Universal Screening for Familial Hypercholesterolemia in Children

Gasper Klančar, BSc,†† Uhr Groselj, MD,† Jernej Kovač, PhD,† Nevenka Bratanič, MD,† Nataša Bratina, MD,† Katarina Trebušak Podkrajsek, PhD,† Tadej Battelino, MD*†

ABSTRACT

BACKGROUND Individuals with familial hypercholesterolemia (FH) who are untreated have up to 100-fold elevated risk for cardiovascular complications compared with those who are unaffected. Data for identification of FH with a universal screening for hypercholesterolemia in children are lacking.

OBJECTIVES This study sought genetic identification of FH from a cohort of children with elevated serum total cholesterol (TC) concentration, detected in a national universal screening for hypercholesterolemia.

METHODS Slovenian children born between 1989 and 2009 (n = 272) with TC > 6 mmol/l (231.7 mg/dl) or > 5 mmol/l (193.1 mg/dl) plus a family history positive for premature cardiovascular complications, identified in a national universal screening for hypercholesterolemia at 5 years of age were genotyped for variants in LDLR, PCSK9, APOB, and APOE.

RESULTS Of the referred children, 57.0% carried disease-causing variants for FH: 38.6% in LDLR, 18.4% in APOB, and none in PCSK9. Nine novel disease-causing variants were identified, 8 in LDLR, and 1 in APOB. Of the remaining participants, 43.6% carried the APOE E4 isoform. Estimated detection rate of FH in the universal screening program from 2009 to 2013 was 53.6% (95% confidence interval [CI]: 34.5% to 72.8%), peaking in 2013 with an upper estimated detection rate of 96.3%. Variants in LDLR, APOB, or the APOE E4 isoform occurred in 48.6%, 60.0%, and 76.5%, respectively, of patients with a family history negative for cardiovascular complications.

CONCLUSIONS Most participants who were referred from a national database of universal screening results for hypercholesterolemia had genetically confirmed FH. Data for family history may not suffice for reliable identification of patients through selective and cascade screening. (J Am Coll Cardiol 2015;66:1250–7) © 2015 by the American College of Cardiology Foundation.

Individuals with untreated familial hypercholesterolemia (FH) have up to 100-fold increased risk for developing atherosclerosis and cardiovascular disease in early adulthood compared with unaffected individuals (1). Early diagnosis and management reduce this risk (2,3). The prevalence of FH in the general population is estimated to be between 1 in 200 (4,5) and 1 in 500 (4). FH is an autosomal dominant disorder clinically diagnosed by elevated concentration of serum total cholesterol (TC) and/or low-density lipoprotein (LDL) cholesterol, possible family history of premature cardiovascular complications, possible presence of xanthomas and corneal arcus, and/or causative variants in genes implicated in FH (4,6). Such variants are found predominantly in the gene encoding the LDL receptor (LDLR), which binds and clears LDL particles from the blood (7). A few patients have a common disease-causing variant of apolipoprotein B (APOB), p.Arg3527Gln, or disease-causing variants in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene (8). APOB is an integral component of LDL particle...
and a ligand for the LDL receptor (9), whereas PCSK9 regulates LDL receptor membrane concentration (10). Additionally, the interaction of common small-effect LDL cholesterol-elevating alleles in various genes may contribute to multifactorial disease development (11,12), with the apolipoprotein E (APOE) E4 isoform being the most recognized multivariant cause of hypercholesterolemia (13).

Various screening strategies have been proposed to identify children with FH. Only implementations of partial (14) and selective screenings primarily on the basis of family history are reported (15). These approaches lack the strength of identifying patients without known family history. Therefore, the National Heart, Lung, and Blood Institute (16), the National Lipid Association Expert Panel (17), and the European Expert Panel (15) proposed universal screening as a preferred method of screening for hypercholesterolemia in primary prevention efforts. Slovenia (population 2 million) started universal screening for hypercholesterolemia in 5-year-old children in 1995 (15). Screening was gradually implemented for hypercholesterolemia in 5-year-old children in population 2 million started universal screening for hypercholesterolemia in 5-year-old children.

Written informed consent was obtained from all parents or legal guardians. Principles of the Declaration of Helsinki were followed, and the Slovenian National Medical Ethics Committee approved the study (numbers 25/12/10 and 63/07/13).

**DNA SEQUENCING AND ANALYSIS.** Genomic DNA was isolated from whole-blood samples according to established laboratory protocols using FlexiGene isolation kit (Qiagen, Hilden, Germany). NGS is a high-throughput method based on simultaneous sequencing of numerous candidate genes or genomic intervals in a parallel fashion, effectively balancing cost and data quality for population-targeted sequencing studies (19). Samples for NGS were prepared following the manufacturer’s protocol using ADH MASTR ready-to-use NGS-based molecular assay (Multiplicom NV, Niel, Belgium) for detection of variants in 4 genes associated with FH (encompassing coding and promoter regions of \( \text{LDLR} \), \( \text{PCSK9} \), APOE and part of \( \text{APOB} \) exon 26). Samples were sequenced using a MiSeq sequencer with MiSeq reagent kit version 2 (Illumina, San Diego, California) following the manufacturer’s protocol, including recommendations for quality control parameters. The presence of copy number variations in \( \text{LDLR} \) was analyzed using NextGENe (Softgenetics, State College, Pennsylvania) and confirmed by Multiplex ligation-dependent probe amplification with an LDLR-P062 kit (MRC-Holland, Amsterdam, the Netherlands). Variants reported in disease-specific databases (20) and the Human Gene Mutation Database (HGMD) (Institute of Medical Genetics, School of Medicine, University of Cardiff, Cardiff, Wales) as unequivocally disease-causing were classified as pathogenic. In silico analysis of novel variants was performed with PolyPhen-2 (Harvard University, Cambridge, Massachusetts), SIFT (J. Craig Venter Institute, San Diego, California), and MutationTaster (University of Medicine, Berlin, Germany) bioinformatic tools. Variants declared to be disease-associated by at least 2 analytical algorithms were obtained at the tertiary pediatric outpatient clinic. Simon Broome Register criteria (1) were used for assessment of family history. Positive family history was defined as myocardial infarction before 50 years of age in any second-degree relative or before 60 years of age in any first-degree relative or as TC >7.5 mmol/l (289.6 mg/dl) in any first- or second-degree relative (first-degree relation included parent, offspring or sibling; second-degree relation included grandparent, grandchild, nephew, niece, or half-sibling).
classified as novel causative variants. Single-nucleotide variants and small duplications/deletions with potentially disease-causing effect were confirmed by targeted Sanger DNA sequencing.

**STATISTICAL ANALYSIS.** Descriptive statistics (means, standard deviations, and ratios) were used to characterize the investigated population. Assumption of equal proportions of detected FH population and total population enrolled in the national universal screening was made on the basis of the reported incidence, number of referred children, and number of live-born children publicly available at the Statistical Office of the Republic of Slovenia to estimate approximate number of children enrolled in the universal screening program in the previous 5 years (2009 to 2013), as exact data were not available. The detection rate of the universal screening in the studied population was evaluated by the number of children
per generation referred to the tertiary outpatient clinic compared to the potential FH population estimated from the registry of live-born children and was reported as 1 in 500 and 1 in 200 incidence of FH (4,5). Data deviation from normal was evaluated with the Agostino and Pearson omnibus normality test. Differences in disease-associated variant accumulation between different groups of participants (sex and cardiovascular-positive family history), corresponding odds ratio (OR) and/or relative risk (RR), were evaluated using Fisher exact test for 2 × 2 contingency tables. Logistic regression model was applied (21) to calculate OR and RR with corresponding 95% confidence intervals (CIs). Genotype-associated differences among TC, LDL, HDL, and non-HDL levels and standard deviation score of body mass index were statistically evaluated using Kruskal-Wallis test with Dunn’s multiple comparisons test. A p value < 0.05 was used as an indicator of statistical significance. All statistical calculations were made in Prism version 6.01 software (GraphPad, Inc., La Jolla, California).

## Results

Altogether, 272 unrelated participants born between 1989 and 2009 were included in the study. Characteristics of participants are shown in Table 1. Serum TC levels at universal screening for hypercholesterolemia at age 5 and fasting lipid profile (TC, LDL, HDL, and TG levels) were measured at first admission to a tertiary pediatric outpatient clinic. Participants were referred to the tertiary pediatric outpatient clinic at 7.3 ± 3.1 years of age. A cardiovascular complications-positive family history according to Simone Broome Register criteria was identified in 33.1% of participants.

### Prediction of National Universal Screening Detection Rate

The national universal screening in Slovenia was introduced gradually, and the referral rate to the tertiary institution reached its expected level only in 2013. An estimated 33,000 to 70,000 of 96,690 live-born children were enrolled at
5 years of age in the national universal screening from 2009 to 2013. Assuming an FH incidence of 1 in 500, the predicted detection rate from the data for the previous 5 years (2009 to 2013) was 53.6% (95% CI: 34.5% to 72.8%), reaching its peak in 2013 (children born in 2008) with an upper estimated detection rate of 96.3%. Assuming a greater incidence of 1 in 200, the upper estimated detection rate was only 38.5%.

**GENETIC CHARACTERIZATION.** Of 272 participants, 105 (38.6%) had heterozygous disease-causing variants in LDLR, 50 (18.4%) in APOB, and none in PCSK9. Twenty-three known (Online Table 1) and 8 novel disease-causing variants (Table 2) were identified in LDLR. Six novel disease-causing variants were missense and 2 were intronic. Four participants had multiple variants, 2 combinations of known disease-causing variants (Online Table 1) and 1 combination of a known and a novel disease-causing variant (Table 2). Copy number variations were identified in 3 patients, 2 had a deletion of exons 2 to 18, and 1 had a deletion of the whole LDLR coding region (Online Table 1). Two known missense variants (Online Table 1) and 1 novel disease-causing variant (Table 2) were identified in APOB. In the remaining participants without disease-causing variants, 51 (18.7%) were carriers of the hypercholesterolemia-associated APOE E4 isoform, whereas 66 participants (24.3%) had conditions that remained genetically undiagnosed (Table 1). A cardiovascular complication-negative family history according to Simone Broome Register criteria (1) was associated with 48.6%, 60.0%, and 76.5% of patients with disease-causing or disease-associated variants in LDLR, APOB, or APOE E4 isoform, respectively (Table 1). Relative risk of having a disease-causing genetic variant when a participant had a positive family history was 1.53 (RR: 1.529; 95% CI: 1.25 to 1.88; p = 0.0001). The OR for having a disease-causing genetic variant in participants with a TC level >6 mmol/l (231.7 mg/dl) was 7.71 compared with an OR of 7.71 (95% CI: 1.75 to 33.35; p = 0.0013) for the participants with TC levels between 5 and 6 mmol/l (193.1 and 231.7 mg/dl, respectively). Among 33 patients with TC levels between 5 and 6 mmol/l (193.1 and 231.7 mg/dl, respectively), 2 had disease-causing variants in LDLR and none in APOB. Among 177 patients with TC levels between 6 and 8 mmol/l (231.7 and 308.9 mg/dl, respectively), 31.1% had disease-causing variants in LDLR and 12.9% in APOB. Carriers of LDLR and APOB disease-causing genetic variants had on average higher TC levels at universal screening for hypercholesterolemia than noncarriers (7.7 ± 1.2 mmol/l vs. 6.6 ± 0.9 mmol/l, respectively [297.3 ± 46.3 mg/dl vs. 254.8 ± 34.8 mg/dl, respectively]; p < 0.0001). Additionally, carriers of LDLR and APOB disease-causing genetic variants had on average higher TC levels at initial evaluation at the tertiary pediatric outpatient clinic than noncarriers. Associations among

### Table 2: Novel Heterozygous Disease-Causing Variants in LDLR and APOB

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of Variants (%)</th>
<th>Nucleotide Change</th>
<th>Protein Change</th>
<th>Localization</th>
<th>Protein Domain</th>
<th>In Silico Novel Variant Analysis</th>
<th>Mutation Taster</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR</td>
<td>2 (1.9)</td>
<td>c.300C→G</td>
<td>p.Asp100Glu</td>
<td>Exon 3</td>
<td>LDL receptor class A2</td>
<td>Deleterious (0)</td>
<td>Probably damaging (0.982)</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.408C→T</td>
<td>p.Asp136Asp1</td>
<td>Exon 4</td>
<td>LDL receptor class A3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.827G→T</td>
<td>p.Cys267Phe</td>
<td>Exon 6</td>
<td>LDL receptor class A7</td>
<td>Deleterious (0)</td>
<td>Probably damaging (0.996)</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.972C→T</td>
<td>p.Thr324Asp</td>
<td>Exon 7</td>
<td>EGF-like 1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.1587→G</td>
<td>p.Arg490Cys</td>
<td>Exon 10</td>
<td>LDL receptor class B3</td>
<td>Deleterious (0)</td>
<td>Probably damaging (1)</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.1587→G</td>
<td>p.Glu20Arg</td>
<td>Intron 10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.1706→A</td>
<td>p.Arg562Cys</td>
<td>Intron 11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LDLR</td>
<td>1 (1.0)</td>
<td>c.1487→A</td>
<td>p.Glu496Glu</td>
<td>Exon 10</td>
<td>LDL-receptor class B3</td>
<td>Deleterious (0.02)</td>
<td>Probably damaging (0.998)</td>
</tr>
<tr>
<td>APOB</td>
<td>2 (2.0)</td>
<td>c.10370C→G</td>
<td>p.5Ser3457Cys</td>
<td>Exon 26</td>
<td>LDLR binding side</td>
<td>Deleterious (0.03)</td>
<td>Probably damaging (0.976)</td>
</tr>
</tbody>
</table>

*Variant c.1587→G > A at the same location is disease-causing according to a disease-specific database (19) and the Human Gene Mutation Database (HGMD; Institute of Medical Genetics, School of Medicine, University of Cardiff, Cardiff, Wales). †Variant c.1706→G > T at the same location are disease-causing according to a disease-specific database (19) and the HGMD database. ‡Variant is predicted to have potential alterations on splicing by Human Splicing Finder. §Known disease-causing variant.

Abbreviations as in Table 1.
This is the first study to evaluate genetic identification of FH by a national universal screening for hypercholesterolemia. Children were screened at 5 years of age, yet the mean referral age to the tertiary pediatric outpatient clinic was 7.3 years of age, indicating the requirement of additional educational intervention to promote more rapid referral of patients from primary care institutions for further clinical evaluation, along with a record of children not referred or whose parents refused referral. Serum TC level discriminates best between people with and without FH in the interval from 1 to 9 years of age, making it the optimal period for universal screening for hypercholesterolemia. Serum TC can identify 88.0% to 96.0% of cases, with a false positive rate between 0.1% and 1.0% (21). In addition, once an affected child is identified, 96.0% of parents with the disorder could be detected by identifying the parent with the higher cholesterol level (child-parent screening strategy) (22). All parents and older siblings from children identified as having FH in our study were referred for evaluation. The simulated detection rate of FH in our national universal screening for hypercholesterolemia on the basis of commonly reported incidence of 1 in 500 was more than 96.0% in the last year and allowed for a reliable identification of the risk population. Nevertheless, when an incidence rate of 1 in 200 is assumed, only 38.5% of the at-risk population was detected. An estimation of the total population enrolled in the national universal screening from 2009 to 2013 indicated that only approximately one-half of the total population was reached. Consequently, continuation and further improvements of screening and referral strategies will provide more data for the real FH incidence in the Slovenian population and improve clinical algorithms in FH risk management. The optimal age for a universal screening for hypercholesterolemia remains to be determined (15). Early dietary and lifestyle intervention along with cholestyramine assessment at 6 years of age (23) and use of statins after 8 years of age (24) in FH are beneficial, and therefore a diagnosis and intervention before pubertal age seems prudent (4,15,18).

A disease-causing variant for FH was identified in 57% of participants referred from the universal screening, with an additional one-fifth identified with the most common multifactorial form of hypercholesterolemia, amounting to >75.0% of referred participants carrying a disease-causing or disease-associated genetic variant. Proportions of disease-causing variants in the present study were on the lower end of reported population data for LDLR and on the higher end in the same reports for APOB. For Czech (25), Polish (26), French (27), Spanish (28), and Italian (29) populations, the reported disease-causing variant frequencies were 23.9%, 45.0%, 73.9%, 96.4%, and 97.4%, respectively, and were 11.8%, 6.0%, 6.6%, 3.5%, and 2.2%, respectively, for APOB. Lack of the disease-causing PCSK9 variant in the Slovenian population was similar to that in the Finnish (30) and Greek (31) populations. The LDLR and APOB spectra of disease-causing variants are diverse. In large countries such as Italy (29), Spain (28), France (27), or Poland (25), more than 100 disease-causing variants contribute to a heterogeneous pool, whereas in populations such as those in Finland (30) or Iceland (32), the diversity of disease-causing variants is smaller, with less than 10 disease-causing variants present. Thirty-three disease-causing variants were identified in LDLR in the Slovenian cohort, 6 of which explained 55.2% of LDLR-positive cases. Two disease-causing variants were identified in APOB. One of these (p.Arg3527Gln) explained 96.0% of APOB-positive cases, probably due to its central European origin and a known migration pattern of carriers (33). Approximately 6.0% of disease-causing variants in LDLR and APOB were novel, confirming the wide variety of variants associated with the disease in other populations (4).

Almost one-fifth of the total cohort was negative for disease-causing variants in LDLR, APOB, and PCSK9 but carried the APOE E4 isoform, emphasizing the potential contribution of APOE genetic variants in the multifactorial cause of hypercholesterolemia (13). The remaining 24.3% of patients in the present study had no disease-causing or disease-associated variants identified, which is in accordance with other reports in which 10.0% to 40.0% (depending on the inclusion criteria) of those with clinical diagnosis of FH were found not to be carriers of disease-causing genetic variants in LDLR, APOB, or PCSK9 and were likely to have a polygenic or multifactorial type of hypercholesterolemia (28,34).

A significant advantage in cost reduction (approximately 3-fold decrease for the central European region) and timely identification of causative genetic variants (approximately 7-fold reduction in turnaround time in our laboratory) with the NGS compared with more widely used genetic screening methods (35) (e.g., denaturing high-pressure liquid chromatography or high resolution DNA melting analysis and Sanger sequencing) were observed.
Moreover, NGS reduced the possibility of human errors due to reduced hands-on time per sample (36).

A 56.0% diagnostic yield and a significant association of tendon xanthoma and LDL-cholesterol level with the disease-causing LDLR or APOB variant has been demonstrated in an adult cohort (34). Comparable diagnostic yield in the adult population with clinical presentation of hypercholesterolemia (34) and our pediatric population without any clinical signs indicates that a universal screening may be an efficacious strategy for early detection of FH and prevention of cardiovascular complications. Importantly, almost one-half of participants with a TC level <6 mmol/l (231.7 mg/dl) carried a disease-causing or disease-associated genetic variant. Furthermore, a cardiovascular complications-negative family history according to Simone Broome Register criteria (1) was observed in one-half to three-quarters of patients with a disease-causing or disease-associated variant in LDLR, APOB, or the APOE E4 isoform. The RR of having a disease-causing variant with a positive family history was significant but relatively low. Data for a family history may not always be a reliable indicator for identification of potential FH patients (37).

**STUDY LIMITATIONS.** This study was limited by a relatively small Slovenian population, as well as gradual implementation of the universal national screening for hypercholesterolemia. More data are required to optimize and evaluate the universal screening criteria and referral strategy. Additionally, a cascade screening for parents and siblings of identified affected children should be envisaged.

**CONCLUSIONS**

Results from the present study demonstrated for the first time that a national universal screening for hypercholesterolemia at 5 years of age identified genetically confirmed FH in more than one-half of the referred participants. Moreover, a genetic variant in APOE associated with hypercholesterolemia was detected in an additional one-fifth of the referred participants. Data for family history may not suffice for reliable identification of patients through selective screening. Our findings support the latest recommendations by professional forums for a universal screening for hypercholesterolemia in children. Coupled with a cascade screening for family members, universal screening for hypercholesterolemia could be a powerful approach for FH detection and prevention of cardiovascular complications. Additional studies are warranted to confirm the results of this study.

**ACKNOWLEDGMENTS** The authors thank the participants in this study. They also thank the primary care pediatricians, the laboratory personnel, and the nursing team who helped in conducting the study.

**REPRINT REQUESTS AND CORRESPONDENCE:** Dr. Tadej Battelino, University Children’s Hospital, Department of Pediatric Endocrinology, Diabetes and Metabolic Diseases, Bohoričeva 20, SI-1000 Ljubljana, Slovenia. E-mail: tadej.battelino@mf.uni-lj.si.

**REFERENCES**


5. Benn M, Watts GF, Tybjærg-Hansen A, et al. Familial hypercholesterolemia in the Danish general population: prevalence, coronary artery...
disease, and cholesterol-lowering medication. J Clin Endocrinol Metab 2012;97:3956-64.


KEY WORDS cholesterol, familial hypercholesterolemia, next-generation sequencing, universal screening

APPENDIX For supplemental tables, please see the online version of this article.