# Molecular diagnosis methods in familial hypercholesterolemia

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## ABSTRACT

Familial hypercholesterolemia (FH) is considered the genetic cause of coronary heart disease and ischemic stroke. FH is mainly an autosomal codominant pattern-based disorder and is primarily determined by point mutations within the low-density lipoprotein receptor, apolipoprotein B, and proprotein convertase subtilisin/kexin type 9 genes, causing increased low-density lipoprotein cholesterol levels in the serum of untreated individuals. The accumulation will eventually lead to atherosclerotic cardiovascular disease. Although clinical criteria comprising several prognosis scores, such as the Simon Broome, Dutch Lipid Clinic Network, Make Early Diagnosis to Prevent Early Death, and the recently proposed Montreal-FH-SCORE, are the conventional basis of diagnosing FH, the genetic diagnosis made by single nucleotide polymorphism genotyping, multiplex ligation-dependent probe amplification analysis, and sequencing (both Sanger and Next-Generation sequencing) offers unequivocal diagnosis. Given the heterogeneity of known mutations, the genetic diagnosis of FH is often difficult to establish, despite the growing evidence of the causative mutations, as well as the polygenic aspect of this pathology and the importance of cascade screening of the FH patient's healthy family members. This review article details different genetic techniques that can be used in FH identification when there is a clinical FH suspicion based on criteria comprised in prognosis scores, knowing that none of these are exhaustive in the diagnosis, yet they efficaciously overlap and complement each other for confirming the disease at the molecular level. *(Anatol J Cardiol 2020; 23: 120-7)*

**Keywords:** familial hypercholesterolemia, hyperlipoproteinemia type II, genetic testing

## Introduction

Familial hypercholesterolemia (FH, Online Mendelian Inheritance in Man #143890) (1) is recognized as the genetic cause of premature atherosclerotic cardiovascular disease (ACD), which clinically manifests as coronary heart disease (CHD) or ischemic stroke (IS). CHD and IS are considered the main causes of worldwide morbidity and mortality (2).

FH is mainly transmitted in an autosomal codominant manner (2) and is primarily (>95%) (3) determined by point mutations causing loss-of-function within the low-density lipoprotein (LDL) receptor (*LDLR*) gene. Further, small or large gene rearrangements total up to an approximate 2900 different *LDLR* variants (4). A large proportion of *LDLR* variants seem to cluster in exon 4 (5), which encodes the ligand-binding domain, resulting in a severe form of FH. FH cases caused by loss-of-function mutations within the apolipoprotein B (*APOB*) gene or gain-of-

function mutations in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene (2) are more infrequent; the latter leading to decreased LDL-receptor recycling and hindering LDL uptake due to lower cell membrane LDL-receptor levels (6). In contrast, in rare FH cases, patients are reported to harbor point mutations in added genes, namely LDL-receptor adaptor protein 1 (*LDLRAP1*), apolipoprotein E (*APOE*), signal-transducing adaptor protein family 1 (*STAP1*), lysosomal acid lipase (*LIPA*), and patatin-like phospholipase-domain-containing family (*PNPLA5*) (4). However, an FH-like phenotype can also be the added as a result of multiple single nucleotide polymorphisms (SNPs) in the common cholesterol-raising variants, each of them incrementally raising the LDL-cholesterol (LDL-C) plasma levels by a small amount (7), thereby leading to the polygenic determination.

Despite the causative genetic mutation, the FH phenotype is caused by defective LDL clearance (8) and is clinically charac-

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terized by increased LDL-C serum levels of 310–580 mg/dL (8–15 mmol/L) in untreated heterozygous mutation carriers (HeFH) (9) (1:200 to 1:300 estimated individuals worldwide) (2) and 460–1160 mg/dL (12–30 mmol/L) in homozygous carriers (HoFH) (1:1,000,000 estimated individuals worldwide) (9). The prolonged increased LDL-C levels will finally lead to the accumulation of cholesterol-rich apoB-containing lipoproteins within the arterial intima (2) and the early occurrence (below the age of 50 years for men and 60 years for women) (10) ACD, as highlighted in prospective epidemiologic studies, Mendelian randomization studies, and randomized intervention trials (2).

Conventionally, clinical criteria comprising several prognostic scores, such as Simon Broome (from UK), Dutch Lipid Clinic Network (DLCN) (from the Netherlands), Make Early Diagnosis to Prevent Early Death (MEDPED) (from the USA) (4), and the recent Montreal-FH-SCORE (11), are used to diagnose FH. The score considers the patient's lipid levels, cutaneous stigmata, and personal and family history of coronary artery disease (CAD) to some extent. In contrast, there is significant dependence on the unequivocal genetic diagnosis, which has been made accessible by emerging technologies, such as polymerase chain reaction (PCR), PCR-based techniques, and sequencing, although genetic testing for FH is not routine (12) in many countries.

In this review, we present a short overview of several molecular diagnosis techniques used for the genetic diagnosis of FH, mainly focusing on the detection rate of each method as well as its applicability; considering that none of the techniques are exhaustive, yet they efficaciously overlap and complement each other for the molecular confirmation of this disease during the clinical suspicion of FH based on criteria comprised in prognostic scores.

### Single nucleotide polymorphism genotyping

Real-time PCR with SNP genotyping can be used to diagnose FH mainly in the *APOB* and *PCSK9* genes, since the known number of definite FH causative mutations (4) compared to *LDLR* is relatively limited.

In a cohort of 235 Slovakian FH patients selected using the MEDPED score, the most frequent FH-causing *APOB* mutation in Caucasians, namely rs5742904 (p.Arg3527Gln, R3527Q or R3500Q), was investigated using TaqMan (Applied Biosystems, Germany) technology, and the authors reported a frequency of 6% (13). This frequency is in line with the results of other European-based studies that reported similar frequencies for the same *APOB* R3500Q SNP (Austria: 4.7%, Hungary: 5.5%, Poland: 6.6%, Czech Republic: 11.8%, and the UK 10.8%) (14-18).

*PCSK9* rs374603772 (R496W) and rs137852912 (p.Asp374Tyr, D374Y) mutations were investigated in a Turkish cohort by High-Resolution Melting SNP genotyping. The study population included 200 patients diagnosed with primary dyslipidemia, and harbored 6% rs374603772 and 7% rs137852912 (19), with heterozygous and mutant homozygous cases. The same *PCSK9* rs137852912 was also investigated in a Portuguese cohort by Medeiros et al. (20), but the prevalence rate was zero in their cohort of 1340 individuals selected based on the Simon Broome FH register criteria. The prevalence of *PCSK9* rs137852912 seems to be (very) low in the Iberian Peninsula, as only one carrier of this mutation was found another study that comprised of 5430 index cases and 2223 family members from Spain selected according to the DLCN criteria (21). In contrast, according to a Japanese study, other mutations in the *PCSK9* gene seem to be more common in the Asian population comprising of 1096 patients (22) selected based on the clinical diagnosis of FH. Using the Invader SNP genotyping assay, Mabuchi et al. (22) found a prevalence of 5.9% for the *PCSK9* rs564427867 heterozygous carriers as well as 13 homozygous patients, their results being similar to those of Miyake et al. (23) that suggested a frequency of 5% *PCSK9* mutations in Japan.

Monogenic FH was also examined in a study of Lye et al. (24), which focused on the Asian population, and investigated by microarray SNP genotyping a number of 1536 polymorphisms previously described in international genetic databases as being implicated in FH etiology in other populations, on a cohort of 141 consecutive patients with clinical diagnosis of FH, selected based on the DLCN criteria and a representative cohort of FH-free control subjects (24). The authors reported 14 SNPs within the *LDLR*, *APOB*, and *PCSK9* genes to be significantly associated with FH, namely 11 SNPs associated with an increased risk for FH and three SNPs associated with a decreased FH risk. Among the 11 risk-increasing SNPs, *LDLR* rs2569556 was the most prevalent, with a frequency of 39% (24).

SNP genotyping can also be used in the evaluation of multiple SNPs within the cholesterol-raising genes, followed by the evaluation of the patient's polygenic score. For example, a European study advocates for a shortlist of only 12 SNPs (25) called "SNP-Score" in common variants that raise LDL-C by the largest amount. This score was developed based on the refinement of the initial finding in 2010 of 95 loci significantly associated with FH, by the Global Lipids Genetics Consortium (GLGC) (26). Using the GLGC "SNP-Score" the authors showed that the mean weighted score was significantly higher in two cohorts of FH patients with no mutation in *LDLR*, *APOB*, and *PCSK9* versus controls (27). Adding more SNPs to the initial 12 did not significantly improve the test's ability to discriminate between FH and healthy subjects; however, in order to improve cost efficiency and ease the SNP-Score calculations, the initial authors came up with an even shorter list of only six GLGC SNPs for the computation of the FH weighted score (Table 1) (28).

In contrast, the study by Ghaleb et al. (29) included over 3000 FH probands from the French Research Network for FH and computed both the 6-SNP and the 12-SNP GLGC score in selected probands and their families, concluding that while these scores were a reliable instrument to quantify the severity of hypercholesterolemia, they were not a reliable tool to distinguish phenocopies within FH families.

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**Table 1. Polygenic FH risk score in variants increasing the LDL-C level in the previously described studies**

| Chromosome | Gene | GLGC 12 SNPs (25) | GLGC 6 SNPs (28) | Mutation weight for risk score calculation (25) (27) (28) | Risk allele/haplotype |
|---|---|---|---|---|---|
| 1 | *PCSK9* | rs2479409 | | 0.052 | G |
| 1 | *CELSR2* | rs629301 | rs629301 | 0.15 | T |
| 2 | *APOB* | rs1367117 | rs1367117 | 0.10 | A |
| 2 | *ABCG8* | rs4299376 | | 0.071 | G |
| 2 | *ABCG8* | | rs6544713 | 0.071 | T |
| 6 | *SLC22A1* | rs1564348 | | 0.014 | T |
| 6 | *HFE* | rs1800562 | | 0.057 | G |
| 6 | *MYLIP* | rs3757354 | | 0.037 | C |
| 11 | *ST3GAL4* | rs11220462 | | 0.050 | A |
| 14 | *KIAA1305* | rs8017377 | | 0.029 | A |
| 19 | *LDLR* | rs6511720 | rs6511720 | 0.029 | G |
| 19 | *APOE* | rs429358 | rs429358 | 0 | Haplotypes |
| | | | | 0.1 | E3/E3 |
| | | | | 0.2 | E3/E4 |
| 19 | *APOE* | rs7412 | rs7412 | | E4/E4 |

### Biochip array technology

The technique was developed by Randox Laboratories (Crumlin, UK) and is able to identify the 40 most common FH-causing mutations in the United Kingdom and Ireland in the *LDLR*, *APOB*, and *PCSK9* genes (30) and has been certified as European Conformity (CE) for In Vitro Diagnosis (IVD) in Europe (30). It is split into two biochip arrays: FH I and FH II (20 mutations per array). It relies on multiplex PCR (2 multiplexes per biochip, to avoid cross-amplification by unspecific primers) and biochip array hybridization. Every mutation is amplified in an allele specific PCR by a pair of primers, with a specific 5′ tail in one primer and a biotin molecule at the 5′ of the other primer (31). In case the mutation is present, the amplicon is hybridized to a complementary oligonucleotide probe spotted on a biochip discrete test region, and using a biotin–streptavidin enzymatic reaction, the chemiluminescent signal is recognized by a charge-coupled device, followed by a scan with the semi-automated Evidence Investigator analyzer (31). Thereafter, a proprietary software is used to issue the result automatically for each analyzed sample.

Randox's FH I and FH II biochip array technology was able to identify 71.3% of all point mutations in a cohort of 465 patients from the UK and Ireland. The included patients were previously diagnosed with FH using Sanger DNA sequencing (31). However, the same study reported a mutation-detection rate of 7.4% in a group of 663 FH patients from throughout the UK and Ireland, who were enrolled based on the Simon Broome criteria (31).

To date, there are no other published data to confirm or infirm the above-mentioned study's results, nor to reveal the detection frequency in other populations, or based on other inclusion criteria (such as DLCN, MEDPED, or Montreal-FH-SCORE).

Nevertheless, predominantly based on the lack of large-population studies, although it is an IVD test, the Randox FH I and FH II biochip technique seems more suitable for the cascade screening (4) of family members once an index case is identified through another method, rather than a first-intention diagnosis method.

### Multiplex ligation-dependent probe amplification

Several authors consider multiplex ligation-dependent probe amplification (MLPA) as the gold standard (32-34) reference method for the investigation of large gene deletions and duplications. However, the assessment of structural variations within the *LDLR* gene with the help of MLPA is usually performed subsequent to a negative testing of small-scale DNA variants using targeted NGS panels (32) or as complementary testing to targeted SNP genotyping.

MLPA testing is straightforward, relying on multiplex PCR with "half-primers" that hybridize immediately adjacent to one another on the same gDNA target and bind together during the ligation step, followed by capillary electrophoresis in denaturing condition settings. Subsequent data interpretation is possible using the MRC-Holland proprietary software Coffalyser.Net or a third-party software. The SALSA MLPA P062 *LDLR* probe mix, CE IVD certified, made by MRC-Holland (Amsterdam, the Netherlands) can be used to detect copy number variations (CNVs) in the *LDLR* gene, using 33 probes located within the gene (investigating exons 1-18) and one probe upstream of the *LDLR* gene (35).

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CNVs within the *LDLR* gene were investigated as probable FH etiology in a Canadian-based study. The study reported a frequency of 6.4% in their cohort of 313 HeFH patients (in 2016) (7) who were included after a probable or definite FH diagnosis made using the DLCN criteria and personal or family history of premature CHD. As a follow-up, in 2017, the same team of researchers published data regarding the enlargement of the initial cohort (now at 388 individuals, selected based on the same criteria) and indicated a frequency of 9.8% CNVs detected using MLPA, detecting both heterozygous deletions and duplications in 15 out of the 18 *LDLR* exons (exons 8, 9, and 10 being unaffected) (32). Similarly, in a UK-based study on the Oxford FH cohort, comprising 289 patients enrolled after the definite or possible FH diagnosis using the Simon Broome diagnostic criteria, Futema et al. (18) found the prevalence of large *LDLR* gene rearrangements to be 10.89%, using the SALSA MLPA P062 probemix. In the study of Medeiros et al. (20), which comprised of 482 index patients diagnosed with FH based on the Simon Broome criteria, two patients were identified to carry large *LDLR* deletions as detected using the MLPA analysis.

On contrast, large deletions and duplications in the *LDLR* gene seem to be not very common determinants of FH, as implied in a number of studies (from Europe and the USA) who reported the investigation of *LDLR* using the MLPA method for their patients clinically diagnosed with FH. Interestingly, the same studies did not mention the prevalence of CNVs detected using MLPA (13, 36, 37). Therefore, we can only speculate that the detection frequency was zero in these studies. Additionally, the same cohorts were investigated with other diagnostic methods, and authors suggested point mutations or polygenic etiology to be responsible for the FH phenotype and not CNVs (13, 36, 37).

### Sanger sequencing

Sanger sequencing can be used for the investigation of large gene fragments (such as exons, introns, promoter regions, and exon-intron boundaries) in uninvestigated patients, or it can be used as a confirmation method for NGS.

Gabčová et al. (13) investigated all 18 exons and exon-intron boundaries, as well as the promoter region of the *LDLR* gene in 235 FH probands, recruited based on the MEDPED criteria and found 37.9% to bear an *LDLR* mutation. Known mutations included substitutions, small insertions, and deletions, besides large rearrangements, while unknown mutations discovered (substitutions - p.Asp90Glu, p.Asp136Tyr, p.Ser177Pro, p.Gly478Glu, p.Leu680Pro, c.314-2A>G; frame-shift insertions and deletions - p.Lys225_Glu228delinsCysLys, p.Gly675Trpfs*42, p.Thr832Argfs*3) were predicted to be pathogenic based on a dedicated software (13).

In another European study, Bertolini et al. (38) discovered a 26% mutation prevalence within the *LDLR* gene, while not discovering any mutations in the other frequently implicated genes, namely *APOB* and *PCSK9*. All patients included in their study had premature CAD, which was highly selected according to the DLCN criteria (38). The researchers indicated several pathogenic mutations within *LDLR*, such as major rearrangements 9.6% (exon deletions and duplications), small mutations 11.5% (deletions, insertions/duplications, and deletions/insertions), point mutations 68.9% (missense, nonsense, and single nucleotide deletions and insertions), and splicing mutations 10% (38).

Rare variants can also be investigated using Sanger sequencing. For example, the study of Reeskamp et al. (39) found a prevalence of 0.24% for the rare intronic *LDLR* c.2140+103G>T variant in a cohort of 1245 FH patients without a previously known FH-causing mutation in the exons of the three major genes (*LDLR*, *APOB*, and *PCSK9*) (39).

One of the first studies to suggest the implication of *PCSK9* in the occurrence of FH was carried out in 2004 in Norway in a cohort of 51 subjects who were clinically diagnosed with FH, but had no mutations in the *LDLR* gene and *APOB* (rs5742904) when the presence of rs137852912 (*PCSK9* p.Asp374Tyr) was identified using Sanger sequencing in a patient harboring autosomal dominant hypercholesterolemia (40). Later on, the same *PCSK9* mutation was observed in other FH cohorts with different ethnic backgrounds (41-43).

Another essential use for Sanger sequencing is confirming the variants detected by NGS (7, 44), as the Sanger technique is still regarded as the "gold standard" (45-47) in base-calling by numerous authors. For example, the study by Wang et al. (48) used Sanger sequencing to confirm the variants obtained with a custom resequencing array, and reported a frequency of 3.2% FH-causing mutations in their cohort that included a small number (156) of highly selected (DLCN - definite FH) hypercholesterolemia patients (48). In contrast, new research is implying that the validation of NGS-derived variants using Sanger sequencing has nowadays a limited utility (49, 50), mainly due to the latest-generation techniques validation rate being almost 100% to that of Sanger sequencing (49).

### Next-generation sequencing

NGS is nowadays considered the main diagnosis technique for FH, mainly due to its massively parallel sequencing capabilities (47) and there are a number of NGS types to choose from: targeted panels for monogenic dyslipidemias [such as the LipidSeq (51)], whole exome sequencing, or the extensive whole-genome sequencing (52).

Whole exome sequencing was used in a single-center US study for the investigation of FH-related mutations, where the authors investigated 50,726 participants. They indicated a frequency of 0.8% in 6,747 participants recruited from the cardiac catheterization laboratory and a frequency of 0.4% in the remaining 43,979 people from the general population (12). With regard to the whole investigated cohort, the main three genes involved in the occurrence of FH surprisingly had the following frequencies: 42.8% *LDLR*, 44.5% *APOB* variants, and 12.7%

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*PCSK9* (12). Similarly, NGS exome sequencing detected definite or likely causal mutations in 47.3% out of 313 patients investigated by Wang et al. (7) from an Ontario-based hypercholesterolemia cohort (it included FH patients diagnosed as "probable" or "definite" using the DLCN score). This study reported the detection of 105 unique mutations, 90 (85.7%) within the *LDLR* gene and the rest in the *APOB* and *PCSK9* genes; 16 mutations were not previously mentioned in literature, and 12 were within the *LDLR* gene, as follows: 2 splicing, 5 frame-shift, and 5 missense mutations (7).

Whole-genome sequencing data from a US-based study on 2.081 patients recruited after early-onset myocardial infarction, irrespective of their lipid status, indicated an FH-causing mutation in 1.7% (52) of cases. Researchers reported several types of mutation within the *LDLR* gene, namely loss-of-function mutations and missense mutations that were previously illustrated as pathogenic in the ClinVar online database, as well as novel missense mutations predicted to be pathogenic by computer software, while no variants were identified in the *APOB* or *PCSK9* genes (52).

Genome sequencing was also used in the study of Reeskamp et al. (39) to investigate the presence of the rare intronic variant *LDLR* c.2140+103G>T (0.23% frequency) in a cohort of 2,154 FH patients that did not carry a known mutation within the exons of *LDLR*, *APOB*, and PCKS9 (39).

NGS data can also be coupled with bioinformatics tools (such as CNV Caller, VarSeq v1.4.3, Golden Helix, Bozeman, MT) for CNV detection, with results similar to MLPA. For example, Iacocca et al. (32) reported a 100% concordance rate in *LDLR* CNV detection between the two methods (NGS vs. MLPA) in a cohort of 388 FH patients from Canada, selected based on DLCN criteria (32).

As NGS technology is being more extensively used, other alternative applications of this scientific know-how are being developed, mainly with the help of bioinformatics. Software tools, such as PLINK (52, 53), which were primarily developed for genome-wide association studies (GWAS), can also be used on NGS whole-genome sequencing data to predict the cumulated effect of risk alleles for each variant and their corresponding weight in the occurrence of FH (better known as polygenic FH

**Table 2. Advantages and limitations of reviewed techniques in FH diagnosis**

| | Advantages | Limitations |
|---|---|---|
| Sanger sequencing | • Time-efficient and cost-effective for low numbers of targets<br>• Single base-pair resolution<br>• 5% minor allele fractions can be detected<br>• Contiguous sequences as long as 1000 bases can be analyzed<br>• Useful in NGS confirmation | • Not cost-effective for high target numbers<br>• Low scalability due to increasing sample input requirements |
| NGS | • Sequencing multiple genes/targets simultaneously<br>• High sample throughput<br>• Single base-pair resolution<br>• Higher probability to find novel variants<br>• Increased sequencing depth resulting in higher sensitivity (down to 1%) | • Less cost-effective and less time-efficient for sequencing low target numbers (up to 20)<br>• Big data interpretation is needed |
| Single nucleotide polymorphism genotyping | • Cost-effective for SNPs<br>• Time-efficient results<br>• Can be scaled up in multiplex SNP assays | • Interrogating one SNP at a time<br>• Investigates point mutations and not DNA aberrations |
| Biochip array technology | • ICD certified (based on the British population)<br>• The mutational status can be determined from a single test | • Fixed number of investigated mutations/SNPs<br>• Limited published data available |
| Multiplex ligation-dependent probe amplification | • Golden standard technique for CNV (microdeletions, microduplications) identification<br>• P-062 investigates 33 *LDLR* targets in one reaction<br>• IVD certified<br>• Multiplex, easy workflow, and low-cost technique | • Investigates only the *LDLR* gene<br>• Unable to detect anomalies at the single cell level<br>• Cannot detect unknown point mutations<br>• Sensitive to novel/undescribed benign polymorphisms at or near a probe ligation site |

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etiology). This hold true even in studies with relatively low number of subjects (compared to GWAS), when the obtained data are compared with previously published genome-wide association studies (52, 54) and then statistically interpreted (55). For example, a recently published study reported a 17.1% frequency of individuals carrying a high polygenic score but no FH-causing mutation in the *LDLR*, *APOB*, or *PCSK9* genes, with an additional 0.2% persons who carried both a high polygenic score and a FH-causing mutation in the above-mentioned genes, all data being obtained from the whole-genome sequencing of 2,081 early-onset myocardial infarction patients (52).

The main advantages as well as limitations that apply to the previously described techniques for the diagnosis of FH are presented in Table 2.

## Conclusion

The genetic diagnosis of FH is important for both patients and their curing physicians, but it is often difficult to be established, mainly due to the heterogeneity of known mutations. Despite the fact that more knowledge is constantly being gained about the FH-causing mutations, we also have to take into account that the FH phenotype may be caused by mutations not yet described in the intronic or regulatory regions of *LDLR*, *APOB*, and *PCSK9* genes, as well as the polygenic aspect of this pathology. Cascade screening of family members of patients with FH and premature CHD is also a priority, which has been made easier by the establishment of international FH registries, such as the FH Studies Collaboration Registry (56).

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