A wide next-generation-sequencing panel improves the molecular diagnosis of dyslipidemias

Carola Giacobbe[1,2], Maria Donata Di Taranto[1,2], Daniela Palma[1,2], Giovanna Maione[1,2], Giovanna Cardiero[1,2], Gabriella Iannuzzo[3], Alessio Buonaiuto[3], Francesco Forte[3], Ilenia Calcaterra[3], Giuliana Fortunato[1,2]

[1]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Napoli

[2]CEINGE S.C.a r.l. Biotecnologie Avanzate, Napoli

[3]Dipartimento di Medicina Clinica e Chirurgia, Università degli Studi di Napoli Federico II, Napoli

ABSTRACT

Introduction: dyslipidemias are common clinical conditions associated to cardiovascular diseases. Among these, Familial Hypercholesterolemia (FH) and severe Hypertriglyceridemia (sHTG) are the most frequent ones. The aim of this study is to evaluate the possible use of a wide next-generation-sequencing (NGS) panel of 28 genes involved in lipid metabolism, to improve the molecular diagnosis of dyslipidemias.

Methods: a reanalysis of 25 patients (21 FH and 4 sHTG) previously analyzed for a few causative genes has been carried on. Patients bearing different types of variants [single nucleotide variants (SNVs) and copy number variations (CNVs)] in different genes, previously analyzed with Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) have been selected. DNA libraries have been prepared using Agilent SureSelect target enrichment protocol; the sequencing has been performed using Illumina MiSeq (V2 150x2 Micro). The results of the sequencing have been evaluated by Agilent SureCall and Agilent Alissa Align&Call and Intrepret pipelines.

Results: all previously identified SNVs and CNVs have been confirmed by NGS. Additional rare variants, not always associated with dyslipidemias, were found in 23/25 patients. An additional pathogenic variant in the *APOB* gene has been identified in a sHTG patient carrying only 1 pathogenic variant in the *APOA5* gene (causative of sHTG).

Conclusions: the NGS-method confirmed all the results obtained with direct sequencing and MLPA methodologies. Additional rare variants were detected, even if most of them turned out to be variant of uncertain significance (VUS). In conclusion, this NGS approach may enhance the molecular diagnosis of different types of dyslipidemias, thereby leading to a better understanding and detection of complex phenotypes.

INTRODUCTION

Dyslipidemias, especially hyperlipidemias, are a common clinical condition and a major risk factor for cardiovascular diseases (1,2). Dyslipidimias are usually due to genetic causes; however the presence of secondary factors (mainly the life style) can play an important role in their clinical manifestations (2). Among the different genetic forms of hyperlipidemias, Familial Hypercholesterolemia (FH) and Severe Hypertriglyceridemia (sHTG) are the most frequent and studied ones (3) and are characterized by elevated levels of cholesterol (CHOL) and triglycerides (TG), respectively.

FH shows an autosomal dominant pattern and is considered the most common genetic dyslipidemia (4). FH is mainly due to the presence of loss-of-function variants in the low-density lipoprotein receptor (*LDLR*), while loss-of-function variants in apolipoprotein B-100 (*APOB*) and gain-of-function variants in pro-protein convertase subtilisin/kexin type 9 (*PCSK9*) are less frequent (5). Two forms of FH are known: heterozygous FH (HeFH), with a frequency of ~1:200 (6), characterized by the presence of a single pathogenic variant in one of its causative genes and homozygous FH (HoFH), much rarer than HeFH (~1:300.000) (7-9) characterized by the presence of one pathogenic variant at homozygous status or of two pathogenic variants at compound heterozygous status.

Corrispondenza a: Giuliana Fortunato, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, via S. Pansini 5, 80131 Napoli, Tel +39-081.746.4200; E-mail fortunat@unina.it

Ricevuto: 20.01.2020 Revisionato: 23.03.2020 Accettato: 08.04.2020

Pubblicato on-line: 08.07.2020 DOI: 10.19186/BC_2020.038

More recently, two additional genes, the *APOE* and *STAP1* genes, encoding the apolipoprotein E and the signal-transducing adaptor family member 1, respectively, have been identified as being causative of autosomal dominant FH (5). Finally, several pathogenic variants in the low density lipoprotein receptor adaptor protein 1 (*LDLRAP1*) have been identified as causative of a recessive form of FH (ARH) (10). In a patient with clinical suspicion of FH but negative for pathogenic variants in the above genes, hypercholesterolemia could be due to the contemporary presence of multiple common variants in different genes; this form is known as polygenic hypercholesterolemia. Six single-nucleotide polymorphisms (SNPs) are used to calculate the genetic risk score of polygenic hypercholesterolemia, known as the LDL-cholesterol (LDL-C) score (11).

sHTG, also known as familial chylomicronemia syndrome (FCS) is a very rare (~1:500.000/ ~1:1.000.000) inherited autosomal recessive disease characterized by TG levels higher than 10 mmol/L and recurrent pancreatitis (12); it is caused by the presence of biallelic (homozygous or compound heterozygous) pathogenic variants in *LPL* (lipoprotein lipase) and other genes encoding the proteins required for LPL activity, such as *APOC2* (apolipoprotein C2), *APOA5* (apolipoprotein A5), *GPIHBP1* (glycosylphosphatidylinositol-anchored HDL binding protein 1), and *LMF1* (lipase maturation factor 1) (12,13). It has been reported that the phenotype of severe hypertriglyceridemia may be frequently caused either by the contemporary presence of heterozygosity for rare variants in causative FCS genes, or by the presence of rare or common functional variants in non-canonical triglyceride-raising genes or secondary factors. This latter condition is known as multifactorial chylomicronemia syndrome (MCS) (14).The aim of this study was to evaluate the possible use of a wide next-generation-sequencing (NGS) panel of 28 genes involved in lipid metabolism to improve the molecular diagnosis of dyslipidemias.

METHODS

Patients

25 patients have been analyzed: 21 with a clinical suspicion of FH and 4 of sHTG recruited at the Lipid Clinic of the University Hospital Federico II in Naples, that is the reference center for dyslipidemias of Campania Region.

Informed consent for the study was obtained from each patient. The study was performed according to the current version of the Helsinki Declaration and was approved by the Ethical Committee of the University Hospital Federico II (Number 262/17, November 29, 2017).

FH adult patients were enrolled based on LDL-C levels above 4.9mmol/L and hypercholesterolemia or premature cardiovascular disease in first degree family members, while FH pediatric patients were enrolled as previously described (15).

sHTG patients have been enrolled according to the following criteria: TG serum levels >10 mmol/L, absence of secondary causes of hypertriglyceridemia, and at least one episode of pancreatitis.

Genetic screening

Genomic DNA was isolated from peripheral blood using the ReliaPrep™ Blood gDNA Miniprep System (Promega, Madison,Wisconsin, USA) according to the manufacturer's instructions. DNA quantity was evaluated using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Twenty-one FH patients were genetically screened as previously described (15).

In 4 sHTG patients, genetic screening was performed by direct sequencing of the coding region and the exon-intron junctions of the *LPL, APOA5, APOC2, GPIHBP1* and *LMF1* genes, while multiplex ligation-dependent probe amplification was performed to search for copy number variants (CNVs) in the *LPL* gene (16).

Identified variants were checked against pathogenic variants databases: the Leiden Open Variation Database (LOVD) and the Human Gene Mutation Database (HGMD). For variants not present in these databases or never reported in the literature as being causative of FH or sHTG, pathogenic evaluation was performed according to the American College of Medical Genetics and Genomics Guidelines (ACMG) (17). To verify the minor allele frequency (MAF), these variants were searched for in NGS databases: Exome Aggregation Consortium, Genome Aggregation Database, Exome Variant Server and 1000 genomes. Rare variants are defined as variants with a MAF lower than 1%. Variants are reported according to the Human Genome Variation Society nomenclature.

The results of the traditional screening performed in 25 patients used to validate this NGS panel are shown in Table 1.

Reanalysis with Next-Generation-Sequencing

We reanalyzed 25 patients, previously screened with traditional sequencing and multiplex ligation-dependent probe amplification (MLPA).

These patients were selected since they were carriers of different types of variants (missense variants, splicing variants, frameshift variants and CNVs) in different genes.

Patients without causative variants were also included in the present study in order to verify if they could carry a causative variant in other genes not previously analyzed with traditional sequencing and MLPA.

Table 1
Genotype of the 25 patients used for validation of the Next Generation Sequencing panel

Patients	Gene analyzed with direct sequencing	Gene analyzed with MLPA	Molecular diagnosis
sHTG1	*LPL, APOA5, APOC2, LMF1, GPIHBP1*	*LPL*	heterozygosity for 1 missense pathogenic variant in *LPL* gene (genetic data are not enough to make a molecular diagnosis)
sHTG2	*LPL, APOA5, APOC2, LMF1, GPIHBP1*	*LPL*	heterozygosity for 1 missense pathogenic variant in *LPL* gene (genetic data are not enough to make a molecular diagnosis)
sHTG3	*LPL, APOA5, APOC2, LMF1, GPIHBP1*	*LPL*	heterozygosity for 1 missense pathogenic variant in *APO5* gene (genetic data are not enough to make a molecular diagnosis)
sHTG4	*LPL, APOA5, APOC2, LMF1, GPIHBP1*	*LPL*	double heterozygosity for 1 missense likely pathogenic variant in *LPL* gene and 1missense VUS in *APOA5* gene (genetic data are not enough to make a molecular diagnosis)
FH1	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH2	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH3	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH4	*LDLR*	-	HeFH: heterozygosity for 1 null pathogenic variant in *LDLR* gene
FH5	*LDLR*	*LDLR*	HoFH: compound heterozygosity for 1 CNV and 1 missense pathogenic variant in *LDLR* gene
FH6	*LDLR*	*LDLR*	HeFH: heterozygosity for 1 CNV in *LDLR* gene
FH7	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	double heterozygosity for 1 CNV in *LDLR* and 1 missense pathogenic variant in *PCSK9* gene
FH8	*LDLR*	-	HeFH: heterozygosity for 1 missense pathogenic variant in *LDLR* gene
FH9	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH10	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH11	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH12	*LDLR*	-	HeFH: heterozygosity for 1 missense pathogenic variant in *LDLR* gene

Table 1
Continues

Patients	Gene analyzed with direct sequencing	Gene analyzed with MLPA	Molecular diagnosis
FH13	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH14	*LDLR*	-	HeFH: heterozygosity for 1 missense pathogenic variant in *LDLR* gene
FH15	*FLDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH16	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH17	*LDLR*	-	HeFH: heterozygosity for 1 null pathogenic variant in *LDLR* gene
FH18	*LDLR*	-	HeFH: heterozygosity for 1 null pathogenic variant in *LDLR* gene
FH19	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found
FH20	*LDLR*	-	HeFH: heterozygosity for 1 missense pathogenic variant in *LDLR* gene
FH21	*LDLR, APOB* [exon 26 (c.9670_c.11916) and exon 29], *PCSK9*	*LDLR*	No rare variants found

sHTG, severe hypertriglyceridemia; FH, Familial Hypercholesterolemia; MLPA, Multiplex ligation-dependent probe amplification; VUS, variant of uncertain significance; CNV, copy number variants.

We designed a custom panel of genes involved in lipid metabolism and known in literature as being causative of or associated to different forms of dyslipidemia: *LPL, APOA5, APOC2, GPIHBP1, LMF1, GPD1, CREBL3, APOC1, APOC3, APOC4, APOA1, APOA4, ANGPTL3, GCKR, LRP1, CYP27A1, LDLR, APOB, PCSK9, LDLRAP1, APOE, STAP1, LIPA, ABCG5, ABCG8, MTTP, CH25H, HMGCR*. For each gene we included the coding regions, 50 bp in each of the intronic boundaries, the 5'UTR and the 3'UTR regions. The panel also included 6 SNPs (rs629301, rs1367117, rs4299376, rs6511720, rs429358 and rs7412) used to calculate the LDL-C genetic risk score as previously described (11).

gDNA was sheared and captured using the SureSelect QXT target enrichment system (Agilent, Santa Clara, CA, USA), according to the manufacturer's instructions and 2 sequencing runs were performed with the micro v2 flow cell (300 cycles) on a MiSeq platform (Illumina Inc., SanDiego, CA, USA) with paired-end reads (2x150 base pairs). In particular, two pools were prepared containing 13 libraries (12 samples and 1 reference sample) and 14 libraries (13 samples and 1 reference sample), respectively.

The obtained FASTQ files were aligned against the hg19 genome assembly (GenBank: GCA_000001405.1); variant alignment and calling was performed by Alyssa Align and Call (Alissa v5.2.6 - Agilent, Santa Clara, CA, USA) based on a quality score ≥30, whereas variant filtering was performed by Alyssa Interpret software (Alissa v5.2.6 - Agilent, Santa Clara, CA, USA). The analysis of CNVs of the target region was performed by pair analysis with the SureCall software (Agilent, Santa Clara, CA, USA), comparing each DNA sample with a Human Reference DNA (Agilent, Santa Clara, CA, USA, cat.n. 5190-4371).

All additional rare variants identified with NGS analysis were confirmed by Sanger sequencing. The pathogenicity of rare variants identified was assessed according to the ACMG guidelines.

Statistical Analysis

The normality of variable distribution was evaluated using the Kolmogorov-Smirnov test. Parametric variables are reported as mean SD. Data were analyzed using the Predictive Analytics SoftWare 18.0 (SPSS Inc.).

RESULTS

This study included a total of 25 patients (21 FH and 4 sHTG). Their clinical and biochemical characteristics are shown in Table 2 and in Table 3, respectively. All SNVs and CNVs previously detected with Sanger sequencing and MLPA analysis were confirmed by NGS and the average read depth obtained was >200X in all samples; an average of 150 SNVs was found in each sample analyzed, most of these were common polymorphic variants with a MAF>1%. As for the analysis with traditional sequencing, 66 additional rare SNVs were detected with NGS analysis in 23 out of 25 reanalyzed patients, while no additional CNVs were found. All the additional detected variants were present in genes or gene regions not previously analyzed. The complete list of additional rare SNVs and their pathogenicity classification is shown in Table 1S. In particular, we found 20 (30.3%) missense variants, 8 (12.1%) synonymous variants, 21 (31.8%) intronic variants, while the remaining 17 (25.8%) were found in the 5' UTR and 3'UTR regions (4 in 5'UTR and 13 in 3'UTR, respectively). Among these variants, 52 (78.8%) were classified as variants of uncertain significance (VUS), 13 (19.7%) as benign or likely benign, while only 1 (1.5%) was classified as likely pathogenic. This latter variant was a missense variant in the *APOB* gene c.10672C>T- p.(Arg3558Cys), found at heterozygous state in the sHTG3 patient.

NGS analysis allowed us to calculate the LDL-C score for diagnosing polygenic familial hypercholesterolemia in FH patients without causative variants; the resulting score was > 0.73 in 6 out of 11 FH patients without causative variants. The APOE genotype in the 4 sHTG patients was E2/E3 in 3/4 (75%) patients, while it was E3/E3 in the remaining ones.

Table 2
Demographic, anamnestic and biochemical features of the Familial Hypercholesterolemia (FH) reanalyzed patients divided by age.

Parameter	FH pediatric patients n=5	FH adult patients n=16
Age (year range)	1-11	24-67
Gender (n males and %)	4 (80.0%)	7 (43.8 %)
Total cholesterol (mmol/L)	8.4 (2.6)	10.8 (3.2)
LDL-cholesterol (mmol/L)	5.4 (3.7)	7.6 (2.1)
HDL-cholesterol (mmol/L)	1.5 (0.1)	2.0 (0.7)
LDL/HDL ratio	4.6 (2.2)	4.4 (2.0)
Xanthomas n (%)	0 (0%)	2 (12.5%)
Premature CAD n (%)	0 (0%)	1 (6.3%)
Premature CAD in first degree relatives,n (%)	0 (0%)	2 (12.5%)
Hypercholesterolemia in first-degree relatives, n (%)	2 (40.0%)	4 (25.0%)

Values are reported as mean (SD)
CAD: coronary artery disease

Table 3
Demographic, anamnestic and biochemical features of the Severe Hypertriglyceridemia (sHTG) reanalyzed patients

Parameter	sHTG patient 1	sHTG patient 2	sHTG patient 3	sHTG patient 4
Age (years)	33	25	39	46
Gender	female	female	male	female
Tryglicerides (mmol/L)	16.4	33.9	16.2	28.0
Total cholesterol (mmol/L)	10.5	-	5.6	10.9
HDL-cholesterol (mmol/L)	0.5	-	0.3	0.6
Pancreatitis	yes	yes	yes	yes
Abdominal pains	yes	yes	yes	yes
Diabetes secondary to pancreatitis	yes	no	no	yes

The analysis of the 25 patients enrolled in this study using traditional approach (Sanger sequencing and MLPA analysis) required about 2 months, while the analysis by NGS required about 15 days.

DISCUSSION

In this study, we assess the great advantage of a wide custom NGS panel to enhance the molecular diagnosis of different forms of dyslipidemias. The genes included in our custom panel are genes known as being causative of FH, sHTG and other dyslipidemias such as Sitosterolemia, Cerebrotendinous Xanthomatosis, Hypobetalipoproteinemia and Lysosomal Acid Lipase Deficiency. Six SNPs used to obtain the LDL-C score and determine the risk of Polygenic hypercholesterolemia were also added.

This evaluation was carried out performing the reanalysis of 25 patients (21 FH and 4 sHTG), previously analyzed using Sanger sequencing for the detection of causative SNVs, and MLPA for the detection of CNVs.

All variants previously detected were confirmed and 66 additional rare SNVs were found in genes or regions not previously analyzed. The pathogenicity of these rare variants was evaluated according to the ACMG guidelines and 1 variant out of 66 was classified as likely pathogenic, while 65 were classified as VUS and benign/likely benign (52 and 13 respectively).

The only variant classified as likely pathogenic was the c.10672C>T- p.(Arg3558Cys) variant in the *APOB* gene, found at heterozygous state in sHTG3 patient. The presence of a likely pathogenic variant in the *APOB* gene, together with the pathogenic variant in the *APOA5* gene previously detected (18), could better define the genetic diagnosis of the patient. Actually, an accumulation of variants in lipid-related genes is considered to be the genetic basis of MCS (19). The *APOB* variant was described in the HGMD database as associated to Familial ligand-defective apolipoprotein B with incomplete penetrance (20,21), a phenomenon often observed for other APOB variants (22,23).

After performing NGS reanalysis, we inferred that all sHTG patients could be classified as MCS patients, since the presence of a rare causative variant, at heterozygous state, together with common variants in the *APOA5* gene associated to hypertriglyceridemia, and with presence of the E2 allele in the *APOE* gene, could contribute to the hypertriglyceridemic phenotype.

As for VUS, a functional study should be carried out to evaluate their potential impact on protein structure and function. Several studies have reported about the relevance of functional characterization of potentially causative rare variants, especially for assessing FH causative variants (24-28).

In FH patients without causative variants, 6 SNPs were used to calculate the LDL-C score, which was previously suggested linked to the presence of polygenic hypercholesterolemia (11). Eleven out of 21 FH reanalyzed patients in our study were negative for causative variants of FH. Since 6 patients (54.5%) presented a LDL-C score >0.73, the hypercholesterolemic phenotype in these patients could be of polygenic origin. In the remaining 5 FH patients negative for causative variants and for the LDL-C score, the hypercholesterolemic phenotype may be due to causative variants in other genes not yet known as being associated to FH.

From the reported results, it may be concluded that:

- this genetic screening with NGS has proved to be a useful and reliable methodology to simultaneously analyze a panel of genes involved in lipid metabolism in a great number of patients, since FH and sHTG are extremely heterogeneous from a genetic point of view (29-31);
- this methodology allows us to genotype numerous interesting SNPs in a single reaction, thus saving time and costs compared to standard methodologies such as Sanger sequencing or real time Taqman (32-35);
- the used NGS panel also allows for the detection of causative variants in rare and severe dyslipidemias such as Cerebrotendinous Xanthomatosis and Lysosomal acid lipase deficiency, for which prenatal diagnosis could also be envisaged, in order to prevent clinical complications (36,37);
- NGS could enhance genetic diagnosis and, as a result, improve the clinical management of the affected patients. Furthermore, patients could be entered in nationwide registers used for identification of treatment gaps and for integration of clinical and genetic data (38-40).

This genetic workflow analysis could be used for the diagnosis of different types of dyslipidemias, leading to a better understanding and detection of complex phenotypes.

CONFLICT OF INTEREST

None.

REFERENCES

1. Kopin L, Lowenstein C. Dyslipidemia. Ann Intern Med 2017;167:ITC81-ITC96.
2. Ramasamy I. Update on the molecular biology of dyslipidemias. Clin Chim Acta 2016;454:143-85.
3. Berberich AJ, Hegele RA. The role of genetic testing in dyslipidaemia. Pathology 2019;51:184-92.
4. Benito-Vicente A, Uribe KB, Jebari S, et al. Familial Hypercholesterolemia: The Most Frequent Cholesterol Metabolism Disorder Caused Disease. Int J Mol Sci 2018;19.
5. Di Taranto MD, Giacobbe C, Fortunato G. Familial hypercholesterolemia: A complex genetic disease with variable phenotypes. Eur J Med Genet 2019;103831.
6. Benn M, Watts GF, Tybjaerg-Hansen A, et al. Familial Hypercholesterolemia in the Danish General Population: Prevalence, Coronary Artery Disease, and Cholesterol-Lowering Medication (vol 97, pg 3956). J of Clin Endocrinol Metab 2014;99:4758-9.

7. Cuchel M, Bruckert E, Ginsberg HN, et al. Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. Eur Heart J 2014;35:2146-57.
8. Sjouke B, Hovingh GK, Kastelein JJP, et al. Homozygous autosomal dominant hypercholesterolaemia: prevalence, diagnosis, and current and future treatment perspectives. Curr Opin Lipidol 2015;26:200-9.
9. Di Taranto MD, Giacobbe C, Buonaiuto A, et al. A Real-World Experience of Clinical, Biochemical and Genetic Assessment of Patients with Homozygous Familial Hypercholesterolemia. J Clin Med 2020;9.
10. Fellin R, Arca M, Zuliani G, et al. The history of Autosomal Recessive Hypercholesterolemia (ARH). From clinical observations to gene identification. Gene 2015;555:23-32.
11. Futema M, Shah S, Cooper JA, et al. Refinement of variant selection for the LDL cholesterol genetic risk score in the diagnosis of the polygenic form of clinical familial hypercholesterolemia and replication in samples from 6 countries. Clin Chem 2015;61:231-8.
12. Chyzhyk V, Brown AS. Familial chylomicronemia syndrome: A rare but devastating autosomal recessive disorder characterized by refractory hypertriglyceridemia and recurrent pancreatitis. Trends Cardiovasc Med 2020;30:80-5.
13. Surendran RP, Visser ME, Heemelaar S, et al. Mutations in LPL, APOC2, APOA5, GPIHBP1 and LMF1 in patients with severe hypertriglyceridaemia. J Intern Med 2012;272:185-96.
14. D'Erasmo L, Di Costanzo A, Cassandra F, et al. Spectrum of mutations and long-term clinical outcomes in genetic chylomicronemia syndromes. Arterioscler Thromb Vasc Biol 2019;39:2531-41.
15. Di Taranto MD, de Falco R, Guardamagna O, et al. Lipid profile and genetic status in a familial hypercholesterolemia pediatric population: exploring the LDL/HDL ratio. Clin Chem Lab Med 2019;57:1102-10.
16. Peterfy M, Bedoya C, Giacobbe C, et al. Characterization of two novel pathogenic variants at compound heterozygous status in lipase maturation factor 1 gene causing severe hypertriglyceridemia. J Clin Lipidol 2018;12:1253-9.
17. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.
18. Evans D, Aberle J, Beil FU. Resequencing the apolipoprotein A5 (APOA5) gene in patients with various forms of hypertriglyceridemia. Atherosclerosis 2011;219:715-20.
19. Moulin P, Dufour R, Averna M, et al. Identification and diagnosis of patients with familial chylomicronaemia syndrome (FCS): Expert panel recommendations and proposal of an "FCS score". Atherosclerosis 2018;275:265-72.
20. Pullinger CR, Hennessy LK, Chatterton JE, et al. Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. J Clin Invest 1995;95:1225-34.
21. Olfson E, Cottrell CE, Davidson NO, et al. Identification of Medically Actionable Secondary Findings in the 1000 Genomes. PLoS One 2015;10:e0135193.
22. Garcia-Garcia AB, Ivorra C, Martinez-Hervas S, et al. Reduced penetrance of autosomal dominant hypercholesterolemia in a high percentage of families: importance of genetic testing in the entire family. Atherosclerosis 2011;218:423-30.
23. Ruotolo A, Di Taranto MD, D'Agostino MN, et al. The novel variant p.Ser465Leu in the PCSK9 gene does not account for the decreased LDLR activity in members of a FH family. Clin Chem Lab Med 2014;52:e175-8.
24. Di Taranto MD, Benito-Vicente A, Giacobbe C, et al. Identification and in vitro characterization of two new PCSK9 Gain of Function variants found in patients with Familial Hypercholesterolemia. Sci Rep 2017;7:15282.
25. Di Taranto MD, D'Agostino MN, Fortunato G. Functional characterization of mutant genes associated with autosomal dominant familial hypercholesterolemia: integration and evolution of genetic diagnosis. Nutr Metab Cardiovasc Dis 2015;25:979-87.
26. Romano M, Di Taranto MD, D'Agostino MN, et al. Identification and functional characterization of LDLR mutations in familial hypercholesterolemia patients from Southern Italy. Atherosclerosis 2010;210:493-6.
27. Romano M, Di Taranto MD, Mirabelli P, et al. An improved method on stimulated T-lymphocytes to functionally characterize novel and known LDLR mutations. J Lipid Res 2011;52:2095-100.
28. Benito-Vicente A, Uribe KB, Jebari S, et al. Validation of LDLr Activity as a tool to improve genetic diagnosis of familial hypercholesterolemia: a retrospective on functional characterization of LDLr variants. Int J Mol Sci 2018;19.
29. Rubba P, Gentile M, Marotta G, et al. Causative mutations and premature cardiovascular disease in patients with heterozygous familial hypercholesterolaemia. Eur J Prev Cardiol 2017;24:1051-9.
30. Pirillo A, Garlaschelli K, Arca M, et al. Spectrum of mutations in Italian patients with familial hypercholesterolemia: New results from the LIPIGEN study. Atheroscler Suppl 2017;29:17-24.
31. 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.
32. Di Taranto MD, Staiano A, D'Agostino MN, et al. Association of USF1 and APOA5 polymorphisms with familial combined hyperlipidemia in an Italian population. Mol Cell Probes 2015;29:19-24.
33. Calcagno G, Staiano A, Fortunato G, et al. A multilayer perceptron neural network-based approach for the identification of responsiveness to interferon therapy in multiple sclerosis patients. Inform Sciences 2010;180:4153-63.
34. Staiano A, Di Taranto MD, Bloise E, et al. Investigation of Single Nucleotide Polymorphisms Associated to Familial Combined Hyperlipidemia with Random Forests. Smart Innovation, Systems and Technologies 2013;19:169-78.
35. Labruna G, Pasanisi F, Fortunato G, et al. Sequence Analysis of the UCP1 Gene in a Severe Obese Population from Southern Italy. J Obes 2011;2011:269043.
36. Di Taranto MD, Gelzo M, Giacobbe C, et al. Cerebrotendinous xanthomatosis, a metabolic disease with different neurological signs: two case reports. Metab Brain Dis 2016;31:1185-8.
37. Maruotti GM, Frisso G, Calcagno G, et al. Prenatal diagnosis of inherited diseases: 20 years' experience of an Italian Regional Reference Centre. Clin Chem Lab Med 2013;51:2211-7.
38. Perez de Isla L, Alonso R, Mata N, et al. Predicting Cardiovascular Events in Familial Hypercholesterolemia: The SAFEHEART Registry (Spanish Familial Hypercholesterolemia Cohort Study). Circulation 2017;135:2133-44.

39. Averna M, Cefalu AB, Casula M, et al. Familial hypercholesterolemia: The Italian Atherosclerosis Society Network (LIPIGEN). Atheroscler Suppl 2017;29:11-6.
40. Amrock SM, Duell PB, Knickelbine T, et al. Health disparities among adult patients with a phenotypic diagnosis of familial hypercholesterolemia in the CASCADE-FH patient registry. Atherosclerosis 2017;267:19-26.