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Family hypercholesterolemia and LDLR mutations in communities of european origin in southern Brazil: a prospective observational cohort study

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Abstract

Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by high levels of circulating low-density lipoprotein (LDL) and premature coronary heart events. A quarter of the population of Southern Brazil is affected by hypercholesterolemia and the region has the highest mortality rates due to cardiovascular disease in the country. The aim of this study was to describe LDLR mutations in European descendants with FH living in Southern Brazil. Ten mL of venous blood were taken from 40 patients and used for DNA extraction and subsequent Polymerase Chain Reaction (PCR). The DNA fragments were sequenced and analyzed and the data obtained were compared to reference values from the University of California Santa Cruz (UCSC) Genome Browser. A total of 15 mutations were identified in 38 patients (95% of the total samples). These mutations were located in exons 11 (P518L) and 15 (D727G) in Italian, Portuguese and Spanish descendants and the *105T>G mutation, still undescribed, should be critically evaluated by means of mRNA alteration studies. The present study demonstrated for the first time the presence of the P518L mutation located in exon 11 of the LDLR gene in European descendants living in southern Brazil. This mutation has a high potential to be pathogenic since it is located in a domain responsible for LDLR release from the endoplasmic reticulum (ER).

Keywords: Familial Hypercholesterolemia. Genetic polymorphism. LDL receptors. European Ancestry. Mutation.

Introduction

Familial Hypercholesterolemia (FH) is a common autosomal dominant genetic disease caused by mutations in three genes (LDL-C, APOB, and PCSK9) affecting the plasma clearance of LDL-cholesterol (LDL-C) **[1]**. Among FH patients where a mutation can be found, approximately 93% of these mutations occur in the LDLR gene, and to date, more than1200 different mutations of all types have been reported **[2]**.

The frequency of clinical homozygous FH has been estimated at 1 in 1,000,000 and the frequency of heterozygous FH at 1 in 500 individuals, who are at high risk of coronary events **[3]**. Both are at high risk of premature heart disease. Brazil, it is estimated that 40% of the population has high cholesterol levels (>200 mg/dL) **[4]**. In Southern Brazil, a region settled by European families in the nineteenth century, almost a quarter of its population is affected by hypercholesterolemia, with one of the highest mortality rates in Brazil due to cardiovascular disease **[5]**. FH is caused primarily by altered synthesis or functional capacity of low-density lipoprotein receptors (LDLR) due to mutations **[6]**.

Defects in LDLR are divided into 6 classes affecting different aspects of its function. In practice, it is simpler to classify mutations into 2 groups: LDL receptor-deficient mutations (i.e. null alleles that do not produce LDL receptor protein) and LDL receptor–defective mutations (i.e. gene variants that affect function such as the interaction with the ligandbinding domain of LDL) **[7]**.

According to the World Health Organization **[8]**, in

Brazil there are about 320,000 individuals with heterozygous FH. In this country, the molecular studies of defects of the LDLR gene began with the identification of the "Lebanese allele", a nonsense C660X mutation located in exon 14 of the LDLR gene, which results in the production of a truncated protein without activity. This mutation was found in 14 of the 16 families participating in the study **[9,10]**.

Due to the large concentration of people of Lebanese descent in Brazil, particularly in the southeast region, the C660X mutation is probably one of the most important causes of HF among Brazilian patients. This high frequency is attributed to a founder effect plus a high rate of inbreeding among first-generation immigrants. Salazar et al. **[11]** defined the molecular profile of a group of FH patients living in São Paulo, all of them European descendants, identifying 15 mutations in the LDLR gene, seven of which were novel mutations, five non-synonymous and two frameshift types. Inocêncio et al. **[12]** identified eight novel mutations in the LDLR gene from 32 index cases and their families in the same plasma samples investigated by Alberto et al. **[10]** and Figueiredo et al. **[9]**, whose studies focused only on the Lebanese mutation C660X. The importance of the mutation C660X was confirmed in a large study conducted in the southeast of Brazil by Jannes et al **[13]**. The prevalence of mutations of the LDLR gene in Latin America has been analyzed by Mehta et al **[14]**.

The present study described a new class 2 mutation in three Brazilian patients from the southern region, and other variants more distant from intron-exon junctions that affect splicing.

Methods

Study Design

The rules for a prospective observational cohort study were followed (The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies). Available in: https://www.equator-network.org/reporting-guidelines/strobe/. Accessed at 2023/04/10.

Ethical Approval

The subjects gave written informed consent and the study was approved by the Ethics Committee of the Medical School of Ribeirão Preto, University of São Paulo, Brazil.

Participants and Samples

A total of 40 patients with the HF phenotype were selected from the records of health care services in the State of Rio Grande do Sul for participation in the study. The inclusion criteria followed the guideline adapted from the Simon Broome Heart Research Trust **[15]**. All patients were from families of European descent belonging to the immigration movements to Southern Brazil: 7 Germans, 12 Portuguese, 8 Italians, 5 Spaniards, 5 Poles, and 3 Latvians. Samples of venous blood (10 mL) were taken from each patient, added to EDTA- containing tubes, and centrifuged for 20 min at 2,500g.

DNA Extraction

Genomic DNA was extracted from peripheral blood leukocytes using a Super Quickgene-rapid DNA isolation kit (Promega), according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

LDLR exons 3 to 18 were amplified using specific primers as described in **Table 1**.

Table 1. Primers used to sequence the LDLR gene exons 3 to 18, annealing temperature and size of amplified fragments.

Exons	Primer names	Sequences 5′ → 3′	Ann. Temp. (°C)	Product (bp)	size
3	LDLrEx3F	CTCGGCTCAGTGGGTCTTTC	57	260	
	LDLrEx3R	GAGCAGGACCCCGTAGAGACAAA			
4	LDLrEx4F	AATGGGCTGGTGTTGGGAGACTTC	60	497	
	LDLrEx4R	GAGCCCAGGGACAGGTGATAGGAC			
5	LDLrEx5F	AGGCCCTGCTTCTTTTTCTCT	56	414	
	LDLrEx5R	CTGCCGTGAGCTGTGATTGTG			
6	LDLrEx6F	GTGCCCGACGCGTTTTCTTA	59	699	
	LDLrEx6R	ATCCACCGCGCCCAGCATC			
7	LDLrEx7F	GGCGAAGGGATGGGTAGGGGC	59	236	
	LDLrEx7R	GGTGCCATGTCAGGAAGCGC			
8	LDLrEx8F	TGTCTCCTGGCTGCCTTCGAA	58	406	
	LDLrEx8R	GGCAAGCCCAAGTCCTAACA			
9	LDLrEx9F	TCCATCGACGGGTCCCCTCTGACCC	53	272	
	LDLrEx9R	AGCCCTCATCTCACCTGCGGGCCAA			
10	LDLrEx10F	ATGCCCTTCTCTCCTCCTGCCTCAG	63	280	
	LDLrEx10R	AGCCCTCAGCGTCGTGGATAGGCAC			
11	LDLrEx11F	CCAGCAGGACTATTTCCCAA	58	346	
	LDLrEx11R	GGAAACCTTCAGGGAGCAGCTT			
12	LDLrEx12F	AGGCTCACATGTGGTTGGAG	54	360	
	LDLrEx12R	GCTTGAGTGATCTATAGTCTGTGT			
14	LDLrEx14R	GGAGGGGGCAGTTGGAGGACAC	60	661	
15	LDLrEx15F	GAGACTTTCGTCATTAGGCG	54	435	
	LDLrEx15R	GTTATTAGACCCACTTTACAG			
16	LDLrEx16F	GTCCTACAACCTCGATAACTCAC	56	401	
	LDLrEx16R	GAGGCCGTGGGGGTGATAAAGGAC			
17	LDLrEx17F	CCACAAGGCGATCTCTAAAC	56	491	
	LDLrEx17R	TGCTTATCCAACATTCCTCTACAC			
18	LDLrEx18F	TGCCAGGCCCAGAAGGTGAGAA	60	355	
	LDLrEx18R	CCCCCGGGCCTGTTTCTCTTAT			

Ann. Temp. = annealing temperature; F=Forward; R=Reverse

All PCR procedures were carried out under the following conditions: 2.5mM of each primer, 2.5 mM dNTPs, 2.5 µL of buffer 1x (Biotools), 1U of Taq polymerase (Biotools); 200 ng of genomic DNA, and 14 µL distilled water, in a final reaction volume of 25 µl. PCR was performed according to the following program: an initial denaturation for 4 min followed by 35 cycles of 40s at 94°C, 50s at annealing temperatures specific for each reaction (Table 1), 50 cycles at 72°C (extension), and a final extension at 72°C for 10 min.

Sequencing Analysis

DNA fragments amplified by PCR were sequenced directly with the automated capillary sequencing system MegaBACE 1000 DNA Sequencing System® (MolecularDynamics & Amersham Life Science), using the DYEnamic ET terminator Sequencing System® kit and the same PCR primers according to the manufacturer's instructions. The sequencing results were analyzed with the Polyphred software (Geospiza Inc.) and the data obtained were compared to the reference values of the UCSC Genome Browser **[16]**. The nomenclature used to classify the genotypes was that proposed by La Du et al **[17]**.

Results

A total of 15 mutations were identified in 38 patients (95% of the original sample tested) belonging to six groups of European descent (**Table 2**).

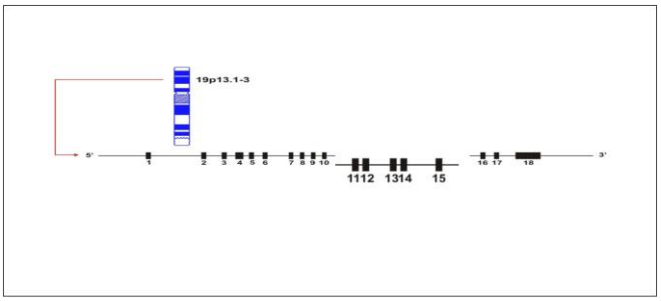
Table 2. Frequencies of the mutations identified in the present study.

Regions	Mutations		Type	European descendants						Total
	Nucleotide	Amino Acid		PT N=12	IT N=8	DE N=7	ES N=5	PL N=5	LV N=3	
Exons										
4	c.408C>T	D115D	S	1				1		2
11	c.1616C>T	P518L	NS	1	2					3
12	c.1773C>T	N570N	S	4	2	5	1	3	2	17
15	c.2243A>G	D727G	NS	5	3	1	3	2		14
Introns										
6	IVS6+171G>A			2	1					3
6	IVS6+36G>A			2	1	1				4
11	IVS11+56C>T			4	3		3	1		11
11	IVS11-69G>T			3	3	1	2	1		10
11	IVS11-55A>C			5	2	1		1		9
15	IVS15-136A>G			10	5	4	3	2	3	27
16	IVS16+46C>T			3	2	1	1	1		8
17	IVS17-42A>G			7	5	3	2	2	2	21
3'UTR										
	*52G>A			5	2	1	2	1		11
	*105T>G			4	3	2	1	2	2	14
	*141G>A			2	1					3

Legend: PT: Portuguese, IT: Italian, DE: German, ES: Spanish, PL: Polish, LV: Latvian, N = number of patients. Type of variation: S = synonymous; NS = non-synonymous

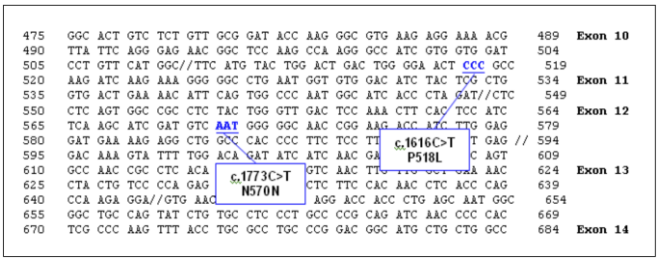
A total of 8 mutations were found within the intronic region, four in the coding region, and three in the 3' untranslated region (3' UTR). Eight mutations have not been previously described; two of them are missense and located in exons 11 (P518L) and 15 (D727G). The LDLR contains a domain homologous to the precursor of epidermal growth factor (EGF) (**Figure 1**), which is important for the release of the receptor from the endoplasmic reticulum (ER). A mutation in this domain blocks the proper transport to the Golgi complex and causes protein accumulation in the ER.

Figure 1. LDLR gene and its 18 exons showing region domain class 2 (exons 11-15), in which mutations block the transport of protein from the endoplasmic reticulum to the Golgi complex.



The frequency of the nonsynonymous mutation D727G was higher among the Spanish subjects (60%), with homozygous cases (57%) predominating over heterozygous ones (43%). Despite its high frequency, the D727G mutation is not pathogenic because it is located in a region that does not affect protein function. However, the same cannot be said for the P518L mutation, which was found in subjects of Italian and Portuguese descent (**Figure 2**). In addition, it would be important to perform a functional analysis to validate its pathogenicity.

Figure 2. Pro518Leu position of the LDLR mutation in exon 11.



Discussion

This is the first study aiming to identify the variations of the LDLR gene in patients of European descent with FH who live in southern Brazil. These

variations are shown in **Table 3** together with those previously identified by Alberto et al. **[10]**, Figueiredo et al. **[9]**, Inocêncio **[12]**, Salazar, et al. **[11]**, and van de Kerkhof et al. **[13]** regarding the Southeast región of Brazil.

Table 3 Mutations identified in Brazil.

Gene region/ Reference	Mutation Nucleotide	Amino acid position	Brazilian region	Population	Reference (previously mentioned)[1]
Exon 1					
Salazar et al.(2002)	c.4G>C	G(-20)R	Southeast	Caucasian	New
Exon 4					
Salazar et al.(2002)	c.337G>T	E92X	Southeast	Caucasian	Hobbs, Brown e Goldstein (1992)
Inocêncio (2003)	c.532G>T	D157Y	Southeast	Caucasian	New
Werutsky (Present Study)	c.408C>T	D115D	RS	Polish#	
Exon 5					
Salazar et al.(2002)	c.769C>T	R236W	Southeast	Caucasian	Amsellem and Benlian (2000)
Intron 6					
Werutsky (Present Study)	IVS6+36G>A		RS	Portuguese #	
Werutsky (Present Study)	IVS6+171G>A		RS	Portuguese #	
Exon 7					
van de Kerkhof et al.(2003)	c.977C>G	S305C	Southeast	Lebanese and Italian	New
Salazar et al. (2002)	c.1027G>A	G322S	Southeast	Caucasian	Hobbs, Brown and Goldstein (1992)
Intron 7					
Inocêncio (2003)	IVS7+10C>G		Southeast	Caucasian	Dedoussis et al. (2004)
Exon 8					
Salazar et al.(2002)	c.1118G>A	G352D	Southeast	Caucasian	Bertolini et al. (1999)
	c.1171G>A	A370T	Southeast	Caucasian	Kotze et al. (1989)
	c.1176C>A	C371X	Southeast	Caucasian	Langenhoven et al. (1996)
Exon 10					
Salazar et al. (2002)	c.1489A>G	T476P	Southeast	Caucasian	New
	c.1571T>G	V503G	Southeast	Caucasian	New
Exon 11					
Werutsky (Present Study)	c.1616C>T	P518L	RS	Italian#	
Intron 11					
Inocêncio (2003)	IVS11-10G>A		Southeast	Caucasian	Cenarro et al. (1998)
Werutsky (Present study)	IVS11+56C>T		RS	Spanish#	Amsellem et al. (2002)
	IVS11-69G>T		RS	Spanish#	Amsellem et al. (2002)
	IVS11-55A>C		RS	Portuguese #	
Exon 12					
Salazar et al. (2002)	c.1801G>C	D580H	Southeast	Caucasian	New
Werutsky (Present study)	c.1773C>T	N570N	RS	Latvian#	Boright et al. (1998)
Exon 14					
Figueiredo et al. (1992) and Alberto et al.(1999)	c.2043C>A	C660X	Southeast	Lebanese	New
Salazar et al.(2002)	c.2017A>C	S652R	Southeast	Caucasian	New
	c.2088C>G	C675W	Southeast	Caucasian	Arca e Jokinen (1998)
	c.2093G>A	C677Y	Southeast	Caucasian	Schmidt e Kostner (2000)
Exon 15					
Present Study	c.2243A>G	D727G	RS	Spanish#	
Intron 15					
Werutsky (Present Study)	IVS15-136A>G		RS	Portuguese #	
Exon 16					
Salazar et al.(2002)	c.2333insC	FsR757	Southeast	Caucasian	New
Intron 16					
Werutsky (Present Study)	IVS16+46C>T		RS	Italian and Portuguese #	Gen Bank
Exon 17					
Salazar et al. (2002)	c.2546delC	FsS828	Southeast	Caucasian	New
Intron 17					
Werutsky (Present Study)	IVS17-42A>G		RS	Latvian#	Amsellem et al. (2002)
3'UTR (*Untranslated Region*)					
Inocêncio (2003)	*52G>A		Southeast	Caucasian	Gen Bank
Werutsky (Present Study)	*52G>A		RS	Portuguese #	
Werutsky (Present Study)	*105T>G		RS	Latvian#	
Werutsky (Present Study)	*141G>A		RS	Portuguese #	Amsellem et al. (2002)

Legend: http://www.ucl.ac.uk/fh/, # Most prevalente ethnic group , RS= Rio Grande do Sul.

The N570N mutation in exon 12 was the most prevalent in all ethnic groups, confirming the relatively high heterozygosity of the LDLR gene. D115D and N570N are silent mutations but can modify protein function if they create a new splicing site or even if they affect mRNA stability.

The P518L and N570N mutations belong to Class 2 (endoplasmic accumulation of LDLR because of deficient transportation) and the D115D mutation belongs to Class 3 (deficient allele linkage) and they may result in functional impairment of LDLR. The D727G mutation in

exon 15 was the most prevalent among subjects of Spanish descent (60%), with a predominance of homozygous cases (57%) over heterozygous ones (43%). This mutation, as well as P518L, detected among Italians and Portuguese, is of the missense type with a greater chance to affect LDLR functionality, although functional studies are still needed to determine its role in FH development.

Most of the new variations described in the present study occurred in intronic regions due to two main factors. First, direct DNA sequencing using a new set of primers has permitted the tracking of these regions to a greater extent. Second, the detection of a larger number of mutations is expected in intronic regions compared to the promoter and coding regions, since they would be selectively neutral, except for those located in consensus regions directly involved in the splicing process. It is estimated that mutations at splicing sites may correspond to approximately 15% of disease-causing mutations in humans **[18]**.

In a study of French patients carrying FH, Amsellem et al. **[19]** observed that mutations located outside donor and acceptor splicing regions may exert a regulatory effect on gene expressions, such as the activation of critical splicing sites or partial alteration of splicing efficiency. Therefore, these mutations can be causative factors of FH, even if they are not located in the exon-intron junctions. One Portuguese patient and one Italian patient, individually, were the carriers of the greatest number of mutations **[10]**, followed by a Spanish patient and two more Italian patients with seven mutations each, showing a frequency of the order of 66% and 46%, respectively.

Notably, few mutations in introns that are outside the exon-intron junctions of any genes have been described as causes of human genetic disorders. They occur in highly conserved intronic sequences near donor and acceptor splicing sites **[19-21]**. The current genomic screening strategy using new primers that release the intronic sequence has significantly improved the detection of disease-causing variations in patients with FH. In half of the patients studied by Amsellem et al. **[19]** who carried mutations, these mutations were not detected using the denaturing gradient gel electrophoresis technique and DNA sequencing with usual primers. Inocêncio **[12]** performed an analysis of prediction to create alternative sites in introns that could affect the normal mRNA processing using specific computer programs for these sites **[22]**. The creation of one more splicing location in the intron central segment of the LDLR gene should be another cause of the disease. Recently, such an intronic mutation resulted in the aberrant inclusion of a pseudoexon in the Duchenne muscular dystrophy (DMD) gene responsible for the clinical symptoms of the disorder **[23]**.

Thus, the screening procedures used to detect variations in the DNA sequence could not omit the intronic mutations. Amsellem et al. **[19]** showed that *Alu* repetitions represent 65% of LDLR gene intronic sequences, 85%of which are distant from exon-intron junctions. It has been demonstrated that alternative splicing in farther regions of the exon-intron junction may affect the pre-mRNA transcription depending on where polyadenylation begins and where it ends **[24]**.

Regarding the mutations in the 3'UTR that were identified after exon 18, the *141G>A and the *52G>A mutation have been previously mentioned at http://www.ucl.ac.uk/fh **[25]**, the latter having also been mentioned by Inocêncio **[12]**. These mutations, together with the *105T>G mutation, still undescribed, should be critically evaluated in studies of mRNA alterations. Jensen et al. **[26,27]** as well as Dedoussis et al. **[28]** indicated the need for functional evaluation of mutations in the 3'UTR and of those located in the promoter region and the splicing site junctions.

Cotton and Scriver **[29]** suggested some criteria to define whether a particular mutation modifies the phenotype, leading to the onset of a given disease, or is a neutral variation and common in the population (polymorphism). According to the use and accessibility, criteria such as analysis of the type of mutation, analyzed sequence region and size, analysis of phenotypic segregation, affected amino acid, prevalence of such mutation, functional expression analysis and the relative importance of mutation type may be chosen. In our study, possible errors and ambiguities were minimized by the purification of fragments amplified by PCR. The manual verification of the bases identified in the chromatogram permitted the exclusion of exon 3 due to poor sequencing quality so that its variations could be mistaken as artifacts. No mutations were detected in exons 5, 7, and 8 because the bases were verified manually one by one. Another possible source of error lies in the analysis of a small region of the gene of interest to identify mutations associated with certain diseases (analysis of specific exons). However, a mutant allele cannot be responsible for disease onset but may be located next to the allele of interest that was not analyzed. Thus, analysis of the entire gene or its largest possible region is recommended **[30]**. In the present study, most of the LDLR gene was examined, including the intronic region (from exon 3 to 3'UTR).

The criterion of family segregation analysis is characterized by the comparison of affected subjects with unaffected relatives. Since FH is an autosomal

dominant disorder, if a certain mutation causes this condition, it will be always present in affected subjects and absent in unaffected relatives. Furthermore, the mutation should be analyzed in normolipidemic subjects from the source population of patients with FH. Similarly, if the mutation provokes FH or it is closely related to this disease, its frequency should be null or much lower than that observed in patients with FH. For example, the T7051 mutation (FHParis 9) was first described as a mutation that caused FH **[27]** but subsequent studies demonstrated that this mutation also occurs in normocholesterolemic subjects **[31]**.

Since this is the first study of the molecular basis of FH performed in Rio Grande do Sul, the 15 variations identified will require future studies of expression analysis (alteration of phenotype), the measurement of biological activity, and segregation analysis with its prevalence in normolipidemic subjects, according to criteria of Cotton and Scriver **[29]**. To determine the corresponding phenotype, environmental factors should be implicated, and other genes such as the cholesteryl ester transfer protein (CETP) gene, the lipoprotein lipase (LPL) gene, the apolipoprotein E gene, and, recently, the PCSK9 gene, whose mutations are associated with the reduction of serum LDL-cholesterol levels and the reduction of cardiovascular diseases. Mutations in these genes could explain the clinical manifestation of the disease in two of the key cases studied here, which had no mutations in the LDLR gene **[32]**.

The non-sequencing of the promoter region, exons 1 and 2 of the LDLR gene, the lack of functional analysis of mutations detected, and patients' life habits may be considered to be limitations of our study. These mutations preliminarily identified in Rio Grande do Sul raise issues such as the difficulty in differentiating between common variants (polymorphisms) and disease-generating mutations. Patients with no functional mutation in LDLR may have other variants more distant from intron-exon junctions that affect splicing by influencing cis-elements, such as splicing enhancers, exonic and intronic splicing enhancers or splicing silencers, and exonic and intronic splicing silencers, which usually interact with splicing stimulating or inhibiting factors **[20]**.

Conclusion

The present study demonstrated for the first time the presence of the P518L mutation, located in exon 11 of the LDLR gene, in subjects of European descent from southern Brazil. This mutation has a high potential to be pathogenic since it is located in a domain responsible for LDLR release from the endoplasmic reticulum ER. Thus, because of the diversity of mutations and, probably, of genes that cause FH in Brazil, the molecular basis of the disease should continue to be investigated to better explain the transmission of the FH trait in more than 7% of dyslipidemia Brazilians **[8]** and its phenotypic expression in Rio Grande do Sul, which has one of the highest mortality rates due to cardiovascular diseases in Brazil.

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Ethical Approval

The subjects gave written informed consent and the study was approved by the Ethics Committee of the Medical School of Ribeirão Preto, University of São Paulo.

Informed consent

It was applied to all participants.

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Data sharing statement

No additional data are available.

Conflict of interest

The authors declare no conflict of interest.

Similarity check

It was applied by Ithenticate@.

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