⁹ It would be interesting to perform a large genetic study to track the origin of the Brazilian patients with FXII-HAE and to compare their ethnic makeup with that of these patients in a European population; however, this was beyond the objectives of the present study. Although the existence of different genetic markers in the European and Brazilian populations may account for our results, we could not rule out a role for phenotype-modifying environmental factors in the expression of the disease.

The proportion of patients reporting a hormonal influence on their symptoms in the present study (68.6%) was lower than that previously described in French (94%), German (83%), and Spanish studies (96% and 81.3%).^{6,13,24,25} Estrogen has been recognized as a regulator of the expression of genes encoding crucial proteins in the bradykinin release pathway, including the *F12* gene, but this regulation is not completely understood.⁴⁰⁻⁴² A study of the polymorphism *F12*-46C/T (rs1801020), located in the promoter region of the *F12* gene, was performed in 258 patients with C1-INH-HAE. The T allele, associated with

decreased FXII plasma levels,⁴³ was also associated with a delay in disease onset and to less need for long-term treatment.⁴⁴ In the present study, no association was found between this polymorphism and mean age at disease onset.

Our data suggest that the current major criterion for the diagnosis of FXII-HAE, family history, should be only one of the criteria for suspecting a case of FXII-HAE. Overall, family history was reported in only 58% of patients with HAE with normal C1-INH; conversely, 42% did not have a family history. This may be because of the high proportion of asymptomatic carriers with FXII mutations in families of patients with FXII-HAE, ranging from 23.9% in our own study to as much as 83% in European studies.^{13,24,25} Moreover, we found that it was very important to carefully ask about family history, particularly about the presence of mild symptoms or symptoms that might not prompt clinical suspicion of HAE, including recurrent episodes of abdominal pain. There is a debate between countries on when to genotype patients clinically suspected of HAE with normal C1-INH, and this may account for the variable frequency of detection of FXII mutations.⁴⁵ Our data indicate that the absence of a family history should not prevent a practicing physician from pursuing diagnosis of FXII-HAE by genetic testing.

Considering the lack of specific biochemical assays to detect FXII-HAE and the high frequency of asymptomatic carriers of *F12* gene mutations, a genetic study of the relatives of patients with FXII-HAE is recommended, especially before starting treatment with eOC, HRT, and/or ACEI. Indeed, sequencing of exon 9 of the *F12* gene is a fast and objective method to diagnose the disease, and it may help guide treatment in uncertain cases. In one example, genetic screening of a single family following diagnosis of the index case, who was participating in the present study, allowed the identification of a total of 19 individuals bearing the *F12* gene mutation T328K who had not been previously diagnosed with HAE. In addition to the index case (Figure 3), 8 patients were symptomatic (6 females, 2 males). Four were paucisymptomatic, including the 2 men; however, 4 patients presented recurrent episodes of moderate to severe abdominal pain and/or subcutaneous angioedema in the lips, hands, or feet that requires many visits to emergency departments or unscheduled doctors' consultations. Specific treatments comprising withdrawal of eOC or progestin and tranexamic acid therapy were prescribed for 3 patients, 1 of whom received icaltiban for an acute episode of abdominal pain, and one 9-year-old girl with recurrent abdominal attacks who was treated with tranexamic acid showed marked clinical improvement.

Limitations to this study are as follows: (1) The precise level of penetrance could not be established because of the lack of information on relatives of some of our study participants. Genetic analysis of 164 individuals with consistent clinical information indicated that 65% carried *F12* mutations; there was no difference by sex. However, 80.5% of females carrying *F12* gene mutations were symptomatic compared with 51.7% of males. Interpretation of these data may be limited by the fact that we tested 2.5 times more females than males in this study, despite inviting all susceptible individuals to be genetically screened. Nevertheless, in the 4 studies listed in Table 1, a total of 8 symptomatic FXII-HAE males in 69 families were reported,^{6,13,24,25} whereas 16 symptomatic FXII-HAE males in 42 families were identified in the present study. (2) Only patients with HAE with normal C1-INH and mutations in the *F12* gene (FXII-HAE) were included in our study. We did not study patients with HAE with normal C1-INH of unknown cause (U-HAE) because of the very small number of patients who fulfilled the criteria for this clinical condition. It would have been interesting to compare frequencies and characteristics of FXII-HAE and U-HAE among Brazilian patients; however, several factors prevented this analysis, including uncertain family histories, lack of data on the response to 4-fold doses of antihistamines, and inconsistent laboratory results. Recent reports identifying mutations in genes encoding angiotensinogen⁴⁶ and plasminogen⁴⁷ in individuals with a family history of U-HAE highlight the rapid advances in identifying genetic variants of HAE that allow a prediction of clinical outcomes, such as potentially severe tongue swelling in patients with HAE with a mutation in the plasminogen gene.⁴⁷ (3) Although this cohort of patients with FXII-HAE was large enough to allow for clinical characterization, sample size may have been a limitation in identifying associations between polymorphisms and clinical symptoms.

Our study determined clinical features in a large cohort of patients with FXII-HAE from 42 unrelated families that allowed for a comparison with European FXII-HAE cohorts described in

the literature. Findings of a larger number of symptomatic males and less estrogen influence in triggering symptoms in female patients than found in previous studies suggest that FXII-HAE is not a disease that only affects women as initially believed and that the disease should be considered in men with recurrent angioedema episodes. These differences between our population and others may be used to increase physicians' awareness and encourage them to search for a precise diagnosis by screening for genetic variants of the *F12* gene, and recently identified mutations in angiotensinogen and plasminogen genes in patients with HAE with normal C1-INH. Another contribution of our study is its demonstration of the importance of active genetic screening of families with FXII-HAE to identify both asymptomatic carriers of *F12* gene mutations and symptomatic patients for whom the diagnosis of HAE had not been considered.

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Initial evaluation for HAE patients

Personal Information

Date: ____/____/____ Code: _____

Name: _____

Birth: ____/____/____ Age: _____ Nationality: _____

Ancestry:

☐ European: _____ ☐ Amerindian ☐ Afrodescendant ☐ Oriental: _____

Race:

☐ White ☐ Black ☐ Brown ☐ Yellow ☐ Indigenous ☐ Other: _____

MD requester: _____ Institution: _____

e-mail: _____

CLINICAL DATA

Age at onset of symptoms: _____ ☐ Asymptomatic

Mean frequency of attacks before treatment: _____

Angioedema and associated symptoms (during entire life)

☐ Hands	☐ Lips	☐ Abdominal pain	☐ Other
☐ Feet	☐ Eyes	☐ Abdominal distension	☐
☐ Arms	☐ Tongue	☐ Diarrhea	☐
☐ Legs	☐ Uvula	☐ Nausea	☐
☐ Genitals	☐ Larynx	☐ Vomit	☐
☐ Face	☐ Dyspnea	☐ Urticaria	☐
☐ Neck	☐ Dysphagia	☐	☐
☐ Ears	☐ Erythema Marginatum	☐	☐

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FIGURE E1. Protocol for acquisition of clinical information on patients with FXII-HAE and family members. Information was obtained on demographic characteristics and clinical data including age at disease onset; frequency, severity, and location of HAE symptoms; diagnosis and treatment of HAE; trigger factors, comorbidities, laboratory tests, and family history. ER, Emergency room; FFP, fresh frozen plasma.

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Location	Frequency of symptoms			
	None	< 6 times/year	6-11 times/year	> 1 time/month
Hands				
Feet				
Arms				
Legs				
Genitals				
Face				
Neck				
Ears				
Lips				
Eyes				
Tongue				
Uvula				
Larynx				
Dyspnea				
Dysphagia				
<i>Erythema Marginatum</i>				
Abdominal pain				
Abdominal distension				
Nausea				
Vomit				
Diarrhea				

Dose:

Without prophylaxis:

without prophylaxis: ☐ 1 day ☐ 2 days ☐ 3 - 5 days ☐ 5 days ☐ > 5 days

With prophylaxis:

☐ 1 day ☐ 2 days ☐ 3 - 5 days ☐ 5 days ☐ > 5 days

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FIGURE E1. (CONTINUED).

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Severity of attacks

MILD	Discomfort noticed, but no disruption of normal daily activity
MODERATE	Discomfort sufficient to reduce or affect normal daily activity
SEVERE	Inability to work or perform daily activity. Emergency treatment: conservative, substitutive (C1-INH, FFP) Emergency treatment: invasive (intubation, tracheotomy)

Severity of attacks **BEFORE** treatment:

- ☐ Mild
☐ Moderate
☐ Severe

Severity of attacks **AFTER** treatment, **IN THE PAST YEAR**:

- ☐ Asymptomatic
☐ Mild
☐ Moderate
☐ Severe

Trigger Factors (before treatment)

- ☐ Stress ☐ Trauma ☐ Infections ☐ Dental Procedures ☐ Pregnancy ☐ Intercourse ☐ Exercise

☐ Food (specify): _____

☐ Medicine (specify): _____

☐ Surgeries (specify): _____

☐ Contraceptive pills or hormonal reposition (specify): _____

☐ Other _____

Prodrome:

- ☐ Yes ☐ No

Describe: _____

How long before the attacks?

- ☐ < 2 hs ☐ 2-24 hs ☐ Days _____

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FIGURE E1. (CONTINUED).

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Other diagnosis:

TREATMENT OF ATTACKS

- ☐ Treatment at home ☐ ER. How many times? _____
☐ Hospitalization. How many times? _____
☐ Intensive Care Unit, how many times? _____
☐ Invasive procedures. How many times? _____
☐ Intubation
☐ Tracheostomy ☐ Laparotomy
- ☐ Hydration ☐ Epinephrine ☐ Fresh frozen plasma
☐ Pain killers ☐ Tranexamic acid ☐ Icatibant (Firazyr®)
☐ Sedation ☐ E-aminocaproic ☐ C1-INH replacement (Berinert®, Cynriz®)
☐ Antihistamines ☐ Danazol ☐ _____
☐ Corticosteroid ☐ _____

PROPHYLAXIS

Drug	Dose	Administration Frequency
Tranexamic acid		
Danazol		
Aminocaproic acid		
Oxandrolone		
C1 Inhibitor		
Progestin		
Other		

Pregnancy☐ Yes ☐ No

Intercurrences: _____

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FIGURE E1. (CONTINUED).

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Comorbidities

☐ Cancer ☐ Diabetes ☐ Hypertension ☐ Thyroid disease ☐ Heart disease
☐ Asthma ☐ Rhinitis ☐ Drug allergies ☐ Other: _____

Drugs in use

Drug	Specify	Dose
Angiotensin-converting enzyme inhibitors (ACEI)		
Non-steroidal anti-inflammatories		
Angiotensin receptor blockers (ARBs)		
Oral contraceptives		
Other		

Biochemistry Measurements (without prophylaxis)

Exams	Data	Results	Reference value	Measured during attacks (yes or no)
C1-INH quantitative	___/___/___			
C1-INH functional	___/___/___			
C4	___/___/___			
C1q	___/___/___			
C1-INH quantitative	___/___/___			
C1-INH functional	___/___/___			
C4	___/___/___			
C1q	___/___/___			
C1-INH quantitative	___/___/___			
C1-INH functional	___/___/___			
C4	___/___/___			
C1q	___/___/___			
	___/___/___			
	___/___/___			
	___/___/___			
	___/___/___			

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FIGURE E1. (CONTINUED).

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Family History

Do you have relatives with HAE symptoms: ☐ Yes ☐ No
Who? _____

Relatives diagnosed with HAE by physicians: ☐ Yes ☐ No
Who? _____

FAMILY PEDIGREE

☒ ☒ Symptomatic male/female
☐ ☐ Asymptomatic male/female

Registered in ABRANGHE: ☐ Yes ☐ No

DIAGNOSTIC HYPOTHESIS

☐ HAE type I ☐ ACEI induced angioedema
☐ HAE type II ☐ Idiopathic histaminergic angioedema
☐ HAE with normal C1-INH ☐ Idiopathic non-histaminergic angioedema
☐ Angioedema due to acquired C1-INH deficiency ☐ _____

Observations:

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FIGURE E1. (CONTINUED).

FIGURE E2. Pedigrees of 19 families with FXII-HAE. Nineteen families from whom consistent data on family pedigrees were available underwent further analysis. This subgroup of 20 families comprised 419 individuals with information available and 164 underwent genetic analysis. Homozygous *F12* gene mutation T328K was present in family 2 (patient II.2) and family 3 (patient IV.32).²¹ *The following obligatory carriers were identified by analysis of the family pedigree: II.2, II.3, III.2, III.4, III.5, IV.1, IV.22, and V.40.

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FIGURE E2. (CONTINUED).

ANEXO 6

CLINICS

REVIEW ARTICLE

Brazilian Guidelines for Hereditary Angioedema Management - 2017 Update Part 1: Definition, Classification and Diagnosis

Pedro Giavina-Bianchi,^{I,*} Luisa Karla Arruda,^{II} Marcelo V. Aun,^I Regis A. Campos,^{III} Herberto J. Chong-Neto,^{IV} Rosemeire N. Constantino-Silva,^V Fátima R. Fernandes,^{VI} Maria F. Ferraro,^{II} Mariana P.L. Ferriani,^{II} Alfeu T. França,^{VII} Gustavo Fusaro,^{VIII} Juliana F.B. Garcia,^I Shirley Komninakis,^V Luana S.M. Maia,^{II} Eli Mansour,^{IX} Adriana S. Moreno,^{II} Antonio A. Motta,^I João B. Pesquero,^X Nathalia Portilho,^I Nelson A. Rosário,^{IV} Faradiba S. Serpa,^{XI} Dirceu Solé,^{XII} Priscila Takejima,^I Eliana Toledo,^{XIII} Solange O.R. Valle,^{VII} Camila L. Veronez,^X Anete S. Grumach^V

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Giavina-Bianchi P, Arruda LK, Aun MV, Campos RA, Chong-Neto HJ, Constantino Silva RN, et al. Brazilian Guidelines for Hereditary Angioedema Management - 2017 Update Part 1: Definition, Classification and Diagnosis. Clinics. 2018;73:e310

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Hereditary angioedema is an autosomal dominant disease characterized by recurrent angioedema attacks with the involvement of multiple organs. The disease is unknown to many health professionals and is therefore underdiagnosed. Patients who are not adequately diagnosed and treated have an estimated mortality rate ranging from 25% to 40% due to asphyxiation by laryngeal angioedema. Intestinal angioedema is another important and incapacitating presentation that may be the main or only manifestation during an attack. In this article, a group of experts from the "Associação Brasileira de Alergia e Imunologia (ASBAI)" and the "Grupo de Estudos Brasileiro em Angioedema Hereditário (GEBRAEH)" has updated the Brazilian guidelines for the diagnosis and treatment of hereditary angioedema.

KEYWORDS: Hereditary Angioedema; Angioedema; C1 Inhibitor Deficiency; Coagulation Factor XII Mutations; Management, Guidelines.

■ WHY STUDY HEREDITARY ANGIOEDEMA?

Since the first Brazilian Hereditary Angioedema Guidelines were published in 2011, the body of knowledge regarding hereditary angioedema (HAE) has increased, and its management has improved (1-6). More patients have been diagnosed with HAE, and new treatments for the disease have become available in our country. In this article, a group of experts in HAE from the Brazilian Association of Allergy

and Immunology (Associação Brasileira de Alergia e Imunologia - ASBAI) and the Hereditary Angioedema Brazilian Study Group (Grupo de Estudos Brasileiro em Angioedema Hereditário - GEBRAEH) have updated the guidelines for the diagnosis and therapy of hereditary angioedema.

Prof. William Osler, who established the hereditary nature of HAE, stated that "Medicine is both a science and an art". Developing and applying evidence-based guidelines is fundamental for the practice of medicine as a science; however, assisting individual patients with their biopsychosocial features in a personalized manner is practicing medicine as an art.

HAE remains unknown to and underdiagnosed by many health care providers. The long periods from the onset of symptoms to the diagnosis of the disease and from diagnosis to access to therapy increase HAE-related morbidity and mortality, thus affecting the quality of life (QoL) of patients and their families (1,2,5-9). HAE patients visit an average of

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4.4 physicians before receiving a correct diagnosis, and 65% of these patients are misdiagnosed (10,11). Although HAE accounts for a minority of cases of angioedema, and the initial estimate of its prevalence was 1:50,000 (ranging from 1:10,000 to 1:150,000), new subsets of patients with the disease have been described; thus, the disease is more common than previously thought (6,12-15). Physicians and other health professionals should be aware of the clinical presentation of HAE and of screening laboratory tests whose results may be suggestive of its diagnosis. In addition, allergy and immunology specialists must be updated with information on the diagnosis and management of patients with HAE.

The two most severe clinical manifestations of HAE are related to the upper airways and intestinal angioedema. Patients who are not properly treated have estimated mortality rates of 25% to 40% due to asphyxiation by laryngeal angioedema (3,5,12,16). In the United States, HAE accounts for 15,000-30,000 emergency room visits per year. These visits often lead to hospitalization and admission to intensive care units (17,18). Incapacitating intestinal angioedema is another major manifestation of HAE, and the intestine can be the main or only organ system involved in an attack. Such patients are often misdiagnosed as having an acute surgical abdomen and undergo unnecessary surgeries (12,19,20). It is estimated that patients with HAE experience some degree of disability for 20-100 days per year (4,9).

■ WHAT IS HEREDITARY ANGIOEDEMA?

Angioedema is a localized, self-limiting, asymmetric and disfiguring non-inflammatory edema of the deep dermis or subcutaneous or submucosal tissues that occurs as a result of vasodilation and increased vascular permeability. HAE comprises a group of diseases characterized by recurrent angioedema caused by excess bradykinin production, with an autosomal dominant inheritance pattern.

■ WHAT ARE THE TYPES OF HEREDITARY ANGIOEDEMA?

In an attempt to standardize nomenclature, an international panel of HAE experts issued a consensus statement in which they proposed a classification for "Angioedema without wheals" based mainly on the presence or absence of C1-INH deficiency (4). The following three forms of HAE have been defined:

1) HAE with quantitative C1-INH deficiency (previously designated C1-INH-HAE Type-I)

HAE characterized by a quantitative decrease in C1-INH expression to levels below 50% of the normal value and, consequently, a decrease in functional protein activity. This phenotype is the most prevalent form of HAE (80-85% of cases of the disease are caused by C1-INH deficiency) (21).

2) HAE with C1-INH dysfunction (previously designated C1-INH-HAE Type-II)

HAE in which C1-INH levels are normal or elevated, but the function of the protein is impaired (22).

3) HAE with normal C1-INH (previously designated HAE Type III)

This form of HAE, which was identified more recently than the other forms of the disease, affects mainly women

but has also been identified in men. This form is characterized by clinical symptoms similar to those of HAE with C1-INH deficiency, along with heritability and normal C1-INH levels and function (15,23). HAE with normal C1-INH has been associated with increased serum estrogen levels (pregnancy and exogenous administration) and mutations in the gene encoding FXII in a subset of patients (FXII-HAE). However, in a significant percentage of cases, the genetic defect remains unknown. Therefore, the following two subtypes have been recognized: HAE with normal C1-INH and FXII mutation (FXII-HAE) and HAE with normal C1-INH and unknown genetic defect (U-HAE) (1,2,5-9,24). It has been recommended that clinicians no longer use the term HAE type III because it is not associated with C1-INH deficiency.

■ WHAT IS THE CAUSE OF HEREDITARY ANGIOEDEMA?

Quincke first described HAE as a clinical entity in 1882 (25-27), and Osler established its hereditary nature in 1888 (28-32). The first biochemical change associated with the disease, C1 inhibitor (C1-INH) deficiency, was not identified until 75 years later. Patients with HAE due to C1-INH deficiency present with a quantitative or qualitative defect of the protein, a serine protease inhibitor from the SERPIN superfamily (33-35). The protein inhibits the first components of the complement system-classical pathway, C1r and C1s esterases, which bind to and activate C1q (36). In the absence of such inhibition, the complement system becomes excessively activated. C1-INH has also been recognized as an inhibitor of several proteases, including plasma kallikrein, coagulation factors XII (FXII) and XI, plasmin and MASP-1 and MASP 2, which are components of the complement system-lectin pathway. Therefore, C1-INH inhibits the complement system and participates in regulating the contact, coagulation and fibrinolysis systems (37-39).

Episodes of angioedema were initially attributed to factors formed during complement system activation, including a C2 fragment (C2 kinin) associated with vasodilation and increased permeability. Subsequent studies have revealed that C1-INH deficiency results in over-activation of the contact system (kallikrein-kinin system) and increased bradykinin production (Figure 1). Bradykinin binds to its B2 receptor, which is constitutively expressed on endothelial cells, and decreases endothelial junctions, thereby increasing vascular permeability and inducing angioedema (40,41). Bradykinin also stimulates nitric oxide production, leading to vasodilation induced by contraction of the cytoskeleton (42). Evidence indicates that bradykinin is the main HAE mediator (43,44).

In 1999, Nussberger et al. assessed plasma samples from patients with HAE and observed that bradykinin levels were higher in blood drawn from edematous sites than in blood drawn from other sites that were not affected by the disease (45). Another study showed that vascular permeability was lower in knockout mice with concomitant C1-INH and bradykinin receptor B2 (BDKRB2) deficiencies than in mice with only C1-INH deficiency, demonstrating that the bradykinin/BDKRB2 pathway plays an important role in angioedema (46). The investigation and development of new treatments, such as bradykinin receptor antagonist and kallikrein inhibitors, has reinforced the role of bradykinin as the main mediator of HAE (47,48).

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Figure 1 - Kallikrein-kinin system.

In conclusion, HAE with C1-INH deficiency occurs due to decreased levels of the protein or as a consequence of the production of a dysfunctional protein. Among patients with HAE, the levels of functional C1-INH typically range from 5% to 30% of the normal value. One would expect the functional level of the protein to reside at approximately 50% of the normal value because almost all patients are heterozygous for the mutation and thus bear a functioning allele of the C1-INH gene (4,12). The reasons for the lower-than-expected functional activity of C1-INH remain unclear. The above findings suggest that additional mechanisms may underlie HAE development.

In 2000, Bork et al. identified a new subset of patients with HAE with normal C1-INH levels (15). Mutations of the gene encoding coagulation factor XII (FXII), which segregated within the families of patients with HAE with normal C1-INH levels, were subsequently described. This HAE type was designated FXII-HAE (49). FXII plays a central role in the early stages of contact system activation by increasing bradykinin synthesis (50). Initial observations suggested that

mutations in FXII lead to gain-of-function changes and subsequent increases in bradykinin production; however, subsequent studies have failed to confirm these earlier findings, and the functional role of the observed mutations remains unclear (51,52). By generating recombinant natural and mutated variants of FXII, studies have recently shown that FXII-HAE mutations introduce new sites that are sensitive to enzymatic cleavage by plasmin, thus rendering FXII mutants abnormally sensitive to plasmin. The FXII mutants rapidly activate after cleavage by plasmin and escape from inhibition through C1-INH, leading to excessive bradykinin production. Interestingly, the authors provided evidence that lysine analogs, including tranexamic acid and epsilon aminocaproic acid, may attenuate these changes. This finding explains the therapeutic value of these agents in patients with FXII-HAE (53). However, not all patients with HAE with normal C1-INH levels bear a mutation in FXII and, thus, have HAE of unknown cause (U-HAE) (24). As is the case in HAE with C1-INH deficiency, HAE with normal C1-INH levels appears to be mediated by bradykinin.

Figure 2 - A: Warning Signs. B: HAAAAE for Heridity, recurrent Angioedema, recurrent Abdominal pain, Absence of urticaria, Absence of response to antihistamines and association with Estrogen.

Patients with HAE do not present urticaria, experience worsening symptoms with angiotensin-converting enzyme inhibitors (ACEi) and estrogen treatments, fail to respond to antihistamines and corticosteroids and improve with the use of the bradykinin receptor B2 blocker (4,23). Affected patients with HAE, especially those with normal C1-INH levels, show worsening symptoms with exogenous estrogen intake, such as that which occurs during contraception or hormone replacement therapy (23,54). The mechanisms underlying this deterioration are only partially understood. The promoter region of the FXII gene contains an estrogen responsive element, and increases in FXII mRNA transcription in response to estrogen have been demonstrated (55). It is likely that estrogen also contributes to the regulation of B2 bradykinin receptor expression, modulates the kallikrein-kinin cascade and reduces C1-INH levels (56,57).

■ WHAT ARE THE TYPICAL CLINICAL MANIFESTATIONS OF HEREDITARY ANGIOEDEMA?

The clinical history is an important component of the diagnosis of HAE, and it has been well characterized in the cohorts studied by Profs. Agostoni, Cicardi and Bork (2,3,5,11). Patients with HAE suffer from recurrent attacks of edema involving the skin and submucosa of several organs. HAE is not associated with urticaria and is nonpruritic, but patients sometimes describe experiencing a burning sensation in edematous regions. The sites most commonly affected are the face, extremities, genitalia, oropharynx, larynx and digestive system. However, rare clinical manifestations of the disease, such as intense headaches caused by brain edema, urinary retention and acute pancreatitis, may also occur (58).

The frequency and severity of the above clinical manifestations vary among individuals. It has been reported that 5% of patients with HAE are asymptomatic and that 25% develop sporadic symptoms (5,6,13,15). A retrospective study analyzing 131,110 attacks in 221 patients with HAE reported that laryngeal edema occurred in less than 1% of attacks, although over 50% of patients enrolled in the study had previously experienced this particular manifestation at least once (5).

HAE attacks typically last 48-72 hrs if left untreated and do not improve with administration of antihistamines, corticosteroids or epinephrine. Although many attacks occur spontaneously, several factors that trigger them have been identified, including minor trauma, stress, infection, menstruation, pregnancy, alcohol consumption, extreme temperature changes,

exercise, ACEi use and estrogen use (contraceptives and hormone replacement therapy) (2,3,5,11).

In some patients, erythema *serpiginosus* can occur as a prodromal manifestation preceding an HAE attack, but the concomitant presence of pruritic urticaria favors the diagnosis of histaminergic angioedema, making the diagnosis of HAE unlikely (5). Nevertheless, some cases of HAE accompanied by urticaria have been reported. In addition to the above phenomenon, irritability, weakness, nausea and "flu sensations" have also been reported as prodromal manifestations of the disease.

During adolescence, patients may experience substantial increases in disease activity. In particular, girls may experience increases in disease activity due to menstruation or the use of contraceptives containing estrogen. A family history of angioedema is suggestive of a diagnosis of HAE; however, this history may be absent in up to one-fourth of affected patients.

Although the clinical manifestations of HAE with normal C1-INH are similar to those of other types of HAE, there are some differences between them. Symptom onset generally occurs later in this type of HAE, and the course of the disease tends to be more benign than that of others. Moreover, tongue involvement is common. Bruising is occasionally seen at sites affected by angioedema. However, the most striking characteristic of HAE with normal C1-INH is its association with female gender and estrogen intake (24). We have generated a list of warning signs of the disease and have also devised an acronym to increase awareness of HAE. These items are presented in Figure 2.

■ HOW CAN LABORATORY TESTS CONFIRM THE DIAGNOSIS OF HEREDITARY ANGIOEDEMA?

Individuals clinically suspected of having HAE and those with a family history of HAE should be investigated (Figure 3). Serum C4 levels can be used as a screening test because quantitative or qualitative C1-INH deficiency leads to permanent complement system activation, which is accompanied by C4 consumption, even when patients are not experiencing an angioedema attack. C4 levels normalize during the inter-crisis period in only 2-5% of patients with HAE (3,5,10,16). However, determining C3 levels is unnecessary, as C3 levels are normal in patients with HAE because C3 has greater turnover than C4, and C1-INH does not directly regulate its activation. Additionally, C3 levels and activity are regulated by other factors, such as factors H and I from the alternative complement pathway.

Figure 3 - Algorithm of HAE diagnosis.

In addition to performing measurements of serum C4 levels, quantitative and functional evaluations of C1-INH should be performed. All health professionals and family members involved in providing care for patients with HAE must ensure that such tests are available. Although quantitative determination of C1-INH levels is relatively easy, evaluations of functional C1-INH activity (qualitative tests) must be performed at referral laboratories (12,14,18). Ideally, these tests should be performed immediately after sample collection to avoid degradation. However, as this is not feasible in most cases, reliable test results can be achieved when samples are properly stored and the tests performed with adequate methodology. When using the chromogenic functional assay, it is critical that samples are kept at -20°C in all steps of the process, including storage and transportation, for accurate results (59,60). It is mandatory to avoid freezing and thawing the same sample more than once for functional C1-INH evaluation. A functional activity test is usually performed only when the quantitative determination of C1-INH is normal (Figure 3). However, some studies suggest that functional activity could be a suitable screening test in addition to C4 level measurement, considering that functional activity would be decreased in all patients with HAE due to C1-INH deficiency (types I and II) (60).

If the clinical suspicion of HAE due to C1-INH deficiency remains in the presence of normal C4 levels, the test should be repeated during an episode of angioedema whenever possible, as C4 levels are occasionally (2-5%) normal between attacks (61). If the test result is again normal, and quantitative and qualitative levels of C1-INH are normal, a diagnosis of HAE with normal C1-INH is suggested, a condition in which all of these biochemical parameters are normal (17).

Analysis of *SERPING1*, the gene encoding C1-INH, may be performed in cases of undefined diagnoses or for research purposes. Mutations can be identified in one of the eight exons or exons/introns adjacent regions of the gene, which affect the production of the protein and/or its function. Not all mutations detected by routine genetic testing are disease-causing, and genetic testing of other affected and disease-free

family members is sometimes needed. Genotyping may be recommended whenever a discrepancy exists between a patient's clinical history and laboratory test results and in newborns or infants or specific situations, such as when patients with late-onset disease or no family history of disease present with clinical symptoms suggestive of HAE (62,63).

Factor XII gene mutations have been identified in a subset of patients with HAE with normal C1-INH. Initially, genetic sequencing detected two different missense mutations in exon 9: one encodes a threonine-to-lysine substitution (p.Thr328Lys), and the other encodes a threonine-to-arginine substitution (p.Thr328Arg) (49). Novel mutations have subsequently been published: a mutation featuring a deletion of 72 base pairs (c.971_1018 + 24del72), which was reported in a family originating from Turkey; and a mutation featuring a duplication of 18 base pairs (c.892_909dup), which has been described in patients from Hungary (64,65).

In acquired angioedema with C1-INH deficiency (C1-INH AAE), a condition associated with lymphoproliferative or autoimmune diseases, paraproteins induce the activation and consumption of complement components. Therefore, determining C3 and C1q levels, the latter of which are reduced in 75% of patients, may aid clinicians in differentiating between HAE and AAE. In addition to C1q consumption, anti-C1-INH antibodies may be observed in AAE associated with autoimmune disease (66).

■ WHAT ARE THE DIAGNOSTIC CRITERIA FOR HAE?

Criteria with which the diagnosis of HAE with C1-INH deficiency can be standardized have been proposed (Table 1). According to those criteria, the diagnosis of HAE is confirmed when patients meet one primary clinical and one biochemical disease criterion (12,19,20). It should be highlighted that the criteria are not absolute and that clinical history takes precedence over laboratory findings, particularly in localities in which laboratory tests are not available. In selected cases, a therapeutic test can aid in establishing the diagnosis of HAE.

The diagnosis of HAE with normal C1-INH is considered in patients with recurrent angioedema not associated with urticaria and with normal C1-INH activity and plasma protein levels. A history of one or more family members also being affected by the disease, along with a history of the disease occurring predominantly in female family members, makes the diagnosis more likely. At this time, the only confirmatory test for HAE with normal C1-INH is the study of the *F12* gene to identify one of the mutations that has been described (FXII-HAE) (9,17).

Table 1 - Diagnostic Criteria for Hereditary Angioedema with C1-INH Deficiency (12,26).

I – Primary clinical criteria
a) Non-inflammatory subcutaneous angioedema lasting longer than 12 hrs
b) Abdominal pain with no specific etiology lasting longer than 6 hrs
c) Laryngeal edema
II – Secondary clinical criteria
a) Family history of hereditary angioedema
III – Biochemical criteria
a) Quantitative C1-INH deficiency (<50%, in two distinct samples)
b) Functional C1-INH deficiency (<50%, in two distinct samples)
c) Mutation in the gene encoding C1-INH (<i>SERPING1</i>)

■ WHAT IS NOT HEREDITARY ANGIOEDEMA?

Two major pathophysiological mechanisms underlying the development of angioedema have been described. Angioedema can be induced by the activation of mast cells and/or basophils, resulting in the release of histamine and other mediators (histaminergic angioedema), and by an excess of bradykinin (bradykinin-mediated or non-histaminergic angioedema), as seen in HAE, acquired angioedema with C1-INH deficiency (AAE) and angioedema induced by ACEi and other drugs involved in bradykinin metabolism (4,29). A scheme in which angioedema is classified according to endotype has recently been proposed and may provide clinicians with insights into the etiology of the disease and/or the pathophysiological mechanism underlying the development of the different types of this clinical entity (Figure 4) (25,27,33-35).

The differential diagnosis for HAE encompasses other types of angioedema, mainly those with chronic or recurrent presentations. The most frequent type of recurrent angioedema is the histaminergic type, which is usually associated with urticaria. This type can be induced or exacerbated by the use of non-steroidal anti-inflammatory drugs (NSAIDs) (28,30-32,36). The presence of urticaria, the improvement of the disease with the administration of antihistamines, and the triggering of symptoms by NSAIDs intake make the

Figure 4 - Classification of Angioedema by Endotypes (25,27).

diagnosis of HAE less likely in a patient with angioedema. However, chronic histaminergic angioedema can present without urticaria, and NSAIDs are one of the major causes of angioedema without urticaria (25). In contrast, it is not necessary to avoid prescribing NSAIDs for patients with HAE, as the pathophysiological mechanisms underlying the development of angioedema are different.

The current guidelines for treating chronic spontaneous urticaria/angioedema highlight the fact that some patients will not respond to conventional doses of antihistamines and may need their dose increased to levels as high as four times the recommended daily doses to control their symptoms. Therefore, a trial with an antihistaminic agent administered at four times the recommended dose is warranted. This trial must last for a period of time sufficient to determine whether the angioedema has been controlled to confirm or rule out its histaminergic nature. The safety of up-dosing antihistamines, including cetirizine, levocetirizine, desloratadine, fexofenadine and rupatadine, has been documented in previous studies (67).

It is very important to ask patients about the use of ACEi when attempting to diagnose acquired forms of bradykinin-mediated angioedema. As ACE is the major enzyme involved in bradykinin degradation, its inhibition leads to increased serum concentrations of this mediator and may cause angioedema. ACEi should be withdrawn in all patients with recurrent angioedema, even if the angioedema developed several years after treatment onset. Up to 0.7% of individuals who take ACEi present with recurrent angioedema, and the risk of the disease is increased among African-descendants, smokers, elderly patients and females (68,69). ACEi-induced angioedema more frequently involves the face, tongue, neck and larynx than other sites; however, sporadic cases of abdominal attacks have been reported. The mean time for the onset of angioedema symptoms is 1.8 years after treatment initiation; however, symptoms occur in the first month post-treatment initiation in 25% of cases and may occur up to 10 years after ACEi treatment initiation (70). Although symptoms of angioedema induced by ACEi may resemble those of HAE, affected patients will have normal C4 and C1q levels and normal C1-INH levels and function. More rarely, angiotensin II receptor antagonists (ARBs) and gliptins, which are oral hypoglycemic agents that inhibit dipeptidyl peptidase IV, another enzyme involved in bradykinin catabolism, may also induce angioedema (71).

AAE is another form of angioedema characterized by C1-INH deficiency (C1-INH AAE); however, this disease is not inherited. In C1-INH AAE, the onset of symptoms occurs later, there is no family history of angioedema and the disease is caused by the consumption of C1-INH or the production of C1-INH-neutralizing autoantibodies, which are associated with lymphoproliferative disorders or autoimmune diseases, respectively (12,72,73). As a consequence, C1-INH activity is low, the complement system is activated and C1q levels are usually decreased, a particular feature that can aid disease diagnosis (74,75). Moreover, C1-INH function decreases to levels less than 50% of normal, and C1-INH antigen levels are generally decreased, although the presence of cleaved C1-INH may result in apparently normal C1-INH antigen levels in approximately 20% of patients (74,76). C1-INH AAE with autoantibodies and C1-INH AAE with lymphoproliferative diseases largely overlaps and should be considered the same disease (4).

Idiopathic non-histaminergic angioedema is characterized as non-hereditary and is diagnosed in cases in which all

known causes of angioedema have been excluded. Its symptoms persist despite treatment with high doses of antihistamines, doses that are typically as high as 4 times the standard doses of second-generation, non-sedating antihistamines. Some evidence exists indicating that bradykinin may be the mediator involved in the development of idiopathic non-histaminergic angioedema; however, this evidence is not definitive, as other vasoactive mediators, including cysteinyl-leukotrienes, prostaglandins and platelet-activator factor, may play a role in the development of the disease in some patients. Treating patients with idiopathic non-histaminergic angioedema is often a challenge in clinical practice, and no definitive recommendations for the treatment of such patients are reliable. The capacity of tranexamic acid and icatibant to alleviate symptoms has been reported in individual cases. Cyclosporine and omalizumab have also been used to treat affected patients, indicating that this group of patients is heterogeneous. We speculate that this classification may include patients with HAE with normal C1-INH levels and without a family history of the disease and patients with chronic spontaneous urticaria/angioedema resistant to antihistamines, given that up to 10% of these patients present with recurrent angioedema without urticaria (4,27,67).

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AUTHOR CONTRIBUTIONS

All authors meet the following three conditions: 1) All authors made substantial contributions to the conception of the study; 2) All authors contributed to drafting the manuscript and its critical revision for important intellectual content; 3) All authors provided final approval of the version to be published.

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ANEXO 7

PRACTITIONER'S CORNER

Life-threatening Reaction to Iron Dextran: Protocol for Induction of Tolerance

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Palabras clave: Alergia, Anafilaxia, Desensibilización, Dextran, Hierro.

Adverse reactions to iron dextran are usually delayed and consist of joint pain, vomiting, nausea, headache, and fever. Life-threatening reactions to iron dextran have been reported [1,2].

We report the case of a 48-year-old woman who experienced a severe reaction to iron dextran and who was able to receive the drug after undergoing a series of tolerance induction protocols.

The patient had celiac disease and could not absorb oral iron; therefore, she required parenteral iron. At the time of her first reaction, she had been pretreated with 10 mg of prednisolone and 10 mg of loratadine 1 hour before administration of parenteral iron. After receiving 300 mg of iron dextran she experienced a severe reaction consisting of maculopapular rash and hypotension. Administration was stopped. As part of the allergy workup, we performed a skin prick test and intradermal test with ferrous sulfate, ferrous lactate, and iron sucrose based on a previously reported protocol [3]. The results were negative.

Because the patient needed iron and there was no alternative route of administration, she was treated with iron dextran utilizing the protocol of Altman et al [4], with some modifications. The patient received the following drugs and doses: intravenous methylprednisolone 50 mg at 13 hours, 7 hours, and 1 hour before administration of iron dextran; intramuscular diphenhydramine (50 mg) and ephedrine (25 mg) 1 hour before iron dextran; and 20 mL of intravenous dextran (150 mg/mL) immediately before iron dextran. The modification consisted of 180 mg of fexofenadine without ephedrine. The target dose was approximately 2 g in 3 days. At the beginning of the protocol, iron dextran (50 mg in 100 mL of normal saline [NS]) was administered intravenously at 20 mL/h. Subsequent doses were administered at 22 hours (100 mg in 250 mL of NS at 40 mL/h), 28 hours (200 mg in 250 mL of NS at 50 mL/h), 47 hours (400 mg in 250 mL of NS at 60 mL/h), 52 hours (500 mg in 250 mL of NS 60 mL/h), 71 hours (500 mg in 250 mL of NS at 80 mL/h), and 75 hours (250 mg in 250 mL of NS at 80 mL/h).

The patient tolerated the first and second day with the increasing doses of iron dextran. On the third day, after the third hour of the infusion (60 mg/h), she experienced a maculopapular vasculitic reaction and edema in her legs. We stopped the protocol and treated the reaction with antihistamines and systemic corticosteroids. The skin lesion had resolved completely 24 hours later (Figure).

We then adjusted the protocol several times until we were able to administer iron dextran in NS every 3-4 months without a reaction. Our protocol required 10 doses, administered as follows: first dose, 0.1 mg in 0.5 mL; second dose, 0.2 mg in 1 mL; third dose, 0.5 mg in 2.5 mL; fourth dose, 1 mg in 5 mL; fifth dose, 2 mg in 10 mL; sixth dose, 5 mg in 25 mL; seventh dose, 10 mg in 50 mL; eighth dose, 20 mg in 100 mL; ninth dose, 50 mg in 250 mL; tenth dose, 100 mg in 500 mg. The total dose was 188 mg in 944 mL of NS. The first 7 doses were administered continuously for 15 minutes each. The eighth dose was administered over 30 minutes, the ninth over 60 minutes, and the tenth over 90 minutes (total time 4 hours and 45 minutes).

Administration of iron is sometimes necessary, even when the patient experiences severe reactions. Altman et al [4], reported the case of a 26-year-old man with chronic malabsorption and gastrointestinal blood loss resulting in iron deficiency anemia and anaphylaxis to iron dextran. Hickman et al [5] reported the case of a 58-year-old woman who developed irritable bowel syndrome after subtotal gastrectomy and severe iron deficiency anemia because of intolerance to impaired absorption of oral iron. The patient experienced a life-threatening reaction after intravenous infusion of iron. The same protocol was used in both cases, and the patients were able to receive 2 g of iron dextran without experiencing a reaction.

In the case we report, serum ferritin levels were 6 ng/mL (15-186 interval range) and hemoglobin 7 mg/dL before the protocol of Altman et al [4] was applied. During administration, the patient received a total dose of 800 mg of iron dextran. After

Figure. A, Skin reaction during the protocol. B, Patient's skin 24 hours after infusion of iron dextran.

2 months, ferritin levels were 106.39 ng/mL and hemoglobin levels were 14 mg/dL, although we were unable to reach our goal of 2 g because the patient experienced a severe reaction. We had to modify the protocol several times before we were finally able to administer iron dextran without a reaction. The patient has subsequently received iron dextran on 3 occasions without experiencing new reactions.

It is difficult to define the mechanism or mechanisms involved in the reaction observed in the present case. Skin test results were negative, although the low sensitivity of the test prevents us from ruling out the involvement of IgE. Other potential mechanisms include formation of antibodies against dextran and a T-cell response or mast cell activation. Slow administration after premedication reduces the severity of a reaction by any of these mechanisms, although it is sometimes necessary to adjust the dose according to the patient's tolerance.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Perception of Bronchodilation Assessed by Visual Analog Scale in Asthmatics: A Real-life Study

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The visual analog scale (VAS) is a psychometric response scale used to assess subjective characteristics or attitudes in numerous diseases. The continuous (or "analog") aspect of the VAS differentiates it from discrete scales such as the Likert scale. The validity of the VAS was previously documented in the measurement of asthma symptoms in adults and children in both experimental and clinical studies [1-4].

Reversibility of airflow obstruction is pathognomonic of asthma, although bronchodilation (BD) testing is usually performed only in specialized clinics. We hypothesized that the patient's perception of his/her breathing assessed using the VAS was correlated with the response to BD. It was recently reported that the patient's perception of breathing assessed using the VAS might be considered an initial tool for assessment of the response to BD in children with asthma, mainly in those with overt bronchial obstruction [5]. Thus, in the present study, we investigated whether assessment of the patient's perception of breathing using the VAS could be useful when evaluating the response to BD testing in adults with asthma.

We performed a cross-sectional study of 73 consecutive patients with asthma (28 males; mean age, 39.1 years). The local ethics committee approved the study procedure, and all patients gave their written informed consent.

The inclusion criterion was a documented diagnosis of asthma, according to the GINA guidelines (<http://www.ginasthma.org>). The exclusion criteria were recent asthma exacerbation and the presence of acute upper and/or lower respiratory infections. Patients discontinued use of long-acting and short-acting bronchodilators, respectively, 12 and 4 hours before BD.

Spirometry was performed using a computer-assisted spirometer (Pulmolab 435-Spiro 235, Morgan; predictive values based on ECCS 1993) according to guidelines [6,7]. BD testing was performed with 400 µg of salbutamol; airflow obstruction was considered reversible if an increase in FEV₁ of at least 12% and 200 mL over baseline was achieved [7].

The VAS evaluation system comprised a 10-cm segment, upon which the patient is asked to indicate his/her perception of breathing by marking a point along the segment [7]. In this study, 0 corresponded to the worst breathing, while 10 corresponded to optimal, symptom-free breathing. The segment was without interval markers.

The Wilcoxon signed rank test was applied to assess any difference between the pretest and post-test VAS score. A receiving operator characteristic (ROC) curve was used to define a predictive optimal cutoff for the percentage increment in the VAS score for detecting positive BD response. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp).

Airflow obstruction was reversed after BD testing in 24 patients. The patients were therefore subdivided into those with a positive response (BD+) and those with a negative response (BD-).

The VAS score both at baseline and after BD testing was significantly higher in BD- patients than in BD+ patients. The difference in the VAS score was lower in the BD- group

(1.1 [1.5]) than in the BD+ group (2.1 [1.9]) (Figure). In both groups, a significant increase in the VAS score was observed after BD ($P<.001$).

The ROC curve analysis revealed a predictive optimal increase in the VAS score of 12.5% for BD+ patients. Sensitivity was 83.33%, specificity 55.10%, diagnostic accuracy 64.38%, and the area under the curve 0.71 (0.58-0.83) ($P=.004$) (Figure, D). The positive likelihood ratio was 1.86 and the negative likelihood ratio was 0.30, with a statistically significant ($P=.003$) diagnostic odds ratio of 6.14 (1.83-20.62). Therefore, patients with an increase in the VAS score of $\geq 12.5\%$ had a 6-fold greater probability of a positive response than those with an increase of $<12.5\%$.

Most studies that have investigated perception of respiratory symptoms were conducted in experimental settings, for example, patients with symptoms induced by bronchoconstrictor stimuli. Very few studies have evaluated the response to BD testing using the VAS score [4,5,8]. The first study to investigate the perception of airway obstruction induced by methacholine challenge and BD testing was

Figure. A, VAS values at baseline and after BD testing in patients with a negative response (BD-). B, VAS values at baseline and after BD testing in patients with a positive response (BD+). C, Difference in VAS values after BD testing in patients with a negative or a positive response. D, ROC curve for defining a cut-off VAS value to predict the response to BD testing. VAS indicates visual analog score; BD, bronchodilation; ROC, receiver operating characteristic.

performed by Baker et al [8]. The second study was performed on adult patients with allergic rhinitis [4]. A more recent study conducted in asthmatic children revealed that assessment of the patient's perception of breathing using VAS was a reliable tool for predicting bronchial reversibility [5]. Therefore, the present study was designed to confirm the possibility of using VAS to assess the response to BD testing in adult asthma patients in a real-life setting, namely, consecutive patients referred to an asthma clinic.

Our findings showed that VAS values increased significantly after BD in all patients. BD+ patients had more impaired lung function than BD- patients. This functional difference was well perceived by the patients: in effect, BD+ patients reported lower baseline VAS values than BD- patients. Interestingly, a significant increase in the VAS values was perceived after BD, even though there was a significant difference in the VAS score between the groups. This finding may prove to be clinically relevant, as it underlines the subjective ability to perceive variations in breathing.

The most important outcome of the present study was the definition of a cut-off value for changes in the VAS score after BD that enables us to predict bronchial reversibility. In this regard, an increase of 12.5% may reliably predict reversibility of airflow obstruction (area under the curve, 0.71; odds ratio, 6.14).

Assessment of BD testing using the VAS could make it possible to distinguish between patients with a positive and negative response to BD both at home and at the doctor's office. Therefore, a >12.5% increase in VAS values after BD could indicate that the patient should attend a specialized center for further assessment. In fact, many primary care centers do not have a spirometer or staff trained in the proper use of the device and interpretation of results.

Our study is limited by its cross-sectional nature and the fact that it was conducted in a real-life setting. The number of patients was also low. Therefore, further studies addressing these issues should be conducted.

In conclusion, the present study demonstrated that assessment of the perception of breathing using the VAS could be an initial step in the assessment of the response to BD in adult patients with asthma at home or in the doctor's office.

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Conflicts of Interest

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The Addition of Benzylpenicillin Does Not Increase the Skin Test Sensitivity Obtained With Classic β -Lactam Determinants

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The high prevalence of β -lactam allergy, together with the diversity of this family of antibiotics, all of which are capable of inducing allergic reactions, is an issue of worldwide concern [1,2]. It is essential to improve the accuracy of diagnostic methods, since most patients could be proven tolerant to β -lactams if appropriately evaluated [1]. Guidelines recommend skin testing with the major determinant of benzylpenicillin (BP), penicilloyl-polylysine (PPL), and the minor determinant mixture (MDM). In addition, the European Academy of Allergy and Clinical Immunology (EAACI) has proposed skin testing with amoxicillin (AX) and the culprit β -lactam, when this was neither BP nor AX [2]. In addition, owing to the increased prescription of the combination of AX with clavulanic acid (CLV) and the increasing frequency of selective reactions to this compound [3], it is recommended to use CLV for diagnosis.

Interest in skin testing using BP has recently grown, nourished by the limited availability of the main reagents (PPL and MDM) and the removal of the BP component from the MDM. Indeed, MDM, which was originally composed of BP, the hydrolysis product of BP (benzylpenicilloate), and its decarboxylated product (benzylpenilloate) was recently substituted in Europe by a purer and more stable product composed exclusively of benzylpenilloate (named DM) in order to comply with new regulations [4].

In previous studies, skin testing with BP appeared to improve sensitivity for the diagnosis of penicillin allergy [5,6]. However, the positive predictive value of this approach determined using a drug provocation test has not been established. The aim of the present study was to determine the diagnostic value of skin testing with BP in patients with a consistent clinical history of β -lactam allergy. We performed a prospective study of 97 consecutive patients evaluated for a history of β -lactam allergy (March-October 2014). Diagnosis was based on general EAACI guidelines [2], and

patients were classified as immediate reactors (<1 hour) or nonimmediate reactors (>1 hour) and as selective reactors (responding to 1 β -lactam, tolerating BP and/or AX) or cross-reactors (responding to BP). The institutional review boards approved the study, and patients gave their informed consent to participate.

Skin testing was performed using the prick method; if the result was negative, intradermal testing was performed [2] using PPL 1.08×10^{-3} mM, DM 1.6 mM, AX 20 mg/mL, CLV 20 mg/mL (Diater-Ferrer), and cephalosporins (CEP) 2 mg/mL. Skin testing with BP (Normon) at 10 000 IU/mL was performed in all patients and in 20 nonallergic controls with proven tolerance to BLs. ImmunoCAP (IDD Thermo Fisher Scientific) with BP and AX and the basophil activation test with BP, AX, CLV, and CEP were performed as described elsewhere [7]. Single-blind placebo-controlled drug provocation testing was performed using BP, penicillin V (PV), AX, AX-CLV, and/or CEP, as previously reported [2]. Regardless of the result of skin testing with BP, all cases with negative results to PPL and DM underwent a drug provocation test with BP (600 000 U). If the result was negative, the patient received a 2-day course of outpatient therapy with PV.

Of the 97 patients included, 23 were confirmed as allergic (23.7%) (Table). The median time between the last reaction and the study was 9.5 months, and the culprit drugs were AX-CLV in 15 cases, AX in 3, CEP in 2, BP in 1, and an undetermined BL in 2. Twenty-two patients experienced an immediate reaction presenting mainly as anaphylaxis (59.1%) or urticaria (36.4%). Among the immediate reactors, 12 had positive skin test results (6 AX, 2 CLV, 2 DM, 1 PPL, and 1 CEP), 9 had a positive basophil activation test result (5 CLV, 2 AX and 2 CEP), 8 had a positive drug provocation test result (4 BP and 4 AX), and 1 had a positive ImmunoCAP result to AX. Only 1 patient had a nonimmediate reaction (maculopapular exanthema 24 hours after administration of AX-CLV), which was diagnosed by a positive delayed intradermal test reading with AX. The diagnoses were cross-reactive BL allergy (7 patients [30.4%]), selective allergy to AX (9 patients [39.1%]), selective allergy to CLV (5 patients [21.7%]), and selective allergy to CEP (2 patients [8.7%]).

Skin testing with BP was positive in 2 patients, both of whom tolerated administration of BP and PV in the drug provocation test. Of these, 1 was finally considered nonallergic and the other (Table, patient 6) was an immediate selective reactor to CLV. These results indicate that skin testing with BP can induce false-positive results in patients with a history of BL allergy.

In the group of patients with cross-reactions and, therefore, allergic to BP ($n=7$), the sensitivity was 0.0%, as no patient was diagnosed based on a positive result in skin testing with BP. The specificity obtained from the control group was 100%. The positive predictive value and negative predictive value of skin testing were 0.0% and 92.6%, respectively.

In this prospective study, we provide a reliable estimation of the value of skin testing with BP in patients with BP allergy (cross-reactive group) in a population where AX and AX-CLV were the drugs involved in 78.3% of the confirmed reactions. Although the 2 cases with positive results in skin testing with BP found here were similar to those reported elsewhere [5], we found that these patients tolerated BP

Table. Clinical Characteristics and Diagnostic Test Results for Patients With Confirmed Allergy

Patient	Age, y	Culprit Drug	Initial Reaction	Interval Reaction-Event, mo	Type	Skin Test	ImmunoCAP	BAT	DPT	Allergic Diagnosis
1	57	AX-CLV	Urticaria	3	Immediate	(+) MDM	(-)	(-)	ND	CR
2	18	AX-CLV	MPE	10	Nonimmediate	(+) AX	ND	ND	(-) BP	SAX
3	40	AX	Anaphylaxis	120	Immediate	(-)	(-)	(-)	(-) BP (+) AX	SAX
4	55	AX-CLV	Urticaria	9	Immediate	(-)	(-)	(-)	(-) BP (+) AX	SAX
5	57	AX-CLV	Anaphylaxis	24	Immediate	(+) CLV	(-)	(+) CLV	(-) BP (-) AX	SCLV
6	22	AX-CLV	Anaphylaxis	1	Immediate	(+) BP	(-)	(+) CLV	(-) BP (-) AX	SCLV
7	38	BL	Urticaria	60	Immediate	(-)	(-)	(+) CLV	(-) BP (-) AX	SCLV
8	26	AX-CLV	Urticaria	3	Immediate	(-)	(-)	(+) CLV	(-) BP (-) AX	SCLV
9	47	AX-CLV	Anaphylaxis	1	Immediate	(+) AX	(-)	(+) AX	(+) BP	CR
10	67	AX	Anaphylaxis	60	Immediate	(+) PPL	(-)	(-)	ND	CR
11	66	BL	UD	UD	Immediate	(+) MDM	(-)	(-)	ND	CR
12	61	AX-CLV	Urticaria	1	Immediate	(-)	(-)	(-)	(+) BP	CR
13	52	AX-CLV	Anaphylaxis	0.25	Immediate	(-)	(-)	(+) AX	(+) BP	CR
14	45	AX-CLV	Anaphylaxis	1	Immediate	(+) AX	(-)	(-)	(-) BP	SAX
15	69	AX-CLV	Urticaria	264	Immediate	(+) AX	(-)	(-)	(-) BP	SAX
16	65	BP	Urticaria	168	Immediate	(-)	(-)	(-)	(+) BP	CR
17	19	AX-CLV	Anaphylaxis	12	Immediate	(+) CLV	(-)	(+) CLV	(-) BP (-) AX	SCLV
18	52	AX-CLV	Anaphylaxis	24	Immediate	(-)	(-)	(-)	(-) BP (+) AX	SAX
19	35	CEP	Anaphylaxis	406	Immediate	(-)	(-)	(+) CEP	(-) BP (-) AX	SCEP
20	32	CEP	Anaphylaxis	2	Immediate	(+) CEP	(-)	(+) CEP	(-) BP (-) AX	SCEP
21	50	AX-CLV	Anaphylaxis	6	Immediate	(+) AX	(-)	(-)	(-) BP	SAX
22	18	AX-CLV	Anaphylaxis	12	Immediate	(-)	(-)	(-)	(-) BP (+) AX	SAX
23	31	AX	Urticaria	8	Immediate	(+) AX	(+) AX	(-)	(-) BP	SAX

Abbreviations: AX, amoxicillin; BAT, basophil activation test; BL, β -lactams; BP, benzylpenicillin; CEP, cephalosporin; CLV, clavulanic acid; CR, cross-reactive; DPT, drug provocation test; MDM, minor determinant mixture; MPE, maculopapular exanthema; ND, not done; PPL, penicilloyl-polylysine; SAX, selective amoxicillin; SCEP, selective cephalosporins; SCLV, selective clavulanic acid; UD, undetermined.

and PV. This false-positive rate should be highlighted, since the results could lead to avoidance of penicillin, with the subsequent costs and potential side effects that result from the use of other non- β -lactam antibiotics [1]. Avoiding unnecessary treatment with β -lactams impacts patient morbidity by increasing the risk of multiresistant bacteria [8] and increases health care costs. This approach has been estimated to cost Can \$326.50 per patient in a hospital setting when antibiotics other than standard-of-care penicillin are used [9]. Moreover, as the negative predictive

value of skin testing with BP (92.6%) is lower than that of PPL (99%) [10], the usefulness of this approach should be reconsidered. It is important to remember that skin testing with all the reagents used in this study only confirmed the diagnosis in 47.8% of cases; in the remaining 52.2%, the basophil activation test, ImmunoCAP, or drug provocation testing was necessary, thus emphasizing the need to use a full assessment to confirm β -lactam allergy.

To conclude, we found no clear benefits of adding skin testing with BP to a diagnostic algorithm that already included

PPL and MD for a population where AX/AX-CLV were the main drugs involved in reactions.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Potentially Inappropriate Prescription in Patients With a History of Allergy to β -Lactam Antibiotics: A Health Care Challenge

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Palabras clave: Antibióticos betalactámicos. Prescripción inapropiada. Alergia a medicamentos. Antibióticos de amplio espectro. Diagnóstico de alergia.

β -Lactam allergy is the most widely reported type of drug hypersensitivity [1]. However, 80%-95% of patients with suspected β -lactam allergy have a negative result in diagnostic tests and, therefore, cannot be considered allergic [2]. Furthermore, the frequency of cross-reactivity between β -lactams is relatively low (approximately 10%), while allergies to specific drugs are frequently diagnosed [3].

Patients with suspected β -lactam allergy are often treated with broad-spectrum antibiotics such as vancomycin and fluoroquinolones, leading to unnecessary overprescription of these drugs. Overprescription in turn leads to an increase in the emergence of resistant strains [4], such as multiresistant *Pseudomonas* species, owing to the use of fluoroquinolones, or outbreaks of vancomycin-resistant *Enterococcus* species and methicillin-resistant *Staphylococcus aureus* [5]. Overprescription of second-line antibiotics is also risky (nephrotoxicity due to vancomycin or colitis induced by *Clostridium difficile* related to the use of fluoroquinolones) and expensive.

Despite the availability of validated diagnostic tests, many patients with suspected β -lactam allergy are classified as allergic without undergoing diagnostic tests [6].

According to international recommendations for the study of β -lactam allergy [7], skin tests are the primary diagnostic tool, complemented with *in vitro* tests such as the measurement of total and specific IgE and the basophil activation test. Furthermore, drug provocation testing is still necessary to reach a definitive diagnosis [8].

The aims of our study were as follows: (1) to confirm the real prevalence of β -lactam allergy in our setting; (2) to quantify overprescription of second-line antibiotics in patients with a history of β -lactam allergy; and (3) to assess the cost of replacing β -lactams with second-line antibiotics.

The study was conducted in 2 stages:

(1) Retrospective observational study in a tertiary hospital. Medical records of adult patients admitted during 2012 who had been prescribed levofloxacin and/or vancomycin (main alternatives in patients with β -lactam allergy) were reviewed. Moreover, we verified whether the prescription was a consequence of the suspected allergy or was indicated for the patient's condition. Patient data were reviewed and screened using the institutional electronic prescribing system (SAP Medication).

(2) In order to calculate the actual prevalence of allergy in our setting, the Allergy Unit conducted a protocol-based allergy study [7] in a representative sample of patients with suspected β -lactam allergy.

The assessment of the impact on costs (€/patient/day) due to the overuse of second-line antibiotics was calculated by taking into account the direct costs of the standard treatment (according to our institutional therapeutic protocols) and its alternative.

We reviewed 3668 medical records of inpatients admitted during 2012 who received levofloxacin and/or vancomycin. β -Lactam allergy was reported in 10.5% (n=368), although allergy was confirmed in the clinical history of only 3.26% of patients. Among these patients, 55% (n=212) were prescribed a second-line antibiotic as a consequence of the suspected allergy, while in the remainder, prescription was indicated for the patient's condition according to our hospital therapeutic protocols.

Among patients with suspected allergy (368 patients), a representative sample of 26 (calculated using the Wilson method, assuming a prevalence of 5%-10%, a confidence interval of 95% and an accuracy of 10%) was selected for the protocol-based study. The clinical history was taken, and blood sampling and the study were performed in the Allergy Unit.

Of the 26 patients, only 2 (7.7%; 95%CI, 6.2%-20.6%) had a positive diagnosis of β -lactam allergy; both patients were allergic to amoxicillin. Therefore, the real prevalence of β -lactam allergy calculated in the total sample of patients (n=3668) was 0.7%-2.1%.

Furthermore, potentially inappropriate prescription of second-line antibiotics, considering the upper limit of the confidence interval (20.6%), was close to 80%. In other words, 170/212 patients were prescribed a broad-spectrum antibiotic such as vancomycin and levofloxacin when a β -lactam could have been used as first-line treatment.

We also found that diagnostic tests are rarely used in our setting (3.26% of all patients with suspected allergy). The Figure shows the distribution of patients in the study.

As for cost, which was calculated in 191/212 patients (conditions with a defined standard therapy in our setting), the use of broad-spectrum antibiotics instead of first-line drugs led to a median increase of 49.3% in the cost of treatment (difference of €9.95/patient/day), which is consistent with results from other, similar studies [9]. However, our calculation was based only on direct costs and did not take into account costs derived from possible antimicrobial resistance or therapeutic drug monitoring.

Even though the emergence of multidrug-resistant bacteria is becoming a serious health problem, few studies have measured the real percentage of patients with allergy to

Figure. Distribution of Patients in the Study. PAS indicates protocolized allergy study; PIP, potential inappropriate prescription (ie, patient treated with vancomycin and/or levofloxacin who could have been treated with a β -lactam).
*Calculated by extrapolation of the lower and upper limit of the 95% confidence interval (6.2%-20.6%).

β -lactam antibiotics. In this sense, our study provides evidence from a specific setting. The study was limited to the evaluation of patients who were prescribed vancomycin and levofloxacin, because these are the main therapeutic alternative in β -lactam allergy; consequently, the total number of allergic patients might be higher. In addition, the protocol-based study was performed in a representative sample of patients and not in the entire population with a history of allergy.

Despite these limitations, the percentage of β -lactam allergy found (both real and estimated) is consistent with the prevalence rates published by other authors.

Our findings confirm that the real prevalence of β -lactam allergy among hospitalized patients is low, while the unnecessary prescription of broad-spectrum antibiotics in patients with suspected allergy is very high.

Park et al [10] performed a prospective study with the aim of assessing collaboration between allergists and pharmacists to reduce overprescription and concluded that interdisciplinary collaboration improves patient safety and optimizes antibiotic prescription. Similarly, Wall et al [6] found that the skin tests recommended by hospital pharmacists in patients with a history of β -lactam allergy can help to prevent inappropriate use of second-line antibiotics.

Therefore, our findings and the serious consequences of overprescription of second-line antibiotics indicate that multidisciplinary collaboration should be promoted to increase safe β -lactam prescription.

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Conflicts of Interest

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Genetic Analysis as a Practical Tool for Diagnosis of Hereditary Angioedema With Normal C1 Inhibitor: A Case Report

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Palabras clave: Angioedema hereditario. Angioedema hereditario con C1 inhibidor normal. Bradiquinina. Factor de coagulación XII. Gen *F12*.

Hereditary angioedema (HAE) is an autosomal dominant disease characterized by episodes of edema of the face, extremities, and larynx and recurrent attacks of severe abdominal pain due to edema of the intestinal mucosa [1]. Classic HAE is caused by deficiency of C1 inhibitor (C1-INH), leading to increased activation of the kallikrein-kinin pathway, with overproduction of bradykinin, which increases vascular permeability and induces angioedema [1]. Mutations in the gene coding for coagulation factor XII (*F12*) have been identified in a subset of patients who present a dominantly inherited disease with features similar to those of patients with HAE due to C1-INH deficiency (C1-INH-HAE) [2]. These patients have no abnormalities in complement parameters and no mutations in *SERPINC1*, the gene coding for C1-INH [2]. Symptoms are often worse in women taking estrogen-containing medications and pregnant women in C1-INH-HAE as well as in HAE with normal C1-INH [2]. We report the case of a patient with HAE and normal C1-INH and no family history of angioedema who remained undiagnosed for 19 years.

The patient was a 35-year-old woman with recurrent episodes of abdominal pain that started at the age of 16 years. The episodes were of mild to moderate intensity, although during the past 5 years, they had become severe and were associated with vomiting and diarrhea. The patient had to attend the emergency department on several occasions and required intravenous medication. On 2 occasions, abdominal computed tomography revealed ascites and edema of the intestinal wall (Figure, A and B). Despite being evaluated by several gastroenterologists and undergoing a comprehensive workup, the patient remained undiagnosed, and the abdominal pain attacks were attributed to irritable bowel syndrome. During these attacks, her blood tests showed normal amylase and lipase levels, elevated white cell counts, and

elevated C-reactive protein concentrations. No eosinophilia, anemia, or other abnormalities were present. Endoscopy revealed esophagitis involving the distal esophagus and mild pancreatitis; colonoscopy findings were normal. During the previous 5 years, she had also experienced episodes of edema of the lips and face lasting 48 to 72 hours, despite treatment with intravenous corticosteroids and antihistamines (Figure, C and D). She had received many prolonged courses of oral corticosteroids and continuous treatment with antihistamines, which did not prevent attacks. She had 1 episode of tongue edema, but no respiratory discomfort during the attacks.

Figure. Manifestations of HAE with normal C1-INH. A, Computerized tomography scan showing ascites (arrow). B, Large amount of fluid (**) in the pelvis. C, Angioedema of lips and face. D, Patient during attack-free period.

At the time she was attending our Allergy Clinic, she experienced episodes of abdominal pain and/or facial or lip edema every 2 weeks that required constant visits to the emergency department, with a considerable impact on her quality of life. When asked about use of oral contraceptives, she acknowledged that worsening of symptoms showed a relationship with the start of a combined estrogen/progesterone contraceptive. Her medical history revealed asthma during childhood, and current mild symptoms of allergic rhinitis. She was prescribed pantoprazole and pinaverium bromide by her gastroenterologist, with no improvement in the abdominal symptoms.

We considered a diagnosis of HAE very likely, discontinued the estrogen-containing contraceptive, and switched her contraception to a progestogen-only pill (desogestrel). This measure resulted in complete remission of symptoms (none during the past 12 months). C1-INH and C4 levels were normal, and C1-INH activity in the intervals between attacks was 109% by the chromogenic method (reference value, 70%-130%). A genetic analysis was performed based on allelic discrimination and sequencing of exon 9 and the adjacent intronic regions of *F12*, and the patient was found to be positive for the Thr328Lys mutation. Three of her family members were also found to carry Thr328Lys: her father and her 40-year-old sister were asymptomatic for angioedema and/or abdominal attacks, although her 24-year-old nephew had presented episodes of abdominal pain of mild to moderate intensity since childhood.

It is not unusual for patients with HAE to remain undiagnosed for several years after onset of symptoms [3]. The findings in the present case lead us to recommend the inclusion of HAE in the diagnosis of recurrent abdominal pain, which can be the initial clinical presentation in patients with HAE [4,5], and to point out the difficulties in diagnosing patients with HAE with normal C1-INH. According to a recently updated classification, the patient we report fulfilled the criteria for factor XII-HAE (FXII-HAE) [6]. This term has been recommended not only when there is family history of angioedema, but also in the absence of a family history. However, a major limitation of identifying *F12* mutations as a routine diagnostic test is the fact that mutations are not present in all cases of HAE with normal C1-INH [7,8]. In the absence of an *F12* mutation, the diagnostic possibilities would include HAE of unknown origin (U-HAE), when a family history is present but markers of genetic disease are absent, and idiopathic nonhistaminergic acquired angioedema (InH-AAE), which is characterized by the absence of a family history and of an underlying genetic defect [6]. However, a U-HAE patient could found an HAE family, and a lack of family history does not rule out this possibility, thus hampering confirmation of diagnosis. Although the pathogenic mechanisms for FXII-HAE, U-HAE, and InH-AAE are not fully understood, evaluation of patients categorized as HAE or AAE of unknown origin revealed that those with HAE experience earlier onset of symptoms and a higher frequency of laryngeal and abdominal attacks, whereas patients with acquired forms more often present angioedema of the face and oral mucosa, suggesting that these conditions may be intrinsically different [6].

In Brazil, we detected a higher frequency of the *F12* mutation in patients with HAE with normal C1-INH than previously reported (20%-30%) [7]. The Thr328Lys mutation was found in 17/40 (42.5%) nonrelated families with HAE and normal C1-INH levels and activity. Five of these families were previously described [8], including 4 with at least 1 member carrying the Thr328Lys mutation.

At individual patient level, definitive diagnosis is important for advice on treatment of acute attacks and prophylaxis, genetic counseling, and preventive measures [9]. No controlled studies have addressed treatment of patients with HAE and normal C1-INH, although case reports have shown success in treating acute attacks of angioedema with C1-INH concentrate, icatibant, ecallantide, progesterone, danazol, and tranexamic acid, which are used in classic HAE, thus reinforcing the likely role of bradykinin in the pathogenesis of HAE with normal C1-INH [2,10].

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Does the Effect of Smoking on Fractional Exhaled Nitric Oxide Vary by Race in Adults in a US National Survey?

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Palabras clave: Óxido nítrico exhalado. Fumar. Raza negra. Asma.

Asthma and chronic obstructive pulmonary disease are important causes of mortality and morbidity in adults [1,2]. Inflammation of airways is an important mechanism leading to airway obstruction in these common conditions [3]. Asthma mortality and morbidity are higher in black than white adults; some asthmatics are smokers. In atopic asthma, inducible nitric oxide (NO) synthetase is upregulated in respiratory epithelium and produces increased NO concentrations in exhaled air [4]. Alvarez-Puebla et al [5] have reported on considerations in interpreting fractional exhaled nitric oxide (FeNO), which has gained favor in identifying and monitoring eosinophilic airway inflammation in patients with asthma or chronic obstructive pulmonary disease being considered for inhaled corticosteroid therapy [5]. Little information is available on the racial differences in the well-known lowering effect of cigarette smoking on FeNO [4]. To estimate the difference in association between current smoking and FeNO between non-Hispanic black and white US adults, this study examined data from a large, US national sample.

The National Health and Nutrition Examination Survey (NHANES) is designed to assess the health and nutritional status of adults and children in the United States [6,7]. Response rates for 2-year study cycles exceed 75%. All participants/agents provided written informed consent. Serum cotinine was analyzed using an isotope dilution liquid chromatography tandem mass spectrometry method. Participants were categorized as not smoking if the serum cotinine level was less than 10.0 ng/mL and currently smoking if it was 10.0 ng/mL or greater. FeNO was measured using the Aerocrine NIOX MINO, a portable, handheld NO analyzer (Aerocrine AB). This analysis uses the average of 2 reproducible measurements [6,7]. A 2-sided *t* test was used to compare the means with 95% CIs of ln FeNO values. Multivariable linear regression modeling using Stata Version 11 assessed smoke exposure by history as a predictor of ln FeNO. Data analyses using Stata Version 11 accounted for the complex survey design.

Between 2007 and 2012, NHANES interviewed 30 442 participants and 29 353 underwent examination. Of these 17

404 were aged 20 and over, and 13 207 had 2 reproducible FeNO measurements. Those 12 557 who also had complete data on demographics and serum cotinine levels formed the analytic sample. Mean ln FeNO among non-Hispanic whites was 2.698 in nonsmokers and 2.132 (difference, 0.565) in smokers with no history of asthma. Mean ln FeNO among non-Hispanic blacks was 2.715 in nonsmokers and 2.232 (difference, 0.483) in smokers with no history of asthma. In weighted linear regression analyses controlling for age, gender, and asthma history, the interaction term for smoking and black race was not significant ($P=.357$). This finding was unchanged in sensitivity analyses excluding those with a history of asthma, smoking within 1 hour of testing, and controlling for consuming NO-rich foods before the test, or a respiratory infection in the week before the test.

The current study found that cigarette smoking was associated with lower FeNO compared with not smoking. However, the strength of this association did not differ between black and white non-Hispanics. The mechanism by which tobacco smoke exposure lowers FeNO may be that it increases arginase activity and therefore decreases arginine availability for NO production in the airways [8]. Others have suggested negative feedback of NO in smoke on inducible NO synthase [9]. A polymorphism in the NO synthase and related genes may modify the effect of smoking [9,10]. Strengths of the study include the large, representative sample of the US population, oversampling of blacks, and standardized measurement protocols with quality control checks. Limitations include lack of data on some possible confounders, such as recent exposure to ozone air pollution. In conclusion, a model for mean ln FeNO controlling for age, gender, and asthma history testing the interaction of cigarette smoking with race did not provide evidence for significant variation of the effect of smoking on FeNO between blacks and whites.

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Home-Based Oral Immunotherapy With a Baked Egg Protocol

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The increasing prevalence of food allergy means that allergen avoidance alone is no longer a viable management approach. There is an increasing need for widely available disease-modifying interventions [1]. Oral immunotherapy (OI) is one such approach, with promising results in milk, egg, and peanut allergy [2].

Egg allergy is common in young children and has been shown to be more prevalent and persistent than previously thought [3]. OI to hen's egg has been administered using raw egg [4], powdered or pasteurised egg white [5], dehydrated egg white [6], and lyophilized egg protein [7]. Despite allergenic differences between these preparations (processing and cooking alter the allergenicity of egg), the various forms enabled over 80% of patients to be successfully desensitized. However, these methods are more suited to research settings and are consequently not widely available in clinical practice. In addition, very low doses and specific flavorings or vehicles were required to enhance palatability [5].

Baked egg and cooked egg provide a more practical and palatable option, although their efficacy for developing tolerance to more allergenic forms of egg is uncertain [8]. Cooked egg has been used in OI either alone [9] or after powdered egg, which was deemed unsuitable for large daily doses over long periods [10]. Baked egg has not yet been used in OI. It is possibly more palatable to egg-allergic children, who generally do not like the texture or taste of whole egg, and more practical than whole egg, as it is eaten in biscuits or cakes and can therefore be stored for longer and served in small doses.

We developed a recipe for an egg-containing biscuit to be used in baked egg OI as proof of concept and report on its efficacy and safety in home-based, slow up dosing OI in children with persistent egg allergy.

We made square biscuits measuring about 1 cm thick and weighing 100 g, which were then baked at 150°C for 15 minutes and cut into squares measuring 1 cm × 1 cm. Each square weighed about 1 g. The recipe required 5 stages to allow for gradual increases in the amount of egg ingested at each successive dose without having to eat too large a portion of biscuit. The ingredients were plain flour (40 g at stages 1-4 and 80 g at stage 5), sugar (40 g at all stages), margarine (25 g

at stages 1-3, 15 g at stage 4, and none at stage 5), and egg (0.1 mL at stage 1, 0.5 mL at stage 2, 1.0 mL at stage 3, 10 mL at stage 4, and 50 mL at stage 5).

The first session was supervised in hospital with subsequent up dosing at home. The initial dose was equivalent to 125 µg of egg protein, which was increased daily over 60 days to a target maximum dose of 6.25 g of egg protein (Table). Once the maximum dose was achieved, patients underwent an open challenge with boiled egg to assess whether whole egg was also tolerated.

Fifteen children with IgE-mediated egg allergy (9 boys; median age, 11 years and 2 months; range, 6-17 years) were recruited at routine clinical appointments. All 15 children fulfilled the inclusion criteria of age >5 years and persistent IgE-mediated egg allergy with a positive skin prick test (ALK Abelló) result to egg white and egg yolk (>3 mm) and symptoms after eating baked egg evidenced by an allergic reaction in the previous 6 months or a positive open food challenge result. Additional atopic conditions reported included other food allergies (12 patients), allergic rhinitis (11 patients), eczema (9 patients), and asthma (4 patients). The worst previous symptoms with egg were predominantly oral (itchy mouth or throat), cutaneous (urticaria and angioedema), and gastrointestinal (abdominal pain, nausea, and vomiting). Only 1 patient experienced upper airway symptoms (throat tightness), and 1 experienced lower airway symptoms (cough and wheeze).

Eight of the 15 patients (53%) completed the OI program: 4 in the protocol target of 60 days, as they had no adverse effects or allergic symptoms, and 4 between 80 and 270 days. Of the remaining 7 patients, 2 could not tolerate the first dose without symptoms and thus remained on strict egg-free diets, and 5 achieved partial tolerance at days 10 to 47, allowing them to include previously avoided trace amounts of egg into their diets. All adverse reactions were mild and could be controlled by oral antihistamines where needed.

OI provides protection against egg allergy despite variations in the form of egg, although protocols based on uncommon forms (egg powder, liquid raw egg) are not suitable for clinical use, and rapid up dosing regimens are more commonly associated with allergic adverse reactions [10]. We sought to develop a therapy for egg allergy that would be safe and effective and immediately and widely available to patients in our clinical setting.

Baked egg (ie, egg cooked with wheat) has not been used as an up dosing preparation in egg OI. If effective as an OI preparation, it offers potential advantages. Perhaps the most obvious advantage is that the baked egg-allergic patient, who was traditionally instructed to avoid all forms of egg in his or her diet, is now instructed to do exactly the opposite. Eating eggs therefore in a form that they are most likely to encounter provides visual reassurance, where OI is successful, that the food is safe. Furthermore, as baked egg is a less allergenic form of egg, it is possible that a greater percentage of patients who have to avoid all types of egg will be successfully treated. Whilst baked egg tolerance does not provide protection against raw egg, regular ingestion of baked egg accelerates the resolution of egg allergy and can lead to tolerance of less well-processed and more allergenic

Table. Protocol for oral immunotherapy up dosing with baked egg biscuits

Egg Volume ^a	Day	No. of Biscuit Squares ^b	Amount of Egg Protein	Egg Volume	Day	No. of Biscuit Squares	Amount of Egg Protein
0.1 mL		Stage 1 ^c				Stage 4	
	1	1	125 (μg)	10 mL	32	2	25.00 (mg)
	2	2	250		33	3	37.50
	3	3	375		34	4	50.00
	4	4	500		35	6	75.00
	5	5	625		36	8	100.00
	6	6	750		37	10	125.00
	7	7	875		38	12	150.00
	8	8	1000		39	14	175.00
0.5 mL	9	9	1125		40	16	200.00
		Stage 2			41	20	250.00
					42	¼ biscuit	312.50
	10	2	1.250 (mg)		43	30	375.00
	11	3	1.875		44	35	437.50
	12	4	2.500		45	40	500.00
	13	5	3.125		46	½ biscuit	625.00
	14	6	3.750			Stage 5 (Fraction of biscuit) ^d	
	15	7	4.325	50 mL ^d	47	2/16	780.00
16	8	5.000	48		3/16	1.17 (g)	
17	9	5.625	49		¼ biscuit	1.56	
18	10	6.250	50		5/16	1.95	
1.0 mL		Stage 3				6/16	2.34
	19	6	7.500		51	7/16	2.73
	20	7	8.750		52	8/16	3.12
	21	8	10.00		53	½ biscuit	3.51
	22	9	12.50		54	9/16	3.56
	23	10	13.75		55	10/16	4.29
	24	11	15.00		56	11/16	4.68
	25	12	16.25		57	¾ biscuit	5.07
	26	13	17.50		58	13/16	5.46
	27	14	18.75		59	14/16	6.25
	28	15	20.00		60	Full biscuit	
	29	16	21.25				
	30	17	22.50				
31	18	23.75					

^aVolume of egg in stage recipe.^bBiscuit in stages 1 to 4 cut into squares measuring 1 cm x 1 cm.^cOne biscuit for stages 1 and 2, 2 biscuits for stage 3, 8 for stage 4, and 16 for stage 5.^dBiscuit in stage cut into quarters, then half, and then half again to 1/16 pieces.

forms of egg [1]. Indeed, such was our experience, as, at the end of our baked egg up dosing protocol, all patients who completed the protocol were also able to eat—and were therefore tolerant to—whole egg.

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Conflicts of Interest

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Different Clinical Phenotypes in 2 Siblings With X-Linked Severe Combined Immunodeficiency

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Severe combined immunodeficiency (SCID) is a heterogeneous group of disorders characterized by profound impairment of cellular and humoral immunity [1]. X-linked SCID (XSCID) is the most common form of SCID and is caused by mutations in *IL2RG*, the gene encoding the common γ chain (γ c) shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Patients with XSCID are characterized by very low numbers of circulating T and NK cells and an increased proportion of B cells. Although maternal T-cell engraftment is the most common reason for the presence of circulating T cells in XSCID patients [2], atypical cases of XSCID with mild disease and normal numbers of autologous T and/or NK cells have been reported. Patients were found to carry either specific missense mutations [3-5] or splice-site mutations [6,7] leading to severely reduced levels of functional γ c protein, although residual cytokine signaling and development of peripheral T and/or NK cells were also observed. In addition, spontaneous reversion of genetic defects was recently demonstrated to be the basis for the milder immunological phenotypes in patients with primary immunodeficiency [8].

We report on 2 siblings with atypical XSCID who showed different clinical and immunological features. They were born from nonconsanguineous Japanese parents. The clinical and immunological data of patient 1 have been reported elsewhere [9]. Briefly, patient 1 developed a generalized erythematous rash and alopecia at 1 month of age, followed by persistent cough, fever, hepatosplenomegaly, and lymphadenopathy. He was hospitalized because of failure to thrive, protracted diarrhea, and hypoproteinemia at 5 months of age. His clinical and laboratory findings (erythroderma, lymphadenopathy, hepatosplenomegaly, eosinophilia, low serum IgG, elevated serum IgE, and the presence of activated T cells) (Figure. A) were reminiscent of Omenn syndrome.

Figure. A, Immunophenotyping of lymphocyte subsets. B, Analysis of γ c expression on lymphocyte subsets. Solid peaks indicate control antibody. Open peaks defined by bold lines represent anti-CD132 monoclonal antibody. P1 indicates patient 1; P2, patient 2.

Microsatellite analysis ruled out maternal T-cell engraftment. A thymic shadow was present on the chest radiograph. The patient exhibited revertant T-cell mosaicism caused by a second-site mutation predominately in the skin [9]. Once the diagnosis of atypical XSCID (T⁺ NK⁺ B⁺) was confirmed using molecular techniques, patient 1 underwent cord blood transplantation but died of complications at 10 months of age.

The younger brother of patient 1 (patient 2) was diagnosed with XSCID at birth because of a positive family history and defective γ c expression in cord blood lymphocytes. A chest X-ray revealed no thymic shadow. Patient 2 had autologous NK cells but no T cells (T⁻ NK⁺ B⁺) (Figure, A) and, unlike patient 1, showed no manifestations of Omenn syndrome. He was free from infections on prophylactic antibiotic treatment and underwent successful cord blood transplantation at 46 days of age. He has been in good health with no evidence of disease for 5 years.

Both patients carried the same splice-site mutation at the donor site of intron 1 in the *IL2RG* gene (IVS1+5G>A). This mutation was listed in the *IL2RG* database (<http://research.nhgri.nih.gov/apps/scid/IL2RGbase.shtml>), and the mother was a heterozygous carrier. The mutation caused most of the mRNA to be incorrectly spliced but produced normally spliced transcripts in lesser amounts [9]. In the incorrectly spliced transcripts, the first 28 nucleotides from intron 1 were incorporated into exon 2 due to a cryptic 5' splice site in intron 1, resulting in a frameshift mutation and premature termination. Accordingly, the lymphocytes showed the residual γ c protein that likely enabled T- and/or NK-cell development to occur (Figure, B). Similar results were obtained in another case study of atypical XSCID with poorly functioning peripheral T cells [6]. As in the cases we report here, the patient in question carried a splice site mutation located at the end of exon 1 (129G>A). This mutation caused the same aberrant mRNA splicing, resulting in the generation of both an abundant, nonfunctional isoform containing the first 28 bp of intron 1

using the same cryptic 5' splice site and a normally spliced, functional isoform with a missense mutation (D39N) in limited amounts.

The levels of residual γ c expression were similar in B and NK cells from both patients (Figure, B). T cells from patient 1 also showed γ c expression at levels comparable to those of his B and NK cells. The reason underlying the appearance of autologous T cells in the older brother only is unclear. Peripheral T cells obtained from patient 1 at 7 months of age showed normal absolute numbers ($3.7 \times 10^9/L$), an activated phenotype, and a moderately restricted T-cell receptor repertoire. However, analysis of T-cell receptor excision circles (TRECs) by real-time polymerase chain reaction revealed undetectable levels of TRECs in peripheral blood from patient 1 (<100 copies/ μ g DNA; normal range, 3500–8100) [10]. As expected, TRECs were not detected in patient 2 (T⁻ NK⁺ B⁺ SCID phenotype). These results suggested that the T cells of patient 1 were generated and expanded from a few T-cell progenitors, resulting in a low level of thymic output at 7 months of age.

At 1 month of age, patient 1 developed Omenn syndrome-like manifestations, which might have resulted from both impaired immune tolerance and defective immune regulation. As discussed in our previous report [9], his complicated condition, including the presence of autologous γ c-defective T and NK cells in peripheral blood and the expansion of revertant γ c-expressing T cells in the skin, does not allow us to draw clear conclusions about which aspects are responsible for the development of the Omenn syndrome phenotype. Whatever causal mechanism underlies this phenotype, it is important to note that patient 2 had no circulating mature T cells or revertant mosaicism and did not develop any Omenn syndrome-like manifestations.

In summary, our study demonstrated that the same γ c splice-site mutation caused different leaky phenotypes presenting as either T⁻ NK⁺ B⁺ or T⁺ NK⁺ B⁺ SCID within 1

family. Leaky $\gamma\epsilon$ mutations may lead to a diverse spectrum of clinical and immunological phenotypes.

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Conflicts of Interest

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Successful Capecitabine Desensitization for a Delayed-Type Hypersensitivity Reaction

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Palabras clave: Hipersensibilidad a capecitabina. Desensibilización.

Fluorouracil (5-FU)-based chemotherapies are used to treat many types of cancer, including advanced colorectal, gastric, and breast cancer. With the development of capecitabine, an oral 5-FU drug, many chemotherapy regimens changed to oral regimens since outpatient treatments are more convenient and cheaper [1,2].

The most common cutaneous adverse effect of capecitabine is hand-foot syndrome, also known as palmar-plantar erythrodysesthesia. Alopecia, nail changes, skin discoloration, cutaneous hyperpigmentation, photosensitivity, and radiation recall reactions have also been reported [2,3]. However, delayed-type hypersensitivity reactions such as maculopapular eruptions have not yet been described.

A 74-year-old man, diagnosed with stage III colon cancer, was prescribed a treatment plan of 6 cycles of the XELOX regimen, which consists of capecitabine (2000 mg/m² twice daily) in D1-14/q21 day cycles and oxaliplatin (130 mg/m²) as an adjuvant treatment after surgery. The patient experienced generalized pruritus followed by maculopapular eruptions on the trunk and extremities on the second day of capecitabine, 8 hours after the last dose, and was subsequently referred to our outpatient allergy clinic. Laboratory tests including liver and renal function tests, as well as a complete blood count, were normal. Capecitabine was discontinued and the patient was treated with corticosteroids for 5 days and antihistamines for the following 10 days. After the resolution of all clinical symptoms, intradermal tests with oxaliplatin (1/1000, 1/100 and 1/10 dilutions) and patch tests with both oxaliplatin and capecitabine were performed. For the patch tests, the commercialized forms of capecitabine and oxaliplatin were diluted at a concentration of 30% in petrolatum. Results were read 20 minutes after application, as well as on the second and fourth days, as suggested [4]. The delayed readings of the intradermal tests with oxaliplatin and the patch tests were negative. A parenteral drug provocation test with oxaliplatin was performed at 30-minute intervals with increasing doses of 13 mg (1/10 of the total dose), 32.5 mg (1/4), and the remaining dose of 84.5 mg. No reactions were observed during the test

or in the following 2 days. As maculopapular eruptions due to capecitabine had not been previously reported, an oral drug provocation test was performed to confirm the diagnosis. A 125-mg dose, corresponding to a quarter of the standard dose, was planned to be given in 4 repeated doses at 3-hour intervals. One hour after the second dose, the patient reported itching all over his body. Although pruritus is a subjective sign, considering the previous pruritic rash experienced by the patient, the test was considered positive and stopped. A 180-mg dose of fexofenadine was given to relieve the symptoms. As capecitabine was considered to be essential for the survival of the patient, a desensitization protocol was prepared. A 2000 mg/m² daily dose of capecitabine had been recommended for his treatment.

The desensitization protocol began with a 25-mg dose (1/20 of one 500-mg Xeloda tablet), which corresponds to approximately 1/100 of the total dose. As the patient's previous reaction had been a delayed-type hypersensitivity reaction, desensitization was performed using slow incremental doses over 16 days. Each day, 2 doses were given at 3-hour intervals. On the eighth day, a total of 2000 mg was planned to be given in 2 half doses to the patient. However, 2 hours after the first dose of 1000 mg, the patient experienced pruritus without eruptions. Consequently, the second dose was not given and daily antihistamine treatment was initiated. Over the following 6 days, the last tolerated dosage of 1200 mg per day was repeated, and the desensitization was successfully completed in a total of 16 days. The suggested final desensitization protocol is illustrated in the Table.

Table. Recommended Capecitabine Desensitization Protocol for Patients With Delayed-Type Hypersensitivity Reactions

Day	First Dose	Second Dose	Daily Cumulative Dose
1	25 mg	50 mg	75 mg
2	75 mg	75 mg	150 mg
3	150 mg	150 mg	300 mg
4	300 mg	300 mg	600 mg
5	300 mg	300 mg	600 mg
6	300 mg	300 mg	600 mg
7	600 mg	600 mg	1200 mg
8	600 mg	600 mg	1200 mg
9	600 mg	600 mg	1200 mg
10	600 mg	600 mg	1200 mg
11	600 mg	600 mg	1200 mg
12	600 mg	600 mg	1200 mg
13	600 mg	600 mg	1200 mg
14	750 mg	750 mg	1500 mg
15	750 mg	750 mg	1500 mg
16	1000 mg	1000 mg	2000 mg

According to the XELOX regimen, capecitabine is given at 7-day intervals for 14 days. However, we continued treatment with a daily half dose to maintain tolerance during the interval period. After the successful desensitization, the patient continued to take the drug for 84 days, as prescribed by his oncologist. No hypersensitivity or toxic reactions occurred during the course of treatment. A control positron emission tomography/computed tomography scan performed after discontinuation of treatment revealed no sign of disease.

We have presented the first case of maculopapular eruptions caused by capecitabine and have described a successful desensitization protocol for this drug. Use of capecitabine in different malignancies has increased because of the advantages of outpatient treatment, and has led to increased treatment adherence, reduced treatment costs, and higher quality of life [1].

To determine which drug caused the maculopapular reaction, oxaliplatin and capecitabine were both evaluated using currently suggested methods. Although the patch tests with both drugs were negative, the provocation tests confirmed capecitabine as the culprit drug. As the alternatives are not sufficiently effective, a desensitization procedure with capecitabine was performed. The literature on desensitization is less extensive for delayed-type reactions than for immediate reactions, and it is therefore important to establish a protocol according to the drug and the patient's previous reactions [5]. In patients with exanthems, slow protocols with gradually increasing doses, which can last from hours [6] to several weeks [7], have been reported. Some patients who initially failed a rush protocol were successfully desensitized with a modified slower protocol [8]. The first clinical symptoms of a potential renewed hypersensitivity reaction may not occur for 2 to 3 days. For this reason, long protocols with repetitive, slowly increasing doses have been recommended for patients with delayed-type reactions [5]. We therefore designed a capecitabine desensitization protocol spanning 16 days for our patient.

To our knowledge, this is the first desensitization protocol described for capecitabine. Although it took 16 days to complete, the approach was safe and successful. A similar desensitization approach could be safely attempted for delayed-type hypersensitivity reactions to other oral chemotherapy agents.

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Conflicts of Interest

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Anaphylaxis Due to Oat Ingestion

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Palabras clave: Anafilaxia. Avena. Hipersensibilidad IgE mediada. SDS-PAGE inmunoblotting. Proteína de 48 kDa.

Common oat (*Avena sativa*) is a cereal grain widely used due to its high nutritional value. It belongs to the *Poaceae* family, together with wheat, rice, barley, rye, and maize. Most patients with celiac disease can tolerate moderate amounts of pure oats, but they are advised to undergo both initial and long-term assessments by a health professional [1].

The allergic response to the inhalation of proteins from oat and cereal flour and dust (generally known as bakers' asthma and rhinitis) has been known since ancient times. Nowadays, this response is well characterized and constitutes one of the most important occupational allergies in many countries [2,3]. Contact allergy and urticaria by cutaneous sensitization due to the use of emollients/moisturizers containing oat extracts have been also described [4,5]. Patients with bakers' asthma, urticaria, and contact allergy due to oat or wheat protein usually tolerate the ingestion of oat and wheat. In the case of wheat allergy in adults, the allergen most frequently related to anaphylaxis is ω -5 gliadin [6]. However, no allergens have been described in anaphylaxis due to oat ingestion only.

We report a case of an anaphylactic reaction following oat consumption by an adult probably caused by a 48-kDa serpin protein; no allergy symptoms were observed with the ingestion of any other cereals.

The patient was a 70-year-old man with a history of essential hypertension and rheumatoid arthritis who was evaluated in our allergy unit following an acute episode of palmoplantar pruritus, generalized exanthema, nausea, vomiting, dysphonia, thoracic and laryngeal oppression, and hypotension 10 minutes after the ingestion of oat milk (200 mL). He was evaluated within an hour of the onset of symptoms. In the physical examination, the patient was conscious and oriented; he had a generalized pruritic rash, a blood pressure of 100/70 mm Hg, and a heart rate of 120 beats per minute. He was treated initially with epinephrine (1 mg/mL), dexamethasone (5 mg), and hydrocortisone (100 mg). Due to the persistence of symptoms, a new dose of epinephrine was required 20 minutes later. The patient

was taken to the emergency room and stayed there until he recovered (12 hours). He had previously tolerated oat meals and no cofactors were identified in the anaphylactic episode.

Blood cell count, biochemistry, and coagulation values were in normal ranges. Skin prick tests (SPTs) performed with the most common aeroallergens in our area and common food allergen extracts (milk, egg white and yolk, shellfish, fish, apple, nuts, tomato, garlic, and *Anisakis simplex*) were all negative.

Prick by prick tests with oat milk (8 mm) and oat seed (10 mm) were both positive. Oat seed and oat milk extracts were prepared by homogenization in phosphate buffer saline, dialysis, and lyophilization. SPTs with oat seed extract (9 mm) and oat milk extract (8 mm) were positive; the same tests were negative in control individuals. SPTs with wheat, rice, barley, rye, and maize flour were negative.

Specific IgE determination (ImmunoCAP) to oat, barley, rye, and grass pollen were all negative (<0.35 kU/L). Serum specific IgE levels against oat seed and oat milk extracts were measured by means of the enzyme allergosorbent test (EAST method) following the manufacturer's instructions (specific IgE ELA kit HYTEC, HYCOR BioMedical Ltd). The values obtained were 0.4 kU/L and less than 0.35 kU/L, respectively. An allergen microarray immunoassay with 112 allergens (including rTri a 14, rTri a 19, and nTri a aA_T1) (ImmunoCAP ISAC, Phadia, Thermo Fisher Scientific) was performed, with negative results in all cases.

The protein profiles of oat seed and oat milk extracts were analyzed by SDS-PAGE immunoblotting, as described by Laemmli [7]. The results showed proteins in the range of 90

Figure. SDS-PAGE immunoblotting results in nonreducing conditions (without 2-mercaptoethanol). A, Oat milk extract. B, Oat seed extract. Lane P, patient serum; Lane C, control serum; Lane M, molecular mass marker.

to 14 kDa. SDS-PAGE IgE immunoblotting was carried out in nonreducing conditions and revealed a 48-kDa binding band in both the oat seed and oat milk extracts (Figure). The band was not detected when the assay was performed in reducing conditions (with 2-mercaptoethanol) (results not shown), suggesting the presence of disulphide bridges maintaining the 3-dimensional structure of this protein.

The 48-kDa-IgE-binding band from oat seed extract was extracted from the gel, digested with trypsin, and the protein was identified by mass spectrometry (MS) using LC-ESI-IT (LC-MS/MS), as previously described [8]. Sequence analysis of several internal peptides was carried out using the following sequences: DGSSVSGEEAEGHANAQVQVVLADASAAGGPR, DGVFVDASLPLQSFPR, and LTTPTDVSLSPLANQTR. Research conducted with protein databases identified the IgE-binding band detected as a serpin, as the protein peptide sequence yielded a strong match with serpins from related species, such as *Triticum aestivum*, *Triticum urartu*, *Oryza sativa*, and many others.

We have presented a case of anaphylaxis due to oat milk ingestion in an adult, with demonstration of an IgE-mediated mechanism and absence of cross-sensitization to other cereal antigens. The positive skin test results, as well as the positive serum specific IgE values against oat seed, demonstrated the involvement of an IgE-mediated mechanism. The results of the proteomic study pointed to a 48-kDa serpin protein from oat seed as the probable cause of the anaphylactic reaction after oat milk ingestion. The low concentration of this protein in the oat seed extract could be the cause of the low specific IgE value detected against this extract using the EAST method, and the negative result obtained in ImmunoCAP.

In one case of anaphylaxis due to oat ingestion in a child previously described by Inuo et al [9], positive results were detected against oat and wheat seed extracts in prick tests, serum specific IgE, and IgE immunoblotting. In our case, skin tests and serum specific IgE to other cereals were negative. None of the molecular masses of the IgE binding bands detected in the case described by Inuo et al were similar to the one detected by us. Furthermore, the episode of anaphylaxis occurred the first time the child consumed oat. The above facts lead us to think that the primary sensitization could be due to a cereal other than oat, unlike in our case, in which sensitization was exclusive to oat.

To our knowledge, this is the first report of anaphylaxis after the ingestion of oat with no concomitant allergy to other cereals. The results suggest that a 48-kDa serpin protein was the probable cause of this episode.

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Previous Presentation

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Filaggrin Polymorphism Pro478Ser Is Associated With the Severity of Atopic Dermatitis and Colonization by *Staphylococcus aureus*

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Palabras clave: Dermatitis atópica. Mutación de la filagrina. Polimorfismo P.Pro478Ser. *Staphylococcus aureus*. Gravedad.

Loss-of-function mutations in the filaggrin (*FLG*) gene are associated with increased severity of atopic dermatitis (AD) [1]. Common variants, such as p.Arg501Ter and 2282del4 may be present in up to 50% of Northern European AD patients and absent in Southern European patients [2]. rs11584340 (p.Pro478Ser) is a single-nucleotide polymorphism (SNP) of *FLG* that is located at codon 478. It is associated with skin barrier disruption, since the 478 serine residue may hinder the action of protease cleavage, thus affecting the rate of aggregation between *FLG* and keratin filaments [3,4]. Despite having a minor allele frequency of 0.34 worldwide [5], the polymorphism was found to be associated with an increased risk of AD (odds ratio, 1.87) [6,7]. Previous studies have reported that patients with null mutations in *FLG* have increased transepidermal water loss and increased skin pH, both of which facilitate bacterial growth [8]. However, it remains unknown how p.Pro478Ser affects predisposition to skin colonization by *Staphylococcus aureus* in AD patients.

We performed a cross-sectional analysis to evaluate the association between disease severity/colonization of the skin by *S. aureus* and the polymorphism p.Pro478Ser and the null mutations in *FLG* (p.Arg501Ter and 2282del4).

Patients older than 12 years and diagnosed with AD according to the criteria of Hannifin and Rajka provided their written informed consent to participate in the study. In the case of minors, consent was given by the parents, caretakers, or guardians. The Ethics Committee of Porto University, Portugal approved the study. Participants with severe skin disease other than AD, secondary infection (bacteria, fungi, or viruses), or

any major systemic disease were excluded. Sample size was calculated based on minimal clinically important differences in the SCORing Atopic Dermatitis (SCORAD) score [9], and post hoc statistical power was set at 95.6% ($P=.05$) based on the prevalence of *FLG* mutations in a previous study of Southern European AD patients [2]. We analyzed data from 73 patients (mean age, 30 [13] years; 61% female; 77% atopic) with AD for a mean (SD) of 16 years. Severity was classified based on the SCORAD score as mild (≤ 15), moderate (16-40), and severe (≥ 41). Genomic DNA was extracted from peripheral blood samples and analyzed using PCR and direct DNA sequencing for the presence of the 2 null mutations in *FLG* and the p.Pro478Ser polymorphism. The microbiological profile was assessed in the right and left elbow creases, left and right popliteal creases, and neck region (area, 25 cm²). The number of colony forming units (CFU)/cm² of total staphylococci and *S. aureus* was determined (Baird-Parker Agar [Lab M] for total staphylococci and Mannitol Salt Agar [Lab M] for *S. aureus*). The serum biomarkers assessed were total IgE, eosinophil cationic protein, and specific IgE to a mixture of inhalant allergens (Phadiatop), *S. aureus* enterotoxins (A, B, C, and TSST), and *Malassezia* species (ImmunoCap). The Mann-Whitney test or Fisher exact test was used as appropriate (IBM SPSS Statistics for Windows [Version 20.0], IBM Corp).

FLG mutations were present in 15% of patients (9 with p.Arg501Ter and 2 with c.2282del4) and p.Pro478Ser in 38% (3 homozygotes, 25 heterozygotes). p.Pro478Ser was in linkage disequilibrium with the null mutations, and 3 patients with the p.Arg501Ter mutation also had p.Pro478Ser. The presence of p.Pro478Ser was associated with more severe disease, as reflected by the higher SCORAD score and severity class as well as by increased use of oral corticosteroids (Table). Furthermore, significantly more extensive colonization of *S. aureus* on 3 of the 5 sampled regions and a higher value of IgE to *S. aureus* enterotoxin A were observed. Homozygosity for p.Pro478Ser was not an additional risk factor in this particular group of patients. There were no differences between patients with and without the *FLG* null mutations in terms of AD severity, inflammatory allergic markers, and colonization by *S. aureus*.

The novel finding of this study is that, in contrast with the 2 *FLG* null mutations, p.Pro478Ser was significantly associated with more severe disease and greater skin colonization with *S. aureus* in AD patients. The 478 serine residue can increase skin permeability, leading to greater skin penetration by bacteria and conferring susceptibility to AD [4]. In addition, the presence of an unrecognized functional mutation at or adjacent to *FLG*, which is in linkage disequilibrium with p.Pro478Ser, could increase the risk for AD [10]. Therefore, our findings indicate that this SNP may have clinically relevant implications with respect to increased bacterial colonization of skin and more severe disease in AD patients.

The limitations of this study are as follows. First, the absence of healthy controls restricts us to speculation on the role of this SNP in patients with AD. Nevertheless, our objective was to study the association between this SNP and bacterial load in patients and not the role of the SNP as a risk factor for AD, in which case it would have been mandatory to include healthy controls. Second, the prevalence of *FLG*

mutations in the Portuguese population as a whole and in AD patients in Portugal is unknown. However, the sample size calculations showed that 42 patients were needed to detect a significant difference in the SCORAD score, and we were able to include more patients to overcome the level of uncertainty regarding the prevalence of genetic mutations.

Importantly, this is the first study to show an association between the presence of p.Pro478Ser and severity of AD and bacterial load in European patients with long-term AD. Only 3 previous studies have investigated this SNP, although these were in Asian patients, suggesting that it confers susceptibility to AD, particularly in patients with high IgE levels [3,6,7]. The low prevalence of *FLG* null mutations in our study is consistent with the wide variation in this

gene mutation across the globe and the lower prevalence in Southern European countries. The lack of an association with clinical, microbiological, and allergic parameters reinforces the fact that genetic markers other than *FLG* mutations should be studied.

In conclusion, genetic factors can affect the severity of AD and skin microbiota. Our study shows that the presence of p.Pro478Ser may be related to both increased disease severity and bacterial colonization in patients with long-term AD.

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Table. Characteristics of Patients With Atopic Dermatitis According to Filaggrin Genotype^a

	FLG Null Mutations Mp.Arg501Ter or C.2282del4			FLG Polymorphism Pro478Ser		
	Yes, n=11	No, n=62	P Value	Yes, n=28	No, n=45	P Value
Age, y	32 (6.1)	29.6 (1.5)	.91 ^b	34.1 (2.7)	27.3 (1.8)	.03 ^{b,d}
Female sex, No. (%)	7 (63.6)	38 (61.5)	.22 ^c	16 (57.1)	28 (62.2)	.42 ^c
Disease duration, y	15.9 (10.5)	16.3 (10.4)	.23 ^b	18.4 (2.3)	14.8 (1.3)	.32 ^b
SCORAD (0-103)	50.2 (30.9)	41.3 (22.6)	.72 ^b	51.8 (4.2)	36.0 (3.4)	<.01 ^{b,d}
SCORAD severity, No. (%)						
Mild	2 (18.2)	5 (8.1)	.28 ^c	2 (7.1)	5 (11.1)	.40 ^c
Moderate	3 (27.3)	26 (41.9)	.81 ^c	6 (21.4)	23 (51.1)	.02 ^{c,d}
Severe	6 (54.5)	31 (50.0)	.52 ^c	20 (71.4)	17 (37.8)	.01 ^c
Oral corticosteroids, No. (%)	3 (27.3)	30 (48.4)	.22 ^c	17 (60.7)	16 (35.6)	.03 ^{c,d}
Atopic, No. (%)	6 (54.5)	50 (79)	.53 ^c	22 (78.6)	34 (75.6)	.52 ^c
Asthmatic, No. (%)	4 (36.4)	36 (58.1)	.64 ^c	14 (50.0)	26 (57.8)	.31 ^c
Median (IQR) total IgE, IU/mL	2185 (71.4-5308)	4183 (97.3-3607.8)	.08 ^b	6520 (113.6-7935.0)	2240 (88.6-1151.5)	.08 ^b
Median Phadiatop, kU _A /L median (P ₂₅₋₇₅)	248.6 (4.5-565.9)	529.9 (0.54-441.0)	.12 ^b	763 (9.6-1115.9)	315 (0.4-283.5)	.13 ^b
ECP, µg/L	20.7 (14.9)	35.2 (29.1)	.56 ^b	37.2 (34.2)	30.5 (21.1)	.52 ^b
Specific IgE, kU _A /L						
Enterotoxin A	0.37 (0.2)	2.4 (1.3)	.79 ^b	4.5 (13.9)	0.5 (0.9)	.05 ^{b,d}
Enterotoxin B	0.6 (0.3)	1.5 (0.5)	.42 ^b	2.4 (5.1)	0.6 (1.3)	.23 ^b
Enterotoxin C	1.3 (0.5)	2.2 (0.5)	.38 ^b	2.7 (3.5)	1.6 (3.1)	.06 ^b
Enterotoxin TSST	0.5 (0.2)	1.4 (0.6)	.52 ^b	2.4 (6.7)	0.4 (0.8)	.08 ^b
Malassezia species	6.2 (5.8)	4.2 (1.1)	.78 ^b	7.2 (13.4)	3.3 (8.7)	.23 ^b
<i>Staphylococcus aureus</i> , CFU/cm ²						
Right arm	9471.1	78 152.7	.48 ^b	178 083.3	8002.3	.01 ^{b,d}
Left arm	158 909.9	70 271.9	.58 ^b	142 859.2	48 310.3	.92 ^b
Right leg	23 454.4	39 728.2	.91 ^b	89 778.9	8 386.7	.04 ^{b,d}
Left leg	162 754.4	359 865.8	.96 ^b	759 552.7	95 528.5	.02 ^{b,d}
Neck	8 994.9	30 732.6	.74 ^b	48 538.3	16 244.8	.80 ^b

Abbreviation: ECP, eosinophil cationic protein; SCORAD, SCORing Atopic Dermatitis.

^aResults are presented as mean (SD) unless stated otherwise.

^bMann-Whitney test.

^cFisher exact test.

^dStatistically significant.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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