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Founder effects facilitate the use of a genotyping-based approach to molecular diagnosis in Swedish patients with familial hypercholesterolaemia

P. Benedek[1,2], H. Jiao[3,4], K. Duvefelt[4], T. Skoog[3], M. Linde[4], P. Kiviluoma[4], J. Kere[3,4,5,6], M. Eriksson[1,4] & B. Angelin[1,2]

From the [1]Cardiometabolic Unit, Clinical Department of Endocrinology, Department of Medicine, Karolinska Institutet at Karolinska University Hospital; [2]Department of Medicine, Integrated Cardiometabolic Center (ICMC), Karolinska Institutet at Karolinska University Hospital; [3]Department of Biosciences and Nutrition, Karolinska Institutet; [4]Clinical Research Center, Karolinska University Hospital, Huddinge, Sweden; [5]Folkhälsan Research Center; and [6]Stem Cells and Metabolism Research Program, University of Helsinki, Helsinki, Finland

Abstract. Benedek P, Jiao H, Duvefelt K, Skoog T, Linde M, Kiviluoma P, Kere J, Eriksson M, Angelin B (Karolinska Institutet at Karolinska University Hospital Huddinge, Huddinge; Karolinska Institutet Huddinge, Huddinge; Karolinska University Hospital Huddinge, Huddinge, Sweden; Folkhälsan Research Center, Helsinki; University of Helsinki, Helsinki, Finland; and Karolinska Institutet at Karolinska University Hospital Huddinge, Huddinge, Sweden). Founder effects facilitate the use of a genotyping-based approach to molecular diagnosis in Swedish patients with familial hypercholesterolaemia. *J Intern Med* 2021; **290**: 404–415. https://doi.org/10.1111/joim.13287

Aim. To investigate whether genotyping could be used as a cost-effective screening step, preceding next-generation sequencing (NGS), in molecular diagnosis of familial hypercholesterolaemia (FH) in Swedish patients.

Methods and results. Three hundred patients of Swedish origin with clinical suspicion of heterozygous FH were analysed using a specific array genotyping panel embedding 112 FH-causing mutations in the *LDLR*, *APOB* and *PCSK9* genes. The mutations had been selected from previous reports on FH patients in Scandinavia and Finland. Mutation-negative cases were further analysed by NGS. In 181 patients with probable or definite FH using the Dutch lipid clinics network (DLCN) criteria (score ≥ 6), a causative mutation was identified in 116 (64%). Of these, 94 (81%) were detected by genotyping. Ten mutations accounted for more than 50% of the positive cases, with *APOB* c.10580G>A being the most common. Mutations in *LDLR* predominated, with (c.2311+1_2312-1)(2514)del (FH Helsinki) and c.259T>G having the highest frequency. Two novel *LDLR* mutations were identified. In patients with DLCN score < 6, mutation detection rate was significantly higher at younger age.

Conclusion. A limited number of mutations explain a major fraction of FH cases in Sweden. Combination of selective genotyping and NGS facilitates the clinical challenge of cost-effective genetic screening in suspected FH. The frequency of *APOB* c.10580G>A was higher than previously reported in Sweden. The lack of demonstrable mutations in the *LDLR, APOB and PCSK9* genes in ~1/3 of patients with probable FH strongly suggests that additional genetic mechanisms are to be found in phenotypic FH.

Keywords: Familial hypercholesterolaemia, genotyping, next-generation sequencing, precision medicine, *APOB*, *LDLR*, *PCSK9*.

Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant disease characterized by elevated levels of LDL cholesterol leading to a markedly increased risk of early-onset cardiovascular disease [1]. Although the true prevalence of FH is

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uncertain, it is estimated to be approximately 1/250 worldwide [1–4], making it one of the most common monogenic diseases. FH is most often caused by mutations in the LDL receptor (*LDLR*; NM_000527) gene, but can also result from mutations in the apolipoprotein B (*APOB;* NM_000384) gene, affecting the binding of LDL to its receptor, or from gain-of-function mutations in the propeptide convertase subtilisin/kexin type 9 (*PCSK9*; NM-174936.3) gene, leading to enhanced degradation of the LDLR protein. FH remains heavily underdiagnosed globally, and many cases are still not discovered until the first cardiovascular event. At least 10% of hypercholesterolaemic patients with myocardial infarction carry an FH-causing mutation [5,6].

First-degree relatives of patients with FH have a 50% risk of carrying the disease, and it is now strongly recommended that they should be screened for lipid levels [1–8]. Despite much effort, it is clear that most patients with FH still remain undiagnosed, meaning that a substantial number of individuals are not offered the now well-established benefits of risk reduction by lipid-lowering drugs [9]. Although such screening should be of particular importance in younger subjects, this is generally more challenging due to the less clearly established lipid phenotype in children [8]. Several diagnostic algorithms are in use for FH, the most common being the Dutch lipid clinics network (DLCN) criteria [1]. However, these are not applicable in children and younger patients, and furthermore clinical data are often missing making the use of the algorithm incomplete.

The identification of a disease-causing mutation in a patient with suspected FH is of considerable clinical value. Not only does it facilitate further genetic screening of the family, especially in children with borderline LDL cholesterol levels, but it has also been shown to increase compliance to preventive measures including pharmacological treatment [3,8]. In some countries, genetic diagnosis is also required for access to more advanced therapies. A genetic disease caused by a specific pre-defined mutation can generally be detected at a low cost by genotyping. However, since more than 1500 pathogenic mutations have been described in the *LDLR* [10], in addition to several ones in *APOB* and *PCSK9*, next-generation sequencing (NGS) of DNA has become a preferred method for molecular diagnosis. By sequencing these three genes, causative mutations are generally detected in approximately 60% of individuals that present with a well-defined phenotype of FH [2,3]. For a common disease like FH, the total cost of genetic screening on a national level becomes substantial, and the need for robust and cost-effective approaches is thus imperative.

In many populations, the spectrum of mutations may be relatively narrow due to founder effects. Thus, 14 different mutations explained more than 70% of all genetically investigated cases of FH in a Danish study [11] whilst 10 mutations represented 93 % of the cases in a corresponding report from Norway [12]. A corresponding but different pattern has been reported from Finland, where 7 mutations covered approximately 80% of the mutation spectrum [13]. Although early data from Sweden indicated that the pattern here may be more heterogeneous [14–16], we wanted to pursue the hypothesis that a limited number of mutations predominated also in subjects of Swedish origin. We therefore explored whether genotyping rather than NGS could be used as a first line of diagnosis in FH, with extensive DNA gene sequencing only applied in individuals with a negative result when genotyped for the pre-defined mutations on the panel. Our results clearly show that such a strategy can be successfully applied in Swedish patients with suspected FH, and we propose that a similar approach could be taken on national or regional levels to develop rapid and cost-effective diagnostic tools also in other populations.

Patients and methods

Study subjects

Three hundred unrelated patients of Swedish origin from the Stockholm area referred to the Lipid Out-Patient Clinic at Karolinska University Hospital Huddinge because of suspected FH were included (Table 1). After giving their written informed consent to participate in the study, which had been approved by the Ethics Committee of Karolinska Institutet, physical status, lipoprotein pattern and family history were registered, and their DLCN scores were calculated. The latter are based on LDL cholesterol levels before treatment, presence of early onset of cardiovascular disease, physical signs such as tendon xanthomas or arcus corneae and a family history of early cardiovascular disease and/or hypercholesterolaemia [1]. Of the 300 patients included, 119 had scores of 3–5 ('possible' FH), 151 of 6–8 ('probable' FH) and 30 of > 8 ('definite' FH).

Table 1 *General characteristics of the study group*

	All	Mutation (+)	Mutation (-)
Subjects *n* (%)	300	139 (46%)	161 (54%)
Age years (mean)	54.4	50.0	57.8
Female *n* (%)	165	75 (45%)	90 (55%)
Male *n* (%)	135	64 (47%)	71 (53%)
LDL-C mmol/L (SD)	6.7 ± 1.5	7.3 ± 1.5	6.1 ± 1.4
TG mmol/L (SD)	1.62 ± 0.64	1.52 ± 0.07	1.71 ± 0.57
ACS n (%)	48/300(16%)	15/48	33/48
Early ACS[a] *n* (%)	33/48 (69%)	11/33	22/33
Diabetes II *n* (%)	11 (4%)	3/11	8/11

ACS, acute coronary syndrome; LDL-C, LDL cholesterol; TG, triglycerides: (untreated levels).
[a]Early ACS: men < 55 years and women <60 years.

Construction of the genotyping panel

We performed an extensive search for previously published results from mutation analysis, as well as from existing databases of FH mutations obtained from Scandinavian investigators. All of the mutations previously found in Swedish FH studies [14–16], and most of the common mutations in Denmark, Finland and Norway were selected to form the genotyping panel. Altogether, 124 FH mutations were selected for direct genotyping.

Genotyping

Genotyping was performed at the Mutation Analysis Facility, Clinical Research Centre, at Karolinska University Hospital Huddinge, using the Agena MassARRAY (MassARRAYAgena Bioscience, San Diego, California, USA) technology with iPLEX® Gold chemistry [17], according to the manufacturer's instructions. Of the 124 chosen variants, 117 were straightforward to implement. The FH Helsinki deletion was analysed using a previously published design [18], with the addition of a positive control assay that enables discrimination of homozygous deletions versus no amplification of the target region. During the study, it became evident that five mutations originally reported to be pathogenic had been re-classified as benign, resulting in a total of 112 mutations being analysed (Table S1). The genotyping was validated using a set of 14 trio families, in total 42 individuals, with genotype data available through the HapMap consortium. Concordance analyses with the HapMap data as well as analysis of the parent–offspring compatibility were performed. The genotyping platform was also validated by analysing DNA samples from 21 FH individuals where mutations represented on the platform had been identified using NGS at the Department of Medicine, Sahlgrenska University Hospital, Göteborg (positive controls). All those were identified using our genotyping procedure.

DNA sequencing

DNA samples from all patients in whom no mutation was detected using the genotyping panel were then subjected to NGS. Two different methods of sequencing were used in this study, exome sequencing and Amplicon sequencing (Fig. 1).

Exome sequencing was performed at Science for Life Laboratory, Stockholm, Sweden. DNA libraries for each sample were prepared from genomic DNA, and SureSelect Human All Exon V5 target enrichment kit (Agilent Technologies, Santa Clara, CA, USA) was used. The sequencing runs were performed on an IlluminaHiSeq2500 instrument (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Major computations were performed at UPPMAX (Uppsala Multidisciplinary Center for Advanced Computational Science, Uppsala, Sweden). For a complete description of the data processing and analysis, see online Supplementary methods.

Amplicon sequencing was performed with Progenikas's Familial Hypercholesterolaemia Genetic Analysis (SEQPRO LIPO S® platform (Progenika Biopharma, Derio, Spain) according to manufacturer's instructions, with the exception that the *STAP1* gene was not analysed [19]. For a complete description of the data processing and analysis, see online Supplementary methods.

Role of the funding source

The study was designed, conducted, analysed, interpreted and reported by the investigators independently of all funding bodies.

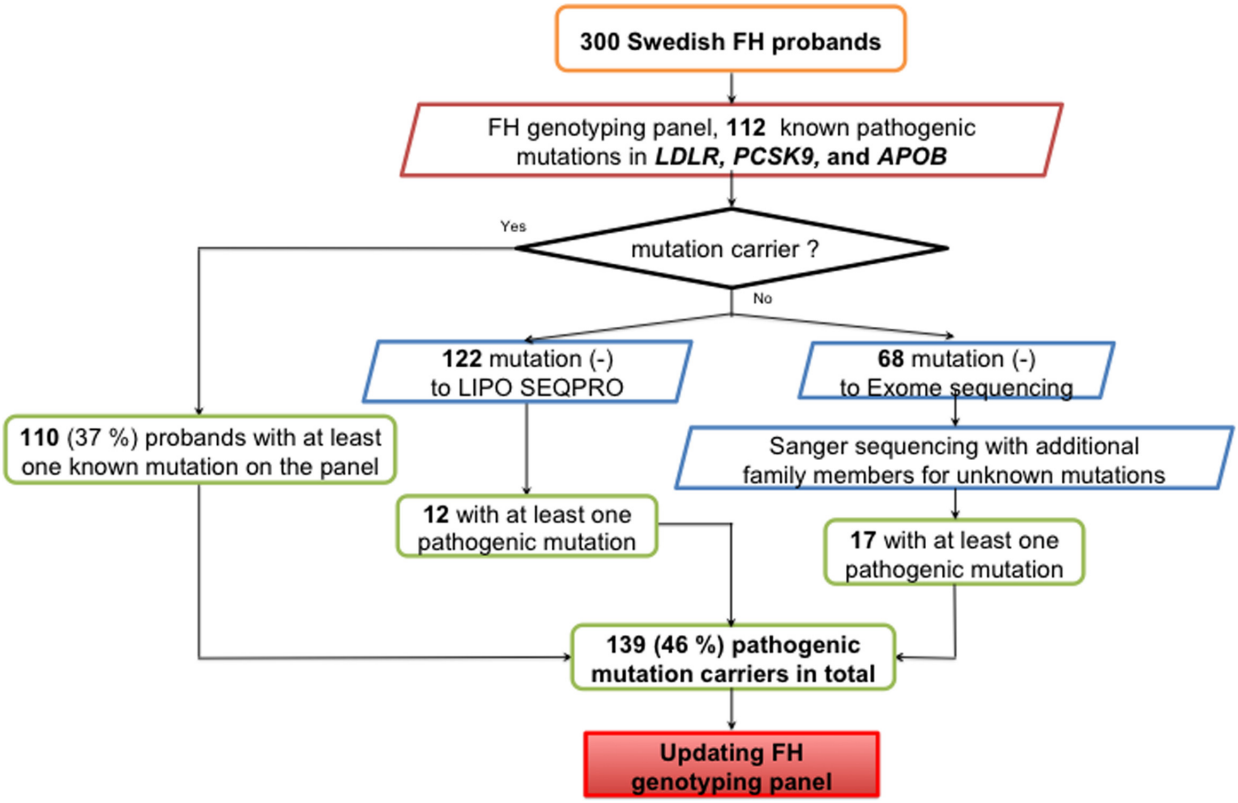

Fig. 1 *Workflow of genetic investigation of Swedish FH cohort.*

Statistics

The significance of differential age distributions between pathogenic mutation carriers and non-carriers in two DLCN groups was calculated by using unequal sample size *t*-tests, and *p* values were based on two-tailed hypothesis.

Results

A selective two-step approach was used to conduct the genetic tests of this study (Fig. 1). In the first phase, all 300 patients underwent genotyping using the designed panel and 110 of them were identified to carry at least one of the 112 mutations represented in the panel. The remaining 190 mutation-negative patients then underwent either exome sequencing ($n = 68$) or targeted Amplicon sequencing ($n = 122$) in the second step of the analysis, which led to the identification of 29 additional carriers of pathogenic mutations.

Mutation spectrum in Swedish FH

A total of 55 different pathogenic mutations were identified in 139 unrelated carriers in this Swedish FH cohort. Of these, 34 were detected with the genotyping panel (110 patients) and 21 by NGS (29 patients) (Table 2). These data indicate clear founder effects in the Swedish population, with a large portion of pathogenic variants limited to a few frequently appearing ones. Twenty-six such recurrent (freq. >1) mutations explained the genetic cause of 81% of mutation carriers and the top 10 most common mutations (freq. ≥5) accounted for 53% of all FH patients (Table 2 and Fig. 2).

LDLR mutations constituted 96 % of the spectrum in this Swedish FH cohort (Table 2). The distribution of these mutations covered most exons and several splicing sites (Fig. 3). Somewhat unexpectedly compared to our previous work [14,15], the most common mutation was *APOB* c.10580G>A*:* p. Arg3527Gln, which was found in 11 patients (8% of

Table 2 *Pathogenic mutations identified in the 300 FH patients*

Gene	Region	Nucleotide change	Amino acid change	Mutation type	No. of carrier	Detection method
APOB	Exon 26	c.10580G>A	p. Arg3527Gln	Missense	11	Genotyping
LDLR	16-18	c.(2311+1_2312-1)_ ([a]2583?) del	p. Ala771Glufs[a]9	Deletion	10	Genotyping
LDLR	Exon 3	c.259T>G	p. Trp87Gly	Missense	10	Genotyping
LDLR	Exon 5	c.782G>T	p. Cys261Phe	Missense	7	Genotyping
LDLR	Exon 9	c.1246C>T	p. Arg416Trp	Missense	7	Genotyping
LDLR	Intron 8	c.1187-10G>A	-	Intron variant	7	Genotyping
LDLR	Exon 9	c.1222G>A	p. Glu408Lys	Missense	6	Genotyping
LDLR	Intron 9	c.1359-1G>A	-	Splice acceptor variant	6	Sequencing
LDLR	Exon 13	c.1979A>G	p. Gln660Arg	Missense	5	Genotyping
LDLR	Intron 3	c.313+1G>A	-	Splice donor variant	5	Genotyping
LDLR	Exon 4	c.662A>G	p. Asp221Gly	Missense	4	Genotyping
LDLR	Exon 8	c.1097A>G	p. Gln366Arg	Missense	3	Genotyping
LDLR	Exon 3	c.296C>G	p. Ser99Ter	Stop Gained	3	Genotyping
LDLR	Exon 4	c.564C>G	p. Tyr188Ter	Stop Gained	3	Genotyping
LDLR	Exon 6	c.888C>A	p. Cys296Ter	Stop Gained	3	Genotyping
LDLR	Exon 8	c.1174T>C	p. Cys392Arg	Missense	2	Genotyping
LDLR	Exon 9	c.1268T>C	p. Ile423Thr	Missense	2	Genotyping
LDLR	Intron 11	c.1706-1G>C	-	Splice acceptor variant	2	Sequencing
LDLR	Exon 12	c.1735G>A	p. Asp579Asn	Missense	2	Genotyping
LDLR	Exon 14	c.2043C>A	p. Cys681Ter	Stop Gained	2	Genotyping
LDLR	Exon 4	c.429C>A	p. Cys143Ter	Stop Gained	2	Genotyping
LDLR	Exon 4	c.463T>G	p. Cys155Gly	Missense	2	Genotyping
LDLR	Exon 4	c.681C>G	p. Asp227Glu	Missense	2	Sequencing
LDLR	Exon 6	c.925_931delCCCATCA	p. Pro309LysfsTer59	Frameshift variant	2	Genotyping
LDLR	Exon 18	c. (2547 + 1-2548-1)_ ([a]2583_?)del		Deletion	2	Sequencing
LDLR	Exon 4	c.691T>G	p. Cys231Gly	Missense	2	Genotyping
LDLR	Exon 11	c.1690A>C	p. Asn564His	Missense	1	Genotyping
APOB	Exon 26	c.10579C>T	p. Arg3527Trp	Missense	1	Genotyping
LDLR	Exon 8	c.1069G>A	p. Glu357Lys	Missense	1	Sequencing
LDLR	Exon 8	c.1103G>T	p. Cys368Phe	Missense	1	Sequencing
LDLR	Exon 8	c.1135T>C	p. Cys379Arg	Missense	1	Sequencing
LDLR	Exon 9	c.1291G>A	p. Ala431Thr	Missense	1	Sequencing
LDLR	Exon 2	c.131G>A	p. Trp44Ter	Stop Gained	1	Genotyping
LDLR	Exon 10	c.1474G>A	p. Asp492Asn	Missense	1	Genotyping
LDLR	Intron 11	c.1705+1G>T	-	Splice donor variant	1	Genotyping
LDLR	Exon 12	c.1730G>A	p. Trp577Ser	Stop Gained	1	Sequencing
LDLR	Exon 12	c.1739C>A	p. Ser580Tyr	Missense	1	Sequencing
LDLR	Exon 12	c.1784G>A	p. Arg595Gln	Missense	1	Genotyping
LDLR	Intron 12	c.1846-1G>A	-	Splice acceptor variant	1	Genotyping

Table 2 (*Continued*)

Gene	Region	Nucleotide change	Amino acid change	Mutation type	No. of carrier	Detection method
LDLR	Exon 13	c.1966C>A	p. His656Asn	Missense	1	Sequencing
LDLR	Exon 14	c.2054C>T	p. Pro685Leu	Missense	1	Genotyping
LDLR	Exon 15	c.2252G>A	p. Arg751Gln	Missense	1	Genotyping
LDLR	Intron 16	c.2390-2A>G	-	Splice acceptor variant	1	Genotyping
LDLR	Exon 17	c.2416dupG	p. Val806Glyfs	Frameshift variant	1	Genotyping
LDLR	Exon 3	c.271G>A	p. Gly91Ser	Missense	1	Sequencing
LDLR	Exon 4	c.514G>A	p. Asp172Asn	Missense	1	Sequencing
LDLR	Exon 2	c.(67+1_68-1)_(190+1_191-1)del	p. Gly24_Leu64del	Deletion	1	Sequencing
LDLR	Exon 5	c.798T>A	p. Asp266Glu	Missense	1	Sequencing
LDLR	Exon 7	c.1012T>A	p. Cys338Ser	Missense	1	Genotyping
LDLR	Exon 6	c.859G>T	p. Gly287Cys	Missense	1	Sequencing
LDLR	Exon 6	c.907C>T	p. Arg303Trp	Missense	1	Sequencing
LDLR	Exon 6	c.913T>G	p. Trp305Gly	Missense	1	Sequencing
LDLR	Intron 6	c.940+2T>G	-	Splice donor variant	1	Sequencing
LDLR	Exon 1-3	c.(1-?_313+1_314-1)del		Deletion	1	Sequencing
PCSK9	Exon 2	c.385G>A	p. Asp129Asn	Gain of function/ Missense	1	Sequencing

[a]NM/NP version for three genes: NM_174936.3/ NP_777596.2 (PCSK9); NM_000384.3/ NP_000375.2 (APOB); and NM_000527.4/ NP_000518.1 (LDLR).

mutation positives). Only one causative mutation in *PCSK9:*(c.385G>A p. Asp129Asn) was identified.

Most of the pathogenic mutations discovered by NGS were only detected in single patients, except for a splicing variant 1359-1G>A in *LDLR* which appeared in six subjects, making it amongst the most frequent in this cohort. Moreover, we identified two novel rare missense mutations, c.1103G>T; p. Cys368Phe, c.913T>G; p. Trp305Gly in *LDLR* by exome-sequencing (Figure S1). Those variants have not been described previously as causative for FH. Further investigations in additional family members showed that these variants co-segregated with FH in the corresponding families (Figure S2), strongly suggesting – but not proving – that they are causative.

Since NGS was not performed in patients where a causative mutation was identified through genotyping, the diagnosis of compound or double heterozygotes may not be complete. However, combined heterozygosity (*LDLR* c.1174T>C+*LDLR* c.691T>G and *LDLR* c.782G>A+*APOB* c.10580G>A, respectively) was observed in two patients based on genotyping. These individuals had a more pronounced lipid phenotype and required more intensive therapy to reach goal levels of LDL cholesterol.

When compared to the reported frequencies of FH-causing mutations from other Scandinavian FH cohorts, those most common in Norway and Denmark were also prevalent amongst Swedish FH patients, whereas FH Helsinki was the only Finnish mutation observed.

Effectiveness of panel for genetic test in relation to clinical score

Out of the 112 selected mutations on the genotyping panel, 34 were discovered in the studied FH cohort (Table 2). Twenty-two of them were recurrent mutations, accounting for 90% of panel mutation-positive patients, and the six most common mutations were found in more than half of the

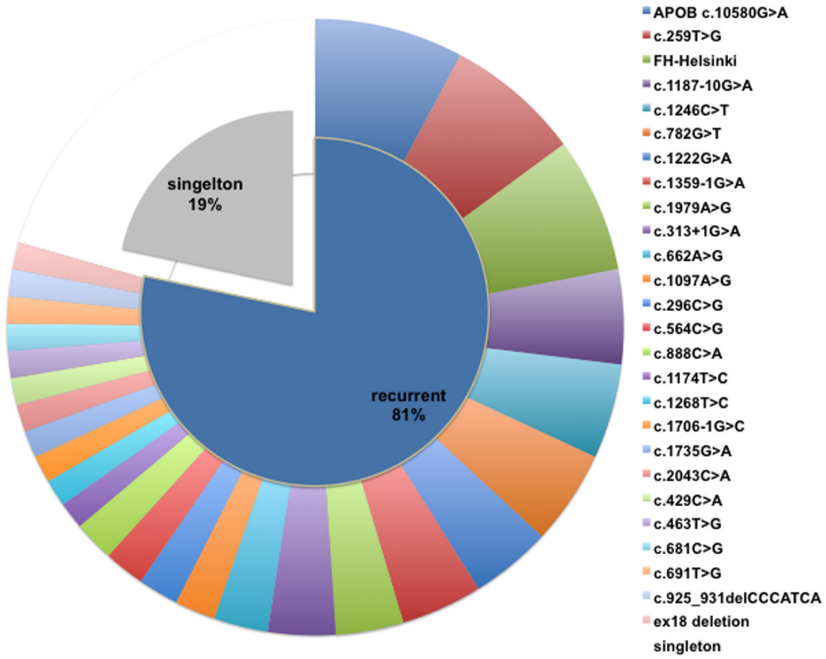

Fig. 2 *Spectrum and proportions of pathogenic mutations in Swedish FH cohort. The mutations were descripted based on NM_000384.3/ NP_000375.2 (APOB) and NM_000527.4/ NP_000518.1 (LDLR). Mutations without gene names are from LDLR.*

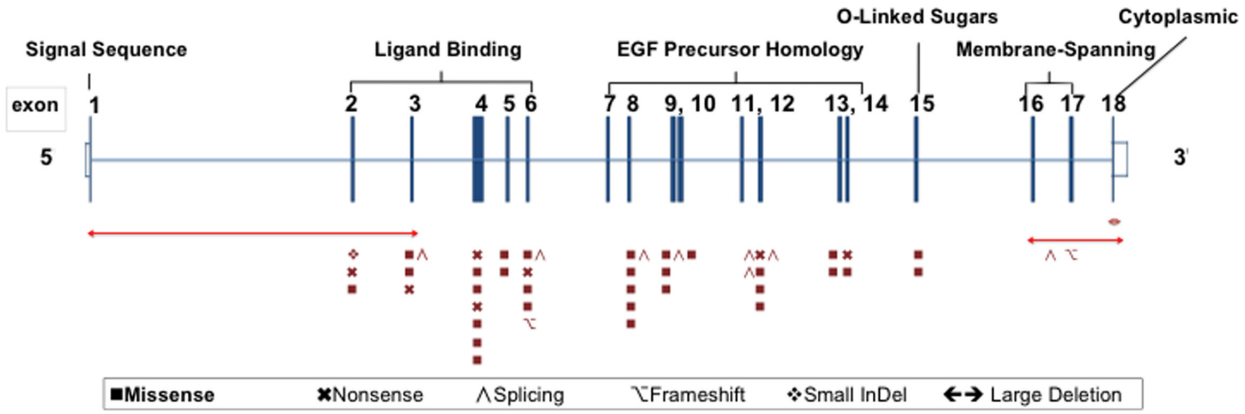

Fig. 3 *Distribution of pathogenic mutations of LDLR detected in FH patients of Swedish origin.*

positives. This indicates that due to founder effects, only a small set of recurrent mutations would represent the causal factor in the majority of FH patients of Swedish origin. Out of the total 139 identified pathogenic mutation carriers, 110 were uncovered using the genotyping panel, accounting for 79 % of the mutation-positive patients (Table 3).

The overall genetic discovery rate was about 50% in this study, which is similar to what has been reported from other studies [2,3,8,20,21]. However, the detection rate varied considerably with DLCN scores, being higher in those with high scores. Higher concordances were shown in probable and definite FH groups, with a discovery rate of 61% and 77%, respectively (Table 3). As

Table 3 *Summary of genetic testing results by genotyping panel and NGS sequencing*

		Genotyping panel		Sequencing	Combined methods		Panel Detection rate
DLCN-Score	No. of patients	Mutation carrier	Discovery rate[a] (%)	Mutation carrier	Total mutation carrier	Total discovery rate (%)	(%)[b]
<6 P	119	17	14	7	24	20	71
6–8 P	151	74	49	18	92	61	80
>8 p	30	19	63	4	23	77	83
All	300	110	37	29	139	46	79

[a]Percentage of patients in whom a FH-causing mutation was identified, by the genotyping panel alone and in combination with NGS.
[b]Percentage of the total number of mutation carriers that were detected by genotyping.

expected, the mean LDL cholesterol was higher in the mutation-positive patients, whereas manifestations of cardiovascular disease tended to be lower. This probably reflects the fact that clinical signs influence the clinical attention (and the DLCN score); a similar explanation is probably behind the finding of more type 2 diabetes in the mutation negatives. Following this observation, we hypothesized that age could be influential on the mutation discovery rate in the group with 'possible' FH (DLCN score 3–5), since not only elevated LDL cholesterol but also clinical manifestations are less prominent in children and adolescent patients with FH. We therefore predicted that the frequency of identified mutations would be higher amongst the younger individuals in this group. As seen in Fig. 4, this was actually the case: the age distribution was different between mutation carriers and non-carriers in those with scores of <6 and ≥ 6 (p-value $<1 \times 10^{-5}$ Fig. 4). The discovery rate in those with possible FH <45 yrs was thus higher (57%) than in those ≥ 45 years (10%).

Discussion

Our results demonstrate that selective genotyping can successfully be used as a first step in the genetic analysis of Swedish patients with FH. Using a genotyping platform covering a limited number of mutations expected to be present in Sweden, we were able to identify more than 80% of the causative mutations in *LDLR*, *APOB* or *PCSK9* in patients with probable or definite FH. This corresponds to a discovery rate of more than 50%, and by performing NGS in those negative when genotyped, this was further increased to 64%. This is in good agreement with other studies when NGS has been used [2,3,8,20,21]. A limited number of mutations explained the phenotype in more than half of the patients, indicating the presence of founder effects also in the Swedish population. Several of those mutations have been reported to be frequent in Danish and Norwegian FH patients, including the *APOB* mutation known to be common in European countries [22]. In the light of the need for rapid and cost-effective methods for establishing a genetic diagnosis in members of families with FH, we propose that application of selective genotyping based on information on the national or regional mutation spectrum should be useful also in other populations.

The present study cohort was based on patients of Swedish origin from the Stockholm area. This represents a mixed population, with a substantial number of Finns who immigrated in the 1950s to 1970s. This explains the relative enrichment of the FH Helsinki mutation, both compared to Norway and Denmark and to other parts of Sweden [14,15]. Whilst the *APOB* c.10580G>A; p. Arg3527Gln is supposed to be very rare in Finland (1/25116, gnomAD) and was not frequently found in our previous studies [14,15], it now emerged as the most common mutation. This is in consonance with its high frequency in both Denmark and Norway [11,12,23] and with the fact that it is believed to have a central European origin [22]. Whilst the phenotype of this mutation has sometimes been described as being milder compared to that in *LDLR* mutations, this was not evident in our patients, who all had DLCN scores of 6 or higher. The mutation pattern in Swedish patients was

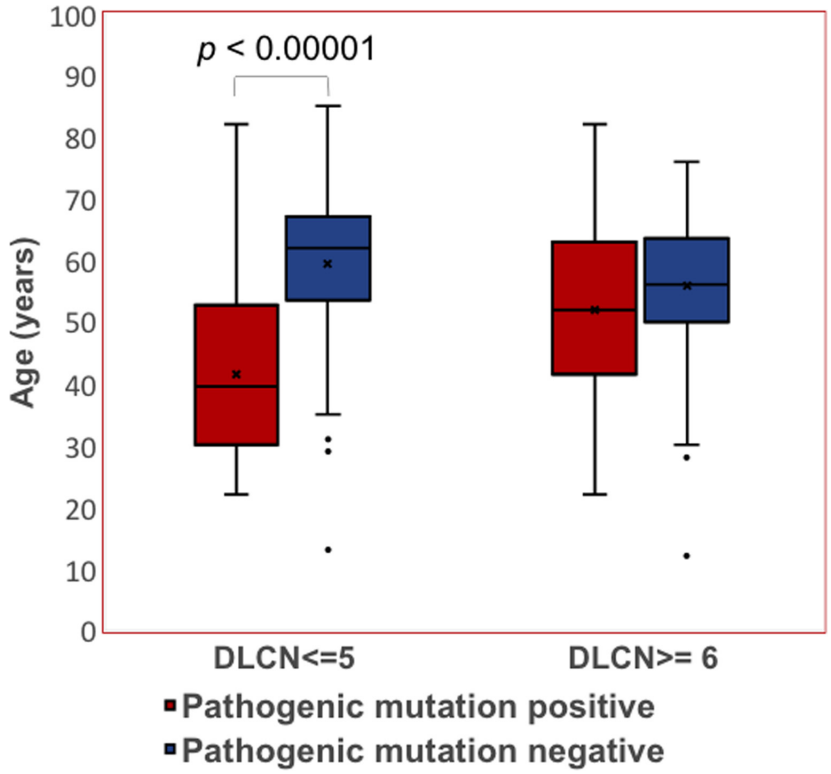

Fig. 4 *Age distribution of pathogenic mutation carriers and non-carriers in two DLCN groups. DLCN score: Dutch lipid clinics network diagnostic criteria for FH.*

generally more similar to that seen in Norway and Denmark, a finding in accordance with previous work on genetic variation in the Nordic countries [24–26]. As seen in those studies, the Swedish population is more genetically diverse, and it is important to note that our present work needs to be expanded to investigate if more pronounced regional variations may occur for patients with FH in other parts of Sweden.

Most of the mutations identified by NGS had been described previously and were seen in single patients. A notable exception was *LDLR* 1359-1G>A which accounted for 4%, making it a common cause of FH in Sweden. This variant is rare, with a minor allele frequency (MAF) of 0.00012 1/8559 (from NHLBI Exome sequencing project, European American ancestry) and pathogenic according to ACMG Guidelines, 2015. Only two novel missense mutations in the *LDLR* gene were discovered and could be confirmed in first-degree relatives. These variants will now be included in our next version of the genotyping platform (see below).

The present study has clearly shown that a rapid and cost-effective genetic diagnosis can be made in a substantial fraction of patients with suspected heterozygous FH by using a carefully designed genotyping platform based on the spectrum of variants in the population under study. An important question is how this technology can best be applied in clinical practice, and the answer may differ in relation to the reasons for performing genetic analysis. Cascade screening in FH is generally initiated by analysis of lipid levels, clinical status and exclusion of secondary hyperlipidaemias, and establishment of a genetic diagnosis is considered to be of value in the early work-up of a family [3,4,7,8,27,28]. If the goal is to identify a causative mutation in the three classical FH genes, *LDLR*, *APOB* and *PCSK9*, initial genotyping based on a population-related panel would be diagnostic in ~ 2/3 of the patients without utilizing NGS. This

corresponds to a detection rate of up to 80%, assuming that all are identified by NGS. As expected, the detection rate was highest in the patients with probable or definite FH. The fact that the detection rate was higher in the younger patients with possible FH probably reflects that those were mainly referred based on their family history, whilst their scoring values were not very high since these are heavily influenced by disease manifestations. By performing further analysis with NGS in patients negative on the mutation platform, causative mutations could only be found in an additional 10% of the patients. By not performing NGS in panel mutation-positive patients, all compound or double heterozygotes will not be identified, which may be of value in explaining difficulties to reach treatment goals and motivate more intense therapies [29].

In many countries, genetic testing for FH is not readily available, mainly for economic reasons [4,7]. We propose that tailor-made genotyping platforms based on the mutation spectrum in the population are a cost-effective first approach, in particular if a founder effect pattern has been established. Even if this is not the case, it is not unlikely that some mutations will be predominant. Currently, the cost of performing NGS is more than tenfold higher than using a genotyping platform and even if the cost for NGS will continue to decrease, this difference will remain substantial. When comparing the cost of the two methods, it is important to consider additional costs that are associated with NGS, such as Sanger confirmation of a detected mutation or further investigation of pathological copy number variation with multiplex ligation-dependent probe amplification. A rough estimate would indicate that only genotyping in 1000 patients with DLCN $\geq$6 (identifying 80%) would have a similar cost as performing only NGS (identifying all) in 100 patients. Using the approach described in the present work, all mutations would be identified at the same cost in about 300 patients. It is obvious that the cost of finding the remaining 20% is relatively high. A tentative pragmatic approach could be to limit the use of NGS to (a) screening-negative subjects highly suspected to have dominant hypercholesterolaemia; (b) patients with a known mutation detected by the genotyping panel but who show an extreme phenotype including insufficient response to therapy (possible compound heterozygosity); (c) patients with a mutation not having a hyperlipidaemic phenotype (possible role of modulating gene variants). Considering the rapid development of NGS, the choice of whole-exome or whole-genome sequencing would probably be preferred over sequencing only target genes as a second step, since acquiring more complete information can be expected to become important in understanding how other genetic variants contribute to the phenotype.

The present study confirms that no genetic explanation can be found in a substantial number of families with a pedigree compatible with dominant hypercholesterolaemia. This was even seen in more than one-fifth of those with DLCN scores >8. Although variants in the classical genes may still have eluded discovery, it is reasonable to hypothesize that additional genes governing cholesterol metabolism are afflicted in some of these cases. The possible importance of polygenic hypercholesterolaemia [21,30] as an explanatory factor in some of these families is still not clear, although such patterns do not seem to be of major relevance in the 'mutation-negative' FH families so far studied by us (unpublished data). This probably heterogeneous group of patients will be of great interest to characterize further. If mutations in new genes can be identified – particularly if relatively frequent – their incorporation into genotyping platforms will further increase the usefulness of that strategy. Meanwhile, 'mutation-negative' patients with suspected FH should obviously be treated on the basis of lipid levels with the same approach as used for heterozygous FH [4,8].

The genotyping platform described here is now being further improved based on our present experience (www.maf.ki.se). First of all, the mutations identified by NGS in the 'platform-negative' patients will be incorporated into its next version, without an increase in the cost of the assay. It may also be useful to include modifying gene variants, such as the *APOE* polymorphism and the common loss-of-function mutations in *PCSK9*, particularly for the interpretation of complex phenotypes in family studies. The increasing number of Scandinavian FH patients of other ethnic origins, in whom the mutation spectrum is different, and the frequency of homozygous and compound heterozygous individuals often high, would motivate the development of alternative DNA panels for genotyping.

Even if genotyping cannot replace NGS in the molecular diagnostics of FH, its use should be highly cost-effective leading to better capacity to

screen the large numbers of families where cascade screening is indicated. Considering the complexity of FH, it needs to be pointed out that the disease can only be confirmed but never excluded through genetic diagnosis and that exploration, treatment and follow-up based on lipid phenotype should not be dismissed in families where causative mechanisms have not yet been identified. An important continuation of our work will now be to focus on identifying novel pathogenic mechanisms for dominant hypercholesterolaemia by family studies in our 'mutation-negative' patients. This will be done by whole-exome sequencing in these families, which will also permit a better characterization of how other known lipid-modulating genes may influence phenotypic expression.

In conclusion, we propose that a first step of genotyping using an analysis platform based on knowledge of the regional mutation spectrum is a rapid and cost-effective tool for the important diagnosis of genetic mechanisms in FH. The application of such strategy should substantially improve our possibility for early and life-saving prevention of cardiovascular disease in FH.

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Author contribution

Peter Benedek: Conceptualization (supporting); Data curation (lead); Investigation (lead); Project administration (lead); Writing-original draft (lead); Writing-review & editing (equal). **Hong Jiao:** Data curation (equal); Methodology (equal); Project administration (equal); Visualization (equal); Writing-original draft (supporting); Writing-review & editing (equal). **Kristina Duvefelt:** Data curation (equal); Methodology (equal); Project administration (equal); Writing-review & editing (equal). **Tiina Skoog:** Data curation (supporting); Project administration (supporting); Validation (supporting); Writing-review & editing (supporting). **Malin Linde:** Data curation (supporting); Investigation (equal); Project administration (equal). **Päivi Kiviluoma:** Data curation (supporting); Investigation (equal); Project administration (equal). **Juha Kere:** Conceptualization (equal); Methodology (supporting); Supervision (equal); Writing-review & editing (equal). **Mats Eriksson:** Conceptualization (equal); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (equal). **Bo Angelin:** Conceptualization (lead); Funding acquisition (lead); Supervision (lead); Writing-original draft (equal); Writing-review & editing (lead).

Conflict of interests

PB has received grants from Vinnova and personal fees from Amgen. ME has received grants from Vinnova; advisory board fees from Amgen, Sanofi and Akcsea; and consultant fees from Novartis. KD has received grants from Vinnova. BA has served as consultant to and received grants from Albireo and AstraZeneca (ICMC) and a competitive grant from Amgen. HJ, TS, PK ML and JK declare no competing interests.

References

1 Nordestgaard BG, Chapman MJ, Ginsberg HN, Masana L, Descamps OS, Wiklund O, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur Heart J.* 2013;**34:**3478–90.
2 Berberich AJ, Hegele RA. The complex molecular genetics of familial hypercholesterolaemia. *Nat Rev Cardiol.* 2019;**16:**9–20.
3 Watts GF, Gidding SS, Mata P, Pang J, Sullivan DR, Yamashita S, et al. Familial hypercholesterolaemia: evolving knowledge for designing adaptive models of care. *Nat Rev Cardiol.* 2020;**17:**360–77.
4 Wilemon KA, Patel J, Aguilar-Salinas C, Ahmed CD, Alkhnifsawi M, Almahmeed W, et al. Reducing the clinical and public health burden of familial hypercholesterolemia: A global call to action. *JAMA Cardiol.* 2020;**5:**217–229.
5 Benedek P, Eriksson M, Duvefelt K, Freyschuss A, Frick M, Lundman P, et al. Genetic testing for familial hypercholesterolemia among survivors of acute coronary syndrome. *J Intern Med.* 2018;**284:**674–84.

6 Amor-Salamanca A, Castillo S, Gonzalez-Vioque E, Dominguez F, Quintana L, Lluís-Ganella C, et al. Genetically confirmed familial hypercholesterolemia in patients with acute coronary syndrome. *J Am Coll Cardiol*. 2017;**70:**1732–40.

7 Collaboration EASFHS, Vallejo-Vaz AJ, De Marco M, Akram A, Freiberger T, Hovingh GK, et al. Overview of the current status of familial hypercholesterolaemia care in over 60 countries - The EAS Familial Hypercholesterolaemia Studies Collaboration (FHSC). *Atherosclerosis*. 2018;**277:**234–55.

8 Wiegman A, Gidding SS, Watts GF, Chapman MJ, Ginsberg HN, Cuchel M, et al. Familial hypercholesterolaemia in children and adolescents: gaining decades of life by optimizing detection and treatment. *Eur Heart J*. 2015;**36:**2425–37.

9 Luirink IK, Wiegman A, Kusters DM, Hof MH, Groothoff JW, de Groot E, et al. 20-Year follow-up of statins in children with familial hypercholesterolemia. *N Engl J Med*. 2019;**381:**1547–56.

10 Leigh S, Futema M, Whittall R, Taylor-Beadling A, Williams M, den Dunnen JT, et al. The UCL low-density lipoprotein receptor gene variant database: pathogenicity update. *J Med Genet*. 2017;**54:**217–23.

11 Brusgaard K, Jordan P, Hansen H, Hansen AB, Horder M. Molecular genetic analysis of 1053 Danish individuals with clinical signs of familial hypercholesterolemia. *Clin Genet*. 2006;**69:**277–83.

12 Leren TP, Tonstad S, Gundersen KE, Bakken KS, Rodningen OK, Sundvold H, et al. Molecular genetics of familial hypercholesterolaemia in Norway. *J Intern Med*. 1997;**241:**185–94.

13 Vuorio AF, Aalto-Setala K, Koivisto UM, Turtola H, Nissen H, Kovanen PT, et al. Familial hypercholesterolaemia in Finland: common, rare and mild mutations of the LDL receptor and their clinical consequences, Finnish FH-group. *Ann Med*. 2001;**33:**410–21.

14 Lind S, Eriksson M, Rystedt E, Wiklund O, Angelin B, Eggertsen G. Low frequency of the common Norwegian and Finnish LDL-receptor mutations in Swedish patients with familial hypercholesterolaemia. *J Intern Med*. 1998;**244:**19–25.

15 Lind S, Rystedt E, Eriksson M, Wiklund O, Angelin B, Eggertsen G. Genetic characterization of Swedish patients with familial hypercholesterolemia: a heterogeneous pattern of mutations in the LDL receptor gene. *Atherosclerosis*. 2002;**163:**399–407.

16 Ekstrom U, Abrahamson M, Sveger T, Lombardi P, Nilsson-Ehle P. An efficient screening procedure detecting six novel mutations in the LDL receptor gene in Swedish children with hypercholesterolemia. *Hum Genet*. 1995;**96:**147–50.

17 Jurinke C, van den Boom D, Cantor CR, Koster H. Automated genotyping using the DNA MassArray technology. *Methods Mol Biol*. 2002;**187:**179–92.

18 Lahtinen AM, Havulinna AS, Jula A, Salomaa V, Kontula K. Prevalence and clinical correlates of familial hypercholesterolemia founder mutations in the general population. *Atherosclerosis*. 2015;**238:**64–9.

19 Loaiza N, Hartgers ML, Reeskamp LF, Balder J-W, Rimbert A, Bazioti V, et al. Taking one step back in familial hypercholesterolemia: STAP1 does not alter plasma LDL (Low-Density Lipoprotein) cholesterol in mice and humans. *Arterioscler Thromb Vasc Biol*. 2020;**40:**973–85.

20 Besseling J, Reitsma JB, Gaudet D, Brisson D, Kastelein JJ, Hovingh GK, et al. Selection of individuals for genetic testing for familial hypercholesterolaemia: development and external validation of a prediction model for the presence of a mutation causing familial hypercholesterolaemia. *Eur Heart J*. 2017;**38:**565–73.

21 Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, 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.

22 Miserez AR, Keller U. Differences in the phenotypic characteristics of subjects with familial defective apolipoprotein B-100 and familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol*. 1995;**15:**1719–29.

23 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.

24 Salmela E, Lappalainen T, Liu J, Sistonen P, Andersen PM, Schreiber S, et al. Swedish population substructure revealed by genome-wide single nucleotide polymorphism data. *PLoS One*. 2011;**6:**e16747.

25 Lappalainen T, Hannelius U, Salmela E, von Döbeln U, Lindgren CM, Huoponen K, et al. Population structure in contemporary Sweden–a Y-chromosomal and mitochondrial DNA analysis. *Ann Hum Genet*. 2009;**73:**61–73.

26 Salmela E, Lappalainen T, Fransson I, Andersen PM, Dahlman-Wright K, Fiebig A, et al. Genome-wide analysis of single nucleotide polymorphisms uncovers population structure in Northern Europe. *PLoS One*. 2008;**3:**e3519.

27 Ajufo E, Cuchel M. Improving the yield of genetic testing in familial hypercholesterolaemia. *Eur Heart J*. 2017;**38:**574–6.

28 Kerr M, Pears R, Miedzybrodzka Z, Haralambos K, Cather M, Watson M, et al. Cost effectiveness of cascade testing for familial hypercholesterolaemia, based on data from familial hypercholesterolaemia services in the UK. *Eur Heart J*. 2017;**38:**1832–9.

29 Cuchel M, Bruckert E, Ginsberg HN, Raal FJ, Santos RD, Hegele RA, 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.

30 Brandts J, Dharmayat KI, Ray KK, Vallejo-Vaz AJ. Familial hypercholesterolemia: is it time to separate monogenic from polygenic familial hypercholesterolemia? *Curr Opin Lipidol*. 2020;**31:**111–8.

Correspondence: Peter Benedek, Department of Medicine C2:94, Karolinska Institutet at Karolinska University Hospital, S-141 86 Stockholm, Sweden.
(e-mail: peter.benedek@ki.se).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Material■