ELSEVIER

CrossMark

Atherosclerosis Supplements 29 (2017) 17–24

ATHEROSCLEROSIS SUPPLEMENTS

www.elsevier.com/locate/atherosclerosis

Spectrum of mutations in Italian patients with familial hypercholesterolemia: New results from the LIPIGEN study

Angela Pirillo [a,*], Katia Garlaschelli [a], Marcello Arca [b,1], Maurizio Averna [c,1], Stefano Bertolini [d,1], Sebastiano Calandra [e,1], Patrizia Tarugi [f,1], Alberico L. Catapano [g,h,1] on behalf of the LIPIGEN Group[2]

[a] *Center for the Study of Atherosclerosis, E. Bassini Hospital, Cinisello Balsamo, Milan, Italy*
[b] *Department of Internal Medicine and Medical Specialties, Sapienza University, Rome, Italy*
[c] *Biomedical Department of Internal Medicine and Specialistics (DIBIMIS), University of Palermo, Palermo, Italy*
[d] *Department of Internal Medicine, University of Genoa, Genoa, Italy*
[e] *Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy*
[f] *Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy*
[g] *Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy*
[h] *IRCCS MultiMedica, Sesto S. Giovanni, Milan, Italy*

Abstract

Background: Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by elevated plasma levels of LDL-cholesterol that confers an increased risk of premature atherosclerotic cardiovascular disease. Early identification and treatment of FH patients can improve prognosis and reduce the burden of cardiovascular mortality.

Aim of this study was to perform the mutational analysis of FH patients identified through a collaboration of 20 Lipid Clinics in Italy (LIPIGEN Study).

Methods: We recruited 1592 individuals with a clinical diagnosis of definite or probable FH according to the Dutch Lipid Clinic Network criteria. We performed a parallel sequencing of the major candidate genes for monogenic hypercholesterolemia (*LDLR, APOB, PCSK9, APOE, LDLRAP1, STAP1*).

Results: A total of 213 variants were detected in 1076 subjects. About 90% of them had a pathogenic or likely pathogenic variants. More than 94% of patients carried pathogenic variants in *LDLR* gene, 27 of which were novel. Pathogenic variants in *APOB* and *PCSK9* were exceedingly rare. We found 4 true homozygotes and 5 putative compound heterozygotes for pathogenic variants in *LDLR* gene, as well as 5 double heterozygotes for *LDLR/APOB* pathogenic variants. Two patients were homozygous for pathogenic variants in *LDLRAP1* gene resulting in autosomal recessive hypercholesterolemia. One patient was found to be heterozygous for the ApoE variant p.(Leu167del), known to confer an FH phenotype.

Conclusions: This study shows the molecular characteristics of the FH patients identified in Italy over the last two years. Full phenotypic characterization of these patients and cascade screening of family members is now in progress.

Keywords: Familial hypercholesterolemia; *LDLR*; *PCSK9*; *APOB*; Pathogenic variants

* Corresponding author. Center for the Study of Atherosclerosis, E. Bassini Hospital, Via M. Gorki 50, Cinisello Balsamo, Milan, Italy.
E-mail address: angela.pirillo@guest.unimi.it (A. Pirillo).

[1] Members of the LIPIGEN Steering Committee.

[2] The Members of the LIPIGEN Steering Committee are listed in Appendix section.

http://dx.doi.org/10.1016/j.atherosclerosissup.2017.07.002

1. Introduction

At present three canonical genes underlie familial hypercholesterolemia (FH) with co-dominant transmission (autosomal dominant hypercholesterolemia, ADH): *LDLR* (ADH-type 1), *APOB* (ADH-type 2) and *PCSK9* (ADH-type 3). Other genes, like *STAP1* (ADH-type 4) and *APOE* (ADH-type 5) have been implicated in some rare forms of FH with dominant transmission [1–3]. In all molecular surveys performed so far in different cohorts of FH patients, mutations in *LDLR* gene were found in more than 85–90% of FH cases. More than 1700 mutations of this gene have been reported so far and annotated in the UCL database [4]. Few mutations in *APOB* underlie ADH-2, two of which are predominant [5]. Gain-of-function (GOF) mutations of *PCSK9* causing FH are exceedingly rare [6]. Only a single mutation in *APOE* gene [p.(Leu167del)] was found to be the cause of a dominant FH phenotype [3]. FH-like phenotypes with a recessive transmission are extremely rare. This group of disorders includes the classic autosomal recessive hypercholesterolemia (ARH) caused by mutations in *LDLRAP1* gene [7], sitosterolemia caused by mutations in *ABCG5/ABCG8* genes [8], and lysosomal acid lipase deficiency (LAL-D) due to mutations in *LIPA* gene [9].

With regard to the prevalence of FH with co-dominant transmission (ADH), recent data based on the determination of the carrier status of pathogenic variants in *LDLR* and *APOB* genes indicate a frequency of 1:217 for heterozygotes in the general population [10]. An extensive molecular study conducted in the Netherlands in genotyped FH patients indicated a prevalence of heterozygous ADH of 1:244 individuals and a prevalence of homozygous FH of 1:300,000 [11]. Several reports have shown that a variable percentage (20–40%) of patients with the clinical diagnosis of heterozygous FH do not carry mutations in the candidate genes mentioned above [12]. Some of these patients were found to carry a burden of LDL-cholesterol (LDL-C) increasing gene variants which cumulatively raise LDL-C levels into the heterozygous FH range [12,13]. However, there are patients in whom the polygenic effect does not explain the high LDL-C levels, indicating the possible presence of other genes yet to be identified [13].

In a previous study [14] we have characterized the molecular bases of ADH in a cohort of Italian FH patients attending three Lipid Clinics in Italy and performed a systematic analysis of the clinical features of mutation-positive ADH patients. This study paved the way for the construction of the LIPIGEN network, as an integrated network aimed at the early clinical/molecular identification and treatment of patients with genetic dyslipidemias (Ref. Averna M. et al. in this issue). The first aim of LIPIGEN was to improve the identification and molecular characterization of FH patients thanks to a collaboration among a large number of Lipid Clinics distributed over the entire country. In this brief report we show the results of the molecular characterization of more than 1000 patients carried out during the first two years of activity of the LIPIGEN network.

2. Materials and methods

2.1. Subjects

For this analysis, 1592 unrelated patients from 20 outpatient clinics with clinical diagnosis of FH and a Dutch Lipid Clinic Network (DLCN) score ≥ 6 were genetically tested for the presence of variants in FH causing genes. Informed consent was obtained from all subjects. The project was approved by the Ethics Committees of each participating institution.

2.2. DNA isolation and sequencing

Genomic DNA was extracted using the Flexigene DNA kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Appropriate primers were used for the PCR amplification of exons and exon-intron junctions of the following genes: *LDLR* (18 exons), *PCSK9* (12 exons) and *LDLRAP1* (9 exons), as well as regions in *APOB* exons 26 and 29 involved in the binding to LDLR, exon 4 of *APOE* gene and *STAP1* gene (9 exons). As internal control, regions of chromosome 21 were amplified. Fifty-eight regions of interest (20 Kb) were amplified from genomic DNA in five different multiplex PCR reactions. A second amplification was then performed in order to include an 8nt index sequence used for sample identification, as well as the adaptors used for sequencing on MiSeq (Illumina) equipment. Before sequencing, purified amplified samples were quantified and diluted to the same concentration. The genetic analysis, including the complete sequence of genes, was performed by Next Generation Sequencing (see above) followed by the Sanger sequence to confirm the presence of the identified variant. Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used to detect copy number variations.

In silico analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein (http://sift.jcvi.org/) and Mutation Taster (www.mutationtaster.org/). The potential effect of an intronic variant on pre-mRNA splicing was assessed by Human Splicing Finder (http://www.umd.be/HSF3/HSF.html), NetGene2 (http://www.cbs.dtu.dk/sevices/NetGene2/), BDGP Splice Site prediction (http://www.fruitfly.org/seq_tools/splice.html), Splice Port (http://spliceport.cbcb.umd.edu/) and Splice Site Score Calculation (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html).

3. Results

In a series of 1592 FH index subjects, 1076 (67.6%) were found to carry at least one variant of the investigated

genes. Most of them (1016 subjects, 94.4%) carried at least one variant in the *LDLR* gene; a total number of 213 variants of *LDLR* gene have been identified. More than 90% of patients were carriers of definite pathogenic/likely pathogenic *LDLR* gene variants. Most *LDLR* variants were single nucleotide substitutions, mainly in the coding sequence. Large gene deletions were found in 53 subjects, whereas single nucleotide or minute deletions were found in 36 and 45 subjects respectively. Single nucleotide or minute duplications were found in 21 subjects.

The most common mutations in *LDLR* gene were [c.1646G>A, p.(Gly549Asp)] (107/1016), [c.662A>G, p.(Asp221Gly)] (84/1016), [c.1775G>A, p.(Gly592Glu)] (45/1016) and [c.1567G>A, p.(Val523Met)] (44/1016), all previously reported in Italian FH patients [14]. Thirty-two *LDLR* gene variants found in this survey had not been reported previously. *In silico* analysis indicated that 27 of them could be regarded as pathogenic/likely pathogenic variants (Table 1).

Pathogenic *APOB* gene variants located in exon 26 (Table 2) were found in 34 subjects.

Four *PCSK9* variants were found in 36 subjects (Table 3). The most common were: i) the [c.137G>T, p.(Arg46Leu)] variant (found in 15 subjects), which is a known loss-of-function variant (LOF) associated with reduced LDL-C levels, and ii) c.60_65dupGCTGCT [p.(Leu21tri)] (in 17 subjects), which is regarded to be a GOF variant and a possible cause of FH [6].

Twelve *LDLRAP1* gene variants were identified in 18 subjects. Five variants were pathogenic, 4 unlikely pathogenic and 3 of unknown significance (Table 4). Among the identified LDLRAP1 variants, 4 have not been reported previously. However, only 1 of these new variants can be assumed to be pathogenic.

Table 1
New *LDLR* gene variants.

Gene variant (c.DNA)	Type of variant	Clinical significance	Protein change
c.-97G>A	Promoter	Reduced transcription?	
c.3G>A	Aa change	Pathogenic	p.(Met1Ile)
c.38_58del21	Deletion	Pathogenic?	p.(Ala13_Ala19del)
c.68-?_190+?dup	Exon 2 duplication	Pathogenic	p.(Gly24_Leu64dup)
c.94T>C	Aa change	Pathogenic	p.(Phe32Leu)
c.191-?_2311+?dup	Exons 3−15 duplication	Pathogenic	p.[Ala771Val; Ser65_Ala771dup]
c.246delC	Deletion	Pathogenic	p.(Cys82*)
c.313+4_313+16del13	Affects donor splice site	Pathogenic (new donor splice site in exon 3)	p.(Arg88Serfs*25)
c.314-?_2583+?del	Exons 4−18 deletion	Pathogenic	p.0
c.316_328delCCCAAGACGTGCT	Deletion	Pathogenic	p.(Lys107Argfs*95)
c.363C>A	Nonsense	Pathogenic	p.(Cys121*)
c.620_626delGCGAGTG	Deletion	Pathogenic	p.(Gly207Alafs*56)
c.641G>A	Aa change	Pathogenic	p.(Trp214*)
C.688A>G	Aa change	Unlikely pathogenic	p.(Asn230Asp)
c.698T>C	Aa change	Unlikely pathogenic	p.(Val233Ala)
c.906C>A	Aa change	Pathogenic	p.(Cys302*)
c.920A>C	Aa change	Pathogenic	p.(Asp307Ala)
c.926C>A	Aa change	Pathogenic	p.(Pro309His)
c.1037delT	Deletion	Pathogenic	p.(Leu346Argfs*24)
c.1061-1G>T	Acceptor splice site broken	Pathogenic (skipping Exon 8)	
c.1171delG	Deletion	Pathogenic	p.(Ala391Profs*22)
c.1413_1414delAGinsGGACAT	Insertion/deletion	Pathogenic	p.(Gln474Hisfs*63)
c.1470G>T	Aa change	Pathogenic	p.(Trp490Cys)
c.1491delT	Deletion	Pathogenic	p.(Val498Serfs*9)
c.1498delG	Deletion	Pathogenic	p.(Val500Leufs*7)
c.1587-?_2583+?del	Exons 11−18 deletion	Pathogenic	p.0
c.1686G>T	Aa change	Pathogenic	p.(Trp562Cys)
c.1943_1944delCCinsG	Deletion	Pathogenic	p.(Ser48Cysfs*17)
c.2120A>C	Aa change	Pathogenic	p.(Asp707Ala)
c.2257C>G	Aa change	Unlikely pathogenic	p.(Pro753Ala)
c.2311_2311+15del16	Donor splice site broken	Pathogenic	
c.2299A>G	Aa change	Possibly pathogenic	p.(Met767Val)

In silico analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein (http://sift.jcvi.org/) and Mutation Taster (www.mutationtaster.org/). The potential effect of an intronic variant on pre-mRNA splicing was assessed by Human Splicing Finder (http://www.umd.be/HSF3/HSF.html), NetGene2 (http://www.cbs.dtu.dk/sevices/NetGene2/), BDGP Splice Site prediction (http://www.fruitfly.org/seq_tools/splice.html), Splice Port (http://spliceport.cbcb.umd.edu/) and Splice Site Score Calculation (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html).

Table 2
Pathogenic variants in *APOB* gene.

Gene variant (c.DNA)	Type of variant	Clinical significance	Protein change
c.10579C>T	Aa change	Pathogenic	p.(Arg3527Trp)
c.10580G>A	Aa change	Pathogenic	p.(Arg3527Gln)
c.10672C>T	Aa change	Pathogenic	p.(Arg3558Cys)

We found 17 subjects carrying two variants in *LDLR* gene (Table 5). Only 8 of these subjects carried five combinations of two variants with pathogenic effect. No formal family study was performed to assess whether the two variants were on the same or on different alleles. However it can be reasonable assumed that carriers of two pathogenic variants (8 patients) might be compound heterozygotes.

Nineteen subjects were double heterozygotes for LDLR/PCSK9 variants (Table 6). However only seven of these subjects were found to carry a putative or definite GOF variant of PCSK9 expected to contribute to the FH phenotype. Five subjects were double heterozygotes for pathogenic variants of *LDLR* and *APOB* genes (Table 7).

We also identified 5 double heterozygotes for LDLR/LDLRAP1 variants; all of them carried a pathogenic

Table 3
Variants in *PCSK9* gene.

Gene variant (c.DNA)	Type of variant	Clinical significance	Protein change
c.137G>T	Aa change	LOF variant[a]	p.(Arg46Leu)
c.-331C>A[b]	Promoter	GOF variant [a]; 2,5-fold increase transcription	
c.60_65dupGCTGCT (c.61_63triCTG)	Aa change	GOF variant[a]	p.(Leu21tri)
c.1327G>A	Aa change	Unlikely pathogenic	p.(Ala443Thr)

[a] Dron JS, Hegele RA. Curr Opin Lipidol. 2017; 28: 161–9.
[b] Previously reported as -332C>A by Blesa S et al. J Clin Endocrinol Metab 2008; 93: 3577–83.

Table 4
Variants in *LDLRAP1* gene.

Gene variant (c.DNA)	Type of variant	Clinical significance	Protein change
c.-17_-12dupGGCGGC	–	Unknown significance	
c.65G>A	Nonsense	Pathogenic	p.(Trp22*)
c.89-1G>C	Acceptor splice site broken	Pathogenic	p.(Lys30Thrfs*3)
c.457A>G **NEW**	Aa change	Unknown significance	p.(Met153Va)
c.569G>C	Aa change	Unlikely pathogenic	p.(Gly190Ala)
c.603dupC	Duplication	Pathogenic	p.(Ser202Leufs*19)
c.605C>A	Aa change	Pathogenic	p.(Ser202Tyr)
c.653C>T	Aa change	Unlikely pathogenic	p.(Thr218Ile)
c.748-2A>G **NEW**	Acceptor splice site broken	Pathogenic	p.(Glu250Glyfs*4)
c.811G>A	Aa change	Unknown significance	p.(Val271Ile)
c.888C>G **NEW**	Aa change	Unlikely pathogenic	p.(Asp296Glu)
c.896G>A **NEW**	Aa change	Unlikely pathogenic	p.(Gly299Asp)

Table 5
Heterozygous carriers of two *LDLR* gene variants.

LDLR gene 1st variant	Protein	Clinical significance	*LDLR* gene 2nd variant	Protein	Clinical significance
c.1587-?_2583+?del (2 subjects)	p.0	Pathogenic	c.1336C>G	p.(Leu446Val)	Unlikely pathogenic
c.68-?_1845+?del	p.(Val23Glyfs*29)	Pathogenic	c.2257C>G	p.(Pro753Ala) **NEW**	Unlikely pathogenic
c.1775G>A	p.(Gly592Glu)	Pathogenic	c.2299A>G	p.(Met767Val) **NEW**	Unlikely pathogenic
c.2072C>T	p.(Ser691Leu) **NEW**	Pathogenic	c.38_58del21	p.(Ala13_Ala19del) **NEW**	Unknown significance
c.352G>T	p.(Asp118Tyr)	Pathogenic	c.1211C>T	p.(Thr404Ile)	Pathogenic
c.58G>A	p.(Gly20Arg)	Unknown significance	c.1474G>A	p.(Asp492Asn)	Pathogenic
c.58G>A	p.(Gly20Arg)	Unknown significance	c.1257C>G	p.(Try419*)	Pathogenic
c.662A>G	p.(Asp221Gly)	Pathogenic	c.698T>C	p.(Val233Ala) NEW	Unlikely pathogenic
c.665G>A	p.(Cys222Tyr)	Pathogenic	c.815A>C	p.(Asn272Thr)	Unknown significance
c.858C>A	p.(Ser286Arg)	Pathogenic	c.2054C>T	p.(Pro685Leu)	Pathogenic
c.898A>G (4 subjects)	p.(Arg300Gly)	Pathogenic	c.907C>T	p.(Arg303Trp)	Pathogenic
c.304C>T	p.(Gln102*)	Pathogenic	c.718G>A	p.(Glu240Lys)	Pathogenic
c.418G>T	p.(Glu140*)	Pathogenic	c.352G>T	p.(Asp118Tyr)	Pathogenic

In silico analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein (http://sift.jcvi.org/) and Mutation Taster (www.mutationtaster.org/).

Table 6
Double heterozygotes for *LDLR* and *PCSK9* gene variants.

LDLR gene variant	Protein	Clinical significance	*PCSK9* gene variant	Protein	Clinical significance
c.1187-?_2140+?dup	p.(Gly396_Thr713dup)	Pathogenic	c.1327G>A	p.(Ala443Thr)	Unlikely pathogenic
c.1246C>T (2 subjects)	p.(Arg416Trp)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.1257C>G	p.(Try419*)	Pathogenic	c.60_65dupGCTGCT (c.61_63triCTG)	p.(Leu21tri)	GOF variant
c.1257C>G	p.(Try419*)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.126C>A	p.(Try42*)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.1646G>A	p.(Gly549Asp)	Pathogenic	c.60_65dupGCTGCT (c.61_63triCTG)	p.(Leu21tri)	GOF variant
c.1783C>T	p.(Arg595Trp)	Pathogenic	c.60_65dupGCTGCT (c.61_63triCTG)	p.(Leu21tri)	GOF variant
c.1846-?_2583+?del (2 subjects)	p.0	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.2215C>T	p.(Gln739*)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.2312-3C>A	p.(Ala771_Ile796del)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.352G>T	p.(Asp118Tyr)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.373C>T (HO)	p.(Gln125*)	Pathogenic	c.60_65dupGCTGCT (c.61_63triCTG)	p.(Leu21tri)	GOF variant
c.418G>T	p.(Glu140*)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.418G>T	p.(Glu140*)	Pathogenic	c.60_65dupGCTGCT (c.61_63triCTG)	p.(Leu21tri)	GOF variant
c.662A>G	p.(Asp221Gly)	Pathogenic	C.137G>T	p.(Arg46Leu)	LOF variant
c.788A>G	p.(Asp263Gly) **NEW**	Pathogenic	c.-331C>A	2,5-fold increase transcription	GOF variant
c.1547G>A	p.(Gly516Asp)	Unlikely pathogenic	c.60_65dupGCTGCT (c.61_63triCTG)	p.(Leu21tri)	GOF variant

GOF: gain-of-function; LOF: loss-of-function.
In silico analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein (http://sift.jcvi.org/) and Mutation Taster (www.mutationtaster.org).

Table 7
Double heterozygotes for *LDLR* and *APOB* gene variants.

LDLR gene variant	Protein	Clinical significance	*APOB* gene variant	Protein	Clinical significance
c.1470G>T	p.(Trp490Cys) **NEW**	Pathogenic	c.10579C>T	p.(Arg3527Trp)	Pathogenic
c.1735G>A	p.(Asp579Asn)	Pathogenic	c.10580G>A	p.(Arg3527Gln)	Pathogenic
c.662A>G	p.(Asp221Gly)	Pathogenic	c.10580G>A	p.(Arg3527Gln)	Pathogenic
c.662A>G	p.(Asp221Gly)	Pathogenic	c.10580G>A	p.(Arg3527Gln)	Pathogenic
c.662A>G	p.(Asp221Gly)	Pathogenic	c.10580G>A	p.(Arg3527Gln)	Pathogenic

Table 8
Double heterozygotes for *LDLR* and *LDLRAP1* gene variants.

LDLR gene variant	Protein	Clinical significance	*LDLRAP1* gene variant	Protein	Clinical significance
c.858C>A	p.(Ser286Arg)	Pathogenic	c.457A>G	p.(Met153Val)	Unknown significance
c.38_58del21+ c.2072C>T	p.(Ala13_Ala19del)+ p.(Ser691Leu)	Pathogenic	c.653C>T	p.(Thr218Ile)	Unlikely pathogenic
c.682G>A	p.(Glu228Lys)	Pathogenic	c.748-2A>G	p.(Glu250Glyfs*4)	Pathogenic
c.1775G>A	p.(Gly592Glu)	Pathogenic	c.811G>A	p.(Val271Ile)	Unknown significance
c.662A>G	p.(Asp221Gly)	Pathogenic	c.888C>G	p.(Asp296Glu)	Unlikely pathogenic

variant in *LDLR* gene, but only one was carrier of a pathogenic variant of *LDLRAP1* gene (Table 8).

Seven true homozygous patients were detected; 5 of them were homozygous for *LDLR* gene variants and 2 for *LDLRAP1* gene variants. (Table 9).

Finally, one subject was found to carry the *APOE* gene variant c.500_502delTCC [p.(Leu167del)], known to be associated with a dominant FH phenotype.

4. Discussion

In this preliminary report we describe the molecular findings in 1592 patients suspected to have definite or probable familial hypercholesterolemia identified through a national collaborative detection program. Only patients with a Dutch score $\geq$ 6 were admitted to the molecular investigation program. The exclusion of patients with

Table 9
Homozygous subjects for *LDLR* and *LDLRAP1* gene variants.

LDLR gene variant	Protein	Clinical significance
c.1060+10G>A	?	Unknown significance
c.662A>G	p.(Asp221Gly)	Pathogenic
c.681C>G	p.(Asp227Glu)	Pathogenic
c.788A>G	p.(Asp263Gly)	Pathogenic
c.373C>T+(PCSK9 c.60_65dupGCTGCT, GOF)	p.(Gln125*)+p.(Leu21tri)	Pathogenic
***LDLRAP1* gene variant**	**Protein**	**Clinical significance**
c.65G>A	p.(Trp22*)	Pathogenic
c.89-1G	p.(Lys30Thrfs*3)	Pathogenic

possible FH (Dutch score 3–5) who might be carriers of pathogenic variants in FH candidate genes [11] was due to funding limitations, organization problems and to the deliberate intent to concentrate our efforts on patients who are more likely to have a monogenic disorder. We used targeted next generation sequencing which allowed the parallel sequencing of the major genes known to be responsible for monogenic forms of hypercholesterolemia.

The overall detection rate of pathogenic/likely pathogenic variants was around 60%, a figure which is comparable to that obtained in other studies [12,13]. The assignment of pathogenicity was based on *in silico* analysis using several algorithms [4] and information available in the published variant databases.

In accordance with findings from our previous study [14], more than 90% of variant-positive patients were carriers of pathogenic or likely pathogenic variants in the *LDLR* gene. Among these patients we discovered 4 true FH homozygotes carrying known pathogenic variants and 5 patients likely be to be compound heterozygotes (as they were carriers of two known pathogenic or likely pathogenic variants of *LDLR* gene). In this survey we discovered 32 *LDLR* gene variants not reported previously in FH patients. A stringent *in silico* analysis [15] indicated that most of these variants can be assumed to be pathogenic or likely pathogenic.

As previously observed [14], only few patients were found to be heterozygous carriers of one of the three known *APOB* pathogenic variants. None of the patients was found to be homozygous/compound heterozygous for any of these variants. However some patients resulted to be double heterozygous for pathogenic *LDLR/APOB* variants. Overall, these findings confirm that *APOB* variants are a rare cause of FH in Italy.

Few patients were found to be heterozygous for putative pathogenic GOF variants of *PCSK9* gene. Surprisingly, none of them carried one of the three GOF variants reported previously in Italian FH patients [14] or one of those reported in a recent large survey of FH patients carrying PCSK9 variants [16]. As a matter of fact, a convincing demonstration of pathogenicity of one putative GOF variant found in our FH patients [c.60_65dupGCTGCT (c.61_63triCTG), p.(Leu21tri)] is not yet available. The same caution applies also to patients found to be double heterozygotes for *LDLR* and *PCSK9* variants (Table 6). Interestingly, most of these double heterozygous patients (carrying *LDLR* pathogenic variants) were found to carry the common polymorphic p.(Arg46Leu) variant, which is known to have an LDL-C lowering effect in normolipidemic as well as in FH patients [17–19].

We sequenced also the *LDLRAP1* gene whose pathogenic variants are the cause of autosomal recessive hypercholesterolemia (ARH), an exceedingly rare disorder in most countries with exception of the Italian island of Sardinia [7]. Two patients of our series were found to have ARH due to pathogenic variants of *LDLRAP1* while one patient was found to be double heterozygous for *LDL*R and *LDLRAP1* pathogenic variants.

This survey included also the sequence of *APOE* gene, in the light of previous studies suggesting that some rare apoE variants might be associated with an FH-like phenotype. We found only one patient heterozygous for a known apoE variant [p.(Leu167del)], considered the cause of FH with dominant transmission [3]. Recently a large study conducted in 228 Spanish FH patients in whom the *LDLR, APOB* and *PCSK9* pathogenic variants had been excluded, showed that nine patients (3.1%) were carriers of the p.(Leu167del) variant [20]. This variant was found to segregate with FH phenotype among index cases' family members. In view of these findings, *APOE* gene should be included among the genes responsible for FH [21].

This molecular survey paves the way to two main actions: i) a deep phenotypic analysis of the molecularly characterized patients, including response to treatment with hypolipidemic drugs; ii) implementation of the cascade screening of index patients' family members to identify carriers of pathogenic variants and characterize their phenotype. In view of our previous study, the cascade screening is expected to lead to the identification of at least 2000 carriers of pathogenic variants who may be unaware of the burden of having high LDL-C from birth and may benefit from early treatment and genetic counselling.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

Appendix

MEMBERS OF THE LIPIGEN STEERING COMMITTEE: Arca Marcello[1], Averna Maurizio[2], Bertolini Stefano[3], Calandra Sebastiano[4], Catapano Alberico Luigi[5], Tarugi Patrizia[4]; **Principal Investigators: Coordinator center:** Pellegatta Fabio[6]; **Participant Centers:** Angelico Francesco[1], Arca Marcello[1], Averna Maurizio[2], Bartuli Andrea[7], Biasucci Giacomo[8], Biolo Gianni[9],

Bonanni Luca[10], Bonomo Katia[11], Borghi Claudio[12], Bossi Antonio Carlo[13], Branchi Adriana[14], Carubbi Francesca[15], Cipollone Francesco[16], Citroni Nadia[17], Federici Massimo[18], Ferri Claudio[19], Fiorenza Anna Maria[20], Giaccari Andrea[21], Giorgino Francesco[22], Guardamagna Ornella[23], Iannuzzi Arcangelo[24], Iughetti Lorenzo[25], Lupattelli Graziana[26], Mandraffino Giuseppe[27], Marcucci Rossella[28], Mombelli Giuliana[29], Muntoni Sandro[30], Pecchioli Valerio[31], Pederiva Cristina[32], Pipolo Antonio[33], Pisciotta Livia[34], Pujia Arturo[35], Purrello Francesco[36], Repetti Elena[37], Rubba Paolo[38], Sabbà Carlo[39], Sampietro Tiziana[40], Sarzani Riccardo[41], Tagliabue Milena Paola[42], Trenti Chiara[43], Vigna Giovanni Battista[44], Werba Josè Pablo[45], Zambon Sabina[46], Zenti Maria Grazia[47]; **Participant Laboratories:** Montali Anna[48], Noto Davide[49], Bertolini Stefano[3], Calandra Sebastiano[4], Fortunato Giuliana[50]; **Collaborators: Coordinator center:** Grigore Liliana[6]; **Participant Centers:** Del Ben Maria[1], Maranghi Marianna[1], Cefalù A. Baldassarre[2], Buonuomo Paola Sabrina[7], Capra Maria Elena[8], Vinci Pierandrea[9], D'Addato Sergio[12], Galbiati Stella[13], Nascimbeni Fabio[15], Bucci Marco[16], Spagnoli Walter[17], Cardolini Iris[18], Cervelli Nazzareno[19], Emanuela Colombo[20], Vinsin A. Sun[21], Laviola Luigi[22], Bello Francesca[23], Chiariello Giuseppe[24], Predieri Barbara[25], Siepi Donatella[26], Saitta Antonino[27], Giusti Betti[28], Pavanello Chiara[29], Lussu Milena[30], Prati Lucia[31], Banderali Giuseppe[32], Balleari Giulia[34], Montalcini Tiziana[35], Scicali Roberto[36], Gentile Luigi[37], Gentile Marco[38], Suppressa Patrizia[39], Sbrana Francesco[40], Cocci Guido[41], Benso Andrea[42], Negri Emanuele Alberto[43], Ghirardello Omar[44], Vigo Lorenzo[45], Zambon Alberto[46], Bonora Enzo[47]; **Participant Laboratories:** Minicocci Ilenia[48], Spina Rossella[49], Orlando Camilla[3], Tarugi Patrizia[4], Di Taranto Maria Donata[50]; **STUDY CENTRAL LABORATORY AND ANALYSIS GROUP:** Catapano Alberico Luigi[5], Casula Manuela[51], Chiodo Lorenzo[51], Garlaschelli Katia[52], Manzato Enzo[53], Tragni Elena[51]

Affiliations: [1]Dipartimento di Medicina Interna e Specialità Mediche "La Sapienza", A.O. Policlinico Umberto I, Rome, Italy; [2]UO Medicina Interna e Malattie Metaboliche, Centro di riferimento regionale per la prevenzione, diagnosi e cura delle malattie rare del metabolismo e delle dislipidemie genetiche, A.O.U. Policlinico "P. Giaccone", Palermo, Italy; [3]Centro Ambulatorio Dislipidemie – U.O. Clinica di Medicina Interna 1,.O. Universitaria San Martino, Genua, Italy; [4]Laboratorio Sequenziamento Genomico, Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Modena, Italy; [5]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, and IRCCS Multimedica, Milan, Italy; [6]Centro per lo Studio dell'Aterosclerosi, IRCCS Multimedica, Sesto San Giovanni, Italy; [7]Ambulatorio Polispecialistico per le Malattie Rare, IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy; [8]Centro Dislipidemie in Età Evolutiva U.O. Pediatria e Neonatologia, Ospedale G. da Saliceto, Piacenza, Italy; [9]S.S. Diabetologia e Malattie Metaboliche, U.C.O. Clinica Medica Generale, Azienda Ospedaliera Universitaria OORR, Ospedale Maggiore, Trieste, Italy; [10]Ambulatorio Dislipidemie, UO Medicina Interna, Ospedale dell'Angelo di Mestre, Venice, Italy; [11]AOU San Luigi Gonzaga, Orbassano, Turin, Italy; [12]U.O. di Medicina Interna, Centro Aterosclerosi, Ambulatorio Dislipidemie, Ospedale Policlinico S. Orsola-Malpighi, Bologna, Italy; [13]U.O.C. Malattie Endocrine e Centro regionale per il Diabete (Diabetologia), Ospedale "Treviglio-Caravaggio" di Treviglio, Bergamo, Italy; [14]Dipartimento di Scienze Cliniche e di Comunità, Università degli Studi di Milano and Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Italy; [15]U.O. Medicina ad indirizzo metabolico-nutrizionistico, Centro dislipidemie e centro di riferimento regionale per le malattie metaboliche rare, Nuovo Ospedale S. Agostino Estense (NOCSAE), Modena, Italy; [16]Centro di alta specializzazione per la prevenzione dell'arteriosclerosi, centro di eccellenza ESH per l'ipertensione arteriosa, centro di riferimento regionale per le Dislipidemie, Ospedale Policlino S.S. Annunziata, Chieti, Italy; [17]Centro Dislipidemia, UO Medicina Interna, Ospedale Santa Chiara, Trento, Italy; [18]Dipartimento Medicina Interna – Centro per l'Aterosclerosi, Policlinico Universtario "Tor Vergata", Rome, Italy; [19]Centro Ipertensione Arteriosa e Prevenzione Cardiovascolare, UOC Medicina Interna e Nefrologia, l'Aquila, Italy; [20]Dip. Medicina Interna, Centro Prevenzione e Cura dell'aterosclerosi, A.O. "Guido Salvini", Garbagnate Milanese, Milan, Italy; [21]UOC Endocrinologia e Malattie del Metabolismo, Policlinico Gemelli, Rome, Italy; [22]U.O. Endocrinologia, Ambulatori di Diabetologia e Malattie Metaboliche, A.O. Universitaria Policlinico Consorziale, Università degli Studi di Bari "Aldo Moro", Bari, Italy; [23]U.O. Dislipidemie e Prevenzione Cardiovascolare, Ospedale Regina Margherita, Turin, Italy; [24]U.O. Medicina Interna 5, Centro per le malattie da arteriosclerosi, AORN Cardarelli, Naples, Italy; [25]U.O. Clinica Pediatrica, Policlinico di Modena, Modena, Italy; [26]U.O. Medicina Interna Angiologia, Malattie da Arteriosclerosi, Ambulatorio di malattie del ricambio lipidico, Ospedale Santa Maria della Misericordia, Perugia, Italy; [27]Dipartimento di Medicina Interna e Terapia Medica, Centro per la Diagnosi e Cura della Dislipidemia e Prevenzione dell'Aterosclerosi, A.O. Universitaria Policlinico "G.Martino", Messina, Italy; [28]Ambulatorio Malattie Aterotrombotiche, AOUC Azienda Ospedaliero-Universitaria Careggi, Florence, Italy; [29]Centro Universitario Dislipidemie "E. Grossi Paoletti", A.O. Ospedale Niguarda Ca' Granda, Milan, Italy; [30]Centro per le Malattie Dismetaboliche e l'arteriosclerosi, Associazione ME.DI.CO Onlus, Cagliari, Italy; [31]UOSD 'Prevenzione cardiovascolare', Dipartimento di Scienze Mediche, Azienda Sanitaria Locale Frosinone, Frosinone, Italy; [32]U.O. Clinica Pediatrica, Servizio Clinico Dislipidemie per lo Studio e la Prevenzione dell'Aterosclerosi in età pediatrica, Ospedale San Paolo, Milan, Italy; [33]AOU San

Giovanni di Dio e Ruggi d'Aragona, Salerno, Italy; [34]U.O. Clinica di Medicina Interna 1, Ambulatorio Dislipidemie, IRCCS – A.O.U. San Martino – IST, Genoa, Italy; [35]A.O.U. Mater Domini, Catanzaro, UOC di Nutrizione Clinica, Ambulatorio Dislipidemie, Catanzaro, Italy; [36]U.O. Medicina Interna, Ospedale "Garibaldi Nesima", Catania, Italy; [37]Società di Diabetologia e Malattie Metaboliche, Asti, Italy; [38]Centro Coordinamento regionale per le Iperlipidemie, AOU Policlinico Federico II, Naples, Italy; [39]U.O. di Medicina Interna "Frugoni" e Centro di Assistenza e Ricerca Malattie Rare, A.O. Universitaria Policlinico Consorziale, Università degli Studi di Bari "Aldo Moro", Bari, Italy; [40]U.O. Lipoaferesi, Centro Regionale di Riferimento per la diagnosi e cura delle Dislipidemie Ereditarie, Fondazione Toscana "G. Monasterio", Pisa, Italy; [41]Clinica di Medicina Interna e Geriatria, Centro di riferimento regionale ipertensione arteriosa e malattie cardiovascolari, INRCA Ospedale "Sestilli" e Azienda Ospedaliero-Universitaria Ospedali Riuniti di Torrette di Ancona, Ancona, Italy; [42]SCDU Endocrinologia, Diabetologia e Metabolismo, Dipartimento di Scienze Mediche, Università di Torino, Turin, Italy; [43]Arcispedale S. Maria Nuova – Azienda ospedaliera di Reggio Emilia, Reggio Emilia, Italy; [44]U. O Medicina Interna Universitaria, Centro per lo Studio delle Dislipidemie e dell'Aterosclerosi Azienda Ospedaliero-Universitaria di Ferrara, Polo di Cona, Ferrara, Italy; [45]U.O. Ambulatorio Prevenzione Aterosclerosi IRCCS Cardiologico Monzino, Milan, Italy; [46]U. O. Clinica Medica 1, Centro Dislipidemie e Aterosclerosi, A.O. di Padova, Padua, Italy; [47]U.O. Endocrinologia, Diabetologia e Malattie del Metabolismo, Centro regionale specializzato per la diagnosi e terapia delle dislipidemie e aferesi terapeutica, A.O. Universitaria Integrata di Verona, Verona, Italy; [48]Centro per l'Arteriosclerosi, Dipartimento di Medicina Interna e Specialità Mediche, Università di Roma "La Sapienza" – Azienda Policlinico Umberto I, Rome, Italy; [49]Centro di riferimento regionale per la prevenzione, diagnosi e cura delle malattie rare del metabolismo, O.S di Medicina Interna e Malattie metaboliche, Università degli Studi di Palermo, Palermo, Italy; [50]Laboratorio di screening di Malattie Metaboliche, CEINGE – Biotecnologie Avanzate, Dipartimento di Biochimica e Biotecnologie Mediche, Azienda Ospedaliera Universitaria "Federico II", Naples, Italy; [51]Centro Universitario di Epidemiologia e Farmacologia Preventiva (SEFAP), Dipartimento di Science Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy; [52]Centro per lo Studio dell'Aterosclerosi, Ospedale E. Bassini, Cinisello Balsamo, Milan, Italy; [53]Dipartimento di Medicina (DIMED), Sezione Geriatrica, Università di Padova, Padua, Italy.

References

[1] Sharifi M, Futema M, Nair D, Humphries SE. Genetic architecture of familial hypercholesterolaemia. Curr Cardiol Rep 2017;19:44.

[2] Fouchier SW, Dallinga-Thie GM, Meijers JC, et al. Mutations in STAP1 are associated with autosomal dominant hypercholesterolemia. Circ Res 2014;115:552–5.

[3] Marduel M, Ouguerram K, Serre V, et al. Description of a large family with autosomal dominant hypercholesterolemia associated with the APOE p.Leu167del mutation. Hum Mutat 2013;34:83–7.

[4] Leigh S, Futema M, Whittall R, et al. The UCL low-density lipoprotein receptor gene variant database: pathogenicity update. J Med Genet 2016;54:217–23.

[5] Andersen LH, Miserez AR, Ahmad Z, Andersen RL. Familial defective apolipoprotein B-100: a review. J Clin Lipidol 2016;10:1297–302.

[6] Dron JS, Hegele RA. Complexity of mechanisms among human proprotein convertase subtilisin-kexin type 9 variants. Curr Opin Lipidol 2017;28:161–9.

[7] Fellin R, Arca M, Zuliani G, Calandra S, Bertolini S. The history of Autosomal Recessive Hypercholesterolemia (ARH). From clinical observations to gene identification. Gene 2015;555:23–32.

[8] Lu K, Lee MH, Hazard S, et al. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. Am J Hum Genet 2001;69:278–90.

[9] Reiner Z, Guardamagna O, Nair D, et al. Lysosomal acid lipase deficiency–an under-recognized cause of dyslipidaemia and liver dysfunction. Atherosclerosis 2014;235:21–30.

[10] Benn M, Watts GF, Tybjaerg-Hansen A, Nordestgaard BG. Mutations causative of familial hypercholesterolaemia: screening of 98 098 individuals from the Copenhagen General Population Study estimated a prevalence of 1 in 217. Eur Heart J 2016;37:1384–94.

[11] Sjouke B, Kusters DM, Kindt I, et al. Homozygous autosomal dominant hypercholesterolaemia in The Netherlands: prevalence, genotype-phenotype relationship, and clinical outcome. Eur Heart J 2014;36:560–5.

[12] Talmud PJ, Shah S, Whittall R, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet 2013;381:1293–301.

[13] Wang J, Dron JS, Ban MR, et al. Polygenic versus monogenic causes of hypercholesterolemia ascertained clinically. Arterioscler Thromb Vasc Biol 2016;36:2439–45.

[14] Bertolini S, Pisciotta L, Rabacchi C, et al. Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy. Atherosclerosis 2013;227:342–8.

[15] Calandra S, Tarugi P, Bertolini S. Impact of rare variants in autosomal dominant hypercholesterolemia causing genes. Curr Opin Lipidol 2017;28:267–72.

[16] Hopkins PN, Defesche J, Fouchier SW, et al. Characterization of autosomal dominant hypercholesterolemia caused by PCSK9 gain of function mutations and its specific treatment with alirocumab, a PCSK9 monoclonal antibody. Circ Cardiovasc Genet 2015;8:823–31.

[17] Cohen JC, Boerwinkle E, Mosley Jr TH, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006;354:1264–72.

[18] Berge KE, Ose L, Leren TP. Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. Arterioscler Thromb Vasc Biol 2006;26:1094–100.

[19] Saavedra YG, Dufour R, Davignon J, Baass A. PCSK9 R46L, lower LDL, and cardiovascular disease risk in familial hypercholesterolemia: a cross-sectional cohort study. Arterioscler Thromb Vasc Biol 2014;34:2700–5.

[20] Cenarro A, Etxebarria A, de Castro-Oros I, et al. The p.Leu167del Mutation in APOE Gene Causes Autosomal Dominant Hypercholesterolemia by Down-regulation of LDL Receptor Expression in Hepatocytes. J Clin Endocrinol Metab 2016;101:2113–21.

[21] Wintjens R, Bozon D, Belabbas K, et al. Global molecular analysis and APOE mutations in a cohort of autosomal dominant hypercholesterolemia patients in France. J Lipid Res 2016;57:482–91.