

A single nucleotide deletion at the C1 inhibitor gene as the cause of hereditary angioedema: insights from a Brazilian family

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Abstract

Background: Hereditary angioedema is an autosomal dominant disease characterized by episodes of subcutaneous and submucosal edema. It is caused by deficiency of the C1 inhibitor protein, leading to elevated levels of bradykinin. More than 200 mutations in C1 inhibitor gene have been reported. The aim of this study was to analyze clinical features of a large family with an index case of hereditary angioedema and to determine the disease-causing mutation in this family.

Methods: Family pedigree was constructed with 275 individuals distributed in five generations. One hundred and sixty-five subjects were interviewed and investigated for mutation at the C1 inhibitor gene. Subjects reporting a history of recurrent episodes of angioedema and/or abdominal pain attacks underwent evaluation for hereditary angioedema.

Results: We have identified a novel mutation at the C1 inhibitor gene, c.351delC, which is a single-nucleotide deletion of a cytosine on exon 3, resulting in frameshift with premature stop codon. Sequencing analysis of the hypothetical truncated C1 inhibitor protein allowed us to conclude that, if transcription occurs, this protein has no biological activity. Twenty-eight members of the family fulfilled diagnostic criteria for hereditary angioedema and all of them presented the c.351delC mutation. Variation in clinical presentation and severity of disease was observed among these patients. One hundred and thirty-seven subjects without hereditary angioedema did not have the c.351delC mutation.

Conclusion: The present study provides definitive evidence to link a novel genetic mutation to the development of hereditary angioedema in patients from a Brazilian family.

Hereditary angioedema (HAE) is an autosomal dominant disease characterized by recurrent episodes of facial or peripheral angioedema and/or attacks of abdominal pain secondary to intra-abdominal edema. In addition, occasional episodes of laryngeal swelling during the course of disease may be life

threatening. The incidence of HAE is estimated at 1:50,000 (1–3).

Most patients with HAE are heterozygous carriers of a defective C1 inhibitor gene (*SERPING1*), and present decreased levels and/or function of C1 inhibitor (C1-INH) and low levels of C4. C1-INH is a serine protease inhibitor (serpin), which has a crucial role in downregulating the activity of proteases from the complement, contact, and fibrinolytic systems (1, 4). Mutations at the *SERPING1* gene may affect several pathways; however, there is strong evidence for

Abbreviations

HAE, hereditary angioedema; C1-INH, C1 inhibitor; *SERPING1*, C1 inhibitor gene; Serpin, serine protease inhibitor.

the central role of elevated bradykinin in pathogenesis of swelling in patients with HAE, as a result of insufficient inhibition of bradykinin-forming enzymes by decreased C1-INH activity (5, 6). The nonapeptide bradykinin, a powerful vasodilator and inducer of increased vascular permeability and hypotension, was discovered by the Brazilian physician scientist Rocha e Silva, while working with the venom of the jararaca snake (7). Successful treatment of acute attacks of angioedema with a bradykinin B2 receptor antagonist (Icatibant, Firazyr[®], Shire, Laupheim, Germany) or with a kallikrein synthesis inhibitor (Ecallantide, Kalbitor[®], Dyax, Cambridge, MA, USA) in patients with HAE has been recently reported (8, 9), providing further support for the importance of bradykinin in the pathogenesis of this disease.

Nearly 200 different mutations in the *SERPING1* gene have been identified in patients with HAE, including *de novo* mutations, which occur in approximately 25% of patients (10–13). The 17 kb gene encoding C1-INH has been mapped to chromosome 11q12–q13.1 and presents eight exons (3). The two main types of HAE, type I (accounting for 85% of cases) and type II (15% of cases), are indistinguishable in clinical presentation but have distinct patterns of C1-INH mutations. Type I HAE is caused by mutations that occur throughout the gene and results in truncated or misfolded proteins that are not efficiently secreted, leading to decrease in both antigenic and functional levels of C1 inhibitor. Type II HAE most frequently results from mutations involving exon 8, at or near the active site; the protein is secreted, and antigenic C1 inhibitor levels are normal, however, its functional activity is low (1). Another type of HAE (type III HAE) has been described in approximately 1% of patients, who are mostly women (14, 15). These patients present symptoms that are similar to those seen in types I and II, however, C1-INH concentration and activity are normal. Factor XII mutations have been described in a subgroup of patients with HAE type III, which may result in greater-specific activity of coagulation factor XIIa, leading to increase in bradykinin formation (16, 17).

Identification of structural alterations affecting function of the C1-INH protein has provided evidence for strong linkage of specific mutations and presence of HAE (18); however, definitive proof could be best obtained by segregation studies in large kindreds (11). In the present study, we report a novel mutation at the *SERPING1* gene in a large Brazilian family. Sequencing analysis revealed that this mutation causes premature termination of C1-INH protein synthesis, in a way that it lacks the serpins domain essential for biological activity of the protein. Evaluation of 165 members of the family allowed us to establish that this mutation is the definitive cause of HAE in this family.

Methods

Subjects

Family pedigree was constructed with 275-related individuals distributed in five generations. Of those, 165 individuals with ages ranging from 1 to 72 years old were interviewed and had

blood samples collected and analyzed for DNA mutation. Subjects reporting history of recurrent episodes of angioedema and/or abdominal pain attacks were evaluated for HAE, including measurements of C4 and C1-INH on at least two occasions, and diagnosis was established according to consensus criteria (2, 3). Information on additional 110 subjects was obtained from close family members who were interviewed. This study was approved by the Ethics Committee of the Clinical Hospital of the School of Medicine of Ribeirão Preto (protocol number 1594/2009), and all subjects or caregivers gave written informed consent to participate in the study.

HAE severity score

A simplified HAE severity score was constructed based on the score proposed by the Third C1 Esterase Inhibitor Deficiency Workshop (2). Episodes of angioedema were classified according to average frequency and intensity of symptoms since onset of disease; to improve accuracy, mean values were obtained for the past 5 years for patients with long-term histories. Frequency of symptoms was quantitated as follows: more than one episode a month, three points; between 6 and 11 episodes a year, two points; <6 episodes a year, one point; and no symptoms of angioedema, zero point. Intensity of symptoms was classified as: presence of discomfort but no disruption of daily activity, 2 points; discomfort reducing normal daily activity, 4 points; and inability to work or perform daily activity and/or necessity of hospital care, 5 points. The sum of the frequency and intensity scores was used to classify severity of disease as: severe (≥ 7 points); moderate (5 or 6 points); mild (≤ 4 points); and asymptomatic (0 point).

Levels of C1-INH and C4

Serum levels of C1-INH and C4 and were determined by nephelometry (Dade Behring, Newark, DE, USA), with normal values ranging from 0.21 to 0.39 g/l (mean normal value 0.3 g/l) and 0.1 to 0.4 g/l, respectively.

Identification of DNA mutation

Genomic DNA was extracted from peripheral blood using the DNA Wizard[®] Genomic DNA Purification Kit (Promega, Madison, WI, USA). Complete sequencing of the coding region of the *SERPING1* gene (all the 8 exons) was carried out in two patients. Partial sequencing of exon 3 was carried out in 165 members of the family. Briefly, amplification of a 466 bp DNA fragment of exon 3 was performed by PCR using the primers 5'-TCCAGCTTGCCGACAAC-CAACTCA-3' and 5'-TACAGCTTCTTCCCAGAGGC-ATGG-3' and the following conditions: final volume of 25 microliters containing 1× PCR buffer (50 mM KCl, 20 mM Tris HCl pH 8.5), 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.5 mM of MgCl₂, 10 pmol of each primer, 1.0 unit of DNA polymerase (Promega) and 200 ng of genomic DNA. The cycling conditions were 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 61°C for 30 s and 72°C for 45 s; final extension was performed for 2 min at 72°C.

PCR products were evaluated on 1% agarose gels, purified by the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the Big Dye™ Terminator V3.1 Cycle Sequencing kit (Applied Biosystems).

Statistical analysis

Association of severity of disease and gender was evaluated by the Fisher exact test. Analysis of the association of C1 inhibitor levels and severity of disease was carried out by adjusting a linear regression model using the Proc GLM program of the software SAS 9.0.

Results

Single-nucleotide deletion of a cytosine in the *SERPING1* gene was the cause of HAE

Complete sequencing of the 8 exons of the *SERPING1* gene carried out in two patients with diagnosis of HAE revealed a heterozygous deletion of one cytosine, which could be located at positions 2493, 2494 or 2495 on exon 3, according to the GenBank sequence of *SERPING1* gene (NCBI accession number X54486) (Fig. 1A, B). We have designated this novel mutation as c.351delC, according to the cDNA numbering recommended by the Human Genome Variation Society. Deletion of the cytosine resulted in a frameshift, leading to a stop codon 30 codons downstream the mutation.

PCR-based sequencing carried out in 165 members of the family revealed that 28 presented the c.351delC mutation and fulfilled diagnostic criteria for type I HAE (2, 3). The other 137 subjects did not have HAE and did not present the c.351delC mutation. Information from relatives was available for the additional 110 individuals. Of those, 106 had no history of angioedema and/or recurrent abdominal pain, and 4 subjects (all deceased) had positive history of HAE symptoms.

Family pedigrees

The first generation of individuals from whom we had available information comprised nine descendants, two of whom presented symptoms suggestive of HAE (Fig. 2A). The family pedigrees of descendants of these two individuals, encompassing all patients with HAE, are presented in Fig. 2B, C.

Clinical features of patients with HAE

Among the 28 subjects who presented the c.351delC mutation, 23 were patients with clinical diagnosis of HAE and five (17.8%) were individuals who have never had angioedema and/or abdominal pain attacks, but presented with decreased levels of C1-INH and C4 on at least two occasions and had parents or siblings diagnosed with HAE and with the c.351delC mutation. In keeping with a recently published international consensus diagnostic algorithm (3), these individuals could be diagnosed with type I HAE and were designated as having asymptomatic HAE. Of these five subjects, one is an 8-year-old girl (subject IV-11, Fig. 2B) and 4 were males, with ages of 2, 6, 26 and 53 years old (IV-10, IV-12,



Figure 1 Localization of the c.351delC mutation on exon 3 of the *SERPING1* gene. (A) Complete nucleotide and amino acid sequence of exon 3, which encodes the final 5 amino acids of the 22-residue signal peptide, the whole 112 amino acids of the N-terminal domain, and the first 49 amino acids of the 366-residue serpin domain of the C1-INH protein. The cytosine that underwent dele-

tion could be any of the three cytosines within the codon for amino acid 95 of the mature protein (indicated in red); the first amino acid residue of the mature protein (N) is shown in blue, and the region where the serpin domain initiates is outlined in green. (B) Schematic structure of the C1-INH protein; the red arrow indicates the site of the mutation.

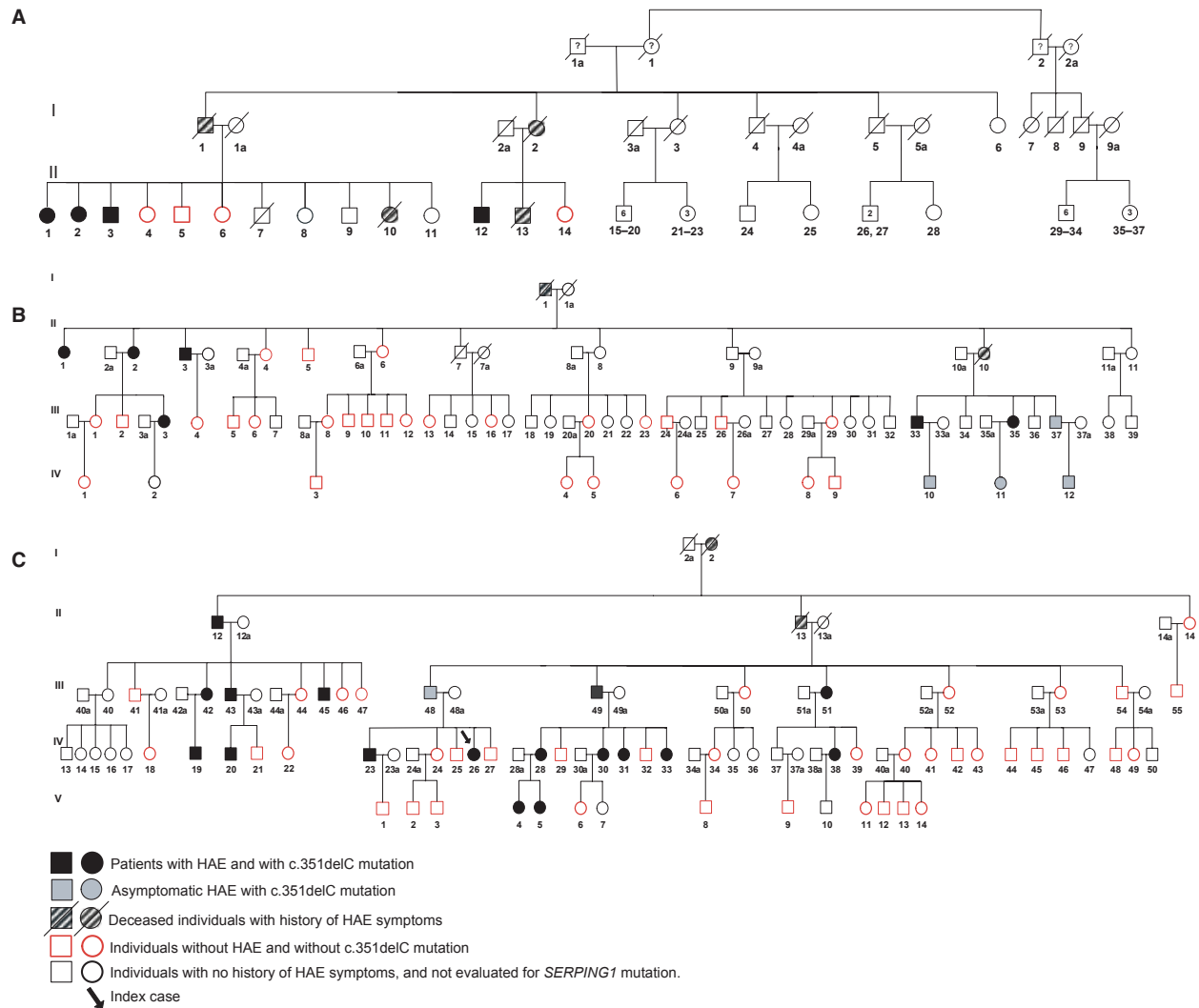


Figure 2 Family pedigree. (A) The family pedigree shows the nine members of the first generation (I). Two of them transmitted the disease to six members of the next generation (II). (B) Family pedigree showing the descendants (II, III and IV generations) of

individual I-1, indicated in Fig. 2A. (C). Family pedigree showing the descendants (II, III, IV and V generations) of individual I-2, indicated in Fig. 2A.

III-37 and III-48, respectively, Fig. 2B, C). Mean age of patients with the c.351delC mutation was 30.9 years (range 2–72 years), and 13 (46.4%) were males.

Clinical features of the 28 patients who fulfilled diagnostic criteria for HAE are presented in Table 1. Mean age at onset of symptoms was 14.5 years (median 10 years), ranging from 3 months to 42 years of age. According to severity score, five patients (17.8%) had mild disease; 7 (25%) had moderate disease; 11 (39.2%) had severe disease; and 5 (17.8%) were asymptomatic. Female patients presented with more severe disease as compared with male patients, comprising 81.9% of severe cases, 57.2% of moderate cases, 20% of mild cases and 20% of asymptomatic cases ($P = 0.04$).

Subcutaneous angioedema was the most frequent clinical presentation of disease (22/23, 95.6%), associated with recurrent abdominal pain in 10 patients (43.4%), with laryngeal

edema in two patients (8.6%) or both in four patients (17.3%). One patient (4.3%) presented recurrent abdominal pain as the only symptom (Table 1). Serum levels of C1-INH were 9% to 33% (mean 20.5%) of the mean normal value, and no correlation was found of C1-INH levels and clinical severity of disease.

A total of 13 patients (56% of the symptomatic patients) are currently being treated with danazol; 12 of them present very good control of symptoms with few adverse effects, with doses of 100 mg twice or three times a week, which are lower than the usually recommended doses. Danazol action in HAE seems to involve up-regulation of the C1-INH gene expression at the normal allele, leading to increased production of C1-INH. Two children were receiving tranexamic acid with partial control of symptoms (Table 1). In keeping with studies in other populations (19), common exogenous triggers

Table 1 Clinical features of patients with HAE

Id	Age (years)	Gender	Age at onset of symptoms	Clinical score	Ab pain	SC angio edema	Laryngeal edema	C1-INH levels* (%)	Prophylactic treatment
III-33	31	M	13 years	Moderate	–	X	–	10	Danazol
II-12	70	M	30 years	Mild	X	X	–	33	None
IV-30	22	F	6 years	Severe	X	X	–	20	Danazol
III-49	51	M	23 years	Mild	–	X	–	13	None
IV-26	28	F	3 months	Severe	X	X	X	22	Danazol
V-5	8	F	5 years	Moderate	X	–	–	16	None
V-4	10	F	6 years	Moderate	–	X	–	10	Tranexamic acid
III-45	38	M	3 years	Mild	–	X	–	30	None
III-43	43	M	10 years	Severe	X	X	X	26	Danazol
IV-31	16	F	10 years	Severe	X	X	–	26	Danazol
IV-23	33	M	8 years	Moderate	X	X	–	13	None
II-2	57	F	32 years	Severe	X	X	X	30	Danazol
III-51	46	F	21 years	Severe	–	X	X	16	Danazol
IV-38	19	F	11 years	Moderate	X	X	–	13	None
II-3	55	M	42 years	Moderate	X	X	–	9	Danazol
III-3	24	F	5 years	Severe	X	X	X	20	Danazol
III-42	46	F	10 years	Severe	X	X	–	23	Danazol
IV-33	10	F	3 years	Moderate	X	X	–	23	Tranexamic acid
IV-28	30	F	19 years	Severe	–	X	X	31	Danazol
IV-20	15	M	3 years	Severe	X	X	–	20	Danazol
III-35	29	F	16 years	Severe	X	X	–	10	Danazol
II-1	72	F	40 years	Mild	–	X	–	20	None
IV-19	18	M	17 years	Mild	–	X	–	23	None
III-48	53	M	–	Asymptom	–	–	–	10	None
IV-10	2	M	–	Asymptom	–	–	–	30	None
IV-11	8	F	–	Asymptom	–	–	–	30	None
III-37	26	M	–	Asymptom	–	–	–	26	None
IV-12	6	M	–	Asymptom	–	–	–	23	None

Id, patient number same as in Fig. 2; Ab, abdominal; SC, subcutaneous; Asymptom, asymptomatic; (X) presence (–) absence.

*Expressed as % of the normal mean.

of angioedema episodes in our patients included use of ACE inhibitors, use of estrogen, menstruation, emotional stress, dental work and physical trauma (data not shown).

Patients with other types of angioedema and/or abdominal pain attacks

Among the 165 individuals of the described pedigree who were interviewed, there were 23(14%) who reported at least one episode of angioedema (14 patients with subcutaneous angioedema, including one with laryngeal angioedema) and/or abdominal pain attack (11 patients), but showed normal levels of C1-INH protein and normal C4 in serum. These patients did not bear the c.351delC mutation. Careful clinical evaluation revealed allergic angioedema (nine patients), drug-induced angioedema (two patients), hypothyroidism (one patient), idiopathic angioedema (two patients), infection with *Strongyloides stercoralis* (one patient), gastritis (one patient) and lactose intolerance (one patient) as the cause of symptoms in these patients; seven patients had no apparent cause for the reported episodes of abdominal pain and are being followed over time.

Discussion

In the present study, we have evaluated a family living in a small town in Southeast Brazil (Carmo do Rio Claro, State of Minas Gerais), with an index case of HAE diagnosed in our clinic in 2003. The index case was a 21-year-old woman, who presented with history of weekly episodes of swelling of the face, tongue and extremities with average duration of 3 days, since childhood. Clinical evaluation revealed C1-INH levels of 31% of the mean normal value and decreased C4 levels, in addition to positive family history of angioedema, which confirmed the diagnosis of type I HAE. Treatment was started with danazol, with excellent control of symptoms. Sequencing analysis of *SERPING1* gene revealed a heterozygous single-nucleotide deletion on exon 3, which results in premature interruption of protein synthesis. The c.351delC mutation was detected in 28 patients in the family; all of them fulfilled diagnostic criteria for HAE including five patients (17%) with asymptomatic HAE. Frequency of asymptomatic adult individuals carrying a mutation in the *SERPING1* gene has been estimated as 5% (2); our rate of 17% could be explained in part by the young age of the

individuals (three of them are children 8 years of age or under) who could still develop symptoms of HAE. To our knowledge, this is the largest family reported with data which strongly support a pathogenic role of a novel mutation of the *SERPING1* gene in the development of HAE, and this study constitutes the first molecular analysis of HAE described in Latin America.

Diagnosis of HAE is usually established by characteristic clinical symptoms confirmed by complement testing. Although identification of the disease-causing mutation is not essential for diagnosis, mutation analysis of *SERPING1* gene establishes the genetic determinant of the low C1-INH function and segregation of the marker within the affected family, allowing for predictive genetic counseling and early identification and treatment of symptoms. In particular, diagnosis based on the disease-causing mutation may offer advantages in infants; at this age group, biochemistry tests are often less valid. Additionally, genetic counseling may be helpful to those individuals who do not present the mutation, eliminating concerns about developing the disease or transferring a mutated gene to their descendants.

With very rare exceptions (20–22), all C1-INH deficient patients are heterozygous for the genetic defect; therefore, functional C1-INH levels in patients with type I or type II HAE would be expected to be around 50% if the normal allele is functioning properly. However, blood levels are typically <50% in HAE patients. One possible explanation is that decreased inhibitor level results in increased activation of proteases regulated by C1-INH, which could in turn consume C1-INH, producing a plasma level below 50% of normal (23). It is also possible that the abnormal mRNA or protein may produce a suppressive effect on either transcription or translation (24, 25). We have found that patients with HAE in our study presented C1-INH levels ranging from 9% to 31% of the mean normal value for the protein. In addition, there was no significant linear correlation of C1-INH antigenic levels and disease severity scores, in accordance with earlier reports (20, 26). Interestingly, a recent study has investigated the association of severity of HAE to C1-INH functional levels, as determined by a commercial ELISA that tests the complex formation between C1s and C1-INH. A significant correlation of disease severity scores and functional C1-INH levels was found in a group of 110 patients with type I HAE at baseline, before any treatment was initiated, whereas no correlation was found to antigenic C1-INH levels, C4 or CH50 measurements (27). Female patients in our study presented with more severe disease, despite similar incidence of HAE between genders, highlighting the role of hormonal influences in disease severity.

Although symptoms tended to occur initially in childhood or early teenage years, some of our patients started with symptoms when they were older than 40 years, supporting the notion that onset of symptoms at a later age should not rule out the diagnosis of HAE. Another relevant observation was that one child presented recurrent abdominal pain as her only symptom of HAE. Although there may be several etiolo-

gies for recurrent abdominal pain, attacks of abdominal pain may precede other symptoms of HAE for a number of years (28); therefore, we feel that HAE should be included in the differential diagnosis of recurrent abdominal pain, particularly in patients with positive family history for angioedema.

In conclusion, sequencing analysis enabled us to establish a causal link between a novel mutation on the *SERPING1* gene and the development of type I HAE in members of a Brazilian family. The genetic defect described here fully explains the nature of the disease. Considerable variation in clinical presentation of HAE was observed among patients of this family who have the same genetic defect. Future gene segregation studies in this large family could be important to further investigate the basis of variability in clinical presentation of HAE.

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Authors' contributions

MFF, WS and LKA conceived the study. MFF interviewed all family members; MFF, WS and LKA performed clinical evaluation of the patients. MFF, ASM, APL and LKA were responsible for collection of patients' samples. MFF, ASM, ECC, EAD, APL and KB carried out the molecular analysis. MP and HA developed the molecular model. MFF and LKA did the statistical analysis and wrote the manuscript. The final version of the article was approved by all authors.

Conflict of interest

None of the authors present any conflicts of interest which could potentially influence the content, presentation and analysis of the present work. LKA has received research support from FAPESP and CNPq, honoraria for lectures from Novartis on subject unrelated to the present study, and financial support to attend scientific meeting from Mantecorp.

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