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Unusual genetic variants associated with hypercholesterolemia in Argentina

Pablo Corral [a], Andrew S. Geller [b], Eliana Y. Polisecki [b], Graciela I. Lopez [c], Virginia G. Bañares [d], Leonardo Cacciagiu [c], Gabriela Berg [c], Robert A. Hegele [e], Ernst J. Schaefer [b], Laura E. Schreier [c, *]

[a] Departamento de Investigación, Facultad de Medicina, Universidad FASTA, Buenos Aires, Argentina
[b] Boston Heart Diagnostics, Framingham, MA, USA
[c] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Bioquímica Clínica, Laboratorio de Lípidos y Aterosclerosis, INFIBIOC-UBA, Buenos Aires, Argentina
[d] Departamento de Genética Experimental, Centro Nacional de Genética Médica "Dr Eduardo Castilla", Administración Nacional de Laboratorios e Institutos de Salud "Dr Carlos Malbrán", Buenos Aires, Argentina
[e] Robarts Research Institute, University Western Ontario, London, Ontario, Canada

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ABSTRACT

Background and aims: Marked hypercholesterolemia, defined as low density lipoprotein cholesterol (LDL-C) levels ≥ 190 mg/dL, may be due to *LDLR, APOB,* and *PCSK9* variants. In a recent analysis, only 1.7% of cases had such variants. Our goal was to identify other potential genetic causes of hypercholesterolemia.
Methods: In a total of 51,253 subjects with lipid testing, 3.8% had elevated total cholesterol >300 mg/dL and/or LDL-C≥190 mg/dL. Of these, 246 were further studied, and 69 without kidney, liver, or thyroid disease and who met Dutch Lipid Clinic Network criteria of ≥6 points had DNA sequencing done at the *LDLR, APOB, PCSK9, APOE, LDLRAP1, STAP1, ABCG5, ABCG8, CYP27A1, LIPA, LIPC, LIPG, LPL,* and *SCARB1* gene loci and also had 10 SNP analysis for a weighted high LDL-C genetic risk score.
Results: In the 69 subjects with genetic analyses, the following variants were observed in 37 subjects (53.6%): *LDLR* (n = 20, 2 novel), *ABCG5/8* (n = 7, 2 novel), *APOB* (n = 3, 1 novel), *CYP27A1* (n = 3, 1 novel), *LIPA* (n = 2, 1 novel), *APOE* (n = 2), *LIPC* (n = 1, novel), *LIPG* (n = 1, novel), and *SCARB1* (n = 1); 14 subjects (20.3%) had a high polygenic score, with 4 (5.8%) having no variants.
Conclusions: Our data indicate that in addition to variants in *LDLR, APOB, PCSK9, APOE, LDLRAP1,* and *STAP1,* variants in *ABCG5/8, CYP27A1, LIPA, LIPC,* and *LIPG* may be associated with hypercholesterolemia and such information should be used to optimize therapy.

1. Introduction

Familial hypercholesterolemia (FH) is one of the most common monogenic metabolic diseases and is characterized by lifelong elevations in plasma low-density lipoprotein cholesterol (LDL-C) levels and premature coronary heart disease (CHD). The inheritance is autosomal dominant, mainly caused by variations in the genes of LDL Receptor *(LDLR),* Apolipoprotein B *(APOB)* or Proprotein Convertase Subtilisin Kexin type 9 (*PCSK9*) [1]. Heterozygous variants of *LDLR* are present in about 90% of FH cases, while *APOB* and *PCSK9* were found in 5% and 1%, respectively [2].

Despite great progress on FH through the last years, the disease is still underestimated, underdiagnosed, and thus undertreated worldwide, being crucial its early detection [2]. There is a lack of data on the prevalence of FH in several countries, including ours, because there are no national policies with regard to registers or screening strategies [3].

Diagnosis of FH is based on the evaluation in clinical practice and/or genotyping. Clinical assessment takes into account LDL-C levels, the presence of premature CHD in patients or in first-degree relatives, as well as clinical examination for tendon

* Corresponding author. Laboratorio de Lípidos y Aterosclerosis, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina.
E-mail address: lschreier@ffyb.uba.ar (L.E. Schreier).

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xanthomas and corneal arcus at a young age [4]. The Dutch Lipid Clinic Network (DLCN) Criteria are widely used and recommended for FH diagnosis [5].

The genetic analysis of the phenotypic FH patients has become widely applied [6]. However, variant detection rates vary considerably when only classic genes such as *LDLR, APOB* or *PCSK9* are assessed. Between 20 and 40% of individuals with phenotypic heterozygous FH have no variants [7,8].

An extended panel of genes related to increased LDL-C such as *APOE, ABCG5, ABCG8, LIPA, CYP27A1* or *STAP1*, in addition to other genes related to lipid metabolism, for example *LIPG and LIPC*, could explain some of the hypercholesterolemia cases, beyond the traditional genes. In addition, other patients could carry a high burden of multiple small-effect common variants (SNP) that raise LDL-C similar to FH levels, but are the result of polygenic causes [8,9].

The aim of this study was to assess the epidemiology and genetics of hypercholesterolemia in a defined district of Argentina in order to detect potential FH index cases and to ascertain genetic causes using an extended gene panel plus a polygenic score.

2. Patients and methods

Patients were selected from a database of 51,253 subjects over the age of 18 years provided by the Secretary of Health from the district of General Pueyrredón, Buenos Aires, Argentina. Subjects were seen at the Centro de Especialidades Médicas Ambulatorias from July 2013 to February 2016, with data on serum total cholesterol and/or LDL-C values and contact information being provided. Participants were eligible to participate in this study if they had a total serum cholesterol level >300 mg/dL and/or a LDL-C >190 mg/dL. A total of 1967 patients (3.84%) met these inclusion criteria, and up to the moment 246 subjects agreed to participate in further studies. Informed consent was obtained from all patients using a protocol and consent form approved by the Ethics Committee of the University of Buenos Aires (Res CD 4705/14).

In these 246 subjects, an extensive history was obtained with a special focus on cardiovascular disease (CVD) (angina pectoris, myocardial infarction, angioplasty, coronary artery bypass grafting, stroke, and/or peripheral vascular or carotid disease), hospitalizations, hypertension, diabetes mellitus, and current lipid-lowering treatment. A careful physical examination was carried out with special care being taken to detect the presence of tendon xanthomas and/or corneal arcus. Medical records of the index cases, and when available, of their first degree relatives (parents, offspring and siblings) were obtained and examined. Premature CVD was defined as the presence angina, myocardial infarction, coronary angioplasty or coronary artery bypass, or other vascular disease occurring prior to age 55 years in male index cases and family members, and prior to age 65 years in female index cases and family members. The score according to DLCN criteria was calculated in each patient considering the highest LDL-C value noted [5]. A total of 98 subjects (40%) were receiving lipid-lowering treatment, and when LDL-C data off medication were not available, a LDL-C correction was applied based on the dose and potency of the statin being used [10]. In addition 21 subjects were excluded: 8 (3.3%) had a TSH value > 10 mUI/L, 4 (1.6%) had a serum creatinine >1.7 mg/dL, 2 (0.8%) had an alkaline phosphatase >200 U/L, and 1 (0.4%) had serum triglyceride levels >1000 mg/dL. Three (1.2%) women were excluded because of pregnancy, and 3 subjects (1.2%) declined to have their blood drawn. Therefore, a total of 225 subjects with potential FH were included in the research analysis. All these subjects had their blood drawn in the non-fasting state, and samples were sent to the Lipid and Atherosclerosis Laboratory at the University of Buenos Aires for biochemical analysis.

Total cholesterol, triglycerides, HDL-cholesterol, direct LDL-C, creatinine, and alkaline phosphatase were measured in serum samples using standardized enzymatic methods with assay kits (Roche Diagnostics, Mannheim/Germany) using a Cobas C-501 autoanalyzer. Mean coefficients of variation (CV) values for these parameters were< 2.3% for intra-assay CVs and <3.0% for inter-assay CVs. Serum apolipoproteinB (apoB) levels were determined using Roche immunoturbidimetric assays on the same automated analyzer, with intra-assay and interassay CVs of <2.5%. TSH was measured by chemiluminescence (DPC, Immulite, Los Angeles, CA, USA) with intra- and inter-assay CVs of <3.5%.

Genomic DNA was extracted from whole blood by the Salting Out protocol [11]and library preparation was performed using Nextera Rapid Capture Custom Enrichment kit (Illumina, San Diego, CA). Next generation DNA sequencing was carried out on all subjects that had a DLCN score of ≥6. DNA sequencing was carried at the following gene loci (exons and intron padding): *LDLR, APOB, PCSK9, LDLRAP1, STAP1, ABCG5, ABCG8, APOE, LIPA, CYP27A1, LIPC, LIPG, LPL*, and *DHCR24* as previously described [12]. In addition, 10 SNPs (rs6544713 in *ABCG8*, rs515135 in *APOB*, rs12740374 in *CELSR2*, rs3846663 in *HMGCR*, rs2650000 in *HNF1*, rs6511720 in *LDLR*, rs6102059 in *MAFB*, rs10401969 in *NCAN*, rs11206510 in *PCSK9*, and rs1501908 in *TIMD4)* were included for the calculation of a Genetic Risk Score developed to assess polygenic contribution to increased LDL-C as previously described [6].

Sequencing was performed on an Illumina MiSeq Dx instrument using 2 × 150 paired end reads. The median read depth was 600X, with 100%sensitivity and specificity for SNV's and 100% sensitivity and 91% specificity for Indels. FASTQ files were processed using a custom workflow in CLC Biomedical Genomics Workbench (v3.2, Qiagen). All mapping, variant calling and filtering, and quality control parameters were performed using validated protocols. VCF files containing all identified variants in targeted regions were annotated using Ingenuity Variant Analysis (Qiagen) with a customized filtering cascade. The variants were classified according to the American College of Medical Genetics and Genomics (ACMG) [13,14]. In addition, assessment of CNV's and large structural variants was performed using built in tools from Biomedical Genomics Workbench and VarSeq CNV Analysis from Golden Helix.

With regard to novel variants, further analysis was sometimes required to determine whether these variants were pathogenic. Software used for these assessments included: 1) Genomic Evolutionary Rate Profiling (GERP) scores to evaluate conservation, 2-DANN pathogenicity scores (which use computational deep learning methodologies to classify variants), and 3- dbNSFP which aggregates several informatics prediction algorithms and uses a proportion to indicate the number of scores predicting the mutation to be deleterious versus the number predicting low functional impact. The variant classifications were based on ACMG guidelines, and were made using aggregated data from public databases and computational tools in VarSome (Saphetor) and Ingenuity Variant Analysis (Qiagen). Calculation of the Polygenic Risk Score (GRS) at particular loci associated with LDL-C levels applied for each SNV using previous methods. The cutoff score for the 90th percentile was 1.96, with maximum GRS of 2.42 as described [15].

All data are expressed as median (range) according to their distribution and percentage or prevalence. The statistical analysis was performed using SPSS 19.0. Frequency differences between ranges were assessed by Chi2 test. A $p < 0.05$ was considered statistically significance.

3. Results

In the population studied (n 51,253 subjects), 1967 or 3.8% had a total cholesterol >300 mg/dL and/or an LDL-C ≥190 mg/dL.

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Moreover, in this population of subjects who chose to be further analyzed (n = 246), after exclusions, the median age was 46 years, and 69% were female. Of these patients, 45 had 1st degree relatives with CVD, 32 had LDL-C values >95th percentile value, 23 had corneal arcus before 45 years, 8 had tendon xanthomas, 4 had premature coronary artery disease and 4 had premature cerebral artery disease. Median laboratory values in mg/dL were: LDL-C 198 (122–414), HDL-C 48 (27–112), triglycerides 175 (50–420), and apoB 140 (68–287). It should be noted that many of these cases were on lipid lowering treatment, mainly statin therapy. Using DLCN scoring, there were 22 definite FH cases (score >8, range: 9–14), 47 probable FH cases (score 6–8) and 105 possible cases (score 3–5). Patients with a score <3 were categorized as unlikely to have FH. Considering only the definite cases, FH prevalence was estimated at 1/291.

Subjects with a DLCN score of ≥6, consistent with the clinical diagnosis of FH (n = 69), underwent DNA sequencing, with 28 (40.6%) having no variants identified. The remaining 41 patients (59.4%) were found to have variants or a high polygenic score or a combination of these two factors. A total of 37 patients (53.6%) were found to have variants affecting LDL-C levels at the following gene loci with some patient overlap: *LDLR* (n = 20), *ABCG5/8* (n = 7), *APOB* (n = 3), *CYP27A1* (n = 3), *LIPA* (n = 2), *APOE* (n = 2), *LIPC* (n = 1), and *LIPG* (n = 1). Moreover, 20% (n = 14) had a high polygenic score, and in 4 it was the only abnormality found. All variants by patient and their classification are shown in Tables 1 and 2, and summarized below.

LDLR variants were found in 20 patients: 1 patient was heterozygous for a novel insertion predicted to affect splicing, 1 patient was heterozygous for a deletion of exons 16–18, 1 patient was heterozygous for a duplication of exons 2–8, 4 patients were heterozygous for G343S, 2 patients were heterozygous for V523M, 1 patient was heterozygous for R744Q, 1 patient was heterozygous for G373D, 1 patient was heterozygous for S326C, 1 patient was heterozygous for E228K, 1 patient was heterozygous for C143F (novel mutation), 1 patient was heterozygous for F282L, 1 patient was heterozygous for E228D and also had a high polygenic score, 1 patient was heterozygous for N632I and also had a high polygenic score, 1 patient was heterozygous for D492G and also had a high polygenic score, 1 patient was heterozygous for R595Q and also was heterozygous for A98G at the *ABCG5* locus, and 1 patient was heterozygous for C216R and also was heterozygous for G181D at the *LPL* locus.

APOB variants were found in 3 patients: 1 patient was heterozygous for R3527Q, 1 patient was heterozygous for V3822V introducing an exonic splice site enhancer, and 1 patient was heterozygous for T3388I (novel mutation) and also was heterozygous for L323P at the *APOE* locus, and also had a high polygenic score. Therefore, of the 69 patients with DNA data, 20 patients (29.0%) had *LDLR* variants, 4 of which were novel, and 4.3% had *APOB* variants, 1 of which was novel, for a total of 33.3% or one third having variants in classical FH genes. Moreover, 4 of these patients had a high polygenic score. In addition, 1 patient was heterozygous for L323P at the *APOE* locus and also had a high polygenic score, for a total of 23 patients (34.8%) with variants linked to elevated LDL-C (Table 1).

A surprising finding in this study was that 6 patients (8.7%) had *ABCG5* and/or *ABCG8* variants known to be associated with sitosterolemia: 3 patients were heterozygous for A98G at *ABCG5,* 1 of whom was also heterozygous for T378M at *SCARB1,* 1 patient was heterozygous for G79R at ABCG5, 1 patient was heterozygous for a novel *ABCG5* variant and heterozygous for R152C at *ABCG8,* and 1 patient was heterozygous for C287C causing an exonic splice site enhancer at *ABCG5* and heterozygous for P415H at *ABCG8*. Therefore the last two patients had genetics consistent with sitosterolemia since they were compound heterozygotes at ABCG5/8.

Another surprising finding in this study was that 3 patients (4.3%) were heterozygous for variants at the *CYP27A1* locus linked to CTX: one patient was heterozygous for T339M, 1 patient was heterozygous for P384T (novel mutation) and also had a high polygenic score, and 1 patient was heterozygous for F240F causing an alteration of an exonic splice site enhancer, and also had a high polygenic score.

A third surprising finding was that 2 patients (2.9%) were heterozygous for variants at the *LIPA* gene locus associated with lysosomal acid lipase deficiency: 1 patient was heterozygous for

Table 1
Subjects with variants in FH genes (n = 23).

#	Variant name(s)	Gene(s)	ID/Novel	Type	Genotype	Classification	GRS
1	c.1118G > A (p.G373D)	*LDLR*	rs879254797	SNV	HTZ	Pathogenic	–
2	c.977C > G (p.S326C)	*LDLR*	rs879254747	SNV	HTZ	Pathogenic	–
3	g.(11200292_11210898)_(11222316_11223953)dup (Ex2-8dup)	*LDLR*	No CNV ID	Duplication	HTZ	Pathogenic	–
4	c.191-3_191-2insA	*LDLR*	Novel	Insertion	HTZ	Pathogenic	–
5	g.39215_47749del8535 (Ex16-18del)	*LDLR*	nsv1067857	Deletion	HTZ	Pathogenic	–
6	c.682G > A(p.E228K)	*LDLR*	rs121908029	SNV	HTZ	Pathogenic	–
7	c.428G > T (p.C143F)	*LDLR*	Novel	SNV	HTZ	L Pathogenic	–
8	c.684G > C (p.E228D)	*LDLR*	No rs ID	SNV	HTZ	L Pathogenic	+
9	c.1027G > A (p.G343S)	*LDLR*	rs730882096	SNV	HTZ	L Pathogenic	–
10	c.1027G > A (p.G343S)	*LDLR*	rs730882096	SNV	HTZ	L Pathogenic	–
11	c.1027G > A (p.G343S)	*LDLR*	rs730882096	SNV	HTZ	L Pathogenic	–
12	c.1027G > A (p.G343S)	*LDLR*	rs730882096	SNV	HTZ	L Pathogenic	–
13	c.846C > A (p. F282L)	*LDLR*	rs730882090	SNV	HTZ	L Pathogenic	–
14	c.1567G > A (p.V523M)	*LDLR*	rs28942080	SNV	HTZ	L Pathogenic	–
15	c.1567G > A (p.V523M)	*LDLR*	rs28942080	SNV	HTZ	L Pathogenic	–
16	c.1895A > T(p.N632I)	*LDLR*	No rs ID	SNV	HTZ	L Pathogenic	+
17	c.971A > G(p.D492G)	*LDLR*	rs879254918	SNV	HTZ	L Pathogenic	+
18	c.2231_2232delGAinsAG (p.R744Q)	*LDLR*	rs137853963	Substitution	HTZ	VUS	–
19	c.1784G > A(p.R595Q) (;) c.293C > G(p.A98G)	*LDLR(;)ABCG5*	rs201102492(;)rs145164937	SNV(;)SNV	HTZ(;)HTZ	Pathogenic(;)VUS	–
20	c.646T > C(p.C216R) (;) c.542G > A(p.G181D)	*LDLR(;)LPL*	rs879254610	SNV(;)SNV	HTZ(;)HTZ	Pathogenic(;)VUS	–
21	c.10580G > A (p.R3527Q)	*APOB*	rs5742904	SNV	HTZ	Pathogenic	–
22	c.11466G > A (p.V3822V)	*APOB*	rs755842633	SNV	HTZ	VUS	–
23	c.10163C > T(p.T3388I) (;)c.890T > C(p.L297P)	*APOB(;)APOE*	Novel(;)rs1039600156	SNV(;)SNV	HTZ(;)HTZ	VUS(;)VUS	+

GRS: Genetic Risk Score, +: ≥1,96, 90th percentile, -: < 1,96, 90th percentile, SNV: single nucleotide variants, HTZ: heterozygous, VUS: variant of uncertain significance, L Pathogenic: likely pathogenic.

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Table 2
Subjects with variants in Non-FH genes (n = 14).

#	Variant name(s)	Gene(s)	ID/Novel	Type	Genotype	Classification	GRS
24	c.890T > C (p.L297P)	*APOE*	rs1039600156	SNV	HTZ	VUS	+
25	c.1244C > A (p.P415H)(;)c.861C > T (p.C287C)	*ABCG8(;)ABCG5*	Novel (;)rs530120175	SNV(;)SNV	HTZ(;)HTZ	VUS(;)VUS	–
26	c.293C > G (p.A98G)(;)c.1133C > T (p.T378M)	*ABCG5(;)SCARB1*	rs145164937(;)rs748231262	SNV(;)SNV	HTZ(;)HTZ	VUS(;)VUS	–
27	c.454C > T (p.R152C)(;)c.402+1G > A	*ABCG8(;)ABCG5*	rs762452685(;)Novel	SNV(;)SNV	HTZ(;)HTZ	VUS(;)VUS	–
28	c.235G > A (p.G79R)	*ABCG5*	rs142125966	SNV	HTZ	VUS	–
29	c.293C > G (p.A98G)	*ABCG5*	rs145164937	SNV	HTZ	VUS	–
30	c.293C > G (p.A98G)	*ABCG5*	rs145164937	SNV	HTZ	VUS	–
31	c.380G > A(p.R127Q)	*LIPA*	rs544080483	SNV	HTZ	VUS	–
32	c.1141C > G (p.L381V)	*LIPA*	Novel	SNV	HTZ	VUS	–
33	c.1016C > T (p.T339M)	*CYP27A1*	rs121908102	SNV	HTZ	L Pathogenic	–
34	c.1150C > A (p.P384T)	*CYP27A1*	Novel	SNV	HTZ	VUS	+
35	c.720C > T (p.F240F)	*CYP27A1*	rs748075933	SNV	HTZ	VUS	+
36	c.724G > A (p.D242N)	*LIPC*	Novel	SNV	HTZ	VUS	+
37	c.3G > T (p.M1I)	*LIPG*	Novel	SNV	HTZ	Pathogenic	+

GRS: Genetic Risk Score, +: ≥1,96, 90th percentile, -: < 1,96, 90th percentile, SNV: single nucleotide variants, HTZ: heterozygous, VUS: variant of uncertain significance, L Pathogenic: likely pathogenic.

R127Q and 1 patient was heterozygous for L381V. Therefore, a total of 13 patients (18.8%) presenting with elevated LDL-C had genetic evidence of sterol or lipid storage disorders. We also found 1 patient heterozygous for D242N at the *LIPC* (hepatic lipase) gene locus who also had a high polygenic score, and one patient who was heterozygous for M1I at the *LIPG* (endothelial lipase) gene locus who also had a high polygenic risk (Table 2). In addition, we have tabulated all novel variants (Table 3).

Regarding the presence of physical stigmata in relation to type of gene variants, 8 tendon xanthomas were observed: 4 in patients with variants (1 *LDLR*, 1 *LDLR* + *LPL*, 1 *LDLR* + *LIPA* and 1 *LIPC*) and 4 without variants; 23 patients with corneal arcus: 11 with variants (1 *APOB*, 2 *CYP27A1,* 1 *APOE,* 4 *LDLR*, 2 *ABCG5*, 1 *LDLR* + *LIPA)* and 12 without variants. Four of these patients have both xanthoma and corneal arcus. It must be taken into account that many patients with FH at the present time do not have signs as tendon xanthomas because of their treatment with statins and ezetimibe.

4. Discussion

This study shows the first results of the FH detection program carried out in Argentina. Considering the definite phenotypic FH cases, after excluding hypothyroidism, renal, and/or liver disease, the prevalence estimated in a population of a defined district was 1/291. Molecular genetic analysis of index cases with a DLCN score ≥6, targeted to an expanded gene panel, showed 33.3% of patients with variants in traditional FH genes (*LDLR* and *APOB*), and 20% of patients with variants in other genes related to elevated LDL-C, lipoprotein metabolism disorders and/or polygenic causes.

A recent large analysis of the Copenhagen General Population Study genotyping *LDLR* [W23X; W66G; W556S] and *APOB* [R3500Q] was carried out in 98,098 subjects who account for 38.7% of pathogenic FH variants in Copenhagen. The authors concluded that FH causing variants are estimated to occur in 1:217 in the general population, and that they are best identified by a definite or probable phenotypic diagnosis of FH based on the DLCN criteria or an LDL-C value > 170 mg/dL. In the present study, accounting for definite index cases according to DLCN score, in order to estimate a preliminary prevalence, reveals a proportion close to what was reported by Benn et al. [16].

Santos and colleagues examined data available on the genetics of FH in IberoAmerica including Argentina [17]. In these countries, 10–15 variants are responsible for 30–47% of all FH cases. The most common *APOB* variant was R3527Q, which we also found in one subject. The six most common *LDLR* variants were S177L, G592Q, A431T, Q92E, V429M, and S326C [17]. We also found one subject with the S326C variant. In these analyses, other genes are not mentioned. Bañares and colleagues, co-investigators on this study, studied *LDLR, APOB, APOE, PCSK9*, and *LDLRAP1* genetic variants in FH cases in Argentina [18]. Genetic variants were identified in 73% of the patients, showing a wide heterogeneity except one relatively common allele, the Lebanese mutation. In these 24 subjects, 2 carried the *APOB* R3527Q variant. They also found 19 LDLR variants, 4 of which were novel and 2 were also found in this study (E228D and N632I). No subjects with *APOE, PCSK9,* or *LDLRAP1* variants were identified. They did find 2 true homozygotes and 3 compound heterozygotes with *LDLR* variants, and 1 double heterozygote with an *LDLR* variant and the *APOB* R3527Q variant [18].

In the most recent American Heart Association statement on familial hypercholesterolemia in 2015, no mention is made of other disorders such as sitosterolemia, cerebrotendinousxanthomatosis (CTX), or lysosomal acid lipase deficiency as being potential causes of markedly elevated LDL-C levels [2]. However, in their 2003 review Pullinger et al. indicated that five monogenic disorders need

Table 3
Novel variants.

Gene	Type	Nucleotide	Protein	GERP Score	DANN Score	dbNSFP predictors (Damaging/Tolerated)	ACMG classification (evidence)
LDLR	SNV	c.428G > T	p.C143F	5.9999	0.9919	9/0	LP (PM1,PM5,PP2,PP3)
	Insertion	c.191-3_191-2insA	–	–	–		VUS (PM2)
LIPA	SNV	c.1141C > G	p.L381V	4.0399	0.9899	3/6	VUS (PM1,PM2,BP4)
CYP27A1	SNV	c.1150C > A	p.P384T	5.76	0.9977	8/1	VUS (PM2,PP2,PP3)
ABCG8	SNV	c.1244C > A	p.P415H	4.2699	0.9957	2/7	VUS (PM2,BP4)
ABCG5	SNV	c.402+1G > A	–	5.42	0.9951	3/0	VUS (PM2,PP3)
APOB	SNV	c.10163C > T	p.T3388I	3.8199	0.9984	3/6	VUS (PM2,BP1)
LIPC	SNV	c.724G > A	p.D242N	5.67	0.9993	9/0	VUS (PM2,PP3,BP1)
LIPG	SNV	c.3G > T	p.M1I	4.55	0.9919	6/3	P (PVS1,PM2,PP3)

SNV: single nucleotide variants, VUS: variant of uncertain significance, LP: Likely Pathogenic, P: pathogenic.

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to be considered in patients with LDL-C ≥ 190 mg/dL: 1) FH due to *LDLR* variants, 2) FH due to *APOB* variants, 3) autosomal recessive hypercholesterolemia due to *LDLRAP1* variants, 4) sitosterolemia due to *ABCG5/8* variants, and 5) cerebrotendinous xanthomas due to *CYP27A1* variants [19]. Moreover, according to Dron and Hegele, variants at the *APOE, PCSK9, STAP1,* and *LIPA* gene loci, as well as a polygenic etiology, should also be considered [20]. Defesche and colleagues in 2017 reviewed FH genetics and indicated that the following number of gene variants had been reported as being causative: *LDLR*> 2000, *APOB* 32, *PCSK9* 23, *STAP1* 4, *ABCG5/8* 2, *APOE* 1, and *LIPA* 1 [21]. Such counts may not be representative because many studies have only sequenced *LDLR, APOB*, and *PCSK9* genes such the study by Khera and colleagues [22]. These investigators concluded that among individuals with LDL-C ≥190 mg/dL, gene sequencing identified an FH mutation in <2%. However, for any given observed LDL-C, FH mutation carriers were at substantially increased risk for CVD, as compared to non-carriers. While this study was very large, subjects were not characterized with regard to the presence of xanthomas or thyroid function, or mode of inheritance, and only three genes were examined [22].

Our report adds another seven *ABCG5/8* variants to this list, two of which are novel. Although they are classified as VUS, we suggest that sterol disorders, such as sitosterolemia with variants in *ABCG5/G8*, need to be considered in the differential of patients with familial increases in LDL-C. In fact, in a recent report, Lamiquiz-Moneo et al. propose that *ABCG5/G8* gene variation would play a role in the pathogenesis of genetic hypercholesterolemia, unrelated to *LDLR, APOB PCSK9* and *APOE* [23]. Measurement of surrogate markers of intestinal cholesterol absorption could contribute to establish the functionality of *ABCG5/G8* variations.

Accordingly, there are sterol disorders where affected subjects may have normal or elevated LDL-C levels. These disorders include sitosterolemia, CTX, and lysosomal acid lipase deficiency. Patients with sitosterolemia have marked intestinal hyperabsorption of β-sitosterol, campesterol, and cholesterol, and very high plasma levels of β-sitosterol and campesterol [24,25]. Such patients may have xanthomas and premature CVD, and have defects in the ABCG5/8 transporters due to homozygous or compound heterozygous variants in *ABCG5* and/or *ABCG8* [27]. Patients with CTX may present with chronic diarrhea, cognitive dysfunction, premature cataracts, xanthomas, significant neurologic disease, and premature CVD [26]. This disease is due to defective sterol 27-hydroxylase enzyme activity caused by defects in the *CYP27A1* gene, with a lack of chenodeoxycholic acid formation and overproduction of plasma cholestanol. These patients have markedly elevated cholestanol levels in plasma and cerebrospinal fluid, resulting in cholestanol deposition in their tendons and brain. Many of the complications of the disease can be prevented and the plasma cholestanol levels normalized by treatment with chenodeoxycholic acid [25]. Herein, we observed 3 *CYP27A1* variations, one was classified as likely pathogenic for CTX. Cholestanol was not measured, and the carriers, apart from high LDL-C, two of them showed corneal arcus. However, until now, the evidence on the relationship between *CYP27A1* variations and hypercholesterolemia is unclear [26].

In addition, two *LIPA* variants were also observed, one of which is novel. Moreover, we add 2 *APOE* variants to the list, as well as indicate a potential linkage of elevated LDL-C with both *LIPC* (hepatic lipase) and *LIPG* (endothelial lipase) variants. In many of these cases a high polygenic risk score was also present making such associations less certain of causality.

Patients with a deficiency of lysosomal acid lipase (LAL), encoded by the *LIPA* gene, present with various types of dyslipidemia including hypercholesterolemia, hepatomegaly, and liver fibrosis [27]. LAL catalyzes the intracellular hydrolysis of cholesteryl esters and triglycerides in hepatocytes and macrophages. *LIPA* defects cause accumulation of these lipids in lysosomes, especially cholesteryl esters in the liver. If the disease is not treated, these patients develop liver failure; they can be successfully treated with enzyme replacement therapy [27]. Measurement of lysosomal lipase activity in the patients with *LIPA* variant could contribute to understand the role of the variants.

Overall these data indicate that markedly elevated LDL-C levels are not only observed in subjects with *LDLR, APOB, APOE*, and *PCSK9* variants, but also in subjects with variants at the *ABCG5/8*, *CYP27A1*, and *LIPA* gene loci. However, in contrast to classical autosomal dominant FH, these patients are found in kindreds with an autosomal recessive mode of inheritance. A form of FH with an autosomal recessive mode of inheritance has also been identified due to variants at the *LDLRAP1* gene locus [28]. Therefore, this mode of inheritance does not automatically exclude FH.

We consider that the present study contributes by expanding the panel of genes, given that genes such as ABCG5/8 or CYP27A1 are often not examined in hypercholesterolemic patients. However, we assume that the pathogenicity for many of these variants is weak and has to be confirmed with further studies. We postulate that pathogenicity determination should be independent of interpreting the cause of disease in a given patient and it should be determined by the entire body of evidence in aggregate. On the other hand, we do not rule out that many of the detected variants may be incidental findings.

The study has some limitations, this was a well-planned systematic study in Argentina that includes a complete panel of genes; however, an increased number of FH subjects in the population studied would add more information to confirm the current results. The primary database failed to indicate the patients who were under hypolipidemic treatment, therefore, in the selection process, some patients were undiagnosed and not be taken into account in this study. Regarding the significance of the variants in *ABCG5/8* and also in *LIPA*, *LIPC/G* a question raised by our data is whether these heterozygotes patients with these variants are biochemically and clinically affected.

Finally, our results are consistent with observations that in cohorts with both LDL >190 mg/dL and clinically suspected FH, there is a high prevalence of monogenic disease-causing variants - up to 50% of patients or more [9] - compared to a very low rate of these variants- < 2% in unselected populations with LDL>190 mg/dL [22], indicating that a careful clinical assessment increases ascertainment of such pathogenic variants.

Conflicts of interest

P.C. has received honoraria for consultancy, membership on advisory boards and/or speakers' bureaus for Aegerion, Amgen, Sanofi, R.A.H. has received honoraria for consultancy, membership on advisory boards and/or speakers' bureaus for Aegerion, Amgen, Boston Heart Diagnostics, Gemphire, Ionis, Lilly, Merck, Pfizer, Regeneron, Sanofi and Valeant. E.J.S. has received honoraria for consultancy, membership on advisory boards and/or speakers' bureaus for Amarin, Amgen, Denka-Seiken, Kastle, and Merck. L.S. has received honoraria for consultancy and membership on advisory boards for: Sanofi, Aegerion, and Boston Heart Diagnostics. The other authors have no conflicts of interest to disclose, except that Drs. Geller, Polisecki, and Schaefer are or have been employees of Boston Heart Diagnostics.

Author contributions

Conception and design: P.C., E.Y.P., E.J.S., and L.S.; data acquisition on population: P.C., V.B.,G.L., G.B., L.C., and L.S.; data acquisition on genetic testing: A.S.G. and E.Y.P.; data analysis and

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interpretation: P.C., A.S.G., E.Y.P., R.A.H., E.J.S., and L.S.; manuscript preparation: E.J.S., P.C., A.S.G., E.Y.P., R.A.H., and L.S. All authors have read and approved the final manuscript.

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