

# Genetic Testing of Familial Hypercholesterolemia in a Hospital-Based Population of the City of Buenos Aires

*Estudio genético de hipercolesterolemia familiar en una población hospitalaria de la Ciudad de Buenos Aires*

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## ABSTRACT

**Background:** Familial hypercholesterolemia is a primary hyperlipidemia. It is an autosomal dominant genetic disorder of lipoprotein metabolism, characterized by elevated plasma concentration of low-density lipoprotein cholesterol and presence of tendon xanthomas, and is associated with early cardiovascular disease.

**Objectives:** The aim of this study was to investigate the presence of mutations in the main gene associated with the development of familial hypercholesterolemia (LDLR) in a group of patients identified as “index cases” attending the Lipid Clinic of Hospital Universitario Fundación Favaloro with clinical diagnosis of familial hypercholesterolemia, and to determine the ancestral composition of the study population.

**Methods:** We evaluated 38 patients with clinical diagnosis of familial hypercholesterolemia. Mutation screening of the LDLR gene coding region and adjacent intronic areas was performed using Sanger sequencing. The ancestral component of the study population was investigated using 46 ancestry-informative markers (AIM-Indel).

**Results:** Fifty different variants were identified, 48% of which were considered pathogenic. A genotype-phenotype severity correlation was established in 60.5% of the patients evaluated. The ancestral component of the study population was predominantly European, followed by native-American and African in a lower proportion.

**Conclusions:** Genetic testing by LDLR gene sequencing in patients identified as “index cases” with clinical diagnosis of familial hypercholesterolemia allows to correlate the genetic information with the severity of the clinical phenotype and to perform a cascade screening of the family members presenting the inclusion criteria considered.

**Key words:** Cholesterol –Risk Factor - Receptors, LDL - Hyperlipidemias - Anticholesteremic Agents

## RESUMEN

**Introducción:** La hipercolesterolemia familiar es una hiperlipidemia primaria. Se trata de un trastorno genético autosómico dominante del metabolismo de las lipoproteínas, caracterizado por concentraciones plasmáticas elevadas de colesterol unido a lipoproteínas de baja densidad y presencia de xantomas tendinosos, y está asociado con el desarrollo prematuro de enfermedad cardiovascular.

**Objetivos:** Investigar la presencia de mutaciones en el principal gen asociado al desarrollo de hipercolesterolemia familiar (LDLR) en un grupo de pacientes identificados como “casos índices”, de entre aquellos que concurren al Servicio de Lípidos del Hospital Universitario Fundación Favaloro con diagnóstico clínico de hipercolesterolemia familiar. Determinar la composición ancestral de la población estudiada.

**Material y métodos:** Se estudió una población de 38 pacientes con diagnóstico clínico de hipercolesterolemia familiar. La región codificante y las zonas intrónicas adyacentes del gen LDLR se secuenciaron automáticamente por el método de Sanger. Se investigó el componente ancestral de la población estudiada a partir del análisis de 46 marcadores informativos de ancestralidad (AIM-Indel).

**Resultados:** Se identificaron 50 variantes diferentes, de las cuales el 48% se consideraron patogénicas. Se logró establecer una correlación genotipo-gravedad del fenotipo en el 60,5% de los pacientes estudiados. El componente ancestral de la población estudiada fue predominantemente europeo, seguido de un componente nativo-americano y, en menor proporción, africano.

**Conclusiones:** El análisis genético por secuenciación del gen LDLR en pacientes identificados como “casos índices” con diagnóstico clínico de hipercolesterolemia familiar permite correlacionar el dato genético con la gravedad del fenotipo observado clínicamente y efectuar un diagnóstico en cascada en los miembros de la familia que presentan los criterios de inclusión considerados.

**Palabras clave:** Colesterol– Factores de riesgo -Receptores de LDL– Hiperlipidemias - Anticolesterolemiantes

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## Abbreviations

<b>FH</b>	Familial hypercholesterolemia	<b>HDL-C</b>	High density lipoprotein-cholesterol
<b>LDL-C</b>	Low density lipoprotein-cholesterol	<b>Lp(a)</b>	Lipoprotein(a)

## INTRODUCTION

Familial hypercholesterolemia (FH, OMIM #143890) is an autosomal dominant genetic disorder, characterized by a reduction in atherogenic low-density lipoprotein (LDL) clearance by the liver, elevating LDL-cholesterol (LDL-C) levels. Consequently, FH causes premature morbidity and mortality due to cardiovascular events. (1) The clinical diagnosis of FH can be made using a score based on LDL-C plasma levels, tendon xanthomas, premature corneal arcus, premature cardiovascular disease and family history of FH. The prevalence of FH is between 1/200 and 1/500 and may be even higher in populations with founder effects. (2-3)

Familial hypercholesterolemia is mostly caused by defects in the gene encoding the LDL receptor (LDLR) protein. Other less common causes of FH include mutations in apolipoprotein B (APOB) that affect the LDL receptor-binding domain, and in proprotein convertase subtilisin/kexin type 9 (PCSK9), involved in recycling of LDL receptors. (2-5) Pathogenic LDLR mutations can affect different stages of the LDL receptor cycle: protein synthesis, protein maturation, expression at the cell surface and correct insertion in the cell membrane, or LDL binding, internalization or recycling. The most severe defects are those that lead to no protein production. These are usually mutations affecting protein expression and synthesis (e.g. when a premature stop codon is introduced or a promoter mutation is detected). (1)

As previously demonstrated, a patient's phenotype can be determined by how much the mutation affects the LDL-receptor activity. (6) However, LDL-receptor activity can be influenced by environmental factors or by other genetic mutations involved in lipid metabolism. (7) Therefore, a direct association between the receptor activity and the phenotype is not always present.

The aim of this study was to investigate the presence of mutations in the LDLR gene in a population with clinical diagnosis of FH. We also investigated the existence of a correlation between the genotype and phenotype and the presence of new genetic variants in an Argentine population sample and thus contribute to the registry of known mutations of this disease.

## METHODS

### Patient selection

All patients with severe hypercholesterolemia (LDL-C  $\geq$ 190mg/dL) attending our Lipid Clinic between January and December 2015 were considered for this study. The Dutch Lipid Clinic Network score (DLCNS) was used for the clinical diagnosis of FH in index cases with suspected FH. (9) All

patients with score  $>$ 8 were considered as cases of definite FH and were invited to participate in the study. (9) Patients with a clear cause of secondary hypercholesterolemia (hypothyroidism, chronic kidney failure, nephrotic syndrome or cholelithiasis), severe hypertriglyceridemia and those with lack of reliable clinical data about the family group were excluded from the study.

### DNA extraction, amplification and sequencing

A 10 ml blood sample was withdrawn from an antecubital vein and collected in a tube with EDTA as anticoagulant agent. DNA extraction was performed using the salting-out method and was quantified by fluorometry (Quantus™ Fluorometer, Promega) using the QuantiFluor™ DNA System (Promega) kit.

The oligonucleotide primers for polymerase chain reaction (PCR) amplification and for sequencing the 18 exons and the adjacent intronic areas of the LDLR gene (MN\_0000527.4) were designed using the Primer-BLAST platform (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). These oligonucleotides were synthesized by Invitrogen by Life Technologies in a scale of 25 nmol and were purified by desalting. Amplified products were analyzed by electrophoresis on a 2% agarose gel and were then purified by enzymatic method using ExoSAP-IT® (Affimetrix, USB). LDLR full gene sequencing reaction was performed using Big Dye® Terminator v1.1 (Applied Biosystems). Amplification and sequencing were done with a Gene Amp® PCR System 9700 (Applied Biosystems). Sequenced products were purified by alcohol precipitation with ethanol-isopropanol. Blood samples from 10 normolipidemic volunteers were used for internal monitoring of the sequencing process and for technique fine-tuning.

### Sequencing and fragment analysis

The resulting sequences were analyzed with the Sequencing Analysis 5.3.1 software (Applied Biosystems) and were aligned with the reference sequence of the LDLR gene (NM\_0000527.4), (10) using the SeqScape 2.5 software (Applied Biosystems). The LDLR gene variants identified in the study population were checked against the Human Gene Mutation Database (HGMD™) and ClinVar archive (NCBI). The following prediction tools were used, as applicable: Mutation Taster (<http://www.mutationtaster.org>), Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2>), Mutation Assessor (<http://mutationassessor.org/r3/>), and SIFT ([www. http://sift.jcvi.org](http://sift.jcvi.org)). The Admixture software (<https://www.genetics.ucla.edu/software/admixture/>) was used to estimate the miscegenation of Native American, European and African parental populations in the study population. The Plink software (<http://pngu.mgh.harvard.edu/~purcell/plink/>) was used to represent the distribution of samples in a principal component analysis (PCA).

### Analysis of InDel sequences

The ancestral component of the samples was evaluated using the protocol proposed by Pereira et al. (11) A set of 46 insertion/deletion ancestry-informative markers was used, in-

**Table 1.** Differential characteristics of patients with clinical diagnosis of familial hypercholesterolemia associated with the presence or absence of pathogenic variants in the LDLR gene

	FH associated with mutation in the LDL receptor (n=23)	FH without mutation in the LDL receptor (n=15)	p value
<b>Clinical characteristics</b>			
Male gender (%)	78.3%	33.3%	0.0007
Age (years)	45.9 ± 12.5	39.6 ± 14.6	NS
DLCNS	12 ± 2.9	12.7 ± 3	NS
<b>CV risk factors</b>			
Hypertension (%)	21.7	13.3	NS
Diabetes (%)	8.7	6.7	NS
Current smoker (%)	13	0	NS
Former smoker (%)	0	20	NS
Family history (%)	65.2	66.7	NS
<b>Lipid panel</b>			
LDL-C (mg/dL)	338.8 ± 89.8	298.1 ± 66.6	NS
HDL-C (mg/dL)	41.6 ± 8.4	57.4 ± 17.7	< 0.001
Triglycerides (mg/dL)	149.5 ± 57	111.4 ± 50.4	0.04
Lp(a) (mg/dL)	71.7 ± 57	177.2 ± 147.2	0.02
<b>Personal history of CVD</b>			
AMI	30.4	6.7	NS
Stroke	4.3	0	NS
CABGS	17.4	13.3	NS
PCI	21.7	6.7	NS
Subclinical carotid artery atherosclerosis	73.7	28.6	NS
Subclinical abdominal atherosclerosis	42.1	28.6	NS
Subclinical peripheral atherosclerosis	66.7	35.7	NS
<b>Lipid panel after treatment</b>			
LDL-C (mg/dL)	159.1 ± 84.6	125.7 ± 51.6	NS
HDL-C (mg/dL)	44.6 ± 10.7	58.4 ± 16.3	0.006
Triglycerides (mg/dL)	127.6 ± 58.9	88.9 ± 20.1	0.03

DLCNS: Dutch Lipid Clinic Network score. CV: Cardiovascular. LDL-C: Low-density lipoprotein cholesterol. HDL-C: High-density lipoprotein cholesterol. Lp(a): Lipoprotein(a). CVD: Cardiovascular disease. AMI: Acute myocardial infarction. CABGS: Coronary artery bypass graft surgery. PCI: Percutaneous coronary intervention.

cluding three parental populations (African, European and Native American). All the markers were analyzed in short fragments (<230 pb) through a single PCR followed by capillary electrophoresis to separate fragments. The result of this analysis was compared with the parental populations in order to estimate the ancestral composition of each subject evaluated.

#### Statistical analysis

Discrete variables were expressed as numbers and percentages and continuous variables as mean ± standard deviation. Groups were compared using Student's t test or the Wilcoxon test for continuous variables and the chi square test or Fisher's exact test for categorical variables, as applicable. A p value <0.05 was considered statistically significant.

#### Ethical considerations

All patients gave their informed consent before blood sample collection. Data was retrieved from the clinical records of the patients or was obtained by direct interrogation at the mo-

ment the samples were taken. Anonymity of personal data and confidentiality of the results were maintained throughout all the phases of the study. The study was approved by the *Fundación Favaloro* Bioethics Committee.

#### RESULTS

Thirty-eight patients were analyzed as index cases of FH. Mean age was 43.4 ± 13.5 years, 68.4% were men and mean DLCNS was 12.4 ± 3.3. Other cardiovascular risk factors were identified in addition to dyslipidemia: hypertension in 7 patients (18.4%) and type 2 diabetes mellitus in 3 (7.9%); 3 patients were current smokers (7.9%) and 3 were former smokers (7.9%). History of cardiovascular disease in first-degree relatives was present in 65.8% of the population studied (25 patients). Mean maximum LDL-C level reported by the patients (without pharmacological therapy) was 322.3 ± 82.7 mg/dL. High-density lipoprotein cholesterol (HDL-C) level was 48 ± 15 mg/dL and triglycer-

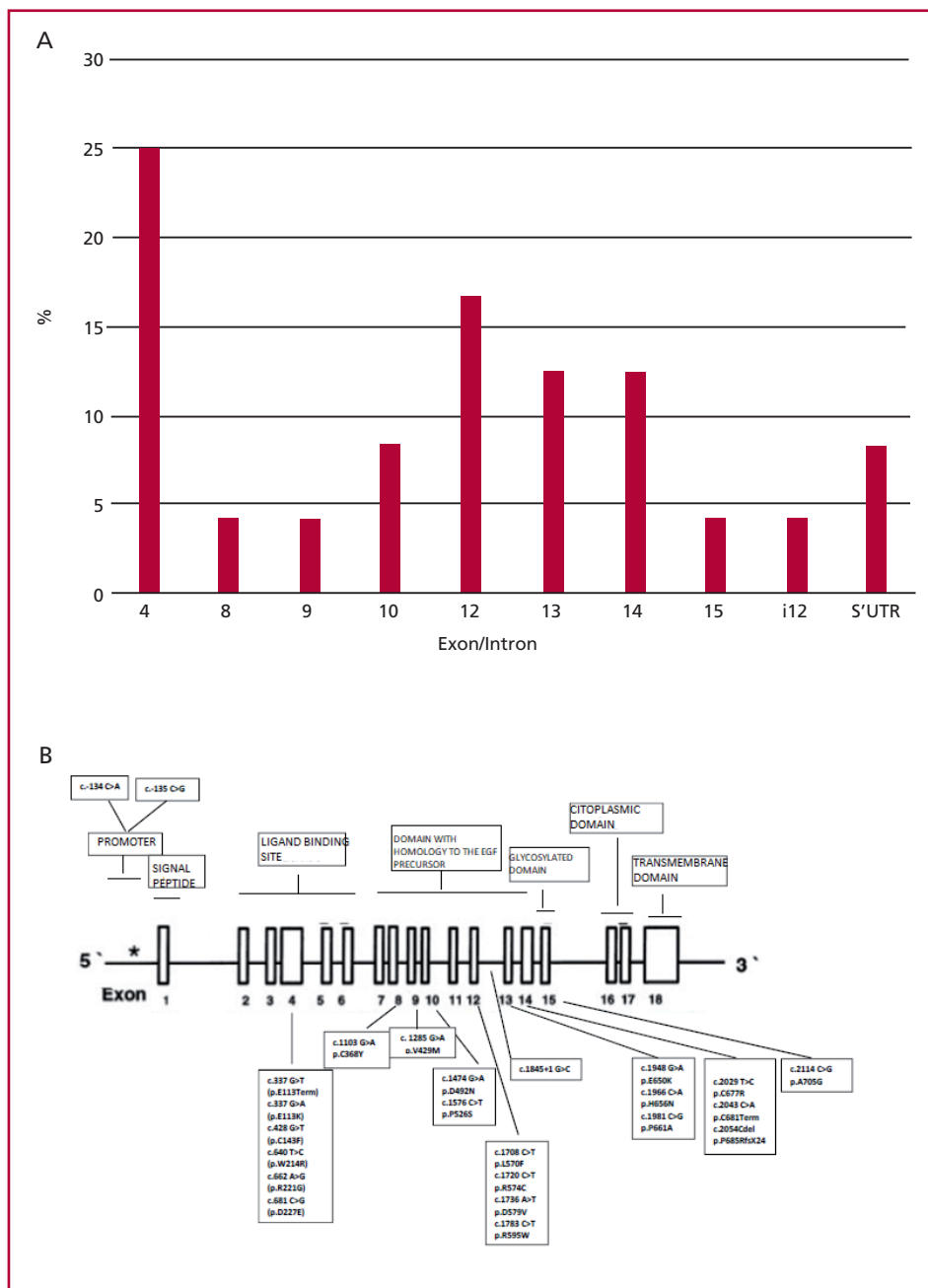


Fig. 1. A. Percent distribution of LDLR gene variants. B. Topological detail of the LDLR gene variant distribution.

ide level was  $134 \pm 55.7$  mg/dL. Lipoprotein(a) [Lp(a)] levels were estimated in 71.2% (n=27) of the patients, with a mean value of  $113.9 \pm 113.2$ . Only one patient was not receiving statins at the moment of data collection due to muscular statin intolerance. Rosuvastatin was the statin most commonly used, followed by atorvastatin and simvastatin. Twenty-eight patients were taking ezetimibe 10 mg/day, 4 patients required cholestyramine and 5 were receiving fenofibrate. Under this regime, mean LDL-C level was  $145.9 \pm 74.3$  mg/dL, mean HDL-C level was  $50 \pm 14.7$  mg/dL and mean triglyceride level was  $112.4 \pm 50.8$  mg/dL.

After sequencing the 18 exons and the adjacent intronic areas of the LDLR gene, 50 variants were identified

and 24 (48%) of them were considered pathogenic by the predictors used, affecting 23 patients. Table 1 summarizes the clinical characteristics associated with the presence of LDLR gene mutations.

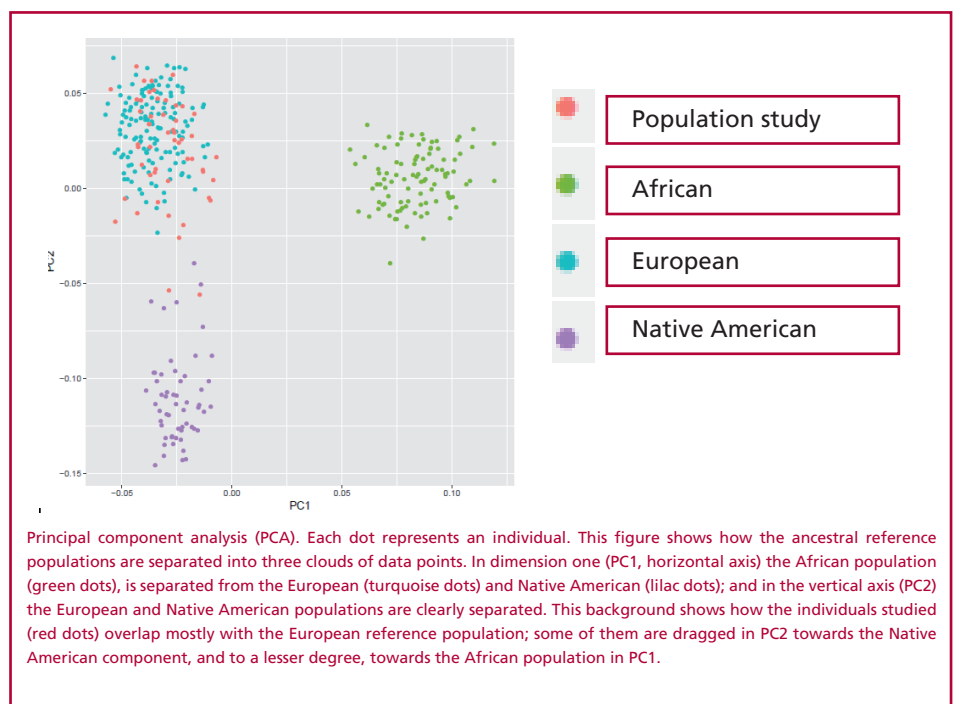
The distribution of variants in the LDLR gene were located in exon 4 (25%), exon 12 (16.7%), exon 13 (12.5%), exon 14 (12.5%), and exon 10 (8.3%). The remaining variants were distributed in exons 8, 9 and 15, in intron 12 and in the 5'UTR region (Figures 1A and 1B). Eight of these variants (33.3%) had not been reported in the databases searched (Table 2).

The analysis of a 46 InDel-type ancestry informative marker panel, using African, European and Native American parental populations revealed that the

**Table 2.** Description of the new variants of the LDL gene

VARIANT	EFFECT PREDICTION	Exon/ Intron	TYPE OF VARIANT	MUTATION TASTER/POLYPHEN2/SIFT	ACMG CLASSIFICATION	SUBCLINICAL ATHEROMATOSIS	FAMILY HISTORY OF CVD	LDL-C (mg/dl)	DLCN SCORE
c.428 G>T	p.Cys143Phe	e4	Nonsense	Disease causing/Benign/Tolerated	Pathological (Moderate)	NO	YES	331	15
c.640 T>C	p.Trp214Arg	e4	Nonsense	Disease causing/Probably damaging/Tolerated	Pathological (Moderate)	YES (CAR/AORT/LL)	YES	350	14
c.1708 C>T	p.Leu570Phe	e12	Nonsense	Disease causing/Benign/Tolerated	Pathological (Moderate)	YES (CAR/AORT/LL)	YES	349	12
c.1736 A>T	p.Asp579Val	e12	Nonsense	Disease causing/Probably damaging/Deleterious	Pathological (Moderate)	YES (CAR/AORT/LL)	YES	356	15
c.1981 C>G	p.Pro661Ala	e13	Nonsense	Disease causing/Benign/Tolerated	Pathological (Moderate)	NE	NO	441	17
c.2114 C>G	p.Ala705Gly	e14	Nonsense	Disease causing/Benign/Tolerated	Pathological (Moderate)	NE	NO	441	17
c.-134 C>A	-	5'UTR	Regulatory	Disease causing/NA/NA	Pathological (Moderate)	NO	YES	267	11
C.-135 C>G	-	5'UTR	Regulatory	Disease causing/NA/NA	Pathological (Moderate)	YES (CAR/AORT/LL)	NO	425	9

**Fig. 2.** Diagram of the ancestral component of the study patients.



population studied had a predominantly European ancestral component (Figure 2).

**DISCUSSION**

In our population, 60.5% of the patients presented an LDLR gene mutation. This frequency varies among the different studies evaluating the genetic causes of

FH. (12, 13) The analysis of LDLR gene mutations may be negative for many reasons. Firstly, some new genes have been implicated in the diagnosis of the disease in recent years. The LDLRAP1 gene encodes an adaptor protein required for the internalization of LDL in the hepatocytes, and thus mutations of this gene explain autosomal recessive FH. (14) Mutations

in the STAP1 gene, probably related with the regulation of cholesterol production, produce types of FH that are clinically indistinguishable. (15) When gene variants that cannot generate monogenic FH come together, they can produce a similar phenotype. Several genes have been involved in this phenomenon (LIPA, APOE, ABCG5/8). (16)

Of importance, the most frequent mutations were observed in exon 4 of the LDLR gene (Figure 1B), in agreement with observations reported by some European studies. (13, 17) This data is consistent with the ancestry testing. Ancestry markers are similar to those previously reported for Argentina, which demonstrates that our dyslipidemic patients have homogeneous ancestral origins in relation to the national population. (18, 19)

The clinical characteristics of mutation carriers are highly relevant; in our study, these subjects exhibited a worse risk profile, even with a similar diagnostic score. (Table 1) In this sense, Khera et al. (20) reported that participants with LDL-C  $\geq 190$  mg/dl and FH mutation had an OR of 22.3 ( $p < 0.0001$ ) compared with non-carriers and LDL-C  $< 130$  mg/dL. In addition, the risk of coronary artery disease was two to three times greater in mutation carriers compared with non-carriers, even with similar LDL-C levels. (20) Therefore, the presence of FH mutations indicates a higher risk for this population.

Many patients with FH are underdiagnosed; thus, cascade genetic screening is a cost-effective tool for the analysis of first-degree relatives. Effective and early treatment can be associated with a 75% reduction in lifetime risk for cardiovascular mortality. (21)

Lipoprotein(a) is an independent risk factor for cardiovascular disease. (22) Some studies have suggested that LDLR gene mutations could be related with elevated Lp(a) plasma levels. Thus, elevated Lp(a) levels would similarly contribute to the risk for myocardial infarction in subjects with and without FH. Subjects with elevated Lp(a) levels could contribute to overestimate the prevalence of FH if Lp(a) levels are not measured and its contribution to the apparent LDL-C is not considered. (23)

This may explain the elevated Lp(a) levels in subjects without LDLR gene mutation in our study and could be the reason why we observed an increase in Lp(a) in our population in those individuals without mutation.

Lack of APOB and PCSK9 gene testing is a limitation of our analysis. Yet, these mutations represent less than 10% of mutations responsible for the disease. (24) In fact, population-based studies only analyze LDLR gene mutations. (25, 26)

The detection of large mutations is performed using multiplex ligation-dependent probe amplification (MLPA) when no point mutations are found. Although this technique was not included in our analysis, the presence of this type of mutations is rare to explain FH; however, its deleterious effect on the normal func-

tion of the LDL receptor protein is well known. This type of gene disorder represents 1.4% in the American College of Medical Genetics and Genomics database. (25)

In conclusion, the identification of new gene variants in a local population with clinical diagnosis of HF emphasizes the importance of continuing the study of the genetic basis of this disease in Argentina. Moreover, considering that the ancestral component analysis proved to be mainly European, it could be erroneously assumed that the carrier population presents a spectrum of known and studied gene variants. The identification of a point gene variant for each index case will allow cascade genetic screening for FH with a simple and inexpensive approach. We believe that the use of these diagnostic methods can be very useful for the early identification of patients with FH, contributing to reduce the risk of developing this metabolic disease.

#### Conflicts of interest

None declared. (See authors' conflicts of interest forms on the website/Supplementary material).

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