ORIGINAL ARTICLE

Targeted sequencing of a gene panel in patients with familial hypercholesterolemia from Southern Poland

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KEY WORDS

familial hypercholesterolemia, FH genetics, next generation sequencing

ABSTRACT

INTRODUCTION Familial hypercholesterolemia (FH) is an autosomal dominant monogenic lipid metabolism disorder characterized by a significantly elevated level of low-density lipoprotein (LDL) cholesterol and leading to premature ischemic heart disease. FH is caused by mutations in the *LDLR*, *APOB*, and *PCSK9* genes; however, these mutations account for only about 40% of FH cases. In order to obtain a genetic diagnosis of FH, sequencing of other genes involved in the lipid metabolism might be useful.

OBJECTIVES This study aimed to describe genetic variants in genes associated with FH in a group of patients from the Małopolska province in Southern Poland, using the targeted next generation sequencing (NGS) technology.

PATIENTS AND METHODS The study involved 90 unrelated adults (age range, 18–70 years) with FH diagnosed clinically according to the Simon Broome Register criteria. A custom-designed capture assay and the Illumina MiSeq platform were used. The panel included exons and exon/intron boundaries of known FH-causing genes: *LDLR*, *APOB*, and *PCSK9*, as well as genes previously associated with high cholesterol levels: *APOE*, *ABCG5*, *ABCG8*, *LPL*, *NPC1*, *LDLRAP1*, *LIPC*, *STAP1*, and *CELSR2*. Genetic variants were classified based on in silico predictions and ClinVar reports.

RESULTS We detected 4 patients with variants in the *LDLR* and *APOB* genes that had not been previously linked to FH in ClinVar. We also found *APOB* mutations outside the common LDL receptor–binding region, in exons 26 and 29. Interestingly, we observed a high frequency of pathogenic variants in exon 4 of the *APOE* gene: rs7412, probably damaging (4 patients) and rs429358, benign (16 patients).

CONCLUSIONS NGS is a useful and reliable method to detect new variants in genes related to FH. In addition, the results enable the detection of FH phenocopies and introduction of appropriate treatment.

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INTRODUCTION Familial hypercholesterolemia (FH) is a common monogenic disorder characterized by elevated low-density lipoprotein cholesterol (LDL-C) levels and an increased risk of premature cardiovascular disease.[1] To date, 3 different genes have been causally linked to autosomal dominant FH: the low-density lipoprotein receptor (*LDLR*) gene, the apolipoprotein B-100 (*APOB*) gene, and the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene. To date, more than 1700 different causal mutations in the *LDLR* gene have been identified, so screening for these mutations to establish a molecular diagnosis represents a methodological challenge. Various mutations in the *APOB* gene, especially mutations in the LDLR-binding domain of *APOB*, are also related to FH. Mutations in other regions of the *APOB* gene are much rarer and usually difficult to screen for using DNA sequencing.[2,3] There is evidence that FH patients with monogenic mutations, even

WHAT'S NEW?

In this study, we aimed to describe the genetic background of familial hypercholesterolemia, a common monogenic disorder leading to premature cardiovascular disease, in a population of patients from the Małopolska province in Poland, using the next generation sequencing (NGS) method. We found pathogenic variants and polymorphisms in the *LDLR* and *APOB* genes, and described variants in other genes associated with low-density lipoprotein cholesterol levels, such as *APOE*, *ABCG5*, *ABCG8*, and *LDLRAP1*. We conclude that NGS is an efficient and reliable method to screen the genetic background of lipid disorders and diagnose the patients.

if treated, have a significantly higher mortality rate than those with polygenic hypercholesterolemia.[4] In light of these findings, early diagnosis and treatment of patients with FH are necessary to prevent cardiovascular complications. Therefore, comprehensive screening for known as well as rare or unknown mutations is required. Moreover, the detection rate of pathogenic mutations in the *LDLR*, *APOB*, and *PCSK9* genes ranges from 40% to 60%, so there is a need for identifying new genes responsible for FH.[5]

The increasing availability and decreasing cost of next generation sequencing (NGS) make it a suitable tool for diagnosing monogenic dyslipidemias. NGS is also useful in identifying novel genes in LDL metabolism that possibly contribute to the FH phenotype, which may open new possibilities in the diagnosis and treatment of patients with FH.[5-8]

In this study, we employed NGS to describe genetic variants affecting cholesterol levels associated with FH and variants in other genes affecting LDL-C levels in a group of patients from the Małopolska province in Poland.

PATIENTS AND METHODS The study comprised patients with a clinical diagnosis of FH based on the Simon Broome Register.[9] According to these criteria, definite FH is defined as a total cholesterol (TC) level greater than 6.7 mmol/l or LDL-C level greater than 4 mmol/l in a child younger than 16 years, or a TC level greater than 7.5 mmol/l or LDL-C level greater than 4.9 mmol/l in an adult (levels either before treatment or highest on treatment), plus tendon xanthomas in the patient or a first-degree (parent, sibling, child) or second-degree relative (grandparent, uncle, aunt). Alternatively, it can be defined as the presence of DNA-based evidence of a *LDLR* mutation, familial defective *APOB*-100, or a *PCSK9* mutation.

Possible FH is defined as a TC level greater than 6.7 mmol/l or LDL-C level greater than 4 mmol/l in a child younger than 16 years, or a TC level greater than 7.5 mmol/l or LDL-C level greater than 4.9 mmol/l in an adult (levels either before treatment or highest on treatment), and meeting at least 1 of the following criteria: family history of myocardial infarction (at the age <50 years in a second-degree relative or <60 years in a first-degree relative) or family history of hypercholesterolemia (>7.5 mmol/l in an adult first- or second-degree relative or >6.7 mmol/l in a child or sibling aged <16 years).

Patients with either a definite or a probable diagnosis of FH were included in the study. They were recruited from among the inhabitants of the Małopolska province in Poland who had been referred to an outpatient lipid clinic at the University Hospital of the Jagiellonian University Medical College due to severe hypercholesterolemia. We also included the data of 2 hypercholesterolemic children, members of family 418 (Supplementary material, *Table S1*), treated at the Department of Endocrinology, Institute of Pediatrics of the Jagiellonian University Medical College, who were diagnosed with FH according to the criteria proposed by Kwiterowich.[10] Secondary causes of hyperlipidemia were excluded in all patients. The participants filled out a standardized questionnaire concerning family history, past medical history, smoking habits, and treatment. All patients gave their written informed consent prior to the inclusion, and the study was approved by the Jagiellonian University Bioethical Committee (KBET/34/B/2012).

Blood samples were collected after an overnight fast. In all patients, the fasting serum lipid levels were analyzed by enzymatic methods. The apolipoprotein (apo) A1 level was determined by immunoturbidimetry (APTEC Diagnostics nv, Sint-Niklaas, Belgium), and the level of apo B-100, by sandwich enzyme-linked immunosorbent assay kits (Human apo B-100: SEA603Hu Cloud-Clone Corp., Houston, Massachusetts, United States), according to the manufacturers' instructions. Serum lipoprotein (Lp) (a) concentration was determined by immunoturbidimetry.

DNA sequencing The DNA was extracted from whole blood samples and collected into ethylenediaminetetraacetic acid tubes with the Maxwell 16 Blood DNA Purification Kit on a Maxwell device (Promega, Madison, Wisconsin, United States). The isolated DNA quantity was measured on the Quantus fluorometer (Promega). To detect mutations, we used a SureSelect custom-designed sequencing panel (Agilent, Santa Clara, California, United States) consisting of probes targeting exons of genes causative for monogenic FH: *LDLR, APOB*, and *PCSK9*, as well as genes with a recently proved causality or having smaller, quantitative effects on FH: apolipoprotein E (*APOE*), ATP-binding cassette subfamily G member 5 (*ABCG5*), ATP-binding cassette subfamily G member 8 (*ABCG8*), lipoprotein lipase (*LPL*), NPC intracellular cholesterol transporter 1 (*NPC1*), low-density lipoprotein receptor adaptor protein 1 (*LDLRAP1*), lipase C, hepatic type (*LIPC*), cadherin EGF LAG seven-pass G-type receptor 2 (*CELSR2*), as well as the signal transducing adaptor protein 1 gene (*STAP1*), whose causality in FH has been recently negated.[11] Library preparation was performed using SureSelect Target Enrichment (Agilent) reagents, according to the manufacturer's

protocol. Pooled DNA libraries were prepared using pair-end sequencing (75 bp) with v3 reagents on the MiSeq platform (Illumina, San Diego, California, United States), following the manufacturer's instructions.

Sequencing data analysis Raw reads were processed with the Illumina software via generating demultiplexed fast files with base calls and corresponding base-call quality scores. These files were then processed through a custom pipeline that had been described in detail in our previous publication.[12] Briefly, the quality of the reads was assessed with FastQC v.11.5 (Babraham Bioinformatics, Cambridge, United Kingdom). Raw reads were aligned to the human reference genome GRCh37 (hg19) using the BWA-MEM algorithm v.0.7.5. Realignment across indels and base quality recalibration were performed with a Genome Analysis Toolkit v.3.7 (GATK). Duplicated reads were filtered out with a SAMtools v.0.1.19 (https://www.htslib.org/).

Variants were called using a GATK Haplotypecaller and filtered out using recommended hard filtering parameters based on GATK Best Practices. The filtered variants were annotated with SnpEff and VEP (https://pcingola.github.io/SnpEff), then prioritized according to the GEnome MINIng (GEMINI, version 0.18.3) impact severity classification. Subsequently, the variants were filtered against the frequencies in known databases (minor allele frequency [MAF] <1%): 1000 Genomes, ESP, and ExAC. Pathogenicity prediction scores, such as CADD, Fitcons, Sorting Intolerant From Tolerant (SIFT2), Polyphen 2, MutationTaster2, Protein Variation Effect Analyzer (PROVEAN), and FATHMM-XF,[13-15] as well as clinical significance (ClinVar) were used to evaluate pathogenicity of the detected variants.[16-18] The variants identified as pathogenic according to at least 3 of the 5 metrics described above were considered pathogenic. As a control of NGS reliability, DNA from FH patients with a known mutation in *APOB* (1 person) and *LDLR* (6 persons) were included.

Statistical analysis Statistical analysis included calculations of means and SD for variables with a normal distribution, and medians and interquartile ranges for those with a nonnormal distribution. The *t* test and the Mann–Whitney test were used for comparisons between sexes. Qualitative characteristics were presented as numbers and percentages. The data of pediatric patients are not included in the table presenting the characteristics of the study group (**TABLE 1**). The calculations were performed using the statistical package STATISTICA v. 13.3 (StatSoft, Kraków, Poland). *P* values lower than 0.05 were considered significant.

RESULTS We examined a total of 90 unrelated patients, including 35 men (38.9%) and 55 woment (61.1%). Among them, 12 patients (13.5%) had coronary artery disease and 7 (7.9%) had a history of myocardial infarction. **TABLE 1** presents the characteristics of the study population according to sex. The mean (SD) age of the patients was 43.8 (14.1) years, and the mean (SD) LDL-C level was 4.95 (2.1) mmol/l. Overall, 60.1% of the patients were treated with statins.

TABLE 2 presents variants in the *LDLR* and *APOB* genes found in the examined group. All the known control *LDLR* and *APOB* gene mutations were identified in the NGS analysis, thus confirming the reliability of this procedure in our center. We found 13 known pathogenic variants and 3 polymorphisms in the *LDLR* gene, as well as 3 variants that were probably disease-causing. The most common mutation was p.Cys34Gly in exon 2, found in 5 patients. Three pathogenic variants were observed in exon 4, and 3 in exon 9 of the *LDLR* gene. One variant of uncertain significance (VUS) / pathogenic was found in exon 10. **TABLE 2** also presents variants that were not previously linked to FH in ClinVar, which were detected in the *LDLR* gene in 3 patients and in the *APOB* gene in 1 patient. Lipid characteristics of the single patient harboring the new variant of *APOB* and his family are presented in Supplementary material, *Table S2*. The mutation c:119dupT (pSer41fs) in exon 2 is predicted to result in a frame shift and change of serine to leucine in the 11th position of the LDLR protein.

Interestingly, we found the same mutation (c.100T>G [p.Cys34Gly]) in exon 2 of the *LDLR* gene in 2 patients, and homozygosity for this mutation in their children (a son and a daughter). The son's lipid values on irregular drug treatment were as follows: TC, 11.59 mmol/l; non–high-density lipoprotein cholesterol (HDL-C), 10.83 mmol/l; HDL-C, 0.76 mmol/l; LDL-C, 10.41 mmol/l; and triglycerides, 0.92 mmol/l. High cholesterol levels were detected at the age of 4 years due to knee xanthomas, which disappeared after hypolipidemic treatment. Physical examination revealed corneal arcus (Supplementary material, *Figure S1*) and Achilles tendon xanthoma, whereas echocardiography showed thickening of the right leaflet of the aortic valve. He and his father had also increased Lp(a) levels (1.04 g/l). Lipid values of the father during treatment with atorvastatin 40 mg and ezetimibe 10 mg were as follows: TC, 6.6 mmol/l; LDL-C, 5.71 mmol/l; HDL-C, 0.77 mmol/l; triglycerides, 0.77 mmol/l; Lp(a), 0.97g/l; apo B, 1.64 g/l; and apo A1, 0.89 g/l. The genealogical tree of family 418 and lipid levels of individual family members are presented in Supplementary material, *Figure S2* and *Table S1*, respectively.

In the *APOB* gene, we observed 3 pathogenic variants, 14 VUSs, and 3 polymorphisms. We also found 1 variant in exon 26 not previously annotated in ClinVar that was probably disease-causing. The most common pathogenic mutation in the *APOB* gene, p.Arg3527Gln (rs5742904) in exon 26, was found in 9 patients. Four variants in the *APOB* gene were found, including 3 in exon 26 (p.Arg3527Trp, p.Leu3377Leu, and p.Asp2213del)

TABLE 1 Characteristics of the study group according to sex[a]

Parameter	Total (n = 90)		Men (n = 35)		Women (n = 55)		*P* value
	n	Mean (SD) or median (IQR)	n	Mean (SD) or median (IQR)	n	Mean (SD) or median (IQR)	
Age, y	87	43.8 (14.1)	34	39.2 (12.03)	53	46.7 (14.66)	0.01
TC, mmol/l	88	7.22 (2.07)	33	6.88 (2.32)	55	7.42 (1.9)	0.23
HDL-C, mmol/l	88	1.57 (0.46)	33	1.38 (0.44)	55	1.69 (0.43)	0.002
LDL-C, mmol/l	88	4.95 (2.01)	33	4.94 (2.34)	55	4.95 (1.81)	0.99
TG, mmol/l	87	1.24 (0.94–2.04)	32	1.35 (1.01–2.1)	55	1.19 (0.91–1.95)	0.38
Apo A1, mg/dl	60	143.64 (41.69)	24	141.55 (39.22)	36	145.03 (43.76)	0.75
Apo B, mg/dl	60	104.3 (19.93)	24	103.65 (20.62)	36	104.72 (19.74)	0.84
Lp(a), g/l	60	0.05 (0.03–0.16)	24	0.03 (0.03–0.08)	36	0.08 (0.03–0.22)	0.01
Glucose, mmol/l	78	5.01 (4.5–5.4)	29	5.02 (4.7–5.52)	49	5 (4.5–5.37)	0.63
Creatinine, mg/l	82	65 (58–75.5)	33	74 (62–78)	49	63 (58–68)	0.02
BMI, kg/m^2	89	26.18 (4.48)	35	26.11 (3.69)	54	26.23 (4.97)	0.89
Waist circumference, cm	88	86.98 (12.56)	35	90.63 (11.76)	53	84.57 (12.6)	0.02
WHR	86	0.87 (0.09)	35	0.91 (0.07)	51	0.84 (0.08)	<0.001
ALT, U/l	80	26 (19–41)	30	43.5 (26–53)	50	23 (16–28)	<0.001
GGTP, U/l	65	21 (14.5–39)	24	38.55 (26–68)	41	18 (12–22.6)	<0.001
SBP, mm Hg	87	132.7 (17.38)	34	136 (15.77)	53	130.5 (18.17)	0.15
DBP, mm Hg	87	83.2 (12.19)	34	83 (12.21)	53	83.3 (12.3)	0.9

Variables with a nonnormal distribution are presented as median with interquartile range (IQR) and were compared using the Mann–Whitney test. Variables with a normal distribution are presented as mean (SD) and were compared using the *t* test.

a Data of pediatric patients analyzed in the study (n = 2) are not presented in the Table.

SI conversion factors: to convert apo A1 and apo B to g/l, multiply by 0.01; creatinine to µmol/l, by 88.4, ALT and GGTP to µkat/l, by 0.0167.

Abbreviations: ALT, alanine aminotransferase; apo, apolipoprotein; BMI, body mass index; DPB, diastolic blood pressure; GGTP, γ-glutamyltranspeptidase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WHR, waist-to--hip ratio

in the LDLR-binding region, and 1 in exon 14 (c.2068-4T>A). We also observed other *APOB* gene variants, outside of exon 14 and the LDLR--binding region, in exons 1, 5, 12, 20, and 25. In exon 12 of the *APOB* gene, a VUS / polymorphism (p.Arg532Trp) was found. A c64_66delCTG (p.Leu22del) variant in exon 1, probably not pathogenic, was detected in 1 patient. A p.Glu2566Lys polymorphism in exon 26, probably not pathogenic, was found in 10 patients.

TABLE 3 presents selected variants in other genes associated with LDL-C levels. Interestingly, we observed a high frequency of pathogenic variants of the *APOE* gene, potentially influencing LDL-C levels. The *APOE* variant rs7412 (p.Arg176Cys// c.526C>T; Cgc/Tgc), probably pathogenic according to in silico prediction, was present in 4 patients. The most common *APOE* gene variant, p.Cys130Arg/c.388T>C (rs429358; Tgc/Cgc), probably benign, was observed in 16 patients and was also located in exon 4. This single-nucleotide polymorphism (SNP) affects the amino acid at position 130 of the resulting protein (TABLE 3).

Three of our patients with a FH phenotype carried no variants in the FH genes, but instead were carriers of rare variants of the *ABCG5* gene. We also found 2 variants of the *ABCG8* gene (TABLE 3). The 3 *ABCG5* variants, namely, pArg446* in exon 10, p.Arg198Gln in exon 5, and p.Ala98Gly in exon 3, are likely to be pathogenic. Variants in gene *ABCG8*, p.Trp361* in exon 7 and p.Leu572Pro in exon 11, are also potentially pathogenic.

Interestingly, in 2 patients, we found a likely pathogenic missense variant (pathogenic score of 0.933 according to MutationTaster2) of the *STAP1* gene: c.120+6T>C (TABLE 3). This variant was located in exon 1, and affected a highly-conserved amino acid. The LDL-C concentration in this *STAP1* variant carrier was 4.5 mmol/l. We also found a missense variant in exon 6 of the *STAP1* gene, p.Asp207Asn (c.619G>A), probably not pathogenic. This variant was predicted to possibly affect the protein function.

Of note, in the majority of patients with mutations in the *LDLR* or *APOB* gene, variants of other genes affecting the LDL-C level, such as *ABCG5*, *ABCG8*, *LPL*, *LRP1*, and *APOE* were also detected, which might influence the response to treatment.

DISCUSSION NGS is replacing older forms of genetic diagnosis in clinical practice. In the present study, targeting the genes responsible for FH was

TABLE 2 Pathogenic, likely pathogenic, and variants of unknown significance identified in the *LDLR*, *APOB*, and *PCSK9* genes in the cohort of 90 patients with familial hypercholesterolemia from Southern Poland

Gene	Mutation	Amino acid change	Exon	Patients with allele, n	dbSNP	ClinVar
LDLR	NM_000527.4:c.58G>A	p.Gly20Arg	1	1	rs147509697	LB, VUS
LDLR	NM_000527.4:c.100T>G	p.Cys34Gly	2	5	rs879254405	LP, P
LDLR	NM_000527.4:c.313+1G>T	–	3	1	rs112029328	LP
LDLR	NM_000527.4:c.442T>C	p.Cys148Arg	4	1	rs879254525	LP, P
LDLR	NM_000527.4:c.530C>T	p.Ser177Leu	4	1	rs121908026	LP, P
LDLR	NM_000527.4:c.666C>A	p.Cys222*	4	1	rs756613387	P
LDLR	NM_000527.4:c.782G>T	p.Cys261Phe	5	1	rs121908040	LP, P
LDLR	NM_000527.4:c.798T>A	p.Asp266Glu	5	1	rs139043155	LB, LP, P
LDLR	NM_000527.4:c.986G>T	p.Cys329Phe	7	2	rs761954844	LP
LDLR	NM_000527.4:c.1061–8T>C	–	7	1	rs72658861	B, LB, LP, VUS
LDLR	NM_000527.4:c.1222G>A	p.Glu408Lys	9	1	rs137943601	LP, P
LDLR	NM_000527.4:c.1223A>T	p.Glu408Val	9	1	rs879254838	LP
LDLR	NM_000527.4:c.1328G>A	p.Trp443*	9	1	rs879254866	P
LDLR	NM_000527.4:c.1449G>T	p.Trp483Cys	10	1	rs879254907	P, VUS
LDLR	NM_000527.4:c.1705+1G>A	–	11	1	rs875989926	LP, P
LDLR	NM_000527.4:c.1775G>A	p.Gly592Glu	12	2	rs137929307	LP, P
LDLR	NM_000527.4:c.1862C>G	p.Thr621Arg	13	1	rs879255058	LP, P
LDLR	NM_000527.4:c.2390–16G>A	–	16	1	rs183496025	LB
LDLR	NM_000527.4:c.119dupT	p.Ser41fs	2	1	NA	DC (P >0.99)[a]
LDLR	NM_000527.4:c.787G>A	p.Asp263Asn	5	1	rs750900506	DC (P = 0.99)[a]
LDLR	NM_000527.4:c.1486G>A	p.Gly496Ser	10	1	NA	DC (P = 0.73)[a]
PCSK9	NM_174936.3:c.60_65dupGCTGCT	p.Leu21_Leu22dup	1	1	rs35574083	B
APOB	NM_000384.2:c.12382G>A	p.Val4128Met	29	1	rs1801703	LB, VUS
APOB	NM_000384.2:c.11833A>G	p.Thr3945Ala	27	1	rs1801698	VUS
APOB	NM_000384.2:c.10708C>T	p.His3570Tyr	26	1	rs201736972	P
APOB	NM_000384.2:c.10580G>A	p.Arg3527Gln	26	9	rs5742904	LP, P
APOB	NM_000384.2:c.10579C>T	p.Arg3527Trp	26	1	rs144467873	LP, P, VUS
APOB	NM_000384.2:c.10131G>A	p.Leu3377Leu	26	1	rs1799812	VUS
APOB	NM_000384.2:c.8462C>T	p.Pro2821Leu	26	2	rs72653095	LB, VUS
APOB	NM_000384.2:c.8353A>C	p.Asn2785His	26	1	rs2163204	B, LB, VUS
APOB	NM_000384.2:c.7696G>A	p.Glu2566Lys	26	10	rs1801696	LB, VUS
APOB	NM_000384.2:c.7615G>A	p.Val2539Ile	26	1	rs148170480	VUS
APOB	NM_000384.2:c.6639_6641delTGA	p.Asp2213del	26	1	rs541497967	LB, VUS
APOB	NM_000384.2:c.3122–6G>A	–	20	1	rs72653071	VUS
APOB	NM_000384.2:c.2068–4T>A	–	14	1	rs41291161	LB
APOB	NM_000384.2:c.1594C>T	p.Arg532Trp	12	1	rs13306194	B, LB, VUS
APOB	NM_000384.2:c.538–9C>T	–	5	1	rs1800478	B, LB
APOB	NM_000384.2:c.13181T>C	p.Val4394Ala	29	1	rs12720843	VUS
APOB	NM_000384.2:c.11087T>C	p.Ile3696Thr	26	1	rs370096275	VUS
APOB	NM_000384.2:c.10032A>C	p.Lys3344Asn	26	1	rs757857092	VUS
APOB	NM_000384.2:c.7640T>C	p.Val2547Ala	26	1	NA	Polymorphism (P = 0.99)[a]
APOB	NM_000384.2:c.3851G>A	p.Arg1284Gln	25	1	rs372154910	VUS
APOB	NM_000384.2:c.64_66delCTG	p.Leu22del	1	1	rs773844839	B

a Variants that were not previously linked to familiar hypercholesterolemia in ClinVar, with pathogenicity probability calculated by MutationTaster2

Abbreviations: *APOB*, apolipoprotein B-100 gene; B, benign; dbSNP, database of single-nucleotide polymorphism; DC, disease-causing; LB, likely benign; *LDLR*, low-density lipoprotein receptor gene; LP, likely pathogenic; NA, not available; P, pathogenic; *PCSK9*, proprotein convertase subtilisin/kexin type 9 gene; VUS, variant of unknown significance

TABLE 3 Selected variants in other genes related to lipid levels

Gene	Mutation	Amino acid change	Exon	Patients with allele, n	MutationTaster2	Polyphen-2	SIFT	dbSNP
LDLRAP1	NM_015627.2:c.672C>T	p.Ser224Ser	7	1	DC	–	T	rs41291054
STAP1	NM_012108.2:c.120+6T>C	–	1	2	DC	–	–	rs187909999
STAP1	NM_012108.2:c.619G>A	p.Asp207Asn	6	2	DC	B	T	rs146545610
ABCG5	NM_022436.2:c.1336C>T	p.Arg446*	10	1	DC	–	–	rs199689137
ABCG5	NM_022436.2:c.593G>A	p.Arg198Gln	5	2	DC	ProD	D	rs141828689
ABCG5	NM_022436.2:c.293C>G	p.Ala98Gly	3	2	DC	PosD	D	rs145164937
ABCG8	NM_022437.2:c.1083G>A	p.Trp361*	7	1	DC	–	–	rs137852987
ABCG8	NM_022437.2:c.1715T>C	p.Leu572Pro	11	1	DC	ProD	D	rs769576789
APOE	NM_000041.2:c.388T>C	p.Cys130Arg	4	16	–	ProD	D	rs429358
APOE	NM_000041.2:c.526C>T	p.Arg176Cys	4	4	–	B	T	rs7412

Abbreviations: *ABCG5*, ATP-binding cassette subfamily G member 5 gene; *ABCG8*, ATP-binding cassette subfamily G member 8 gene; *APOE*, apolipoprotein E gene; D, damaging; *LDLRAP1*, low-density lipoprotein receptor adaptor protein 1 gene; PosD, possibly damaging; ProD, probably damaging; SIFT, Sorting Intolerant from Tolerant; *STAP1*, signal transducing adaptor protein 1 gene; T, tolerated; others, see **TABLE 2**

carried out by designing probe panels targeting the sequence of genes of interest, such as *LDLR*, *APOB*, and *PCSK9*, as well as monogenic mutations in other genes related to high LDL-C levels, such as *APOE*, sterol regulatory element binding transcription factor 2 (*SREBP2*), and lipase A, lysosomal acid type (*LIPA*).[19]

In accordance with our previous report,[20] we found a great heterogeneity of mutations in the *LDLR* gene. In the present study population, most mutations in the *LDLR* gene were located in exon 4, similarly to what had been observed in the Czech population.[21] In our previous cohort from the Małopolska province, the majority of mutations were located in exon 2.[20] The most common mutation in the *LDLR* gene in our patients was p.Gly592Glu, which was also most frequently found in another Polish study[22] and in the Slovak population.[23]

In our study, we confirmed that the most common mutation responsible for FH was the mutation p.Arg3527Gln in the *APOB* gene. It was observed in 10% of the examined group, which is in line with our previous report.[20] In other publications analyzing the Polish population this mutation was observed in about 4% of the examined patients,[21,24] and the frequency was similar in other Slavic populations[25] as well as other European countries.[26-29] We did not find the Thr3492Ile variant of the *APOB* gene in any of our patients.[24] However, we identified *APOB* gene variants that had not been previously linked to FH in ClinVar. Routine analysis tests for the presence of mutations in the LDLR-binding region of the *APOB* gene (amino acids 3441–3615), while NGS enabled the analysis of the whole *APOB* gene, thus allowing us to describe new *APOB* variants in FH patients.

We observed *APOB* variants in exons 29 (2 persons), 25, 27, 20, 14, 12, 5, and 1. The *APOB* gene mutations located outside the LDLR-binding region had been described previously.[30-32] Alves et al[30] used a flow cytometry assay and fluorescently-labelled LDL from individuals with *APOB* gene mutations in exons 22 and 29 and showed that carriers of p.Arg1164Thr (exon 22) and p.Gln4494del (exon 29) were marked by a 40% decrease in internalization of LDL in lymphocytes and HepG2 cells, similarly to persons with an *APOB*-3527 mutation.

No FH-causing gain-of-function mutations in the *PCSK9* gene were found in the group of patients analyzed in the present study.

Mutations in the *STAP1* gene were described by Fouchier et al[33] as being associated with FH. However, the causative role of *STAP1* gene mutations in the context of FH is still under debate and, according to some authors, these mutations should not be considered disease-causing until more data appear.[34-36] In a study by Loaiza et al,[35] *STAP1* gene variants did not alter plasma LDL-C levels. We found a variant in the *STAP1* gene that was pathogenic according to in silico prediction, and it was associated with high LDL-C levels in the affected individuals. However, a family segregation study and functional tests are required to confirm its pathogenicity.

In our cohort of patients, we observed a relatively high frequency of *APOE* gene variants. *APOE* variants rs429358 and rs7412 are included by the Global Lipid Genetics Consortium among 12 SNP alleles showing a LDL-C–rising effect.[37] A benign *APOE* gene variant rs429358 was found in 16 patients, which corresponded to almost 20% of our group. We did not find the *APOE* Leu167del mutation, which had been identified as disease-causing in the Italian and French populations.[38-41]

Interestingly, NGS data showed that a few patients with FH-like phenotypes carried mutations in the *ABCG5*, *APOE*, and *LIPA* genes, which are the causative genes for classical sitosterolemia, dysbetalipoproteinemia, and lysosomal acid lipase deficiency, respectively.[41-44] Tada et al[43] observed variants of the *ABCG5* gene in FH patients without mutations in the genes associated with the FH phenotype; however, in our study, all the patients

with an *ABCG5* mutation carried mutations also in other LDL-C–rising genes, such as *APOE* or *LDLRAP1*.[43] The available literature data suggest that deleterious mutations in the *ABCG5/ABCG8* genes could contribute to worsening of FH or mimicking of the disease.[43,44]

The main limitation of the study is that we did not perform functional tests for the newly detected potentially pathogenic variants. Of note, all the existing algorithms predicting pathogenicity (including those used in the present study) had been reported to have limited sensitivity and / or specificity. Therefore, a lot of effort is still required from researchers to prove that the variants described as pathogenic indeed play a functional role in cholesterol metabolism.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

ARTICLE INFORMATION

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CONTRIBUTION STATEMENT MW-M conceived the concept of the study, performed clinical examinations, analyzed the data, and wrote the manuscript. JT-Ż and MW-M contributed to the design of the research. JT-Ż and MK analyzed the data. EK performed statistical analysis. All authors were involved in data collection and contributed to manuscript revision.

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