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Genetics of familial hypercholesterolemia: New insight - volume II

Edited by
Alpo Juhani Vuorio, Uma Ramaswami and Kirsten B. Holven

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Genetics of familial hypercholesterolemia: New insight - volume II

Topic editors

Alpo Juhani Vuorio — University of Helsinki, Finland

Uma Ramaswami — Royal Free London NHS Foundation Trust, United Kingdom

Kirsten B. Holven — University of Oslo, Norway

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Table of contents

05 Editorial: Genetics of familial hypercholesterolemia: New insight—Volume II
Alpo Vuorio, Uma Ramaswami and Kirsten B. Holven

09 Two Novel Disease-Causing Mutations in the LDLR of Familial Hypercholesterolemia
Haochang Hu, Tian Shu, Jun Ma, Ruoyu Chen, Jian Wang, Shuangshuang Wang, Shaoyi Lin and Xiaomin Chen

18 Low Detection Rates of Genetic FH in Cohort of Patients With Severe Hypercholesterolemia in the United Arabic Emirates
Antoine Rimbert, Hinda Daggag, Peter Lansberg, Adam Buckley, Martijn Viel, Roan Kanninga, Lennart Johansson, Robin P. F. Dullaart, Richard Sinke, Alia Al Tikriti, Jan Albert Kuivenhoven and Maha Taysir Barakat

25 Familial Hypercholesterolemia Prevalence Among Ethnicities—Systematic Review and Meta-Analysis
Frida Toft-Nielsen, Frida Emanuelsson and Marianne Benn

35 Assessment of Associations Between Serum Lipoprotein (a) Levels and Atherosclerotic Vascular Diseases in Hungarian Patients With Familial Hypercholesterolemia Using Data Mining and Machine Learning
Ákos Németh, Bálint Daróczy, Lilla Juhász, Péter Fülöp, Mariann Harangi and György Paragh

45 Fast and Easy Nanopore Sequencing Workflow for Rapid Genetic Testing of Familial Hypercholesterolemia
Muhidien Soufi, Simon Bedenbender, Volker Ruppert, Bilgen Kurt, Bernhard Schieffer and Juergen R. Schaefer

54 Familial Hypercholesterolemia: Real-World Data of 1236 Patients Attending a Czech Lipid Clinic. A Retrospective Analysis of Experience in More than 50 years. Part I: Genetics and Biochemical Parameters
Veronika Todorovova, Tereza Altschmiedova, Michal Vrablik and Richard Ceska

63 Familial Hypercholesterolemia: Real-World Data of 1236 Patients Attending a Czech Lipid Clinic. A Retrospective Analysis of Experience in More than 50 years. Part II. Clinical Characteristics
Tereza Altschmiedova, Veronika Todorovova, Michal Vrablik and Richard Ceska

73 LDL-C Concentrations and the 12-SNP LDL-C Score for Polygenic Hypercholesterolaemia in Self-Reported South Asian, Black and Caribbean Participants of the UK Biobank
Jasmine Gratton, Chris Finan, Aroon D. Hingorani, Steve E. Humphries and Marta Futema

82 Effects of Different Types of Pathogenic Variants on Phenotypes of Familial Hypercholesterolemia
Hayato Tada, Nobuko Kojima, Kan Yamagami, Akihiro Nomura, Atsushi Nohara, Soichiro Usui, Kenji Sakata, Noboru Fujino, Masayuki Takamura and Masa-Aki Kawashiri

89 Familial Hypercholesterolemia and Elevated Lipoprotein(a): Cascade Testing and Other Implications for Contextual Models of Care
Wann Jia Loh, Dick C. Chan, Pedro Mata and Gerald F. Watts

104 Long-Term Cardiovascular and Cerebrovascular Challenges Posed by COVID-19 in Patients With Familial Hypercholesterolemia
Alpo Vuorio, Frederick Raal, Petra Ijäs, Markku Kaste and Petri T. Kovanen

109 The Progression of Treatment for Refractory Hypercholesterolemia: Focus on the Prospect of Gene Therapy
Zhi-Fan Li and Na-Qiong Wu

123 The Role of Registers in Increasing Knowledge and Improving Management of Children and Adolescents Affected by Familial Hypercholesterolemia: the LIPIGEN Pediatric Group
Marta Gazzotti, Manuela Casula, Stefano Bertolini, Maria Elena Capra, Elena Olmastroni, Alberico Luigi Catapano and Cristina Pederiva

130 The Prevalence and Genetic Spectrum of Familial Hypercholesterolemia in Qatar Based on Whole Genome Sequencing of 14,000 Subjects
Ilhame Diboun, Yasser Al-Sarraj, Salman M. Toor, Shaban Mohammed, Nadeem Qureshi, Moza S. H. Al Hail, Amin Jayyousi, Jassim Al Suwaidi and Omar M. E. Albagha

142 Do Biobank Recall Studies Matter? Long-Term Follow-Up of Research Participants With Familial Hypercholesterolemia
Miriam Nurm, Anu Reigo, Margit Nõukas, Liis Leitsalu, Tiit Nikopensius, Marili Palover, Tarmo Annilo, Maris Alver, Aet Saar, Toomas Marandi, Tiia Ainla, Andres Metspalu, Tõnu Esko and Neeme Tõnisson

153 The risk of various types of cardiovascular diseases in mutation positive familial hypercholesterolemia; a review
Anders Hovland, Liv J. Mundal, Marit B. Veierød, Kirsten B. Holven, Martin Prøven Bogsrud, Grethe S. Tell, Trond P. Leren and Kjetil Retterstøl

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Jordi Pérez-Tur,
Institute of Biomedicine of Valencia (CSIC), Spain

*CORRESPONDENCE
Alpo Vuorio,
alpo.vuorio@gmail.com

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Editorial: Genetics of familial hypercholesterolemia: New insight—Volume II

Alpo Vuorio[1,2]*, Uma Ramaswami[3,4] and Kirsten B. Holven[5,6]

[1]Mehiläinen Airport Health Centre, Vantaa, Finland, [2]Department of Forensic Medicine, University of Helsinki, Helsinki, Finland, [3]Lysosomal Disorders Unit, Royal Free London National Health Service Foundation Trust, London, United Kingdom, [4]Genetics and Genomics Department, University College London, London, United Kingdom, [5]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, [6]Norwegian National Advisory Unit on Familial Hypercholesterolemia, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital, Oslo, Norway

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Editorial on the Research Topic
Genetics of familial hypercholesterolemia: New insight—volume II

The second volume of Research Topic "Genetics of Familial hypercholesterolemia: New Insight—Volume II" attracted nearly 100 authors from 16 countries to publish their research articles and again we achieved our goal of bringing together researches of familial hypercholesterolemia (FH) worldwide and to increase FH related awareness of genetics, diagnostics, and risk factors. In addition, Research Topic like future therapies for the treatment of homozygous form of FH (HoFH) gene therapy are discussed in this Research Topic (Li and Wu).

The systematic review and meta-analysis in this Research Topic by Toft-Nielsen et al. showed that heterozygous familial hypercholesterolemia (HeFH) is a common genetic disease with an estimated prevalence of about 1 in 300 persons meaning that over 25 million individuals are affected by HeFH worldwide. Interestingly in this meta-analysis estimated FH prevalence varied across ethnicity and ranged from 1:400 to 1:192 being highest among black and brown individuals. Unfortunately, HeFH has remained an underdiagnosed and undertreated disease (Representatives of the Global Familial Hypercholesterolemia Community, 2020).

While it is important to know the worldwide prevalence of FH it is also important to know the local prevalence of FH in different countries. In this Research Topic (Diboun et al.) for the first time found out the prevalence of HeFH in Qatar. Their results revealed an estimated prevalence of HeFH in Qatar to be 0.8% (1:125) for definite/probable cases. The two most common mutations in Qatar were in *LDLR* but additionally it was found mutations also in *APOB* and *PCSK9*. No HoFH patients were found in this study carried out in a Qatar. In a study by (Rimbert et al.) the prevalence of genetically confirmed HeFH was studied in an Emirati population sample. Rimbert and co-authors (2022) recruited

229 patients with serum low-density cholesterol (LDL-C) over the 95th percentile and used next generation sequencing to screen *LDLR, APOB, PCSK9* and *LDLRAP1*. In this study, the prevalence of genetically confirmed HeFH was 7% with marked hypercholesterolemia as determined by correcting LDL-C for the use of lipid-lowering treatment. Rimbert et al. argue that the results of this study are helpful when planning to cascade screening programs in the United Arab Emirates.

Finding new mutations causing FH remains important because in the majority of countries FH remains vastly underdiagnosed and only about 1% or less of the potential FH cases have been found (Nordestgaard et al., 2013). In a study by Hu et al. the whole-exome sequencing and Sanger sequencing revealed two *LDLR* missense mutations *LDLR* c.226 G > C and c. 1003 G > T in one family, causing most probably LDLR uptake dysfunction. Hu and co-authors (2021) remind us that finding new FH-causing mutations helps to carry out diagnostic screening at population level and start early prevention of vascular disease. In fact, a recent recommendation underscores that especially screening of paediatric FH patients is efficient and cost-effective in preventing vascular disease (Gidding et al., 2022).

Tada et al. studied missense variants in HeFH patients (which can estimate residual LDLR activity) and protein truncating variants (PTVs), which may have lost their LDL function. Based on variant data the authors analysed the occurrence of major adverse cardiac events (MACE). Both PTVs and missense variants were significantly associated with MACEs (hazard ratio [HR] = 1.58, 95% confidence interval [CI] = 1.08–2.08, $p = 0.0033$ and HR = 3.24, 95% CI = 2. 12–4.40, $p = 3.9 \times 10^{-6}$, respectively). The authors conclude that genetic testing is useful to further stratify the risk among HeFH patients.

Gratton et al. discuss in their article the importance of polygenic cause for elevated LDL-C. The diagnosis of polygenic hypercholesterolemia is in this study based on a 12-single nucleotide polymorphism (SNP) score and this score was evaluated among South Asian (SA) and Black and Caribbean (BC) ethnicities. Gratton and co-authors (2022) found differences in LDL-C and 12-SNP score distribution between ethnicities. The authors conclude that there is a need to carry more research to find out benefits and risks of returning polygenic information to patients.

The role of registers improving management of HeFH children was showed by (Gazzotti et al.). In this Italian LIPIGEN register study analysis of about 1,600 HeFH children were analysed, and untreated low-density lipoprotein cholesterol (LDL-C) was about 220 mg/dl which in other European HeFH children's cohorts is on a range of LDL-C 188–220 mg/dl (Futema et al., 2020). Importantly Futema et al., 2020 showed in their European countries analysis that the age of FH diagnosis varies significantly across countries and alarmingly almost three-quarters of the over 10-year-old untreated HeFH children have their LDL-C over the treatment target limit. This result by Futema and co-authors (2020) is particularly unfortunate because statins has shown to be an effective and safe treatment for HeFH in children (Luirink et al., 2019; Vuorio et al., 2019).

In recall-by genotype (RbG) studies, people who carry genotypes of special interest are recalled from a biobank for more detailed investigations. In an Estonian Biobank (EstBB) RbG long-term follow up study on lipid lowering treatment (LLT) adherence of 34 recalled HeFH patients was compared to 291 controls having the same mutations causing HeFH (Nurm et al.). It was shown in this study that the recalled group had significantly more LLT users compared to the non-recalled group (79% vs. 53%, $p < 0.005$). This HeFH RbG study showed positive impact on LLT treatment among EstBB participants.

(Soufi et al.) demonstrated in their study the advantages of Oxford Nanopore technology sequencing compared to Sanger and NextGen sequencing regarding FH diagnostics. Based on their experience, Oxford Nanopore technology sequencing is easy to operate, low-cost, and allows parallel sample sequencing. As an example of costs, they mention the following comparison. Genetic analysis of the *LDLR* of one patient would cost $ 1.060 using Sanger sequencing and $890 using Next Gen sequencing while using the new Nanopore technology the cost is only $ 109.

Recent studies have shown that among FH patients elevated lipoprotein (a) [Lp(a)] is associated with an increased risk of atherosclerotic vascular disease (ASCVD) (Bogsrud et al., 2019; Vuorio et al., 2020; Kronenberg et al., 2022). In a very large Hungarian population-based study, Németh et al. included 590,500 patients and used the Dutch Lipid Clinic Network scores and machine learning to identify probable and definite HeFH patients. They found 459 HeFH patients of which 221 had serum Lp(a) measurements available. Patients with HeFH had significantly higher serum Lp(a) levels compared to non-FH subjects [236 (92.5; 698.5) vs 167 (80.2; 431.5) mg/L, $p < 0.$ 01]. Additionally, 35% of HeFH patients had serum Lp(a) levels over 500 mg/L. Atherosclerotic complications were more often present in FH patients compared to non-FH subjects (46.6 vs 13. 9%). Currently lowering serum Lp(a) by about 30% is possible by using proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors (Spolitu et al., 2019). New drugs, such as antisense oligonucleotides against Lp(a) and small interfering RNA lower serum Lp(a) by about 80–90% (Vuorio et al., 2020).

Because Lp(a) has shown to be a crucial risk factor for FH patients it is also important to diagnose those FH patients having elevated serum Lp(a). To this end, Loh et al. suggest an assessment of Lp(a) as a part of FH cascade screening. They have estimated that those probands with HeFH and hyper-Lp(a), the yield of detection of hyper-Lp(a) is approximately 1 individual for every 2.1–2.4 relatives tested. The yield to find both HeFH and high Lp(a) is 1 individual for every 3–3. 4 relatives screened. Therefore, the combined screening for FH and Lp(a) is very promising (Chakraborty et al., 2022).

There are two retrospective studies in this Research Topic in which a group of 1,236 HeFH patients (841 women; 395 men; and 243 relatives; mean age 44.8 ± 16.7) were followed over 50 years in the General University Hospital in Praque (Altschmiedova et al.; Todorovova et al.). Todorova and co-authors (2022) found that during the follow-up 50 years decrease of serum LDL-C levels was more than 50% when compared to the initial LDL-C values in the beginning of the follow-up in this Czech Lipid Clinic study. Altschmiedova and co-authors (2022) found in this same HeFH group of patients that serum baseline Lp(a) and triglycerides had a strong correlation with ASCVD. Based on these two retrospective analyses authors conclude that HeFH patients benefit from follow-up and treatment in specialized lipid centers.

Follow-up studies of severe acute respiratory syndrome (SARS) caused by the coronavirus (SARS-CoV) epidemic between 2002 and 2003 show deterioration of general health during 24 months after the infection (Ngai et al., 2010; Zhang et al., 2020). Additionally, lipid and glucose metabolism may also remain altered for a long time after a SARS-CoV infection (Wu et al., 2017). In FH with COVID-19 the pre-existing endothelial dysfunction could increase long-term vascular complications (Vuorio et al., 2021). So far it has been already found that COVID-19 illness increases cardiovascular risk of HeFH patients with or without ASCVD (Myers et al., 2021). In their opinion article, Vuorio et al and co-authors (2022) express concern about FH patients who have suffered SARS-CoV-2 infection because post-infection hypercoagulable states may persist even longer among patients with FH compared to non-FH patients. This concern is based on the notion that FH endothelium in these patients is exposed to a lifelong high serum LDL-C and often also to elevated serum Lp(a) levels, which jointly worsen endothelial dysfunction (Vuorio et al., 2021). Therefore, the clinicians need to consider the effective LLT in FH patients during and after a SARS-CoV-2 infection.

Author contributions

AV: writing the first draft. AV, UR, and KV: reviewing and editing to produce the final draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

AV has received consultation fees Amgen and Novartis, none of which are related to the content of this manuscript. KH has received research grants or honoraria from Mills AS, Tine SA, Olympic Seafood, Amgen, Sanofi, none of which are related to the content of this manuscript.

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Two Novel Disease-Causing Mutations in the LDLR of Familial Hypercholesterolemia

Haochang Hu[1†], Tian Shu[1†], Jun Ma[2†], Ruoyu Chen[1], Jian Wang[1], Shuangshuang Wang[1], Shaoyi Lin[1] and Xiaomin Chen[1]**

[1]Department of Cardiology, Ningbo First Hospital, Ningbo, China, [2]Department of Medical Ultrasonics, First Affiliated Hospital of Jinan University, Guangzhou, China

As an autosomal dominant disorder, familial hypercholesterolemia (FH) is mainly caused by pathogenic mutations in lipid metabolism-related genes. The aim of this study is to investigate the genetic mutations in FH patients and verify their pathogenicity. First of all, a pedigree investigation was conducted in one family diagnosed with FH using the Dutch Lipid Clinic Network criteria. The high-throughput sequencing was performed on three family members to explore genetic mutations. The effects of low-density lipoprotein receptor (*LDLR*) variants on their expression levels and activity were further validated by silico analysis and functional studies. The results revealed that LDLC levels of the proband and his daughter were abnormally elevated. The whole-exome sequencing and Sanger sequencing were used to confirm that there were two *LDLR* missense mutations (*LDLR* c.226 G > C, c.1003 G > T) in this family. Bioinformatic analysis (Mutationtaster) indicated that these two mutations might be disease-causing variants. *In vitro* experiments suggested that *LDLR* c.226 G > C and c.1003 G > T could attenuate the uptake of Dil-LDL by LDLR. In conclusion, the *LDLR* c.226 G > C and c.1003 G > T variants might be pathogenic for FH by causing uptake dysfunction of the LDLR.

Keywords: familial hypercholesterolemia, low-density lipoprotein cholesterol, low-density lipoprotein receptor, disease-causing mutations, function

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Muhammad Ajmal, COMSATS University, Pakistan

****Correspondence:***
Shaoyi Lin
shaoyi_lin@hotmail.com
Xiaomin Chen
cxmdoctor@126.com

[†]These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Familial hypercholesterolemia (FH) is a common autosomal genetic disorder mainly caused by pathogenic mutations in genes encoding low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*ApoB*) and proprotein convertase subtilisin kexin 9 (*PCSK9*) (Benn et al., 2016). A meta-analysis of 11 million subjects illustrated that the prevalence of FH in the general population is 0.32%, while the prevalence of FH in ischemic heart disease and premature ischemic heart disease patients is 3.2 and 6.7%, respectively (Beheshti et al., 2020). Due to lifelong exposure to extremely high levels of low-density lipoprotein cholesterol (LDLC), FH is characterized by xanthomas, corneal arcus, and early-onset cardiovascular disease (Peterson et al., 2021). European and American guidelines suggest that FH patients should be identified as early as possible so that LDLC lowering treatment can be started early in life in order to improve the patient's prognosis (Piepoli et al., 2016; Grundy et al., 2019).

As a membrane protein on the surface of liver cells, LDLR is the key point of LDLC metabolism. LDLR could combine with LDLC and transport it to the lysosome for metabolism. The LDLR subsequently returns to the surface of liver cells for recycling (van de Sluis et al., 2017; Chemello et al., 2021). Therefore, the pathogenic variants in *LDLR* could directly lead to protein dysfunction and

LDLC metabolic disorders. Previous studies suggested that the disease-causing variants in *LDLR* might account for about 90% of FH (Gidding et al., 2015; Iacocca and Hegele, 2017). Reeskamp *et al.* performed targeted next-generation sequencing on 1,528 patients with LDLC greater than 5 mmol/L. The results illustrated that 227 cases (14.9%) were heterozygous carriers of pathogenic variants in *LDLR* (80.2%), *APOB* (14.5%), and *PCSK9* (5.3%) (Reeskamp et al., 2021b). Furthermore, a retrospective study pointed out that LDLC gradually increased from patients with no pathogenic variants to patients with a defective variant, patients with a null variant, and patients with two variants (Di Taranto et al., 2021). Therefore, it is imperative to perform genetic diagnoses of FH patients to distinguish between different genetic states or variant types.

According to the UCL *LDLR* gene variant database, 3779 *LDLR* mutations have been reported so far; of which 77% are substitutions, 16% are deletions, and 5% are duplicates (Leigh et al., 2017). Regrettably, the pathogenicity of several *LDLR* variants has not been authenticated. It is crucial to research the functional verification of mutations to better clarify the molecular mechanism of FH.

In this study, a FH family was included, and high-throughput sequencing was used to explore pathogenic mutations. The effects of *LDLR* variants on the expression level and function of LDLR were verified in cellular experiments.

MATERIALS AND METHODS

Patients

The study enrolled one FH family consisting of three members in Ningbo First Hospital. The Dutch Lipid Clinic Network criteria (DLCN) was used to diagnose FH patients (Wang et al., 2019). A detailed information collection form was designed based on the characteristics of FH patients. The content mainly included the patient's general information, family history, personal history, past history, treatments, the results of the auxiliary examination, etc.

Whole-Exome Sequencing and in Silico Analysis

Whole blood samples of the three participants were collected in EDTA tubes (Gongdong Company, China) and stored in a refrigerator at -80 °C (ThermoFisher Scientific, United States). Omega Blood DNA Kit (D3392-02, Omega bio-tek, United States) was used to extract genomic DNA. High-throughput whole-exome sequencing for DNA samples was completed on the BGISEQ-500 platform (Huada Gene Technology Co. Ltd., China). First, the genomic DNA was randomly broken into fragments of about 200–300 bp, and a complete fragment library was established after PCR amplification. The quality of DNA was then inspected before sequencing, and the number of original bases (raw data) obtained by sequencing each sample should meet the standard. Clean data was subsequently obtained by removing low-quality reads from raw data. Finally, we compared the sequencing data with the human reference genome hg19 to obtain high-confidence mutations.

As one of the standard bioinformatic tools, Mutationtaster (http://www.mutationtaster.org) was applied to evaluate the disease-causing potential of DNA variants (Schwarz et al., 2014). The transcript ID of the LDLR was ENST00000558518 (NCBI Reference Sequence: NM_000,527.5) in the manuscript.

Sanger Sequencing

The PCR amplification method was utilized to amplify the participants' DNA. PCR primers were designed based on the exon fragment where the mutation was located. The sequences of primers were shown as follows: *LDLR* (exon 3): 5′-TGACAGTTC AATCCTGTCTCTTCTG (upstream), 5′-ATAGCAAAGGCA GGGCCACACTTAC (downstream); *LDLR* (exon 7): 5′- AGT CTGCATCCCTGGCCCTGCGCAG (upstream), AGGGCT CAGTCCACCGGGGAATCAC (downstream) (Du and Huang, 2007). Sanger sequencing was conducted to verify the variants in each participant by the Biosystems® 3730 DNA analyzer. The sequencing results were analyzed by the Chromas software.

Cell Culture and Plasmid Transfection

HEK293 cells were derived from the cell bank of the Shanghai Chinese Academy of Sciences and were cultured in a DMEM medium (Hyclone, United States) with 10% fetal bovine serum (ThermoFisher Scientific, United States). The DMEM used in the current study included 4.00 mmol/L L-glutamine, 4500 mg/L glucose, and sodium pyruvate. After the cells were grown to about 80–90% in a 37 °C incubator containing 5% CO_2, they were subcultured at a ratio of 1:3.

For plasmid transfection, HEK293 cells in good growth condition were plated in a six-well plate. In this study, wild-type (WT) and mutant plasmids were constructed by chemical synthesis. Lipofectamine 2000 reagent (ThermoFisher Scientific, United States), Opti-MEM medium (Gibco, United States), and plasmid DNA were mixed and incubated at room temperature and then added to the cells. The cells were cultured in 5% CO_2 at 37 °C for 6–8 h.

We divided the cells into the mutant group (HEK293 cells transfected with LDLR mutant plasmids), the WT group (HEK293 cells transfected with LDLR wild-type plasmids), and the NC group (HEK293 cells transfected with empty plasmids).

Expression of LDLR Variants

The expression level of LDLR variants was detected by Western Blot 48 h after plasmid transfection as previously described (Hu et al., 2021). The samples were lysed in RIPA buffer (Solarbio, China) containing protease and phosphatase inhibitors. Protein levels were quantified by the BCA protein assay kit (Cwbio, China). The samples containing equal amounts of protein were separated by 5x SDS-PAGE gel and transferred onto the PVDF membrane. After blocking with 5% milk, the samples were incubated with the primary antibodies: LDLR [Mouse monoclonal to LDL Receptor (Abcam, United States)] and β-actin [β-actin Rabbit mAb (Abclonal, China)] overnight at 4 °C, and secondary

TABLE 1 | Clinical data of FH patients and family members.

Characteristics	I-1	I-2	II-1
Gender	Male	Female	Female
Age (year)	52	53	27
Triglycerides (mmol/L)	1.42	1.43	0.46
Total cholesterol (mmol/L)	7.64	3.78	7.36
High-density lipoprotein cholesterol (mmol/L)	0.91	1.49	1.95
Low-density lipoprotein cholesterol (mmol/L)	5.62*	2.25	5.50
ApoA1 (g/L)	0.97	1.68	1.50
ApoB (g/L)	1.64	0.64	1.42
Lipoprotein a (mg/dl)	36.90	42.40	15.50
Carotid plaque	Yes	No	No
Carotid stenosis	No	No	No
Aortic valve Calcification	Yes	No	No
Left ventricular ejection fraction (%)	68	70	70
Corneal arcus	Yes	No	No
Xanthoma	Yes	No	No
Coronary artery disease	Yes	No	No

The asterisk indicates that the patient is taking a lipid-lowering drug (atorvastatin 20 mg).

antibodies [peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research, United States) and peroxidase-conjugated anti-rabbit IgG (Jackson Immuno Research, United States)] for 1 h at room temperature. Lastly, the signals were analyzed using the ImageJ Software.

Uptake of DiI-LDL

After treatment with a serum-free medium of 0.3% bovine albumin (Solarbio, China) for 12 h, the transfected cells were incubated with 5 μg/ml DiI-LDL (Thermo Scientific, United States) for 4 h. The labeled LDL was used to study LDL uptake through endocytosis and its trafficking throughout the cell, which can be detected via fluorescence microscopy. After washing the cells with PBS, they were fixed with 4% paraformaldehyde for 10–20 min and then stained with DAPI. The fluorescence intensity of the cells was observed using a confocal laser scanning microscope (LEICA TCS SP8, Germany). The ImageJ software was used to quantitatively analyze the fluorescence intensity.

Statistical Analysis

All statistical analyses were performed by the SPSS 24.0 software (SPSS Inc., Chicago, IL, United States). GraphPad Prism 8 (GraphPad Software, La Jolla, CA) was used for plots. $p < 0.05$ was considered statistically significant.

RESULTS

The Clinical Information of the Proband and Pedigree Investigation

A 52 year-old man was diagnosed with coronary heart disease at Ningbo First Hospital. His LDLC level was abnormally elevated at 5.62 mmol/L under the treatment of atorvastatin 20 mg. Corneal arcus and xanthomas on the skin of the elbow were observed since adolescence. The results of coronary angiography illustrated 95% localized stenosis of the proximal segment of the left anterior descending branch, 90% localized stenosis of the proximal segment of the diagonal branch, and subtotal occlusion of the proximal segment of the right coronary artery. Echocardiography displayed hypertrophy of the ventricular septum, aortic valve calcification, and mild mitral regurgitation. Carotid ultrasound showed multiple plaque formation in bilateral carotid arteries.

We tracked three members in two generations of this family, including the proband's wife and the proband's daughter. As shown in **Table 1**, the blood lipid level of the proband's wife (I-2) was normal. However, the proband's daughter (II-1) had an increased level of LDLC (5.50 mmol/L) without corneal arcus or xanthomas. Before performing DNA analysis, the proband and his daughter were diagnosed as FH, according to the DLCN criteria.

Mutational Analysis, in Silico Analysis, and Sanger Sequencing

High-throughput sequencing was performed on all members of this FH family, and the mutations of four FH-related genes (*LDLR, APOB, PCSK9, LDLRAP1*) were analyzed (Tada et al., 2019). There were five *LDLR* variants (*LDLR* c.226 G > C, c.1003 G > T, c.1413A > G, c.1617 C > T, and c.2232A > G) in the proband, five *LDLR* variants (*LDLR* c.1413A > G, c.1617 C > T, c.1773 C > T, c.1959T > C, and c.2232A > G) in the proband's wife and five *LDLR* variants (*LDLR* c.1003 G > T, c.1413A > G, c.1773 C > T, c.1959T > C, and c.2232A > G) in the proband's daughter (**Figure 1**). However, no suspicious disease-causing variant was identified in the other three genes.

The impact of *LDLR* variants on the receptor function was investigated by the bioinformatic tool Mutationtaster. The results revealed that *LDLR* c.1413A > G, c.1617 C > T, c.1773 C > T, c.1959T > C, and c.2232A > G were synonymous mutations, while *LDLR* c.226 G > C and c.1003 G > T were missense mutations. The amino acid sequences of these two sites were highly conserved in various species (**Supplementary Figure S1**).

In addition, both variants could cause changes in amino acid sequence. LDLR c.226 G > C (exon 3) caused the 76th amino acid to change from glycine to arginine, namely p. Gly76Arg. Meanwhile, LDLR c.1003 G > T (exon 7) caused the 335th amino acid to change from glycine to cysteine, namely p. Gly335Cys.

Subsequently, Sanger sequencing was performed to verify the existence of the two variants in the corresponding family members (**Figure 2**). The proband had two variants (*LDLR* c.226 G > C and c.1003 G > T), and his daughter had one variant (*LDLR* c.1003 G > T). Therefore, we speculate that the FH family in this study may be caused by pathogenic mutations in the *LDLR*.

The Expression of LDLR Variants and Uptake of DiI-LDL

The expression level of *LDLR* in the mutant group, WT group, and NC group was detected by Western Blot in HEK293 cells. The two bands detected in the gel map corresponded to the mature type and the precursor type LDLR, respectively. The results

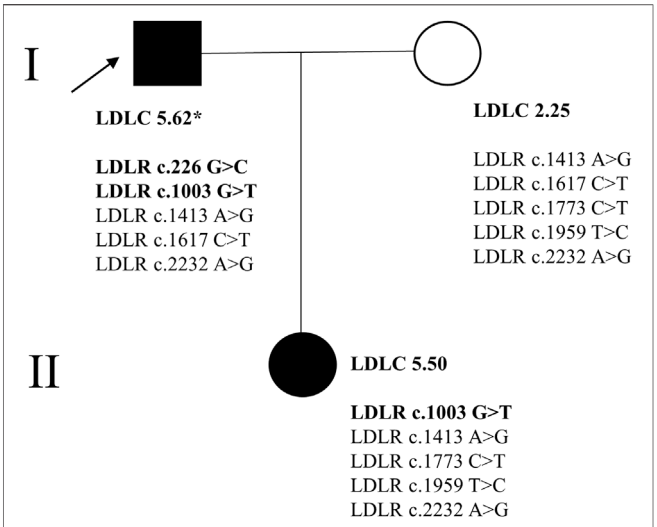

FIGURE 1 | The family tree of the proband. * signified that the patient was taking the lipid-lowering drug (atorvastatin 20 mg); → represented the proband. In the current study, the background of the FH patients' (I-1, II-1) picture was blackened. The disease-causing variants in LDLR were bolded.

demonstrated that there was no significant difference between the mutant group (*LDLR* c.226 G > C) and the WT group (**Figure 3**). Compared with the WT group, less mature LDLR was detected in the *LDLR* c.1003 G > T group.

The HEK293 cells carrying the *LDLR* c.226 G > C and c.1003 G > T variants and the WT *LDLR* were incubated in Dil-LDL medium for 4 h. The ability of mutant LDLR (*LDLR* c.226 G > C, c.1003 G > T) to take up LDL was significantly lower than that of WT LDLR (*LDLR* WT: 100%, *LDLR* c.226 G > C: 63.04%, *LDLR* c.1003 G > T: 42.54%, $p < 0.01$, **Figure 4**, **Figure 5**).

The Correlation Between the Phenotype and the LDLR Mutation

Judging from the FH patients' clinical phenotype, the proband's blood lipid level, corneal arcus, xanthoma, and atherosclerosis were severe. According to the sequencing results, the proband was a compound heterozygote (*LDLR* c.226 G > C and c.1003 G > T), and his daughter was a heterozygote (*LDLR* c.1003 G > T). This finding partly explained that the clinical phenotype of compound heterozygous patients was more severe than that of heterozygous.

DISCUSSION

As a typical disease of abnormal cholesterol metabolism, FH is a significant risk factor in the occurrence and development of cardiovascular disease (Hu et al., 2020). With the advancement of molecular technology, some FH patients have undergone genetic testing to elucidate the pathogenic mechanism. However, the current diagnostic rate of FH in most countries is extremely low, at < 1% even. Hence, it is crucial to carry out screening of high-risk populations in order to improve the diagnostic rate. Herein, a detailed pedigree investigation and the genogram of the proband and his family members were conducted. Two *LDLR* variants (*LDLR* c.226 G > C and c.1003 G > T) were discovered in this family by whole-exome sequencing. Functional prediction through the bioinformatic software showed that both variants might impact the expression or function of LDLR. The *in vitro* analysis confirmed that *LDLR* c.226 G > C and c.1003 G > T could diminish the ability of LDLR to uptake LDL.

By consulting the literature and the UCL database on LDLR mutations (http://www.lovd.nl/LDLR), we confirmed that both variants (*LDLR* c.226 G > C and c.1003 G > T) were not previously identified. The *LDLR* c.226 G > C variant (exon 3) is located in the coding region of the LDLR ligand-binding domain, which consists of 292 amino acids, including seven repeats (Strom et al., 2020). In the process of LDL metabolism, the variants including *LDLR* c.226 G > C in the LDLR ligand-binding domain might result in the LDLR being unable to reach the cell surface or in its inability to bind to LDL. This can eventually lead to hyperlipemia (Pamplona-Cunha et al., 2020). Our functional studies corroborated that *LDLR* c.226 G > C did not affect its expression. Instead, it caused the ability of LDLR to uptake LDL to decrease. In addition, another mutation, *LDLR* c.226 G > T (p. Gly76Trp), was also found at the exact location (Bourbon et al., 2008; Usifo et al., 2012). However, the expression and LDL internalization by *LDLR* c.226 G > T were similar to the WT, which was defined as likely benign (Benito-Vicente et al., 2015). It implies that different variants in the same position may function differently.

The *LDLR* c.1003 G > T variant (exon 7) is located in the homology domain of the EGF precursor, which consists of 406 amino acids, contains three EGF-like repeat units, and one β-propeller domain (Springer, 1998). It might result in the synthesized LDLR protein not being released from the endoplasmic reticulum to the cell surface, in that the receptors that reach the cell surface cannot bind to LDL, or in that the receptor cannot be recycled (Austin et al., 2004). Jeenduan *et al.* identified that LDLR p. D151Y and M391T located in the homology domain of EGF precursor significantly reduced the expression level of LDLR on the cell surface to 18 and 38%, respectively. Additionally, the amount of LDL uptake by LDLR was reduced to 15 and 71%, respectively (Jeenduang et al., 2010).

Our study showed that the other variant *LDLR* c.1003 G > T might affect the expression of the LDLR protein and impair its ability to uptake LDL (reduced to 42.54%). Previous studies have also reported the variant *LDLR* c.1003 G > A (p. Gly335Ser), and bioinformatics predicted that this variant was likely pathogenic (Wang et al., 2001; Laurie et al., 2004; Bertolini et al., 2013; Narang et al., 2020). Interestingly, two different variants in the translation initiation codon of LDLR (*LDLR* c.1A > T and c.1A > C) encoded the same amino acid (LDLR p.Met1Leu), but they cause different degrees of damage to its expression and activity (Graca et al., 2021). Therefore, functional experiments are paramount to clarify whether the mutation is pathogenic or not.

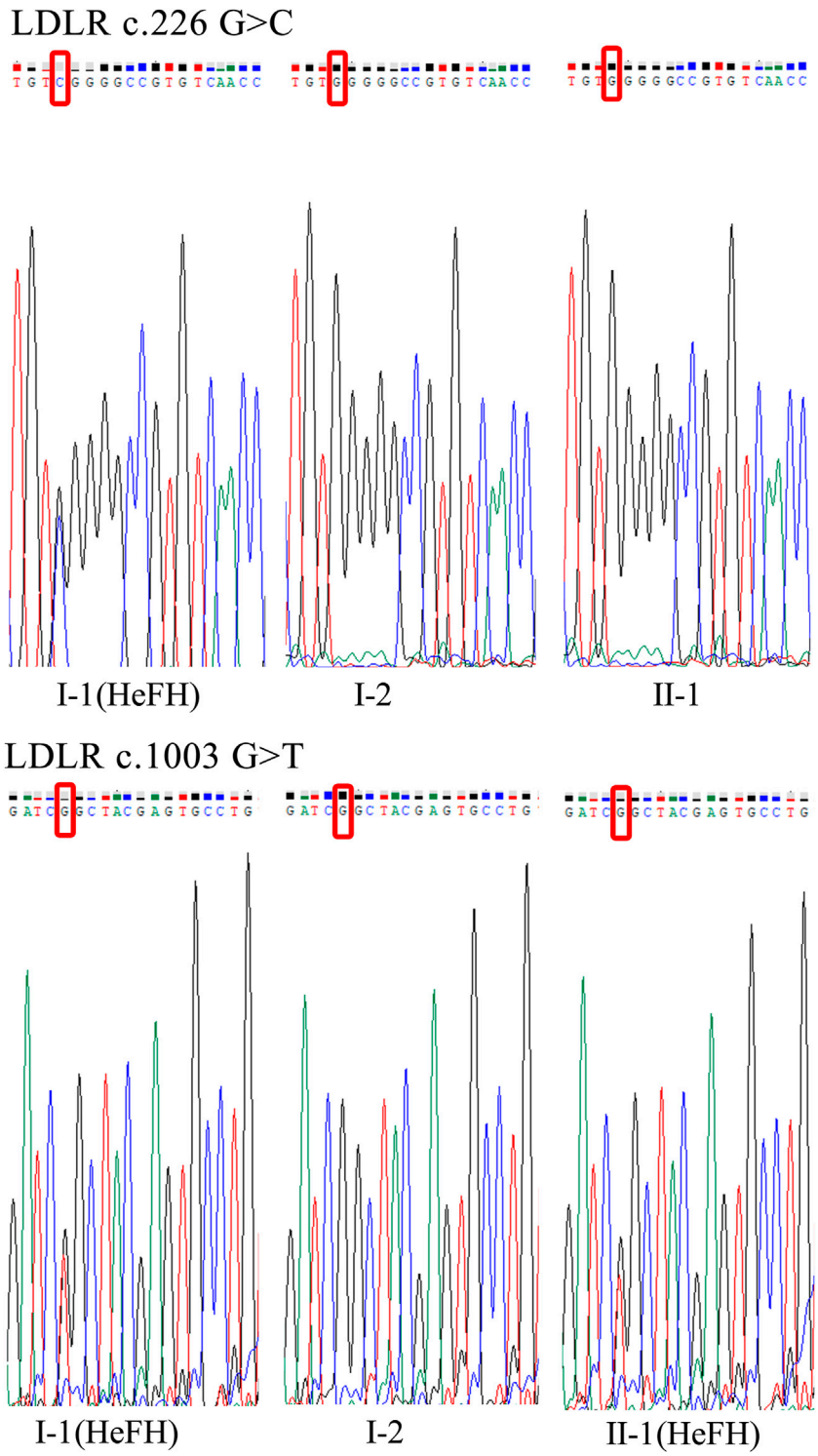

FIGURE 2 | Target sequences on LDLR by Sanger sequencing. Two disease-causing LDLR variants (LDLR c.226 G > C, c.1003 G > T) were found in the proband (I-1). The LDLR c.1003 G > T variant was found in the proband's daughter (II-1).

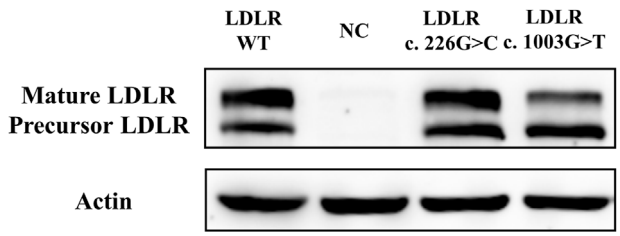

FIGURE 3 | The effect of LDLR variants on LDLR expression.

Ana Catarina Alves *et al.* found that a missense mutation (*LDLR* p. Asp601Val) might cause the loss of LDLR mature form and a complete impairment of its activity (Alves et al., 2021). The variant *LDLR* c. 2389 G > A might cause the erroneous cleavage of messenger RNA to retain the mutant LDLR in the Golgi apparatus (Shu et al., 2021). Existing evidence indicates that missense mutations in LDLR could affect its expression and cause dysfunction of LDLR protein through a variety of mechanisms. Regrettably, our research cannot identify the mechanism of the diminished ability of LDLR by two variants. Therefore, future research should focus on exploring the mechanism of FH caused by its variants.

Generally, the clinical phenotype of homozygous FH patients is more severe than that of heterozygous patients, whereby some patients might eventually suffer from cardiovascular events in adolescence or even childhood (Sanchez-Hernandez et al., 2016). Moreover, the double heterozygous carriers of autosomal

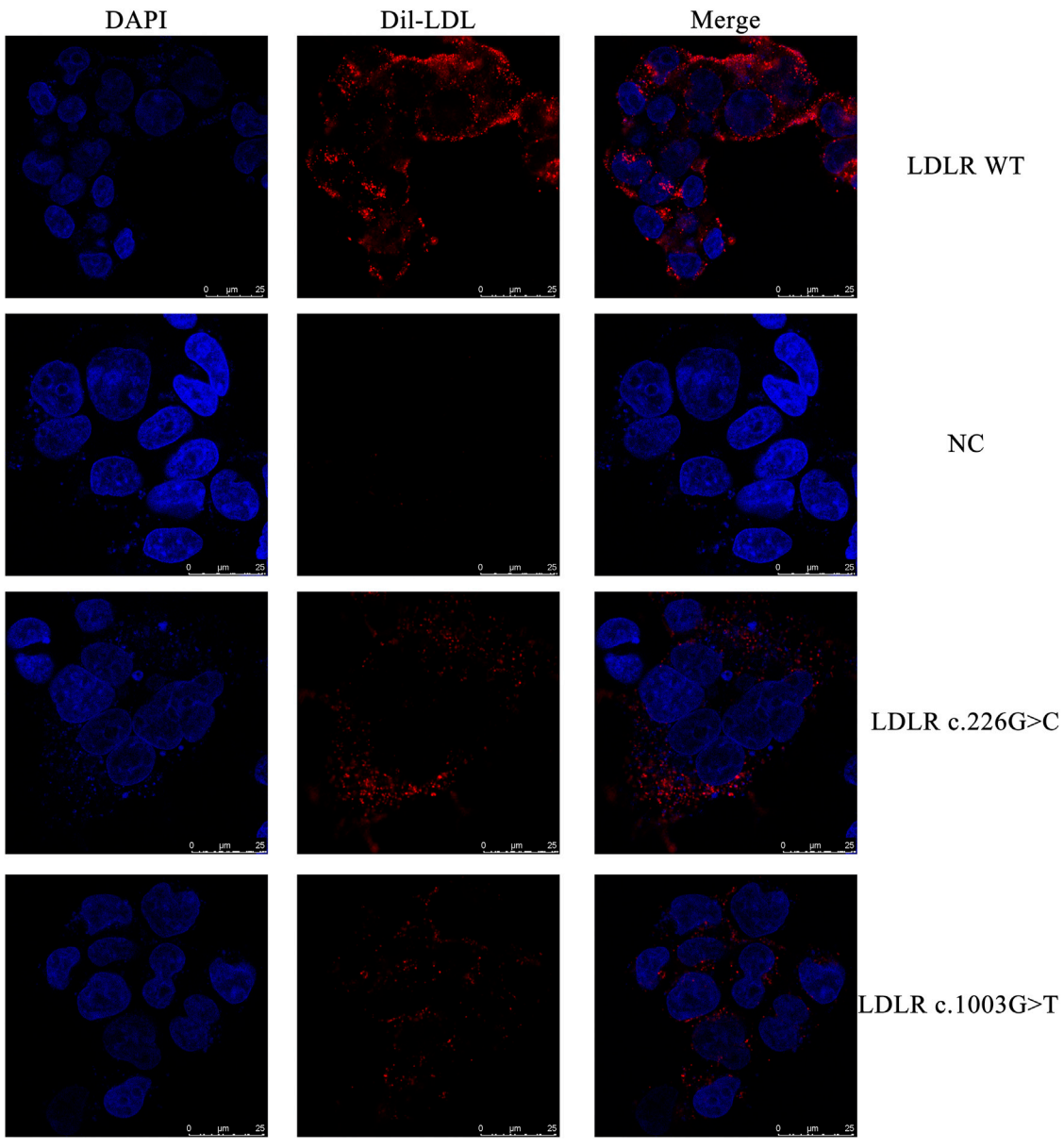

FIGURE 4 | The representative confocal microscopy images. Blue fluorescence represented DAPI, while red fluorescence represented Dil-LDL.

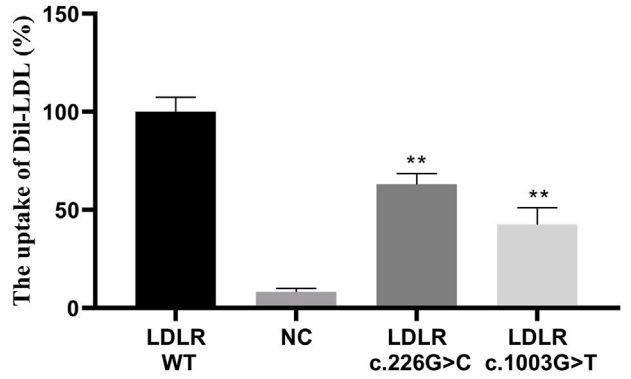

FIGURE 5 | The LDL uptake in different LDLR groups. ** indicates statistical difference between mutant group and WT group ($p < 0.01$).

dominant hypercholesterolemia gene mutations have an intermediate phenotype compared with heterozygous and homozygous/compound heterozygous carriers (Sjouke et al., 2016). In this study, the proband was a compound heterozygous FH, and his daughter was a heterozygous FH. Due to being exposed to high levels of LDLC at birth, FH patients often exhibit severe atherosclerosis with the time response and dose effect of LDLC (Ference et al., 2017). The LDLC level of the proband was exceptionally high, even after drug therapy, and there were severe manifestations such as corneal arcus, xanthomas, carotid artery stenosis, coronary artery stenosis, and aortic valve calcification. Comparatively, the daughter of the proband only had hypercholesterolemia without any other clinical phenotypes. The lipids*age was proposed as an indicator to predict the risk of arteriosclerotic cardiovascular disease (Ference et al., 2018). The influence of age should be considered on the FH patient's phenotype. Furthermore, early introduction of lipid-lowering treatment and long-term medical management could reduce the occurrence of cardiovascular events for the proband's daughter.

There are some limitations in the current study. On the one hand, only three members of one family participated in the study. On the other hand, we only detected the expression of LDLR in the whole cell lysate but did not detect the number of LDLR on the cell surface. In the future, attention should be paid to the effect of LDLR mutation on the remaining activity of LDLR to unravel the damage of LDLR mutation to its function. Finally, we mainly focused on the variants in the exon regions of LDLR in our study. It has been shown that the mutations in the intron regions of LDLR may affect the splicing of mRNA precursors and lead to the occurrence of FH (Reeskamp et al., 2021a). Further studies should be performed to explore the mechanism of LDLR intron region variation in the occurrence and development of FH.

In conclusion, two novel variants, *LDLR* c.226 G > C and c.1003 G > T, might be pathogenic for FH by causing LDLR uptake dysfunction.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because according to the requirements of the Institutional Ethics Committee, the sharing of genomic data in the public domain is not allowed. Requests to access the datasets should be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Ningbo First Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XC, SL, and HH contributed to the conception, design, and final approval of the submitted version. HH, TS, JM, SW, RC, and JW contributed to completing the experiments, writing and revising the paper. All the authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.762587/full#supplementary-material

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Low Detection Rates of Genetic FH in Cohort of Patients With Severe Hypercholesterolemia in the United Arabic Emirates

Antoine Rimbert[1†], Hinda Daggag[2†], Peter Lansberg[1], Adam Buckley[2], Martijn Viel[3], Roan Kanninga[3], Lennart Johansson[3], Robin P. F. Dullaart[4], Richard Sinke[3], Alia Al Tikriti[2], Jan Albert Kuivenhoven[1‡] and Maha Taysir Barakat[2‡]*

[1]Department of Paediatrics, Section Molecular Genetics, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands, [2]Imperial College London Diabetes Centre, Abu Dhabi, United Arab Emirates, [3]Department of Genetics, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands, [4]Department of Endocrinology, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands

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Edited by:
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Poland

***Correspondence:**
Jan Albert Kuivenhoven
j.a.kuivenhoven@umcg.nl

[†]These authors share first authorship

[‡]These authors share last authorship

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Background: Programs to screen for Familial hypercholesterolemia (FH) are conducted worldwide. In Western societies, these programs have been shown to be cost-effective with hit/detection rates of 1 in 217–250. Thus far, there is no published data on genetic FH in the Gulf region. Using United Arab Emirates as a proxy for the Gulf region, we assessed the prevalence of genetically confirmed FH in the Emirati population sample.

Materials and Methods: We recruited 229 patients with LDL-C >95th percentile and employed a customized next generation sequencing pipeline to screen canonical FH genes (*LDLR, APOB, PCSK9, LDLRAP1*).

Results: Participants were characterized by mean total cholesterol and low-density lipoprotein cholesterol (LDL-c) of 6.3 ± 1.1 and 4.7 ± 1.1 mmol/L respectively. Ninety-six percent of the participants were using lipid-lowering medication with mean corrected LDL-c values of 10.0 ± 3.0 mmol/L 15 out of 229 participants were found to suffer from genetically confirmed FH. Carriers of causal genetic variants for FH had higher on-treatment LDL-c compared to those without causal variants (5.7 ± 1.5 vs 4.7 ± 1.0; p = 3.7E-04). The groups did not differ regarding high-density lipoprotein cholesterol, triglycerides, body mass index, blood pressure, glucose, and glycated haemoglobin.

Conclusion: This study reveals a low 7% prevalence of genetic FH in Emiratis with marked hypercholesterolemia as determined by correcting LDL-c for the use of lipid-lowering treatment. The portfolio of mutations identified is, to a large extent, unique and includes gene duplications. Our findings warrant further studies into origins of hypercholesterolemia in these patients. This is further supported by the fact that these patients are also characterized by high prevalence of type 2 diabetes (42% in the current study cohort) which already puts them at an increased risk of atherosclerotic cardiovascular disease. These results may also be useful in public health initiatives for FH cascade screening programs in the UAE and maybe the Gulf region.

Keywords: familial hypercholestelemia, genetics, screening, prevalence, United Arab Emirates (UAE)

INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of death in the Middle East (Bamimore et al., 2015). Hypercholesterolemia, characterised by increased levels of low-density lipoprotein cholesterol (LDL-c), is a major established risk factor for ASCVD (Nordestgaard et al., 2013; Ference et al., 2017). It is well-established that functional mutations in *LDLR* (Brown and Goldstein, 1974), *APOB* (Innerarity et al., 1987) and *PCSK9* (Abifadel et al., 2003) can cause autosomal dominant hypercholesterolemia and that mutations in *LDLRAP1* (Ceska et al., 2019) can cause autosomal recessive hypercholesterolemia. Combined, this set of genetic disorders is generally referred to as familial hypercholesterolemia (FH). Since patients with FH are characterized by a 22-fold increased risk for ASCVD, compared to non-carriers with comparable LDL-c concentrations (Abul-Husn et al., 2016; Khera et al., 2016), knowing the aetiology of severe hypercholesterolemia is of major importance for an early diagnosis and pro-active cardiovascular healthcare.

Programs to identify patients with FH are conducted worldwide to improve management and enable early prevention through cascade screening (Vallejo-Vaz et al., 2018; Ceska et al., 2019). These initiatives (reviewed in (Knowles et al., 2017)) have been shown to reduce the average age at which individuals with genetic FH are diagnosed, to improve treatment initiation/adherence (Perez de Isla et al., 2016), and reduce LDL-c and ASCVD in a cost-effective way (Wonderling et al., 2004; Nherera et al., 2011).

The prevalence of clinically defined FH is currently estimated to be 1:217–250 across different Caucasian populations (Benn et al., 2016; de Ferranti et al., 2016). In patients who are referred to specialized lipid clinics, the proportion of genetically confirmed cases can be 50% or more depending on the inclusion criteria (Wang et al., 2016).

A reportedly high prevalence of clinical FH in the Arabian Gulf region (Al-Rasadi et al., 2018a) recently increased awareness of severe dyslipidaemias and a need for cascade screening. Several promising large-scale initiatives are currently under way, but to date publicly available data on a genetic diagnosis of FH in this part of the world are very limited (Bamimore et al., 2015; Al-Rasadi et al., 2018a).

In this study, we screened patients that were referred to the Imperial College London Diabetes Centre in Abu Dhabi (ICLDC), for LDL-C above the 95% percentile. Ninety-six percent of the patients were using lipid-lowering medication while 42% were suffering from type 2 diabetes. The mean LDL-C value corrected for lipid-lowering medication was 10 mmol/L but we could only identify a genetic origin for FH in 7% of the patients.

MATERIAL AND METHODS

Participants

229 unrelated Emiratis were recruited using the LDL-C cut-off levels used in the Gulf FH Registry final protocol conditions (https://gulfheart.org) [(Al-Rasadi et al., 2018a; Al-Rasadi et al., 2018b) and Supplemental methods]. In brief, adult patients were recruited by Imperial College London Diabetes Centre in Abu Dhabi (ICLDC), presenting with plasma LDL-c concentrations higher than 4.9 mmol/L (with or without lipid lowering treatment) and plasma triglycerides <5 mmol/L. Patients suffering from history of untreated hypothyroidism; history of proteinuria ≥1g/L; history of obstructive liver disease; history of chronic renal failure; human immunodeficiency virus infection or on immunosuppressant or steroid or psychiatric medications, were excluded. Data on family history and physical examination were not available. Ethical approval was obtained from ICLDC Research Ethics Committee. Participants were consented for research by a research patient recruitment officer and were asked for additional blood samples during their regular clinical visit to ICLDC.

TABLE 1 | Baseline parameters of 229 participants with a diagnosis of clinical FH.

	All (n = 229)
Sex	118 W, 111 M
Age at consent (years), [Mean, (±SD)]	46 (±10)
Plasma lipids	
Total Cholesterol (mmol/L), [Mean, (±SD)]	6.3 (±1.1)
LDL cholesterol (mmol/L), [Mean, (±SD)]	4.7 (±1.1)
HDL cholesterol (mmol/L), [Mean, (±SD)]	1.3 (±0.3)
Triglycerides (mmol/L), [Mean, (±SD)]	2.0 (±0.9)
Corrected LDL cholesterol (mmol/L), [Mean, (±SD)]	10.0 (±3.0)
Lipid lowering drugs[$] [n, (%)]	219 (96%)
Anthropometric data	
BMI (kg/m2), [Mean, (±SD)]	31 (±6)
BP Systolic (mmHg), [Mean, (±SD)]	125 (±17)
BP Diastolic (mmHg), [Mean, (±SD)]	74 (±10)
Glucose (mmol/L), [Mean, (±SD)]	8.3 (±4.2)
HbA1c (%), [Mean, (±SD)]	7.2 (±2.1)
Diabetes status	
Non diabetes [*n*, (%)]	81 (35%)
Pre-diabetes [*n*, (%)]	48 (21%)
Type 2 Diabetes Mellitus [*n*, (%)]	96 (42%)
Type 1 Diabetes [*n*, (%)]	4 (2%)

Legend and abbreviations: LDL-c, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; LDL-c plasma levels were calculated using Friedewald's formula (Friedewald et al., 1972); SD, standard deviation; BMI, body mass index; $, lipid-lowering medication includes atorvastatin, Rosuvastatin or Simvastatin and/or Ezetimibe; Correction factors can be found in ***Supplementary Table S1****; BP, blood pressure; HbA1c, glycated haemoglobin; On the basis of current ADA criteria (American Diabetes Associa, 2018), patients presenting HbA1c<5.7% were considered as non-diabetes, 5.7 ≤ HbA1c ≤ 6.4% as pre-diabetes and HbA1c>6.4% as type 2 diabetes mellitus.*

Molecular Genetics Analysis

All samples were analysed for autosomal dominant or autosomal recessive hypercholesterolemia (*LDLR*, *APOB*, *PCSK9*, *LDLRAP1*) using a custom targeted next generation sequencing gene panel. The analysis pipeline focused on: 1- rare genetic variants in the general population [including a control population from ME countries (Scott et al., 2016)]; 2- genetic variants affecting coding and splicing regions of targeted genes or located in promoter regions of *LDLR*. Pathogenicity of identified variants was determined using clinical genetic databases and *in silico* prediction algorithms (see **Supplemental Methods**). The targeted genes were tested for copy number variations (CNV) using the same analysis pipeline (Johansson et al., 2016; Balder et al., 2018) and validated using the Infinium Global Screening

TABLE 2 | FH mutations identified in 229 participants with clinical FH.

Patients IDs	Chrom_pos_ref_alt	Gene Symbol	Coding	Protein	Automatic pathogenic	ClinVar	Predicted Damaging	Publishedevidences
53	Chr19_11216114_G_A	*LDLR*	c.532G > A	p.Asp178Asn			Yes	
	Chr19_11217274_G_A	*LDLR*	c.728G > A	p.Cys243Tyr			Yes	
99	Chr19_11224051_C_G	*LDLR*	c.1284C > G	p.Asn428Lys			Yes	
218	Chr19_11224281_G_A	*LDLR*	c.1429G > A	p.Asp477Asn			Yes	
352	Chr19_11200090_C_G	*LDLR*	c.-135C > G			Yes		Yes
391	Chr19_11221366_C_T	*LDLR*	c.979C > T	p.His327Tyr			Yes	
423	Chr1_55509631_T_G	*PCSK9*	c.323T > G	p.Leu108Arg		Yes		
	Chr19_11224281_G_A	*LDLR*	c.1429G > A	p.Asp477Asn			Yes	
622	Chr19_11224061_C_G	*LDLR*	c.1294C > G	p.Leu432Val			Yes	
634	Chr19_11226789_TG_T	*LDLR*	c.1610delG	p.Gly537fs	Yes			
662	Chr2_21224256–21286077_Dup	*APOB*			Yes			
689	Chr19_11224407_C_T	*LDLR*	c.1555C > T	p.Pro519Ser		Yes		
720	Chr19_11216085_ACAACGAC_A	*LDLR*	c.505_511delAACGACC	p.Asn169fs	Yes			
750	Chr19_11224233_G_A	*LDLR*	c.1381G > A	p.Gly461Ser				Yes
808	Chr19_11224407_C_T	*LDLR*	c.1555C > T	p.Pro519Ser		Yes		
813	Chr19_11200105_C_T	*LDLR*	c.-120C > T			Yes		Yes
851	Chr19_11215960_C_A	*LDLR*	c.378C > A	p.Phe126Leu			Yes	

Legend and abbreviations: Genomic coordinates of identified variants are reported with Chromosome, Position, Reference Allele and Alternative Allele observed (Chrom_Pos_Ref_Alt) related to GRCh37 genome Human built 19. All variants listed are heterozygous. LDLR, low density lipoprotein receptor (NM_000527); PCSK9, *Proprotein convertase subtilisin/kexin type 9 (NM_174936.3); UTR, untranslated regions; LDL-c, low density lipoprotein cholesterol, M, males; F, females; dup, duplication. LDL-c plasma levels were corrected using the Friedewald's formula (Friedewald et al., 1972).* LDLR, *p.Cys243Tyr, p.Asn169fs and p.Gly537fs and* APOB_*dup are reported for the first time.*

Array (Illumina®). Full details of the technical approach are provided in the supplementary methods.

Statistics

T-test was used to compare the lipid parameters between carriers of causal variants in *LDLR, APOB* and *PCSK9* and non-carriers and Chi^2 test was used to compare the proportion of participants between subgroups.

RESULTS

Characteristics of Study Participants

Baseline characteristics of the 229 participants (118 women and 111 men) are shown in **Table 1**. Mean age of the participants at consent was 46 years (±10). Patients presented with average body mass index of 31 (±6). On treatment plasma lipids and lipoproteins were as follows: Total cholesterol 6.3 mmol/L (±1.1), LDL-c: 4.7 mmol/L (±1.1), High-density lipoprotein cholesterol (HDL-c): 1.3 mmol/L (±0.3); Triglycerides: 2.0 mmol/L (±0.9). Ninety-six percent of the patients were using lipid-lowering drugs (Rosuvastatin (n = 106), Atorvastatin (n = 71), Pitavastatin (n = 6); Simvastatin (n = 2), Rosuvastatin+Ezetimibe (n = 22), Atorvastatin+Ezetimibe (n = 10), Simvastatin+Ezetimibe (n = 2); and Ezetimibe (n = 1). When corrected for the reported use of lipid-lowering drugs (conversion factors are given in **Supplementary Table S1**), the corrected LDL-c was on average 10 mmol/L (±3.0).

Genetics of Hypercholesterolemia

Targeted sequencing rendered a mean coverage depth of 678X (±263) for each base with 98% (±0.01) of the targeted regions covered at least 30 times. This result allowed for an efficient and robust detection of heterozygous variants as well as copy number variations calling.

Prevalence of Genetic FH

Fifteen out of 229 participants (7%) were diagnosed with genetically defined FH (FH^+). Thirteen variants were located in coding (missense or frameshift mutations) or promoter regions of *LDLR* gene; one in *APOB* and one in *PCSK9* (**Table 2**). Two rare causal variants were identified only twice which does not support a hypothesis of large impact of founder mutations in Emiratis, as is the case in the Lebanese population (Abifadel et al., 2009). We did not observe known causal rare variants in the *APOB* gene which in Western countries accounts for 13% of the FH cases (Abul-Husn et al., 2016; Khera et al., 2016) but we did identify 28 rare *APOB* variants of unknown clinical significance (**Supplementary Table S2**). Follow-up studies are needed to better understand and determine the potential causality of these variants.

Novel Mutations

Of the mutations identified, none were previously reported in the Middle East so far (Bamimore et al., 2015). Among the fifteen identified causative FH mutations, *LDLR* p. Asn169fs, p. Cys243Tyr and p. Gly537fs are reported for the first time worldwide according to current publicly available information.

Compound Heterozygous FH

Two participants were identified as compound heterozygotes. The first case (**Table 2**; ID:#53), a 41 years old woman with LDL-c of 9.4 mmol/L on lipid-lowering treatment (Simvastatin 20 mg + Ezetimibe 10 mg) (corrected LDL-c = 18.8 mmol/L), carried two mutations in *LDLR* (p.Asp178Asn and p.

TABLE 3 | Differences between participants with (FH^+) and without (FH^-) rare causal variants in *LDLR*, *APOB*, and *PCSK9*.

	FH^- (n = 214)	FH^+ (n = 15)	P value
Sex	112 W, 102 M	6 W, 9 M	0.51
Age at consent (years), [Mean, (±SD)]	46 (±10)	41 (±11)	0.08
Plasma lipids			
Total Cholesterol (mmol/L), [Mean, (±SD)]	6.2 (±1.1)	7.2 (±1.8)	0.0021
LDL cholesterol (mmol/L), [Mean, (±SD)]	4.7 (±1.0)	5.7 (±1.5)	0.00037
HDL cholesterol (mmol/L), [Mean, (±SD)]	1.3 (±0.3)	1.2 (±0.3)	0.28
Triglycerides (mmol/L), [Mean, (±SD)]	2.0 (±0.9)	1.7 (±0.7)	0.18
Corrected LDL cholesterol (mmol/L), [Mean, (±SD)]	9.8 (±2.7)	13.9 (±4.5)	0.00000021
Lipid lowering drugs[$] [*n*, (%)]	204 (95%)	15 (100%)	NA
Anthropometric data			
BMI (kg/m2), [Mean, (±SD)]	31 (±6)	30 (±4)	0.46
BP Systolic (mmHg), [Mean, (±SD)]	125 (±17)	120 (±13)	0.23
BP Diastolic (mmHg), [Mean, (±SD)]	74 (±10)	72 (±7)	0.48
Glucose (mmol/L), [Mean, (±SD)]	8.4 (±4.2)	7.2 (±4.1)	0.29
HbA1c (%), [Mean, (±SD)]	7.2 (±2.1)	6.6 (±2.0)	0.36
Diabetes repartition			
Non diabetes [*n*, (%)]	74 (35%)	7 (47%)	0.34
Pre-diabetes [*n*, (%)]	45 (21%)	3 (20%)	0.92
Type 2 Diabetes Mellitus [*n*, (%)]	91 (42%)	5 (33%)	0.49
Type 1 Diabetes [*n*, (%)]	4 (2%)	0 (0%)	NA

Legend and abbreviations: FH, familial hypercholesterolemia; FH^- non genetically defined FH, FH^+, genetically defined FH; SD, standard deviation; BMI, body mass index; LDL-c, low-density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; $: lipid-lowering medication includes atorvastatin, rosuvastatin or simvastatin and/or Ezetimibe. Correction factors can be found in ***Supplementary Table S1****. LDL-c plasma levels were calculated using the Friedewald's formula (Friedewald et al., 1972). NA, non applicable. T-test was used to compare the lipid parameters between FH mutation carriers and non-carriers and Chi2 test was used to compare the proportion of participants between groups.*

Cys243Tyr). The genetic analysis performed does not allow for determining if these mutations are located on the same allele. The second case (**Table 2**; ID:#423), a 45 years old man with LDL-c of 6.8 mmol/L on lipid-lowering treatment (Rosuvastatin 20 mg + Ezetimibe 10 mg) (corrected LDL-c = 18.2 mmol/L), carried a variant in *LDLR* (p.Asp477Asn) and one in *PCSK9* (p.Leu108Arg), both previously reported as FH causal mutations.

Duplication of APOB

Here we report a duplication of the entire gene *APOB* in a 29 year old man (**Table 2**; ID:#662) with LDL-c of 6.5 mmol/L under lipid-lowering treatment (Rosuvastatin 20 mg; corrected LDL-c = 17.5 mmol/L). This ~1.2 Mb long duplication [chr2: 21,130,084–22,324,616 (Hg19)] has been validated using Global Screening Array (**Supplemental Methods** and **Supplementary Figure S1**). Whether 3 *APOB* gene copies can cause FH is topic of on-going studies, but this specific case illustrates the potential of next generation sequencing to improve our understanding of previously unexplained FH.

FH^- Versus FH^+

Compared to FH^-, the FH^+ group had significantly higher total cholesterol (7.2 vs. 6.2 mmol/L; $p = 2.1e-03$), on treatment LDL-c (5.7 vs 4.7 mmol/L; $p = 3.7e-04$), and corrected LDL-c (9.8 mmol/L (±2.7) vs. 13.9 mmol/L (±4.5); $p = 2.1E-07$). The FH^- and FH^+ group were similar with regard to gender distribution, age, BMI, blood pressure, plasma glucose concentration, HbA1c, HDL-c, and triglycerides plasma levels (**Table 3**).

Percentages of Individuals With FH Within Different LDL-C Ranges

We further evaluated the proportion of patients with FH within different LDL-c ranges using on-treatment or corrected LDL-c values. We observed that the chance of identifying monogenic origins of hypercholesterolemia only tends to increase with on treatment LDL-c levels (**Figure 1A**). In a second step, we used LDL-c values, corrected for lipid-lowering medication with the following cut-offs: <8 mmol/L, >8 to 12 mmol/L, >12 mmol/L (**Figure 1B**). Here, we show that the chances of finding patients with genetic FH increase significantly over the three groups ($p = 3.6E-3$). In the group with corrected LDL-c above 12 mmol/L, 21% was found to be mutation positive.

DISCUSSION

The current study demonstrates that in the studied cohort of Emirati participants, we have been able to identify a genetic origin of FH in 1 out of 15 individuals with an LDL-c>95th percentile.

It is tempting to speculate that the low prevalence of FH in our study is related to the fact that these patients are characterized by not only hypercholesterolemia but also increased triglycerides and low HDL-c and recruited at the Imperial College London Diabetes Centre in Abu Dhabi, a specialised diabetes centre. In

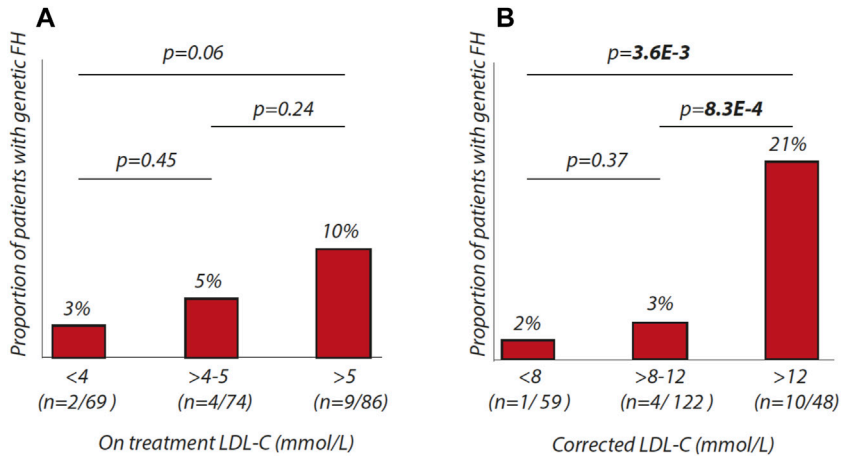

FIGURE 1 | Percentages of patients with genetic FH in different LDL-C ranges. **(A)** proportion of patients with genetic FH according to on-treatment LDL-C ranges (<4 mmol/L, > 4 to 5 mmol/L, > 5 mmol/L). **(B)** proportion of patients with genetic FH according to corrected-LDL-C ranges (<8 mmol/L, > 8 to 12 mmol/L, > 12 mmol/L).

our study, participants have an average BMI of 31, fasting glucose of 8.3 mmol while 57 % were on anti-diabetic medication. Similar characteristics were seen in the Gulf FH registry (Al-Rasadi et al., 2018b). We believe, however, that it is unlikely that inclusion of patients with pre-diabetes or type 2 diabetes in our study can explain the low prevalence of genetic FH compared to studies in Western countries as insulin-resistance is generally not characterised by high LDL-c concentrations but increased levels of triglycerides and decreased levels of HDL-c (Feingold et al., 1992). On the other hand, carriers of FH causal variants are typically characterized by an isolated high LDL-c. It appears that FH causing variants in our study confer classic hypercholesterolemia on top of an unfavourable metabolic plasma lipid phenotype. This is illustrated by the fact that FH^+ and FH^- patients are similar when it comes to age, BMI, blood pressure, high sensitivity CRP, plasma glucose concentration, HbA1c, HDL-c, and triglycerides plasma levels. This observation may warrant attention as patients referred to a diabetes centre can also suffer from hypercholesterolemia which likely puts them at an even higher risk of ASCVD which is especially true for identified with causal mutations in LDL genes.

Taken the lifelong genetic burden of increased LDL-c in the 15 identified FH^+ patients combined with their detrimental metabolic phenotype; the cardiovascular consequences are anticipated to be more serious than observed in classical FH. The relatively young age of our study cohort (45 yrs), however, makes it difficult to evaluate their atherosclerotic burden and cardiac complications but it is noteworthy that ASCVD-associated mortality rate in the Middle East is one of the highest worldwide while the mean age of patients suffering from myocardial infarction in this part of the world is 10–12 years younger when compared to Western countries (Ramahi, 2010; Gehani et al., 2014).

This brings us to the FH^- patients for which there is no explanation for their hypercholesterolemia. Since in this study FH^+ and FH^- patients have similar metabolic phenotypes, it appears unlikely that this trait would drive hypercholesterolemia in FH^- patients. Having excluded causal canonical gene defects, one can imagine that a better understanding of factors driving hypercholesterolemia in our FH^- Emirati cohort may provide novel insights into lipid metabolism. Additional studies are in this regard needed to address the origin of hypercholesterolemia in the FH^- patients to search for novel genes or other factors that can explain their phenotype.

Our study presents limitations. First of all, we had to account for the absence of pre-treatment LDL-c levels while 96% of our study participants were using lipid-lowering medication. Using widely used adjustment criteria for various lipid-lowering drugs (Wensel et al., 2010; Haralambos et al., 2015), we calculated pre-treatment LDL-c levels to be on average 10 mmol/l in our study cohort which we consider a solid basis to start screening for mutations in genes involved in LDL metabolism. To illustrate this point, the DLCN indicates that only LDL-c values above 8.5 mmol/l can be categorized as probable FH. This suggests that even in the case that adjustments would overestimate pre-treatment LDL-c levels, it is likely that true pre-treatment LDL-c levels are still well above 8 mmol/l. Our study also provides support that use of the corrections for LDL-c values are valid as we show that the chance of finding a mutation in LDL candidate genes increases significantly over three strata of estimated pre-treatment LDL-c (**Figure 1**). We were furthermore unable to retrieve information on family history and physical characteristics to complete the DLCN score. These parameters would, however, only have increased the DCLN scores which would further underline the need for molecular diagnostics in our cohort.

In conclusion, this study into the genetics of hypercholesterolemia in the selected Emirati population shows that chances of finding FH causing mutations are low (7%) when standard criteria are used, despite marked hypercholesterolemia. Taking into consideration the limitations of the study, further investigations are needed to explain the discrepancy with similar

studies in Western societies. The study, however, warrants further consideration in regard to FH screening initiatives in the Gulf region and further highlights the health risks in both FH^- and FH^+ individuals with hypercholesterolemia, obesity and type 2 diabetes.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

This study was reviewed and approved by ICLDC Research Ethics Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HD and AA collected data; AR, MV, RK, and LJ were involved in the analysis; AR, RD, AB, HD, PL, RS, and JK were involved in design of the study, data interpretation and drafting paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.809256/full#supplementary-material

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Familial Hypercholesterolemia Prevalence Among Ethnicities—Systematic Review and Meta-Analysis

Frida Toft-Nielsen[1,2], *Frida Emanuelsson*[1] and *Marianne Benn*[1,2]*

[1]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark, [2]Department of Clinical Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

Background: Heterozygous familial hypercholesterolemia (FH) is a common genetic disorder leading to premature cardiovascular disease and death as a result of lifelong high plasma low-density lipoprotein cholesterol levels, if not treated early in life. The prevalence of FH varies between countries because of founder effects, use of different diagnostic criteria, and screening strategies. However, little is known about differences in FH prevalence according to ethnicity. We aimed to investigate the ethnic distribution of FH in diverse populations and estimate the prevalence of FH according to ethnicity.

Methods: We performed a systematic review and meta-analysis, searching PubMed and Web of Science for studies presenting data on the prevalence of heterozygous FH among different ethnicities in non-founder populations. Studies with more than 100 individuals, relevant data on prevalence, ethnicity, and using the Dutch Lipid Clinical Network Criteria, Simon Broome, Making Early Diagnosis Prevents Early Death, genetic screening, or comparable diagnostic criteria were considered eligible for inclusion.

Results: Eleven general population studies and two patient studies were included in a systematic review and 11 general population studies in a random-effects meta-analysis. The overall pooled FH prevalence was 0.33% or 1:303 in 1,169,879 individuals (95% confidence interval: 0.26–0:40%; 1:385–1:250). Included studies presented data on six ethnicities: black, Latino, white, Asian, brown, and mixed/other. Pooled prevalence was estimated for each group. The highest prevalence observed was 0.52% or 1:192 among blacks (0.34–0.69%; 1:294–1:145) and 0.48% or 1:208 among browns (0.31–0.74%; 1:323–1:135) while the lowest pooled prevalence was 0.25% or 1:400 among Asians (0.15–0.35; 1:500–1:286). The prevalence was 0.37% or 1:270 among Latino (0.24–0.69%; 1:417–1:145), 0.31% or 1:323 among white (0.24–0.41%; 1:417–1:244), and 0.32% or 1:313 among mixed/other individuals (0.13–0.52%; 1:769–1:192).

Conclusion: The estimated FH prevalence displays a variation across ethnicity, ranging from 0.25% (1:400) to 0.52% (1:192), with the highest prevalence seen among the black and brown and the lowest among the Asian individuals. The differences observed suggest that targeted screening among subpopulations may increase the identification of cases and thus the opportunity for prevention.

Keywords: familial hypercholesterolemia, ethnicity, race, epidemiology, general population

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***Correspondence:**
Marianne Benn
marianne.benn@regionh.dk

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INTRODUCTION

Familial hypercholesterolemia (FH) is a genetic disorder of lipoprotein metabolism, known to increase levels of total and low-density lipoprotein (LDL) cholesterol in plasma. FH is caused by various mutations, with the majority of known mutations affecting the LDL receptor, apolipoprotein B, and proprotein convertase subtilisin/kexin 9 (Rader et al., 2003; Austin et al., 2004). Without treatment, the lifelong increased LDL-cholesterol levels result in a high risk of premature atherosclerotic cardiovascular disease and death (Nordestgaard et al., 2013a). The diagnosis of FH is confirmed either by applying one of the several clinical criteria, the most common being Dutch Lipid Clinical Network Criteria (DLCN), Simon Broome (SB), and Making Early Diagnosis Prevents Early Death (MEDPED), or by genetic screening.

In a general population setting, FH has recently been estimated to affect 1:313 individuals worldwide, making FH one of the most common genetic disorders in the world (Beheshti et al., 2020). In a Danish study investigating the general population, the estimated prevalence was 1:137 (Benn et al., 2012), suggesting that, in a general population setting, FH is underdiagnosed, emphasizing the importance of efficient FH screening to identify individuals at risk (Beheshti et al., 2020). Identification of subgroups at high risk of FH may facilitate a targeted screening worldwide.

Recent studies have shown that the prevalence of FH varies between countries because of founder effects, use of different diagnostic criteria, and differences in screening for the disease (Beheshti et al., 2020), (Hu et al., 2020). However, no previous study summarized differences in the prevalence of FH among ethnic groups (Harada et al., 2018).

We performed a systematic review and meta-analysis to examine the prevalence of FH among different ethnicities.

METHODS

Search Strategy and Screening Process

PubMed and Web of Science were searched for possible eligible studies. The last search was made on 30 December 2021. The following MeSH terms were used to search the databases: "Familial Hypercholesterolemia," "Prevalence," and "Ethnicity." In the Web of Science database, two separate search strategies were used: "Familial Hypercholesterolemia,"

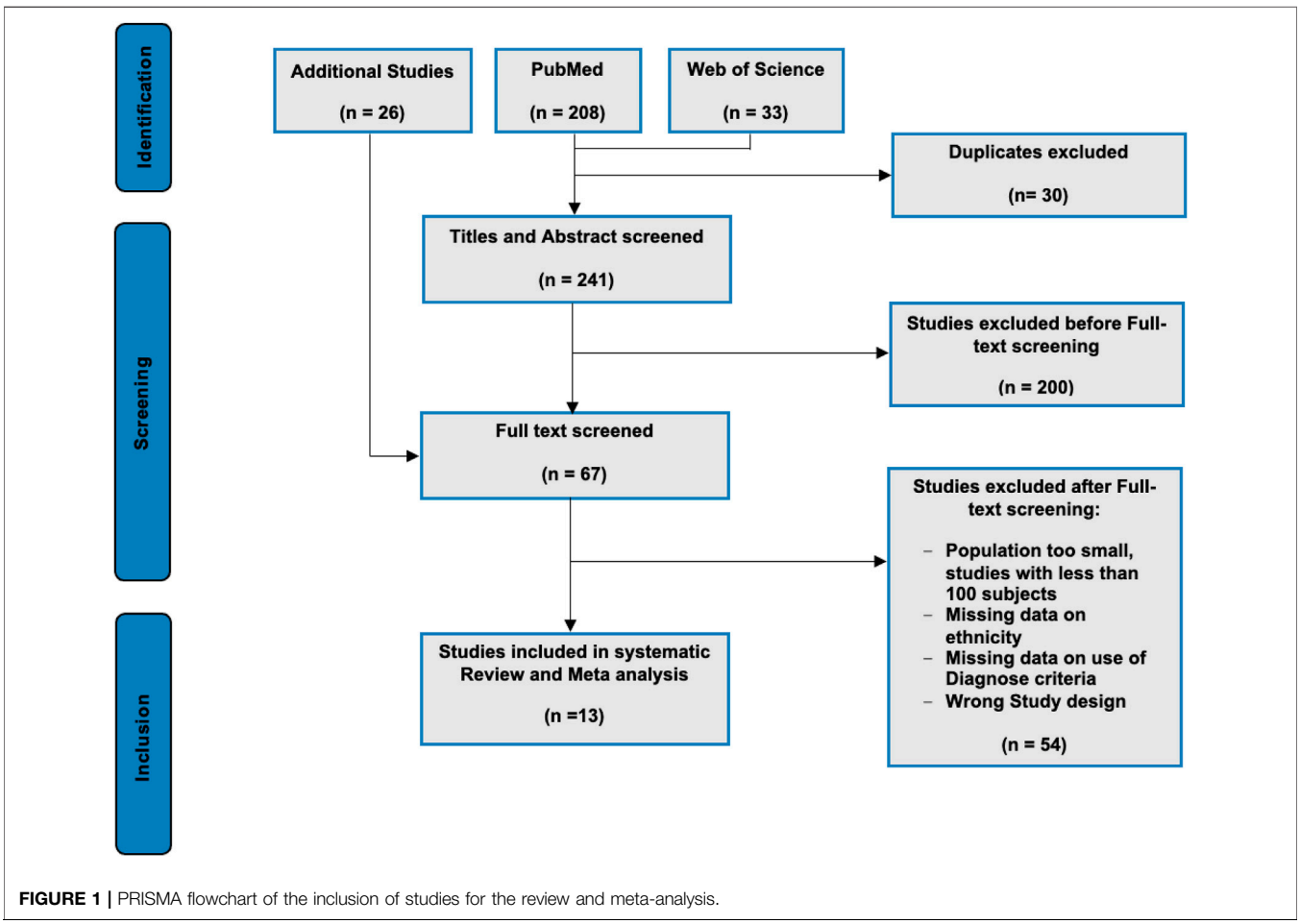

FIGURE 1 | PRISMA flowchart of the inclusion of studies for the review and meta-analysis.

"Prevalence," and "Ethnicity"; and "Familial Hypercholesterolemia," "Prevalence," and "Ethnic groups" (**Figure 1**).

Data were managed in the systematic review software Covidence (Covidence, 2021). Duplicates were removed, and the studies were initially screened based on the study's title/abstract, and irrelevant publications were excluded (**Figure 1**). Systematic reviews and meta-analyses were excluded, and only full-research articles were included for further screening. The remaining publications were subsequently full-text-screened. Additionally, we screened the reference lists from excluded articles/studies with relevant data, and qualified studies were added directly to the full-text screening.

Studies were defined as eligible if they met any of the following pre-defined inclusion criteria:

1) The study cohort: cohorts representative of the general population and patient cohorts with more than one ethnicity were defined as eligible.
2) The size of the study cohort: only studies of a minimum of 100 subjects were included.
3) The use of the following diagnostic criteria: DLCN, SB, MEDPED, genetic screening, or similar criteria where all individuals in the study were diagnosed using the same criteria.
4) Information on ethnicity.
5) Reporting FH prevalence: the prevalence of individuals diagnosed with FH in the full sample and among the different ethnicities was either reported or could be calculated from the available data.
6) Language: only English language studies were included.

Studies that did not meet the inclusion criteria or that met any of the following exclusion criteria were excluded:

1) Deficient data on the ethnic distribution in the study cohort or unclear definition of the diagnostic criteria used.
2) Studies with FH screening of populations with known founder mutations because the FH prevalence in these populations is known to be higher among specific subpopulations.

In cases where more than one study used the same cohort, we included the publication with the most available data on ethnicity and FH prevalence. An attempt was made to contact authors if relevant studies were lacking important data.

The included studies were divided into two categories: "general population" and "patient cohorts." Studies were categorized as patient studies if the subjects in the cohort were included from lipid clinics and hospitals or if the subjects were included based on elevated lipid levels or premature coronary artery disease. Studies with subjects included from the general population, stated by the authors, were categorized as general population studies. The following data were extracted: author, publication year, country, sex, mean age, diagnostic criteria used, total number of subjects, overall FH prevalence, the ethnic distribution of the full study cohort (black, Latino, white, brown, Asian, mixed/other), the prevalence of FH among ethnicities (**Table 1**).

Diagnostic Criteria for Familial Hypercholesterolemia

Studies classifying FH according to either DLCN, SB, MEDPED, similar clinical criteria, or genetic examination were included. The DLCN determines the likelihood of an individual having FH based on family history, own clinical history, a physical examination, LDL-cholesterol concentration, and a genetic examination. Depending on the total score, individuals are categorized into having unlikely FH (score <3), possible FH (score 3–5), probable FH (score 6–8), or definite FH (score ≥8). In the present review and meta-analysis, we considered individuals with probable and definite FH (score ≥6) as having FH (Nordestgaard et al., 2013a) (**Supplementary Table S1**).

The SB criteria predict the risk of an individual having FH based on clinical and genetic factors and family history (**Supplementary Table S2**). MEDPED determines the probability of a patient having FH based on age, family history, and total cholesterol levels (Birnbaum et al., 2021) (**Supplementary Table S3**).

Ethnicity

In order to compare prevalence among ethnicities, the following six groups were defined: black, Latino, white, brown, Asian, mixed/other, on behalf of the available data. The ethnicities presented in each study were then allocated into one of the defined groups.

Prevalence

Data were summarized using prevalence estimates to facilitate the comparison between different ethnicities. If the prevalence was not directly available for data extraction, it was calculated from the size of the total study cohort, the number of individuals with FH, and ethnic distribution in the study. Confidence intervals for prevalence were calculated by score (Wilson) (Nyaga et al., 2014).

Statistical Analyses

We used StataSE 16.1. to examine differences in prevalence among ethnicities and the *metaprop* command to estimate the prevalence of studies combined (Nyaga et al., 2014). Two-sided *p*-values for the difference between the overall population prevalence compared to the prevalence in each ethnic group were calculated using the *prtest* of proportions in Stata, where p-values < 0.05 were considered statistically significant (Acock, 2018). In the meta-analysis, between-study heterogeneity was assessed by I (Rader et al., 2003) statistics (Higgins et al., 2003). A random-effect model was chosen to accommodate potential between-study heterogeneity due to the inclusion of studies using different inclusion criteria.

RESULTS

Of the 267 screened publications, a total of 13 studies, comprising 1,175,249 individuals, were included (**Figure 1**). Characteristics

TABLE 1 | Characteristics of all studies included in the systematic review and meta-analysis.

Study	Author	Year	Country	Source	Mean age	Sex, %	FH criteria	Sample size, N	Total FH cases, N	Total prevalence, %	Ethnicities	Prevalence and ethnicity %
Studies including more than one ethnicity												
NHANES de Ferranti et al. (2016)	De Ferranti et al.	2016	United States	GP	46.8 years	F: 51.4 M: 48.6	DLCN	36,949	148	0.4	Black, Latino, white, mixed/ other	Black: 0.46 Latino: 0.37 white: 0.40 mix./ oth.: 0.28
ELSA-Brasil Harada et al. (2018)	Harada et al.	2018	Brasil	GP	HeFH: 55 years	F: 54 M: 46	DLCN	14,460	55	0.38	16% black, 53% white, 29% mixed	Black: 0.67, white: 0.25, brown: 0.48
The Cape Town Experience Firth and Marais (2008)	Firth et al.	2008	South Africa (Cape Town)	Patients from a lipid clinic	HeFH: 44 years	F: 53 M: 47	Clinical equal to modified DLCN	4,494	1,029	23	Black, white, Asian colored	Black: 4.90, white: 32.2, Asian: 19.8 mix./oth.: 17.2
MyHEBAT FH Study, Malaysia Chua et al. (2021)	Chua et al.	2021	Malaysia	GP	53.7 years	F: 62.6 M: 38.4	DLCN	5,135	55	1.1	Malay, Chinese, Indian, Others	Asian: 1.2%, Others: 0.4%
The Young MI Registry Singh et al. (2019)	Singh et al.	2019	United States	Young adults with MI	45 years	F: 19.1 M: 80.9	DLCN	1,996	180	9	Black, hispanic/ Latino, white, Asian, mixed/ other	Black: 9.8, Latino: 14.0.98 white: 8.79, Asian: 4.3 mix./oth. : 8.1
Studies including one ethnicity												
Copenhagen City Heart Study, DK Tybjaerg-Hansen et al. (2005)	Tybjærg-Hansen et al.	2005	Denmark	GP	53 years	F: 55 M: 45	DLCN	9,255	15	0.17	100% white	White: 0.17
Copenhagen General Population Study Benn et al. (2016)	Benn et al.	2016	Denmark	GP	NA	F: 55 M: 45	Genetic	98,098	450	0.46	100% white	White: 0.46
LIFE Child Health Cohort, GE Dathan-Stumpf et al. (2016)	Dathna-Stumpf et al.	2016	Germany	GP	0–16 years	F:52.3 M: 47.7	Clinical HeFH	2,571	6	0.23	Caucasian	White: 0.23
MyCode cohort, US Abul-Husn et al. (2016)	Abul-Husn et al.	2016	United States	GP	61 years	F: 59.2, M: 40.8	Genetic	43,979	172	0.39	98.4% Caucasian	White: 0.39
Allina Health ambulatory Facility, US Knickelbine et al. (2016)	Knickelbine et al.	2016	United States	GP	FH:53 No FH: 54.1 years	F: 55 M: 45	Clinical National Lipid Assoc. Guidelines	391,166	841	0.21	90% white	White: 0.21
Jiangsu Nutrition Study Wijesekera et al. (2014)	Shi et al.	2014	China	GP	57 years	NA	DLCN	9,324	26	0.28	Asian	Asian: 0.28
Kumamoto Health Care, Japan Ohta et al. (2002)	Ohta et al.	2002	Japan	GP	18 months	NA	Clinical HeFH family study	56,181 adj. size: 47,877	91	0.19	Asian	Asian: 0.19
Korean Meta. Syndrome Mortality Study Jung et al. (2018)	Jung et al.	2018	Korea	GP	44.3 years	F: 42.6 M: 57.4	MEDPED	502,966	540	0.11	Asian	Asian: 0.19

GP, general population; F, female; M, male; HeFH, heterozygous familial hypercholesterolemia; DLCN, Dutch Lipid Clinic Network Criteria; MEDPED, Make Early Diagnosis Prevent Early Death; NA, not available.

of the included studies are shown in **Table 1**. Five studies reported FH prevalence among more than one ethnicity, whereas the remaining eight studies reported FH prevalence in one specific ethnicity (**Table 1**). The included studies originated from North America (four studies from the United States), Asia (four studies from China, Malaysia, Japan, and Korea), Europe (two studies from Denmark and Germany), South America (Brazil), and South Africa (Cape Town).

Studies included in the meta-analysis utilized both clinical and genetic screening to estimate FH prevalence. Seven studies applied the DLCN (or modified versions), one study applied the MEDPED, two studies applied genetic screening, and the remaining three studies applied another clinical screening method. Studies included were published between 2002 and 2019.

Eleven studies reported the prevalence of FH in general populations, including information on ethnicity, while two studies estimated the FH prevalence across ethnicity in patient cohorts (**Table 1**).

The following studies estimated the FH prevalence in more than one ethnicity: NHANES, the ELSA-Brasil study, Cape Town Experience, MyHEBAT FH study, and the YOUNG-MI Registry. NHANES represented the ethnicities: non-Hispanic black (considered as black individuals), non-Hispanic white (considered as white individuals), other race/multiracial (considered as mixed/other individuals), Mexican American, and other Hispanics (considered as Latino). The ELSA-Brasil study reported on the following ethnicities: black, white, Asian, and brown, whereas the MyHEBAT study represented Malay, Chinese, and Indian individuals (all considered Asian) and other individuals (de Ferranti et al., 2016; Harada et al., 2018; Chua et al., 2021).

The two patient cohort studies reported on the following ethnicities: black, white, Asian, and colored (considered as mixed race/other) in the Cape Town Experience; and black, Hispanic/Latino (considered as Latino), white, Asian, and mixed/other (considered as mixed race/other) in the YOUNG -MI Registry (Firth and Marais, 2008; Singh et al., 2019).

The remaining eight studies included only one ethnicity: the Copenhagen City Heart Study and the Copenhagen General Population Study with 100% white individuals, the Life-Child cohort reported all as being Caucasian, My Code Cohort as 98.4% Caucasian, and the Alina Health ambulatory as 90% white (Tybjaerg-Hansen et al., 2005; Abul-Husn et al., 2016; Benn et al., 2016; Dathan-Stumpf et al., 2016; Knickelbine et al., 2016). These individuals were all considered white. Individuals included in the Jiangsu Nutrition Study, Kumamoto Health Care, and Korean Metabolic Syndrome Mortality Study were considered Asian (Ohta et al., 2002; Wijesekera et al., 2014; Jung et al., 2018).

Prevalence of FH in Studies Including More Than One Ethnicity

Of the five included studies reporting FH prevalence among more than one ethnicity, three studies were performed in a general population setting and two studies in patient cohorts (**Table 1**). In the NHANES, the prevalence was 0.40% or 1:250 and in the ELSA-Brasil, 0.38% or 1:263 (**Figure 2**). In both NHANES and ELSA-Brasil, the prevalence of FH was high among black individuals compared to the overall population prevalence, with a prevalence in black of 0.46% or 1:249 in NHANES and 0.64% or 1:156 in ELSA-Brasil. In ELSA-Brasil, the prevalence was also higher for individuals identified as brown: 0.48% or 1: 208, compared to the overall population prevalence. In NHANES, individuals identified as mixed/other had a lower prevalence than the overall population: 0.28% or 1:357, while in ELSA-Brasil, individuals identified as white had a lower FH prevalence of 0.25% or 1:417 compared to the overall population prevalence. In the MyHEBAT study, including Asian and mixed/other individuals, the overall FH prevalence was 1.1% or 1:100, with a prevalence of 1.2% or 1:83 in Asian individuals and of 0.4% or 1: 250 in mixed/other individuals.

In the patient cohort studies, the overall reported prevalence of FH was 23% or 1:4 in the Cape Town Experience and 9% or 1:11 in the YOUNG-MI Registry (**Figure 2**). In the Cape Town Experience, the estimated prevalence among the white individuals of 32% or 1:3 was high compared to the overall population prevalence. In comparison, the prevalence in individuals identified as black (4.9% or 1:20), Asian (20% or 1: 5), and mixed/other (17% or 1:6) had a lower prevalence compared to the overall study prevalence. In the YOUNG-MI Registry, individuals identified as Latino (14% or 1:7) had a high prevalence compared to the overall FH prevalence, while Asian individuals had a low prevalence of 4.3% or 1:23 compared to the overall FH prevalence.

Pooled Prevalence of FH in General Population Studies

Eleven studies were included in the meta-analysis of general population studies, and a pooled prevalence for each of the six ethnicities (black, Latino, white, Asian, brown, and mixed/other) was calculated (**Figure 3**). The overall FH prevalence in the 1,169,879 individuals from the 11 general population studies was 0.33% or 1: 303 (95% confidence interval: 0.26–0:40%; 1:385–1:250). The pooled FH prevalence according to ethnicity ranged from 0.25% or 1:400 in Asian individuals to 0.52% or 1:192 in black individuals. The prevalence of 0.52% or 1:192 (0.34–0.69; 1:294–1:145) in black individuals was higher compared to the overall FH prevalence ($p = 0.007$), while the prevalence of 0.31% or 1:323 (0.24–0.41; 1: 417–1:244) in white individuals and 0.25% or 1:400 (0.15–0.35; 1: 500–1:286) in Asian individuals was lower compared to the overall FH prevalence ($p = 0.03$ and $p < 0.001$, respectively). The pooled prevalence of 0.37% or 1:270 (0.24–0.69; 1:417–1:145) in Latinos, 0.48% or 1:208 (0.31–0.74; 1:323–1:135) in brown individuals, and 0.32% or 1:313 (0.13–0.52%; 1:769–1:192) in individuals defined as mixed/other did not differ significantly from the overall prevalence (all $p > 0.09$).

DISCUSSION

In the current study, we estimated the overall FH prevalence to 1: 303 in the general population. This is very similar to recent

estimations presented in larger studies of 1:313 (Beheshti et al., 2020) and 1:311 (Hu et al., 2020). The pooled prevalence by ethnicity ranged from 0.25% (1:400) to 0.52% (1:192), showing an increase in prevalence from Asian to white to brown to black, suggesting that prevalence differs among ethnicities and that some ethnic groups have a higher risk of FH.

Studies included in the meta-analysis utilized both clinical and genetic screening to estimate FH prevalence. Seven studies applied the DLCN (or a modified version), one study the MEDPED, two studies genetic screening, and the remaining three studies another clinical screening method. The use of clinical screening has several advantages. It may be preferable in areas with limited access to genetic testing and healthcare facilities and may easily be applied in large general population studies, as information about premature coronary artery disease, family history, and clinical measurements is readily obtainable. However, the use of clinical screening may result in an overestimation of FH prevalence, as individuals with other types of dyslipidemias or high lipoprotein (a) may score high in the diagnostic criteria without having genetic FH, resulting in false positives (Hu et al., 2020) although these individuals are at high risk of cardiovascular disease. Similarly, common confounding cardiovascular risk factors such as obesity, diabetes, hypothyroidism, and a high alcohol intake may all cause dyslipidemia, also resulting in false-positive cases and an overestimation of FH prevalence (Nordestgaard et al., 2013b). Differences in these common cardiovascular risk factors together with differences in access to appropriate health care may also explain the differences in the prevalence of FH observed in the present study, although we cannot present data to support this.

As also shown in other studies, the prevalence of FH is significantly higher among patients with premature coronary artery disease and elevated cholesterol concentrations compared to the prevalence observed in general population studies (Beheshti et al., 2020; Hu et al., 2020). In the daily clinic cascade, testing of first-degree relatives is recommended, and it has been suggested that an approach combining contact to relatives through index cases (indirect testing) and direct contact from health care professionals to the relatives (direct testing) may improve the proportion of tested individuals. We did not have information on screening strategy in the patient cohorts included in the present review. However, the choice of strategy may have biased prevalence estimates among ethnicities within the single study (Leonardi-Bee et al., 2021).

The clinical criteria used in FH studies are designed primarily to detect FH in Western populations, and the DLCN, SB, and MEDPED might be less applicable in other populations. This may affect the validity of estimated prevalence in these countries and impact the diagnostic validity in different populations and among ethnicities (Hu et al., 2020).

Genetic screening is considered the most accurate method for diagnosing FH, as the detection of an established disease-causing mutation equals a definite FH diagnosis (Cuchel et al., 2014). Genetic screening is most often applied in patient cohorts to verify the cause of dyslipidemia or establish the presence of a mutation in cascade screening. However, genetic screening is not without limitations. It is time-consuming, is costly, and requires access to health facilities, making it less applicable in less affluent parts of the world (Hu et al., 2020). Moreover, studies using genetic screening report low prevalence estimates of FH, which may be due to not all FH-causing mutations having been identified or included in diagnostic testing panels for FH. Some individuals may present with a polygenic rather than a monogenic cause of FH, which is not always detected (Nordestgaard et al., 2013b; Talmud et al., 2013). In addition, genetic screening often requires evidence from functional studies for further classification of the associated variant as pathogenic or likely pathogenic (Chora et al., 2018). Furthermore, functional characterization of the variant is important for choosing the most effective treatment strategy (Di Costanzo et al., 2021).

In the present study, the estimated prevalence is the highest among black individuals: 0.52% (1:192). However, white individuals had an estimated prevalence of 0.31% (1:323), which is close to the overall estimate. In contrast to this, the prevalence by ethnicity reported in the patient cohort the Cape Town Experience (Firth and Marais, 2008), where 32% of the FH patients were white and 4.9% were black, suggest a bias in access to study participation, potential dissimilarities, or bias in screening strategies.

To successfully investigate differences in prevalence of FH among different ethnicities, a definition of ethnicity was needed. In the included studies, the ancestry of the individuals was either described through race or ethnicity. In four out of five studies representing more than one ethnicity, both terms were used (de Ferranti et al., 2016; Harada et al., 2018). The two concepts are both related to the ancestry of individuals, as race is often described as the distinctive physical trait of an individual, that is, the biology, while the ethnicity is more individual or subjective and is acquired through a cultural identification of the individual. Both terms are considered social concepts, meaning that there are no links between genes and ethnicity. In the present study, the categories of ethnicities were given and limited by the studies included.

The major limitations of the present study are the limited availability of data on prevalence among ethnicities and heterogeneity in diagnostic criteria used. A very limited number of studies reported data on the distribution of FH across ethnicities. White and black individuals were included in most studies, while the inclusion of other ethnicities was limited, potentially introducing bias of estimates of prevalence among less represented ethnicities. Only one study reported FH prevalence among Latino and brown individuals, respectively, and a pooled prevalence could not be estimated for these subgroups.

The 13 studies included used different diagnostic criteria, which might bias and contribute to the heterogeneity of the estimated pooled prevalence. Four out of seven studies applying DLCN used a modified version of the screening method (Wijesekera et al., 2014; Benn et al., 2016; de Ferranti et al., 2016; Harada et al., 2018). The modifications made were due to a lack of data, such as missing information on xanthomas and corneal arcus, or insufficient information on family history. These limitations might have led to an underestimation of the FH prevalence in these studies (Wijesekera et al., 2014; Benn

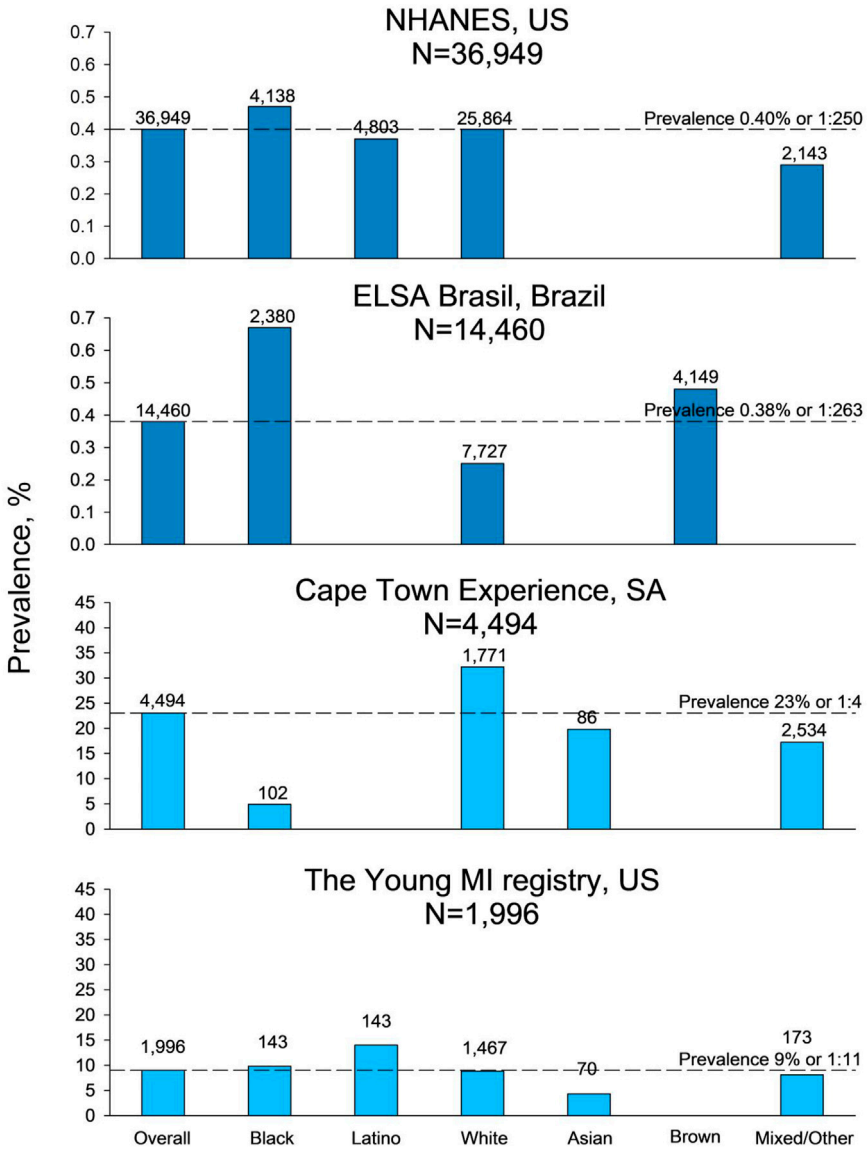

FIGURE 2 | Prevalence of familial hypercholesterolemia in studies including more than two ethnicities. Prevalence shown with dark blue bars is from general population studies, and prevalence shown with light blue bars is from patient cohort studies. For information on ethnicity, refer to Methods. N, number of participants in the study, US, United States of America, SA, South Africa.

et al., 2016; de Ferranti et al., 2016; Harada et al., 2018). However, minor modifications of the criteria were accepted, and the given studies were still considered eligible for inclusion.

The studies included represent different cohorts, with variations in sex distribution and mean age. These differences might further contribute to between-study heterogeneity. Two out of the 11 general population studies examined the FH prevalence in pediatric cohorts (Ohta et al., 2002; Abul-Husn et al., 2016). Clinical screening of FH among children may have to be interpreted differently compared to the results presented in studies examining adult populations. Children might present with lower LDL-cholesterol levels than adults, and a family history of premature myocardial infarction may not be available, as the parents of younger children may be too young to have signs of myocardial infarction. These factors may contribute to underestimating FH prevalence (Pang et al., 2016). However, the prevalence estimated in the two pediatric cohorts was similar to other FH prevalence estimates presented within the ethnic group.

Another limitation might be the variations in the representation of men and women within the different study cohorts. The YOUNG-MI Registry cohort consisted of 80.9% men compared to 19.1% women (Singh et al., 2019). This underrepresentation of women might affect the estimated FH prevalence, especially when clinical screening methods, such as DLCN, are applied. The DLCN includes own myocardial

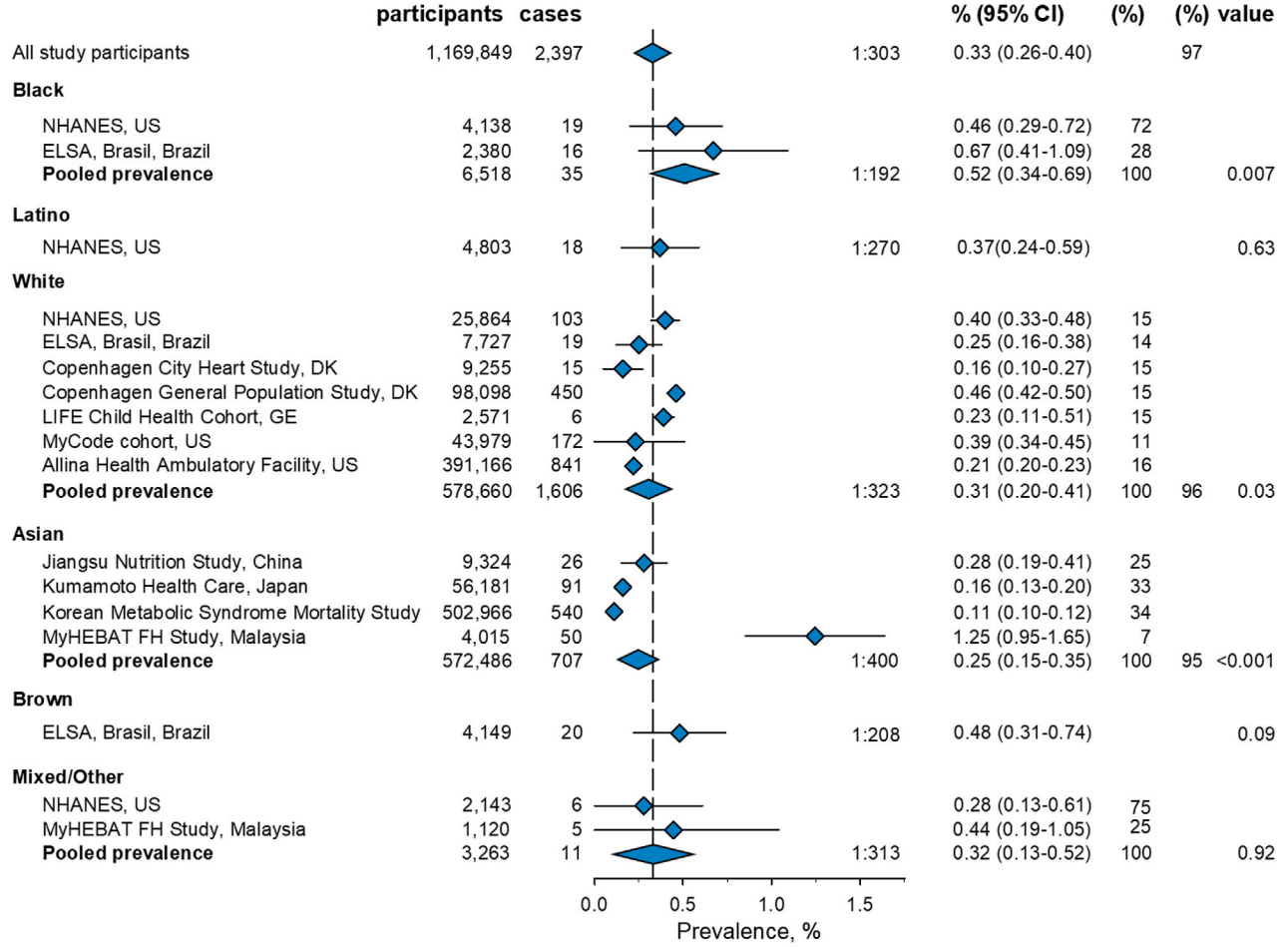

FIGURE 3 | Prevalence of familial hypercholesterolemia among ethnicities in 11 general population studies included in a meta-analysis. Small diamonds represent point estimates for individual studies. Large diamonds represent pooled meta-analysis estimates. Weight (%) is the weight of the study within each meta-analysis. Test for heterogeneity [I (Rader et al., 2003)] was significant for all meta-analyses (all $p < 0.001$). p-value is for comparison of the estimates for each ethnicity compared to the overall estimate (represented by the vertical broken line) of all participants. For information on ethnicity, refer to Methods. N, number, FH, familial hypercholesterolemia, CI, confidence interval.

infarction as a criterion, and as women in general have myocardial infarction at an older age compared to men, an underestimation of FH prevalence may be seen in cohorts with less women compared to men. This could also explain the differences seen among the two included patient studies, as the estimated FH prevalence in the YOUNG-MI Registry is significantly lower than the FH prevalence measured in the Cape Town Experience (Firth and Marais, 2008).

Robust examination of prevalence by ethnicity requires large studies with sufficient data on both FH prevalence in the entire study cohort and among ethnicities. In the present study, study cohorts originating from different countries have been included. For future studies, the ideal setting would be to investigate the prevalence of FH by ethnicity within one country, representing an admixed and diverse society but within the same healthcare system and using the same clinical FH criteria. Focus on aligning sex and age to minimize study heterogeneity may also be important. This set-up would reduce potential bias and give an opportunity to further explore potential differences in FH prevalence according to ethnicity and potential inequalities in access to and participation in screening programs, helping to guide targeted screening and prevention.

In this systematic review and meta-analysis, we found that FH prevalence varies across different ethnicities, ranging from 0.25% (1:400) to 0.52% (1:192), with the highest prevalence seen among black and brown individuals and the lowest prevalence estimated among Asian individuals. There is a need for studies investigating FH prevalence according to ethnicity to establish potential benefits of intensified FH screening in certain population subgroups and further explore potential biases and inequalities in current FH diagnostic criteria and screening programs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FT-N, FE, and MB contributed substantially to the conception and design of the work; acquisition, analysis, and interpretation of data; drafting the work; and revising it critically for important intellectual content. They provided approval for publication of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work were appropriately investigated and resolved.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.840797/full#supplementary-material

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Assessment of Associations Between Serum Lipoprotein (a) Levels and Atherosclerotic Vascular Diseases in Hungarian Patients With Familial Hypercholesterolemia Using Data Mining and Machine Learning

*Ákos Németh[1,2], Bálint Daróczy[3,4], Lilla Juhász[1,2], Péter Fülöp[1], Mariann Harangi[1] and György Paragh[1]**

[1]Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, [2]Doctoral School of Health Sciences, Faculty of Public Health, University of Debrecen, Debrecen, Hungary, [3]Institute for Computer Science and Control, Hungarian Academy of Sciences, (MTA SZTAKI), Budapest, Hungary, [4]Université Catholique de Louvain, INMA, Louvain-la-Neuve, Belgium

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***Correspondence:**
György Paragh
paragh@belklinika.com

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Background and aims: Premature mortality due to atherosclerotic vascular disease is very high in Hungary in comparison with international prevalence rates, though the estimated prevalence of familial hypercholesterolemia (FH) is in line with the data of other European countries. Previous studies have shown that high lipoprotein(a)- Lp(a) levels are associated with an increased risk of atherosclerotic vascular diseases in patients with FH. We aimed to assess the associations of serum Lp(a) levels and such vascular diseases in FH using data mining methods and machine learning techniques in the Northern Great Plain region of Hungary.

Methods: Medical records of 590,500 patients were included in our study. Based on the data from previously diagnosed FH patients using the Dutch Lipid Clinic Network scores (≥7 was evaluated as probable or definite FH), we trained machine learning models to identify FH patients.

Results: We identified 459 patients with FH and 221 of them had data available on Lp(a). Patients with FH had significantly higher Lp(a) levels compared to non-FH subjects [236 (92.5; 698.5) vs. 167 (80.2; 431.5) mg/L, $p < .01$]. Also 35.3% of FH patients had Lp(a) levels >500 mg/L. Atherosclerotic complications were significantly more frequent in FH patients compared to patients without FH (46.6 vs. 13.9%). However, contrary to several other previous studies, we could not find significant associations between serum Lp(a) levels and atherosclerotic vascular diseases in the studied Hungarian FH patient group.

Conclusion: The extremely high burden of vascular disease is mainly explained by the unhealthy lifestyle of our patients (i.e., high prevalence of smoking, unhealthy diet and physical inactivity resulting in obesity and hypertension). The lack of associations between

serum Lp(a) levels and atherosclerotic vascular diseases in Hungarian FH patients may be due to the high prevalence of these risk factors, that mask the deleterious effect of Lp(a).

Keywords: lipoprotein(a), familial hypercholesterolemia, cardiovascular risk, data mining, atherosclerosis

INTRODUCTION

In familial hypercholesterolemia, significantly elevated low-density lipoprotein-cholesterol (LDL-C) levels increase cardiovascular risk. A previous study in Norway showed that the life expectancy of individuals with heterozygous familial hypercholesterolemia (FH) was 15 years shorter than that of the average Norwegian population (Mundal et al., 2014). Different study pointed out that 25% of women and 50% of men with heterozygous FH had cardiovascular complications (McCrindle and Gidding, 2016). Compared to healthy individuals the risk of coronary artery disease is 3.5–16 times higher in patients with FH (Hovingh and Kastelein, 2016), while the risk of peripheral vascular disease was found to be elevated by 5–10 times in these individuals (Kroon et al., 1995; Hutter et al., 2004). In 1963, during his research on blood group antigens, Köre Berg discovered a new lipoprotein system, later named lipoprotein(a) (Lp(a)) (BERG, 1963). Lp(a) is an LDL-like lipoprotein particle produced by the liver. Its major lipoprotein is apolipoprotein B100 (apoB100), to which an apo (a) glycoprotein is covalently bound (Marcovina and Koschinsky, 1998; Anuurad et al., 2006). Association with proteoglycan and fibronectin molecules on the surface of endothelial cells Lp(a) can enter the subendothelial space (Stulnig et al., 2019). Phospholipids on the surface of Lp(a) can be oxidized by reactive free radicals produced by lipoxygenases, myeloperoxidases, and nicotinamide adenine dinucleotide phosphate oxidase (Ferretti et al., 2018). Oxidized phospholipids bound to Lp(a) enhance the production and expression of inflammatory cytokines and chemokines in vascular wall cells promoting the accumulation of monocytes from the circulation in the subendothelial space. Oxidized phospholipids trigger the endocytosis of Lp(a) through the binding to scavenger receptors of macrophages, as well as the migration of vascular smooth muscle cells from the media to the intima, ultimately leading to endothelial dysfunction (Wu et al., 2004; Tsimikas and Witztum, 2008). In addition, Lp(a) competitively inhibits plasminogen plasmin conversion and its binding to fibrin, and thus thrombolysis. Apolipoprotein (a) decreases plasminogen activator-1 levels by increasing the expression of the plasminogen activator-1 inhibitor. Thus, Lp(a) elicits proatherogenic, proinflammatory and prothrombotic effects (van der Valk et al., 2016). Analysis of 31 prospective studies showed a 1.5-fold increase of relative cardiovascular risk in individuals with Lp(a) levels in the upper third compared with those in the lower third (Bennet et al., 2008). A meta-analysis of 36 prospective studies involving 126,634 individuals showed that Lp(a) concentration was associated with the risk of cardiovascular disease as well as stroke (Erqou et al., 2009). Nordestgaard et al. recommended that Lp(a) should be measured in patients with moderate to high cardiovascular risk. (Nordestgaard et al., 2010). After LDL reduction, a reduction in Lp(a) serum levels below 50 mg/dl (500 mg/L) is recommended as a secondary priority. Previous studies have suggested that high levels of Lp(a) are more common in individuals with FH, further increasing the cardiovascular risk in these patients (Clarke et al., 2009; Kamstrup et al., 2009). FH is still underestimated and underdiagnosed in the regions of Central, Eastern and Southern Europe. However, during the last few years, the international ScreenPro Project achieved significant improvement of screening, diagnosis, and treatment of FH in these countries (Ceska et al., 2019). Based on medical and statistical records of two major hospitals in the Northern Great Plain region of Hungary, recently we identified patients with a possible diagnosis of FH using data mining methods. Investigating medical records of 1,342,124 patients the estimated prevalence of FH was found to be 1:340, which is in line with the prevalence data of some other European countries (Paragh et al., 2018).

In the present study, we examined the prevalence of high serum Lp(a) levels and their potential impact on atherosclerotic vascular complications in individuals with FH. We hypothesized that the prevalence of increased serum Lp(a) will be higher in FH patients, which can be associated with higher risk of atherosclerotic vascular complications including cardiovascular (CAD), cerebrovascular (CeVD) and peripheral arterial diseases (PAD), aortic valve stenosis (AoS), and might also be associated with the risk of deep vein thrombosis (DVT).

PATIENTS AND METHODS

Screening Patients for FH Diagnosis

Described in our previous paper (Paragh et al., 2018) data mining methods are, an ideal way to screen for FH in mass hospital data, though the range for potential FH patients was still wide. To narrow our finding, we used cutting edge machine learning techniques. First, we decided to use the Dutch Lipid Network Criteria System (DLNCS) to teach how to recognize FH patients in order to find the most homogeneous patient group. We used the scores of the DLNCS for patient input and four machine learning model groups (feedforward multi-layer perceptron with ReLU (Rectified Linear Unit) activations (Montufar et al., 2014), gradient boosting (Friedman, 2001; Chen and Guestrin, 2016), support vector machines with RBF (radial basis function) kernel (Tan et al., 2016) and binary linear regression (Tan et al., 2016) for training. The training feature space included patient blood test results (with 70 most common test types), diagnostic data (ICD-10 3-digit diagnosis) and textual history data. Boosted trees worked the best similarly to other nonstructural datasets as in (Kerepesi et al., 2018). Then we majorly improved our textual analysis (to collect patient history and family history data more thoroughly and also to get detailed statistics on secondary

TABLE 1 | Clinical and laboratory data of the study populations. Data are presented as median (lower-upper quartile).

	nonFH	FH	*p*
Number of patients	590,041	459	
Age (years)	40.0 (22.2–59.0)	53.2 (39.3–59.7)	<0.05
Male/Female (%)	42.8/57.2	39.2/60.8	n.s.
Cholesterol (mmol/L)	4.94 (4.21–5.73)	7.03 (6.10–8.40)	<0.001
Triglyceride (mmol/L)	1.35 (0.96–1.96)	1.96 (1.41–3.03)	<0.01
LDL-C (mmol/L)	2.95 (2.34–3.62)	4.80 (3.91–5.90)	<0.001
HDL-C (mmol/L)	1.32 (1.09–1.62)	1.41 (1.16–1.72)	<0.05
Urea (mmol/L)	5.10 (4.02–6.50)	5.43 (4.40,6.63)	n.s.
Creatinine (μmol/L)	68.3 (57.1–83.2)	69.1 (58.5–82.2)	n.s.
eGFR (mL/min/1.73m2)	90.0 (78.1–90.0)	88.8 (80.4–90.0)	n.s.
AST (GOT) (U/L)	21.0 (17.5–28.1)	21.8 (18.3–27.5)	n.s.
ALP (GPT) (U/L)	19.6 (14.0–29.2)	24.0 (16.5–33.0)	n.s.
GGT (U/L)	26.1 (16.1–48.2)	32.7 (20.2–58.9)	<0.05
Uric acid (μmol/L)	292 (235–357)	324 (266–384)	<0.05
Lipoprotein(a) (mg/L)	167 (80.2; 431.5)	236 (92.5; 698.5)	<0.01

medical conditions like hypertension or smoking for proper analysis) with Natural Language Processing (NLP) (see details in next subsection).

The best overall training results we achieved with DLNCS scores were 7+ and 8+, so we decided we consider everyone as an FH patient who possessed 7 or more within the score system, which is also entirely in line with the key concept of DLNCS.

Clinical characteristics and laboratory data of the study population are summarized in **Table 1**.

Identifying Cardiovascular Risk Factors and Data on Laboratory Parameters

Since our data set contains several types of data structures, we first created a common representation as a preparation for statistical analysis. The main reason for such a structure is to detect "properties" in any form. For example, high blood pressure could appear in the textual data in several forms as expressions, as a parameter or derived from actual measurements. We developed tools to extract the designated data from the available multiple sources. An additional challenge is data cleansing, especially filling missing or corrupted data parts and treating the various types of corrupted data differently. For example, in case of binary variables filling gaps with the mean value is misleading and should be avoided in any case. These special cases were handled mainly by regular expressions. Given the size of the data, we developed additional serializing and streaming methods to optimize the flexible final query engine which can handle incomplete data and may detect "properties" based on deduction. For textual information included preprocessing steps in the following order: parsing, stemming, stop word filtering, dictionary building with unigrams and bigrams after a by hand cleaning of expressions or terms. The cleaning contained a ranking of terms based on TF-IDF (term frequency-inverse document frequency) and word embeddings. As word embeddings available in Hungarian language are trained on traditional corpora, we needed to build our own language model based on the textual data. For this we have built a Gated Recurrent Unit model (Chung et al., 2014), a special recurrent neural network, based on the cleaned unigrams and bigrams as dictionaries. The final data structure contained the following appearances: a "property" was assigned to a patient if one of the following events happened: either it was detected by a regular expression, or it has high probability given the language model as an expression or based on the laboratory measurements it was directly true.

Lp(a) Measurement

Lp(a) levels were determined from fresh sera with a Cobas c501 analyzer (Roche Ltd., Mannheim, Germany) according to the manufacturer's instructions at the University of Debrecen Department of Laboratory Medicine. The reference range of Lp(a) is <300 mg/L.

Structuring the Study Population

Figure 1 demonstrates a flowchart showing the structure of the study population, number of enrolled patients, and how the patients were divided into the groups, and subgroups.

Statistical Analysis

We used anonymous patient record data from the hospital information system run by University of Debrecen Clinical Center's hospital information system. The data was originally available in an HL7 format and has been already partially cleaned and preprocessed for data mining and machine learning purposes by a contractual cooperating partner of the university (Aesculab Medical Solutions, Black Horse Group Ltd.). We leveraged this database as starting point, so we did not have to deal with the system errors of original hospital data recording). The data included 8 complete years (from 2007 to 2014) and the entire patient record database of the clinical center with all textual, diagnostic and laboratory details. The data was extracted via queries from the PostgreSQL 13.x database and resulted in huge textual files which were the kick off for further statistical analysis. The studied population covered all patients treated in the University of Debrecen during these 8 years resulting in a number of 590,500 patients of which 288,591 had clinical laboratory tests available with an average of 34.03 and a median of 33.0 tests per person. The study data included all departments and all inpatient and outpatient data available during the aforementioned period.

Statistical analysis has been carried out with Python supported data mining packages. Data cleaning and preprocessing were done using Python 3.8, IPython 7.29, Cython 0.29, Pandas 0.23 and Numpy 1.22 under Conda 4.10 environment with Dask. Machine learning for refining data selection and deep textual analysis leveraged SciKit-Learn 1.0 and Pytorch 1.09. In general, for the base for statistical analysis we created a "healthy patient" pool, patients who do not suffer from any conditions (high LDL, low HDL, hypertension, diabetes, obesity, smoking, not following statin treatment) that might be associated with atherosclerotic vascular disease. Statistical significance analysis was performed with unpaired t-tests keeping 95% significance level. Statistical figures have been created with MatPlotLib 3.5 software package.

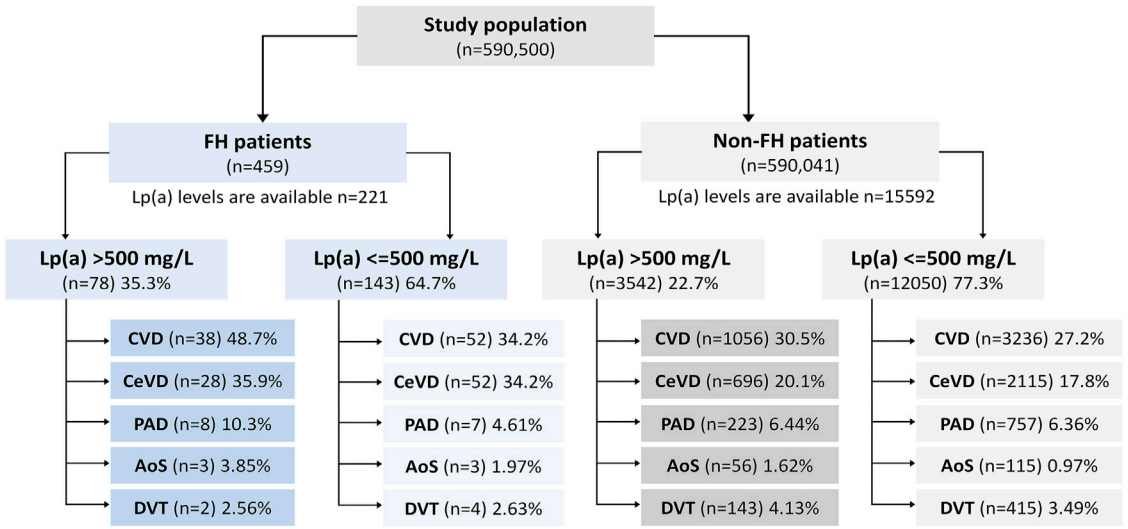

FIGURE 1 | Flowchart showing the structure of the study population, number of enrolled patients, and how the patients were divided into the groups, and subgroups.

RESULTS

We identified 459 patients with FH, out of which 221 had data available on serum Lp(a) levels. Patients with FH had significantly higher Lp(a) levels compared to non-FH subjects [236 (92.5; 698.5) vs. 167 (80.2; 431.5) mg/L, $p < .01$] (**Figure 2**). Significantly higher Lp(a) levels were found in females compared to males with FH [266 (108–875) vs. 182 (73.1–648) mg/L, $p < .05$]. Similar differences were observed in those without FH [179 (81.5–458) vs. 152 (80.7–386) mg/L, $p < .01$]. 35.3% of FH patients had Lp(a) levels exceeding >500 mg/L. Atherosclerotic complications were significantly more frequent in FH patients compared to those without FH (46.6 vs. 13.9%). However, contrary to several other previous studies, we could not find significant associations between serum Lp(a) levels and atherosclerotic vascular diseases in the studied Hungarian FH patient group. Therefore, we determined the prevalence of other cardiovascular risk factors in FH and in non-FH patients. We found the prevalence of hypertension, smoking, obesity, and hyperuricemia extremely elevated in the FH group. Furthermore, the prevalence of diabetes and low HDL-C level were also increased compared to the non-FH population (**Table 2**).

Evaluating this population, the most common risk factors in patients with atherosclerotic vascular disease were hypertension, male gender, age >60 years and the lack of statin treatment. High prevalence of hypertension, male gender, age >60 years and the lack of statin treatment were found in patients with CeVD and PAD. In patients with AoS, the prevalence of hypertension and age >60 were extremely increased. Interestingly, similar risk factor pattern was detected in patients with DVT. The highest prevalence of elevated Lp(a) level was found in patients with AoS (**Table 2**).

We also detected the prevalence of the above-mentioned risk factors in FH patients with low and high Lp(a) levels. Although the prevalence of obesity was increased, and the prevalence of low HDL-C level was decreased in FH patients with high Lp(a) levels, there were no significant differences between the two groups. It must be noted that the ratio of individuals on statin treatment was markedly higher in FH patients with low Lp(a) level. Calculating the prevalence of risk factors, we found that the prevalence of high Lp(a) levels was increased in females, while the prevalence of smoking and hypertension were decreased in males in the non-FH population. In FH patients, the prevalence of high Lp(a) was tended to be augmented in females; however, this difference did not reach statistical significance similarly to the other risk factors (**Table 3**).

We also calculated the impact of risk factors on hazard ratios of cardiovascular diseases. Hypertension and increased LDL-C level were found to have the biggest impact, followed by smoking, diabetes, obesity, and extremely high Lp(a) in non-FH patients. In FH patients, hypertension and smoking increased cardiovascular risk significantly (**Table 4**). Many patients had two or more risk factors. In patients with smoking, obesity, hypertension and diabetes, the risk of CVD was 25.74 times higher; if high LDL-C level was also associated, the risk was even higher: 27.42 (data not shown). Impact of Lp(a) levels were calculated in subgroups with various Lp(a) ranges. The risk of cardiovascular diseases was significantly increased in patients with a Lp(a) level exceeding than 1,000 mg/L (**Figure 3**).

DISCUSSION

Despite the high cumulative LDL-C burden, not all FH patients will develop CVD to the same extend, which results in wide phenotypic heterogeneity (Neefjes et al., 2011). The simultaneous

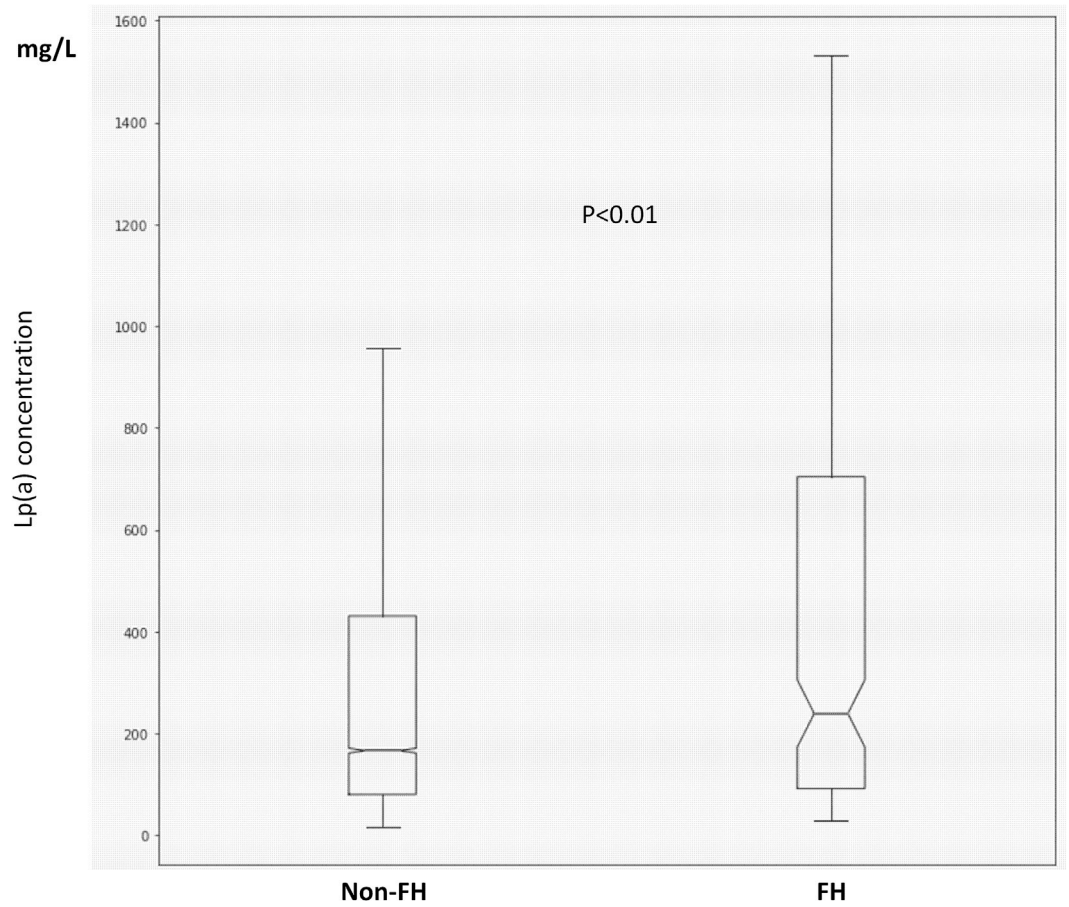

FIGURE 2 | Boxplots and whiskers of serum lipoprotein(a) levels in FH and non-FH patients. The length of the box represents the interquartile range (IQR), the horizontal line in the box interior represents the median, the whiskers represent the 1.5 IQR of the 25th quartile or 1.5 IQR of the 75th quartile.

TABLE 2 | Prevalence of cardiovascular risk factors in the whole study population, in various atherosclerotic vascular diseases and in deep vein thrombosis.

	Non-FH	FH	CVD	CeVD	PAD	AoS	DVT
high Lp(a) (%)	23.0	35.3	25.7	25.8	23.8	33.9	26.2
hypertension (%)	24.5	86.3	77.8	82.6	61.1	85.0	64.2
diabetes (%)	5.16	17.4	19.3	24.4	14.7	23.0	15.7
smoking (%)	9.25	66.4	28.0	32.2	30.6	28.7	25.0
obesity (%)	10.2	42.0	31.4	36.1	26.3	33.5	38.2
hyperuricemia (%)	5.78	41.2	19.2	24.4	21.0	22.1	24.7
low HDL-C (%)	2.12	11.8	8.59	9.23	6.17	9.79	7.96
male gender (%)	42.7	39.2	48.1	46.5	26.6	57.8	42.4
age >60 ys (%)	23.0	20.0	54.8	64.7	33.7	77.8	50.5
no statin therapy (%)	88.3	12.9	46.6	45.3	56.2	35.6	68.8

presence of multiple risk factors has been shown to increase the risk of atherosclerosis. Also, high burden of risk factor clustering might be responsible for phenotypic heterogeneity both in FH and non-FH patients. In a previous study, for index FH cases, the only factor independently associated with increased risk of CV events was the presence of corneal arcus, a known marker of long-term exposition to high levels of LDL-C. In relatives with identified genetic mutations, older age, male sex, hypertension, diabetes, previous CVD, tobacco consumption and corneal arcus were all associated with increased risk of CV events. However, multivariate analysis indicated that only diabetes and tobacco consumption remained significantly associated with the risk of CV events (Silva et al., 2016). Although relation of Lp(a) to CVD and carotid artery stenosis was reported in heterozygous FH patients almost 3 decades ago (Tatò et al., 1993), the significance of elevated Lp(a) concentrations as a risk factor is still not elucidated. Several further studies reported data on cardiovascular risk factor distribution in FH, with conflicting results.

This is the first study aiming to identify CV risk factors in Hungarian FH patients diagnosed with data mining methods. In our FH population, prevalence of hypertension was extremely high (86.3%) compared to the results of some previous studies. Dyrbus et al. also reported increased prevalence of arterial hypertension in Polish patients with definite, probable and possible FH (69.4, 70.7 and 72.6%, respectively) (Dyrbuś et al., 2019). Korneva detected a 59.2% hypertension prevalence in FH patients from Karelia (Korneva et al., 2019). However, Vlad et al. found only a 50.8% prevalence of hypertension in a Romanian FH

TABLE 3 | Prevalence of cardiovascular risk factors in the whole study population, in FH patients and in FH patients with low and high (>500 mg/dl) Lp(a) levels.

	Non-FH	FH	FH with low Lp(a)	FH with high Lp(a)
Whole study population				
high Lp(a) (%)	23.0	35.3	—	—
hypertension (%)	24.5	86.3	86.8	87.2
diabetes (%)	5.16	17.4	16.4	12.8
smoking (%)	9.25	66.4	69.7	76.9
obesity (%)	10.2	42.0	42.8	37.2
hyperuricemia (%)	5.78	41.2	40.8	46.2
low HDL-C (%)	4.63	31.4	31.6	28.3
male gender (%)	42.7	39.2	32.8	27.3
age >60 ys (%)	23.0	20.0	24.7	27.1
no statin therapy (%)	88.3	12.9	16.4	9.0
Males				
high Lp(a) (%)	21.4	29.4	—	—
hypertension (%)	25.8	86.7	87.8	87.8
diabetes (%)	5.94	20.1	20.4	14.7
smoking (%)	11.9	64.5	69.4	78.7
obesity (%)	9.71	46.1	51.0	39.0
hyperuricemia (%)	5.64	43.9	46.9	49.1
low HDL-C (%)	4.58	28.8	30.6	25.4
male gender (%)	—	—	—	—
age >60 ys (%)	23.2	17.2	25.1	27.3
no statin therapy (%)	86.6	11.7	18.4	9.20
Females				
high Lp(a) (%)	24.1	37.9	—	—
hypertension (%)	23.6	86.0	86.4	86.2
diabetes (%)	4.59	15.8	14.6	12.1
smoking (%)	7.27	67.7	69.9	75.9
obesity (%)	10.6	39.4	38.8	36.2
hyperuricemia (%)	5.88	39.4	37.9	44.8
low HDL-C (%)	4.67	33.0	32.0	29.3
male gender (%)	—	—	—	—
age >60 ys (%)	22.9	20.9	24.1	26.9
no statin therapy (%)	89.3	13.6	15.5	8.66

TABLE 4 | Impact of individual risk factors on cardiovascular risk (hazard ratios).

	HR (healthy[a])	HR (healthy FH[b])
smoking	4.9–5.2	1.3–10.6
obesity	1.9–2.2	—
hypertension	10.3–10.4	4.3–9.1
diabetes	2.3–2.7	—
high LDL-C	11.3–11.9	—
extremely high Lp(a) >1,000 mg/L	1.6–6.4	—

[a]*Hazard rations against entirely healthy patients with no overweight, non-smoking, no statin treatment, normal LDL, and HDL levels, no hypertension, no diabetes, no high LPA (392.600 patients).*

[b]*FH patients who are not overweight, non-smoking, no high blood pressure, no diabetes, no high LPA (39 patients).*

patient population (Vlad et al., 2021). Bertolini et al. detected a 16.2% and an 23.8% prevalence of hypertension in Italian FH males and females, respectively (Bertolini et al., 2013). Mehta et al. reported a 17% hypertension prevalence in a Mexican FH cohort (Mehta et al., 2021). In another previous cohort study published by Besseling et al. hypertension was found in only 11% of FH patients (Besseling et al., 2014). We found surprisingly high prevalence of smoking (66.4%), which was similar to that of the Polish FH population mentioned above (59.2, 61.7 and 50.7% in definite, probable and possible FH, respectively) (Dyrbuś et al., 2019), but only 29.5% in the Romanian (Vlad et al., 2021), 16.8% in the Karelian (Korneva et al., 2019) and 16.7% in the Mexican FH populations (Mehta et al., 2021). In a Turkish FH registry, 12.5% of FH patients were smokers, while this number was found to be 20.2% in males according to an Italian FH registry (Bertolini et al., 2013; Kayikcioglu et al., 2018). The prevalence of diabetes in our FH cohort was comparable to the prevalence found in Polish, Romanian and Mexican FH patients (Dyrbuś et al., 2019; Mehta et al., 2021; Vlad et al., 2021), but markedly increased compared to the non-FH population. Vohnout et al. detected a lower, 10.5% prevalence of diabetes in Slovakian FH patients (Vohnout et al., 2018). It must be mentioned that a previous study reported significantly decreased diabetes prevalence in FH patients, and there was an inverse relationship between the severity of the disease-causing mutations and the diabetes prevalence (Besseling et al., 2015). Differences in socioeconomic status and genetic

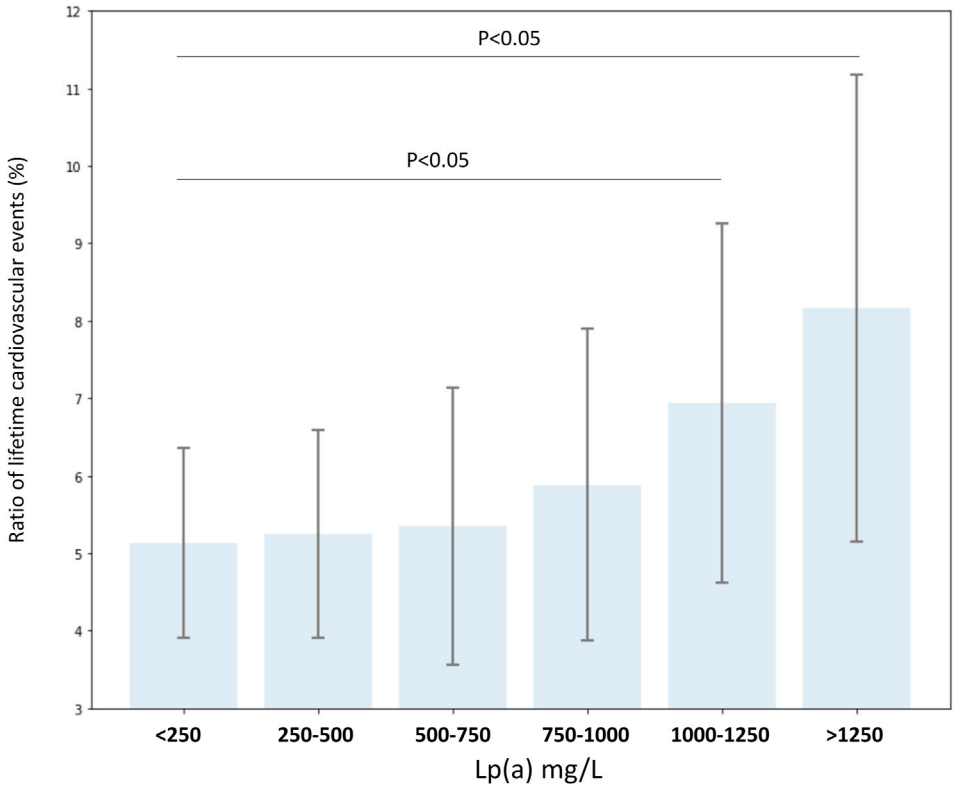

FIGURE 3 | Ratio of lifetime cardiovascular events (%) according to serum lipoprotein(a) level groups in all FH and non-FH patients with known lipoprotein(a) levels.

influences may explain these conflicting results. Additionally, the deficient knowledge of patients and their relatives on FH and its impact on health as a cardiovascular risk factor might contribute to the surprisingly high prevalence of modifiable risk factors including smoking in the studied Hungarian FH patients. Therefore, widespread information, patient education and increased awareness of this condition should be of major importance (Ceska et al., 2019).

Lp(a) levels were detected only in a few previous studies. Mehta et al. found a median Lp(a) level of 30.5 mg/dl (305 mg/L) (Mehta et al., 2021) and other previous European studies reported similar values including the study of Lingenhel et al. (27.7 mg/dl; 277 mg/L) (Lingenhel et al., 1998), Alonso et al. (23.6 mg/dl; 236 mg/L) (Alonso et al., 2014). Our results are in line with these results. Recently, lower mean Lp(a) levels were found in a Japanese cohort (20.8 mg/dl; 208 mg/L) (Naito et al., 2021) and in a previous other Japanese study (21.9 mg/dl; 219 mg/L) (Tada et al., 2018), demonstrating the importance of racial differences. Significantly increased Lp(a) levels in females were previously described in FH (Alonso et al., 2008), as well as in patients with CVD (Virani et al., 2012) and PAD (Forbang et al., 2016), and in the general population (Banerjee et al., 2011). Our data are in line with these observations. The exact cause of higher Lp(a) levels in females is not fully elucidated, but apo(a) expression has been found to be modulated by several hormones including estrogens. The chromosomal region responsible for estrogen response was identified within an apo(a) enhancer located at ~26 kilobases from the apo(a) promoter (Boffelli et al., 1999). In the studied Hungarian population, extremely high Lp(a) levels (>1,000 mg/L) significantly increased the risk of cardiovascular events. Although the relatively low number of FH patients with available Lp(a) values impeded the evaluation of the impact of high Lp(a) concentrations on cardiovascular risk in FH patients, extremely high Lp(a) level might be also a high priority risk factor in FH. Further studies on larger FH patient populations are needed to confirm these conclusions.

In the last few years, many risk equations have been developed in order to determine CV risk associated with FH in various geographic regions. Risk equations specific to FH, such as the SAFEHEART-Risk Equation, have been validated for Spanish (Pérez de Isla et al., 2017) and French (Gallo et al., 2020) populations. The MONTREAL FH score was validated in Canada (Paquette et al., 2017). To develop a similar risk equation in Hungary, data on prevalence of these individual risk factors are essential. Our study is the first one that provides information about CV risk status of a Hungarian FH cohort.

Based on our results, the extremely large burden of vascular disease in Hungarian FH patients is mainly explained by the high

prevalence of several clustered risk factors (i.e., high prevalence of smoking, obesity and hypertension), though extremely high Lp(a) levels are definitely needed to manage. Patients confronting these multiple metabolic risk factors may benefit from exploring new therapeutic frontiers achieving the goal of personalized disease management (Giglio et al., 2021). Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors—both monoclonal antibodies and inclisiran—lower Lp(a) by 26%, but this is insufficient for individuals with very high Lp(a) levels (Spolitu et al., 2019). New agents, such as a N-Acetylgalactosamine (GalNAc) linked antisense oligonucleotides (ASO) against Lp(a) (TQJ230, trade name pelacarsen) and a small interfering RNA (siRNA) compound aimed at reducing apo(a) synthesis (AMG 890, trade name olpasiran) reduce Lp(a) levels by 80–90% with no effect on other variables (Viney et al., 2016). Depending on results of ongoing outcome trials, these agents could be helpful for both FH and non-FH patients with elevated Lp(a) levels. Still, because of the high costs of these novel therapies, strict control of other modifiable risk factors is essential. Indeed, non-smoker patients with well controlled hypertension, diabetes and LDL-C levels, with optimal body weight and diet might have the highest benefit from Lp(a) lowering treatment. These aspects might be considered when novel agents will be prescribed. In summary, despite the technological advances, traditional diligence regarding ruling out secondary factors, encouraging a healthy diet, physical activity and weight loss, along with global CVD risk factor control remain the cornerstones of FH management and cardiovascular prevention (Berberich and Hegele, 2021).

LIMITATIONS

Some limitations of our study must be mentioned. We were unable to assess data of family history and genetic data, moreover, we could not cover 100% of the population as not everybody goes to hospital every year. Furthermore, hospital goers tended to be older and checked more frequently. Oppositely, younger patients usually had less thorough laboratory examinations and their history been asked less frequently. These tendencies mean that identifying FH patients is biased towards the elderly. It must be highlighted that measurement of Lp(a) level is not available for each patient, mostly because of financial causes and technical issues. Furthermore, serum Lp(a) level measurements are usually indicated more frequently in patients with suspected or proved cardiovascular complications.

CONCLUSION

The extremely high burden of vascular disease is mainly explained by the unhealthy lifestyle of our patients (i.e., high prevalence of smoking, unhealthy diet and physical inactivity resulting in obesity and hypertension). The lack of associations between serum Lp(a) levels and atherosclerotic vascular diseases in Hungarian FH patients may be due to the high prevalence of these risk factors masking the deleterious effect of Lp(a). Therefore, encouraging lifestyle interventions, along with global control of CVD risk factors remain the cornerstones of FH management.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of University of Debrecen and the Medical Research Council. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Study design: GP, MH. Development of methodology: ÁN, BD. Collection of data: ÁN, BD. Analysis and/or interpretation of data: GP, MH, PF, ÁN, LJ. Writing (not revising) all or sections of the manuscript: GP, PF, MH. Manuscript review: GP.

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BRIEF RESEARCH REPORT
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Fast and Easy Nanopore Sequencing Workflow for Rapid Genetic Testing of Familial Hypercholesterolemia

Muhidien Soufi[1]†, Simon Bedenbender[1†], Volker Ruppert[2], Bilgen Kurt[2], Bernhard Schieffer[2] and Juergen R. Schaefer[1]*

[1]Center for Undiagnosed and Rare Diseases, University Hospital Giessen and Marburg and Philipps University Marburg, Marburg, Germany, [2]Department of Cardiology, Angiology and Critical Care Medicine, University Hospital Giessen and Marburg and Philipps University Marburg, Marburg, Germany

Familial hypercholesterolemia (FH) is an autosomal dominant lipid metabolism disorder characterized by severely elevated plasma low-density lipoprotein cholesterol levels. The disease is caused by mutations in 3 genes (*LDLR*, *APOB* and *PCSK9*) while over 90% of the mutations are located within the *LDLR* gene. Thus, genetic analysis of the *LDLR* gene is the first step in the genetic diagnosis of FH. However, conventional methods like Sanger and NextGen sequencing are still costly and time-consuming. In contrast, Oxford Nanopore technology sequencing is an emerging third-generation sequencing technology featured by easy operability, low cost, small size and the capability of parallel sample sequencing. Here, we present an easy Nanopore-sequencing-based workflow for the rapid genetic testing of FH taking only 3 days and costing less than $50 per sample without the requirement for deep bioinformatic knowledge. Using our workflow, we were able to identify the underlying pathogenic variants of 10 FH patients including one novel, not yet recorded pathogenic variants. Our workflow allows the rapid evaluation of the pathogenic variants by utilizing detailed variant information from Ensembl. Additionally, our workflow is not restricted to sequencing the *LDLR* gene alone but can be easily adapted to the other FH-causing genes and more importantly, to any desired gene contributing to any hereditary disease. Therefore, our workflow is an attractive opportunity for every diagnostic laboratory to offer fast and easy in-house genetic diagnostics.

Keywords: familial hypercholesterolemia, LDL receptor, rapid genetic testing, oxford nanopore sequencing, long amplicon sequencing, genetic diagnosis, hereditary diseases

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Alpo Juhani Vuorio,
University of Helsinki, Finland

Reviewed by:
Marta Futema,
St George's, University of London, United Kingdom
Hayato Tada,
Kanazawa University, Japan

***Correspondence:**
Muhidien Soufi
soufi@staff.uni-marburg.de

†These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipid metabolism characterized by highly elevated plasma levels of low-density lipoprotein cholesterol (LDL-C). Affected individuals have a high risk for the development of premature atherosclerosis and early onset coronary artery disease due to impaired clearance and accumulation of low-density lipoprotein (LDL) particles in the cardiovascular system (Youngblom et al., 1993; Defesche et al., 2017; Alonso et al., 2018). The estimated prevalence of heterozygous FH in European populations is 1/500 but recent studies suggest a higher prevalence up to 1/200 (Youngblom et al., 1993; Nordestgaard et al., 2013; Defesche et al., 2017). Individuals with heterozygous FH have markedly elevated plasma LDL-C levels and if not adequately treated develop coronary artery disease within the third decade of their

live. Homozygous FH is a very rare condition with a prevalence of 1/1,000,000 in the general population. Similarly, recent studies estimate a much higher prevalence of 1/300,000 (Defesche et al., 2017; Benito-Vicente et al., 2018). Homozygous individuals have extremely elevated plasma LDL-C levels and develop xanthomas, carotid artery stenosis and aortic valve stenosis in the first decade of life. Without intervention in early childhood, these individuals usually die within the first 2 decades of their lives (Youngblom et al., 1993). The molecular basis for FH are mutations in one of three genes: low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*) and protein convertase subtilisin/kexin type 9 (*PCSK9*). The LDLR is responsible for the receptor-mediated endocytosis of LDL particles which consist of APOB, lipids and cholesterol whereas PCSK9 controls the amount of LDLR on the cell surface by increasing LDLR degradation (Benito-Vicente et al., 2018; Roth and Davidson, 2018). The LDLR is expressed mainly in hepatocytes and accounts for the clearance of 70% of all plasma circulating LDL (Brown and Goldstein, 1986). Studies on the prevalence of mutations within these 3 genes showed that the vast majority (93% in UK population (Humphries et al., 2006)) of mutations is located in the *LDLR* gene. To date, more than 3,000 variants within the *LDLR* gene have been identified and reported in ClinVar (Landrum et al., 2018). According to their position within the coding sequence, FH-causing LDLR variants can be categorized into five classes: class 1 (no receptor synthesis), class 2 (impaired intracellular receptor transport), class 3 (defective LDL binding by the receptor), class 4 (defective receptor internalization) and class 5 (impaired receptor recycling) (Hobbs et al., 1990).

Given that the majority of FH cases are associated with pathogenic variants in the *LDLR* gene, genetic testing by sequencing of the entire *LDLR* gene is the first step in genetic diagnosis of FH. However, conventional Sanger sequencing of PCR amplicons still is challenging, costly and time consuming for many routine diagnostic laboratories, especially in cases when numerous FH samples need to be sequenced. Oxford Nanopore technology (ONT) sequencing is a third-generation sequencing technology that enables long-read sequencing in real time. Nanopore sequencing measures the change of an ion current when the DNA is passed through a nanopore by a motor enzyme. This sequence-dependent change in ion current is then used to determine the bases of the analyzed sequence which is called basecalling (Jain et al., 2016; Rang et al., 2018). The ONT MiniON Mk1C device is an easy to operate, low cost and small size sequencing device that allows sequencing of multiple samples in parallel. Due to its low computational system requirements, the ONT MinION sequencing platform is a cost-effective alternative for every diagnostic laboratory (Jain et al., 2016; Petersen et al., 2019).

In this report, we describe a Nanopore sequencing for the genetic analysis of the *LDLR* gene with the ONT MinION Mk1C sequencing device protocol using long-amplicon sequencing with rapid barcoding. Our protocol is fast, easy and allows sequencing of multiple FH samples in parallel. Our workflow includes a simple bioinformatic analysis workflow, which does not require any programming skills to rapidly identify the *LDLR* variants. The results allow fast evaluation of the variants by using information about clinical significance, variant consequence as well as SIFT and PolyPhen2 predictions.

MATERIALS AND METHODS

Patients

For validation experiments of this study, 4 patients (3 male and 1 female) aged 2–36 years were included who had a genetically proven diagnosis of homozygous or heterozygous FH and known disease-causing *LDLR* variants verified by Sanger sequencing. The patient characteristics and ethnicities are stated in **Table 1**.

For the identification of unknown pathogenic variants, 3 patients from a single family (2 male and 1 female) aged 6–40 years and 3 additional unrelated female patients aged 5–43 years with plasma LDL concentration ranging from 5.8 to 18.7 mmol/L and suspected FH based on characteristic symptoms including xanthomas and a familial history of premature coronary artery disease were included. The patient characteristics and ethnicities are stated in **Table 2**.

All patients attended the Lipids Competence Center of the Department of Cardiology at the University Hospital Marburg and gave written informed consent for genetic analysis. All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1996 and the local ethics committee at the Philipps-University, Marburg.

Genomic DNA Isolation and PCR Amplification

Genomic DNA was isolated from whole EDTA blood using the Quick-DNA HMW MagBead Kit (Zymo Research, Freiburg, Germany). The *LDLR* gene was amplified in 5 fragments spanning the promoter region and all 18 exons of the *LDLR* gene (**Supplementary Table S1**). Touchdown PCR reactions were performed in 25 µl reactions containing 12.5 µL LongAmp Taq 2X Master Mix (New England BioLabs, Frankfurt, Germany), 1 µM of each forward primer and reverse primer and 160 ng genomic DNA. PCR cycling conditions were: Initial denaturation for 30 s at 94°C; 20 cycles of 30 s denaturation at 94°C, 30 s annealing at 63–58°C (-0.5°C/2 cycles), 7 min 30 s extension at 65°C; followed by 30 cycles of denaturation at 94°C, 30 s annealing at 58°, 7 min 30 s extension at 65°C and 10 min final extension at 65°C. Purity and correct sizes of the amplicons were verified by DNA agarose gel electrophoresis.

Oxford Nanopore Sequencing

5 µl of each PCR product were treated with the Exo-CIP Rapid PCR Cleanup Kit (New England BioLabs, Frankfurt, Germany). 100 fmol of each fragment were pooled (final volume 25 µl) and used subsequently for library preparation with the SQK-RBK110.96 rapid barcoding kit (Oxford Nanopore Technologies, Oxford, United Kingdom). For addition of rapid barcodes, 2.5 µL of rapid barcode mix and 2.5 µL of nuclease free water were added to 5 µL of the pooled

TABLE 1 | Characteristic of patients selected for the validation experiments and workflow results.

patient ID	1	2	3	4
patient characteristics				
age (years)	36	2	32	23
sex	M	M	M	F
ethnicity	Turkish	Turkish	Turkish	Turkish
TC (mmol/L)	9.18	10.83	8.90	17.93
LDL-C (mmol/L)	7.94	9.18	5.57	16.26
genetic change	c.1729T>C	c.761A>C	c.761A>C	c.1567G>A
exon	12	5	5	10
amino acid change	p.(Trp577Arg)	p.(Gln254Pro)	p.(Gln254Pro)	p.(Val523Met)
protein consequence	missense variant	missense variant	missense variant	missense variant
zygosity (%)	heterozygous (39.3)	homozygous (88.8)	heterozygous (48.4)	homozygous (91.5)
dbSNP reference	rs8792550000	rs879254667	rs879254667	rs28942080
variant calling summary				
total variants	36	22	26	33
recorded in dbSNP	31	16	20	25
not recorded in dbSNP	5	6	6	8
variants in CDS	5	2	3	5
variants in non-CDS	31	20	23	28

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; CDS, coding sequence.

TABLE 2 | Identification of unknown variants in patients with suspected FH.

patient ID	5	6	7	8	9	10
age	38	6	40	44	13	6
sex	F	M	M	F	F	F
ethnicity	German	German	German	German	Turkish	Lebanese
TC (mmol/L)	7.62	14.86	7.88	12.67	14.86	20.61
LDL-C (mmol/L)	5.81	13.52	6.46	9.59	13.26	18.69
genetic change	c.1916T>A	c.1916T>A c.1844A>G	c.1844A>G	c.653del	c.1474G>A	c.1729T>C
exon	13	13/12	12	4	10	12
amino acid change	p.(Val639Asp)	p.(Val639Asp) p.(Glu615Gly)	p.(Glu615Gly)	p.(Gly218ValfsTer47)	p.(Asp492Asn)	p.(Trp577Arg)
protein consequence	missense variant	missense variant	missense variant	frame shift	missense variant	missense variant
zygosity (%)	heterozygous (46.2)	compound heterozygous (49.7/46.6)	heterozygous (46.0)	heterozygous (48.5)	homozygous (95.4)	homozygous (86.6)
dbSNP reference	rs794728584	rs794728584/–	—	rs137853966	rs373646964	rs879255000
pathogenicity	likely pathogenic	likely pathogenic/–	—	pathogenic	pathogenic	pathogenic
SIFT Sim et al. (2012)	deleterious	deleterious/deleterious	deleterious	—	deleterious	deleterious
polyphen2 Adzhubei et al. (2010)	benign	benign/probably damaging	probably damaging	—	probably damaging	probably damaging
PROVEAN protein Choi and Chan (2015)	deleterious	deleterious/deleterious	deleterious	—	deleterious	deleterious
mutation-taster Steinhaus et al. (2021)	disease causing	disease causing/ disease causing	disease causing	disease causing	disease causing	disease causing

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol.

fragment mix. The rapid barcoding reaction mixes were incubated at 30°C for 2 min followed by 2 min at 80°C. Subsequent cleanup, priming and loading of the MinION flow cells (FLO-MIN106D) were performed according to the manufactures protocol. For sequencing with a Flongle flow cell (FLO-FLG001), half of the volumes for addition of rapid barcodes were used. Sequencing runs were performed on a MinION Mk1C instrument (Oxford Nanopore Technologies, Oxford, United Kingdom).

Bioinformatic Analysis

Bioinformatic analysis was performed with Geneious Prime 2022.0.1 (https://www.geneious.com). Barcoded raw reads were mapped against the *LDLR* reference sequence (NG_009060.1) using the MiniMap2 plugin (Li, 2018). Variant calling was performed using the implemented Variations/SNP caller. Found variants were assigned to recorded variants in Ensembl by the annotation comparison tool (for the detailed procedure please refer to the guideline in the **Supplementary Material**).

RESULTS

Validation of the Workflow

We amplified the *LDLR* gene in 5 fragments covering the promoter region and the coding sequences of all 18 exons

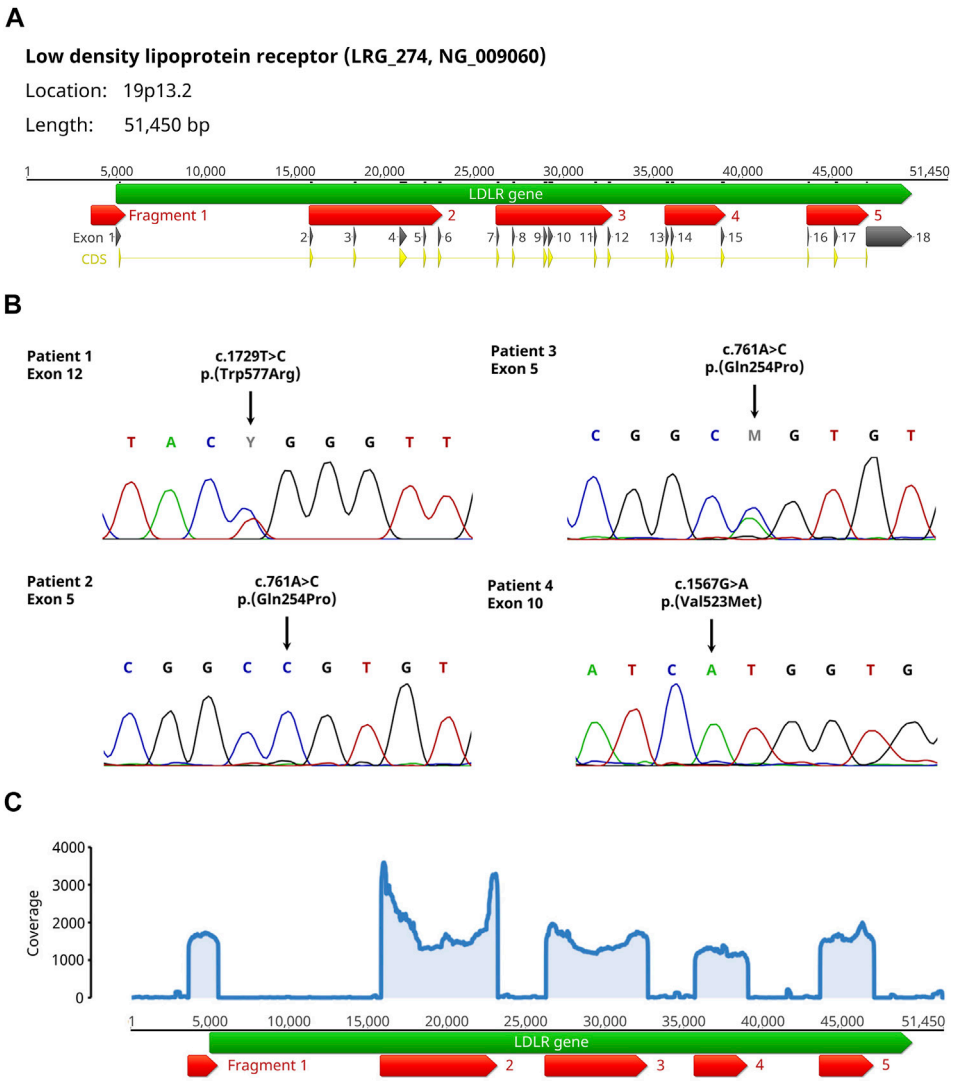

FIGURE 1 | Setup and results of the validation experiments for the Nanopore sequencing workflow of the *LDLR* gene. **(A)** The *LDLR* gene (green arrow) is amplified in 5 fragments (red arrows) covering the promoter region and the coding sequences (yellow arrows) of all 18 exons (dark grey arrows). Please note, that fragment 5 only covers the starting sequence of exon 18 because the remaining part contains only untranslated sequences. **(B)** Sanger sequencing results from the 4 patients with homo- or heterozygous FH showing the pathogenic variants within Exons 5, 10 and 12 including the change on DNA and protein level. **(C)** Coverage plot of the individual fragments showing equal distribution of coverage among all fragments exemplary for patient 4. For all 4 patients, the mean coverage was above 900 with a maximum mean coverage of 2,347.

(**Figure 1A**). Subsequently, an equimolar pooled fragment library was sequenced by Oxford Nanopore Sequencing. We then added all recorded variants in Ensembl (variant source dbSNP/ClinVar) (Howe et al., 2021) within the 5 fragments to the *LDLR* reference sequence [NG_009060.1 (O'Leary et al., 2016)] and mapped 50,000 randomly selected sequence reads to this reference sequence using the Minimap2 plugin (Li, 2018). Using the Geneious Variant caller, variants within the 5 fragments were identified and compared to the recorded variants, thereby differentiating between described variants with their dbSNP reference number and yet undescribed variants. The bioinformatic analysis was very fast taking in average 104 ± 9 *s* for mapping and 19 ± 3 *s* for variant calling.

To validate this workflow, a total of 4 patients were selected [2 related (father and son) and 2 unrelated] which had diagnosed FH with known disease-causing *LDLR* variants (**Table 1**; **Figure 1B**). For all samples, the coverage of the individual fragments was equally distributed, and the mean coverage was always above 900 (**Figure 1C**). In all cases, the variants were identified (**Table 1**). By analyzing the variant frequency, we could also determine the zygosity of the variants (heterozygous: 39%/48%, homozygous: 89%/92%). Analysis of the variant frequency from the two related patients (ID 2 + 3) permitted the clear distinction between heterozygous and homozygous inheritance pattern. We additionally discovered synonymous variants (2–5) in coding regions as well as a high proportion of intron variants

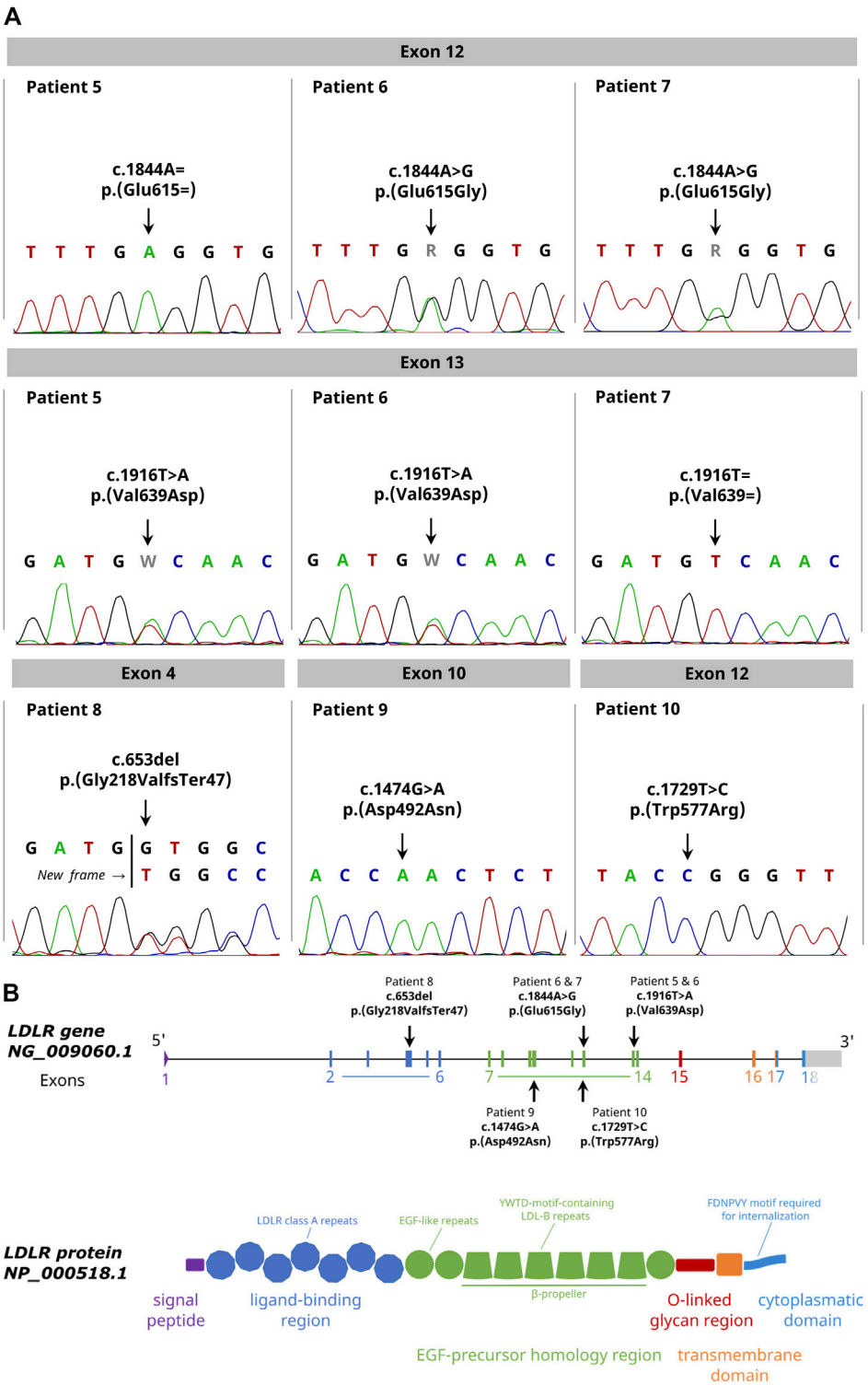

FIGURE 2 | Detection of unknown pathogenic LDLR variants in FH patients with the Nanopore Sequencing workflow. **(A)** Sanger sequencing results confirming all identified variants with indicated change on DNA and protein level. **(B)** Graphical illustration showing the genomic and domain structure of the LDLR and the location of the identified variants within the *LDLR* gene. The exons and the corresponding protein domain are indicated by the same color.

(85–91%) without a reported clinical significance or with benign clinical significance. The majority of the variants found were recorded in Ensembl (72–86%). Because the analysis of 100,000 randomly selected reads did not significantly improve the variant calling results, we conclude that 50,000 reads per patient sample is sufficient for variant analysis.

All pathogenic variants had a strand bias between 52 and 91% and a *p*-value of 0 or close to 0. Because high strand bias values indicate a low confidence hit, we only included variants with a strand bias lower than 100% into the analysis excluding variants with a strand bias of 100% (Guo et al., 2012; Currin et al., 2019).

We noted a high amount of tandem repeat deletions/insertions among the found variants suspecting that this may be caused by sequencing errors. To test this hypothesis, we tested if tandem repeat deletions/insertions are found with a higher frequency in both a healthy control and a FH sample compared to all non-tandem-repeat variants. Fisher's test yielded a $p-value = 0.005044$ indicating that tandem repeat deletions/insertions are caused by errors due to Nanopore sequencing. Consequently, we excluded all tandem repeat deletions/insertions from the further analysis.

Identification of Unknown Pathogenic *LDLR* Variants

To test our workflow on patient samples with unknown pathogenic variants, we selected 3 patients from a single family [father (ID 7), mother (ID 5) and son (ID 6)] aged 6–40 years and 3 additional unrelated female patients (ID 8–10) aged 5–43 years with plasma LDL concentrations ranging from 5.8 to 18.7 mmol/L and suspected FH based on characteristic symptoms including xanthomas and a familial history of premature coronary artery disease.

We detected two heterozygous variants in the family. The father was carrier of the variant c.1844A > G p.(Glu615Gly) in exon 12 and the mother was carrier of the variant c.1916T > A p.(Val639Asp) in exon 13. Both variants were confirmed by Sanger sequencing. The zygosity agreed with the plasma LDL-C concentrations (patient 5: 5.81 mmol/L, patient 7: 6.46 mmol/L) indicating a heterozygous FH in the parents. Consistently with the high plasma LDL-C concentration of 13.52 mmol/L of the son, we identified a compound heterozygous inheritance pattern of both variants in the son (**Table 2**; **Figure 2A**). Notably, the variant c.1844A > G p.(Glu615Gly) of the father was not recorded in any public genetic database (Ensembl, gnomAD and Varsome) so far indicating the identification of a novel variant.

In patient 8, we found the heterozygous frameshift variant c.653del p.(Gly218ValfsTer47) in exon 4 confirmed by Sanger sequencing. This frameshift leads to a stop codon 47 codons downstream of the deletion resulting in a truncated protein which explains the higher plasma LDL-C concentration (9.59 mmol/L) compared to the two other heterozygous variant carriers (patient 5,7).

In the patients 9 and 10, we identified the homozygous variant c.1729T > C p.(Trp577Arg) in exon 12 and c.1474G > A p.(Asp492Asn) in exon 10. The zygosity of both variants in the patients was consistent with the high levels of plasma LDL-C (patient 9: 13.26 mmol/L, patient 10: 18.69 mmol/L)) indicating a homozygous FH.

Due to the prevalence of FH, high sample numbers are not expected in routine diagnosis. Therefore, the use of a single-use Flongle flow cell over a reusable flow cell is more rational. We could also identify the variants of patients 6, 7 and 8, when the samples were sequenced on a Flongle flow cell. Thus, our workflow is also attractive for the analysis of low sample numbers.

DISCUSSION

In this study, we describe a fast and easy feasible workflow for the genetic analysis of the *LDLR* gene using long-amplicon sequencing with rapid barcoding. Mutations in this gene contribute to 90% of all disease-causing variants in familial hypercholesterolemia (Humphries et al., 2006). Our workflow consists of the amplification of the promoter region and the coding sequence of all 18 exons *via* PCR, followed by sequencing using Oxford Nanopore sequencing technology and rapid bioinformatic analysis utilizing the software Geneious Prime. Nanopore sequencing offers several advantages over conventional sequencing technologies such as low cost, fast and easy sample preparation, no need of specialized laboratory requirements, parallel sample sequencing and analysis of long-read sequence libraries. Thus, this technique is an attractive approach for every diagnostic laboratory (Jain et al., 2016; Petersen et al., 2019).

Amplification of long amplicons enabled us to reduce the sequencing effort to the relevant regions by keeping the sample preparation effort to a minimum. Additionally, by mapping the sequence against the *LDLR* reference sequence instead of the chromosome 19 reference sequence or the whole human genome, we minimized the computing time. The subsequent use of the bioinformatic software Geneious Prime allows the bioinformatic analysis of the sequencing data without any programming skills. By adding all variants recorded in Ensembl within the 5 amplicons, we were able to rapidly match found variants to the recorded variants, thereby allowing rapid evaluation of the variants using the recorded information about clinical significance, variant consequence as well as SIFT and PolyPhen2 predictions (Adzhubei et al., 2010; Sim et al., 2012).

Mapping of raw read sequences against the *LDLR* reference sequence was performed using Minimap2 (Li, 2018). We found that 50,000 reads as input data yielded sufficient coverage (>900) identifying all known pathogenic variants. Because increasing the input data to 100,000 reads did not improve the variant calling, we conclude that using 50,000 reads per patient sample is sufficient for accurate genetic analysis. Analysis of the variant frequency allowed the distinct determination of the variant zygosity. We found variant frequencies of 39–48% for heterozygosity and 89–92% for homozygosity agreeing with another study considering variants with variant frequencies of 30–70% as heterozygous (Ahsan et al., 2021).

Strand bias is an indicator for variant calling quality. For the pathogenic variants analyzed, strand bias ranged from 52 to 91%. High strand bias values indicate low confidence calls meaning

that the variant was found on either the forward or reverse strand with a much higher frequency. However, strand bias depends on the peripheral sequence around the variant and can differ between samples, thus filtering of the variant calls by strand bias should be performed with caution. To prevent the unintended exclusion of calls wrongly regarded as false-positive, we only excluded calls with a strand bias of 100% (Guo et al., 2012; Currin et al., 2019). There are two main modes leading to sequencing errors in Oxford Nanopore sequencing: certain sequences with indistinguishable conductance signals and irregular motor enzyme stepping (Noakes et al., 2019). This two modes may be sequence-specific (Currin et al., 2019) which may lead to differing sequences obtained from basecalling, and therefore account for varying strand bias of the variant calls.

Among the found variants, there was a high amount of tandem repeat deletions/insertions. We suspected this to may be caused by sequencing errors. Consequently, we tested if tandem repeat deletions/insertions are found with a higher frequency in both a healthy control and a FH sample compared to all non-tandem-repeat variants (Fisher's test: $p - value = 0.005044$). This suggests that tandem repeat deletions/insertions are caused by errors due to Oxford Nanopore sequencing. Therefore, all tandem repeat deletions/insertions were excluded from the further analysis. It is reported by ONT that the determination of the correct number of bases of homopolymers is an issue with Nanopore sequencing (Brown, 2016). Consistent with other studies, we assume that tandem repeats lead to indistinguishable conductance states due to their inherent nature of repeating identical bases (Rang et al., 2018; Noakes et al., 2019). Additionally, these errors may be caused by sequence-dependent kinetics of the motor enzyme (Craig et al., 2017) complicating the resolution of tandem repeat length (Noakes et al., 2019; Whitford et al., 2021). Consequently, nanopore sequencing is reported to cause an increased frequency of homopolymer deletions (Cretu Stancu et al., 2017; Whitford et al., 2021).

Our study identified a novel variant in the *LDLR* gene which was not listed in the genetic databases (dbSNP, ClinVar, Ensembl, gnomAD and Varsome). A paternal missense variant c.1844A > G p.(Glu615Gly) in exon 12 was detected in a FH family with a compound heterozygous affected child. This variant is located in the EGF-like domain of the LDLR contributing to the receptor dissociation due to the lowered pH in endosomes (**Figure 2B**). Although, no clinical significance is reported for the identified variant, in the same codon, a c.1844A > T p.(Glu615Val) FH-causing variant has been previously reported and classified as likely pathogenic in ClinVar (Marduel et al., 2010). The in-silico mutation prediction tools PolyPhen2, SIFT, PROVEAN and Mutation Taster classified our novel p.(Glu615Gly) LDLR variant as pathogenic. Taken together, we conclude that the identified variant c.1844A > G p.(Glu615Gly) is likely to be pathogenic. In the same family, a maternal heterozygous missense variant c.1916T > A p.(Val639Asp) in exon 13 was identified, also located in the EGF-like domain of the receptor. The variant is reported with conflicting interpretations regarding pathogenicity in ClinVar. However, Nauck et al. (2001) report this variant as pathogenic and also published it in a family with FH.

We identified a heterozygous frameshift variant c.653del p.(Gly218ValfsTer47) in exon 4 in a female FH patient with angiographically proven single-vessel coronary artery disease. The variant is located in repeat 3 of the ligand-binding domain of the LDL receptor and leads to production of a shortened non-functional protein product (Giesel et al., 1995). Finally, our nanopore sequencing workflow identified the underlying homozygous variants in two girls (7 and 10 years old) with clinically proven homozygous familial hypercholesterolemia. In Patient 9, a missense variant c.1729T > C p.(Trp577Arg) was identified in exon 12. This variant lays within the highly conserved YWTD repeats that form the six-bladed ß-propeller domain of the LDLR and is known to produce a class 2 transport-defective LDL receptor (Soufi et al., 2009). In patient 10, a missense variant c.1474G > A p.(Asp492Asn) was detected in exon 10. This variant is also located within the highly conserved YWTD repeats of the six-bladed ß-propeller domain. However, this variant seems to produce a class 5 recycling-defective receptor (Galicia-Garcia et al., 2020).

Although the clinical relevance of genetic analysis of FH is being controversially discussed (Carlson, 2010; Aatre and Day, 2011), our results show that the identification of the underlying variants can have direct impact on the treatment. For example, the identification of the two homozygous YWTD repeat variants in patients 9 and 10 results in a class 2 and class 5 defect (class 2: transport defect and class 5: recycling defect). In our experience, homozygous carriers of these variants neither respond to statin nor PCSK9 inhibitor treatment, requiring LDL-apheresis. For these patients, the direct application of lipid apheresis would spare the patient by preventing unnecessary stress by ineffective treatment as statins can have severe side effects (Bełtowski et al., 2009). In contrast, patients 5–7 with variants affecting receptor dissociation outside of the YWTD repeats may benefit from statin medication as statin-mediated LDLR upregulation may compensate for the reduced receptor recycling. In case of patient 8 with the heterozygous frameshift variant and a higher LDL-C concentration compared to the other heterozygous patients, combination therapy of statin and PCSK9 inhibitors may be sufficient to reduce the LDL-C concentrations to the recommended level (Alonso et al., 2018). The additional inhibition of PCSK9 may increase the presence of the unmutated LDLR by decreasing the turnover rate of the receptor (Roth and Davidson, 2018). Additionally, the psychological effect of a confirming positive result may not be neglected. Studies reported that a definitive positive gene result helped the patients to accept the diagnosis potentially increasing patient's compliance. Also, identification of the genetic cause often led to reassurance because by knowing that the relatives are either unaffected or can be early treated (Aatre and Day, 2011).

Here, we introduced a fast and easy PCR-based sequencing workflow for the rapid detection of pathogenic *LDLR* variants in the diagnosis of familial hypercholesterolemia. Through the use of Oxford Nanopore sequencing as a cost-effective method

without the need for major system requirements and specialized laboratory equipment, our technique is an attractive easy-to-use tool for all diagnostic laboratories (Jain et al., 2016; Petersen et al., 2019). Identification of *LDLR* variants by traditional Sanger sequencing can be still challenging, costly and slow, especially when multiple patient samples need to be analyzed (Whitford et al., 2021). Also, other NextGen sequencing technologies for genetic diagnostics still are expensive (Petersen et al., 2019). For example, genetic analysis of the *LDLR* of one patient by Sanger sequencing would cost $1,060 and $890 by NextGen sequencing (PreventionGenetics, 2021). In contrast, considering the costs of DNA purification, PCR reagents, the Flongle flow cell and the rapid barcoding kit, the costs per sample minus labor are $109. Furthermore, the costs per sample depend on the number of samples sequenced by our workflow leading to a significant reduction if more than one sample is analyzed. So, for 3 patients (e.g., a patient and two family members), the costs per sample analyzed by Sanger sequencing are $1,060 ($3,180 in total) and $557 ($1,670 in total) when analyzed by NextGen sequencing (PreventionGenetics, 2021) while costs per sample analyzed with our workflow are reduced to $49 ($147 in total). Therefore, standard analysis of the patient's family instead of the patient alone would be rational allowing early preventive treatment of affected family members.

Additionally, analysis using our workflow is notably faster and cheaper than analysis by Sanger or NextGen sequencing taking 18 days for standard order or 14 days for STAT order (PreventionGenetics, 2021). In contrast, upon arrival of the blood sample analysis of our workflow is completed within 3 days.

Finally, our workflow is not restricted to sequencing the *LDLR* gene alone but can be easily adapted to the other genes related with FH and most importantly, to any desired gene contributing to any hereditary disease by using our template documents (**Supplementary Templates**). We are currently adapting our workflow to the screening of genes associated with cardiomyopathy. Thereby, our workflow can be used to offer genetic diagnostics for a variety of clinically relevant hereditary diseases. Due to the technique of Nanopore Sequencing, these different samples can even be sequenced in the same run, thereby reducing the time and cost for the analysis. We believe that the workflow presented in this study is an attractive opportunity for every diagnostic laboratory to conduct fast and easy genetic diagnostics especially valuable for clinics to provide fast in-house genetic diagnostics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical commitee of the Department of Medicine of the Philipps University Marburg. Written informed consent to participate in this study was provided by either the participant or the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MS developed the study concept. SB developed the bioinformatic workflow and conducted the bioinformatic analysis. MS and SB performed the laboratory experiments and wrote the manuscript. MS, SB, and VR designed the study and performed the Nanopore Sequencing. BK and JS did the clinical evaluation of the patients and the data collection. BS and JS supervised the study. BS, JS, and VR critically reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.836231/full#supplementary-material

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Familial Hypercholesterolemia: Real-World Data of 1236 Patients Attending a Czech Lipid Clinic. A Retrospective Analysis of Experience in More than 50 years. Part I: Genetics and Biochemical Parameters

Veronika Todorovova, Tereza Altschmiedova, Michal Vrablik and Richard Ceska*

Third Department of Medicine—Department of Endocrinology and Metabolism of the First Faculty of Medicine, Charles University and General University Hospital, Prague, Czechia

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***Correspondence:**
Tereza Altschmiedova
tereza.altschmiedova@vfn.cz

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Introduction: The cause of familial hypercholesterolemia (FH) is defect in LDL receptor or familial defect of apolipoprotein B-100 (FDB) or, rarely, defect in proprotein convertase subtilisin/kexin type 9. Identification and treatment of patients with FH improves their prognosis. Our data represent retrospective analysis of 50 years of specialised care in our center.

Patients and Methods: A group of 1236 FH patients (841 women, 395 men; 993 study subjects and 243 relatives; mean age 44.8 ± 16.7 years) included 154 FDB patients followed at the Lipid Clinic of the General University Hospital in Prague since the mid-1960s to the present. Clinical diagnosis was based on the Dutch Lipid Clinic Network Criteria. Genetic analysis was performed using PCR-RFLP to detect FDB and apolipoprotein E (APOE) polymorphism. Biochemical data were collected and statistically analysed.

Results: At baseline, mean LDL-C and total cholesterol (TC) levels of all FH patients combined were 6.49 ± 1.92 mmol/L and 8.95 ± 1.95 mmol/L, respectively. Their LDL-C levels decreased to 3.26 ± 1.57 mmol/L and TC levels to 5.43 ± 1.69 mmol/L during follow-up. In the subgroup of LDL receptor-mediated FH (non-FDB) patients, baseline LDL-C and TC levels of 6.61 ± 1.95 mmol/L and 9.09 ± 1.97 mmol/L declined to 3.21 ± 1.60 mmol/L and 5.39 ± 1.72 mmol/L, respectively, during follow-up. In the FDB subgroup of patients, baseline levels of LDL-C and TC were 5.57 ± 1.46 mmol/L and 7.88 ± 1.58 mmol/L decreasing to 3.45 ± 0.24 mmol/L and 5.58 ± 1.37 mmol/L, respectively, during follow-up. Differences were also found in the effects of various APOE isoforms on lipid lowering. A significant decrease in lipid parameters was observed with the E2E2 isoform whereas a minimal decrease was seen with the E4E4 and E3E3 isoforms.

Conclusion: Whereas, overall, non-FDB patients had higher baseline lipid levels, these levels declined more appreciably compared with FDB patients during follow-up. Our retrospective analysis also found different effects of APOE isoforms on the decrease in lipid levels.

Keywords: familial hypercholesterolemia, familial defective apolipoprotein B-100, LDL-C, ApoB, ApoE isoform, Lp(a), statin, ASCVD

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant inherited disorder characterised by elevated levels of low-density lipoprotein cholesterol (LDL-C) whose accumulation leads to the development of atherosclerotic cardiovascular disease (ASCVD); moreover, if not treated properly, it may result in premature death (Watts et al., 2016). It is estimated that, while there are 30 million FH patients worldwide, most of them are unaware of their condition (The FH Foundation, 2021). The prevalence of heterozygous FH (HeFH) is 1 per 200 to 250, with their LDL-C levels ranging between 4 and 13 mmol/L (Cuchel et al., 2014; Benn et al., 2016). In homozygous FH patients, the levels of LDL-C are >13 mmol/L and the prevalence of this rare disease is approximately 1 per 160,000 to 300,000 (Cuchel et al., 2014). The diagnosis of FH is established based on the Dutch Lipid Clinic Network Criteria (DLCNC) categorizing patients into definite (>8 points), probable (6–8 points), possible (3–5 points) and unlikely (<3 points) FH groups. The patients are assigned to their respective categories based on each individual´s family history, clinical history, physical examination, levels of LDL-C and, possibly, genetic testing (Benn et al., 2016). The patients are first asked to change their eating habits and increase physical activity. However, lifestyle changes are not always enough and treatment has to be enhanced pharmacologically. Familial hypercholesterolemia patients are most often treated with statins, a class of drugs highly effective in lowering LDL-C levels, especially when combined with ezetimibe (Gagné et al., 2002). A breakthrough in the treatment of FH came with the discovery of PCSK9 inhibitors shown to decrease LDL-C by ≥50% (Watts et al., 2020).

Variants of three genes are a major cause of FH. The most common of these mutations occur in the LDL receptor (*LDLR*) gene and can lead to ligand-binding dysfunction, impaired LDL transport or internalization, recycling, or complete receptor deficiency (Soutar and Naoumova, 2007; Cuchel et al., 2014). Likewise, FH can be caused by mutations in the apolipoprotein B (*APOB*) and the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) (Vrablik et al., 2020). To the best of our knowledge, no mutation in the *PCSK9* gene in the Czech population has been reported to date. An important role is also played by the apolipoprotein E (*APOE*) gene, which affects the levels of LDL-C thus contributing to higher LDL-C levels in FH patients (Pirillo et al., 2017; Rashidi et al., 2017; Khalil et al., 2021).

A mutation in the *APOB* gene causes familial defective apolipoprotein B-100 (FDB), an autosomal dominant disease of lipid metabolism similar to LDL receptor-mediated FH (non-FDB) characterised by elevated plasma LDL-C levels (Vega and Grundy, 1986; Innerarity et al., 1987). The prevalence of FDB varies largely being, e.g., approximately 1 per 209 in Switzerland while the figure for Denmark is 1 per 883 (Miserez et al., 1994; Miserez and Muller, 2000; Benn et al., 2016). Familial defective apolipoprotein B-100 is caused by monogenic variants in the *APOB* gene where a single amino acid, arginine, at position 3527 is replaced, most frequently, by glutamine (p.R3527Q) and, rarely, by tryptophan (p.R3527W) or lysine (p.R3527L) or at position 3558 where arginine is replaced by cysteine (p. R3558C). This replacement leads to other protein conformations disrupting the binding of apolipoprotein B-100 (as a part of LDL particles) to LDLR (Brown and Goldstein, 1986; Whitfield et al., 2004).

The most common APOE isoform is E3E3 with the p.C112 and p.R158 variants (Ferrières et al., 1994; Eichner et al., 2002; Phillips, 2014). A less frequent isoform increasing LDL-C levels and contributing to the risk of developing Alzheimer's disease is E4E4, i.e., the p.C112R and p.R158 variants (Huebbe and Rimbach, 2017; Muñoz et al., 2019). A rare isoform is E2E2 determined by the p.C112 and p.R158C variants.

Familial defective apolipoprotein B-100 is clinically almost indistinguishable from FH; it is easier to identify FDB genetically as a common monogenic variant R3527Q. Unlike FDB, FH can be caused by monogenic variants as well as polygenic forms encountered in approximately 20% of FH patients (Trinder et al., 2019). Although many studies have focused on numerous aspects of FH, data in the relevant literature about the individual FH subgroups and the differences between them are relatively scarce. Still, it is most likely that the clinical features, effect of treatment and inherent risks of the disease are significantly different between the non-FDB and FDB subgroups of patients.

The aim of this retrospective analysis was to analyse data of a large homogeneous group of patients diagnosed to have FH and followed in a single lipid center and, also, to show the benefits of therapy and the results obtained over the course of half of a century in specialised care. This large group was followed and processed in 2 different perspectives. This article (Part I) is focused on differences in the lipid profiles in subgroups of FH patients, i.e. FDB versus non-FDB patients, and in FH patients with different *APOE* genotypes. Concurrent article (Part II) by Altschmiedova et al. (2022) is focused on clinical symptomatology, i.e., on differences between the parameters in patients whose FH is already complicated by overt ASCVD and those without ASCVD in order to identify factors contributing to a complicated course of the disease.

PATIENTS

Characteristics of Individuals Diagnosed With FH

A total of 1236 FH patients (841 women and 395 men; 993 study subjects, 243 relatives; mean age 44.8 ± 16.7 years) attending the Lipid Clinic of the General University Hospital in Prague, Czech Republic, were followed. The diagnosis of FH in our patients was based on the Dutch Lipid Clinic Network Criteria (DLCNC). Genetic analysis including FDB and APOE isoforms was performed in more than 76% of FH patients; however, a mutation in the *LDLR* gene was investigated in only ≥10% of these patients (**Supplementary Figure S1**). Risk factors and clinical complications are summarised in **Table 1** and they are in more detail described in the article about clinical symptomatology by Altschmiedova et al. (2022) Enrolled in the retrospective analysis were patients, both pharmacotherapy-naïve and those treated pharmacologically, and their relatives.

TABLE 1 | Clinical characteristics.

Risk factors, clinical complications	Percentage (%)
DM	6.47
Hypertension	26.70
Smokers	31.39
Arcus lipoides corneae	3.80
Xanthalesma	4.61
Tendon xanthomas	3.32
CAD (MI included)	9.63
Stroke	2.51
PAD	2.59
Death	2.83

DM, diabetes mellitus; CAD, coronary artery disease; MI, myocardial infarction; PAD, peripheral arterial disease.

The first patients of this retrospective analysis have been followed since the mid-1960s when diagnosed with FH based on their clinical symptoms; complete biochemical and genetic data have been available here since 1974. The follow-up has continued to date with the latest biochemical values recorded in late 2020.

MATERIALS AND METHODS

Biochemical Analysis

Blood samples were collected from study subjects. The serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C) and triglycerides (TG) were measured enzymatically on automated analysers (Modular P800, Roche, Basel, Switzerland and UniCel DxC 880i Beckman Coulter, Brea, CA, United States). LDL-C was calculated using the Friedewald formula whereas apolipoprotein B (APOB) and lipoprotein (a) [Lp(a)] were measured by nephelometry and immunonephelometry.

Genetic Analysis

Genomic DNA was isolated from peripheral blood collected into EDTA-anticoagulated tubes using the salting out method proposed by Miller et al., 1998. The concentration and purity of DNA were determined using a spectrophotometer (A260/A280; BioPhotometer Eppendorf 6131, Eppendorf, Germany).

The p.R3527Q (*MluI*) and p.R3558C (*MspI*) variants in the *APOB* gene were detected by polymerase chain reaction (PCR). Oligonucleotides 5′-CTT ACT TGA ATT CCA AGA GCA CCC-3′ and 5′-TGT ACT CCC AGA GGG AAT ATA CGC-3′ were used as the primer set. *MluI* and *MspI* as restriction enzymes were used in PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. Fragments were subsequently separated and visualised by electrophoresis on GelRed-stained 4% agarose gel (MetaPhor-agarose: agarose = 3:1).

The *E2*, *E3*, *E4* variants in the *APOE* gene were detected by PCR. Here, oligonucleotides 5′-TCC AAG GAG CTG CAG GCG GCG CA-3′ and 5′-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A-3′ were used as the primer set. *CfoI* as a restriction enzyme was used in PCR-RFLP analysis. Fragments were subsequently separated and visualised by electrophoresis on GelRed-stained 10% polyacrylamide gel.

Variants in the *LDLR* gene were analysed by Sanger sequencing. The specific primers (**Supplementary Material S1**) were designed according to the sequence of 18 *LDLR* exons. The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit.

Statistical Analysis of Baseline and Follow-Up Data

Statistical analysis was performed using STATISTICA 13 software (TIBCO Software Inc., Palo Alto, CA, United States). Values in the text, tables and figures are means ± standard deviation (SD). The level of statistical significance was set at 5%. Comparison of baseline vs. follow-up was done using the paired *t*-test. The lipid parameters of the two subgroups were compared using the two-sample *t*-test or, when comparing three groups, using analysis of variance (ANOVA).

RESULTS

An appreciable decrease in LDL-C levels, from 6.49 ± 1.92 mmol/L to 3.26 ± 1.57 mmol/L (~49.8%) was observed (**Figure 1**) in all patients. Likewise, a 39.3% decrease, from 8.95 ± 1.95 mmol/L to 5.43 ± 1.69 mmol/L, in TC levels was seen (**Supplementary Figure S2**). The mean levels of other 2 parameters (TG and HDL-C) are clearly shown in **Table 2**. Major reductions were noted in APOB (−38.1%) and TG (−23.8%) levels (**Supplementary Figure S3, S4**). The decrease in HDL-C levels was only a small one (−6.6%) (**Supplementary Figure S5**). The differences between the baseline and follow-up levels of the parameters investigated were statistically significant ($p < 0.001$) except for Lp(a) whose levels remained almost unaltered throughout the retrospective analysis (**Supplementary Figure S6**).

Based on the division of our patients into subgroups according to the use (Y) or non-use (N) of therapy, statistically significant differences ($p < 0.005$) were found between the subgroups in the baseline levels of LDL-C, TC, APOB, TG, and Lp(a). However, statistically significant differences ($p < 0.001$) between the subgroups were found during follow-up in their LDL-C, TC, APOB, and HDL-C levels. When comparing the on-therapy levels of these parameters with baseline, the biggest decreases—except for Lp(a)—were noted in the groups not initiating their therapy until the start of the analysis (N/Y). In this particular group, LDL-C, TC and APOB levels dropped by as much as 55.7, 44.8 and 45.4%, respectively (**Table 3**). A smaller decline (−26.4%) in the N/Y group occurred in TG levels. Decreases in the levels of lipid parameters were likewise observed in the group of patients on pre-existing therapy (Y/Y) whose LDL-C, TC and APOB levels decreased by 49.6, 38.2 and 37.0%, respectively. The Y/Y group showed a smaller decrease in TG levels (−23.4%). As to HDL-C levels, similar to all study parameters except for Lp(a), there was an obvious decrease in the group of patients not receiving therapy throughout the analysis (N/N). The decreases in the levels of LDL-C, TC, APOB, TG

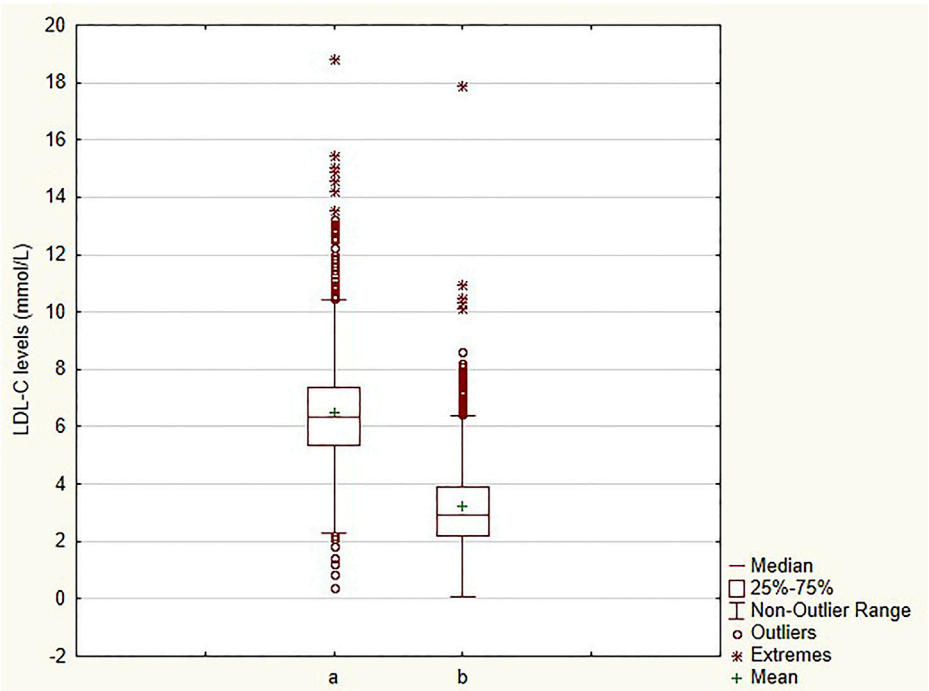

FIGURE 1 | Comparison of LDL-C levels at baseline and at follow-up a—baseline; b—follow-up; LDL-C—low-density lipoprotein cholesterol.

TABLE 2 | Baseline and follow-up lipid levels of FH cohort.

Parameter	Number of patients	Baseline	Follow-up	Difference (%)	*p*-value
		Mean ± SD	Mean ± SD		
LDL-C (mmol/L)	1,049	6.49 ± 1.92	3.26 ± 1.57	–49.8	$p < 0.001$
TC (mmol/L)	1,118	8.95 ± 1.95	5.43 ± 1.69	–39.3	$p < 0.001$
APOB (g/L)	184	1.76 ± 0.56	1.09 ± 0.56	–38.1	$p < 0.001$
TG (mmol/L)	1,108	1.81 ± 1.13	1.38 ± 0.78	–23.8	$p < 0.001$
HDL-C (mmol/L)	1,092	1.67 ± 0.46	1.56 ± 0.46	–6.6	$p < 0.001$
Lp(a) (g/L)	284	0.56 ± 0.74	0.59 ± 0.74	5.4	$p = 0.2706$

SD, standard deviation.

and HDL-C were significant in all groups of patients (categorised by their therapy) throughout the analysis ($p < 0.05$).

Another division of our population of FH patients was based on genetic analysis of *APOE* polymorphisms by individual APOE isoforms, where differences in the levels of individual lipid parameters throughout the analysis were compared (**Table 4**). The biggest decreases in the levels of lipid parameters were seen in patients with the E2E2 isoform, being 75.3, 59.0, 53.5, 56.2, 17.6, and 36.7% in LDL-C, TC, APOB, TG, HDL-C and Lp(a) levels, respectively. In patients with the other isoforms, the decreases in LDL-C, TC, APOB and TG levels were within the ranges of 49.0–57.8%, 36.8–43.9%, 32.3–57.3%, and 22.4–39.4%, respectively, while HDL-C levels remained almost unchanged. A decline in Lp(a) levels was only seen in patients with the E2E2 isoform. The decrease was significant ($p < 0.001$) in only TG levels during follow-up.

A total of 1008 FH patients were genetically tested. Familial defective apolipoprotein B-100 was detected in 154 patients (mean age 40.8 ± 18.1 years; 107 women and 47 men; 117 study subjects and 37 relatives). One of these patients was diagnosed as FDB homozygote. The rest of genetically tested FH patients group consisted of 854 patients (557 women and 277 men; 686 study subjects and 168 relatives) supposed to have mutation in *LDLR* gene (non-FDB) (mean age 44.8 ± 16.0 years). Five patients were diagnosed with homozygous FH due to a mutation in the *LDLR* gene. Overall, another 228 study subjects met the DLCN criteria for FH but genetic testing was not performed and, thus, these subjects were excluded from further analysis.

One of the main subgroups within our study participants was that of non-FDB patients where significant decreases in the levels of LDL-C were noted in 754 patients (–51.1%); TC, in 795

Table 3 | Distribution of FH patients by treatment and effect of treatment on lipid levels.

Parameter	Group	Baseline			Follow-up					
		N	Mean ± SD	*p*	N	Mean ± SD	*p*	N	Diference (%)	*p*
LDL-C (mmol/L)	Y/Y	167	5.76 ± 1.93	$p < 0.001$	166	2.89 ± 1.13	$p < 0.001$	160	−49.6	$p < 0.001$
	N/Y	678	6.83 ± 1.80		678	3.01 ± 1.37		660	−55.7	
	N/N	172	6.35 ± 2.07		97	5.48 ± 2.02		93	−10.7	
TC (mmol/L)	Y/Y	175	8.15 ± 1.98	$p < 0.001$	169	5.03 ± 1.31	$p < 0.001$	169	−38.2	$p < 0.001$
	N/Y	699	9.32 ± 1.83		689	5.16 ± 1.49		689	−44.8	
	N/N	177	8.72 ± 2.07		102	7.84 ± 1.90		102	−8.8	
APOB (g/L)	Y/Y	97	1.57 ± 0.51	$p < 0.001$	65	0.98 ± 0.34	$p < 0.001$	43	−37.0	$p < 0.001$
	N/Y	316	1.86 ± 0.51		190	0.99 ± 0.39		86	−45.4	
	N/N	89	1.85 ± 0.65		40	1.69 ± 0.79		14	−7.9	
TG (mmol/L)	Y/Y	174	1.86 ± 1.17	$p = 0.003$	169	1.41 ± 0.69	$p = 0.792$	168	−23.4	$p = 0.026$
	N/Y	690	1.85 ± 1.17		688	1.37 ± 0.80		680	−26.4	
	N/N	177	1.54 ± 0.84		102	1.38 ± 0.84		102	−11.8	
HDL-C (mmol/L)	Y/Y	174	1.63 ± 0.39	$p = 0.536$	169	1.51 ± 0.40	$p < 0.001$	168	−6.9	$p < 0.001$
	N/Y	687	1.67 ± 0.45		682	1.53 ± 0.44		671	−8.2	
	N/N	175	1.67 ± 0.50		102	1.77 ± 0.54		101	1.9	
Lp(a) (g/L)	Y/Y	154	0.61 ± 0.66	$p = 0.003$	47	0.74 ± 0.67	$p = 0.229$	47	7.7	$p = 0.921$
	N/Y	553	0.44 ± 0.60		181	0.57 ± 0.80		180	4.8	
	N/N	113	0.39 ± 0.62		14	0.38 ± 0.39		13	12.1	

Y/Y, on treatment at baseline and throughout the analysis; N/Y, no treatment at baseline/on treatment throughout the analysis; N/N, no treatment at baseline and throughout the analysis; N, number of patients; SD, standard deviation; p, p*-value.*

patients (−40.7%); APOB, in 132 patients (−40.7%) and TG, in 788 patients (−24.4%). In 781 non-FDB patients, HDL-C levels declined by a mere 7.6% whereas Lp(a) levels remained almost unaltered. The second subgroup consisted of 154 patients with FDB. Their mean values of lipid parameters, both baseline and follow-up, are given in more detail in **Table 5**. Among the FDB patients, appreciable decreases in LDL-C were seen in 120 patients (−37.7%), TC in 131 patients (−30.3%), APOB in 26 patients (−29.2%), and TG in 130 patients (−24.0%). In 127 patients, HDL-C levels decreased by 5.9% during follow-up.

A comparison of non-FDB and FDB patients revealed significant differences ($p < 0.001$) in their baseline levels of LDL-C, TC, APOB and TG whereas the difference versus follow-up levels was significant ($p < 0.001$) only in TG levels. Statistically significant differences ($p < 0.05$) were found between the baseline and follow-up levels of LDL-C, TC, APOB and Lp(a) in both, non-FDB and FDB, subgroups of patients.

At the end of the day, we would like to present the least favourable mean levels of Mr. and Mrs. FH and FDB in our analysis.

- Those of Mr. and Mrs. FH in our group are as follows: LDL-C 6.61 mmol/L, TC 9.09 mmol/L, APOB 1.83 g/L, TG 1.86 mmol/L, HDL-C 1.68 mmol/L and Lp(a) 0.46 g/L and
- Those of Mr. and Mrs. FDB in our group are as follows: LDL-C 5.57 mmol/L, TC 7.88 mmol/L, APOB 1.53 g/L, TG 1.40 mmol/L, HDL-C 1.68 mmol/L and Lp(a) 0.40 g/L.

DISCUSSION

What makes our retrospective analysis actually important is that our data were collected from a large group of more than 1,000 patients with FH attending a single lipid clinic. A positive finding of the long-term follow-up of patients in our center were decreases in the levels of LDL-C by more than 50%, which were not only statistically significant, but, also, clinically beneficial. Of no less importance was the decrease (by as much as 38%) in LDL-C levels in our FDB patients. As suggested by earlier reports, the levels of lipid parameters in FBD patients are generally lower than in those with LDL receptor-mediated FH (Gaffney et al., 2002; Vohnout et al., 2003; Fouchier et al., 2004). Similarly, the disorder diagnosed in our FDB homozygous patient was not as severe as that seen in homozygous individuals with receptor-mediated disorder. This may be explained by the APOE-regulated clearance of very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) particles in FDB patients and the interaction between APOB and LDLR, important for the conversion of IDL to LDL-C (Gaffney et al., 2002; Vohnout et al., 2003).

Our analysis is a retrospective one whose first participants were receiving specialised care in a Prague-based clinic headed by Josef Šobra, with their data collection starting as early as 1960s (Šobra, 1970). Long-term care of these patients succeeded in reducing their cardiovascular risk and the clinic continues to provide individual care to each FH patient. While some of the patients have been taken care of for over 30 years, others have been attending the facility for less than 2 years; nonetheless, their personalised treatment plans have been shown to be beneficial in the long term. This explains the absence of statistically analysed data from the above period. This is partly due to the different numbers of patients and amount of analysed data in the individual subgroups of patients, with some of them referred to other physicians using different procedures, approaches and requirements for lipid parameter determination. However, the differences in the amount of data analysed and presented here are

TABLE 4 | Patients with specific APOE isoforms and effect of treatment on lipid levels.

Parameter	Group	Baseline			Follow-up					
		N	Mean ± SD	*p*	N	Mean ± SD	*p*	N	Diference (%)	*p*
LDL-C (mmol/L)	E3E4	219	6.40 ± 1.80	$p = 0.753$	212	3.09 ± 1.36	$p = 0.034$	202	−50.9	$p = 0.615$
	E2E3	63	6.58 ± 2.22		59	3.27 ± 1.45		58	−50.3	
	E3E3	651	6.49 ± 1.92		603	3.32 ± 1.65		588	−49.0	
	E2E4	9	6.03 ± 2.36		11	2.45 ± 0.77		9	−57.8	
	E4E4	22	5.94 ± 1.97		21	3.02 ± 1.26		21	−49.0	
	E2E2	4	6.51 ± 1.30		6	1.77 ± 0.50		4	−75.3	
TC (mmol/L)	E3E4	230	8.90 ± 1.86	$p = 0.644$	217	5.29 ± 1.46	$p = 0.126$	217	−40.5	$p = 0.202$
	E2E3	65	8.93 ± 2.22		61	5.42 ± 1.62		61	−39.6	
	E3E3	668	8.94 ± 1.97		619	5.48 ± 1.78		619	−38.9	
	E2E4	11	8.29 ± 2.07		11	4.65 ± 0.84		11	−43.9	
	E4E4	22	8.53 ± 1.98		21	5.38 ± 1.47		21	−36.8	
	E2E2	6	9.82 ± 1.98		6	4.02 ± 0.60		6	−59.0	
APOB (g/L)	E3E4	116	1.79 ± 0.53	$p = 0.129$	84	1.09 ± 0.46	$p = 0.361$	47	−37.7	$p = 0.933$
	E2E3	35	1.67 ± 0.62		24	1.13 ± 0.40		14	−32.3	
	E3E3	348	1.80 ± 0.54		182	1.08 ± 0.54		97	−39.8	
	E2E4	6	1.76 ± 0.68		3	0.81 ± 0.10		2	−45.5	
	E4E4	11	1.60 ± 0.34		7	0.78 ± 0.28		3	−57.3	
	E2E2	5	1.22 ± 0.24		2	0.60 ± 0.11		1	−53.5	
TG (mmol/L)	E3E4	228	1.86 ± 1.28	$p < 0.001$	217	1.44 ± 0.77	$p = 0.485$	215	−24.1	$p < 0.001$
	E2E3	65	1.77 ± 1.10		61	1.34 ± 0.54		61	−24.4	
	E3E3	662	1.73 ± 1.08		619	1.34 ± 0.83		613	−23.6	
	E2E4	11	2.30 ± 1.68		11	1.39 ± 0.76		11	−39.4	
	E4E4	22	1.76 ± 0.84		21	1.38 ± 0.72		21	−22.4	
	E2E2	6	4.20 ± 2.50		6	1.84 ± 0.53		6	−56.2	
HDL-C (mmol/L)	E3E4	225	1.71 ± 0.47	$p = 0.141$	214	1.58 ± 0.45	$p = 0.391$	209	−8.3	$p = 0.443$
	E2E3	65	1.59 ± 0.51		60	1.53 ± 0.50		60	−5.1	
	E3E3	662	1.67 ± 0.45		616	1.56 ± 0.47		610	−7.1	
	E2E4	11	1.52 ± 0.53		11	1.57 ± 0.45		11	3.3	
	E4E4	22	1.85 ± 0.49		21	1.78 ± 0.55		21	−4.5	
	E2E2	6	1.73 ± 0.41		6	1.43 ± 0.26		6	−17.6	
Lp(a) (g/L)	E3E4	199	0.52 ± 0.66	$p = 0.219$	61	0.66 ± 0.73	$p = 0.361$	60	−3.8	$p = 0.692$
	E2E3	51	0.32 ± 0.45		12	0.20 ± 0.18		12	10.0	
	E3E3	569	0.44 ± 0.56		173	0.53 ± 0.63		173	13.2	
	E2E4	9	0.42 ± 0.46		4	0.59 ± 0.95		4	51.3	
	E4E4	19	0.48 ± 0.62		5	0.94 ± 0.89		5	3.5	
	E2E2	6	0.16 ± 0.10		1	0.19 ± 0.00		1	−36.7	

N, number of patients; SD, standard deviation; p, p-*value.*

TABLE 5 | FDB and non-FDB patients and effect of treatment on lipid levels.

Parameter	FDB	Baseline			Follow-up					
		N	Mean ± SD	*p*	N	Mean ± SD	*p*	N	Diference (%)	*p*
LDL-C (mmol/L)	+	148	5.57 ± 1.46	$p < 0.001$	124	3.45 ± 0.24	$p = 0.117$	120	−37.7	$p < 0.001$
	−	812	6.61 ± 1.95		779	3.21 ± 1.60		754	−51.1	
TC (mmol/L)	+	153	7.88 ± 1.58	$p < 0.001$	131	5.58 ± 1.37	$p = 0.252$	131	−30.3	$p < 0.001$
	−	840	9.09 ± 1.97		795	5.39 ± 1.72		795	−40.7	
APOB (g/L)	+	85	1.53 ± 0.37	$p < 0.001$	43	1.13 ± 0.38	$p = 0.448$	26	−29.2	$p = 0.023$
	−	430	1.83 ± 0.56		255	1.07 ± 0.51		132	−40.7	
TG (mmol/L)	+	152	1.40 ± 0.98	$p < 0.001$	131	1.07 ± 0.51	$p < 0.001$	130	−24.0	$p = 0.251$
	−	833	1.86 ± 1.17		795	1.42 ± 0.83		788	−24.4	
HDL-C (mmol/L)	+	151	1.68 ± 0.47	$p = 0.960$	129	1.60 ± 0.46	$p = 0.316$	127	−5.9	$p = 0.455$
	−	831	1.68 ± 0.46		790	1.55 ± 0.47		781	−7.6	
Lp(a) (g/L)	+	133	0.40 ± 0.45	$p = 0.229$	33	0.65 ± 0.62	$p = 0.350$	33	61.2	$p < 0.001$
	−	715	0.46 ± 0.60		217	0.54 ± 0.66		216	1.0	

+, FDB; –, non-FDB; N – number of patients; SD, standard deviation; p, p-*value.*

mainly due to FH patients referred from general practitioners to specialised centers; the result is some patients had incomplete baseline data while baseline blood sampling had not been performed in others. Another reason for the incompleteness data of some patients is only one value of some of the lipid parameters was obtained before the patient decided to discontinue follow-up.

A limitation of our analysis is the composition of our entire FH group consisting predominantly of patients attending a lipid clinic with only a small proportion being their family members. While not usual in other countries (Bhatnagar et al., 2000; Jarauta et al., 2016), a small number of relatives is a typical feature in the Czech Republic.

As expected, the differences in the decreases in lipid parameters between the untreated versus treated groups seen during the analysis in our lipid clinic were statistically significant. Nonetheless, the group of patients receiving treatment from practitioners prior to initiation of therapy by a lipid specialist also showed an appreciable decrease in their lipid levels, similar to that seen in patients not starting therapy before admission to our center. The implication is that targeted and proper management of FH patients is of crucial importance and, despite the undeniable role of general practitioners, tailored and specific care provided in lipid clinics is more effective and beneficial.

While it is difficult to identify a specific therapeutic strategy for over more than 50 years, generally, the treatment copied the availability and development of pharmacotherapy. It can be clearly stated that, until 1990, the mainstay of therapy of FH were cholestyramine and colestipol. Since 1990, treatment of FH has been based on statins (always the most efficient statin available, i.e., lovastatin, simvastatin, atorvastatin and rosuvastatin). A combination with ezetimibe has been used since the beginning of the 21st century and the monoclonal antibodies evolocumab and alirocumab have been available since 2018.

Patients with the rare *APOE E2E2* genotype showed an obviously major drop in the levels of LDL-C, TC and TG corresponding the metabolic processing of the E2E2 isoform, where particle clearance does not occur through binding to LDLR but through the LDL-related receptor and heparin sulfate proteoglycans (Phillips, 2014). A similar major decrease was observed in patients with the E2E4 isoform, processed partly in the same way as the above E2E2 isoform. In FH patients, the decreases seen with the E3 and E4 isoforms were smaller, a fact possibly attributable to the clearance of APOE *via* LDLR where binding may be impaired due to the high frequency of *LDLR* gene mutations in FH patients.

Another parameter assessed in our study were Lp(a) levels not showing significant changes in some of our study subgroups. This may be partly explained by the fact that analysis of Lp(a) levels was undertaken in a period when no therapy to modify Lp(a) levels was available yet.

The relationship between high Lp(a) levels and proprotein convertase subtilisin/kexin type 9 (PCSK9) was not investigated until 2018, when Sun et al. reported their data obtained from patients with heterozygous FH; it has been shown only recently that Lp(a) levels can be decreased with the use of PCSK9 inhibitors (Sun et al., 2018). PCSK9 inhibitors were approved for clinical use in the Czech Republic in 2018; hence, the introduction of PCSK9 inhibitors is not significantly reflected in our analysis.

Limitation of Retrospective Analysis

Despite their long-term follow-up, a small group of patients has not had genetic testing, with their diagnosis established solely using the DLCNC. As a result, some of these patients could not be conclusively identified as actually being or not being FDB patients. The relatively small number of mutations detected in the *LDLR* gene is due to the fact that the sequencing technique developed by Sanger was adopted by our lipid clinic only recently. Besides, the technique is also more time-consuming than those of PCR restriction fragment length polymorphism (RFLP) or real-time PCR detecting point mutations. The analysed *LDLR* gene region contains 18 exons which have to be sequenced separately when using Sanger's technique. The proportion of *LDLR* gene mutation analyses is likely to increase in our clinic with the introduction of new generation sequencing (NGS) techniques in the years to come.

Our retrospective analysis provides initial data obtained from a large group of patients attending a single lipid clinic and analysed in terms of the biochemical and genetic characteristics.

CONCLUSION

Using a large group of patients with familial hypercholesterolemia, the present analysis reports data related to lipid and lipoprotein metabolism. The project was designed to assess changes in the levels of these parameters between baseline and follow-up in patients receiving personalised care admitted to our clinic. Our experience gained within the international ScreenPro FH project shows that patient surveillance and long-term follow-up are most beneficial as documented by Ceska et al., 2019. As an extension to the outcome of the present retrospective analysis, clinical data of our FH cohort are reported in Part II by Altschmiedova et al. (2022)

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of General University Hospital, Prague. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

VT processed the data, wrote the article and participated in the interpretation of the analyzed data. TA contributed to the data

processing and TA and MV contributed to the writing and interpretation of the data. RC designed the project, interpreted the data and participated in the writing of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.849008/full#supplementary-material

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Familial Hypercholesterolemia: Real-World Data of 1236 Patients Attending a Czech Lipid Clinic. A Retrospective Analysis of Experience in More than 50 years. Part II. Clinical Characteristics

Tereza Altschmiedova, Veronika Todorovova, Michal Vrablik and Richard Ceska*

Third Department of Medicine - Department of Endocrinology and Metabolism of the First Faculty of Medicine, Charles University and General University Hospital, Prague, Czechia

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Alpo Juhani Vuorio, University of Helsinki, Finland

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***Correspondence:**
Veronika Todorovova
veronika.todorovova@vfn.cz

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Introduction: Patients with familial hypercholesterolemia (FH) are at increased risk of premature atherosclerotic cardiovascular disease (ASCVD).

Aim of study: To perform a retrospective analysis of data to assess the effects of individual lipoproteins and other risk factors (RFs) on the development of ASCVD and to compare these parameters in individuals with versus without ASCVD.

Patients and methods: Our study group included a total of 1,236 patients with FH (395 men and 841 women with a mean age of 44.8 ± 16.7 years) attending a single lipid clinic. The diagnosis of FH was established using the Dutch Lipid Clinic Network score (DLCN). Among the 1236 FH patients, 1,008 of them [854 suspected with LDL receptor-mediated FH and 154 with familial defective apolipoprotein B-100 (FDB)] were genetically analysed. Their RFs were assessed based on the patients' clinical characteristics.

Results: While patients with ASCVD had higher baseline LDL-C, TC, TG and Lp(a) compared with patients without this diagnosis, this ratio was just the opposite by the follow-up. The highest statistically significant differences were seen in the baseline levels of Lp(a) and, quite surprisingly, TG. Except for Lp(a), the levels of all lipid parameters declined significantly over time. While the incidence of diabetes and arterial hypertension was not higher in our group compared with the general population, these patients were at a more significant risk of ASCVD.

Conclusion: Familial hypercholesterolemia is a major RF for the development of ASCVD. While our analysis confirmed the important role of LDL-C, it also corroborated a strong correlation between ASCVD and other lipid parameters, and Lp(a) and TG in particular. Familial hypercholesterolemia is not the only RF and, to reduce cardiovascular risk of their patients, physicians have to search for other potential RFs. Patients diagnosed to have FH benefit from attending a specialized lipid clinic perse.

Keywords: familial hypercholesterolemia, LDL-cholesterol, Lp(a), ASCVD, RWD

INTRODUCTION

With an estimated prevalence of 1 to 200–250, familial hypercholesterolemia (FH) ranks among the most frequent inherited metabolic diseases (Nordestgaard et al., 2013; Beheshti et al., 2020). The typical FH patient is predestined to have high LDL cholesterol (LDL-C) levels since childhood considerably raising the risk of premature atherosclerotic cardiovascular disease (ASCVD) (The Lipid Research Clinic, 1984; Watts et al., 2016; Ference et al., 2017). All patients diagnosed with FH are automatically at least at high risk of developing ASCVD (Visseren et al., 2021). However, we suppose there are differences between individual patients which will decide whether or not ASCVD will eventually develop. All FH patients require, in particular, an early diagnosis and initiation of lipid-lowering therapy as soon as possible. The class of drugs of choice are statins which, by effectively lowering LDL-C levels, significantly reduce cardiovascular morbidity and mortality (Versmissen et al., 2008). To achieve the target levels of LDL-C, combination lipid-lowering therapy is quite often necessary; most often a combination of a statin with ezetimibe or, alternatively, with a PCSK9 inhibitor, is used (Visseren et al., 2021).

AIM OF STUDY

One of the goals of our project was to present the baseline and follow-up clinical and biochemical findings in a large cohort of patients diagnosed to have FH and attending a single lipid center to show that patients do benefit from mere surveillance and highly specialized therapy. In addition to assessing the effects of therapy on pre-defined lipid parameters, we evaluated the effects of individual lipoproteins and other major risk factors on the development of complications associated with the atherosclerotic process. In particular, we focused our attention on differences between the parameters in patients whose FH is already complicated by overt ASCVD and those without ASCVD in order to identify factors contributing to a complicated course of the disease.

PATIENT CHARACTERISTICS AND METHODS

The submitted project is a retrospective analysis of data of a total of 1,236 patients (841 women and 395 men with a mean age of 44.8 ± 16.7 years) with FH on follow-up in a single lipid center.

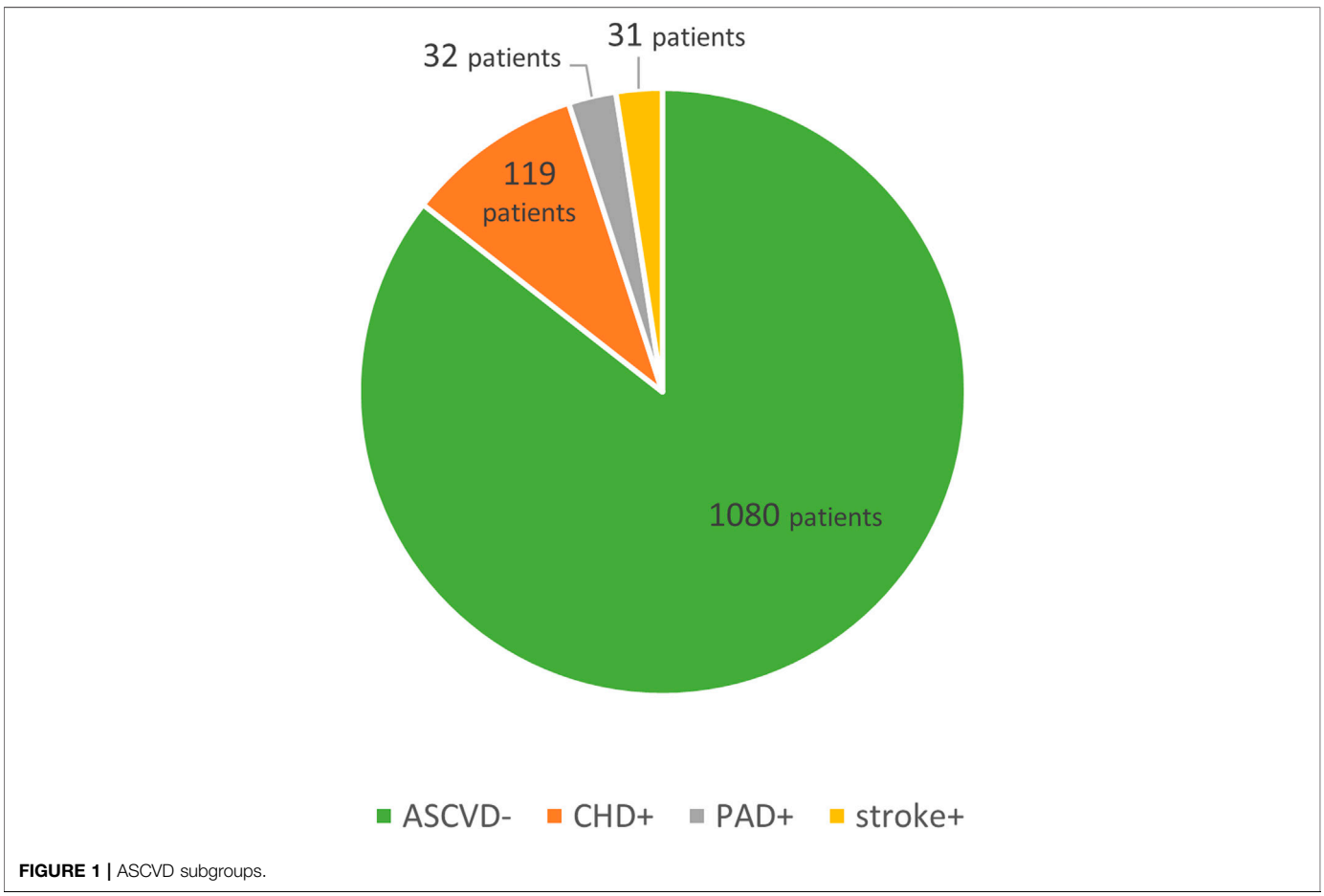

FIGURE 1 | ASCVD subgroups.

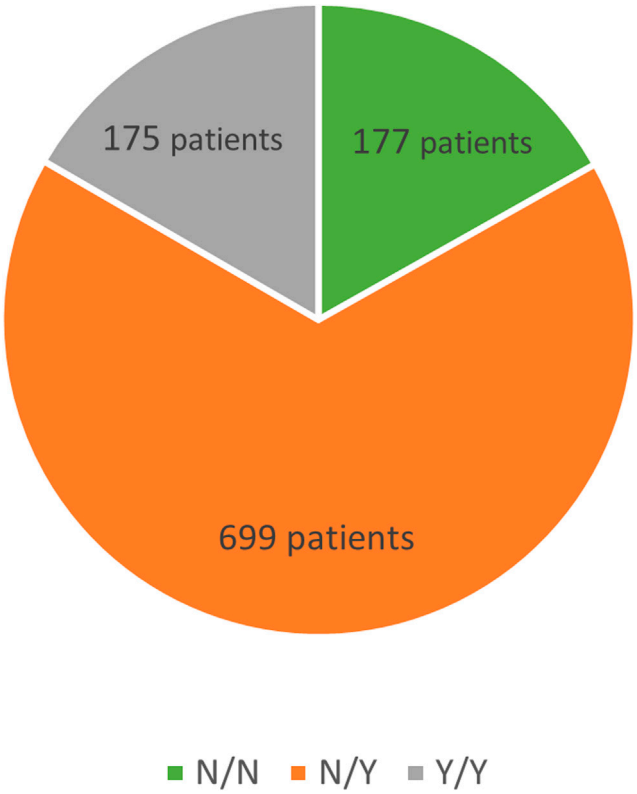

FIGURE 2 | Treatment subgroups.

The period of data collection started in the 1960s and last data were analysed in the 2020. The average follow-up time was not analysed.

Data of a large cohort of patients were analysed using multiple parameters. This article (Part II) focuses on FH clinical symptomatology. The principles of biochemical, statistical and genetic analyses of blood samples and classification of FH patients by the type of gene mutation are addressed in a Part I co-published by Todorovova et al. (2022) hence, they are not discussed more in detail in this article.

The diagnosis of FH was established using the Dutch Lipid Clinic Network score (DLCN). Among the 1,236 FH patients, 1,008 of them [854 supposed to have mutation in *LDLR* gene and 154 with familial defective apolipoprotein B-100 (FDB)] were genetically analysed (Todorovova et al., 2022).

The parameters of lipid and lipoprotein metabolism investigated in our analysis included LDL-cholesterol (LDL-C), total cholesterol (TC), apolipoprotein B (ApoB), HDL-cholesterol (HDL-C), triglycerides (TG) and apolipoprotein Lp(a). Their levels were recorded and analysed in patients at baseline in our clinic and compared with their current or latest available data. Also assessed was the presence of the other major risk factors for atherosclerosis, i.e., arterial hypertension, diabetes mellitus and smoking.

Our group of patients was further subdivided, into subgroups to be compared using several characteristics.

The first division was based on the presence/absence of ASCVD in their history, with patients showing overt complications of the atherosclerotic process further subdivided into three subgroups by the anatomical site involved, i.e., those with coronary heart disease (CHD), ischemic cerebrovascular event (stroke) and peripheral arterial disease (PAD). See **Figure 1**.

Another division, again into three subgroups, was based on differences in drug therapy. The first subgroup was made up of patients not taking any medications both prior to and during follow-up in our clinic, the second subgroup consisted of patients with pharmacotherapy not initiated until the start of follow-up whereas patients in the third subgroup had been on drug therapy already at baseline and continued their pharmacotherapy thereafter. See **Figure 2**.

Data were analyzed using STATISTICA 13 software (TIBCO Software Inc., Palo Alto, CA, United States). The baseline and follow-up levels were compared using the paired *t*-test. In univariate analysis, correlations between the lipid parameters and age were determined using Pearson's correlation coefficients. The tests used when comparing two

TABLE 1 | Patients with/without ASCVD and effect of treatment on lipid levels.

Parameter	ASCVD	Baseline			Follow-up			N	Diference (%)	p
		N	Mean ± SD	p	N	Mean ± SD	p			
LDL-C (mmol/L)	+	146	6.85 ± 2.05	$p = 0.011$	149	2.79 ± 1.54	$p < 0.001$	140	–59.72	$p < 0.001$
	-	1,035	6.42 ± 1.89		937	3.32 ± 1.57		909	–48.09	
TC (mmol/L)	+	156	9.28 ± 2.13	$p = 0.012$	153	4.83 ± 1.69	$p < 0.001$	153	–47.95	$p < 0.001$
	-	1,071	8.86 ± 1.93		965	5.53 ± 1.67		965	–37.84	
ApoB (g/L)	+	77	1.87 ± 0.62	$p = 0.137$	48	0.96 ± 0.43	$p = 0.05$	20	–48.03	$p = 0.219$
	-	525	1.77 ± 0.53		314	1.11 ± 0.50		164	–36.70	
TG (mmol/L)	+	153	2.11 ± 1.36	$p < 0.001$	153	1.39 ± 0.73	$p = 0.905$	150	–34.26	$p < 0.001$
	-	1,064	1.74 ± 1.06		964	1.38 ± 0.79		958	–21.48	
HDL-C (mmol/L)	+	153	1.55 ± 0.42	$p = 0.001$	152	1.43 ± 0.45	$p < 0.001$	149	–8.36	$p = 0.578$
	-	1,057	1.68 ± 0.46		955	1.58 ± 0.46		943	–6.57	
Lp(a) (g/L)	+	108	0.66 ± 0.79	$p < 0.001$	36	0.77 ± 1.08	$p = 0.107$	36	–8.35	$p = 0.123$
	-	844	0.44 ± 0.58		253	0.55 ± 0.67		248	7.62	

N—number of patients; SD, standard deviation; p—p-*value.*

TABLE 2 | Patients with/without CHD/stroke/PAD and effect of treatment on lipid levels.

Parametr	Group	Baseline			Follow-up			N	Diference (%)	p
		N	Mean ± SD	p	N	Mean ± SD	p			
LDL-C (mmol/L)	CHD+	110	6.90 ± 2.21	$p = 0.015$	113	2.82 ± 1.57	$p = 0.002$	105	–59.75	$p < 0.001$
	CHD-	1,071	6.43 ± 1.88		973	3.30 ± 1.57		944	–48.53	
	stroke+	31	6.84 ± 1.65	$p = 0.282$	30	2.57 ± 1.20	$p = 0.016$	30	–62.29	$p = 0.007$
	stroke-	1,150	6.47 ± 1.93		1,056	3.27 ± 1.58		1,019	–49.33	
	PAD+	31	7.14 ± 1.86	$p = 0.051$	32	2.99 ± 1.61	$p = 0.342$	31	–57.61	$p = 0.017$
	PAD-	1,150	6.46 ± 1.92		1,054	3.26 ± 1.58		1,018	–49.46	
TC (mmol/L)	CHD+	119	9.31 ± 2.29	$p = 0.020$	116	4.88 ± 1.73	$p < 0.001$	116	–47.57	$p < 0.001$
	CHD-	1,108	8.87 ± 1.92		1,002	5.50 ± 1.67		1,002	–38.27	
	stroke+	31	9.46 ± 1.78	$p = 0.115$	31	4.60 ± 1.33	$p = 0.005$	31	–51.40	$p < 0.001$
	stroke-	1,196	8.90 ± 1.97		1,087	5.46 ± 1.69		1,087	–38.91	
	PAD+	32	9.54 ± 1.97	$p = 0.069$	32	4.94 ± 1.75	$p = 0.096$	32	–48.16	$p = 0.006$
	PAD-	1,195	8.90 ± 1.96		1,086	5.45 ± 1.68		1,086	–39.00	
ApoB (g/L)	CHD+	57	1.88 ± 0.66	$p = 0.135$	34	0.98 ± 0.44	$p = 0.149$	15	–43.20	$p = 0.741$
	CHD-	545	1.77 ± 0.53		328	1.11 ± 0.50		169	–37.46	
	stroke+	16	1.78 ± 0.40	$p = 0.968$	11	0.79 ± 0.36	$p = 0.039$	4	–68.20	$p = 0.033$
	stroke-	586	1.78 ± 0.54		351	1.10 ± 0.50		180	–37.17	
	PAD+	17	2.01 ± 0.57	$p = 0.081$	9	1.10 ± 0.37	$p = 0.954$	4	–51.01	$p = 0.242$
	PAD-	585	1.77 ± 0.54		353	1.09 ± 0.50		180	–37.57	
TG (mmol/L)	CHD+	118	2.12 ± 1.47	$p < 0.001$	116	1.42 ± 0.78	$p = 0.623$	115	–33.55	$p = 0.002$
	CHD-	1,099	1.75 ± 1.05		1,001	1.38 ± 0.78		993	–22.10	
	stroke+	30	2.02 ± 0.85	$p = 0.235$	31	1.29 ± 0.46	$p = 0.523$	30	–35.74	$p = 0.119$
	stroke-	1,187	1.78 ± 1.11		1,086	1.39 ± 0.79		1,078	–23.13	
	PAD+	31	2.06 ± 0.83	$p = 0.155$	32	1.46 ± 0.67	$p = 0.562$	31	–28.87	$p = 0.365$
	PAD-	1,186	1.78 ± 1.11		1,085	1.38 ± 0.78		1,077	–23.33	
HDL-C (mmol/L)	CHD+	118	1.54 ± 0.43	$p = 0.002$	116	1.42 ± 0.45	$p < 0.001$	115	–7.87	$p = 0.825$
	CHD-	1,092	1.68 ± 0.46		991	1.57 ± 0.46		977	–6.68	
	stroke+	30	1.58 ± 0.44	$p = 0.304$	30	1.48 ± 0.52	$p = 0.355$	29	–7.39	$p = 0.947$
	stroke-	1,180	1.67 ± 0.46		1,077	1.56 ± 0.46		1,063	–6.78	
	PAD+	31	1.57 ± 0.41	$p = 0.236$	32	1.29 ± 0.34	$p = 0.001$	31	–17.22	$p = 0.023$
	PAD-	1,179	1.67 ± 0.46		1,075	1.56 ± 0.46		1,061	–6.51	
Lp(a) (g/L)	CHD+	80	0.72 ± 0.81	$p < 0.001$	25	0.96 ± 1.24	$p = 0.007$	25	0.71	$p = 0.801$
	CHD-	872	0.44 ± 0.58		264	0.54 ± 0.66		259	5.31	
	stroke+	17	0.53 ± 0.84	$p = 0.634$	6	0.46 ± 0.36	$p = 0.677$	6	–53.56	$p < 0.001$
	stroke-	935	0.46 ± 0.60		283	0.58 ± 0.74		278	6.85	
	PAD+	23	0.66 ± 0.65	$p = 0.112$	9	0.43 ± 0.44	$p = 0.521$	9	2.96	$p = 0.916$
	PAD-	929	0.46 ± 0.61		280	0.59 ± 0.74		275	4.67	

N—number of patients; SD, standard deviation; p—p-*value.*

TABLE 3 | Distribution of FH patients by treatment and effect of treatment on lipid levels.

Parameter	Group	Baseline			End of study			N	Diference (%)	p
		N	Mean ± SD	p	N	Mean ± SD	p			
LDL-C (mmol/L)	Y/Y	167	5.76 ± 1.93	$p < 0.001$	166	2.89 ± 1.13	$p < 0.001$	160	–49.6	$p < 0.001$
	N/Y	678	6.83 ± 1.80		678	3.01 ± 1.37		660	–55.7	
	N/N	172	6.35 ± 2.07		97	5.48 ± 2.02		93	–10.7	
TC (mmol/L)	Y/Y	175	8.15 ± 1.98	$p < 0.001$	169	5.03 ± 1.31	$p < 0.001$	169	–38.2	$p < 0.001$
	N/Y	699	9.32 ± 1.83		689	5.16 ± 1.49		689	–44.8	
	N/N	177	8.72 ± 2.07		102	7.84 ± 1.90		102	–8.8	
APOB (g/L)	Y/Y	97	1.57 ± 0.51	$p < 0.001$	65	0.98 ± 0.34	$p < 0.001$	43	–37.0	$p < 0.001$
	N/Y	316	1.86 ± 0.51		190	0.99 ± 0.39		86	–45.4	
	N/N	89	1.85 ± 0.65		40	1.69 ± 0.79		14	–7.9	
TG (mmol/L)	Y/Y	174	1.86 ± 1.17	$p = 0.003$	169	1.41 ± 0.69	$p = 0.792$	168	–23.4	$p = 0.026$
	N/Y	690	1.85 ± 1.17		688	1.37 ± 0.80		680	–26.4	
	N/N	177	1.54 ± 0.84		102	1.38 ± 0.84		102	–11.8	
HDL-C (mmol/L)	Y/Y	174	1.63 ± 0.39	$p = 0.536$	169	1.51 ± 0.40	$p < 0.001$	168	–6.9	$p < 0.001$
	N/Y	687	1.67 ± 0.45		682	1.53 ± 0.44		671	–8.2	
	N/N	175	1.67 ± 0.50		102	1.77 ± 0.54		101	1.9	
Lp(a) (g/L)	Y/Y	154	0.61 ± 0.66	$p = 0.003$	47	0.74 ± 0.67	$p = 0.229$	47	7.7	$p = 0.921$
	N/Y	553	0.44 ± 0.60		181	0.57 ± 0.80		180	4.8	
	N/N	113	0.39 ± 0.62		14	0.38 ± 0.39		13	12.1	

Y/Y—on treatment at baseline and throughout the study; N/Y—no treatment at baseline/on treatment throughout the study; N/N—no treatment at baseline and throughout the study; N—number of patients; SD, standard deviation; p—p-*value.*

TABLE 4 | Patients with/without arterial hypertension and effect of treatment on lipid levels.

Parameter	AH	Baseline			Follow-up			N	Diference (%)	p
		N	Mean ± SD	p	N	Mean ± SD	p			
LDL-C (mmol/L)	+	319	6.64 ± 1.90	$p = 0.073$	322	2.75 ± 1.21	$p < 0.001$	310	–58.17	$p < 0.001$
	-	862	6.41 ± 1.92		764	3.46 ± 1.66		739	–46.06	
TC (mmol/L)	+	332	9.12 ± 1.96	$p = 0.026$	328	4.89 ± 1.35	$p < 0.001$	328	–46.33	$p < 0.001$
	-	895	8.84 ± 1.96		790	5.66 ± 1.76		790	–36.27	
ApoB (g/L)	+	159	1.81 ± 0.57	$p = 0.455$	90	0.96 ± 0.37	$p = 0.003$	48	–46.83	$p = 0.122$
	-	443	1.77 ± 0.53		272	1.14 ± 0.52		136	–35.48	
TG (mmol/L)	+	331	2.09 ± 1.14	$p < 0.001$	328	1.52 ± 0.77	$p < 0.001$	327	–27.38	$p = 0.002$
	-	886	1.67 ± 1.07		789	1.32 ± 0.78		781	–21.49	
HDL-C (mmol/L)	+	327	1.61 ± 0.42	$p = 0.013$	327	1.46 ± 0.42	$p < 0.001$	322	–9.37	$p = 0.032$
	-	883	1.68 ± 0.47		780	1.59 ± 0.47		770	–5.74	
Lp(a) (g/L)	+	250	0.58 ± 0.75	$p < 0.001$	84	0.69 ± 0.92	$p = 0.112$	84	–5.95	$p = 0.056$
	-	702	0.42 ± 0.54		205	0.54 ± 0.64		200	11.20	

AH, arterial hypertension; N—number of patients; SD, standard deviation; p—p-*value.*

TABLE 5 | AH+/AH- patients developing ASCVD.

	AH	ASCVD +	ASCVD -	Total
Count	+	91	241	332
Row Percent (%)		27.41	72.59	
Count	-	65	839	904
Row Percent (%)		7.19	92.81	

and three subgroups in univariate analysis were the two-sample *t*-test and ANOVA test, respectively. We used multivariable logistic regression model to assess the effect of risk factors smoking, diabetes and arterial hypertension for total cardiovascular risk.

RESULTS

The present analysis compared the levels of lipid parameters obtained prior to start of follow-up and the most recent ones available. The primary endpoint LDL-C declined from a baseline mean of 6.49 ± 1.92 mmol/L to 3.26 ± 1.57 mmol/L (by 49.8%). A decrease by 39% was observed in TC levels

TABLE 6 | Patients with/without diabetes and effect of treatment on lipid levels.

Parameter	DM	Baseline			Follow-up			N	Diference (%)	p
		N	Mean ± SD	p	N	Mean ± SD	p			
LDL-C (mmol/L)	+	77	6.52 ± 1.78	$p = 0.827$	78	2.62 ± 1.35	$p < 0.001$	73	−58.95	$p = 0.008$
	-	1,104	6.47 ± 1.93		1,008	3.30 ± 1.58		976	−49.03	
TC (mmol/L)	+	83	9.07 ± 1.77	$p = 0.467$	82	4.74 ± 1.49	$p < 0.001$	82	−47.63	$p < 0.001$
	-	1,144	8.90 ± 1.98		1,036	5.49 ± 1.69		1,036	−38.60	
ApoB (g/L)	+	42	1.89 ± 0.55	$p = 0.166$	19	1.13 ± 0.49	$p = 0.755$	10	−40.21	$p = 0.122$
	-	560	1.77 ± 0.54		343	1.09 ± 0.50		174	−37.75	
TG (mmol/L)	+	83	2.40 ± 1.53	$p < 0.001$	82	1.73 ± 0.95	$p < 0.001$	82	−28.04	$p = 0.026$
	-	1,134	1.74 ± 1.05		1,035	1.36 ± 0.76		1,026	−23.01	
HDL-C (mmol/L)	+	81	1.55 ± 0.41	$p = 0.015$	82	1.37 ± 0.41	$p < 0.001$	80	−11.20	$p = 0.158$
	-	1,129	1.67 ± 0.46		1,025	1.57 ± 0.46		1,012	−6.48	
Lp(a) (g/L)	+	65	0.60 ± 0.84	$p = 0.059$	23	0.88 ± 1.33	$p = 0.042$	23	5.32	$p = 0.819$
	-	887	0.45 ± 0.59		266	0.55 ± 0.66		261	4.53	

DM, diabetes mellitus; N—number of patients; SD, standard deviation; p—p-*value.*

TABLE 7 | DM+/DM-patients developing ASCVD.

	DM	ASCVD +	ASCVD -	Total
Count	+	34	49	83
Row Percent (%)		40.96	59.04	
Count	-	122	1,031	1,153
Row Percent (%)		10.58	89.42	

falling from 8.95 ± 1.95 mmol/L to 5.43 ± 1.69 mmol/L. ApoB showed a decrease from a baseline mean of 1.76 ± 0.56 mmol/L to 1.09 ± 0.56 mmol/L. TG levels declined from a mean baseline of 1.81 ± 1.13 mmol/L to 1.38 ± 0.78 mmol/L. The change in HDL-C levels was 1.67 ± 0.46 mmol/L vs. follow-up levels of 1.56 ± 0.46 mmol/L. All the above differences were significant ($p < 0.001$). Lp(a) was unchanged (0.56 vs. 0.59 g/L, $p = 0.27$).

A total of 156 patients of the entire group (12.6%) had a history of ASCVD (ASCVD+ group; mean age 54.0 ± 12.5; 89 women, 67 men; 75 smokers) in the form of either CHD, stroke or PAD. As a total of 1,080 patients were in primary prevention of ASCVD, atherosclerosis had not yet manifested itself (ASCVD–group; mean age 43.5 ± 16.8; 752 women, 328 men; 313 smokers). The primary outcome was LDL-C declining, in ASCVD+ (ASCVD–) patients, from a baseline 6.85 ± 2.05 (6.42 ± 1.89) mmol/L to 2.79 ± 1.54 (3.23 ± 1.57) mmol/L during follow-up, which was 60% (48%) difference. This trend was seen in TC levels either, which fell in the ASCVD+ (ASCVD–) subgroups by 48% (38%). While the differences between the two subgroups in the baseline levels of ApoB were non-significant, follow-up difference reached statistical significance. The baseline TG levels of patients with a history of ASCVD were higher compared with patients without ASCVD. The TG levels decreased in either subgroup, 34% in ASCVD+, and 21% in ASCVD–patients. Statistically significant were the differences in baseline Lp(a) levels. In ASCVD+ subgroup, Lp(a) levels decreased, whereas in ASCVD-subgroup increased towards follow-up. HDL-C levels decreased over time, the overall change from baseline to follow-up was non-significant. For more details see **Table 1**.

Patients with ASCVD (ASCVD+; $n = 156$) were further subdivided into three subgroups by the anatomical site involved into those with CHD ($n = 119$), stroke ($n = 31$) and PAD ($n = 32$). Some patients were included in more than one subgroup. **Figure 1.**

All results are summarized in **Table 2**. In the CHD+ subgroup, LDL-C levels decreased by 60% from a baseline during follow-up compared with CHD–patients without a history of CHD (CHD–), whose baseline fell by 49%. In the CHD+ (CHD–) subgroups, TC levels decreased by 48% (38%). The differences in the levels of ApoB between the individual subgroups were non-significant both at the start and during follow-up. The baseline TG levels in the CHD+ (CHD–) subgroups were 2.12 ± 1.47 (1.75 ± 1.05) mmol/L to be non-significant during follow-up. Patients in the CHD+ subgroup had lower baseline levels of HDL-C compared with CHD–patients. Lp(a) levels were higher at baseline in CHD+ patients compared with CHD-. These levels rose in both subgroups over time, the changes were not significant ($p = 0.801$).

In patients with a history of stroke (stroke+), no significant differences in the baseline levels were found. The follow-up LDL-C (as well as TC or ApoB) levels in patients stroke+ were lower than in subgroup without this condition (stroke-).

While patients with PAD did not show significant differences in the lipid parameters at baseline, a significant difference was noted over time in HDL-C levels, being lower in PAD+ patients compared with PAD–subgroup.

Among the 1,236 patients, drug-status was available for 1,051 patients, and these were then subdivided into three subgroups based on whether or not the patients had been previously on lipid-lowering therapy and whether or not they were currently being treated with lipid-lowering agents.

The Y/Y subgroup ($n = 175$, lipid-lowering therapy at baseline and during follow-up) had baseline LDL-C levels of 5.76 ± 1.93 mmol/L decreasing to 2.89 ± 1.13 mmol/L (a 50% reduction; $p < 0.001$). The baseline TC levels of 8.15 ±

1.98 mmol/L declined to 5.03 ± 1.31 mmol/L (by 38%; $p < 0.001$) during follow-up, TG levels decreasing by 23% ($p = 0.026$).

The N/Y subgroup ($n = 699$, no therapy at baseline, therapy during follow-up) showed a decrease in LDL-C from baseline of 6.83 ± 1.80 mmol/L to 3.01 ± 1.37 mmol/L (a reduction by 56%; $p < 0.001$). TC levels dropped from 9.32 ± 1.83 mmol/L to 5.16 ± 1.49 mmol/L (down by 45%; $p < 0.001$). The levels of TG declined during follow-up by 26% ($p = 0.026$).

In the N/N subgroup ($n = 177$, therapy-naïve at baseline and no therapy during follow-up), the baseline LDL-C levels of 6.35 ± 2.07 mmol/L declined to 5.48 ± 2.02 mmol/L (11% reduction; $p < 0.001$), TC levels decreased from 8.72 ± 2.07 mmol/L to 7.84 ± 1.90 mmol/L (by 9%; $p < 0.001$) and TG levels reduction was 12% ($p = 0.026$).

More details in **Table 3**. When comparing the values between the three subgroups, the biggest decrease ($p < 0.001$) occurred in the LDL-C, TC, ApoB, HDL-C and TG levels in the N/Y subgroup ($p = 0.026$). The differences in Lp(a) levels were non-significant. The smallest changes were documented among N/N patients showing significantly ($p = 0.003$) lowest baseline TG levels compared with the Y/Y and N/Y subgroups. The follow-up levels of LDL-C, TC and ApoB were highest in the N/N subgroup ($p < 0.001$). All results summarized in a table are available in Todorovova et al., 2022

Our cohort comprised of 332 patients (27%) with arterial hypertension (AH). In the subgroup of patients with this diagnosis (AH+), the baseline levels of lipid parameters were significantly higher than in the subgroup without AH (AH–) such as in TC, TG, Lp(a) and lower in HDL-C. In the AH+ subgroup, the follow-up levels were significantly lower compared with the AH–subgroup in LDL-C (2.75 ± 1.21 vs 3.46 ± 1.66 mmol/L; $p < 0.001$), TC and ApoB, whereas TG levels in the AH+ subgroup showed poorer control (1.52 ± 0.77 mmol/L) than in AH–patients (1.32 ± 0.78 mmol/L). For more details see **Table 4**.

The number of AH+ patients developing ASCVD was significantly higher (27.4%) than of those without it (AH–) (7.2%). For details see **Table 5**. In group AH+ is 2.44 greater chance for KVO (OR = 2.44; CI0.95 = (1.65; 3.63)) than in AH-.

During follow-up, diabetes mellitus was diagnosed in a total of 83 patients (7% of the whole study group; $n = 1,236$). Patients with diabetes mellitus (DM+) showed worse control of lipid parameters than those without this diagnosis (DM–) as reflected in the levels of TG and HDL-C. The differences in the other parameters assessed were non-significant. Follow-up levels of LDL-C and TC in DM+ patients were lower compared with DM–patients. On the other hand, the follow-up levels of TG were higher in the DM+ subgroup than among DM–patients. The difference in ApoB levels was not significant. All pertinent data are shown in detail in **Table 6**.

In the DM+ subgroup ($n = 83$), 34 patients had a history of ASCVD (41%) whereas ASCVD was not present in 49 (59%). Among the DM–patients ($n = 1,153$), ASCVD was present in 122 (10.6%), with 1,031 patients (89.4%) without this diagnosis. The prevalence of ASCVD in DM+ vs. DM-was 41% vs 10,6%; $p < 0.001$ (see **Table 7**). In DM+ is 2.84 greater chance for KVO (OR = 2.84; CI0.95 = (1.67; 4.83)) than in DM-.

The baseline lipid profile in smokers ($n = 389$) differed significantly only in TG levels, which were higher (2.05 ± 1.37 mmol/L) compared with non-smokers (1.66 ± 0.93 mmol/L) and in HDL-C levels (1.60 ± 0.45 vs. 1.69 ± 0.46 mmol/L). During follow-up, TG levels in smokers remained higher (1.54 ± 0.99 vs. 1.3 ± 0.63 mmol/L), with the trend in HDL-C levels also unchanged (1.49 ± 0.47 vs. 1.59 ± 0.45 mmol/L). The follow-up levels of LDL-C and TC were lower in smokers (LDL 3.09 ± 1.47 vs. 3.3 ± 1.62 mmol/L, and TC 5.28 ± 1.65 vs. 5.51 ± 1.7 mmol/L, respectively). In smokers, their LDL-C levels declined by 3.44 mmol/L (3.13 mmol/L in non-smokers), with TC levels decreasing by 3.71 mmol/L (3.41 mmol/L in non-smokers).

Among smokers, 19.3% were classified as ASCVD+ and 80.7% as ASCVD–; the respective figures for non-smokers were 9.6 and 90.4%. In group of smokers is 1.87 greater chance for KVO (OR = 1.87; CI0.95 = (1.29; 2.71)) than in nonsmokers.

The whole group of our patients included 841 women and 395 men. Compared with men, women started follow-up with lower levels of TG (1.7 ± 1.05 mmol/L vs 1.95 ± 1.18 mmol/L; $p < 0.001$) and higher levels of HDL-C (1.77 ± 0.46 mmol/L vs 1.44 ± 0.34 mmol/L; $p < 0.001$). Over time, TG levels were higher in women, 1.35 ± 0.76 mmol/L (1.47 ± 0.83 mmol/L in men; $p = 0.016$) as were HDL-C levels, 1.67 ± 0.46 mmol/L (1.32 ± 0.36 mmol/L in men; $p < 0.001$). The follow-up TC levels were higher in women (5.57 ± 1.67 mmol/L) than in men (5.14 ± 1.68 mmol/L; $p < 0.001$). The changes between the baseline and follow-up levels of the other parameters assessed were non-significant.

Clinical presentation of FH was seen in a total of 145 (12%) patients, with xantelasma palpebrarum diagnosed in 57 cases (5%), arcus lipoides corneae in 47 patients (4%) and tendon xanthomas in 41 patients (3%).

DISCUSSION

The 1,236 patients with analyzed data attended a single Prague-based clinic with a history spanning more than 50 years. The period of data collection is not exactly defined as our project was a retrospective analysis with data of the first patients recorded as early as the 1960s when Šobra founded the Center of Preventive Cardiology (Center hereinafter) (Šobra, 1970). Over the decades, the Center was being attended by a large number of patients with familial hypercholesterolemia; however, the duration of their follow-up has varied substantially as, while some patients have been taken care of for decades, the follow-up period of other patients has not been longer than 2 years.

Needless to say, an ideal scenario would involve a patient referred to the Center by their general practitioner for assessment and subsequent follow-up. In practice, however, some patients presenting for follow-up do not have complete medical records, do not present for routine blood tests or are simply lost to follow-up. This explains the differences in the numbers of patients whose

data were available for analysis. Last but not least, an additional reason may be the different, or inconsistent, approach of individual physicians.

Recent studies have suggested that the only causal factor of ASCVD is dyslipidemia or, more exactly, LDL-C (Borén et al., 2020). In fact, the diagnosis of FH *per se* puts all our 1,236 patients into the category of at least high cardiovascular risk (Visseren et al., 2021); nonetheless, while some of them do develop ASCVD, others do not. This was why our project focused also on the differences between the two major groups (ASCVD+ vs ASCVD–) of FH patients.

During follow-up, all patient subgroups showed a significant decrease in the levels of LDL-C, TC, ApoB and TG. While the reason for the decrease in HDL-C levels over time remains unclear, its follow-up levels (1.56 mmol/L) were within the optimal range (van der Steeg et al., 2008). Until the advent of PCSK9 inhibitors, Lp(a) was traditionally seen as an important player in the atherosclerotic process independent of the other risk factors (O'Donoghue et al., 2019) and not modifiable by drug therapy (Sun et al., 2018). The levels of Lp(a) did not change significantly in our analysis of follow-up data. There is no doubt this is due to the fact that PCSK9 inhibitors were unavailable in the Czech Republic until the summer of 2018; hence, they could not have affected the outcomes of patients on follow-up. Other reasons include the small number of patients with baseline and follow-up data available and, also, the inconsistent approach by physicians many of whom simply failed to focus their attention on a parameter refractory to drug therapy.

As noted above, not all patients with FH develop premature ASCVD. We did suspect that the lipid profile of ASCVD+ patients would be associated with increased risk, which was eventually the case. Patients with ASCVD had higher baseline LDL-C and TC levels and lower HDL-C levels than ASCVD–patients. The most striking differences were observed in the baseline levels of TG and Lp(a), which were again higher in the ASCVD+ subgroup thus corroborating, together with lower HDL-C levels, the importance of residual cardiovascular risk (Hoogeveen and Ballantyne, 2021). The tide turned during follow-up with ASCVD+ patients showing significantly lower levels of LDL-C, TC and ApoB whereas the differences in TG and Lp(a) levels were non-significant. The reasons for the more favorable lipid profile in ASCVD+ patients are multiple. First and foremost, these at-risk patients (category of very high cardiovascular risk according to the guidelines (Visseren et al., 2021)) receive more attention by health care providers. Also, their target levels are more ambitious and, last but not least, patients with a history of cardiovascular disease are more likely to adhere to their recommended therapy and tend to comply with their physicians' advice (Jackevicius et al., 2002).

As in the ASCVD+ subgroup, patients assigned to the CHD subgroup had significantly higher baseline levels of LDL-C, TC, TG and Lp(a) a lower HDL-C levels compared with patients without a history of ASCVD. Except for TG and Lp(a), the follow-up levels in the CHD subgroup were lower (in analogy to ASCVD–vs ASCVD+). A similar trend was noted in the stroke (n = 31) and PAD subgroups (n = 32); however, the differences were non-significant due to the small number of patients on follow-up.

When comparing the subgroups with different therapeutic status (N/N, N/Y, Y/Y), it came as no surprise that the largest decrease in the levels of LDL-C, TC, ApoB and TG was seen in the subgroup with therapy not initiated prior to follow-up in the Center (N/Y). Nonetheless, a significant decrease in the above parameters was also seen in the (Y/Y) subgroup suggesting that patients benefit already from receiving therapy in a specialized center adopting the most recent therapeutic strategies combined with an effort to achieve target levels. Patients not currently on therapy and not treated at the time of starting outpatient follow-up showed minimal decreases in the investigated parameters. The most frequent reason for failure to initiate therapy in a specialized healthcare facility was statin intolerance. The number of patients not receiving therapy after the PCSK9 inhibitors had been approved for the Czech market is currently smaller (Altschmiedova et al., 2020); however, providing more details on this issue is outside the scope of this paper. Other reasons for not instituting therapy drug include the patients' unwillingness and/or reluctance to initiate therapy even after they had been informed about all the risks associated with untreated significant dyslipidemia.

A total of 27% of our patients had a history of arterial hypertension, a condition with a global prevalence estimated at 20–24% in years 1975–2015 (Zhou et al., 2017). In the Czech Republic, according to Cífková et al., the prevalence of hypertension declined from 47.1% in 1985 to 41.5% in 2016/17 (Cífková et al., 2020a). Diabetes mellitus was present in 7% of our cohort. The prevalence of diabetes in the Czech Republic was according to the same author about 8% in men and 5% in women (Cífková et al., 2020b). These results clearly show that familial hypercholesterolemia is a genetic disease whose incidence cannot be linked to a lifestyle. Patients with FH are not in higher risk of development of diabetes and AH. The lipid profile of them with AH and diabetes is worse because of higher TG and lower HDL and we assume that this trend is associated with the lifestyle of individuals.

Smokers totaling 389, i.e., 31% of our whole group of patients, initiated follow-up with higher baseline TG levels and lower levels of HDL-C than non-smokers; this fact remained unaltered during follow-up and is presumably associated with the lifestyle of these patients. However, the follow-up levels of LDL-C and TC were more favorable in smokers. Smokers also tended to respond better to therapy and showed greater decreases in LDL-C, TC and ApoB levels compared with non-smokers, likely due to their higher cardiovascular risk and' consequently, more ambitious LDL-C targets (Visseren et al., 2021).

Patients with FH and a history of arterial hypertension, diabetes or tobacco smoking, experienced more cardiovascular events than those without the above conditions.

At baseline and throughout follow-up, women had lower TG levels and higher HDL-C levels compared with men. These differences may be due to their more consistent adherence of women to a healthy lifestyle. We also assessed overall changes in the investigated parameters prior to and

during follow-up; however, no sex-related statistical significance was demonstrated.

Clinical presentations of FH such as tendon xanthomas, arcus lipoides corneae or xantelasma palpebrarum are currently less frequent than in the past. In a first-ever monograph on FH published in 1970, Šobra reported a 30% incidence of arcus lipoides corneae, 23% incidence of xantelasma palpebrarum and 10% of patients with some form of xanthomatosis (Šobra, 1970). By contrast, in a paper published in 2014 and reporting on patients currently treated in the same center, arcus lipoides corneae, xanthelasma palpebrarum and xanthomatosis diagnosed were in 3, 6, and 5% of patients, respectively (Ceska et al., 2014). The development of these clinical signs is associated not only with cholesterol levels but, also, with the period of time the body is exposed to these levels. Patients with a well-defined treatment plan initiated in a timely manner do not develop these clinical presentations or, in the opposite case, these regress or disappear completely (Ceska et al., 2014; Civeira et al., 2016). If comparing the current therapeutic options with those available more than 50 years ago, it comes as no surprise that tendon xanthomas, arcus lipoides corneae or xantelasma palpebrarum become less frequent. We consider our assessment of the clinical signs in the present paper only an estimate since the figures cover all patients treated since the 1960s and the final number is no doubt confounded by the above regression due to intensive lipid-lowering therapy.

During the 50 + years of follow-up, there have been some deaths; however, the exact numbers are unavailable as some of the deaths may not have been recorded.

CONCLUSION

The present analysis confirmed the well-known fact that, while LDL-C is a causal risk factor of ASCVD, every effort should be made to modulate all the known risk factors posing a residual risk, even after achieving target LDL-C levels. Therapeutic modification of Lp(a) by promising new agents still under development as well as by PCSK9 inhibitors already introduced into clinical practice may have the potential to further reduce cardiovascular risk in the near future. Results of this project have suggested that patients with the diagnosis of FH do benefit from receiving therapy in a specialized center which was confirmed by ScreenPro FH project (Ceska et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the General University Hospital in Prague. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TA interpreted data and wrote the article. VT analysed data of patients and contributed to writing the article MV contributed with interpretation of data and writing the article RC interpretated of data, paticipated on revision, proofreading, professional supervision.

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LDL-C Concentrations and the 12-SNP LDL-C Score for Polygenic Hypercholesterolaemia in Self-Reported South Asian, Black and Caribbean Participants of the UK Biobank

Jasmine Gratton[1], *Chris Finan*[1,2,3], *Aroon D. Hingorani*[1,2], *Steve E. Humphries*[1] and *Marta Futema*[1,4]*

[1]Institute of Cardiovascular Science, Faculty of Population Health, University College London, London, United Kingdom, [2]UCL British Heart Foundation Research Accelerator, London, United Kingdom, [3]Division of Heart and Lungs, Department of Cardiology, University Medical Center Utrecht, Utrecht, Netherlands, [4]Cardiology Research Centre, Molecular and Clinical Sciences Research Institute, St George's, University of London, London, United Kingdom

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***Correspondence:**
Marta Futema
mfutema@sgul.ac.uk

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Background: Monogenic familial hypercholesterolaemia (FH) is an autosomal dominant disorder characterised by elevated low-density lipoprotein cholesterol (LDL-C) concentrations due to monogenic mutations in *LDLR*, *APOB*, *PCSK9*, and *APOE*. Some mutation-negative patients have a polygenic cause for elevated LDL-C due to a burden of common LDL-C-raising alleles, as demonstrated in people of White British (WB) ancestry using a 12-single nucleotide polymorphism (SNP) score. This score has yet to be evaluated in people of South Asian (SA), and Black and Caribbean (BC) ethnicities.

Objectives: 1) Compare the LDL-C and 12-SNP score distributions across the three major ethnic groups in the United Kingdom: WB, SA, and BC individuals; 2) compare the association of the 12-SNP score with LDL-C in these groups; 3) evaluate ethnicity-specific and WB 12-SNP score decile cut-off values, applied to SA and BC ethnicities, in predicting LDL-C concentrations and hypercholesterolaemia (LDL-C>4.9 mmol/L).

Methods: The United Kingdom Biobank cohort was used to analyse the LDL-C (adjusted for statin use) and 12-SNP score distributions in self-reported WB (n = 353,166), SA (n = 7,016), and BC (n = 7,082) participants. To evaluate WB and ethnicity-specific 12-SNP score deciles, the total dataset was split 50:50 into a training and testing dataset. Regression analyses (logistic and linear) were used to analyse hypercholesterolaemia (LDL-C>4.9 mmol/L) and LDL-C.

Findings: The mean (±SD) measured LDL-C differed significantly between the ethnic groups and was highest in WB [3.73 (±0.85) mmol/L], followed by SA [3.57 (±0.86) mmol/L, $p < 2.2 \times 10^{-16}$], and BC [3.42 (±0.90) mmol/L] participants ($p < 2.2 \times 10^{-16}$). There were significant differences in the mean (±SD) 12-SNP score between WB [0.90 (±0.23)] and BC [0.72 (±0.25), $p < 2.2 \times 10^{-16}$], and WB and SA participants [0.86 (±0.19), $p < 2.2 \times 10^{-16}$].

In all three ethnic groups the 12-SNP score was associated with measured LDL-C [R^2 (95% CI): WB = 0.067 (0.065–0.069), BC = 0.080 (0.063–0.097), SA = 0.027 (0.016–0.038)]. The odds ratio and the area under the curve for hypercholesterolaemia were not statistically different when applying ethnicity-specific or WB deciles in all ethnic groups.

Interpretation: We provide information on the differences in LDL-C and the 12-SNP score distributions in self-reported WB, SA, and BC individuals of the United Kingdom Biobank. We report the association between the 12-SNP score and LDL-C in these ethnic groups. We evaluate the performance of ethnicity-specific and WB 12-SNP score deciles in predicting LDL-C and hypercholesterolaemia.

Keywords: FH, ethnicities, UK Biobank, 12-SNP score, LDL-C, polygenic

INTRODUCTION

Pathogenic variants in *LDLR, APOB, PCSK9*, and *APOE* are known to cause autosomal dominant familial hypercholesterolaemia (FH), but such mutations are found in only ~40% of individuals with the clinical diagnosis of FH (Taylor et al., 2010; Motazacker et al., 2012; Futema et al., 2013; Nordestgaard et al., 2013; Mariano et al., 2020). In 2013, Talmud et al. (2013) reported that a high burden of common variants identified from genome-wide association studies (GWAS) was associated with LDL-C concentrations as high or higher than those found in individuals with a FH mutation, suggesting a polygenic cause in mutation negative patients with a biochemical FH phenotype. A score based on 12-single nucleotide polymorphisms (SNP) was developed to suggest a polygenic cause of high LDL-C concentrations for these FH mutation-negative individuals (Talmud et al., 2013). The validity of this score has been confirmed in samples of no-mutation FH adults and children from more than 8 countries with White European populations (Futema et al., 2018). Other studies applied the score to define a polygenic cause of high LDL-C in patients with hypercholesterolaemia (Amor-Salamanca et al., 2017; Saadatagah et al., 2021). Two of the current NHS Genomic Laboratory Hubs (GLHs) (South West and North East) are including the 12-SNP score within their diagnostic pipelines (George et al., 2021), demonstrating feasibility of implementation. Clinicians receiving these reports are strongly supportive of the roll out to all GLHs and find them helpful for patient management because of the benefit of being able to offer a genetic explanation for mutation negative patients with a biochemical FH phenotype, which may motivate adherence to lifestyle interventions and cholesterol-lowering therapy (Kullo et al., 2016). However, the major limitation of the 12-SNP score is that it has not yet been evaluated in two other major ethnic groups in the United Kingdom [South Asian (SA), and Black and Caribbean (BC) individuals], which has led to uncertainty in reporting. In this study, using the United Kingdom Biobank data we analysed the LDL-C and 12-SNP score distributions among self-reported WB, SA, and BC participants; and tested the association between the 12-SNP score and LDL-C concentrations in the three groups. We also performed an out-of-sample validation to compare the use of WB versus ethnicity-specific 12-SNP score deciles applied to SA and BC participants.

MATERIALS AND METHODS

The analysis was performed in individuals of self-reported White (n = 353,166), South Asian (SA) (n = 7,016), and Black and Caribbean (BC) (n = 7,082) ethnicities of the United Kingdom Biobank (project ID 40721). Up to third degree relatives were excluded from the dataset. The following variables had missing data which was singly imputed using predictive mean matching (PMM) with the R package "mice" (van Buuren and Groothuis-Oudshoorn 2011): LDL-C, body mass index (BMI), total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides, and smoking. The data analysis was performed in R version 4.0.2. Measured LDL-C concentrations (UKB data-field 30,690) were adjusted for self-reported statin use by multiplying their measured LDL-C concentrations by the correction coefficient 1.43, as used by Trinder et al. (2020). The weighted 12-SNP scores were calculated for each participant using the previously published SNPs and effect sizes from people of European ancestry (**Supplementary Table S1**) (Talmud et al., 2013). The two *APOE* variants were calculated in an isoform-specific manner as detailed in **Supplementary Table S1** (Bennet et al., 2007). The 10 other variants and their effect sizes (also known as the beta value, which is the incremental increase or decrease in LDL-C concentration per effect allele), originally obtained from the Global Lipids Genetics Consortium (GLGC) GWAS (Teslovich et al., 2010), were calculated by first converting the negative effect sizes to positive ones and inverting the effect and non-effect alleles; and subsequently, the number of alleles multiplied by their positive effect sizes were summed for each individual. The scores obtained from the *APOE* isoforms and the GLGC variants were then added together to obtain the final 12-SNP score for each participant.

The UK Biobank dataset was split 50:50 into a training and a testing dataset. Ethnicity-specific 12-SNP score decile cut-off values were obtained for each self-reported ethnic group using

TABLE 1 | Study participant characteristics stratified by self-reported ethnicity. For continuous variables, *p*-values from the Welch t-statistic tests are reported; while for categorical and binary variables, *p*-values from Pearson's Chi-squared tests are reported. The data presented here is for the training and the test dataset combined. CHD, coronary heart disease; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; SD , standard deviation; SNP, single nucleotide polymorphism.

	White	Black/ Caribbean	South Asian	P-Value: White-Black/ Caribbean	P-Value: White-South Asian
n	353,166	7,082	7,016		
Age (median [IQR])	58 [51, 63]	50 [45, 58]	53 [46, 60]	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Sex (male) (%)	163,063 (46.2)	3,070 (43.3)	3,807 (54.3)	2.5×10^{-6}	$<2.2 \times 10^{-16}$
Body mass index, kg/m2 [mean (SD)]	27.39 (4.76)	29.46 (5.36)	27.31 (4.47)	$<2.2 \times 10^{-16}$	0.11
Smoking status (%)				$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Non-smoker	199,045 (56.4)	5,147 (72.7)	5,646 (80.5)		
Former smoker	129,004 (36.5)	1,357 (19.2)	961 (13.7)		
Light smoker (<10 cigarettes/day)	4,932 (1.4)	240 (3.4)	139 (2.0)		
Moderate smoker (10–19 cigarettes/day)	10,698 (3.0)	257 (3.6)	186 (2.7)		
Heavy Smoker (>20 cigarettes/day)	9,487 (2.7)	81 (1.1)	84 (1.2)		
Blood biomarkers					
LDL-C (unadjusted), mmol/L [mean (SD)]	3.57 (0.87)	3.27 (0.84)	3.33 (0.85)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
LDL-C (adjusted for statin users), mmol/L [mean (SD)]	3.73 (0.85)	3.42 (0.90)	3.57 (0.86)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Total cholesterol, mmol/L [mean (SD)]	5.71 (1.14)	5.26 (1.10)	5.29 (1.11)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Triglycerides, mmol/L [median (IQR)[	1.49 [1.05, 2.16]	1.05 [0.77, 1.50]	1.67 [1.18, 2.40]	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
HDL-C, mmol/L [mean (SD)]	1.46 (0.41)	1.45 (0.41)	1.27 (0.36)	2.4×10^{-4}	$<2.2 \times 10^{-16}$
Non-HDL-C, mmol/L [mean (SD)]	4.26 (1.07)	3.83 (1.01)	4.03 (1.05)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Apolipoprotein A, g/L [mean (SD)]	1.54 (0.27)	1.49 (0.26)	1.40 (0.24)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Apolipoprotein B, g/L [mean (SD)]	1.03 (0.24)	0.97 (0.24)	1.00 (0.23)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Statin use (%)	47,427 (13.4)	873 (12.3)	1,475 (21.0)	7.0×10^{-3}	$<2.2 \times 10^{-16}$
12-SNP score [mean (SD)]	0.90 (0.23)	0.72 (0.25)	0.86 (0.19)	$<2.2 \times 10^{-16}$	$<2.2 \times 10^{-16}$
Prevalent CHD (%)	11,743 (3.3)	115 (1.6)	479 (6.8)	2.5×10^{-15}	$<2.2 \times 10^{-16}$
Incident CHD (%)	14,471 (4.1)	200 (2.8)	491 (7.0)	9.4×10^{-8}	$<2.2 \times 10^{-16}$

the training data. These cut-points were applied to the test data in an ethnicity-specific manner. The WB 12-SNP score decile cut-off values obtained from the training data were also applied to the test data in SA and BC participants to compare the performance of WB and ethnicity-specific deciles. The test data was used for the analyses performed in this study. Regression analyses (linear and logistic) were used to predict LDL-C concentration and hypercholesterolaemia (LDL-C >4.9 mmol/L, one of the FH diagnostic criteria included in the Simon Broome criteria) (Civeira et al., 2004). Individuals were also grouped into low (deciles 1–3), intermediate (deciles 4–5), and high (deciles 6–10) polygenic hypercholesterolaemia groups based on their 12-SNP score. The odds ratio (OR) for hypercholesterolaemia was obtained by setting the intermediate polygenic hypercholesterolaemia group as the reference.

The analysis was also performed in principal component analysis (PCA)-verified White European, Black, and South Asian ancestry groups as defined by Giannakopoulou et al. (2021)(**Supplementary Table S2**).

RESULTS

The study participant characteristics were significantly different for self-reported White (WB), South Asian (SA) and Black and Caribbean (BC) ethnic groups (**Table 1**). WB participants were older (median age of 58 [interquartile range (IQR): 51–63)] and exhibited the highest concentrations of adjusted LDL-C [3.73 mmol/L (standard deviation (SD): 0.85], total cholesterol [5.71 mmol/L (SD: 1.14)], high-density lipoprotein cholesterol (HDL-C) [1.46 mmol/L (SD: 0.41)] and non-HDL-C [4.26 mmol/L (SD: 1.07)] compared to SA and BC individuals. A higher proportion of males was present in the SA ethnic group (54.3%) compared to the WB (46.2%) and BC (43.3%) ethnic groups. 80.5% of SA and 72.7% of BC participants were self-reported non-smokers, while 56.4% WB participants self-reported as non-smoking. Mean body mass index (BMI) was highest in BC participants [29.46 kg/m^2 (SD: 5.36)], and there was no significant difference in mean BMI between WB and SA participants (*p*-value = 0.11) (**Table 1**). Triglyceride levels were highest for SA participants [1.67 mmol/L (IQR: 1.18–2.40)], as well as the use of statins (21%), and the prevalence (6.8%) and incidence (7%) of coronary heart disease (CHD).

When compared between the three ethnic groups, the measured mean (SD) LDL-C concentrations adjusted for statin use was highest in WB participants [3.73 (0.85) mmol/L], followed by SA [3.57 (0.86) mmol/L], and BC [3.42 (0.90) mmol/L] participants (**Figure 1**). These group differences were statistically significant (*p*-value $<2.2 \times 10^{-16}$) (**Table 1**).

The 12-SNP score followed the same pattern, with the mean (SD) score being the highest in WB participants [0.90 (0.23)],

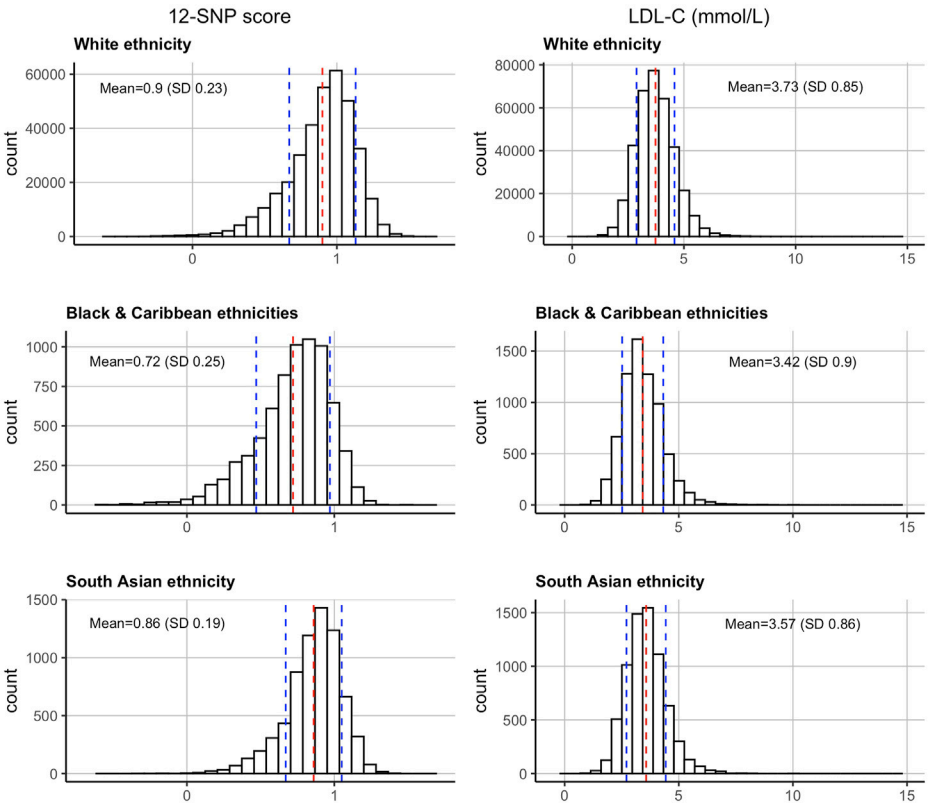

FIGURE 1 | Distributions of the 12-SNP score and LDL-C concentrations in the United Kingdom Biobank in self-reported White, Black & Caribbean, and South Asian ethnicities. The mean and SD are depicted by dotted red and blue lines respectively. LDL-C, low-density lipoprotein cholesterol; SD, standard deviation.

followed by SA [0.86 (0.19)], and BC [0.72 (0.25)] participants (**Table 1** and **Figure 1**). These group differences were also statistically significant (p-value $<2.2 \times 10^{-16}$). The ethnicity-specific 12-SNP score decile cut-off values were therefore different between WB, BC, and SA participants (**Table 2**).

In all three ethnic groups studied, the 12-SNP score was correlated with LDL-C concentrations: the R^2 was equal to 0.067 [95% confidence intervals (CI): 0.065–0.069] in WB, 0.080 (95% CI: 0.063–0.097) in BC, and 0.027 (95% CI: 0.016–0.038) in SA individuals (**Supplementary Table S3** and **Figure 2**). The beta coefficients indicating the increase in LDL-C per unit increase of the score were equal to 0.258 (95% CI: 0.241–0.275) for WB, 0.282 (95% CI: 0.169–0.395) for BC, and 0.163 (95% CI: 0.012–0.315) for SA participants (**Supplementary Table S3** and **Figure 2**). The 12-SNP score had an OR of 11.01 (95% CI: 10.08–12.04) in WB, 10.54 (95% CI: 5.29–21.67) in BC, and 6.64 (95% CI: 2.98–15.22) in SA individuals in predicting hypercholesterolaemia (LDL-C >4.9 mmol/L) (**Supplementary Table S3** and **Figure 2**).

The 12-SNP score was also associated with an increased OR for CHD prevalence and incidence in self-reported WB individuals [CHD prevalence: 1.76 (95% CI: 1.56–1.99); CHD incidence: 1.25 (95% CI: 1.13–1.39)] (**Figure 2** and **Supplementary Table S3**). These observations were also present in self-reported SA and BC participants, but the CI were wide and overlapping with 1 (**Figure 2** and **Supplementary Table S3**).

Considering that the LDL-C and 12-SNP score distributions were significantly different between the three ethnic groups studied, we hypothesised that applying ethnicity-specific 12-SNP score deciles might be more accurate in predicting mean LDL-C concentrations and hypercholesterolaemia (defined as LDL-C >4.9 mmol/L by the Simon Broome criteria). To test this, we split the data 50:50 into a training and a testing dataset and applied the WB and ethnicity-specific decile cut-off values derived from the training data to the test data (**Supplementary Table S4**). We then tested the association between the deciles and the mean adjusted LDL-C concentrations in WB, SA, and BC participants. In all ethnic groups studied, the mean adjusted LDL-C concentration had a positive R^2 value with the ethnicity-specific 12-SNP score deciles: 0.058 (95% CI: 0.056–0.060) for WB, 0.063 (95% CI: 0.047–0.079) for BC, and 0.022 (95% CI: 0.012–0.032) for SA participants (**Supplementary Table S3** and **Figure 3**). The R^2 obtained using the WB 12-SNP score deciles had overlapping confidence intervals with the ethnicity-specific deciles: 0.074 (95% CI: 0.057–0.091) for BC, and 0.021 (95% CI: 0.012–0.030) for SA participants (**Supplementary Table S3** and **Figure 3**). For hypercholesterolaemia (LDL-C >4.9 mmol/L), the area under the curve (AUC) was identical when applying the ethnicity-

TABLE 2 | Outcome data according to self-reported ethnicity-specific 12-SNP score deciles. The results presented here are using the test data. The training data was used to obtain the 12-SNP score decile cut-off values for each ethnic group studied. LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; SNP, single nucleotide polymorphism.

Self-reported ethnicity	12-SNP score decile	Minimum decile value	Maximum decile value	Number of individuals	Mean 12-SNP score	SD 12-SNP score	Mean LDL-C (mmol/L)	SD LDL-C (mmol/L)	Number of individuals with LDL-C >4.9 mmol/L	Percent (%) of individuals with LDL-C >4.9 mmol/L
White	1	−0.54	0.58	17,731	0.42	0.15	3.27	0.78	505	2.8
White	2	0.58	0.73	17,846	0.66	0.04	3.49	0.77	746	4.2
White	3	0.73	0.82	17,457	0.77	0.03	3.61	0.79	1,018	5.8
White	4	0.82	0.88	17,915	0.85	0.02	3.69	0.82	1,320	7.4
White	5	0.88	0.93	17,707	0.91	0.02	3.74	0.82	1,422	8
White	6	0.93	0.98	17,371	0.96	0.01	3.8	0.83	1,633	9.4
White	7	0.98	1.03	17,395	1.01	0.01	3.83	0.85	1758	10.1
White	8	1.03	1.08	17,551	1.06	0.02	3.89	0.84	1983	11.3
White	9	1.08	1.16	17,920	1.12	0.02	3.94	0.86	2,230	12.4
White	10	1.16	1.56	17,150	1.22	0.06	4.03	0.88	2,682	15.6
Black & Caribbean	1	−0.59	0.38	383	0.2	0.17	2.92	0.79	9	2.3
Black & Caribbean	2	0.38	0.53	346	0.46	0.04	3.14	0.77	6	1.7
Black & Caribbean	3	0.53	0.63	340	0.58	0.03	3.25	0.87	9	2.6
Black & Caribbean	4	0.63	0.7	336	0.67	0.02	3.44	0.88	22	6.5
Black & Caribbean	5	0.7	0.76	315	0.73	0.02	3.42	0.85	13	4.1
Black & Caribbean	6	0.76	0.81	336	0.78	0.02	3.42	0.9	21	6.2
Black & Caribbean	7	0.81	0.87	381	0.84	0.02	3.52	0.81	20	5.2
Black & Caribbean	8	0.87	0.93	378	0.9	0.02	3.62	0.88	32	8.5
Black & Caribbean	9	0.93	1	352	0.96	0.02	3.75	1.01	31	8.8
Black & Caribbean	10	1	1.47	325	1.07	0.06	3.76	0.95	40	12.3
South Asian	1	−0.17	0.61	342	0.46	0.12	3.29	0.8	11	3.2
South Asian	2	0.61	0.73	356	0.67	0.04	3.46	0.82	20	5.6
South Asian	3	0.73	0.79	377	0.76	0.02	3.53	0.8	15	4
South Asian	4	0.79	0.84	311	0.82	0.01	3.5	0.84	19	6.1
South Asian	5	0.84	0.88	361	0.86	0.01	3.58	0.8	19	5.3
South Asian	6	0.88	0.93	368	0.91	0.01	3.62	0.84	24	6.5
South Asian	7	0.93	0.96	323	0.95	0.01	3.57	0.81	18	5.6
South Asian	8	0.96	1.01	362	0.99	0.01	3.73	1	40	11
South Asian	9	1.01	1.08	377	1.04	0.02	3.68	0.92	32	8.5
South Asian	10	1.08	1.38	279	1.15	0.06	3.81	0.92	27	9.7

specific deciles or when applying the WB deciles: 0.63 (95% CI: 0.63–0.64) for WB, 0.65 (95% CI: 0.61–0.69) for BC, and 0.59 (95% CI: 0.55–0.63) for SA individuals (**Supplementary Table S3** and **Figure 3**).

When grouping individuals into low (deciles 1-3 of the 12-SNP score), intermediate (deciles 4–5), and high (deciles 6–10) polygenic hypercholesterolaemia categories and using the intermediate group as the reference category, the OR was lowest in the low polygenic hypercholesterolaemia category and highest in the high polygenic hypercholesterolaemia category (**Supplementary Table S3** and **Figure 3**). The values obtained were similar in the three ethnic groups studied: for low polygenic hypercholesterolaemia, the OR (95% CI) was 0.54 (0.51–0.57) for WB individuals, 0.40 (0.24–0.68) for BC individuals, and 0.75 (0.48–1.16) for SA individuals; and for the high polygenic hypercholesterolaemia group, the OR (95% CI) was 1.60 (1.53–1.67) for WB individuals, 1.56 (1.08–2.31) for BC individuals, and 1.50 (1.05–2.20) for SA individuals (**Supplementary Table S3** and **Figure 3**). The OR were similar when applying the ethnicity-specific 12-SNP score decile cut-off values and the WB decile cut-off values: when applying the WB deciles, the OR (95% CI) for BC individuals was equal to 0.56 (0.39–0.80) for the low polygenic hypercholesterolaemia category, and to 1.56 (1.07–2.28) for the high polygenic hypercholesterolaemia category; and for SA individuals, the OR (95% CI) was equal to 0.84 (0.57–1.23) for the low polygenic hypercholesterolaemia category, and to 1.70 (1.22–2.40) for the high polygenic hypercholesterolaemia category (**Supplementary Table S3** and **Figure 3**).

The results for PCA-verified White, Black, and South Asian ancestries were similar to the results obtained for self-reported WB, BC, and SA individuals (**Supplementary Table S2**).

DISCUSSION

In this study, we analysed the adjusted LDL-C concentrations and 12-SNP LDL-C score distributions for three major ethnic groups of the UK Biobank and the UK: White (WB), Black and Caribbean (BC), and South Asian (SA). The rationale for using self-reported ethnicity in our analysis instead of PCA-verified ancestry is to mimic a typical clinical scenario where the

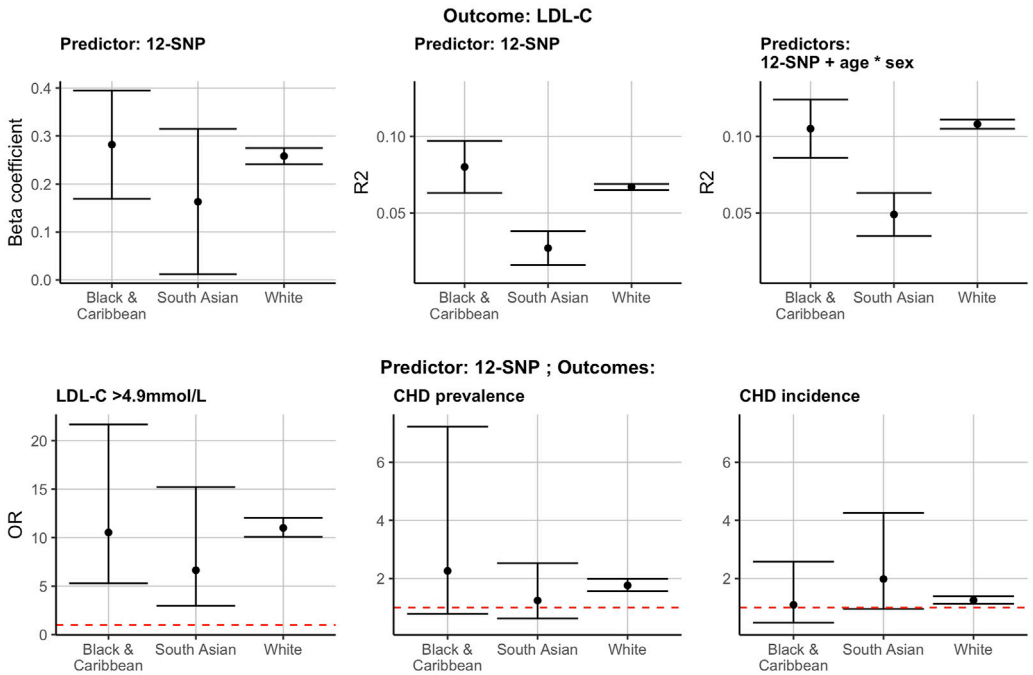

FIGURE 2 | The 12-SNP score in the prediction of LDL-C, hypercholesterolaemia (LDL-C >4.9 mmol/L) and CHD prevalence and incidence in self-reported BC, SA and WB ethnicities. The results presented here are using the test data. * denotes the interaction term in the regression analysis. The red dotted horizontal lines represent an OR of 1. CHD, coronary heart disease; LDL-C, low-density lipoprotein cholesterol; R2, correlation coefficient (R^2); OR, odds ratio; BC, Black & Caribbean; SA, South Asian; WB, White British.

12-SNP score would be applied. However, we also performed the analysis in PCA-verified White, Black, and South Asian ancestries and obtained similar results (**Supplementary Table S2**). We observed that the adjusted LDL-C mean values were significantly different between WB and BC, and between WB and SA ethnicities. This likely reflects both the differences in genetic and environmental factors that influence LDL-C concentrations. One explanation is that several of the participant characteristics that are known to influence LDL-C concentrations (age, sex, and statin use) were significantly different between WB and SA participants, and WB and BC participants.

The 12-SNP score distributions were also significantly different between WB and SA, and between WB and BC participants. These differences in the score distributions are likely to be due to the differences in the minor allele frequencies (MAFs) of the 12 variants included in the score between these ethnic groups (**Supplementary Table S1**). All 12 SNPs showed significant differences in MAF between the three ancestry groups, with for example rs1800562 in the *HFE* gene being tenfold more frequent in WB compared to SA and BC individuals (**Supplementary Table S1**). Since the 12-SNP score variants and weights were derived in a White European population (Teslovich et al., 2010), to further improve the identification of polygenic causes of hypercholesterolaemia among SA and BC patients, and once GWAS increase in diversity and size, the SNPs selected in the score should be re-evaluated to reflect these genetic differences. In a recent report from GLGC, a large-scale multi-ancestry GWAS meta-analysis of lipid levels demonstrated that an LDL-C polygenic risk score (PRS) developed from a diverse population performed better than an ancestry-specific PRS (Graham et al., 2021).

In all three ethnic groups, the 12-SNP score was correlated with LDL-C concentrations, with the highest R^2 obtained for BC, followed by WB, and finally SA individuals, although the confidence intervals were overlapping for BC and WB individuals suggesting that it performed equally well in BC and WB individuals. This also suggests that the 12-SNP score is better suited for individuals of self-reported BC ethnicity compared to individuals of self-reported SA ethnicity, although the variance in LDL-C explained by the 12-SNP score remains low in all ethnic groups (highest estimate obtained: R^2 8%). Including more variants in the score might improve the LDL-C variance observed. A recent study by Wu et al. (2021) showed that a polygenic score for LDL-C containing 8,367 variants had a R^2 of 0.215 (95% CI: 0.207–0.222) in WB individuals and a R^2 of 0.139 (95% CI: 0.125–0.154) in SA individuals of the UK Biobank, although such a large score would be currently less feasible to implement in the clinic. And in the recent GLGC study based on the largest genotype and lipids data to date, several LDL-C PRS were tested and the polygenic predictability of LDL-C adjusted for covariates (adjusted R^2) varied between 0.10 and 0.16 across the five PCA-verified ancestry groups studied (Graham et al., 2021).

The WB 12-SNP score decile cut-off values in this study were almost identical (up until the second decimal place) to the

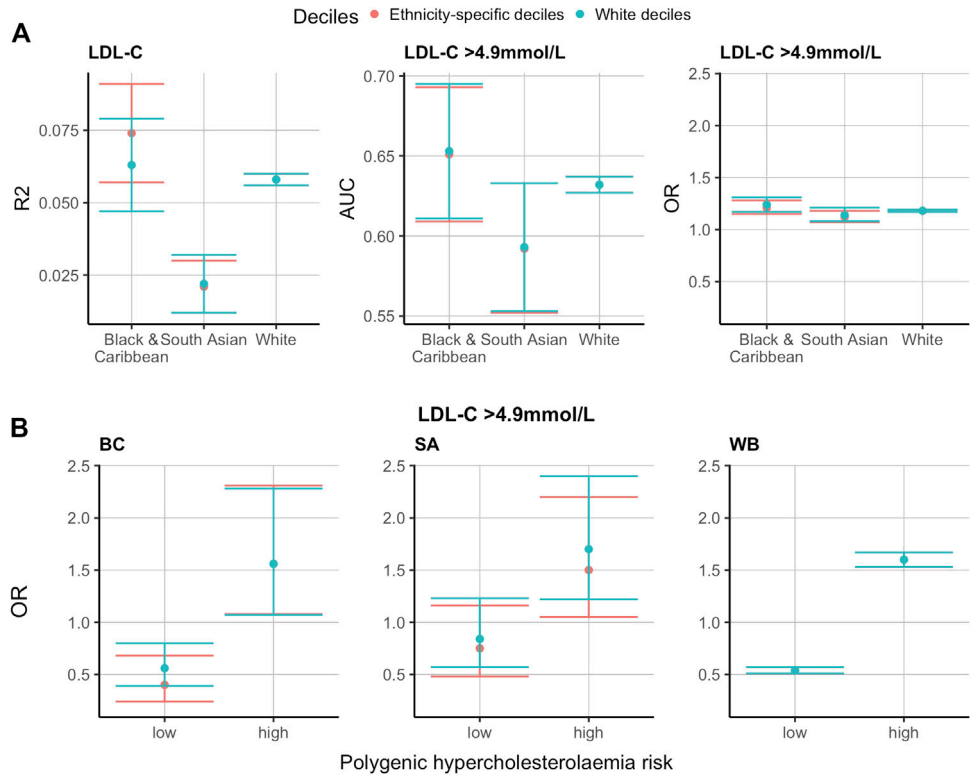

FIGURE 3 | Ethnicity-specific versus WB 12-SNP score decile cut-off values in the prediction of LDL-C and hypercholesterolaemia (LDL-C >4.9 mmol/L) in self-reported BC, SA and WB ethnicities. The results presented here are using the test data. The training data was used to obtain the 12-SNP score decile cut-off values for each ethnic group studied. The red and blue plots refer to ethnicity-specific and White deciles respectively. **(A).** R^2, AUC and OR for the prediction of LDL-C and hypercholesterolaemia (LDL-C >4.9 mmol/L) using ethnicity-specific versus WB 12-SNP score decile cut-off values. **(B)**. Polygenic hypercholesterolaemia risk using ethnicity-specific versus WB 12-SNP score deciles cut-off values for the prediction of hypercholesterolaemia (LDL-C >4.9 mmol/L). Low polygenic hypercholesterolaemia risk refers to the 12-SNP score deciles 1-3, intermediate polygenic hypercholesterolaemia risk represent deciles 4-5, and high polygenic hypercholesterolaemia risk are for deciles 6–10. Intermediate polygenic hypercholesterolaemia risk is the reference group equal to an OR of 1. LDL-C, low-density lipoprotein cholesterol; R2, correlation coefficient (R^2); OR, odds ratio; AUC, area under the curve; BC, Black & Caribbean; SA, South Asian; WB, White British.

published values derived from the UK Whitehall II study (Talmud et al., 2013). This supports the robustness of the 12-SNP decile cut-off values for WB individuals. Since the 12-SNP score is already being reported by several diagnostic labs across the UK, we provide information on how the ethnicity-specific deciles and the WB deciles of the 12-SNP score applied to these ethnic groups perform.

The 12-SNP score decile cut-off values obtained for WB, SA and BC were different. Considering that both the 12-SNP score and LDL-C distributions were significantly different between the ethnic groups studied, we hypothesised that applying ethnicity-specific decile cut-off values might be more appropriate than using WB 12-SNP score decile cut-off values. The out-of-sample validation results suggest that the WB 12-SNP score deciles applied to SA and BC participants, and the ethnicity-specific 12-SNP score deciles perform similarly in SA and BC ethnicities when predicting both the adjusted LDL-C concentrations or hypercholesterolaemia (defined as LDL-C >4.9 mmol/L by the FH Simon Broome diagnostic criteria).

Diagnostic labs in the UK are grouping individuals into low (deciles 1–3), intermediate (deciles 4–5) and high (deciles 6–10) polygenic hypercholesterolaemia score categories in order to simplify the classification of polygenic hypercholesterolaemia (George et al., 2021). This also ensures that the discrepancies in the percent of individuals with LDL-C >4.9 mmol/L in each decile are smoothed for the BC and SA ethnic groups. The grouping into low, intermediate, and high polygenic hypercholesterolaemia categories shows an increase in the odds ratio for hypercholesterolaemia from one category to another in all self-reported ethnic groups when applying the WB 12-SNP score deciles and the ethnicity-specific 12-SNP score deciles. Overall, the odds ratios for hypercholesterolaemia were similar for all ethnic groups (or had overlapping confidence intervals), implying that the ethnic differences in the 12-SNP score might not be as large as the ones reported by other polygenic score studies (Martin et al., 2019), most likely due to the low number of variants in the score, the small sample size of SA and BC individuals, and because the self-reported ethnicities in this study were not PCA verified. Indeed, the 95% confidence intervals obtained using the ethnicity-specific deciles overlapped between the low and high polygenic hypercholesterolaemia categories in SA individuals, and this was also the case (by a value of 0.01) when applying the WB decile cut-off values. However, this was not observed when using PCA-verified SA

ancestry as a study cohort, which might be because the sample size was bigger (8279 PCA-verified individuals of SA ancestry versus 7,016 participants of self-reported SA ethnicity), leading to narrower CI and more accurate estimates (**Supplementary Tables S2, S3**).

A limitation of this study is that monogenic FH cases in the cohort were not identified because the exome data was not readily available for all participants. Based on the estimated prevalence of 1:250 monogenic FH cases in the general population (Akioyamen et al., 2017), we expect roughly 1,469 (0.4%) participants in our cohort to have a monogenic cause for their hypercholesterolaemia, as opposed to a polygenic cause. However, it is unlikely that this relatively small number of monogenic FH cases would significantly influence our results. Overall, environmental variables are likely to have the biggest influence on LDL-C concentrations in all ethnic groups. Another limitation is that the sample size for BC (n = 7,082) and SA (n = 7,016) individuals were small compared to WB (n = 353,166) individuals, resulting in less precise estimates than for WB individuals, and potentially also explaining why the ethnicity-specific deciles were not significantly more accurate in predicting LDL-C concentrations and hypercholesterolaemia. Ideally this analysis would be replicated in an external and larger dataset to get more accurate decile cut-off values and estimates.

The 12-SNP score is currently being used in clinical practice in two GLHs in the UK. The score is being administered to FH mutation-negative patients with hypercholesterolaemia in order to provide them with an explanation for their hypercholesterolaemia (i.e., a polygenic cause or not). The aim of this study was not to evaluate the utility of the score in clinical practice, but to provide information on how this score, which was developed in individuals of White ancestry, performs in individuals of self-reported SA and BC ethnicities. We found that this 12-SNP score translated better to individuals of self-reported BC ethnicity than to individuals of SA ethnicity. We also found that ethnicity-specific deciles of the score performed as well as WB deciles in these two ethnic groups.

Overall, LDL-C concentrations are likely to be heavily influenced by environmental factors, and to varying levels in different ethnic groups. Regardless of cause, the key is to focus on lowering LDL-C concentrations in individuals with hypercholesterolaemia through the means of cholesterol-lowering therapy and by advocating a healthy diet and lifestyle. More research is also needed to fully assess the benefits and risks of returning polygenic information to patients (Adeyemo et al., 2021).

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: UK Biobank application ID 40721. Requests to access these datasets should be directed to, https://www.ukbiobank.ac.uk/enable-your-research/apply-for-access.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the North West Multi-centre Research Ethics Committee (MREC) as a Research Tissue Bank (RTB) approval. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF and SH contributed to the idea of the study. MF, SH, AH, CF, and JG provided critical feedback on the analyses. JG performed the analyses and wrote the manuscript. MF, SH, CF, and AH provided feedback and comments on the written manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.845498/full#supplementary-material

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Effects of Different Types of Pathogenic Variants on Phenotypes of Familial Hypercholesterolemia

Hayato Tada[1], Nobuko Kojima[1], Kan Yamagami[1], Akihiro Nomura[1], Atsushi Nohara[2], Soichiro Usui[1], Kenji Sakata[1], Noboru Fujino[1], Masayuki Takamura[1] and Masa-Aki Kawashiri[1]*

[1]*Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kanazawa University, Kanazawa, Japan,* [2]*Department of Clinical Genetics, Ishikawa Prefectural Central Hospital, Kanazawa, Japan*

Objective: It has been shown that pathogenic variants are associated with poor clinical outcomes in patients with familial hypercholesterolemia (FH). However, data on the effect of different types of pathogenic variants on FH phenotype is limited.

Methods: We retrospectively investigated the associations between genotypes and phenotypes, including low-density lipoprotein (LDL) cholesterol level and the occurrence of major adverse cardiac events (MACEs), defined as cardiovascular death, myocardial infarction, unstable angina, or coronary artery revascularization, in patients with FH (N = 1,050, male/female = 490/560). Based on genotype, the patients were divided into the following three groups: patients without pathogenic variants, patients with missense variants, and patients with protein-truncating variants (PTVs). Cox proportional hazard model was used to identify the factors associated with MACEs.

Results: The median follow-up duration was 12.6 years (interquartile range = 9.5–17.9 years). There were 665 patients with FH-mutation (277 patients with missense variants and 388 patients with PTVs) and 385 patients without FH-mutation. Over the follow-up duration, 175 MACEs were observed. We identified 89 different pathogenic variants in the 665 patients with FH. LDL cholesterol level was found to be significantly higher in patients with PTVs (256 mg/dl) than in patients with missense variants (236 mg/dl) and patients without pathogenic variants (216 mg/dl). It was also found that PTVs and missense variants are significantly associated with MACEs (hazard ratio [HR] = 1.58, 95% confidence interval [CI] = 1.08–2.08, $p = 0.0033$ and HR = 3.24, 95% CI = 2.12–4.40, $p = 3.9 \times 10^{-6}$, respectively), independent of classical risk factors.

Conclusion: Pathogenic variants, especially PTVs, are significantly associated with poor outcomes in patients with FH. Genetic testing is useful for the diagnosis and risk stratification of patients with FH.

Keywords: familial hypercholesterolemia, LDL cholesterol, genetics, LDL receptor (LDLR), protein-truncating variants

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****Correspondence:***
Hayato Tada
ht240z@sa3.so-net.ne.jp

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HIGHLIGHTS

- Pathogenic variants are associated with higher risk for CVD among patients with FH.
- LDL cholesterol level differs according to types of pathogenic variants among patients with FH.
- Prognosis differs according to types of pathogenic variants among patients with FH.

1 INTRODUCTION

Familial hypercholesterolemia (FH) is one of the most common Mendelian disorders, and its incidence in the general population is reported to be one in 300 (Beheshti et al., 2020; Hu et al., 2020). FH is caused by genetic variants associated with low-density lipoprotein (LDL) metabolism, such as the LDL receptor (*LDLR*), apolipoprotein B (*APOB*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*), and *LDLR* adaptor protein 1 (*LDLRAP1*) (Mabuchi, 2017). Typically, FH is diagnosed based on clinical criteria (Austin et al., 2004; Harada-Shiba et al., 2018; Tada et al., 2021a); however, it was reported that pathogenic variants may be associated with increased risk of coronary artery disease (Khera et al., 2016; Tada et al., 2017). Therefore, genetic testing is recommended for the diagnosis and risk stratification of patients with FH. More than 2000 pathogenic variants have been identified worldwide, and most of them are *LDLR* variants (Nohara et al., 2021). Based on genotype, pathogenic variants can be classified as missense variants (which can estimate residual LDLR activity) or protein-truncating variants (PTVs), which may have lost their LDLR function. Only a few studies have investigated the effects of pathogenic variants on the phenotype of patients with FH (Tada et al., 2020). The aim of this study was to investigate the associations between genotypes and phenotypes, including LDL cholesterol level and occurrence of major adverse cardiac events (MACEs) in patients with FH diagnosed using clinical diagnostic criteria.

2 MATERIALS AND METHODS

2.1 Study Population

We evaluated the data of 2011 patients with FH diagnosed clinically using the Japan Atherosclerosis Society (JAS) 2017 criteria at Kanazawa University Hospital between 1990 and 2020. All the patients in this study fulfilled at least two of the three essential clinical criteria stipulated by the JAS for FH diagnosis. The criteria are as follows: 1) LDL cholesterol level ≥180 mg/dl, 2) tendon xanthoma on the backs of the hands, elbows, knees, or other areas; Achilles tendon hypertrophy or Achilles tendon thickness on X-ray ≥ 9 mm; or xanthoma tuberosum, and 3) family history of FH or premature coronary artery disease diagnosed in a first- or second-degree relative. Nine hundred and sixty-one patients were excluded due to missing data (such as data on blood lipids and genetic analysis or data on homozygous and compound heterozygous FH. Finally, 1,050 patients were included in this study (**Supplementaary Figure S1**).

2.2 Clinical Data Assessment

We defined hypertension as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or use of antihypertensive agents. Further, we used the definition of diabetes given by the Japan Diabetes Society (Araki et al., 2020). Smoking status was defined as a current smoking status. Cardiovascular disease (CVD) was defined as angina pectoris, myocardial infarction, or severe stenotic region(s) in the coronary artery (≥75% stenosis), identified on angiography or computed tomography. Serum levels of total cholesterol, triglycerides, and high-density lipoprotein cholesterol were determined enzymatically using automated instrumentation. LDL cholesterol level was calculated using the Friedewald formula if triglyceride level was <400 mg/dl; otherwise, it was determined enzymatically.

2.3 Genetic Analysis

We assessed genotypes using a next-generation sequencer. In brief, the coding regions of *LDLR*, *APOB, PCSK9*, and *LDLRAP1* were sequenced as described in a previous study (Tada et al., 2018). Further, copy number variations at the *LDLR* were assessed using the eXome Hidden Markov Model software as described in an earlier study (Yamamoto et al., 2016). We evaluated the pathogenicity of the genetic variants according to the standard American College of Medical Genetics and Genomics criteria (Richards et al., 2015). We classified pathogenic variants as missense variants or PTVs, which include frameshift variants, large deletion or duplication variants, nonsense variants, and splice site variants.

2.4 Ethical Considerations

This study was approved by the Ethics Committee of Kanazawa University. All procedures were conducted in accordance with the ethical standards of the Human Research Committee (institutional and national) and the Helsinki Declaration (1975, revised in 2008). Informed consent for genetic analysis was obtained from all the study participants.

2.5 Statistical Analysis

Categorical variables were reported as numbers and percentages, and they were compared using Fisher's exact test or chi-square test. Normally distributed continuous variables were reported as means ± standard deviations. Not normally distributed continuous variables were reported as medians and interquartile ranges. Mean values of continuous variables were compared using Student's t-test for independent variables, and median values were compared using non-parametric Wilcoxon–Mann–Whitney rank sum test. Chi-square or Fisher's post-hoc test was used for categorical variables as indicated. Cox proportional hazard model was used to assess relationships between all the variables. Cumulative Kaplan–Meier survival curves starting at baseline were constructed to compare times to the first MACE. All statistical analyses were conducted

TABLE 1 | Baseline characteristics.

Variable	All (N = 1,050)	MACE (N = 175)	NO-MACE (N = 875)	*p*-value
Age (years)	49 ± 16	59 ± 15	46 ± 17	$<2.2 \times 10^{-16}$
Male gender (%)	490 (46.7%)	115 (65.7%)	375 (42.9%)	5.0×10^{-8}
Hypertension (%)	250 (23.8%)	114 (65.1%)	136 (15.5%)	$<2.2 \times 10^{-16}$
Diabetes (%)	83 (7.9%)	45 (25.7%)	38 (4.3%)	0.0016
Smoking (%)	224 (21.3%)	96 (54.9%)	128 (14.6%)	$<2.2 \times 10^{-16}$
Total cholesterol level (mg/dl)	326 [268–365]	340 [280–382]	322 [261–358]	0.00021
Triglyceride level (mg/dl)	113 [76–177]	144 [91–185]	126 [80–171]	0.0045
HDL cholesterol level (mg/dl)	47 [43–51]	45 [41–49]	48 [44–52]	0.0019
LDL cholesterol level (at baseline, mg/dL)	244 [208–279]	256 [218–294]	240 [204–270]	0.0011
LDL cholesterol level (at follow-up, mg/dL)	110 [96–120]	102 [90–116]	112 [96–121]	0.0024
Family history of FH and/or premature CVD (%)	776 (79.9%)	140 (80.0%)	636 (72.7%)	0.055
Pathogenic variants of FH (%)	777 (74.0%)	153 (87.4%)	624 (71.3%)	1.4×10^{-5}
History of CVD (%)	290 (27.6%)	141 (80.6%)	149 (17.0%)	$<2.2 \times 10^{-16}$

MACE, major adverse cardiac event; FH, familial hypercholesterolemia; CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

TABLE 2 | Medical therapies.

Lipid-lowering therapy	All (N = 1,050)	Without pathogenic variants (N = 385)	With missense variants (N = 277)	With PTVs (N = 388)
Statins (%)	1,025 (97.6%)	380 (98.7%)	265 (95.7%)	380 (97.9%)
Ezetimibe (%)	644 (61.3%)	198 (51.4%)	160 (57.8%)	286 (73.7%)
Colestimide (%)	243 (23.1%)	30 (7.8%)	70 (25.3%)	143 (36.9%)
Probucol (%)	2 (0.2%)	0 (0.0%)	1 (0.4%)	1 (0.3%)
PCSK9 inhibitor (%)	45 (4.3%)	3 (0.8%)	13 (4.7%)	29 (7.5%)
LDL apheresis (%)	2 (0.2%)	0 (0.0%)	0 (0.0%)	2 (0.5%)
Fibrates (%)	6 (0.6%)	3 (0.8%)	2 (0.7%)	1 (0.3%)
n-3 PUFAs (%)	10 (1.0%)	5 (1.3%)	1 (0.4%)	4 (1.0%)

PTV, protein-truncating variants; PCSK9, proprotein convertase subtilisin/kexin type 9; PUFA, polyunsaturated fatty acid; LDL, low-density lipoprotein.

using R statistics (https://www.r-project.org). *p*-values < 0.05 were considered statistically significant.

3 RESULTS

3.1 Clinical Characteristics

The clinical characteristics of the study participants are shown in **Table 1**. The mean age of patients was 49 years, and almost half of the patients were men. The median LDL cholesterol level at baseline was 244 mg/dl, and it decreased to 110 mg/dl at follow-up. A total of 776 patients (73.9%) had a family history of FH and/or premature CVD. Furthermore, 290 patients (27.6%) had a history of CVD. Upon division of patients into two groups based on the occurrence of MACEs, we observed significant differences in all variables (except family history of FH and/or premature CVD) between the two groups. In addition, we observed differences in the trend of characteristics; for example, we observed, following division of patients with pathogenic mutations into three groups based on genotype, that the proportion of patients with diabetes decreased (**Supplementary Table S1**). The medical treatments administered during follow-up are summarized in **Table 2**.

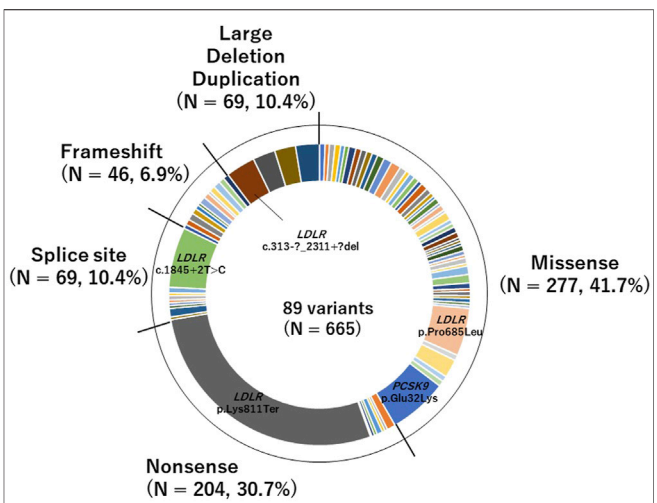

FIGURE 1 | Pie chart of pathogenic variants identified in this study of the pathogenic variants, 277 were classified as missense variants, and 388 were classified as PTVs (46 were frameshift variants, 69 were large deletion or duplication variants, 204 were nonsense variants, and 69 were splice site variants).

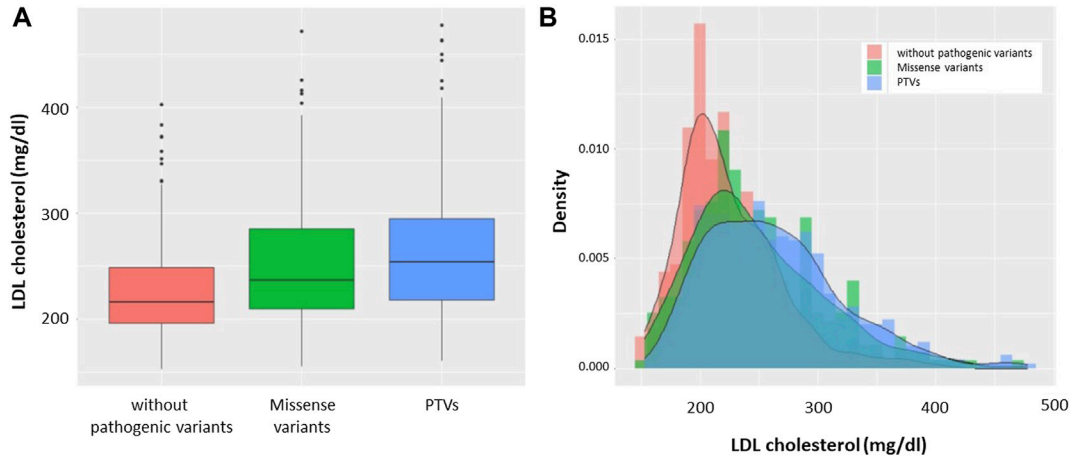

FIGURE 2 | LDL cholesterol levels according to mutation status. **(A)** Boxplots: Red indicates patients without pathogenic variants. Green indicates patients with missense variants. Blue indicates patients with PTVs. **(B)** Histograms with density: Red indicates patients without pathogenic variants. Green indicates patients with missense variants. Blue indicates patients with PTVs.

TABLE 3 | Types of MACEs.

Type of MACE	All (N = 1,050)
CVD-associated death	56 (5.3%)
Myocardial infarction	24 (2.3%)
Unstable angina	33 (3.1%)
Staged revascularization	62 (5.9%)
Total	175 (16.7%)

MACE, major adverse cardiac event; CVD, cardiovascular disease.

Statin therapy, frequently followed by ezetimibe and colestimide therapy, was administered to most of the patients.

3.2 Mutation Distributions

We identified 89 pathogenic variants in 665 patients. Of the pathogenic variants, 277 were classified as missense variants, and 388 were classified as PTVs (46 were frameshift variants, 69 were large deletion or duplication variants, 204 were nonsense variants, and 69 were splice site variants; **Figure 1**). The details are shown in **Supplementary Table S2**.

3.3 LDL Cholesterol Level According to Genotype

Based on genotype, we divided the patients into the following three groups: patients without pathogenic variants, patients with missense variants, and patients with PTVs. We found that LDL cholesterol level was highest in patients with PTVs, and patients with missense variants were found to have higher LDL cholesterol levels than patients without pathogenic variants (**Figure 2A**). The median LDL cholesterol levels of patients without pathogenic variants, patients with missense variants, and patients with PTVs were 216 mg/dl, 236 mg/dl, and 256 mg/dl, respectively. The LDL cholesterol levels of the three groups showed deformed trimodal distributions (**Figure 2B**).

3.4 Factors Associated With MACEs

Over the median follow-up duration of 12.6 years, 175 patients had MACEs, which include CVD-associated death, myocardial infarction, unstable angina, and staged revascularization (**Table 3**). Using Cox proportional hazard model, we assessed factors associated with MACEs and found that age (hazard ratio [HR] = 1.09, 95% confidence interval [CI] = 1.04–1.14, $p = 2.2 \times 10^{-14}$), male gender (HR = 1.58, 95% CI = 1.08–2.08, $p = 0.0079$), hypertension (HR = 3.11, 95% CI = 2.10–4.25, $p = 6.9 \times 10^{-6}$), diabetes (HR = 2.44, 95% CI = 1.46–3.52, $p = 0.0021$), smoking (HR = 2.56, 95% CI = 1.56–3.18, $p = 0.00041$), LDL cholesterol level (per 10 mg/dl; HR = 1.01, 95% CI = 1.00–1.02, $p = 0.022$), and prior CVD (HR = 3.45, 95% CI = 2.02–4.80, $p < 2.2 \times 10^{-16}$) are significantly associated with MACEs (**Table 4**). We also found that missense variants and PTVs are associated with MACEs (HR = 1.58, 95% CI = 1.08–2.08, $p = 0.0033$ and HR = 3.24, 95% CI = 2.12–4.40, $p = 3.9 \times 10^{-6}$, respectively).

3.5 Prognosis According to Genotype

We assessed the survival curve and found that patients with PTVs have the worst outcome of the three groups and that patients with missense variants have worse outcomes than patients without pathogenic variants (**Figure 3**).

4 DISCUSSION

In this study, we evaluated the effects of pathogenic variants on the clinical phenotypes of FH and found that 1) LDL cholesterol level is highest in patients with PTVs and higher in patients with missense variants than in patients without pathogenic variants, 2) prognosis is worst in patients with PTVs and worse in patients

TABLE 4 | Factors associated with MACEs.

Variable	HR	95% CI	*p*-value
Age (per year)	1.09	1.04–1.14	2.2×10^{-14}
Male gender (yes versus no)	1.58	1.08–2.08	0.0079
Hypertension (yes versus no)	3.11	2.10–4.25	6.9×10^{-6}
Diabetes (yes versus no)	2.44	1.46–3.52	0.0021
Smoking (yes versus no)	2.56	1.56–3.18	0.00041
LDL cholesterol level (per 10 mg/dl)	1.01	1.00–1.02	0.022
Prior CVD (yes versus no)	3.45	2.02–4.80	$<2.2 \times 10^{-16}$
Missense variants (versus no pathogenic variants)	1.58	1.08–2.08	0.0033
PTVs (versus no pathogenic variants)	3.24	2.12–4.40	3.9×10^{-6}

HR, hazard ratio; CI, confidence interval; CVD, cardiovascular disease; PTV, protein-truncating variants; LDL, low-density lipoprotein.

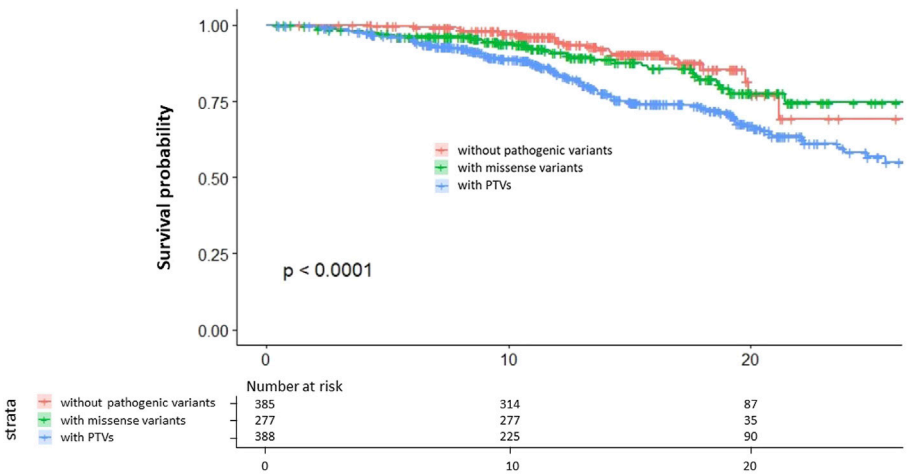

FIGURE 3 | Kaplan–Meier survival curves. Red indicates patients without pathogenic variants. Green indicates patients with missense variants. Blue indicates patients with PTVs.

with missense variants than in patients without pathogenic variants.

There are many factors, including traditional risk factors and genetic factors that have been associated with CVD in patients with FH. Indeed, a clinical risk score named Montreal-FH-score comprising traditional risk factors has been one of the useful tools predicting CVD risk in patients with FH (Paquette et al., 2017). In addition, we and others have shown that rare and common genetic variations contributed to increase or decrease risks for CVD among patients with FH on top of FH-mutation (Tada et al., 2018; Fahed et al., 2020). Based on different standpoints, including diagnosis, cascade screening, and risk stratification, genetic testing is currently recommended for FH (Sturm et al., 2018). Patients with FH are associated with significantly high risk of CVD due to cumulative exposure to elevated LDL cholesterol level (Tada et al., 2021b). Thus, early diagnosis and intervention are considered vital. Previous studies reveal that FH diagnosis is improved by early treatment of patients (Luirink et al., 2019; Tada et al., 2021c). Since patients with FH typically do not have tendon xanthomas (one of the most important clinical diagnostic criteria) in childhood, genetic testing is essential for early diagnosis of FH (Tada et al., 2021d; Matsunaga et al., 2021; Nagahara et al., 2021). Furthermore, given the criteria of pathogenicity of genetic variants and the catalog of pathogenic variants of FH, there is a growing demand for further risk stratification of patients with FH based on genotype (Chora et al., 2022). The growing demand is natural in this personalized medicine era, when phenotypes can be estimated using genotypes and the best treatments for many inherited diseases can be determined based on genotype (Sukrithan et al., 2019; Pujol et al., 2021; Tada, 2021). We expect that patients with PTVs will receive intensive treatment and that cascade screening will be recommended for patients with pathogenic variants, especially PTVs, to identify patients with high CVD risk.

This study has several limitations. First, this is a retrospective study conducted in a single center. Therefore, the study findings may not be applicable to other patients. However, our institute has a long history of treating patients with FH and has one of the largest databases in Japan. Second, we could not account for treatments administered during follow-up, and this may affect the study results. Third, many patients were excluded from analysis due to missing data or loss to follow-up. This exclusion may also affect study results.

When we compared the baseline characteristics (only the key variables, including age, gender, and LDL cholesterol) between study subjects and those excluded. We found that the mean age of the patients excluded from this study was significantly younger than that of patients included in this study, although there were no significant differences between these 2 groups in gender and LDL cholesterol (**Supplementary Table S2**). Fourth, in this study, functional analysis was not performed to validate the pathogenicity of genetic variants. Fifth, polygenic factors were not considered in this study. Sixth, we did not account for functional analyses of the variants, especially in missense variants. In fact, some of the missense variants, such as p.Ser177Leu, p.Glu228Gln, p.Asp266Asn, and p.Val429Leu have been considered as "null alleles" based on functional assays (Hobbs et al., 1992). However, the number of individuals with these variants were so small that it is unlikely to affect our results. In addition, it is difficult to classify all of the variants of this study clearly because of lack of such data. Furthermore, a simple classification of PTVs appears to have great impact on phenotypes in our data. Accordingly, we believe that it is beneficial for us to divide PTVs and missense variants in case of FH. Seventh, we did not fully observe other atherosclerotic disease, such as cerebrovascular disease, peripheral artery disease and aortic valve stenosis unless they exhibited any symptoms suggesting these conditions. Further studies will full assessments of these conditions will be useful to estimate their comprehensive risk assessments.

In conclusion, pathogenic variants, especially PTVs, are significantly associated with poor outcomes in patients with FH. Genetic testing is useful for the diagnosis and further risk stratification of patients with FH.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the IRB of Kanazawa University did not give us an approval to deposit genomic data even in a public repository. Requests to access the datasets should be directed to the corresponding author, HT.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB of Kanazawa University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HT and M-AK conceived of the presented idea. HT, NK, KY, ATN, AKN, SU, KS, NF, MT, and M-AK collected clinical data. HT, NK, KY, and AKN performed genetic analyses. HT performed statistical analyses. MT and M-AK supervised the findings of this work. All authors contributed to write the manuscript. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.872056/full#supplementary-material

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REVIEW
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Familial Hypercholesterolemia and Elevated Lipoprotein(a): Cascade Testing and Other Implications for Contextual Models of Care

Wann Jia Loh[1]*, *Dick C. Chan*[2], *Pedro Mata*[3] and *Gerald F. Watts*[2,4]

[1]Department of Endocrinology, Changi General Hospital, Singapore, Singapore, [2]Medical School, University of Western Australia, Perth, WA, Australia, [3]Fundación Hipercolesterolemia Familiar, Madrid, Spain, [4]Lipid Disorders Clinic, Department of Cardiology and Internal Medicine, Royal Perth Hospital, Perth, WA, Australia

Elevated lipoprotein(a) [Lp(a)], a predominantly genetic disorder, is a causal risk factor for atherosclerotic cardiovascular disease (ASCVD) and calcific aortic valvular disease, particularly in patients with familial hypercholesterolemia (FH), a Tier I genomic condition. The combination from birth of the cumulative exposure to elevated plasma concentrations of both Lp(a) and low-density lipoprotein is particularly detrimental and explains the enhanced morbidity and mortality risk observed in patients with both conditions. An excellent opportunity to identify at-risk patients with hyper-Lp(a) at increased risk of ASCVD is to test for hyper-Lp(a) during cascade testing for FH. With probands having FH and hyper-Lp(a), the yield of detection of hyper-Lp(a) is 1 individual for every 2.1–2.4 relatives tested, whereas the yield of detection of both conditions is 1 individual for every 3–3.4 relatives tested. In this article, we discuss the incorporation of assessment of Lp(a) in the cascade testing in FH as a feasible and crucial part of models of care for FH. We also propose a simple management tool to help physicians identify and manage elevated Lp(a) in FH, with implications for the care of Lp(a) beyond FH, noting that the clinical use of RNA therapeutics for specifically targeting the overproduction of Lp(a) in at risk patients is still under investigation.

Keywords: cascade testing, familial hypercholesterolemia, lipoprotein (a), Lp(a), inherited hypercholesterolemia, hyper-Lp(a), FH model of care

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***Correspondence:**
Wann Jia Loh
loh.wann.jia@singhealth.com.sg

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INTRODUCTION

A century ago, a few case reports of patients with sudden cardiac death and xanthomata by Dr. Francis Harbitz, led to the discovery of a heritable condition, that in the words of Dr. Carl Müller was "inherited as a pronounced dominant quality" (Müller, 1938; Goldstein and Brown, 2009). The condition is now known as Familial Hypercholesterolemia (FH). In the 1970s, Joseph Goldstein and Michael Brown discovered the low density lipoprotein (LDL) receptor and the concept of receptor-mediated endocytosis, which explained that FH was due to defective or deficient LDL receptor function (Goldstein and Brown, 1974).

Another clinically important lipoprotein disorder that is under strong genetic influence is elevated plasma lipoprotein(a) [Lp(a)] concentration. Lp(a) was first discovered in 1963 by Kare Berg as an antigenic variant of LDL (Berg, 1963). Large epidemiological, Mendelian randomization, and genome-wide association studies have shown that elevated levels of Lp(a) increase the risk of

atherosclerotic cardiovascular disease (ASCVD) and calcific aortic valve disease (CAVD) (Kamstrup et al., 2008; Clarke et al., 2009; Emerging Risk Factors et al., 2009; Kamstrup et al., 2009; Kamstrup et al., 2014; Burgess et al., 2018; Langsted et al., 2019; Perrot et al., 2019; Trinder et al., 2020a). Lp(a) is an LDL-like lipoprotein consisting of a single apolipoprotein B-100 (apoB) covalently bound to apolipoprotein(a) [apo(a)] (Schmidt et al., 2016), a preferential carrier of oxidized phospholipids that also has plasminogen-like properties (Boffa and Koschinsky, 2019). Lp(a) particles accordingly have proatherogenic, prothrombotic, and proinflammatory properties (Nordestgaard et al., 2010; Boffa and Koschinsky, 2019).

The ASCVD risk associated with hyper-Lp(a) is accentuated in patients at high-risk of ASCVD, especially FH. Hence, recommendations on measuring Lp(a) in high-risk patient groups have been integrated into recent clinical guidelines and consensus statements (Grundy et al., 20182019; Cegla et al., 2019; Mach et al., 2019; Wilson et al., 2019; Handelsman et al., 2020; Pearson et al., 2021; Reyes-Soffer et al., 2022). In this article, we review hyper-Lp(a) in the context of FH, particularly in respect of testing for Lp(a) during cascade testing of relatives of probands with a definite diagnosis of FH. We also refer to the impact of elevated Lp(a) on the phenotypic diagnosis of FH.

DOUBLE-TROUBLE: FH AND HYPER-LP(A) AS DUAL RISK FACTORS

Two Monogenic Defects in Atherogenic Lipoproteins: FH and Hyper-Lp(a)

FH has long been considered a highly penetrant disease, with rarely anyone having a plasma LDL cholesterol (LDL-C) concentration below the 95th percentile of the general population (Miserez and Keller, 1995), however a recent finding that pathogenic or likely pathogenic FH mutations were present even in subjects with LDL<3.3 mmol/L contributing to 27% of total mutation-positive subjects in a large study suggests large heterogeneity in the clinical expression of monogenic FH (Khera et al., 2016; Sniderman et al., 2022). The majority of FH (80%–85%) is caused by mutations of the *LDLR* gene, with over 1700 mutations identified (Iacocca et al., 2018), causing either deficient or defective LDL receptors (Usifo et al., 2012). Other mutations are *APOB* gene missense mutations (5%–10%), gain-of-function mutations in proprotein convertase subtilisin/kexin type 9 (*PCSK9)* (1%), and mutations of *LDLRAP1* (Nordestgaard et al., 2013; Fellin et al., 2015). The inheritance of FH is autosomal-codominant except for *LDLRAP1* which is autosomal recessive (Fellin et al., 2015). The phenotype of FH is severe in cases with deficient or null LDL receptors, but milder in cases with missense mutations in *LDLR* that do not inactivate LDL receptors completely (Khera and Hegele, 2020), or in familial defective *APOB* mutations (Miserez and Keller, 1995). The cumulative exposure of LDL-C in patients with FH begins in fetal life (Brown et al., 1978). Hence the majority of untreated patients with homozygous FH (HoFH) and heterozygous FH (HeFH) develop symptomatic atherosclerotic coronary artery disease (CAD) before 20 years old and 60 years old respectively (Nordestgaard et al., 2013).

Young men with FH have >25-fold increased relative risk of CAD compared with patients without FH (Hopkins, 2017). A recent meta-analysis highlighted that FH was highly prevalent among patients with ASCVD (1 affected individual in 17 patients), an 18-fold higher frequency than in the general population (Hu et al., 2020). Early initiation of cholesterol-lowering treatment can achieve a 10-fold decreased risk of ASCVD in patients with FH (Perez-Calahorra et al., 2019). However, despite lowering LDL-C to target levels, there is still a residual risk in some patients, a particular culprit being elevated plasma Lp(a) levels (Alonso et al., 2014; Vuorio et al., 2017; Rosenson and Goonewardena, 2021).

Although FH and hyper-Lp(a) are both autosomal co-dominantly inherited, the genetics of FH and hyper-Lp(a) differ. The *LDLR* is located on chromosome 19 and *LPA* on chromosome 6. The *LPA* gene is considered to be fully expressed by 2 years of age, adult plasma Lp(a) concentrations achieved by age of 5 years (Wilson et al., 2019; Strandkjær et al., 2021). However, a very recent report has suggested that Lp(a) increases by ≈ 20% from childhood to adulthood in a large cohort of children with mostly a diagnosis of FH (de Boer et al., 2022). Unlike FH, a pure monogenic disorder, elevated Lp(a) levels are consequent on a combination of heritable factors that contribute to >90% of plasma concentrations. Lp(a) concentrations are mainly determined by copy number variation of the Kringle IV type 2 ($K\text{-}IV_2$) repeats, with over 2000 gene variants in the wider *LPA* gene region (Burgess et al., 2018; Trinder et al., 2020a; Hoekstra et al., 2021; Said et al., 2021), and apoE genotypes (Moriarty et al., 2017). Owing to the inheritance of >40 possible different allelic *LPA* variants, the multiple copies of $K\text{-}IV_2$ domain of the apo(a) isoform result in a widely variable molecular mass of Lp(a) that contributes up to 1000-fold differences in plasma Lp(a) concentrations (Schmidt et al., 2016). Over 500 gene variants in the $K\text{-}IV_2$ repeats region have a major effect on Lp(a) concentration (Coassin et al., 2019). The relationship between Lp(a) genetics and concentration varies with ethnicity (Dumitrescu et al., 2011; Paré et al., 2019). Higher Lp(a) concentrations are observed among people of African descent and Indians, whereas lower concentrations are reported in Chinese and Hispanic populations (Enkhmaa et al., 2016; Paré et al., 2019; Loh et al., 2021; Patel et al., 2021). Studies, in general, support the clinical use of Lp(a) rather than apo(a) isoforms or single nucleotide polymorphisms (SNPs) in predicting the risk of ASCVD (Paré et al., 2019; Trinder et al., 2020a; Page et al., 2020). Phenotype, and hence the penetrance of the genetic defect, appears to be more important in influencing ASCVD risk than genotype with both FH (Perez-Calahorra et al., 2019) and elevated Lp(a) levels (Paré et al., 2019; Trinder et al., 2020a).

The risk of ASCVD is directly related to the plasma concentration of Lp(a). An analysis of 460,506 participants from the United Kingdom Biobank database showed that Lp(a) was linearly associated with ASCVD risk after a median follow up of 11.2 years, with a hazard ratio (HR) of 1.11 per

50 nmol/L increments within each ethnicity (White, South Asian, Black), despite ethnic differences in median Lp(a) values (Patel et al., 2021). Lp(a) concentration ≥150 nmol/L (≈70 mg/dL) was associated with increased incidence of CAD in both primary and secondary prevention groups (HR 1.63 and HR 1.23 respectively), with weaker associations being found with ischaemic stroke (Patel et al., 2021). An early meta-analysis by the Emerging Risk Factors Collaboration of 126,634 people without prior history of CAD or stroke at baseline showed a curvilinear relationship between Lp(a) and cardiovascular outcomes, with the highest risk of CAD and ischaemic stroke being seen at extremely high Lp(a) levels (Emerging Risk Factors et al., 2009). Extremely high plasma Lp(a) concentrations (>95th percentile) are associated with the highest risk of ASCVD (Kamstrup et al., 2008; Burgess et al., 2018; Loh et al., 2021), CAVD (Kamstrup et al., 2014; Guddeti et al., 2020), and heart failure (Kamstrup and Nordestgaard, 2016) in the general population. Mendelian randomization studies employing polygenic risk scores suggest that an extremely elevated Lp(a) (>430 nmol/L or >180 mg/dL) is a risk factor for ASCVD equivalent to HeFH (Burgess et al., 2018; Trinder et al., 2020a).

Is Hyper-Lp(a) More Common in FH?

Plasma Lp(a) concentration above 50 mg/dL (≈100–125 nmol/L), that is above the 80th percentile for a Caucasian population, is commonly accepted in clinical practice as an elevated level (Nordestgaard et al., 2010; Grundy et al., 20182019; Wilson et al., 2019; Pearson et al., 2021), and is used as a clinically meaningful threshold level in many studies. Hyper-Lp(a) is more prevalent in HeFH than in the general population (29.3% vs. 22.2%), as shown in a large study of 2,917 patients with HeFH by (Alonso et al., 2014). The prevalence of hyper-Lp(a) may be as high as 30%–50% of patients with HeFH (Vuorio et al., 2020). Although the genetic control of Lp(a) and FH is a priori orthogonal (Ellis et al., 2019), a defective LDL receptor pathway may contribute to elevating Lp(a) levels. Patients with HoFH due to *LDLR* mutations have been reported to have an almost 2-fold higher Lp(a) level than those with HeFH signifying a gene dosage effect (Kraft et al., 2000), although not all studies are concordant (Sjouke et al., 2017). Also, Lp(a) concentrations were reported to be higher in patients with FH (*LDLR* and *PCSK9* mutations) compared with controls (Tada et al., 2016), and Lp(a) levels are modestly lowered by PCSK9 inhibitors (Bittner et al., 2020; O'Donoghue et al., 2019). Lp(a) levels were numerically higher in FH patients with *LDLR* null mutations compared with those with defective mutations in the SAFEHEART study (Alonso et al., 2014). Although Lp(a) concentrations have been reported to be high in FH, it remains unclear whether this involves decreased Lp(a) clearance via the LDL receptor (Vuorio et al., 2020). Lp(a) levels are not altered or even increased by statin, which upregulates LDL receptors (Tsimikas et al., 2020a). It has been suggested that elevated levels of Lp(a) in FH may be due to ascertainment bias (Trinder et al., 2020b) and population-based studies do not suggest that FH is associated with Lp(a) (Langsted et al., 2016).

Hyper-Lp(a) as a Risk Enhancer in FH

About 10% of patients with HeFH have ASCVD events even after 12 years of high intensity cholesterol-lowering treatment, pointing to significant residual risk (Perez-Calahorra et al., 2019). An important cause is hyper-Lp(a) (Alonso et al., 2014; Vuorio et al., 2017; Rosenson and Goonewardena, 2021; Alonso et al., 2022). Premature ASCVD risk conferred by excessive exposure to LDL-C in patients with FH is further accentuated by elevated Lp(a) levels and this appears to be independent of the type of FH mutation (Alonso et al., 2014; Vuorio et al., 2020). In a Spanish cohort of HeFH patients (SAFEHEART), patients with a combination of null *LDLR* mutation and hyper-Lp(a) > 50 mg/dL had the worst CVD-free survival time compared with patients with non-null *LDLR* mutation and/or Lp(a) < 50 mg/dL (Alonso et al., 2014). The risk of ASCVD or mortality of relatives of patients with FH was also highest in relatives with both FH and hyper-Lp(a) (HR 4.40) than in those with FH alone (HR 2.47), and hyper-Lp(a) alone (HR 3.17) when compared with individuals with neither disorder (**Figure 1**) (Ellis et al., 2019). These studies suggest that hyper-Lp(a) is an independent residual risk factor for ASCVD in FH. Several other prospective studies have also shown a significant association between elevated Lp(a) levels and increased risk of ASCVD in FH and are summarized in **Table 1**.

Lp(a) may be useful in improving ASCVD risk prediction in high-risk groups (Grundy et al., 20182019; Wilson et al., 2019; Cegla et al., 2019; Handelsman et al., 2020; Pearson et al., 2021), particularly in FH (Santos et al., 2016; Pérez de Isla et al., 2017; Paquette et al., 2021). The two major risk equations, the SAFEHEART-RE (Pérez de Isla et al., 2017) and the FH-Risk-Score (Paquette et al., 2021), were derived from large prospective FH populations and included hyper-Lp(a) [Lp(a) > 50 mg/dL (Pérez de Isla et al., 2017) or ≥50 mg/dL (Paquette et al., 2021)] as a predictor of ASCVD risk in patients with the FH in primary (Pérez de Isla et al., 2017; Paquette et al., 2021) and secondary prevention settings (Pérez de Isla et al., 2017). **Figure 2** shows an example of using the SAFEHEART-RE to predict 5- and 10-year risks of developing incident ASCVD in FH. The SAFEHEART-RE risk equation was based on an exclusively genetically defined population, whereas the FH-Risk-Score was based on a population defined using a mixture of genetic and phenotypic criteria. In the multivariate analyses that formulated these two risk equations, the hyper-Lp(a) variable was associated with similar hazard ratios in both studies; HR 1.5 [SAFEHEART RE: HR1.52 (95% CI 1.05–2.21) (Pérez de Isla et al., 2017), FH-Risk-Score: HR 1.53 (1.14–2.04) (Paquette et al., 2021)], **Table 1**. In the FH-Risk-Score, the risk attributed by Lp(a) ≥50 mg/dL as a binary variable (yes/no) carried more weightage than untreated LDL-C 5.5–7.5 mmol/L (Paquette et al., 2021). It follows that extremely elevated Lp(a) concentrations (>95 percentiles, 100 mg/dL or 200 nmol/L) would be strongly predictive of future ASCVD in FH. Conversely, low Lp(a) levels (1–74th percentile, 0–30 mg/dL or 0–60 nmol/L) may contribute to resilience against ASCVD in FH (Perez de Isla et al., 2021).

Hyper-Lp(a) is also an independent risk factor for CAVD in both the general population (Kamstrup et al., 2014; Guddeti et al.,

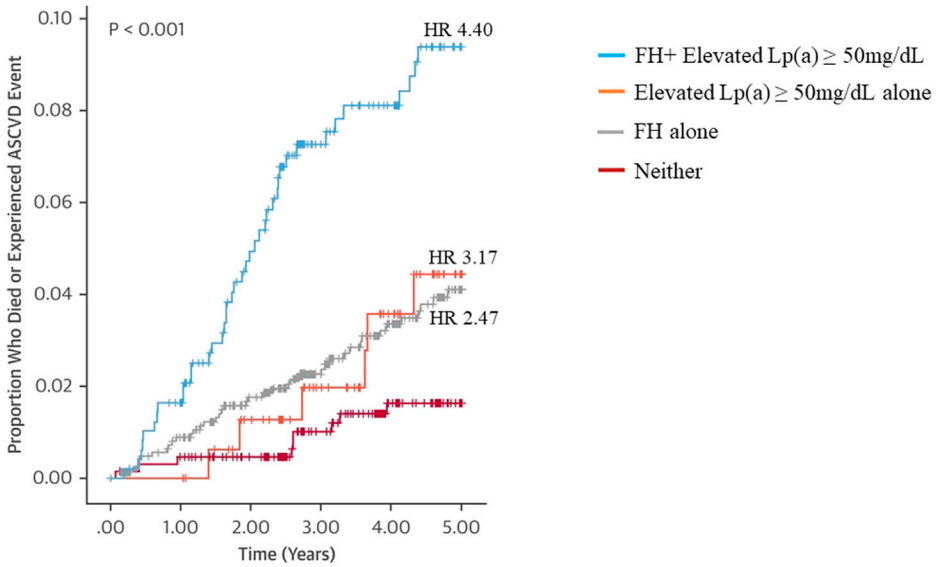

FIGURE 1 | Kaplan-Meier survival analysis showing a significant association between the FH and Lp(a) diagnoses of relatives and ASCVD events. Cox proportional hazard ratio (HR) of patients with dual FH and Lp(a) diagnoses, elevated Lp(a) alone, FH alone compared with individuals with neither disorder are shown in the figure. Modified from Ellis KL et al. Value of Measuring Lipoprotein (a) During Cascade Testing for Familial Hypercholesterolemia. J Am Coll Cardiol. 2019; 73:1029–1039, with permission.

2020) and in patients with FH (Vongpromek et al., 2015; Mata et al., 2021). The mechanisms are unclear, but are likely mediated by oxidized phospholipids carried by Lp(a) (Yeang et al., 2016; Kamstrup et al., 2017), with contributions also from the cumulative burden of LDL-C, increasing age, and a history of hypertension (Mata et al., 2021; Pérez de Isla et al., 2021). CAVD itself is a risk factor for ASCVD, heart failure, and mortality, further contributing to residual risk in FH (Yeang et al., 2016; Kamstrup et al., 2017; Mata et al., 2021). The prevalence of aortic valve calcification is two-fold more common in HeFH than in controls (Ten Kate et al., 2015), and is present in 40%–60% of asymptomatic HeFH (Ten Kate et al., 2015; Vongpromek et al., 2015). In a family study of 17 patients with both calcific aortic valve stenosis and Lp(a) ≥60 mg/dL, (Perrot et al., 2019) showed that their first-degree relatives were at increased risk of aortic valve microcalcification and stenosis compared with a control group of normal Lp(a) levels . Although this was a small and underpowered study, the relatively high yield of detection of 48.5% of aortic valve microcalcification suggests that cascade screening of families for CAVD and elevated Lp(a) may be potentially useful (Perrot et al., 2019), but further studies are required.

Cumulative ASCVD Burden Due to Two Genetic Risk Factors

Although governed by different genetic pathways, the cumulative effects of these two pro-atherogenic risk factors are more than additive. The lifetime cumulative effect of these dual heritable risk factors was recently illustrated by (Vuorio et al., 2017) using the concept of compound LDL-C and Lp(a)-particle cholesterol burden (**Figure 3**). Using the combination of the burden of LDL-C-years and Lp(a)-cholesterol years in FH (Vuorio et al., 2017), an earlier onset of CVD burden of 4 years was estimated in patients with untreated HeFH, comparing patients with Lp(a) levels of 100 mg/dL with patients with levels of 30 mg/dL (Vuorio et al., 2020). Because of the significant contribution to ASCVD and CAVD risk, Lp(a) concentration should be measured on at least one occasion as a priority in patients with FH (Grundy et al., 20182019; Cegla et al., 2019; Wilson et al., 2019; Langsted and Nordestgaard, 2022).

INCORPORATING LP(A) IN SCREENING FOR FH

Cascade Testing for FH

Despite much guidance on best practices for the care of FH, FH remains mainly underdiagnosed (Nordestgaard et al., 2013). One in 250 people has HeFH, while 1 in 300,000 people has HoFH (Nordestgaard et al., 2013; Watts et al., 2015; Akioyamen et al., 2017; Berberich and Hegele, 2019; Hu et al., 2020; Khera and Hegele, 2020). The prevalence of HeFH may be up to 1% in gene founder populations and up to 20% in patients with premature ASCVD (De Backer et al., 2015; Berberich and Hegele, 2019). The Center for Disease Control and Prevention recommends cascade testing for FH (the leading and actionable Tier condition), because early diagnosis can be aided by genetic testing, and implementation of ASCVD prevention measures can have a significant public health impact. Cascade testing for FH has been shown to be highly cost-effective in multiple populations (Marang-van de Mheen et al., 2002; Ademi et al., 2013; Kerr et al., 2017; Knowles et al., 2017; Lázaro et al., 2017), including children (Wald et al., 2016; Ademi et al., 2020; Jackson

TABLE 1 | Selected prospective studies in familial hypercholesterolemia showing the association between lipoprotein(a) levels and atherosclerotic cardiovascular disease risk.

Study population, published year	Characteristics, number of subjects (n)	Outcome, follow up (yr)	Hazard ratios (95% confidence interval) for ASCVD risk multivariable regression analysis		p value
Copenhagen General Population Study Langsted et al. (2016)	Phenotypic Diagnosis of FH using modified DLCN, Simon Broome, MEDPED	MI	Neither disorder (reference), *n* = 35153	1	<0.001 (log-rank trend)
	Probable or definite FH 184	Median follow up: 3.9 years	Lp(a) > 50 mg/dL alone, *n* = 6921	1.4 (1.1–1.7)	
	Possible FH 3082		FH alone, *n* = 2300	3.2 (2.5–4.1)	
	Unlikely to have FH 42934		FH and Lp(a) > 50 mg/dL, *n* = 715	5.3 (3.6–7.6)	
	n = 46200				
SAFEHEART Pérez de Isla et al. (2017) (Spanish)	Genetic diagnosis (includes patients with and without history of ASCVD) *n* = 2404	ASCVD event or death	Lp(a) ≤50 mg/dL (reference)	1	0.028
		Mean follow up 5.5 years	Lp(a) > 50 mg/dL	1.52 (1.05–2.21)	
SAFEHEART Ellis et al. (2019) (Spanish)	Relatives of probands with genetic diagnosis of FH	ASCVD event or death Mean follow up 3.5 years	Neither disorder (reference), n = 780	1	
	Genetically positive FH 1944		Lp(a) ≥50 mg/dL alone, *n* = 203	3.17 (1.16–8.64)	0.024
	No FH 983		FH alone, *n* = 1413	2.47 (1.06–5.74)	0.036
	n = 2927		FH and Lp(a) ≥50 mg/dL, *n* = 531	4.40 (1.92–10.07)	<0.001
Cao et al. (2019) (Chinese)	Phenotypic diagnosis of FH using DLCN score >6 (definite and probable FH) from a patient population with suspected CVD *n* = 393	ASCVD event Mean follow up 3 years	Lp(a) per log unit increase	2.03 (1.28–3.21)	0.002
			Lp(a) Tertile 1 (reference)	1	
			Lp(a) Tertile 2	4.99 (1.36–9.25)	0.015
			Lp(a) Tertile 3	6.96 (2.24–9.32)	0.001
Multicenter study from 5 registries Paquette et al., 2021 (Montreal, British Columbia, United Kingdom Biobank, Ontario, France), 2021	Phenotypic diagnosis of FH (DLCN ≥6 points) or genetic diagnosis (74%), without prior history of ASCVD *n* = 3881	ASCVD Mean follow up 8 years	Lp(a) < 50 mg/dL (reference)	1	0.004
			Lp(a) ≥50 mg/dL	1.53 (1.14–2.04)	

ASCVD, atherosclerotic cardiovascular disease; DLCN, dutch lipid clinic network; FH, familial hypercholesterolemia; Lp(a), lipoprotein (a); MEDPED, make early diagnosis to prevent early death criteria; MI, myocardial infarction.
Reference: refers to this group being the reference group for comparison.

et al., 2021). A related approach is child-parent testing, following the detection of a child at immunization at 2 years old (Wald et al., 2016). Ademi et al. (2020)recently showed that cascade screening in ten-year-old children followed by early initiation of statin in children with HeFH is cost-effective compared with usual care, with a predicted significant lifetime reduction in hospitalization costs. In this study, the prevalence of a positive FH mutation among children detected through cascade screening was 56.8% (Ademi et al., 2020).

Cascade Testing for Both FH and Hyper-Lp(a)

The distribution of Lp(a) in the general population is positively skewed to the right, with hyper-Lp(a) coinciding with the top 20th percentile of the population distribution (Nordestgaard et al., 2010). This equates to 1 in 5 people having hyper-Lp(a) in the general population, although studies have shown that there are ethnic differences in prevalence (Paré et al., 2019; Patel et al., 2021). It is estimated that worldwide hyper-Lp(a) is a common disorder affecting 1.4 billion people and that at least five million have both HeFH and hyper-Lp(a) (Vuorio et al., 2020). Hyper-Lp(a) is, therefore, more common than HeFH and may be more prevalent in patients with HeFH (Alonso et al., 2014) than those without. Cascade testing from affected probands with FH or high Lp(a) levels is useful in identifying these conditions. **Figure 4** shows examples of the pedigrees of three families who were cascade tested for FH and high Lp(a).

Two major studies showed that incorporating Lp(a) into the genetic cascade testing of FH in families of probands with FH mutations is an effective way of identifying hyper-Lp(a) in this high-risk group (Ellis et al., 2019; Chakraborty et al., 2021). In these two studies, the yield of detection of FH *via* cascade

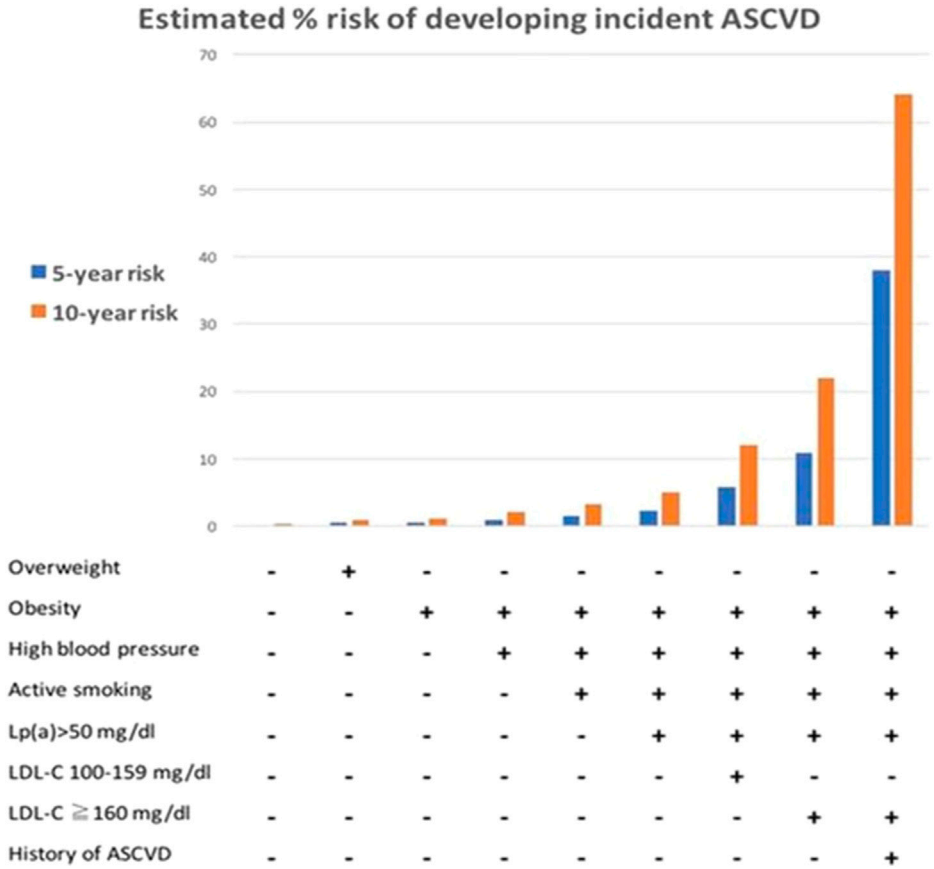

FIGURE 2 | Graphical use of SAFEHEART-RE for predicting 5 and 10-year risks of developing incident atherosclerotic cardiovascular disease (ASCVD) in familial hypercholesterolemia (FH) in primary prevention or secondary prevention settings; an example shown refers to a 66-year-old man with FH and low-density lipoprotein-cholesterol (LDL-C) <100 mg/dl (2.6 mmol/L) in absence or presence of elevated lipoprotein (a) [Lp(a)] > 50 mg/dl. From Perez de Isla L et al. Predicting Cardiovascular Events in Familial Hypercholesterolemia: The SAFEHEART Registry (Spanish Familial Hypercholesterolemia Cohort Study). Circulation. 2017; 135:2133–2144, with permission.

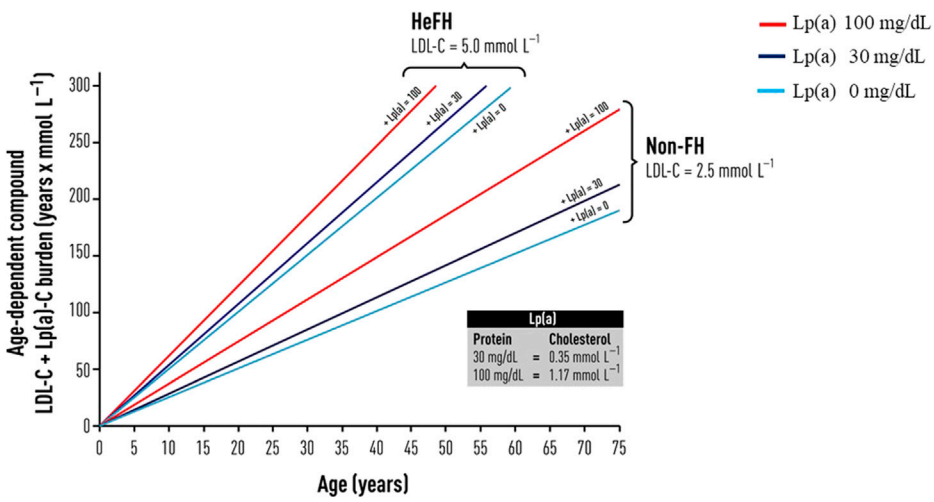

FIGURE 3 | Schematic diagram of the concept of compound LDL- and Lp(a)--cholesterol burden (*y*-axis) that increases with age, in patients with and without FH, with assumption that in untreated heterozygous FH patients (HeFH) and in non-FH individuals. From Vuorio A, Watts GF, Schneider WJ, Tsimikas S, Kovanen PT. Familial hypercholesterolemia and elevated lipoprotein (a): double heritable risk and new therapeutic opportunities. J. Intern. Med. 2020; 287:2–18, with permission.

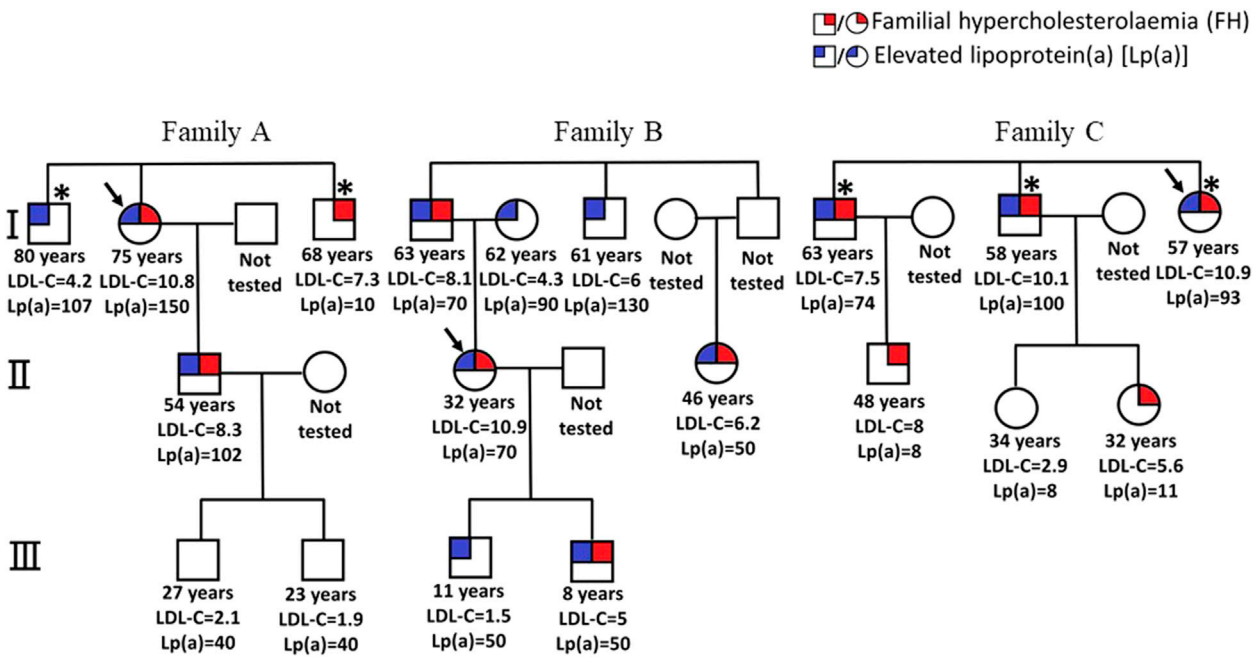

FIGURE 4 | Pedigrees of families (A–C) with familial hypercholesterolemia (FH) and elevated lipoprotein (a) [Lp(a)] ≥ 50 mg/dL, with their LDL cholesterol concentrations (mmol/L) and Lp(a) concentrations (mg/dl). In families (A,C), two new cases of FH and elevated Lp(a) were identified among five relatives tested. In family (B), all six relatives tested were found to have elevated Lp(a) (i.e., one new case of elevated Lp(a) for every relative tested) and three identified with FH. However, there were families where fewer, or no relatives were detected than those shown in these three families. Asterisk * indicates coronary artery disease. From Chakraborty A et al. Cascade testing for elevated lipoprotein (a) in relatives of probands with familial hypercholesterolemia and elevated lipoprotein(a). Atherosclerosis 2021, with permission.

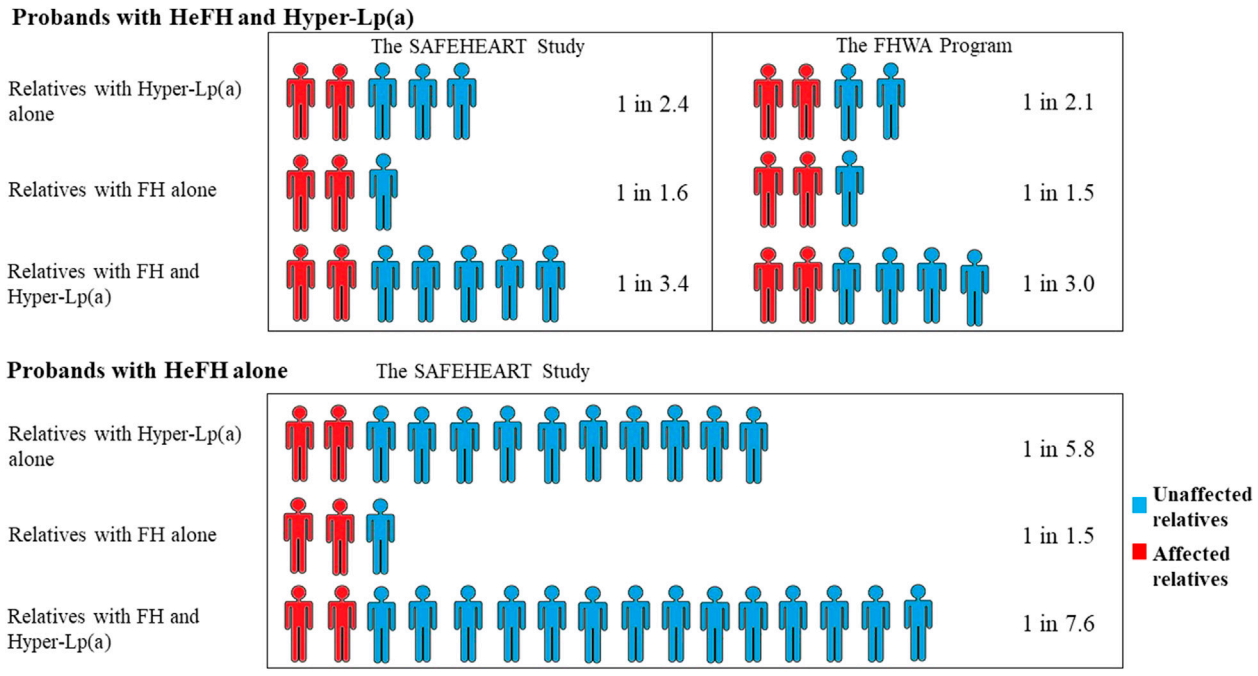

FIGURE 5 | Yield of detection of diagnoses of elevated lipoprotein (a) [Lp(a)] and familial hypercholesterolemia (FH) during cascade screening of relatives from HeFH probands with or without hyper-Lp(a). One individual of hyper-Lp(a) without FH was detected for every 2.1–2.4 relatives screened if the proband had HeFH and hyper-Lp(a), whereas the yield of detection was one individual for every 5.8 relatives screened if the proband had HeFH but not hyper-Lp(a). HyperLp(a) refers to Lp(a)≥ 50 mg/dL. The SAFEHEART Study by Ellis et al., (2019); The FHWA Program by Chakraborty et al., (2021).

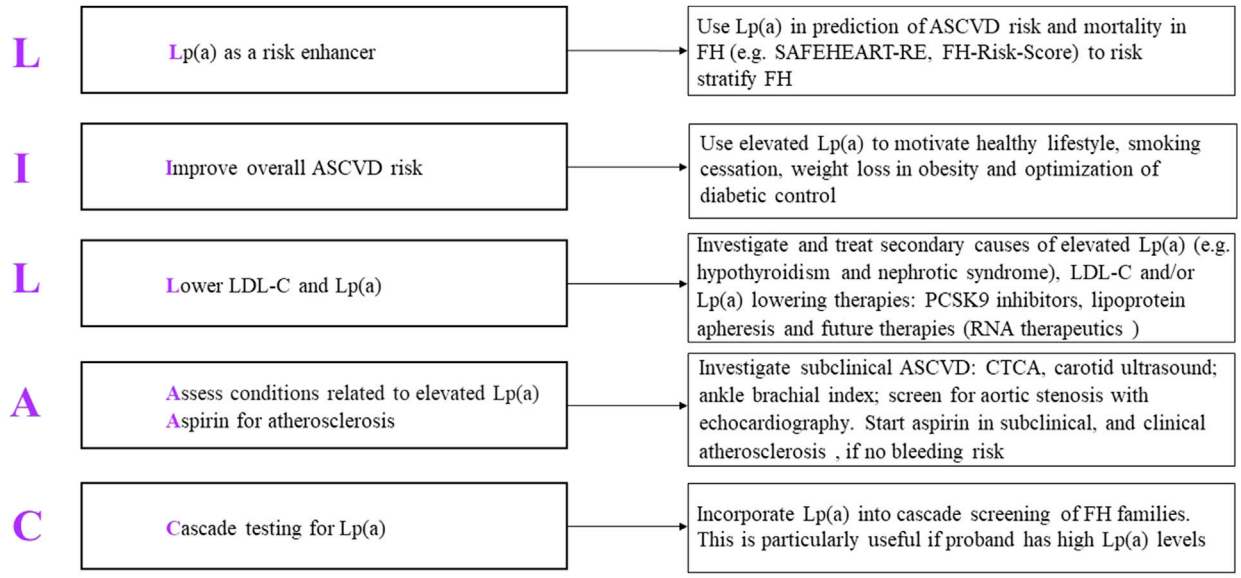

FIGURE 6 | Proposed mnemonic (LILAC) for the management of elevated lipoprotein (a) in familial hypercholesterolemia.

screening was 1 individual for every 1.5–1.6 relatives screened. The yield of detection of hyper-Lp(a) in FH cascade screening was also high: 1 individual of hyper-Lp(a) was detected for every 2.1–2.4 relatives screened, whereas the yield of detection of both hyper-Lp(a) and FH was 1 individual for every 3–3.4 relatives screened (**Figures 5**). Unsurprisingly, screening from probands with FH and hyper-Lp(a) levels compared with screening from probands without hyper-Lp(a) had a higher yield of detection of relatives with hyper-Lp(a) and FH (1 in 3.4 vs. 1 in 7.6) (Ellis et al., 2019). With FH probands in absence of hyper-Lp(a), the yield of detection of hyper-Lp(a) among relatives (1 individual in 5.8 relatives) was similar to the general population (1 in 5 people) (Nordestgaard et al., 2010). Ellis et al. (2019) also showed that the yield of detection of hyper-Lp(a) decreases with increasing generational separation from the proband with hyper-Lp(a): 1 individual in 2 of 1st degree relatives screened have hyper-Lp(a), 1 individual in 2.9 of 2nd degree relatives, and 1 in 3.3 of 3rd degree relatives. Concordance of detection of FH and Lp(a) was low and non-significant, implying that the conditions are genetically orthogonal.

Cascade Testing for Hyper-Lp(a) Alone

The autosomal co-dominant heritability of Lp(a) is confirmed by pedigree studies (Utermann et al., 1987; Genest et al., 1991). Genest et al. (1991) showed that Lp(a) levels were significantly correlated between parents-and-offsprings, whereas Lp(a) levels of proband-and-spouse were not. Studies correlating Lp(a) levels of the younger generation and family history showed that the serum Lp(a) levels of the offspring were significantly and directly associated with a positive CAD history of parents (Kostner et al., 1991; Srinivasan et al., 1991; Routi et al., 1996), but less so with CAD history in second- and third-degree relatives; higher Lp(a) levels in children were associated with a positive CAD history in their grandparents in one study (Wilcken et al., 1993) but not another. (Routi et al., 1996).

Small apo(a) isoforms are associated with higher Lp(a) levels (Kronenberg, 2016). The alleles of genes determining the apo(a) isoform size are randomly inherited from each parent and the expression of the inherited alleles is dominated by the alleles controlling small apo(a) isoform size (high Lp(a) levels) (Kronenberg, 2016). This explains the observation that an offspring with extremely high Lp(a) level may have both or one parent with elevated Lp(a). Children from parents with low Lp(a) levels have low Lp(a) levels, unless there is a secondary cause for elevated Lp(a). When only 1 out of 2 parents has elevated Lp(a), the Lp(a) level of the offspring will on average range from normal to moderately elevated in most instance (Genest et al., 1991).

Non-genetic factors should be considered if the Lp(a) levels of the offspring differ greatly from parents. This also applies to testing adult relatives of the proband with high Lp(a). Acquired causes that could markedly elevate Lp(a) level (up to 3-fold) include nephrotic syndrome, severe renal impairment, use of growth hormones, and pregnancy, while Lp(a) levels may be moderately elevated by 20%–50% from hypothyroidism, menopause, inflammatory conditions, and testosterone deficiency (Kostner et al., 2013). Other causes that markedly lowered Lp(a) include cholestatic liver disease (up to 90% reduction) (Chennamsetty et al., 2011), whereas Lp(a) levels are moderately lowered by PCSK9 inhibition, niacin, post-menopausal hormone replacement therapy (25% reduction)

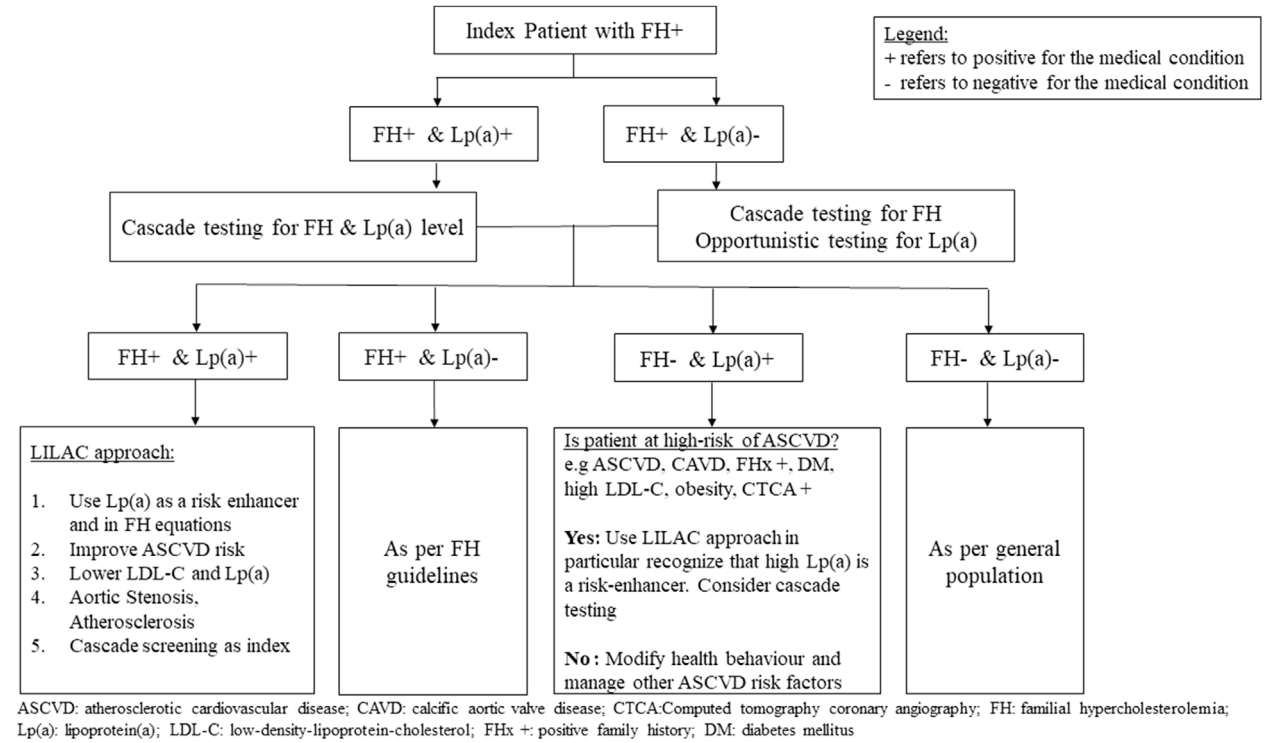

FIGURE 7 | Proposed algorithm for the management of relatives after cascade testing for hyper-lipoprotein (a) from probands with familial hypercholesterolemia.

(O'Donoghue et al., 2019), as well as mildly lowered by other medications e.g. fibrates, aspirin, angiotensin-converting enzyme inhibitor, calcium antagonist (<10% reduction) (Nordestgaard et al., 2010). Owing to a recent observation that Lp(a) may rise with age, especially in children with suspected FH, Lp(a) level should be rechecked in adulthood (de Boer et al., 2022). A probable increase in Lp(a) may relate to the effects of age, endocrine changes, use of statin, and other secondary factors (de Boer et al., 2022).

PROPOSED MANAGEMENT OF ELEVATED LP(A): LESSONS FROM MODELS OF CARE FOR FH

Assessment of Lp(a) is valuable in stratifying the risk of ASCVD in FH (Thanassoulis, 2019; Tsimikas and Stroes, 2020; Watts et al., 2020). We propose the principles of management of patients with elevated Lp(a) according to five salient points, guided by the mnemonic LILAC (**Figure 6**). Starting with detection, Lp(a) should be tested in all patients with FH as this is a necessary step in the risk stratification of ASCVD in FH. Hyper-Lp(a) is defined as ≥50 mg/dL (≈100–125 nmol/L) (Grundy et al., 20182019; Wilson et al., 2019; Pearson et al., 2021), noting that patients with extremely high Lp(a) levels (>150 mg/dL or 300 nmol/L) have the highest risk. Management of hyper-Lp(a) includes a thorough history taking of family and personal history of premature ASCVD, as well as identifying early signs of ASCVD. Knowledge of Lp(a) can be utilized in these specific risk equations for ASCVD (Pérez de Isla et al., 2017; Paquette et al., 2021). Blood testing should exclude secondary causes of elevated Lp(a), such as hypothyroidism, kidney impairment, proteinuria, and cholestatic liver disease. Investigation for subclinical atherosclerosis e.g., CT coronary calcium score may be considered because this can enable decisions to intensity treatment plans (Santos et al., 2016; Gallo et al., 2021). In FH with high Lp(a) levels, an echocardiogram to confirm calcific aortic valve stenosis at an appropriate age is essential. Treatment for modifiable risk factors such diabetes mellitus, reducing obesity, alcohol, and smoking cessation, should be optimized. Healthy lifestyle, heart-healthy diet, and improving modifiable cardiovascular risk factors should be emphasized in patients with FH, as well as in patients with hyper-Lp(a) as this has significant cardiovascular benefit even in the absence of effective Lp(a) lowering treatment (**Figure 7**) (Perrot et al., 2017).

There is currently a lack of effective Lp(a) lowering therapies, and thus a lack of concrete evidence yet showing that specific Lp(a)-lowering can reduce ASCVD risk and mortality. This evidently applies to FH. The HORIZON study is a cardiovascular outcome trial of the antisense oligonucleotide APO(a)-L_{RX} (TQJ230) that targets the mRNA transcript of the *LPA* gene and can lower Lp(a) by 80% (NCT04023552) (Tsimikas et al., 2020b). A phase 2 study of GalNac3-conjugated-siRNA (Olpasiran) is also undergoing (NCT04270760) will proceed to the clinical outcome trial. While we await these studies, the effect of Lp(a) lowering onto ASCVD is estimated to be significant. Mendelian randomization analyses

suggest that Lp(a) lowering of approximately a 65 mg/dL (Lamina et al., 2019) or 100 mg/dL (Emerging Risk Factors et al., 2009) (i.e., >150–200 nmol/dL) is required to achieve to CAD risk reduction equivalent of 1 mmol/L LDL-C lowering. From Danish population-based studies, the authors of another study estimated that an Lp(a) lowering of 50 mg/dL (105 nmol/L) may be needed to reduce CVD risk by 20% in a secondary prevention setting (Madsen et al., 2020).

However, it is likely that even smaller sustained reductions of Lp(a) level would confer significant clinical benefit in ASCVD risk reductions in patients with established ASCVD, as suggested by post-hoc analyses of PCSK9 inhibitor clinical outcome trials (O'Donoghue et al., 2019; Bittner et al., 2020) and lipid apheresis studies (Jaeger et al., 2009; Leebmann et al., 2013; Waldmann and Parhofer, 2016). PCSK9 monoclonal inhibitors have Lp(a) lowering effect of ≈25%. (O'Donoghue et al., 2019; Bittner et al., 2020). In the FOURIER study, investigating evolocumab versus placebo in patients with ASCVD, it was estimated that reduction of Lp(a) level of 34 nmol/L (95% CI 18.5–97 nmol/L) is associated with a relative risk reduction of 20% of cardiovascular events (O'Donoghue et al., 2019). Although lipid apheresis reduces Lp(a) immediately by 60–75%, this effect is not sustained, with the mean inter-apheresis reduction of Lp(a) being approximately 35% for weekly apheresis (Waldmann and Parhofer, 2016). Studies show that patients with elevated Lp(a) who underwent weekly lipid apheresis have a reduced risk of major coronary events through its Lp(a) lowering effect, even in patients who achieve target LDL-C levels (Jaeger et al., 2009; Leebmann et al., 2013).

IMPLICATIONS OF RECENT STUDIES ON LIPOPROTEIN (A): FAMILIAL HYPERCHOLESTEROLEMIA AND BEYOND

In patients with very high Lp(a) levels, Lp(a) cholesterol may contribute to 30%–45% of the measured LDL-C (Yeang et al., 2015). Whilst correction of LDL-C concentrations for the cholesterol content of Lp(a) remains contentious, it is clear that in patients with very high Lp(a) concentrations, the cholesterol content of this lipoprotein particle may confound estimation of LDL-C and hence lead to a false positive clinical diagnosis of FH (Chan et al., 2019). This could result in unnecessary DNA testing in individuals with DLCN scores >5. Clinical and population studies have shown that up to 25% of patients with probable/definite FH can be reclassified by adjusting LDL-C for the cholesterol content of Lp(a) (Langsted et al., 2016; Chan et al., 2019). We have previously demonstrated that the diagnostic accuracy of DLCN and SB criteria in predicting the detection of a pathogenic mutation of FH is reduced when Lp(a) level is very high, and that adjusting LDL-C for Lp(a) cholesterol using a correction factor of 30% greatly improved the specificity of DLCN and SB criteria when Lp(a) > 100 mg/dL except when LDL-C was as high as 6.5 mmol/L or more (Chan et al., 2019). It is clinically relevant to clarify the diagnosis of clinical HeFH because patients with dual diagnosis of clinical HeFH and hyper-Lp(a) have the highest risk of myocardial infarction compared with patients with either diagnosis alone (Langsted et al., 2016) (**Table 1**). The effect of Lp(a) cholesterol on the measured LDL-C also has important clinical implications in achieving the target "LDL-C" in patients irrespective of FH status. However, correction factors use assumptions that a certain percentage of cholesterol makes up Lp(a) mass which can range from 30% – 45%; there are so far no consensus on the usage of correction factor(s) to adjust LDL-C for Lp(a) cholesterol content in clinical practice. Quantification of Lp(a) cholesterol with a recently described assay, which allows for more accurate estimates of true LDL-C, may be recommended over the use of correction factors (Yeang et al., 2021; Yeang et al., 2022).

Studies of cascade testing for high Lp(a) within the context of genetic cascade testing for FH provide proof of principle for the effectiveness of this method for screening for elevated Lp(a) (Ellis et al., 2019; Chakraborty et al., 2021). Cascade testing can identify affected family members with varying risks of ASCVD. Cascade testing for Lp(a) does not require genetic testing for Lp(a) variants or use of a polygenic score (Ellis et al., 2019; Chakraborty et al., 2021). The data also suggest that the yield of identifying relatives with high Lp(a) is directly proportional to the level of Lp(a) in the proband (Ellis et al., 2019; Chakraborty et al., 2021), providing a guide to the design of effective implementation strategies. However, formal health economic evaluations that focus not only on cost-effectiveness, but also on cost-utility analyses are recommended. Studies of patient-reported outcomes and experience measures are also needed. The estimation of ASCVD risk in both probands and affected relatives employing the outcome of measuring Lp(a) can be carried out using a recently validated risk equation (Gallo et al., 2020). The importance of measuring Lp(a) in all patients with FH also relates to predicting increased risk of calcific aortic valve stenosis, which is also related to LDL-C burden and history of hypertension (Pérez de Isla et al., 2021).

While cascade testing for elevated Lp(a), particularly in the context of FH, is likely to be cost-effective, identification of affected probands without FH is essential to trigger the cascade testing of relatives. Universal testing of the population for markedly elevated Lp(a) concentrations above 180 mg/dL (>430 nmol/L) has been promulgated by certain guidelines (Mach et al., 2019; Pearson et al., 2021). An alternative, and not mutually exclusive approach, is to consider the screening of adults with established ASCVD, particularly when testing may lead to the initiation or a change in therapy (e.g., statin, aspirin, antihypertensive treatment), as well as when Lp(a) is being used to restratify ASCVD in people with the following conditions: family history of premature ASCVD (Grundy et al., 20182019; Cegla et al., 2019; Mach et al., 2019; Wilson et al., 2019; Handelsman et al., 2020; Pearson et al., 2021), family history of high Lp(a) (Cegla et al., 2019; Wilson et al., 2019; Handelsman et al., 2020), FH (Grundy et al., 20182019; Cegla et al., 2019; Mach et al., 2019; Wilson et al., 2019; Pearson et al., 2021; Watts et al., 2021), diabetes mellitus, renal impairment or family history of thrombophilia (Tsimikas et al., 2018). Measurement of Lp(a) should also be considered in patients at intermediate or borderline risk of ASCVD, particularly when the decision to

initiate cholesterol-lowering therapy remains undecided (Cegla et al., 2019; Mach et al., 2019; Wilson et al., 2019; Pearson et al., 2021). Testing for Lp(a) should also be considered in patients with suboptimal reduction in LDL-C despite good adherence to statin therapy, as well as in those with a history of progressive CAVD (Cegla et al., 2019; Wilson et al., 2019; Handelsman et al., 2020). This recommendation applies mainly to adults, although consideration may also be given to testing children or adolescents with FH with premature ischemic stroke or within the context of cascade testing for high Lp(a) and FH (Wilson et al., 2021).

Clinical use of Lp(a) has to be seen in the context of other cardiovascular risk factors and, hence, the estimation of an individual's absolute cardiovascular risk in individuals. This implies that health economic evaluations are most likely to be favorable when background risk is increased, as in the presence of established or recurrent ASCVD, FH, diabetes, or multiple cardiovascular risk factors. This notion requires verification.

The detection of Lp(a) seems to be to meet most, but not all, classical criteria for screening for a condition (Wilson et al., 1968). The important ones that are not met are that, with the exception of lipoprotein apheresis, there is no accepted or specific treatment for elevated Lp(a) and hence no agreed policy on who should be treated, noting that policy also depends on a favorable health economic evaluation. Amongst FH patients, the case for offering specific treatment with lipoprotein apheresis relates to is those with recurrent ASCVD and evidence of progressive CAVD. Lowering of Lp(a) with PCSK9 inhibitors has been shown to contribute to a reduction in ASCVD events in post-ACS and high-risk patients (Bittner et al., 2020; O'Donoghue et al., 2019), but trials have not been undertaken specifically in patients with FH. Future studies should address the potential impact of additional therapies that can lower Lp(a), including obicetrapib (a CETP inhibitor) (Hovingh et al., 2015) and resmetirom (a selective thyroid hormone receptor-β agonist) (Harrison et al., 2019). Ongoing studies with RNA based therapies will establish whether specific lowering of Lp(a) of the order of 80% (Tsimikas et al., 2020b), or a mean reduction of 100 mg/dL (Burgess et al., 2018), confers improvement in cardiovascular outcomes in high risk individuals, but none of these trials are specifically being carried out in patients with FH.

CONCLUSION

Towards Implementation of Improved Care for Hyper-Lp(a)

There is clear evidence that elevated Lp(a) is a causal risk factor for ASCVD, CAVD, and cardiovascular and all-cause mortality in both genders and multiple ethnic groups. This risk appears to be particularly markedly increased in patients with FH, in whom the case for cascade testing for elevated Lp(a) extends to the benefit of relatives affected with FH and not affected with FH.

The phenotypic diagnosis of FH may be confounded by the cholesterol content of Lp(a), particularly when this is extremely high and this should be accounted (Langsted et al., 2016; Chan et al., 2019); adjusting for the cholesterol content of Lp(a) is also important when phenotypically cascade testing relatives based on age, gender, and LDL-C levels. Lp(a) is a key component of risk stratification equations in FH (Pérez de Isla et al., 2017; Paquette et al., 2021). A positive association has been reported between coronary artery calcium score and Lp(a) in population studies among people who have not yet developed a coronary event (Gallo et al., 2021). The value of measuring Lp(a) in predicting the risk of aortic stenosis in FH is critically important, particularly so in patients with homozygous FH (Ten Kate et al., 2015; Vongpromek et al., 2015; Pérez de Isla et al., 2021). At present, reduction of Lp(a) in people with FH relies on the use of PCSK9 inhibitors (Bittner et al., 2020; O'Donoghue et al., 2019), noting that niacin is not recommended in European countries. Lipoprotein apheresis is another modality but is expensive, labor-intensive and not widely available. (Waldmann and Parhofer, 2016). Apheresis is reimbursable, however, in certain countries for those with progressive coronary disease and elevated Lp(a) concentration (Leebmann et al., 2013). Antisense oligonucleotide and small interfering RNA approaches are currently being trialed and could in the future be the treatment of choice for patients with elevated Lp(a) and established ASCVD or FH.

While the results of large clinical outcome trials are awaited to fully establish the value of screening for high Lp(a), the use of universal, opportunistic, and selective screening strategies can be enabled by the use of implementation strategies. These are relevant to patients and families with or without FH. Using an isoform independent immunoassay is essential for accurate measurement of Lp(a) (Tsimikas et al., 2018) and this should be a reimbursable test (Pearson et al., 2021). Alerts and interpretive comments on laboratory reports are used to acknowledge the value of high Lp(a) in cardiovascular risk assessment and consideration of referral to or discussion with a specialist. Electronic health records should employ a specific ICD code for elevated Lp(a) which may be utilized to trigger the testing of Lp(a) in clinical practice, as well as linkage studies and economic valuations. The management of Lp(a) at present requires wider promotion and acceptance by practitioners who should develop skills in the clinical use of the test and in shared decision-making concerning risk assessment, cascade testing of first-degree relatives, and modification of behavioral and standard cardiovascular risk factors.

In conclusion, evolving models of care for FH have important lessons that can inform models of care for patients with elevated Lp(a) and other risk factors other than FH, including familial combined hyperlipidemia, diabetes mellitus, and hypertension (Watts et al., 2020). Finally, it is worth emphasizing that most studies to date have been carried out in white, Caucasian populations and further investigation of the significance and management of Lp(a) are needed in other ethnic groups, including black Africans and Southern and Eastern Asians (Tsimikas et al., 2018; Paré et al., 2019).

AUTHOR CONTRIBUTIONS

WL and GW contributed to the conceptualization. WL, DC, and GW drafted the manuscript, and WL, DC, PM, and GW contributed to the revision. All authors contributed to the article and approved the submitted version.

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Long-Term Cardiovascular and Cerebrovascular Challenges Posed by COVID-19 in Patients With Familial Hypercholesterolemia

Alpo Vuorio[1,2]*, *Frederick Raal*[3], *Petra Ijäs*[4], *Markku Kaste*[4] and *Petri T. Kovanen*[5]

[1]Mehiläinen, Airport Health Center, Vantaa, Finland, [2]Department of Forensic Medicine, University of Helsinki, Helsinki, Finland, [3]Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa, [4]University of Helsinki and Helsinki University Hospital, Helsinki, Finland, [5]Wihuri Research Institute, Helsinki, Finland

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***Correspondence:**
Alpo Vuorio
alpo.vuorio@gmail.com

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INTRODUCTION

Follow-up studies have revealed that during the severe acute respiratory syndrome (SARS) caused by coronavirus SARS-CoV epidemic between 2002 and 2003, a significant proportion of those infected had a deterioration in the general health and exercise capacity, especially during the 24 months post-infection (Ngai et al., 2010; Zhang et al., 2020a). Although the emphasis of the follow-up by Ngai and coworkers (2010) was on monitoring alterations in the lung function, a significant impairment in quality of life, as assessed by the SF-36 quality-of-life measures, was reported (Ngai et al., 2010). The long-term effects of SARS-CoV infection have been found to also affect lipid and glucose metabolism in recovered SARS patients (Wu et al., 2017). Thus, the impaired metabolic pathologies were observed 12 years after the infection, and they were considered to be long-term effects of the initial lung damages, and to be potentially also related to the one-month-long high-dose prednisolone treatment given during the acute phase of the disease. Long-term symptoms, usually lasting for about 24 months, have also been reported in survivors of the epidemic influenza A (H7N9) (Chen et al., 2017) and Dengue (Garcia et al., 2011). As could have been envisaged, numerous COVID-19 patients also suffer from long-term impairment of health after an acute SARS-CoV-2 infection, particularly if the symptoms of the disease have been severe and the patients had to be hospitalized (Nalbandian et al., 2021).

In an earlier commentary, we have discussed the acute phase of COVID-19 in patients with familial hypercholesterolemia (FH) (Vuorio et al., 2021a). We now wish to highlight concerns related to the long-term effects of SARS-CoV-2 infections and the increases in the risk for complications and potentially a poor outcome in this group of patients.

FAMILIAL HYPERCHOLESTEROLEMIA AND COVID-19

FH is the most common monogenic inherited metabolic disease worldwide, and it affects an estimated one in every 330 individuals (Beheshti et al., 2020). Accordingly, we can estimate that among the currently reported over 500 million total cases of COVID-19 worldwide, more than one million are likely FH patients. FH patients have a markedly elevated serum low-density lipoprotein cholesterol (LDL-C) already *in utero* which is often accompanied by an elevated level of serum lipoprotein(a) [Lp(a)], and if left untreated, a lifelong dysfunction of the arterial endothelium ensues in these patients (Sorensen et al., 1994; Vuorio et al., 2020; Nurmohamed et al., 2022). In FH patients with COVID-19, the pre-existing endothelial dysfunction is likely to increase the risk of

macrovascular and microvascular thrombosis caused by a direct viral attack of the endothelial cells (endothelitis) and by the cytokine storm typically seen during severe COVID-19 illness (Vuorio et al., 2021b). In FH patients, such compounded endothelial injuries with ensuing thrombosis/thromboembolism pose a danger to cardiovascular health, particularly when they affect the coronary vessels supplying the heart and/or the extracranial and intracranial vessels supplying the brain (Quick et al., 2021). Thus, severe COVID-19 illness has been found to increase the risk of myocardial infarction in FH patients whether they have or have not been diagnosed with atherosclerotic cardiovascular disease (ASCVD) (Myers et al., 2021). Additionally, the elevated risk of ischemic stroke associated with COVID-19 potentially also applies to FH patients with COVID-19 (Vuorio et al., 2021c).

LONG COVID OR POST-COVID SYNDROME

Long COVID or the post-COVID-19 syndrome has been defined as a condition characterized by persistent symptoms lasting more than 2 months after the onset of SARS-CoV-2 infection (Soriano et al., 2021). The severity of the post-COVID syndrome is highly variable, and research suggests that even persons with mild COVID-19, i.e., nearly asymptomatic SARS-CoV-2 infection, may experience long-term symptoms ranging from neuropsychiatric symptoms, such as chronic fatigue, to arterial, venous, and microvascular thrombotic complications in various organs (Di Toro et al., 2021; Nalbandian et al., 2021). In fact, because the long COVID symptoms are frequent among COVID-19 patients (about 30% or more) who have been hospitalized, the American Heart Foundation has initiated an extensive program in order to determine if those individuals who have ASCVD or have survived stroke are more prone to develop long COVID (https://newsroom.heart.org/news/10-million-invested-to-study-long-term-impact-of-covid-19-on-heart-and-brain-health).

POTENTIAL THREATS TO CARDIAC FUNCTION AFTER SARS-COV-2 INFECTION

Although research into the etiological factors of long COVID needs further study, some causal mechanisms potentially relevant also for FH patients with long COVID have already been identified, as discussed below. However, it is important to remember that the data refer to a very wide variety of patients with SARS-CoV-2 infection, and, accordingly, the clinicians need to be careful when trying to generalize the results so far available. Moreover, no long-term cardiovascular or cerebrovascular clinical data on FH patients after COVID-19 have yet been published; so, we have to utilize indirect measures when assessing the significance of the results already obtained in non-FH patients.

One potentially interesting cause of dyspnea due to COVID-19 was found in patients without a known pre-existing cardiopulmonary disease (mean age 51 ± 11 years), and who underwent a 1-year intensive clinical follow-up (Luchian et al., 2022). All of the patients had persistent dyspnea, and in more than a third, the echocardiographic evaluation revealed significant changes in cardiac global constructive work and global work index, suggesting that these patients had decreased myocardial performance and a subclinical cardiac dysfunction. Earlier studies have suggested that dyspnea in the recovered patients who suffered SARS-CoV-2 infection could be related to myocarditis or ischemic injury of the heart (Kotecha et al., 2021; Özer et al., 2021). Regarding FH patients, COVID-19-associated impairment in myocardial performance is highly relevant, particularly among those patients with pre-existing ASCVD, a condition which is often present in untreated FH patients already in early adulthood, even if left undiagnosed (Representatives of the Global Familial Hypercholesterolemia Community, 2020). It is possible that decreased myocardial performance continuing after COVID-19 potentially affects a much wider range of FH patients, including those without clinically evident ASCVD. A very recent study among 46 male FH patients without known ASCVD (and without COVID-19) demonstrated a mildly reduced global longitudinal left ventricular strain when compared with controls (Vartela et al., 2021). This finding suggests that, particularly in FH males, the presence of subclinical vasculopathy adversely affecting the cardiac function might predispose the patients to even greater deleterious post-COVID-19 sequelae.

CONVALESCENT PERIOD OF COVID-19 AND THE POTENTIAL RISK OF ISCHEMIC STROKE AND INTRACRANIAL HEMORRHAGE

In an early study on neurological and psychiatric sequelae of COVID-19, the risk of cerebrovascular events, notably acute ischemic stroke and intracranial hemorrhage, was found to be significantly increased within the first 6 months after COVID-19, particularly in patients who had suffered from severe acute COVID-19 and associated encephalopathy (Taquet et al., 2021). In a study of South Asian males aged 50 years or younger, the estimated annual incidence rate of acute ischemic stroke was significantly higher (82.6 cases per 100,000 persons) in those with COVID-19 infection when compared with historical data (38.2 cases per 100,000 persons) (Tu et al., 2021). Importantly, in this study, acute ischemic stroke was reported to occur during the convalescent phase after an asymptomatic COVID-19 infection, the median time from a positive serological test result to stroke being 55 days (range 0–130 days).

Bikdeli and coworkers (2020) have reported acute ischemic stroke as a secondary wave of complications of COVID-19 and postulated that the prothrombotic state associated with acute COVID-19 may persist long-term (Bikdeli et al., 2020). A recent study utilizing healthcare databases from the US Department of Veterans Affairs indicated that the period of increased risk and burden for cerebrovascular disorders (stroke and transient

ischemic attack) may persist at least for 1 year after the infection (Xie et al., 2022). Thus, the authors reported that persons who survived the first 30 days of COVID-19 exhibited an increased risk of stroke and transient ischemic attack for 12 months after the acute infection. In this register study, the exact mechanisms underlying cerebrovascular events remained undetermined, but the high incidence of high-risk cardioembolic conditions (atrial fibrillation, heart failure, acute coronary syndrome, myocarditis) suggests that strokes may be secondary to cardiac disease, while other COVID-19-related mechanisms (hypercoagulopathy, endotheliitis) likely contribute to the final events.

Regarding hypercoagulability, elevated levels of plasma factor VII and plasminogen activator inhibitor-1 have been shown to persist after SARS-CoV-2 infection (von Meijenfeldt et al., 2021). There is also a concern about coagulopathy and the appearance of antiphospholipid antibodies, which can arise transiently in patients with various infections including COVID-19 (Zhang et al., 2020b). Such antibodies, particularly the anticardiolipin antibodies, may have an acute ischemic stroke risk-impacting effect jointly with other well-recognized risk factors for stroke, such as hypertension, hyperlipidemia, and obesity (Rothstein et al., 2020). What could such scenarios potentially mean in the context of FH? An early study found that untreated FH patients, i.e., those not receiving lipid-lowering therapy, have a markedly increased risk for acute ischemic stroke (Kaste and Koivisto, 1988) More recently, i.e., during the statin era, such increased risk appears to have largely disappeared, most likely reflecting an effective treatment of the hypercholesterolemia (Huxley et al., 2003; Soljanlahti et al., 2005; Hovland et al., 2018; Beheshti et al., 2018). Unfortunately, however, the great majority of FH patients have not been diagnosed, and, accordingly, they remain untreated or, even if correctly diagnosed, remain undertreated (Representatives of the Global Familial Hypercholesterolemia Community, 2020). Therefore, the concern regarding the risk of an acute ischemic stroke in most FH patients with COVID-19 continues.

A PERSISTENT HYPERCOAGULABLE STATE AFTER SARS-COV-2 INFECTION

A recently reported South African study was searching for a common explanator of the large variety of long COVID symptoms (Pretorius et al., 2021). The study included patients who had suffered from long COVID for at least 2 months, and as control groups healthy persons and patients with type 2 diabetes mellitus without known previous SARS-CoV-2 infection. Although the study was small in size, it revealed that the plasma derived from the long COVID patients contained large anomalous deposits of microclots. After trypsinization of the plasma, increased concentrations of several pro-inflammatory molecules such as alpha (2)-antiplasmin, various fibrinogen chains, and serum amyloid A (SAA) were detected in the samples derived from the long COVID patients, but not in those from the healthy or diabetic control subjects. The authors concluded that the clotting proteins were dysfunctional and that an imbalance between the supply and demand of lytic enzymes existed. Three mechanisms were postulated as an explanation of the clotting pathology in the long COVID-19 patients: first, the increased levels of pro-inflammatory molecules in the plasma caused a hypercoagulable state; second, platelets were hyperactivated and led to microclot formation in the circulation, and third, an aberrant fibrinolytic system prevailed. Based on their findings, the authors recommended considering prolonged anticoagulation for COVID-19 patients after discharge from the hospital. Regarding the FH patients, an elevated serum Lp(a) is also a matter of major concern because the unique apolipoprotein(a) component present in the Lp(a) particles prevents fibrinolysis and so tends to promote thrombus growth wherever the thrombus is forming in the vasculature (Vuorio et al., 2020).

CONCLUDING REMARKS

When compared with non-FH patients who have suffered an acute SARS-CoV-2 infection, in FH patients a hypercoagulable state may persist for even longer periods after the infection. This assumption is relevant because the endothelial cells have been exposed to a lifelong high LDL-C concentration, and often also to an elevated Lp(a) level, which jointly cause endothelial dysfunction even in childhood (Vuorio et al., 2021b). This can be particularly harmful in the FH patients whose LDL-C-lowering therapy is lacking or sub-optimal, and among those FH patients who also have a highly elevated serum Lp(a) level. Thrombus formation in an arterial, venous, or microvascular vascular segment, is likely to occur with greater frequency among FH patients not only because of a pre-existing endothelial dysfunction but also as a result of the acute direct viral endothelial damage and the hypercoagulability state during the post-COVID period.

The COVID-19 pandemic will increase health inequalities, and particularly in low-income countries, there is a need to increase vaccination coverage in the most vulnerable patient groups (Vuorio et al., 2022). Significant health inequalities between high-to-low-income countries are demonstrated by the fact that by April 2022, only about 15% of people in low-income countries had received at least one dose of a COVID-19 vaccine (https://www.ourworldindata.org/covid-vaccinations). The COVID-19 vaccination program is important not only to prevent acute SARS-CoV-2 infections but also to potentially decrease long-term COVID-19 complications.

Although reliable epidemiological data are not yet available, it is obvious that the entire FH population, i.e., about 30 million FH patients worldwide, are at an increased risk of serious vascular complications which can occur after COVID-19. Consequently, when a clinician encounters an FH patient with symptoms that match with those typical of the post-COVID syndrome, it is of importance that the clinician ascertains that lipid-lowering pharmacotherapy is being taken regularly, is adequate, and follows the current guidelines. In this context, it is relevant to remember that statins also act as mild antithrombotic

medications (Vuorio et al., 2021d). When appropriate, PCSK9 inhibition may be included in the lipid-lowering therapy even among younger FH patients with COVID-19 and thereafter, as, unlike statins, the PCSK9 inhibitors can also reduce the level of serum Lp(a) (Vuorio et al., 2020).

AUTHOR CONTRIBUTIONS

AV and PK: writing the first draft. AV, FR, PI, MK, and PK: editing to produce the final draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Progression of Treatment for Refractory Hypercholesterolemia: Focus on the Prospect of Gene Therapy

*Zhi-Fan Li and Na-Qiong Wu**

Cardiometabolic Center, National Center for Cardiovascular Diseases, Fuwai Hospital, Chinese Academy of Medical Science, Peking Union Medical College, Beijing, China

Refractory hypercholesterolemia (RH), including homozygous familial hypercholesterolemia (HoFH) and compound heterozygous familial hypercholesterolemia, is characterized by high levels of low-density lipoprotein cholesterol (LDL-C) despite existing cholesterol-lowering methods at maximal tolerable doses. Patients with RH have early onset and higher risk of atherosclerotic cardiovascular disease (ASCVD) under insufficient treatment. Therefore, it is urgent to seek new therapies to maintain the blood lipids in refractory hyperlipidemia at normal levels. Currently, new cholesterol-lowering strategies are on the market, not only at the protein level [i.e., bempedoic acid (inhibiting ATP-citrate lyase), alirocumab and evolocumab (monoclonal antibodies against PCSK9), evinacumab (monoclonal antibody against ANGPTL3)] but also at the transcript level [i.e., mipomersen (antisense oligonucleotide inhibiting ApoB), inclisiran (siRNA targeting PCSK9)], providing more options for RH patients to achieve their lipid-lowering targets. More RNA-based therapies targeting RH-related genes have been designed for the treatment. However, for a proportion of patients, especially those with LDLR deficiency, the available treatments are still insufficient. More recently, emerging genome engineering based on CRISPR/Cas9 techniques, and advanced delivery technologies such as lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, lipid nanoparticles, and exosomes are being rapidly developed and implemented as novel therapies for RH. Gene therapy targeting RH-related genes has been successfully conducted in cells, mice, and non-human primates with high efficacy in lipid lowering and good tolerability. Especially the new generation of genome editing technique, base editing, performed *in vivo* with ideal lipid-lowering effect and limited occurrence of unwanted results. Excitingly, a

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Anna-Kaisa Ruotsalainen, University of Eastern Finland, Finland

***Correspondence:**
Na-Qiong Wu
fuwainaqiongwu@163.com

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Abbreviations: RH, refractory hypercholesterolemia; HoFH, homozygous familial hypercholesterolemia; HeFH, heterozygous familial hypercholesterolemia; LDL-C, low-density lipoprotein cholesterol; ASCVD, atherosclerotic cardiovascular disease; PCSK9, proprotein convertase subtilisin-kexin type 9; ANGPTL3, angiopoietin-like protein 3; mAbs, monoclonal antibodies; Apo, apolipoprotein; siRNA, small interfering RNA; ASO, antisense oligonucleotide; VLDL, very-low-density lipoprotein; Lp(a), lipoprotein (a); Ad, adenovirus; AAV, adeno-associated virus; IDOL, inducible degrader of LDLR; hiPSC, human induced pluripotent stem cell; HMG-COA, 3-hydroxy3-methylglutarate reductase; LNP, Lipid nanoparticle; sgRNA, single guide RNA; CRISPR, Clustering regularly spaced short palindromic repeats; Cas, CRISPR-associated protein; SaCas9, *staphylococcus aureus* Cas9; NmeCas9, neisseria meningitidis Cas9; HLC, hepatocellular like cell; indel, insertion and deletion; CBE, cytosine base-editor; ABE, adenine base-editor; BE3, the third-generation base editor; GPCR, G protein-coupled receptor; USP20, ubiquitin specific peptidase 20.

phase I/II clinical study of LDLR gene replacement has been recently completed in RH patients, likely to be employed in clinical practice in the future. Furthermore, new targets for cholesterol reduction such as REV-ERB, G protein-coupled receptor, Ubiquitin specific peptidase 20 are continually being developed. This narrative review updates recent advances in treatment for RH, summarizes related clinical trials and preclinical studies, especially on the prospect of gene therapy.

Keywords: refractory hypercholesterolemia, lipid lowering strategies, gene therapy, familial hypercholesterolemia, LDL- cholesterol, delivery system, gene editing

INTRODUCTION

Patients with refractory hypercholesterolemia (RH) have significantly elevated low-density lipoprotein (LDL) cholesterol levels despite treatment with lipid-lowering therapies at maximum tolerated doses, associated with a very high risk for atherosclerotic cardiovascular disease (ASCVD) (Mach et al., 2020; Rosenson et al., 2020). From an etiological perspective, this review defines RH as containing homozygous familial hypercholesterolemia (HoFH) and compound heterozygous familial hypercholesterolemia (HeFH), belonging to the category of hereditary metabolic disorders. HoFH is manifested by identical mutations in two alleles, and compound HeFH is manifested by double allele mutations (heterozygous genotypes with two mutant alleles at the same locus of two homologous chromosomes), which are associated with different mean LDL-C levels depending on the types of mutations (Hartgers et al., 2018). Historically, the prevalence of HoFH in the general population was believed to be 1/1,000,000, but recent studies in larger population have found this may be an underestimate (Austin et al., 2004; Cuchel et al., 2014; Beheshti et al., 2020). Using molecular genetic testing, the prevalence of HoFH or compound HeFH was estimated to be 1/300,000 in the Netherlands, at least three times higher than previously assumed (Sjouke et al., 2015; Bell and Watts, 2016). The FH prevalence among population with severe hypercholesterolemia incidence of complex heterozygosis is 23-fold higher than general population (Beheshti et al., 2020), and the clinical phenotype is similar to HoFH. Our team have investigated prevalence of clinical FH according to Dutch Lipid Clinic Network criteria in different Chinese patients, and found the prevalence of definite and probable FH was 3.5% in 8,050 patients with coronary artery disease (CAD) (5.8% in premature CAD) (Li et al., 2017), 3.9% in 1,843 patients with myocardial infarction (MI) (7.1% in premature MI) (Li et al., 2016), 6.5% in 1,093 patients 35 years of age with a first MI (Li et al., 2018), and 0.47% in 13,002 patients with first-onset acute MI (Shi et al., 2020).

Existing cholesterol-lowering methods include statins and ezetimibe, which have poor efficacy in patients with refractory hypercholesterolemia either single-agent or in combination (Hartgers et al., 2015). Proprotein convertase subtilisin-kexin type 9 (PCSK9) and angiopoietin-like protein 3 (ANGPTL3) have emerged as key regulators of LDL-C levels, in which loss-of-function mutation are associated with lower blood lipid levels and lower odds for ASCVD (Brandts and Ray, 2021). In recent years, scientists have developed new cholesterol-lowering drugs targeting different targets at different stages of cholesterol synthesis, transport and metabolism, such as microsomal transfer protein inhibitors (MTP inhibitors) (D'Erasmo et al., 2017), liver selective thyroid hormone mimics (Sjouke et al., 2014), ATP citrate lyase inhibitors (Brandts and Ray, 2020), monoclonal antibodies (mAbs) targeting PCSK9/ANGPTL3, oligonucleotides that inhibit apolipoprotein (Apo) B/ANGPTL3, and small interfering RNA (siRNA) targeting PCSK9/ANGPTL3 (Akoumianakis et al., 2021; Rached and Santos, 2021). Recently marketed new drug PCSK9 mAbs (Alirocumab and Evolocumab) has been gradually promoted and applied in extremely high-risk patients because of its significant reduction ability of LDL-C and ASCVD event risk (Sabatine, 2019). New lipid-lowering drugs including Bempedoic acid (inhibiting ATP-citrate lyase), Inclisiran (siRNA targeting PCSK9), Evinacumab (monoclonal antibody against ANGPTL3) have recently received U.S. Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) approval (Aguilar-Salinas et al., 2021).

However, in clinical practice, patients with genetic LDLR deficiency do not respond well to PCSK9 mAbs, and for some patients with refractory hypercholesterolemia, the reduction of LDL-C still cannot fully meet their requirements of lipid standards despite current methods. Early in 2013, our team reported an 8-year-old HoFH boy with an early onset acute MI, detected very high levels of PCSK9 protein and lipid profile, and used strengthened lipid-lowering measures at that time (rosuvastatin 20 mg and ezetimibe 10 mg daily) to treat. However, we didn't see significant improvement of his serum lipid profile (Wu et al., 2013). And in 2019, we reported a 33-year-old HoFH lady with variants in LDLR exon12: c.1724T.C (p.L575P) (NM_000527) had poor response to alirocumab (Wu et al., 2019). Besides, some drugs such as Mipomersen (antisense oligonucleotide inhibiting ApoB) (Raal et al., 2010) and Lomitapide (MTP inhibitors) (D'Erasmo et al., 2017), both of which can promote liver triglycerides accumulation and increase transaminase, causing nonalcoholic fatty liver disease, have limited clinical application due to side effects (Li et al., 2014; Blom et al., 2016). Therefore, there is a need for continuous research into new cholesterol-lowering targets and to develop new lipid-lowering drugs to meet the clinical needs of these patients.

RNA-based therapies are hot spots in drug development, enabling the desired outcomes such as knockdown of target genes and induced expression of selected proteins, and have been designed for the treatment of RH and other gene-specific diseases (Chi et al., 2017; Gupta et al., 2021). Gene therapy is a promising platform that not only offers an alternative to current lipid-lowering therapies, but may well be the ultimate cure for genetic diseases such as HoFH. However, how to apply this technology more widely depends on the guarantee of its safety and effectiveness (Cao et al., 2021).

Here, we describe recent progression on basic research and treatment of refractory hypercholesterolemia, focusing on advances in gene therapy.

NEW CHOLESTEROL-LOWERING STRATEGIES TARGETING TRANSCRIPTION

Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) can target RNA, induce protein knockdown or restoration by activating RNA degradation or modulate pre-mRNA splicing, thus slowing disease progression. There are 10 approved ASO therapies in Europe and the United States, and a broad pipeline in development (Kuijper et al., 2021).

Mipomersen is an antisense oligonucleotide that binds to ApoB mRNA and subsequently down-regulates ApoB expression and VLDL production via ribosomes. Given subcutaneously 200 mg once a week, mipomersen reduced LDL levels by 21% in HoFH patients and 28% in HeFH patients (Hartgers et al., 2015). Phase III RCT trial studies showed that the addition of mipomersen in the maximum tolerance standard lipid-lowering therapy for HoFH patients significantly reduced the levels of LDL, ApoB and lipoprotein (a) [Lp(a)], by 25%, 27% and 31%, respectively (Raal et al., 2010). The most common adverse events with mipomersen included transient injection-site reactions and influenza-like symptoms, as well as elevated ALT in most patients. In 2013, mipomersen was approved by FDA as adjunctive therapy for HoFH treatment. However, it failed to pass the re-examination of the European Committee for Medicinal Products for Human Use due to concerns about the long-term impacts and adverse effects on the liver. Furthermore, the higher incidence of adverse events and intensive monitoring requirement impacted life quality of patients, thus limiting its clinical use (Chambergo-Michilot et al., 2022).

Angiopoietin-like protein 3 (ANGPTL3) and ANGPTL4 are members of the angiopoietin-like family of secretory factors that target lipoprotein lipase (LPL) and regulate lipid metabolism, primarily expressed in the liver (Ruscica et al., 2020). Functional loss variation in its genes is associated with significantly low plasma LDL and triglyceride levels, and prevention of atherosclerotic cardiovascular disease (Musunuru et al., 2010). Preclinical studies showed the significant effects of ASO targeting hepatic ANGPTL-3 mRNA on lipid metabolism in various mouse models, resulting in significant reductions of blood triglycerides (35%–85%), LDL cholesterol (7%–64%), and triglycerides within LDL particles, very-low-density lipoprotein (VLDL), and intermediate-density lipoprotein (Graham et al., 2017). However, study in cynomolgus nonhuman primates illustrated that platelet decreases were a common side effect of ASOs and might translate to humans (Henry et al., 2017).

Recently, a randomized, double-blind, dose-escalation phase I clinical trial of IONIS ANGPTL3-LRx (a drug targeting hepatic Angptl3 mRNA) was completed in healthy volunteers with elevated TGs and patients with FH to evaluate its safety, tolerability, pharmacokinetics, and pharmacodynamics (NCT02709850). 44 participants were randomly assigned into experimental groups: receiving an antisense oligonucleotide targeting ANGPTL3 mRNA in a single dose (20, 40, or 80 mg) or multiple doses (10, 20, 40, or 60 mg once weekly for 6 weeks), or corresponding placebo-controlled groups (0.9% sterile saline). Significantly reductions in absolute levels of ANGPTL3 protein (46.6%–84.5%, $p < 0.01$ for all doses) and non-HDL cholesterol (10.0%–36.6%) showed in all multiple-dose groups, values increasing corresponding to dose-escalation. Triglycerides (63.1 ± 10.9, $p = 0.01$) and VLDL (60.0 ± 15.5, $p < 0.01$) reached maximum lowering in the dose of 20 mg, and LDL reduced greatest in 60 mg group (32.9 ± 10.4, $p < 0.01$). No serious side effects were observed (Graham et al., 2017). More recently, phase II clinical trial of IONIS ANGPTL3-LRx carried out in participants with HoFH and other types of dyslipidemia. However, the former was withdrawn due to lack of available patients meeting entry criteria (NCT03455777) (**Table 1**). Therefore, whether ASO targeting ANGPTL3 can be applied to RH requires more clinical evidence.

Moreover, recent studies provide new insights into the cholesterol-lowering effect of other regulators targeting LPL such as apolipoprotein (Apo) C-III and ANGPTL4 (Akoumianakis et al., 2021; Singh et al., 2021; Bellosta et al., 2022; Tardif et al., 2022; Xu et al., 2022). Currently, ASOs against Apo-A, and Apo-CIII are under development (Bellosta et al., 2022; Xu et al., 2022). Because of their great advantages in pharmacokinetics such as high affinity for plasma proteins and rapid distribution throughout the body, ASOs are potential cardiometabolic therapeutic strategies for treating refractory hypercholesterolemia and reducing the risk of cardiovascular disease (Bellosta et al., 2022). Further trials are needed to verify the clinical effects and provide detailed indications of these agents.

Small Interfering RNA

In the clinical phase of treating hypercholesterolemia, small interfering ribonucleic acid (siRNA) technology is another important gene silencing approach targeting mRNA to block transcription (Mohamed et al., 2021).

Inclisiran is a small interfering ribonucleic acid (siRNA) that acts on PCSK9. In the phase III LDL-C lowering studies, adults with heterozygous FH (Orion-9 trial, NCT03397121) received a maximum dose of statins with or without ezetimibe and inclisiran (300 mg) subcutaneously on day 1, day 90, day 270 and day 450. LDL-C levels were significantly lower than those in the placebo group (Raal et al., 2020a), and adverse and serious adverse events

TABLE 1 | Registered clinical trials of RNA-based therapy and gene therapy for RH (clinicaltrials.gov; search date: 2022/05/11).

Indication	Title	Study type/CT phase	Therapeutic agent	Route of administration	Identifier	(Estimated) study start date–completion date
FH	Phase I Study of *Ex Vivo* Liver-Directed Gene Therapy for Familial Hypercholesterolemia	Interventional (Phase I)	autologous hepatocytes	Retrovirus LDL iv	NCT00004809	June 1992–1995
HyperTG, FH	Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of IONIS ANGPTL3-LRx in Healthy Volunteers With Elevated Triglycerides and Participants With Familial Hypercholesterolemia	Interventional (Phase I)	IONIS ANGPTL3-LRx	subcutaneous	NCT02709850	30 November 2015–26 June 2017
HoFH	AAV8-mediated Low Density Lipoprotein Receptor (LDLR) Gene Replacement in Subjects With Homozygous Familial Hypercholesterolemia (HoFH)	Interventional (Phase I/II	AAV8-hLDLR	iv infusion	NCT02651675	March 2016–27 November 2020
HoFH	A Long-term Follow-up Study to Evaluate the Safety and Efficacy of RGX-501	Observational	No investigational product, participants have previous received RGX-501(human LDLR Gene Therapy)		NCT04080050	30 September 2019–29 September 2025
HeFH, Elevated Cholesterol	Trial to Evaluate the Effect of Inclisiran Treatment on Low Density Lipoprotein Cholesterol (LDL-C) in Subjects With Heterozygous Familial Hypercholesterolemia (HeFH) (ORION-9)	Interventional (Phase III)	Inclisiran	SC injections	NCT03397121	28 November 2017–17 September 2019
ASCVD, Elevated Cholesterol	Inclisiran for Participants With Atherosclerotic Cardiovascular Disease and Elevated Low-density Lipoprotein Cholesterol (ORION-10)	Interventional (Phase III)	Inclisiran	SC injections	NCT03399370	21 December 2017 - 17 September 2019
ASCVD, ASCVD-Risk equivalents, Elevated Cholesterol	Inclisiran for Subjects With ASCVD or ASCVD-Risk Equivalents and Elevated Low-density Lipoprotein Cholesterol (ORION-11)	Interventional (Phase III)	Inclisiran	SC injections	NCT03400800	1 November 2017–27 August 2019
HCL	A Non-interventional Implementation Study to Evaluate Treatment With Inclisiran (Leqvio®) and Other Lipid Lowering Treatments in a Real-world Setting	Observational	Inclisiran	SC injections	NCT05362903	28 January 2022–31 January 2025
Dyslipidemias, FH, HyperTG	A Phase 1 Single and Multiple Dose Study to Evaluate the Safety, Tolerability, Pharmacokinetics and Pharmacodynamic Effects of ARO-ANG3 in Adult Healthy Volunteers and in Dyslipidemic Patients	Interventional (Phase I)	ARO-ANG3	SC injections	NCT03747224	7 January 2019–17 May 2021
Mixed Dyslipidemia	A Double-blind, Placebo-controlled Phase 2b Study to Evaluate the Efficacy and Safety of ARO-ANG3 in Adults With Mixed Dyslipidemia	Interventional (Phase II)	ARO-ANG3	SC injections	NCT04832971	28 June 2021–30 April 2023
HoFH	Phase 2 Study to Evaluate the Safety and Efficacy of ARO-ANG3 in Subjects With Homozygous Familial Hypercholesterolemia (HOFH)	Interventional (Phase II)	ARO-ANG3	SC injections	NCT05217667	June 2022–October 2023
FH	Exosome-based Nanoplatform for Ldlr mRNA Delivery in Familial Hypercholesterolemia	Interventional (Phase I)	LDLR mRNA exosomes	Intravenous/ peritoneal infusion	NCT05043181	December 2021–December 2026

FH, familial hypercholesterolemia; HyperTG, hypertriglyceridemia; HoFH, homozygous familial hypercholesterolemia; HeFH, heterozygous familial hypercholesterolemia; ASCVD, atherosclerotic cardiovascular disease; HCL, hypercholesterolemia; SC, subcutaneous.

were similar in both groups. Patients with ASCVD (Orion-10, NCT03399370) and patients at equivalent risk for ASCVD or ASCVD (Orion-11, NCT03400800) had elevated LDL-C levels despite receiving the maximum tolerated dose of statin (>100 mg/dl), randomized to inclisiran or placebo, and found that LDL-C levels decreased by approximately 50% in the inclisiran subcutaneously injected every 6 months (Ray et al., 2020). Inclisiran (Leqvio®) was approved by EC in December 2020

for the treatment of hypercholesterolemia and mixed dyslipidemia in adults and was approved by FDA in 2021, becoming the only siRNA drug currently on market to lower LDL-C. Recently, a real-world prospective observational cohort study to evaluate inclisiran (Leqvio®) and other lipid lowering treatments is recruiting, expecting to provide more clinical evidence in this newly initiated siRNA (NCT05362903).

A novel siRNA targeting ANGPTL3 mRNA, named ARO-ANG3, was developed by Arrowhead Pharmaceuticals and recently completed phase I study (NCT03747224) in dyslipidemic patients and in adult healthy volunteers. Results of ARO-ANG3 in hypercholesterolemia patients on a stable lipid-lowing regimen showed ANGPTL3 levels dropped by 79–88% on average, and mean maximum reductions in LDL-C was up to 42% (Arrowhead Reports Interim Clinical Data on Cardiometabolic Candidates Aro-Apoc3 and Aro-Ang3, 2020). In healthy volunteers, the reduction in fasting lipid profile was similar to that reported in ANGPTL3 deficient function carriers. Besides, this study showed good safety and tolerability of ARO-ANG3 (Watts et al., 2019). In HeFH patients, interim findings demonstrated that, from baseline to week 16, LDL-C reductions increased by 23%, 30% and 37% with rising doses (100, 200 and 300 mg), respectively (Watts et al., 2020). Currently, phase II studies of ARO-ANG3 in adults with mixed dyslipidemia (NCT04832971) and in participants with HoFH (NCT05217667) are ongoing, expected to provide more safety and efficacy information (**Table 1**).

GENE THERAPY OF REFRACTORY HYPERCHOLESTEROLEMIA

Virus-Mediated Gene Therapy

Gene therapy may provide a promising approach to treat RH because targeting specific genetic loci can lead to precise results with minimal side effects. Early experiments demonstrated that viral vector-associated gene transfer can up-regulate LDLR expression and control hypercholesterolemia in animal models (Andreoletti et al., 2001), and Grossman et al. first conducted a clinical trial in five HoFH patients using retrovirus-mediated gene therapy (NCT00004809) (**Table 1**). This treatment first transduced recombinant retroviruses carrying healthy LDLR with autologous hepatocytes *ex vivo*, then infused into patients' livers directly. After 4 months, the trial achieved only a small proportion of normal LDLR expression, and a slight decrease in plasma total cholesterol, LDL-C, and ApoB (6–20%, 6–25%, 10–21%, respectively) only in 3 of 5 patients (Grossman et al., 1995). The unsatisfactory result was likely due to low transfection efficiency of retrovirus, thus selecting appropriate viral vectors may improve it.

The use of lentivirus vectors has improved *ex vivo* transduction efficiency, achieving transduction in nearly 90% of cells (Oertel et al., 2003). Ou et al. delivered Ldlr gene using lentiviral vector into FH mice and observed significant decrease of LDL-C by 46%, and amelioration of lipid accumulation (Ou et al., 2016). Hytonen et al. develop different virus-mediated gene transfer system in LDLR deficient rabbit model and found that lentiviral vector-LDLR resulted long-term reduction in lipid profile instead of AAV2, AAV9-LDLR (Hytönen et al., 2019).

Besides retrovirus and lentiviral vectors, adenovirus (Ad) vectors are effective in gene delivery. Transfer of the helper-dependent Ad vector LDLR gene into $LDLR^{-/-}$ mice has been shown to ameliorate lipid profile and produce long-term protection against atherosclerosis (Nomura et al., 2004; Leggiero et al., 2019). Ad are also used in somatic genome editing without adverse consequences reported from Musunuru' lab (Wang et al., 2016; Chadwick et al., 2017). Splicing regulation of ApoB is another approach that can treat FH. ApoB posttranscriptional modification appears to be safe and effective in lowering cholesterol by interfering with VLDL assembly and LDL clearance (Khoo, 2015). The peg technique appears to be useful for additional anti-inflammatory effects, and this modification does not interfere with LDL reduction and atherosclerosis regression induced by helper-dependent Ad vectors (Leggiero et al., 2013).

Adeno-associated virus (AAV) vectors are safe platforms and the most commonly used viral vectors for gene therapy delivery nowadays (Xu et al., 2019). Different serotypes of AAV-based vectors have different organ-specific tropism. Among them, hepatotropic vectors based on AAV serotype 8 (AAV8) have been showed best in aspects of total cholesterol profile reduction and hepatocyte transduction (Lebherz et al., 2004), thus been developed for liver-directed gene therapy including FH.

AAV8-mediated gene therapy is widely used and suitable for somatic genome editing. Inducible degrader of LDLR (IDOL), an E3-ubiquitin ligase that binds LDLR at different locations with PCSK9 can promote receptor ubiquitination and lysosomal degradation, having a positive effect on LDL metabolism through specific amino acid substitution (Yu et al., 2021). A study of humanized mice showed that AAV8-mediated expression of IDOL in the liver resulted in increased LDL dependence on LDL plasma levels (Ibrahim et al., 2016). A recent toxicological study assessed the effect of AAV8 on direct expression of LDLR in rhesus monkeys, suggesting that the treatment is safe except for mild and transient transaminases and immune adaptation responses (Greig et al., 2017a). In addition, AAV8-induced RNA silencing against ApoB (via short hairpin RNA and artificial microRNA) has led to a significant reduction in plasma cholesterol (Maczuga et al., 2014).

Currently, FDA has already approved two therapies of AAV-mediated gene therapy. One is AAV2-mediated delivery of the RPE65 gene in treating confirmed biallelic RPE65-mediated inherited retinal dystrophy in 2017, the other is AAV9-mediated therapy that functionally replaces the mutated survival motor neuron 1 gene in treating spinal muscular atrophy type 1 disease in children under 2 years of age. Clinical trials have confirmed the significant efficiency and safety of these AAV-mediated therapies (Mendell et al., 2017; Russell et al., 2017; Pennesi et al., 2018). The most related clinical trial of RH treatment is AAV8-mediated delivery of LDLR gene for HoFH, which was completed as a phase I/II study in 2020, while no study results were posted yet (NCT02651675) (**Table 1**).

Before initiating of phase I/II clinical trial, Greig et al. (2017b) determined the pharmacology and toxicology of clinical

candidate vector, AAV8.TBG.hLDLR, as well as those expressed mouse LDLR, AAV8.TBG.mLDLR in a specific mouse model of HoFH. They used 280 homozygous double Ldlr/Apobec-1 knockout mice which can develop severe hypercholesterolemia, and divided them into 5 cohorts. Four cohorts used AAV8.TBG.mLDLR in different doses (7.5×10^{11} GC/kg, 7.5×10^{12} GC/kg, and 6.0×10^{13} GC/kg) as vectors and the other used AAV8.TBG.hLDLR (6.0×10^{13} GC/kg). The lowest dose stood for the initial dose of the ongoing clinical trial (NCT02651675), the middle dose represented the highest clinical trial proposed dose, while the highest dose is approximately 8-fold the middle dose to test the safety. Pathology analysis indicated no dose-limiting toxicities even in the highest dose despite mild and transient liver pathology. Therefore, the maximally tolerated dose was higher than 6.0×10^{13} GC/kg while the no-effect dose was equal to or higher than the middle dose of 7.5×10^{12} GC/kg. They also determined the minimally effective dose was 7.5×10^{11} GC/kg, based on measurable reductions in total serum cholesterol, and suggested the therapeutic window for the treatment of HoFH was no less than 80-fold (Greig et al., 2017b). Those findings were considered being clinically significant and provided evidence for proposed clinical trials.

In non-human primate (NHP), PCSK9 knockdown by AAV-delivered meganuclease was demonstrated to be effective, safe and long-term durable (Wang et al., 2018; Wang et al., 2021). Wang et al. designed a combination of AAV8 and engineered meganucleases named M1PCSK9 (first generation) and M2PCSK9 (second generation) to target a sequence in PCSK9 gene exon 7 which was conserved between humans and macaques. After administering AAV8-M1PCSK9 into four macaques, all animals achieved dose-dependent PCSK9 inactivation in liver and serum within 6 weeks as well as stable serum cholesterol decrease up to 11 months. The modest dose (6.0×10^{12} GC/kg) of AAV8-M2PCSK9 injection into two other macaques led to a 34% reduction of serum LDL. Besides, AAV8-M2PCSK9 was more precise in target recognition and had less off-target cleavage (Wang et al., 2018). During the following 3-year monitoring, researchers found the all abovementioned six macaques, and another two treated with the AAV3B-M2PCSK9 vector maintained PCSK9 knockdown by AAV8-M2PCSK9 and serum LDL-C level reduction. Human genetics data indicated therapeutic potential of AAV meganuclease and chimeric liver-humanized mouse model showed similar on-target editing in primary human hepatocytes. No obvious adverse effects except transient transaminitis during early phase were detected, and most hepatocytes sustained stably in histopathology (Wang et al., 2021).

Nonetheless, the possibility of detrimental immune response to virus-mediated gene delivery hampered its use in genetic therapy. The first pilot study in which retrovirus genes were transferred to the liver cells of HoFH patients resulted in a different biochemical response (Grossman et al., 1995). Additionally, mild toxicity of AAVs is reported at high doses from animal studies, hence the need to establish more effective gene therapy approaches (Lau and Suh, 2017).

Non-viral Vectors for Gene Therapy

In order to overcome the difficulties of host immune response of viral vectors, novel vectors with lower toxicity and immunogenicity are under development. Hou et al. demonstrated the creation of multiple minicircle non-viral DNA vectors. After specific modifications and highly efficient liver-specific LDLR gene expression, correction of hypercholesterolemia in LDLR deficient mice has no significant toxicity, thus providing another potential genetic tool for treating FH (Hou et al., 2016). In addition, human induced pluripotent stem cell (hiPSC) technology has shown encouraging results through plasmid vectors (Hou et al., 2016). Recently, a study expressed LDLR cDNA binding microRNA in FH mouse model with episomal non-viral vector, which inhibited the 3-hydroxy3-methylglutarate reductase (HMG-COA) and resulted in lipid level lowering about 32% in animals (Kerr et al., 2016).

Previous studies established that polymer nanoparticles designed for specific purpose are efficient in siRNA, microRNAs and drugs delivery (Maestro et al., 2021). Lipid nanoparticles (LNPs) are formed by amphiphilic lipids, suitable as vehicles for nucleic acid delivery. Yin et al. developed a LNP coencapsulated chemically enhanced single guide RNAs (sgRNA) and Cas9 mRNA to knockout PCSK9 gene in mouse liver, and detected a significant reduction of serum PCSK9 as well as a 35%–40% total cholesterol decrease (Yin et al., 2017). A recent study determined that PCSK9-targeted zinc finger nuclease mRNAs formulated into LNP could effectively delivered to the liver through intravenous injection in mouse models, achieving more than 90% knockout of the PCSK9 gene expression after the first dose. Besides, this method was well tolerated and efficient genome editing continuously increased after repeated dosing (Conway et al., 2019).

Compared with nanoparticles constructed from artificial materials, endogenous nano-sized carriers have the distinct advantage of good *in vivo* biocompatibility, avoiding rapid recognition and clearance by the reticuloendothelial system (Baek et al., 2019). Exosome (diameter of 30–150 nm) is a cell-derived vesicle released by most eukaryotic cells, which is an important medium of cell communication and material transmission by carrying proteins, non-coding RNAs, DNA, and other bioactive substances (Yang et al., 2020). Based on their endogenous, biocompatible and multifunctional properties, exosomes are emerging as a new tool for drug delivery systems and precision therapy. Recently, an exosome-based Ldlr gene therapy for FH successfully implemented in mice. Li et al. generated exosomes encapsulating enriched Ldlr mRNA by forced expression of Ldlr in AML12 cells. After confirming Exo^{Ldlr} could efficiently deliver functional Ldlr mRNA *in vitro*, investigators injected Exo^{Ldlr} in atherosclerotic $Ldlr^{-/-}$ mice. Those exosomes successfully delivered wild-type Ldlr to the liver and expressed LDLR protein, significantly decreasing LDL-C, total cholesterol, and triglyceride levels, without any noticeable adverse effects. Moreover, Exo^{Ldlr} reduced lipid deposition in the liver and atherosclerotic lesions (Li et al., 2021). More recently, a first-in-human study of an exosome-based nanoplatform for Ldlr mRNA delivery is currently in phase

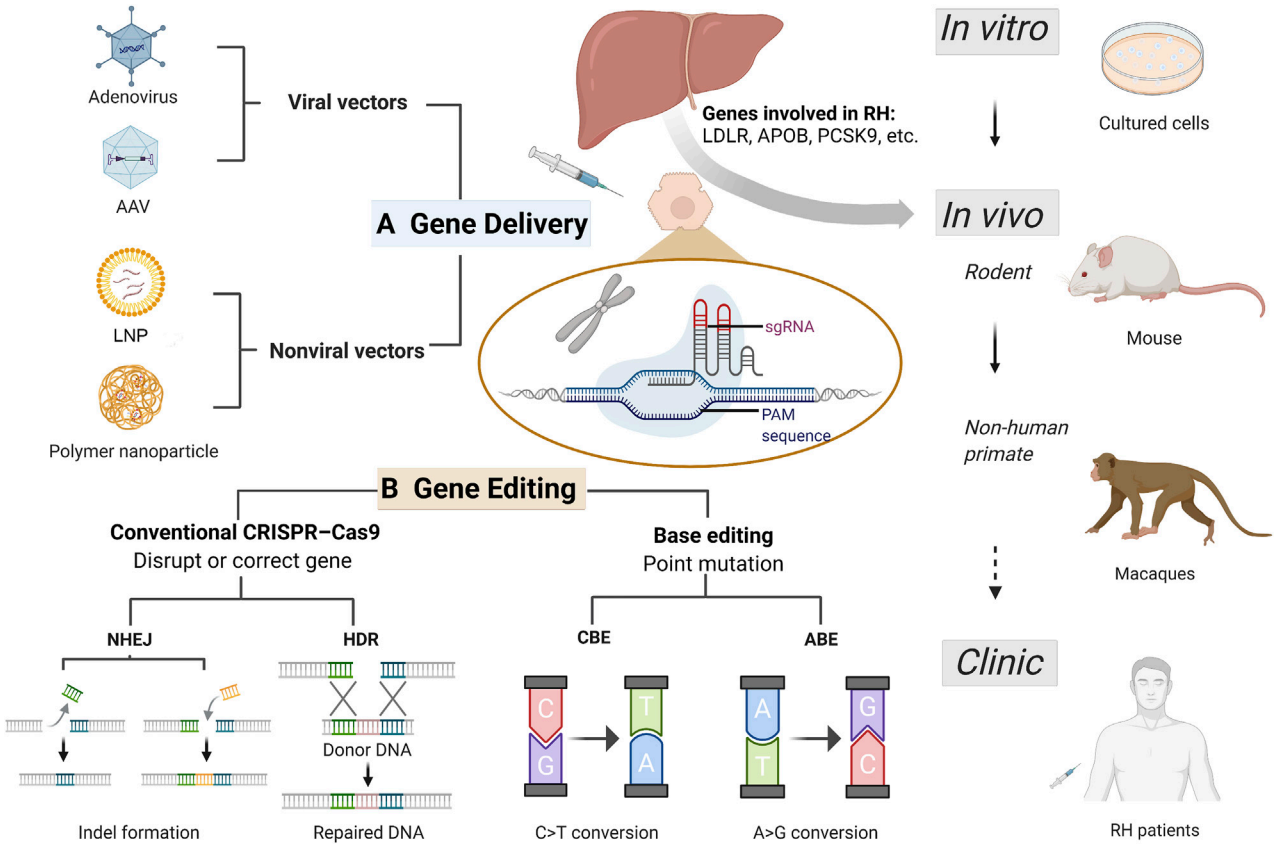

FIGURE 1 | Current CRISPR/Cas9 based gene therapy for refractory hypercholesterolemia. Refractory hypercholesterolemia (RH) including homozygous familial hypercholesterolemia and compound heterozygous familial hypercholesterolemia, associated with mutations in genes encoding LDLR, APOB, PCSK9, etc. Efficient vectors enable delivery of CRISPR progenitors and genetic materials *in vitro* and *in vivo*, and the development of CRISPR technology has improved its efficacy and safety in target gene editing. Gene therapy experiments have been successfully conducted in cells (including human induced pluripotent stem cell), mice and non-human primates, expected to be used for clinical treatment in the future. **(A)** Common delivery vectors used in RH gene therapy. Adenovirus and adeno-associated virus are the most frequently used viral vectors in RH gene therapy. Non-viral vectors including lipid nanoparticle and engineered polymer nanoparticles offer more options for gene delivery with greater capacity and less toxicity and immunogenicity. **(B)** Gene editing based on CRISPR/Cas9 system for treating RH. For RH-related genes, gene disruption and correction can be achieved using conventional CRISPR-Cas9 and base editing techniques. In conventional CRISPR-Cas9, NHEJ can induce indel mutation (insertion or deletion), achieving disruption of pro-atherosclerotic genes such as PCSK9 and ANGPTL3; and HDR can correct a disease-causing mutation from donor DNA. Base editing is a new generation of CRISPR technology that enables single-nucleotide changes without double-strand breaks for precise gene editing. CRISPR-Cas9, clustering regularly spaced short palindromic repeats (CRISPR) and CRISPR-associated protein; RH, refractory hypercholesterolemia; AAV, adeno-associated virus; LNP, lipid nanoparticle; PAM, protospacer-adjacent motif; sgRNA, single guide RNA; NHEJ, non-homologous end joining; HDR, homology-directed repair; CBE, cytosine base-editors; ABE, adenine base-editors.

I clinical trials (NCT05043181), promising to treat HoFH patients (**Table 1**).

Common gene therapy delivery vectors suitable for both *in vitro* and *in vivo* experiments to treat RH were briefly summarized in **Figure 1A**. In conclusion, almost all delivery methods have both advantages and disadvantages. Further efforts are needed to quest for appropriate delivery systems possessing rare toxicity and immunogenicity, high transfection rate efficiency, high specificity to targets, large capacity, and ease of manipulation (Sharma et al., 2021).

CRISPR/Cas9 Gene Editing

Clustering regularly spaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) system is currently the most widely used programmable genome editing tool.

CRISPR/Cas9 system can be oriented to recognize protospacer-adjacent motif (PAM) sequence at the targeted location by an artificial single guide RNA (sgRNA), and cleave DNA strands by the Cas9 nuclease (Liu et al., 2022). Then the double-strand DNA breaks can be repaired by the cell's natural repair machinery, non-homologous end joining (NHEJ), to restore the original DNA sequence or introduce insertion and deletion (indel mutation); or by using another customized DNA repair template to realize homology-directed repair (HDR) (Porteus, 2019) (**Figure 1B**).

Currently, *streptococcus* pyogenes Cas9 has been widely used in genome engineering (Bolukbasi et al., 2016). Other *in vivo* delivery of Cas9 orthologs including *staphylococcus aureus* Cas9 (SaCas9), *campylobacter jejuni* and *neisseria meningitidis* Cas9 (NmeCas9) (Esvelt et al., 2013; Kim et al., 2017a). Ran et al. used a

single AAV vector to deliver SaCas9 and its sgRNA to target mouse liver, and observed more than 40% Pcsk9 gene modification within 7 days after injection, along with substantial decreases in blood Pcsk9 and total cholesterol (Ran et al., 2015). Ibraheim et al. validated the efficiency of *in vivo* delivery of NmeCas9 guided by all-in-one recombinant AAV to target Pcsk9 gene in C57Bl/6 mice, and determined significant reduction in serum cholesterol levels (Ibraheim et al., 2018). Jiang et al. demonstrated a non-viral system, capable of *in vivo* delivering CRISPR/Cas9 components targeting of mouse Pcsk9 gene, and observed a high cutting efficiency in the targeted region, accompanied by a reduction in PCSK9 protein (Jiang et al., 2017).

CRISPR correction of LDLR dysfunction is a promising therapeutic strategy for RH treatment, especially for those HoFH patients bearing a null variant in LDLR gene. Transformed differentiated hepatocellular like cells (HLCs) showed increased LDL uptake and FH phenotypic modification, either by vector or specific genome editing by CRISPR/Cas9 techniques (Kuijper et al., 2021). Okada et al. (2019) studied the recovery of LDLR function and immunogenicity of gene-corrected iPSC-derived HLCs from an HoFH patient harbored a point mutation in exon 6 of LDLR using the CRISPR/Cas9 method. They reprogrammed T-cells of the HoFH patient to iPSCs and then differentiated into HLCs. Both homozygous gene-corrected iPSC-derived HLCs clone and two heterozygous clones successfully gained functional recovery of LDL uptake. Additionally, the immunogenicity after gene correction against the HoFH patient's peripheral blood mononuclear cells was low and similar to that before operation. Therefore, LDL uptake of HoFH-iPSC can be repaired by CRISPR correction without further immunogenicity, suggesting this gene-corrected strategy has clinical potential in the treatment of HoFH (Okada et al., 2019).

A more recent preclinical study applied CRISPR/Cas9 system delivered by AAV to realize gene editing of Ldlr in mutant knock-in mouse model, and determined *in vivo* AAV-CRISPR/Cas9 somatic cell gene editing could correct LDLR mutations and ameliorate hypercholesterolemia. After confirmation of the efficient mouse model generated the E208X nonsense point mutation in the Ldlr gene, researchers delivered elaborate AAV-CRISPR/Cas9 into newborn Ldlr (E208X) mice to correct Ldlr gene mutations in a subset of hepatocytes. When mice matured, the AAV-CRISPR/Cas9 treatment group had approximately 6.7% (6.67 ± 0.64%) correction of the Ldlr alleles, and partially (~18%) expression of LDLR proteins. Compared with same high-fat diet control group, the treated group had greater reductions in total cholesterol, triglycerides, and LDL-C. The symptoms of atherosclerosis were significantly reduced, as were the symptoms of macrophage infiltration and lipid accumulation. No obvious off-target or immune rejection reaction was found in adult mice, indicating the efficacy and safety of the gene editing therapy (Zhao et al., 2020).

The above-mentioned evidences point to CRISPR/Cas9 as a viable genome editing tool against RH, particularly in cases involving LDLR deficiency. However, there are several technical limitations and safety concerns of CRISPR/Cas9 which limit its application in clinical. First, because of DNA double-strand breaks introduced in CRISPR/Cas9 technology, unpredictable repair such as random insertions and deletions (indel) may occur and results in on-target mutagenesis. Additionally, low-rate HDR mediated editing leads to inefficient on-target alterations and is limited to dividing cell types. Besides, off-target mutagenesis due to guide RNA lack of sufficient specificity would increase the therapeutic risks, thus hinder research and clinical application (Fu et al., 2013; Doudna, 2020).

Base Editing

Base editing is a new generation of genome editing techniques and can solve undesired effects of CRISPR/Cas9 (Anzalone et al., 2020). Base editor toolbox utilizes the positioning and cleavage capabilities of the Cas system for direct and precise single base substitution, without inducing double-stranded DNA breaks (Rees and Liu, 2018). Two classes of DNA base-editors have been described: cytosine base-editors (CBEs) and adenine base-editors (ABEs), realizing installation of all four transition mutations (C→T, T→C, A→G, and G→A) (Kantor et al., 2020) (**Figure 1B**).

Previous study demonstrated that the third-generation base editor (BE3), a widely used CBE which converts a targeted C G base pair to a T A base pair, resulted in fewer off-target mutagenesis than standard CRISPR/Cas9 *in vitro* (Kim et al., 2017b). Chadwick et al. established the efficacy of base editing *in vivo* for the first time by delivering base editor to the codon Trp-159 site of the mouse PCSK9 gene. After injecting BE3 with PCSK9-targeting gRNA in adult mice, researchers observed a median rate of 25% base editing in PCSK9 alleles, associated with more than 50% reduction in blood PCSK9 protein levels. Indel mutagenesis rate (approximately 1%) was far lower than that in prior studies of *in vivo* genome editing (~40%) (Wang et al., 2016). Although the degree of plasma cholesterol decrease (~30%) was less than that in standard CRISPR/Cas9 genome editing (35%–40%) (Ding et al., 2014), it is enough to reduce cardiovascular risk when translated to humans. Moreover, no evidence of off-target mutagenesis was obtained in this *in vivo* study.

Following PCSK9, *in vivo* base editing of ANGPTL3 was demonstrated to be successful. Chadwick et al. (Chadwick et al., 2018) identified the codon Gln-135 of ANGPTL3 gene was targetable for base editing. AAV encoding BE3 with and without gRNA targeting Angptl3 Gln-135 was injected in 5-week-old male C57BL/6J mice and BE3-Angptl3 caused a median editing rate of 35% increase in the Angptl3 target site, associated with significantly lower in mean levels of plasma ANGPTL3 protein (50%), triglycerides (35%), and cholesterol (20%) compared to BE3 without gRNA (BE3-control). Besides, BE3-Angptl3 performed better than BE3 targeting Pcsk9 in declining triglycerides. In male hyperlipidemic Ldlr-knockout mice (phenocopy of homozygous FH which PCSK9 loss-of-function has little effect) of the same age, BE3-Angptl3 showed higher efficiency (as compared to the BE3 control), with 56% reduction in triglycerides, and 51% reduction in cholesterol levels (Chadwick et al., 2018). Additionally, there was no evidence of off-target mutagenesis, and decrease of

TABLE 2 | Experimental studies of base editing for RH.

Species	References	Target	Cas nuclease	Model	Delivery
Mouse	Chadwick et al. (2017)	Pcsk9 (Trp-159 site)	BE3	5-week-old male C57BL/6J mice	Adenoviral vector
	Carreras et al. (2019)	mouse Pcsk9 and human PCSK9	BE3	10-week-old male hPCSK9-KI mice and wildtype mice	Adenoviral vector
	Chadwick et al. (2018)	Angplt3 (Gln-135 site)	BE3	5-week-old male C57BL/6J mice	Adenoviral vector
		Angplt3+Pcsk9	BE3	5-week-old male C57BL/6J mice	Adenoviral vector
		Angplt3	BE3	5-week-old male B6.129S7-Ldlrtm1Her/J mice	Adenoviral vector
	Rothgangl et al. (2021)	PCSK9	ABEmax	mice	Lipid nanoparticle
Macaque	Musunuru et al. (2021)	PCSK9	ABE8.8-m	cynomolgus monkeys	Lipid nanoparticle
	Rothgangl et al. (2021)	PCSK9	ABEmax	cynomolgus macaques (Macaca fascicularis)	Lipid nanoparticle

Species	References	Editing rate	Decreased levels of target protein	Degree of plasma cholesterol decrease
Mouse	Chadwick et al. (2017)	Day 5: a median rate of 25% (average rate of 24%, max = 34%); Week 4: median rate of 28%	Day 5: PCSK9 protein (~56%); Week 4: PCSK9 protein (~54%)	Day 5: cholesterol (28%); Week 4: cholesterol (28%)
	Carreras et al. (2019)	most in the human PCSK9 and mouse Pcsk9 were the targeted C to T transitions	Week 3: human PCSK9 protein (~32%); mouse PCSK9 protein (~28%)	significant reductions in total cholesterol
	Chadwick et al. (2018)	Day 7: a median editing rate of 35%	Day 7: mean levels of plasma ANGPTL3 protein (49%)	Day 7: triglycerides (31%); cholesterol (19%)
		-	Day 7: ANGPTL3 protein (~48%); PCSK9 protein (~57%)	Day 7: triglycerides (~27%); cholesterol (~29%)
		-	Day 14: ANGPTL3 protein (~24%)	Day 14: triglycerides (56%); cholesterol (51%)
	Rothgangl et al. (2021)	67%	~95%	LDL-C 58%
Macaque	Musunuru et al. (2021)	almost complete editing of PCSK9	nearly 90%	LDL-C up to 60%
	Rothgangl et al. (2021)	34%	32%	LDL-C 14%

PCSK9, proprotein convertase subtilisin-kexin type 9; ANGPTL3, angiopoietin-like protein 3; BE3, third-generation base editor; ABE, adenine base-editor.

bone marrow hematopoietic stem cells which documented in ANGPTL3 full knock-out mouse (Zheng et al., 2011) was not observed in BE3-Angptl3-treated mice.

Recently, a study in humanized mouse model target human PCSK9 showed similar results. Researchers first generated a knock-in mouse model with liver-specific expression of human PCSK9 (hPCSK9-KI), and compared the lipid lowering efficiency of three treatment: evolocumab, CRISPR/Cas9-mediated genome editing, and base editing using gRNA. All treatments resulted in reduction of cholesterol levels in hPCSK9-KI, whereas base editing was the only one reducing levels of both human and mouse circulating PCSK9 protein (~32% and ~28%, respectively). Compared to Cas9-mediated genome editing, BE3 was more precise with no detectable off-target editing or chromosomal translocations *in vivo*, generated fewer indels, and potentially reduced immune response probability (Carreras et al., 2019).

Genome-wide profiling of ABE showed lower off-target rates compared to canonical SpCas9 (Liang et al., 2019). More recently, Musunuru et al. took a step toward investigating the efficacy of base editing in liver-specific PCSK9 of nonhuman primates. After a single-dose of LNP loaded with ABE injecting into cynomolgus monkeys, researchers observed almost complete editing of Pcsk9 in the liver. Circulating levels of PCSK9 and LDL-C were stably reduced by nearly 90% and up to 60%, and lasted for at least 8 months (Musunuru et al., 2021). Rothgangl et al. detected that ABEmax, a codon-optimized version, delivered by LNP to target liver provided that up to 67% knockdown of Pcsk9 in mice and 34% in macaques, resulting in concomitant reductions in plasma PCSK9 proteins and LDL levels of approximately 95% and about 58% in mice, and 32% and 14% in nonhuman primates, respectively (Rothgangl et al., 2021). Experimental studies of base editing technology for the treatment of RH were shown in **Table 2**.

Base editing functions without inducing double-strand DNA breaks, limiting the occurrence of unwanted mutations and chromosomal abnormalities (Komor et al., 2018). Therefore, CRISPR base editing could be a promising strategy in correcting LDLR gene mutations or multiple genes defects and treating RH, and may help improve the efficacy of current lipid-lowering drugs.

Concerns Beyond Technology of Gene Therapy

Although gene therapy is an emerging therapy with great potential to treat or cure serious diseases, potential risks of novel drugs should always be carefully considered, especially at the aspect of human genome editing.

In 2017, the US National Academies of Sciences, Engineering, and Medicine established an international committee to discuss important issues about human genome editing and noted that somatic cell genome editing was expected to have the most immediate clinical application, but should only be used to treat or prevent disease and disability in accordance with current ethical standards (Human Genome Editing, 2017). In 2020, a consensus from International Commission on the Clinical

Use of Human Germline Genome Editing defined translational pathway of heritable human genome editing to clinical use, and classified its potential application categories. According to the report, RH would fit in category B and suitable for using heritable human genome editing under stringent governance because of disease-causing FH allele both carried by prospective parents (Heritable Human Genome Editing, 2020). Besides, with current guidelines recommending that lower LDL-C is better (Grundy et al., 2019; Mach et al., 2020), gene editing is expected to further reduce LDL-C levels and improve the prognosis of RH patients, especially for those with genetic defects, or those who have had poor results or poor adherence with other treatment.

Another consideration is the cost gap between current therapies and gene therapy. Most studies have reported that gene therapy products were more costly than their comparators (Lin et al., 2018; Cher et al., 2020; Furzer et al., 2020). For example, Strimvelis®, a gene therapy treatment for severe combined immunodeficiency, will cause the incremental cost-effectiveness ratios when compared with hematopoietic stem cell transplants (hematopoetic stem cell transplant-haploidentical donor or hematopoietic stem cell transplant-matched unrelated donor) about £36,360 and £14,645 per quality-adjusted life-year, respectively (South et al., 2019). Although with the higher cost, potentially curative gene therapy offers the possibility of lifelong benefits from a single course of treatment, which could lead to these therapies being more cost-effective than current treatments in the long term (Ho et al., 2021). Therefore, the cost-effectiveness of gene therapy should be carefully considered from clinical trials to clinical practice (Jayasundera et al., 2022).

OTHER NEW TARGETS FOR CHOLESTEROL REDUCTION

Genome wide association study (GWAS) have identified nearly 80 genes with pathogenic variants to be associated with hypercholesterolemia, such as genes encoding LDLR, APOB, PCSK9, APOE, LDLRAP1, and the signal-transducing adaptor family member 1 (STAP1), among which mutations in LDLR account for most cases with FH (~85–90%) (Paththinige et al., 2017; Di Taranto et al., 2020; Jackson et al., 2021). Several targets involved in lipid metabolism are already popular for lipid-regulating therapies, such as HMGCR (statins), NPC1L1 (ezetimibe), PCSK9 (alirocumab, evolocumab, inclisiran), and ANGPTL3 (evinacumab) (Raal et al., 2020b). Recently, novel targets have been recognized to act important function on cholesterol metabolism.

REV-ERB

REV-ERB α, a member of the orphan nuclear hormone receptor superfamily, is one of the ligands activated transcription factors, and its isoform is REV-ERB β(Nr1D2), both of which are rhythmically expressed in supraoptic nucleus, liver and heart tissue. The heme-regulated nuclear receptor can regulate metabolic pathways. Previous studies have shown that treatment with synthetic REV-ERB agonists can inhibit plasma cholesterol levels and liver cholesterol biosynthesis rate-limiting enzyme (HMG CoA reductase) levels in mice. Animal studies have shown that the REV-ERB agonist SR9009 reduced plasma cholesterol levels in wild-type C57Bl/6 and LDLR deletion mice and reduced the expression of a series of genes in the cholesterol biosynthesis pathway. Consistent with these data, increased expression of these genes has been observed in mice with under-expression of REV-ERB α. Analysis showed that REV-ERB binds directly to most of the genes involved in cholesterol biosynthesis and directly inhibits their expression. This study reveals the complex mechanism by which REV-ERB regulates cholesterol biosynthesis directly or indirectly (by inhibiting Srebf2 expression) and provides information on how cholesterol levels are regulated in a circadian manner. This study suggests that targeting REV-ERB may be an effective method to reduce LDL-C levels clinically (Sitaula et al., 2017).

G Protein-Coupled Receptor

Through bioinformatics analysis and functional verification, Han et al. recently found that SNPs rs1997243 was the only pathogenic mutation in the 7p22 region of the genome, which located in the non-coding region of the genome and specifically increased the expression of GPR146. Further study revealed that GPR146 encodes a G-protein-coupled receptor (GPCR), which is located in the plasma membrane of liver cells and activates the camp-PKA-CREb signaling pathway in response to serum stimulation, thereby regulating the balance of liver lipid metabolism and blood cholesterol level. This is the first GPCR that directly regulates blood cholesterol level. GPCR has good drug-forming properties, and more than 1/3 of all the drugs on the market are targeted by GPCR. This study provides a new important target for the development of cholesterol-lowering drugs (Han et al., 2020).

Ubiquitin Specific Peptidase 20

Lu et al. first found that the protein content of the rate-limiting enzyme HMGCR in the cholesterol synthesis pathway significantly increased after eating. Using a cleverly designed *in vitro* biochemical reaction, they cloned and expressed over 70 deubiquitination enzymes one by one, and screened USP20 as hMGCr-specific deubiquitination enzyme. In the feeding state, the deubiquitination enzyme ubiquitin specific peptidase 20 (USP20) stabilized HMG-CoA reductase (HMGCR), a rate-limiting enzyme in the cholesterol biosynthesis pathway. Postprandial increases in insulin and glucose concentrations synergistically activated mTORC1 and phosphorylate USP20 at S132 and S134. USP20 was recruited to the HMGCR complex and antagonized its degradation, up-regulating cholesterol synthesis and converting absorbed glucose and other nutrients into cholesterol. Subsequently, it was also found that long-term high-sugar and high-fat diet induced increased phosphorylation of USP20, stabilized HMGCR protein and increased cholesterol, which caused metabolic diseases. In order to explore whether USP20 can be used as a therapeutic target for metabolic diseases such as obesity, investigators gave USP20 inhibitor to obese mice. They found that USP20 inhibitor can significantly reduce body weight, reduce blood cholesterol and triglyceride levels, and improve insulin sensitivity. Inhibition

of USP20 can promote HMGCR degradation and decrease lipid synthesis. It also caused succinic acid to increase and increased heat production. The improvement of these metabolic indicators is conducive to the treatment of metabolic diseases such as hypercholesterolemia, obesity and diabetes (Lu et al., 2020).

CONCLUSION

In summary, research on therapeutic targets for refractory hypercholesterolemia is being carried out in depth. At the same time, for the treatment of refractory hypercholesterolemia, gene therapy technology is still developing, and is expected to achieve new breakthroughs. In view of these innovative techniques/approaches, more research efforts are currently focused on developing precise, effective and safe gene delivery and editing strategies for genetic therapy of RH. However, potential risks of novel drugs should always be carefully considered, especially at the aspect of human genome editing. Therefore, further studies are needed to induce effective RH-related gene transgenic expression safely, balance the risks and benefits of clinical use, and ultimately achieve sustained reduction and regression of atherosclerosis in humans. Another consideration is the higher cost potential lifelong benefits caused by gene therapy. Therefore, the cost-effectiveness of gene therapy need to be carefully considered. Gene therapy has always been a research hotspot in the field of RH, and it is committed to developing effective and safe treatment methods that can be successfully transformed into clinical application. In addition, new targets such as REV-ERB, GPCR and USP20 are expected to be used in the clinic in the near future. Although the initial results are promising, more, larger and longer clinical trials are needed to determine the exact role of these approaches in the treatment of refractory hypercholesterolemia.

AUTHOR CONTRIBUTIONS

N-QW was the originator, supervisor of the project, and conducted elaborate polishment on the paper. Z-FL collected relevant literature, wrote the first draft of the paper, designed the tables and painted the figures. All authors contributed to the article and agreed with the final manuscript.

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BRIEF RESEARCH REPORT
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The Role of Registers in Increasing Knowledge and Improving Management of Children and Adolescents Affected by Familial Hypercholesterolemia: the LIPIGEN Pediatric Group

Marta Gazzotti[1], *Manuela Casula*[2,3]*, *Stefano Bertolini*[4], *Maria Elena Capra*[5], *Elena Olmastroni*[3], *Alberico Luigi Catapano*[2,3] and *Cristina Pederiva*[6]

[1]SISA Foundation, Milan, Italy, [2]IRCCS MultiMedica, Milan, Italy, [3]Department of Pharmacological and Biomolecular Sciences, Epidemiology and Preventive Pharmacology Service (SEFAP), University of Milan, Milan, Italy, [4]Department of Internal Medicine, University of Genova, Genova, Italy, [5]Centre for Paediatric Dyslipidaemias, Paediatrics and Neonatology Unit, Guglielmo da Saliceto Hospital, Piacenza, Italy, [6]Clinical Service for Dyslipidaemias, Study and Prevention of Atherosclerosis in Childhood, Paediatrics Unit, ASST-Santi Paolo e Carlo, Milan, Italy

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Edited by:
Alpo Juhani Vuorio,
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***Correspondence:**
Manuela Casula
manuela.casula@unimi.it

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Pathology registers can be a useful tool to overcome obstacles in the identification and management of familial hypercholesterolemia since childhood. In 2018, the LIPIGEN pediatric group was constituted within the Italian LIPIGEN study to focus on FH subjects under 18 years. This work aimed at discussing its recent progress and early outcomes. Demographic, biochemical, and genetic baseline characteristics were collected, with an in-depth analysis of the genetic defects. The analysis was carried out on 1,602 children and adolescents (mean age at baseline 9.9 ± 4.0 years), and almost the whole cohort underwent the genetic test (93.3%). Overall, the untreated mean value of LDL-C was 220.0 ± 97.2 mg/dl, with an increasing gradient from subjects with a negative (N = 317; mean untreated LDL-C = 159.9 ± 47.7 mg/dl), inconclusive (N = 125; mean untreated LDL-C = 166.4 ± 56.5 mg/dl), or positive (N = 1,053; mean untreated LDL-C = 246.5 ± 102.1 mg/dl) genetic diagnosis of FH. In the latter group, the LDL-C values presented a great variability based on the number and the biological impact of involved causative variants. The LIPIGEN pediatric group represents one of the largest cohorts of children with FH, allowing the deepening of the characterization of their baseline and genetic features, providing the basis for further longitudinal investigations for complete details.

Keywords: familial hypercholesterolemia, pediatric cohort, genetic diagnosis, pathology register, clinical diagnosis, cardiovascular genetics

INTRODUCTION

Familial hypercholesterolemia (FH) in its heterozygous form is one of the most common inherited metabolic diseases. It is characterized by elevated serum levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) since birth, predisposing to premature atherosclerotic cardiovascular disease (ASCVD) (Fahed and Nemer, 2011; Nordestgaard et al., 2013)

This genetic defect is mainly due to pathogenic variants of the gene encoding for the LDL receptor (*LDLR*), which account for more than 90% of genetically confirmed FH cases and result in reduced uptake and clearance of LDL particles which accumulate in the plasma (Nordestgaard et al., 2013). Less common causes of FH are a few variants of the gene encoding for apolipoprotein B (*APOB*) that interfere with the binding of LDL to its receptor or gain-of-function variants in the gene encoding for proprotein convertase subtilisin/Kexin type 9 (*PCSK9*), a protein involved in the LDLR degradation (Nordestgaard et al., 2013). In the rare autosomal recessive form, variants in the LDL receptor adaptor protein gene (*LDLRAP1*) in the homozygous or compound heterozygous form can also result in an FH phenotype (Fellin et al., 2015; Defesche et al., 2017; Berberich and Hegele, 2019).

A high number of different causative genetic variants with a wide impact on the LDLR expression and function are responsible for the large heterogeneity of LDL cholesterol levels of FH in affected subjects. As such, inheritance of only one mutant allele (heterozygous FH, HeFH) results in a clinical phenotypic expression of variable severity, with physical signs of LDL deposition mainly evident between the third and sixth decade of life (Najam and Ray, 2015), making a prompt diagnosis of FH and treatment more difficult. On the other hand, a genotype with both mutated alleles (homozygous FH and HoFH), either with the same pathogenic variant (true homozygosity) or with different pathogenic variants of the same gene (compound heterozygosity) or of different genes (double heterozygosity), potentially translating into the total absence or defective activity of the LDLR, is characterized by a more severe clinical phenotype and worse prognosis since patients can present with cardiovascular events at a very young age and sometimes also during infancy (Bertolini et al., 2020).

The estimated prevalence of HeFH was reported to be 1:200-500, but recent reports showed a higher prevalence of 1:100-250 albeit not in all populations tested (Akioyamen et al., 2017; Bjornsson et al., 2021). Such a prevalence would expect to yield over 4.5 million patients in Europe and 35 million patients worldwide, of whom 20–25% are children and adolescents. Given the high prevalence of FH, it is estimated that one FH is born every minute (Wiegman et al., 2015; Pyles et al., 2017; Beheshti et al., 2020).

Clinical trials and epidemiology consistently show that lipid-lowering therapies reduce LDL-C and consequently cardiovascular mortality (Mach et al., 2020). Therefore, early detection and treatment in childhood/adolescence are crucial to achieving a normal life expectancy. Familial hypercholesterolemia, however, is still largely underdiagnosed and undertreated (Nordestgaard et al., 2013; Representatives of the Global Familial Hypercholesterolemia Community et al., 2020). Although in adults few diagnostic algorithms are available despite some limitations (Casula et al., 2018), the identification of FH in childhood is made more challenging by the lack of validated diagnostic criteria and the usually less severe physical manifestations of FH in the first decade of life, as a consequence of limited long-life exposure to high concentrations of LDL-C. However, the diagnostic challenges are not the only critical aspect in the management of FH in the pediatric population. Because of their age, children and adolescents need a more dedicated and tailored approach, addressing both the patients and their families, whether it is a dietary approach and lifestyle changes or a pharmacological approach. The need for research to progress both in providing the clinicians with tools to support the diagnosis and in guiding the tailoring of therapeutic interventions is quite compelling (Wiegman et al., 2015).

In the last decade, the implementation of local pathology registers allowed to focus on specific sub-groups as the one including FH subjects under 18 years (Gazzotti et al., 2020; Ramaswami et al., 2020; de Ferranti et al., 2021). National registers of FH play a key role in increasing the detection of patients with FH, understanding gaps in care, and improving management and outcomes. Here, we present the LIPIGEN pediatric group and the early outcomes of its work.

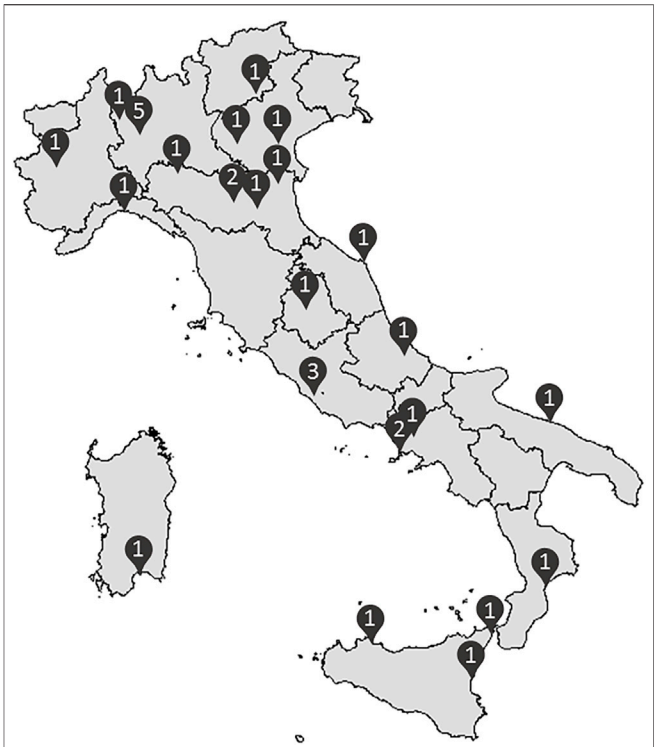

FIGURE 1 | Distribution of the 31 Italian lipid clinics involved in the LIPIGEN pediatric group (number of centers/provinces).

METHODS

In 2018, a subgroup of Italian lipid clinics involved in the LIPIGEN (LIpid TransPort Disorders Italian GEnetic Network) (Averna et al., 2017) study constituted the LIPIGEN pediatric group. The main aim of this sub-study, which includes both pediatric and adult centers that (even occasionally) manage FH subjects under 18 years, was to improve the detection, diagnosis, and management of children and adolescents affected by FH (Averna et al., 2017; Gazzotti et al., 2020).

In October 2021, the LIPIGEN pediatric group accounted for more than 1,600 subjects under 18 years with a clinical and/or genetic diagnosis of FH, followed up by 31 LIPIGEN sites in Italy (five of them specifically dedicated to the pediatric population) (**Figure 1**).

To be enrolled in the LIPIGEN pediatric group, subjects must be under 18 years old at their first presentation at the lipid clinic and receive a clinical and/or genetic diagnosis of FH, according to the specialist's judgment, in compliance with the LIPIGEN study protocol (Averna et al., 2017). As for the LIPIGEN parental study, demographic, anamnestic, biochemical, and genetic data were collected (Averna et al., 2017). Because of many data being retrospectively collected and referred to historical cohorts, the genetic test was not centralized, and the majority of pediatric samples were analyzed in the local laboratory of each lipid clinic.

Up to 2015, genetic analysis was based on Sanger sequencing of *LDLR, PCSK9,* and *APOB* (apolipoprotein B-binding region of the *APOB* gene to the LDLR). Southern blotting or multiplex ligation-dependent probe amplification (MLPA) was used for the detection of major rearrangements of the *LDLR* gene. Since 2015, the next-generation sequencing (NGS) methodology has been introduced for the simultaneous analysis of the major FH candidate genes (Bertolini et al., 2017; Bertolini et al., 2020); for copy number variations and nucleotide variations in splicing sites, the analysis of transcripts was conducted using Northern blotting and RT-PCR (Bertolini et al., 1999). In the case of compound heterozygous patients with two *LDLR* variants, to differentiate variants in *trans* from those in *cis*, the analysis was extended to the patient's first-degree relatives to verify familial segregation of the two variants (Bertolini et al., 2020).

We defined a subject as "positive" to the genetic analysis if at least one causative variant, pathogenic or likely pathogenic, was detected according to the ACMG Guidelines (Richards et al., 2015) and the more recent FH-specific suggestions (Chora et al., 2018; Chora et al., 2022); we defined a subject as "inconclusive" if only variants with uncertain clinical significance (VUS) were identified, and "negative" if only benign/likely benign or no variants in the tested genes were found (Richards et al., 2015; Chora et al., 2022) despite the clinical phenotype.

The assignment of pathogenicity was based, in addition to the aforementioned criteria, on *ex vivo* functional characterization of the LDLR activity using either cultured skin fibroblasts or immortalized blood lymphocytes, or *in vitro* LDLR activity validation in cells transfected with LDLR variants (Benito-Vicente et al., 2018), *in silico* analysis of variants using multiple algorithms, and search in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) and the recently updated *LOVD/LDLR* variant database (https://databases.lovd.nl/shared/genes/LDLR). Furthermore, *LDLR* variants were classified based on their impact on the LDLR activity: values lower than 2–5% were considered to be associated with no receptor activity (null variants), and values higher than 5% were associated with various degrees of reduced receptor activity (defective variants) (Bertolini et al., 1999; Bertolini et al., 2020).

Results for continuous variables are presented as mean (±SD) and median (with IQR), while categorical variables are presented as percentages and numbers. Mean differences between dichotomous variables or among categories were tested using the *t* test or ANOVA, respectively. All analyses were performed using IBM SPSS Statistics 27. Statistical significance was set at the 0.05 level for every analysis performed.

RESULTS

Overall, the LIPIGEN pediatric register included 1,602 subjects under 18 years with at least one untreated LDL-C measurement and genetic testing data available.

The percentages of males and females were comparable (49.9 and 50.1%, respectively). Two-third of the study population were index cases, while the others were identified through cascade screening, with no difference in the age at baseline (9.8 ± 3.9 vs. 10.0 ± 4.3 years, respectively, $p = 0.206$). Overall, the mean (±SD) age at baseline was 9.9 ± 4.0 years, and the study population was composed of 278 (17.3%) subjects within the age class 0–5 years, 605 (37.8%) in the class of 6–10 years, 381 (23.8%) of 11–13 years, and 338 (21.1%) of 14–17 years (**Table 1**).

Among the study cohort, 3.7% of subjects were already on lipid-lowering therapy at the first visit to one of the LIPIGEN centers (**Table 1**). The values of total cholesterol (mean ± SD) were 284.1 ± 98.0 mg/dl among subjects without any lipid-lowering therapy and 255.1 ± 54.2 mg/dl in treated subjects. LDL-C levels (mean ± SD) were 214.0 ± 100.0 mg/dl and 184.4 ± 51.1 mg/dl, HDL-cholesterol levels (mean ± SD) were 53.4 ± 13.6 mg/dl and 54.7 ± 13.5 mg/dl, and triglycerides (median [IQR]) were 75.0 [57.0–100.0] mg/dl and 74.0 [58.0–92.0], respectively (**Table 2**). Overall, the untreated mean value of LDL-C was 220.0 ± 97.2 mg/dl, with about 90% of individuals in each age group presenting untreated LDL-C >130 mg/dl.

Almost the whole cohort, 93.3% underwent genetic testing to identify the presence of variants in major candidate genes. About 70% of tested individuals (N = 1,053) presented at least one causative variant, while 125 individuals had an inconclusive diagnosis of genetically determined FH (VUS), and for 317 children/adolescents, no known genetic variants were identified to explain the clinical phenotype. Subjects with a positive genetic diagnosis presented higher untreated levels of LDL-C than subjects with only VUS or negative diagnosis (246.5 ± 102.1 mg/dl vs. 166.4 ± 56.5 mg/dl vs. 159.9 ± 47.7 mg/dl, respectively; $p < 0.0001$), while no significant differences in age at baseline were detected.

TABLE 1 | Characteristics of the LIPIGEN pediatric cohort.

Male, N (%)	800 (49.9)
Index cases, N (%)	1,093 (68.2)
Age at baseline [years], mean ± SD	9.9 ± 4.0
Age class 0–5, N (%)	278 (17.3)
Age class 6–10, N (%)	605 (37.8)
Age class 11–13, N (%)	381 (23.8)
Age class 14–17, N (%)	338 (21.1)
On lipid-lowering therapy at the first visit at the LIPIGEN center, N (%)	59 (3.7)

TABLE 2 | Lipid profile among untreated and treated subjects at the moment of the first visit at the LIPIGEN center.

	Without any lipid-lowering therapy (N = 1,543)	On lipid-lowering therapy (N = 59)
LDL cholesterol [mg/dL], mean ± SD	214.0 ± 100.0	184.4 ± 51.1
Total cholesterol [mg/dL], mean ± SD	284.1 ± 98.0	255.1 ± 54.2
HDL cholesterol [mg/dL], mean ± SD	53.4 ± 13.6	54.7 ± 13.5
Triglycerides [mg/dL], median [IQR]*	75.0 [57.0–100.0]	74.0 [58.0–92.0]

**IQR, interquartile range.*

TABLE 3 | Mean ± SD untreated LDL-C levels stratified by genetic diagnosis and age classes.

		Genetic diagnosis		
		Positive	Inconclusive	Negative
Untreated LDL-C [mg/dL]	0–5 years (N = 278)	273.1 ± 128.6	189.2 ± 66.0	173.6 ± 59.0
	6–10 years (N = 605)	248.2 ± 110.4	158.5 ± 40.4	159.1 ± 46.5
	11–13 years (N = 381)	237.3 ± 87.0	169.8 ± 49.4	156.2 ± 43.1
	14–17 years (N = 338)	230.3 ± 66.6	161.1 ± 67.0	157.5 ± 48.7
	Total	246.5 ± 102.1	166.4 ± 56.5	159.9 ± 47.7

By stratifying the mean untreated LDL-C levels by genetic diagnosis, values showed variability across age classes despite a significant difference only in the "positive" group ($p < 0.0001$) (**Table 3**).

Among subjects with a positive diagnosis of FH, 1,015 individuals presented one causative variant in *LDLR* (N = 1,000) or *APOB* (N = 15) genes, while in 38 subjects two causative variants were detected (17 *LDLR* homozygotes, 18 *LDLR* compound heterozygotes, and three double heterozygotes [*LDLR*/*APOB*: N = 2; *LDLR*/*PCSK9*: N = 1]) (**Supplementary Tables S1–3**). Among subjects with more than one causative variant, the mean untreated LDL-C value was 731.6 ± 154.0 mg/dl in homozygotes, 633.6 ± 211.3 mg/dl in compound heterozygotes, and 229.9 ± 47.0 mg/dl in double heterozygotes, while heterozygotes had a mean LDL-C level of 231.6 ± 53.4 mg/dl (*LDLR* heterozygotes: 231.9 ± 53.3 mg/dl and *APOB* heterozygotes: 208.5 ± 54.9 mg/dl).

In HeFH for *LDLR*, more than 200 different causative variants were detected, with the three most frequently reported being c.1646G > A p. Gly549Asp (N = 93), c.1775G > A p. Gly592GLu (N = 63), and c.662A > G p. Asp221GLy (N = 61). Within each variant, the mean untreated LDL-C levels were 245.0 ± 50.6 mg/dl (min 118 mg/dl, max 400 mg/dl), 198.7 ± 50.1 mg/dl (min 107 mg/dl, max: 367 mg/dl), and 212.2 ± 41.2 mg/dl (min 133 mg/dl, max 302 mg/dl), respectively. The higher levels of LDL-C in the carrier of the former (**Supplementary Figure S2**, panel A) could be explained by its impact on LDLR residual activity (null variant) compared to the other two receptor-defective variants (**Supplementary Figure S1**, panels B and C); nevertheless, large variability in the LDL-C values was observed even among carriers of the same variant.

By stratifying *LDLR* causative variants by the receptor residual activity, children/adolescent HeFH with a null variant (<5% residual activity) (N = 470, with more than 120 different variants) presented significantly higher levels of LDL-C than the carriers of a defective-receptor variant (N = 530, with more than 85 different variants): 245.1 ± 52.0 mg/dl vs. 220.2 ± 51.8 mg/dl ($p < 0.0001$), with no differences in the age at baseline (10.0 ± 4.3 vs. 9.8 ± 4.1 years, $p = 0.432$).

DISCUSSION

Pathology registers have proved to be very useful in providing evidence that can improve knowledge about the pathophysiological basis of diseases and can support clinicians in diagnosis and treatment (Kindt et al., 2017; Gazzotti et al., 2020); this is even more relevant in children. The detection of a

genetic severe form of hypercholesterolemia in childhood that implies an accelerated atherosclerotic process can lead to a prompt lipid-lowering treatment in order to reduce coronary heart disease in young adult age (Wiegman et al., 2015).

Recently, various pathology-specific registers for genetic dyslipidemia have been implemented (Collaboration et al., 2018). The LIPIGEN pediatric project is part of a well-established National Network (LIPIGEN project) aiming at improving and enhancing the knowledge of genetic dyslipidemia starting from the first decade of life (Averna et al., 2017). Data from our cohort can fully fit into the pathology register landscape (Kodra et al., 2018; Collaboration, 2021) as the UK National Pediatric Familial Hypercholesterolemia Register (Ramaswami et al., 2017), the Czech MedPed registry (Vrablik et al., 2017), or the Greek Pediatric FH Register (Mollaki et al., 2013). These local experiences and their incorporation into international collaborations of country-specific databases, such as the International Pediatric FH register (Ramaswami et al., 2020), result in a large-scale real-world data collection, necessary to fill the gaps in knowledge, and allow to compare different approaches on identification and treatment of FH.

In our cohort, genetic analysis has been performed on 93.3% of subjects. A pathogenic variant has been identified in 70% of children/adolescents, so the percentage of patients under 18 years with a genetic confirmation is comparable to that highlighted in other European pediatric cohorts, such as the UK (67%), but lower than the Czech cohort (85%) and the pooled pediatric data from eight European countries (88%), where 93.5% of patients had genetic analysis performed (Futema et al., 2021). This variability strongly depends on the inclusion criteria and on the screening strategy implemented in each country. In Italy, the current screening strategy is not universal screening but targeted cascade screening for the identification of potential FH subjects.

The characteristics of the Italian cohort are very similar to those of other pediatric cohorts, for example, in terms of the mean age at diagnosis (10 years old in LIPIGEN, a range of 8–11 years in the European cohorts (Ramaswami et al., 2020), and 9 years in the CASCADE-FH Registry youth participants (de Ferranti et al., 2021)). In fact, in these cohorts, subjects are identified both through opportunistic screening and cascade screening starting from index cases in the family. Instead, much lower is the average age at diagnosis in those cohorts that provide for systematic screening in pre-school children, as in Greece (3 years) (Mollaki et al., 2013) and Slovenia (6 years) (Groselj et al., 2018).

Mean levels of untreated LDL-C were also comparable to those of other pediatric cohorts: 220 mg/dl in LIPIGEN, a range of 188–240 mg/dl in the European cohorts (Ramaswami et al., 2020), and 238 mg/dl in the CASCADE-FH Registry youth participants (de Ferranti et al., 2021)). Moreover, also in our study population, the most common cause of FH was the presence of at least one causative pathogenic variant in the *LDLR* gene while the prevalence of carriers of a heterozygous causative variant on the *APOB* gene was 1.4%. The most prevalent variant in our Italian pediatric cohort was c.1646G > A p. Gly549Asp, a disruptive-missense variant that showed reduced LDL uptake in an *in vitro* study (Bertolini et al., 1999; Thormaehlen et al., 2015); this is one of the most frequent variants also among LIPIGEN adults (untreated mean LDL-C 275 mg/dl). In LIPIGEN children carrying this null variant, the mean untreated LDL-C level was 245 mg/dl, similar to those detected in other European cohorts (256 mg/dl) (Futema et al., 2021). A lower mean untreated LDL-C value was detected among LIPIGEN children/adolescents with the defective variant p. Gly592GLu (198 mg/dl) but still comparable with carriers from another European cohort (199 mg/dl). Moreover, the stratification of *LDLR* heterozygous variants by LDLR residual activity confirmed in children a more severe phenotype among carriers of a null variant compared to carriers of a defective variant (Bourbon et al., 2017).

The large sample size is one of the strengths of our study that enables us to provide an accurate report of the Italian scenario. The presence of the LIPIGEN network as the root of the LIPIGEN pediatric group is an added value as the LIPIGEN network is well-established and geographically well-distributed in all the country, involving lipid clinics with long-time experience. In these centers, expert lipidologists have been dealing with genetic dyslipidemias for decades, so they have been able to properly carry on the data collection, support analysis strategies and also suggest modulation of the collection of family and medical history, adding specific pediatric items to the standard clinical and biochemical data collection (e.g., family history extended to second-degree relatives, the lipid profile of the affected parent, and the age of puberty onset). The combination of these new data with the ones that are being collected about the follow-up of FH pediatric patients will also allow evaluating longitudinal outcomes, such as the effect of lipid-lowering therapies in this age group and the response to treatment, in terms of real-life effectiveness and safety.

The main limitation to our register is that by its nature, it is an opportunistic sample and is likely to be biased toward children from more severely affected families than FH children in the general population. Moreover, the partially retrospective nature of the data collection generated a certain number of missing data. However, researchers are actively working toward information retrieval and standardization.

In conclusion, data collected in the LIPIGEN pediatric network are consistent with those of the main pathology specific registers and allow to better focus on children and adolescents with FH, characterizing their baseline and genetic features, providing the basis for further longitudinal investigations and representing an opportunity to deepen the analysis of the genotype and phenotype of children with FH. The collection of follow-up information and the implementation of the register will help us in improving diagnosis and management standards for FH in Italy starting from childhood.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to Manuela Casula, manuela.casula@unimi.it.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Institutional Review Board of IRCSS Multimedica (as coordinating center, on 23/06/2015) and by the Institutional Review Boards of each participating center. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MC, CP, and ALC were responsible for the study concept and design. MG, MC, and MEC were responsible for study management and data collection. EO provided methodological and statistical knowledge and performed the analysis. SB provided genetic counseling for the interpretation of the results. MG and MC wrote the manuscript, and all authors critically revised for important intellectual content and approved the final manuscript.

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The Prevalence and Genetic Spectrum of Familial Hypercholesterolemia in Qatar Based on Whole Genome Sequencing of 14,000 Subjects

*Ilhame Diboun[1,2], Yasser Al-Sarraj[3], Salman M. Toor[1], Shaban Mohammed[4], Nadeem Qureshi[5], Moza S. H. Al Hail[4], Amin Jayyousi[6], Jassim Al Suwaidi[7] and Omar M. E. Albagha[1,8]**

[1]College of Health and Life Sciences (CHLS), Hamad Bin Khalifa University (HBKU), Qatar Foundation (QF), Doha, Qatar, [2]Medical and Population Genomics Lab, Sidra Medicine, Doha, Qatar, [3]Qatar Genome Program, Qatar Foundation Research, Development and Innovation, Qatar Foundation (QF), Doha, Qatar, [4]Department of Pharmacy, Hamad Medical Corporation, Doha, Qatar, [5]Primary Care Stratified Medicine Research Group, Centre for Academic Primary Care, School of Medicine, University of Nottingham, Nottingham, United Kingdom, [6]Department of Diabetes, Hamad Medical Corporation (HMC), Doha, Qatar, [7]Adult Cardiology, Heart Hospital, Hamad Medical Corporation (HMC), Doha, Qatar, [8]Centre for Genomic and Experimental Medicine, MRC Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, United Kingdom

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***Correspondence:**
Omar M. E. Albagha
oalbagha@hbku.edu.qa

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Familial hypercholesterolemia (FH) is an inherited disease characterized by reduced efficiency of low-density lipoprotein-cholesterol (LDL-C) removal from the blood and, consequently, an increased risk of life-threatening early cardiovascular complications. In Qatar, the prevalence of FH has not been determined and the disease, as in many countries, is largely underdiagnosed. In this study, we combined whole-genome sequencing data from the Qatar Genome Program with deep phenotype data from Qatar Biobank for 14,056 subjects to determine the genetic spectrum and estimate the prevalence of FH in Qatar. We used the Dutch Lipid Clinic Network (DLCN) as a diagnostic tool and scrutinized 11 FH-related genes for known *pathogenic* and *possibly pathogenic* mutations. Results revealed an estimated prevalence of 0.8% (1:125) for definite/probable cases of FH in the Qatari population. We detected 16 known *pathogenic/likely pathogenic* mutations in *LDLR* and one in *PCSK9;* all in a heterozygous state with high penetrance. The most common mutation was rs1064793799 (c.313+3A >C) followed by rs771019366 (p.Asp90Gly); both in *LDLR*. In addition, we identified 18 highly penetrant *possibly pathogenic* variants, of which 5 were Qatari-specific, in *LDLR*, *APOB*, *PCSK9* and *APOE*, which are predicted to be among the top 1% most deleterious mutations in the human genome but further validations are required to confirm their pathogenicity. We did not detect any homozygous FH or autosomal recessive mutations in our study cohort. This pioneering study provides a reliable estimate of FH prevalence in Qatar based on a significantly large population-based cohort, whilst uncovering the spectrum of genetic variants associated with FH.

Keywords: dyslipidemias, hypercholesterolemia, familial hypercholesterolemia, monogenic, FH, LDL-C, LDLR

INTRODUCTION

Familial hypercholesterolemia (FH) is a common autosomal disease characterized by elevated levels of low-density lipoprotein cholesterol (LDL-C), leading to an increased risk of atherosclerosis and premature coronary heart disease (CHD) (Bouhairie and Goldberg, 2015). The prevalence of FH in Caucasian populations has been typically considered around 1: 500 (Austin et al., 2004), but more recent estimates show around 1: 310 prevalence, with up to a 20-fold higher prevalence in those with premature CHD (Beheshti et al., 2020; Hu et al., 2020). Indeed, a recent study based on 225 Chinese subjects with premature myocardial infarction revealed up to 23.6% prevalence of FH (Cui et al., 2019). Notably, FH has a strong genetic basis compounded by environmental factors and its prevalence varies amongst different populations. Homozygous FH (HoFH) is rare; typically considered to affect 1 in 1,000,000 worldwide, but recent reports have estimated a higher prevalence of 1 in 160,000–300,000 (Cuchel et al., 2014; Singh and Bittner, 2015; Sjouke et al., 2015). HoFH is characterized by a drastic increase in LDL-C (>13 mmol/L before therapy) and the early development of atherosclerotic complications (Cuchel et al., 2014). Heterozygous FH (HeFH) is more common with an estimated prevalence of 1 in 250 in European populations (Vrablik et al., 2020). However, variations in FH prevalence have been reported in certain populations such as 1 in 137 in the Danish population (Benn et al., 2012), 1 in 270 in the United Kingdom population (Wald et al., 2016), and 1 in 311 in the Russian population (Meshkov et al., 2021). Notably, FH is considerably higher in certain populations such as Ashkenazi Jews (1 in 67) or Christian Lebanese (1 in 85) and South African Afrikaners (1 in 72), attributing to the founder effect which contributes to the significantly higher incidence (Austin et al., 2004; Henderson et al., 2016).

Mutations in *LDLR*, *APOB* and *PCSK9* account for most FH cases and have been reported in both HoFH and HeFH. These three genes are members of the low-density lipoprotein receptor (LDL-R) pathway, which is responsible for maintaining healthy levels of plasma and intracellular cholesterol in the body. The vast majority of *LDLR* variants are deleterious (79%), affecting the splicing or regulatory aspects of early transcription or alternatively, leading to a defective receptor function (Henderson et al., 2016). Owing to the extensive spectrum of *LDLR* variants in FH, mutations in the *LDLR* gene can be population specific (Vrablik et al., 2020). Less stricter forms of FH have been associated with variants in *APOB*, a gene whose protein product helps LDL bind to its receptor LDL-R, but it is observed in about 5% of FH cases in European populations (Henderson et al., 2016; Vrablik et al., 2020). Mutations leading to a gain of function of *PCSK9*, an enzyme that promotes the degradation of LDL-R, could account for <1% of FH cases (Henderson et al., 2016). Less commonly observed variants of FH have been described in the *LDLRAP1* gene causing an autosomal recessive form of the disease termed autosomal recessive hypercholesterolemia (ARH) (Garcia et al., 2001). Interestingly, ARH is characterized with LDL-C levels in between the HeFH and HoFH known levels, and unlike HoFH, the early-onset phenotype is comparatively rare (Henderson et al., 2016). Further to monogenic variants, polygenic effects have also been described to account for hypercholesterolemia (HC) cases (Saadatagah et al., 2021). Notably, more than 900 lipid-associated genomic loci have been identified in genome-wide studies (GWAS) based on investigating genetic variants in diverse ancestries (Graham et al., 2021).

The most widely accepted guidelines for the diagnosis of FH include the Simon-Broome Register criteria (Mortality, 1999), the Dutch Lipid Clinic Network (DLCN) criteria (Umans-Eckenhausen et al., 2001) and the US MedPed program (Williams et al., 1993). The DLCN criteria is a comprehensive tool, which considers multiple parameters in the assessment of FH including patients' personal and family history related to the onset of early cardiovascular (CVD) or HC, untreated LDL-C concentration, disease clinical manifestations (including tendinous xanthomas and arcus cornealis) and functional mutation in a pathogenic FH-related gene (Defesche et al., 2004). However, despite the comprehensiveness of available diagnostic schemes, FH remains largely underdiagnosed.

The prevalence of FH in Qatar is not yet known. However, FH prevalence (probable and definite cases) in neighboring Gulf countries (Saudi Arabia, Oman, United Arab Emirates, Bahrain and Kuwait) was estimated at 1:232 (Al-Rasadi et al., 2020) and subsequently stratified to fulfill the DLCN criteria to reveal a markedly higher prevalence of 1:112, but genetic screening was not performed in this study (Alhabib et al., 2021). The high burden of lipid disorders in the region has also led to continued recommendations to improve the management of lipid disorders (Alsayed et al., 2022). In addition, accumulating studies have also explored the genetic spectrum of FH in the wider Middle Eastern region (Awan et al., 2019), with reports of mutations occurring predominantly in *LDLR* (Alhababi and Zayed, 2018). Notably, a recent study reported mutations in 6 *LDLR* variants in a subset of ~6,000 individuals from Qatar, but no phenotypic analyses or evidence were sought for FH (Elfatih et al., 2021). Moreover, GWAS have also linked specific lipid risk variants to various populations in the region (Hebbar et al., 2019), specifically in Qatar (Thareja et al., 2021). Therefore, comprehensive investigations into FH etiology in Qatar are warranted.

In this study, we estimated the prevalence of FH in Qatar, while uncovering the mutational spectrum and penetrance of population-specific FH-related monogenic variants using whole-genome sequencing of the population-based cohort of Qatar biobank (QBB; n = 13,808). We sought to decipher mutations in genes previously linked with FH pathogenicity and identify highly penetrant Qatari-specific variants. Our findings have the potential to empower the current diagnostic approaches by allowing targeted genetic screening of high-risk individuals and cascade screening of suspected FH cases.

MATERIALS AND METHODS

Study Populations

This study was based on 14,056 Qatari subjects from the population-based Qatar Biobank (QBB) study (Al Kuwari

et al., 2015) and was executed under ethical approvals from the institutional review boards of QBB, Doha, Qatar (Protocol no. E-2020-QF-QBB-RES-ACC-0154-0133) and Hamad Bin Khalifa University (Approval no. 2021-03-081). All participants provided written informed consent prior to sample donation. Participants also underwent a medical examination and filled out an approved and standardized questionnaire, which captured information on medical history. The study cohort is deeply phenotyped, featuring lipid-related measurements including total cholesterol, HDL-C, LDL-C, and triglycerides, and detailed information on diagnosis, comorbidities related to heart disease and administration of cholesterol-lowering medication was also accessible. However, while information on the family history of HC was recorded, lipid measurements from close relatives were not available.

Whole-Genome Sequencing

Whole-genome sequencing of the cohort was available through the Qatar Genome Program (QGP) and was performed as previously described (Mbarek et al., 2022). Briefly, libraries were constructed using the Illumina TruSeq DNA nano kit and indexed using Illumina TruSeq Single Indexes (Illumina, San Diego, CA, United States). Quality-passed libraries were sequenced on an Illumina HiSeq X instrument. The quality metrics for generated Fastq files were assessed using FastQC (v. 0.11.2). The raw reads were trimmed and aligned to hs37d5 reference genome using bwa.kit (v0.7.12) (Li and Durbin, 2009) to generate mapped reads on BAM files. The coverage of each sample was evaluated using Picard (v1.117) (CollectWgsMetrics), while variant calling was performed using base quality score recalibration (BQSR) and intermediate genomic gVCF (gVCF) was generated by running HaplotypeCaller (Genome Analysis Toolkit; GATK). The genotype data were processed for quality control using Hail (Team, 2022) and Plink (Slifer, 2018). Genetic variants with <98% call rate, chi-square test p value for Hardy-Weinberg equilibrium $<1 \times 10^{-10}$, or those with a depth of coverage <10X were removed, while only variants with minor allele frequency <0.005 were included in the analysis, calculated based on estimating FH prevalence of ~1 in 200. Subjects with excess heterozygosity, gender ambiguity, or with call rate <95% were also excluded. The final study cohort comprised 13,808 subjects with both whole-genome sequence data and complete phenotype data.

Familial Hypercholesterolemia Evaluation Criteria

The DLCN scheme draws evidence from high LDL-C levels, the presence of functional mutations in *LDLR*, *APOB* or *PCSK9* genes, xanthomas and corneal deposits of fat, and evidence of coronary and vascular disease in subjects and their close relatives for diagnosing FH (Austin et al., 2004). The assessment of the phenotypic or genetic parameters assigns numerical scores to classify subjects with unlikely FH (DLCN score: <3), possible FH (DLCN score: 3–5), probable FH (DLCN score: 6–8) or definite FH (DLCN score >8) (Austin et al., 2004). We followed these criteria to characterize FH cases, while performing correction of LDL-C levels for cholesterol-lowering medication as defined by Haralambos *et al.* (Haralambos et al., 2015). In instances where the medication dose was not available, we used the minimum dose for correction. Of note, the presence of xanthomas and corneal fat deposition was not recorded by QBB and these parameters were not taken into consideration in our study. Importantly, the remaining criteria were sufficient to classify patients into definite/probable/possible or unlikely FH cases based on DLCN scores.

Variant Annotation and Assessment of Pathogenicity

We used the bcftools software (version 1.10.2) to extract records of variants located in selected FH genes from the variant calling format (VCF) genotype file. The selection of FH genes was based on a literature search and included *LDLR*, *APOB*, *PCSK9*, *APOE*, *LDLRAP1*, *CYP7A1*, *STAP1*, *ITIH4*, *EPHX2*, *GHR* and *PPP1R17* (also known as *GSBS*) genes (Takada et al., 2003; Fujita et al., 2004; Sato et al., 2004; Paththinige et al., 2017; Wang et al., 2020). Variant annotation was performed using SnpEff/SnpSift (v4.3t) (Cingolani et al., 2012) utilizing dbSNP build version 151 (Sherry et al., 1999), ClinVar (Landrum et al., 2018), Human Gene Mutation Database (HGMD) variant categorization (Stenson et al., 2020) and Leiden Open Variation Database (version: 3.0; LOVD3) (Fokkema et al., 2021). Variants were considered pathogenic for FH if reported as *pathogenic/likely pathogenic* or *disease-causing mutation (DM)* or *likely disease-causing mutation (DM?)* by at least two of the databases described earlier. We assessed the penetrance of genetic variants using two approaches; the first was based on the DLCN score (DLCN-penetrance), which takes into consideration multiple phenotypic variables related to FH risk as described earlier. Using this approach, penetrance was defined as the proportion of mutation carriers with a DLCN score ≥3. The second approach was based on LDL-C and self-reported HC (HC-penetrance) by which penetrance was defined as the proportion of mutation carriers with LDL-C > 3.3 mmol/L, or taking cholesterol-lowering medication, or self-reported HC. For variants of uncertain significance (VUS), we considered them *possibly pathogenic* if their HC-penetrance value was >50% and their predictive combined annotation dependent depletion (CADD) (Kircher et al., 2014) score was greater than 20, which represents the top 1% most deleterious mutations in the human genome.

Statistical Analysis

The Hardy-Weinberg equilibrium of genotypes was evaluated by $\chi 2$ tests. Unpaired Student's t test was used for comparison between individuals with definite, probable, and possible FH relative to those with unlikely FH. The prevalence of each definition of FH was estimated as a percentage for all study subjects. Fisher's exact test was used to compare the frequency of factors among the definitions of FH. A gene-based burden test was performed using SAIGE-Gene (Zhou et al., 2020) by collapsing all *pathogenic/possibly pathogenic* variants detected in each gene into a single burden variable, and in this analysis

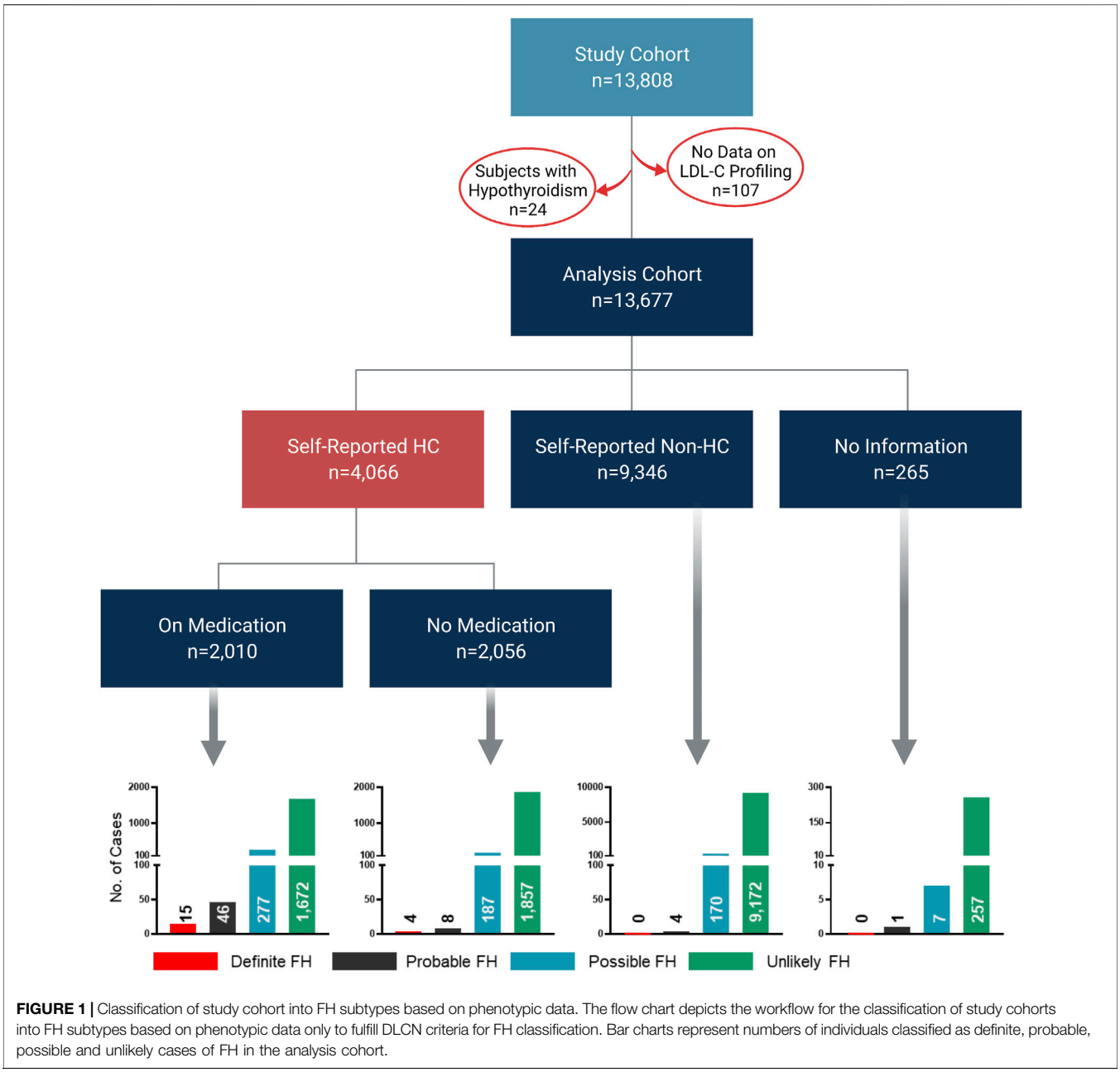

FIGURE 1 | Classification of study cohort into FH subtypes based on phenotypic data. The flow chart depicts the workflow for the classification of study cohorts into FH subtypes based on phenotypic data only to fulfill DLCN criteria for FH classification. Bar charts represent numbers of individuals classified as definite, probable, possible and unlikely cases of FH in the analysis cohort.

model, we adjusted for age, sex, genomic kinship and the first four population principal components.

RESULTS

Dutch Lipid Clinic Network Classification Based on Phenotypic Traits

This study was based on phenotypic and genotypic data from 13,808 individuals. However, the analysis cohort comprised 13,677 subjects since LDL-C profiling measurements were not available for 107 subjects, while additional 24 subjects with hypothyroidism were removed from the analysis (**Figure 1**). We first categorized our cohort based on self-reports of HC; 4,066 subjects declared HC, while 9,346 subjects reported no HC and the remaining 265 subjects did not provide any information. Moreover, among the self-reported HC subjects, 2,010 subjects declared taking cholesterol-lowering medication. Importantly, our initial classifications showed that 19 subjects were classified as definite cases of FH based on the fulfillment of the DLCN criteria (DLCN score >8) by phenotypic evidence only (**Figure 1**). Similarly, probable, possible and unlikely FH cases were also first classified based on phenotypic evidences only. The characteristic features of the overall study cohort classified into FH categories based on phenotypic evidence are listed in

TABLE 1 | Characteristics of Qatar Biobank study subjects according to the Dutch Lipid Clinic Network (DLCN) criteria based on phenotypic traits only.

Phenotypic trait(s)	Definite FH	Probable FH	Possible FH	Unlikely FH
Characteristic features				
n	19	59	641	12,958
Gender (males/females)	7/12	30/29	362/279	5,680/7,278
Age (years)	47.9 ± 12.2*	47.7 ± 11.9*	46.4 ± 11.6*	39.7 ± 13.1
BMI (kg/m^2)	30.3 ± 4.5	30.0 ± 5.8	30.1 ± 5.4*	29.5 ± 6.1
Smoker (%)	6 (31.6%)	8 (13.6%)	145 (22.6%)*	2,175 (16.8%)
Medical history				
Hypertension (%)	4 (21.1%)	23 (39.0%)*	160 (25.0%)*	1989 (15.3%)
Diabetes mellitus (%)	9 (47.4%)*	30 (50.8%)*	197 (30.7%)*	2,563 (19.8%)
Self-reported hypercholesterolemia (HC)	19 (100%)*	53 (89.8%)*	450 (70.2%)*	3,438 (26.5%)
Age at HC diagnosis (years)	35.5 ± 11.6	38.3 ± 10.2	39.6 ± 10.4	40.6 ± 10.6
Cholesterol-lowering medication	15 (78.9%)*	46 (78.0%)*	277 (43.2%)*	1,672 (12.9%)
History of myocardial infarction (MI)	0 (0.0%)	3 (5.1%)*	31 (4.8%)*	35 (0.3%)
Age at MI (years)	n/a	37.3 ± 13.5	44.6 ± 8.0	49.5 ± 14.9
History of angina	0 (0.0%)	0 (0.0%)	21 (3.3%)*	24 (0.2%)
Paternal heart disease (%)	14 (73.7%)*	18 (30.5%)	168 (26.2%)*	2,760 (21.2%)
Maternal heart disease (%)	7 (36.8%)*	12 (20.3%)	109 (17.0%)*	1,615 (12.5%)
Lipid profile				
Total cholesterol (mmol/L)	8.35 ± 2.03*	7.16 ± 1.37*	6.41 ± 1.16*	4.82 ± 0.86
HDL-C (mmol/L)	1.36 ± 0.28	1.30 ± 0.34*	1.29 ± 0.37*	1.41 ± 0.39
LDL-C (mmol/L)	9.83 ± 1.01*	8.46 ± 1.88*	5.51 ± 0.93*	3.02 ± 0.82
Triglycerides (mmol/L)	2.16 ± 1.05*	1.81 ± 0.78*	1.72 ± 0.85*	1.27 ± 0.73
DLCN Score	9 ± 0*	6.8 ± 1.0*	3.5 ± 0.7*	0.4 ± 0.6

†*Continuous traits are given as mean ± standard deviation from the mean.*Statistically significant (P <0.05) compared to unlikely FH.*

Table 1. Of note, the definite FH cases cohort comprised individuals with considerably higher percentages of self-reported HC and a history of paternal and maternal heart disease compared to those in the unlikely FH group (**Table 1**). Moreover, subjects in the definite FH group had a lower age of HC diagnosis (35.5 ± 11.6) compared to subjects in the unlikely FH category (40.6 ± 10.6), but this was of borderline significance (p = 0.06).

Familial Hypercholesterolemia Pathogenic Variants Detected in the Qatari Population

Applying the DLCN criteria for FH diagnosis entails the identification of functional mutations in *LDLR*, *APOB* and *PCSK9* genes. To this end, variants were scrutinized for their pathogenicity using ClinVar, LOVD3 and HGMD significance terms to identify previously reported *pathogenic* variants in the Qatari population. Our search for variants covered 11 FH-related genes (refer to methods). We detected 17 variants that were reported as *pathogenic/likely pathogenic* or *DM/DM?* by at least two of the ClinVar/LOVD3/HGMD databases (**Table 2**); all as heterozygous. Notably, 16 variants were located in *LDLR* (**Figure 2**) with rs1064793799 (c.313+3A>C) being the most frequent mutation (n = 13), followed by rs771019366 (p.Asp90Gly; n = 6) and rs747507019 (p.His327Tyr; n = 4), while one mutation was located in *PCSK9* (rs891322948, n = 2). Moreover, the majority of these were missense mutations, which lead to specific protein changes while others were splice variants or associated with regulatory regions. Most mutations showed high DLCN-penetrance (>50%) and the majority had complete HC-penetrance (100%). We did not detect any previously reported *pathogenic* mutations in *APOB*, *APOE*, *LDLRAP1*, *CYP7A1*, *STAP1*, *ITIH4*, *EPHX2*, *GHR*, or *PPP1R17*.

Possibly Pathogenic Variants in the Qatari Population

We identified 18 *possibly pathogenic* variants as those which were predicted to be in the top 1% of most deleterious mutations in the human genome (CADD score >20) and their HC-penetrance is ≥50% (**Table 3**). These mutations were detected in FH-related genes including *LDLR*, *APOB*, *PCSK9* and *APOE*, and featured a wide range of deleterious effects, including stop-gained and missense, in addition to impacting regulatory features. Five of the 18 detected variants were novel since they were not reported in genetic databases such as dbSNP and the Genome Aggregation Database (gnomAD), and were considered to be Qatari-specific. The most common *possibly pathogenic* variants were located in *APOB* (rs775231207; n = 7, rs13306190; n = 4, and rs12713559; n = 4). Overall, the majority of mutations observed were missense, while one stop-gain was detected in *PCSK9*. Notably, rs1230170597 is located 169 bp upstream of the coding region of *LDLR* and overlaps *LDLR-AS1*, an antisense non-coding RNA predicted to downregulate production of the LDL-R. However, these *possibly pathogenic* mutations were not used in DLCN classification because functional validation will be required to confirm their pathogenicity. Gene-based burden test LDL-R for the *pathogenic* and *possibly pathogenic* variants detected in our cohort showed significant associations with HC for *LDLR* (p <0.0001; Beta (β) = 3.2; standard error (SE) = 0.52), *PCSK9* (p = 0.004; β = 2.6; SE = 1.08), and *APOB* (p = 0.004; β = 1.1; SE = 0.37) but not for *APOE* (p = 0.303). Genetic variations that fit the

TABLE 2 | Known *pathogenic* variants detected in the study subjects.

Gene	SNP	Position	Ref	Alt	Protein change	No. Het	MAF	HGMD class.	LOVD class.	ClinVar sig.	DLCN-penetrance (%)	HC-penetrance* (%)
LDLR	rs879254375	chr19:11089414	C	G	TF-binding	1	3.41×10^{-5}	DM	LP	VUS	100	100
LDLR	rs776421777	chr19:11100246	G	A	p.Glu31Lys	2	6.82×10^{-5}	DM	LP	LP/VUS	0	100
LDLR	rs879254420	chr19:11100324	G	A	p.Asp57Asn	1	3.41×10^{-5}	DM?	LP	P/LP/VUS	100	100
LDLR	rs730882078	chr19:11102714	C	T	p.Arg81Cys	1	3.41×10^{-5}	DM	LP	NR	0	100
LDLR	rs771019366	chr19:11102742	A	G	p.Asp90Gly	6	2.05×10^{-4}	DM	LP	P/LP	83.3	100
LDLR	rs1064793799	chr19:11102789	A	C	Splice variant	13	4.43×10^{-4}	DM	NR	P	84.6	100
LDLR	rs730882090	chr19:11107420	C	A	p.Phe282Leu	1	3.41×10^{-5}	DM	LP	P/LP/VUS	100	100
LDLR	rs112366278	chr19:11110650	A	C	Splice variant	1	3.41×10^{-5}	DM	LP	P/LP	100	100
LDLR	rs746834464	chr19:11110660	G	A	p.Glu317Lys	3	1.02×10^{-4}	DM?	LP	P/LP	0	33.3
LDLR	rs747507019	chr19:11110690	C	T	p.His327Tyr	4	1.36×10^{-4}	DM	LP	VUS	25	100
LDLR	rs752951310	chr19:11111598	G	T	p.Gly382Val	3	1.02×10^{-4}	DM	P/LP	P/LP/VUS	66.7	100
LDLR	rs879254809	chr19:11111607	T	G	p.Leu385Arg	1	3.41×10^{-5}	DM	LP	LP	100	100
LDLR	rs373646964	chr19:11113650	G	A	p.Asp492Asn	1	3.41×10^{-5}	DM	LP	NR	100	100
LDLR	rs758194385	chr19:11116198	A	G	p.Asn564Ser	2	6.82×10^{-5}	DM	LP	P/LP	50	50
LDLR	rs763147599	chr19:11116927	G	A	p.Gly592Arg	2	6.82×10^{-5}	DM	P	P/LP	50	50
LDLR	rs750518671	chr19:11128085	G	A	p.Val797Met	1	3.41×10^{-5}	DM	LP	NR	100	100
PCSK9	rs891322948	chr1: 55059529	G	T	p.Gly516Val	2	6.82×10^{-5}	DM?	LP	NR	100	100

*Alt, alternative allele; DM, disease-causing mutation; DM?, likely disease-causing mutation; FH, familial hypercholesterolemia; Het, heterozygous; HGMD, Human Gene Mutation Database; LP, likely pathogenic; LOVD, Leiden Open Variation Database; MAF, minor allele frequency; NR, not reported; P, pathogenic; Ref, reference allele; VUS, variant of uncertain significance; *HC, high cholesterol (refer to methods of details). Protein positions are in reference to the NCBI sequence NP_000518.1.*

"*possibly pathogenic*" criteria were not detected in the other FH-related genes: *LDLRAP1*, *CYP7A1*, *STAP1*, *ITIH4*, *EPHX2*, *GHR*, or *PPP1R17*.

Comparing Familial Hypercholesterolemia Classification Based on Phenotypic and Genotypic Profiles of Study Subjects

Assessment of QBB study participants using DLCN criteria after adding information from genotypes and known FH-pathogenic mutations revealed 52 subjects as definite cases of FH (**Table 4**). Moreover, the probable, possible and unlikely FH cases classified based on combining phenotypic and genotypic evidence also showed some differences in numbers as opposed to classification based on FH-associated phenotypes only (**Tables 1**, **4**). A number of probable FH (n = 8), possible FH (n = 14) and unlikely FH (n = 10) subjects were re-classified as definite FH because they were found to carry one of the known *pathogenic* mutations listed in **Table 2**. Additionally, 6 subjects who were originally

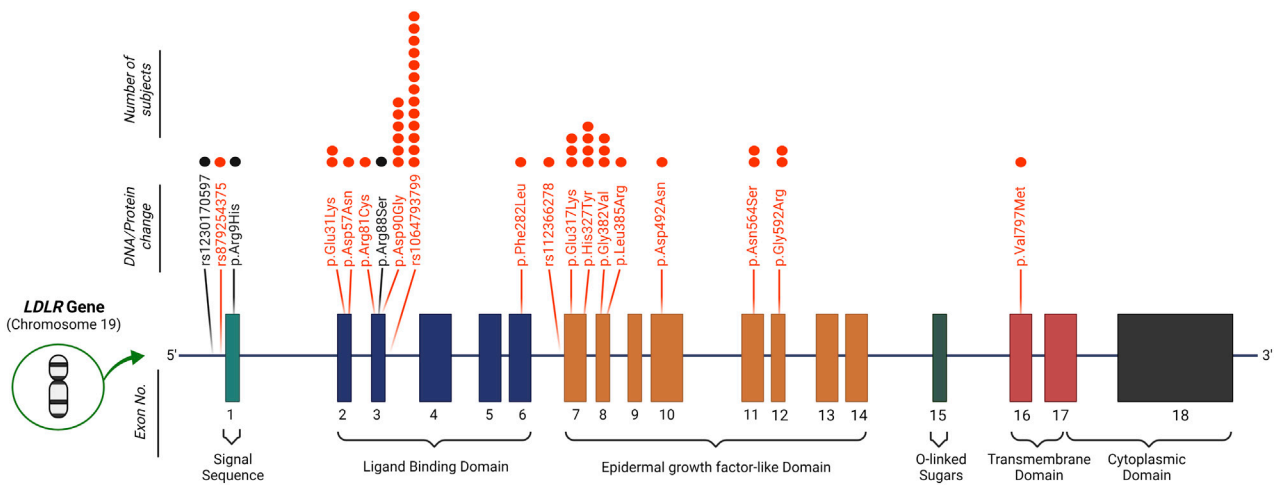

FIGURE 2 | Identified mutations in the *LDLR* gene. 16 known *pathogenic* variants (marked in red) and 3 *possibly pathogenic* mutations (marked in black) were detected in *LDLR* in our study cohort. The number of subjects carrying each mutation is depicted by dots above each variant. Protein change is in reference to the NCBI sequence NP_000518.1.

TABLE 3 | *Possibly pathogenic* variants with high penetrance detected in the study subjects.

Gene	Variant ID	Position	Ref	Alt	Protein change	No. het	MAF	HGMD class.	ClinVar sig.	LOVD class.	HC-penetrance (%)	CADD score
LDLR	rs1230170597	chr19:11089380	G	C	Promoter	1	3.41×10^{-5}	NR	VUS	NR	100	20.5
LDLR	**NR**	**chr19:11089574**	**G**	**A**	**p.Arg9His**	**1**	**3.41×10^{-5}**	**NR**	**NR**	**NR**	**100**	**22.8**
LDLR	rs879254454	chr19:11102737	G	T	p.Arg88Ser	1	3.41×10^{-5}	NR	VUS	NR	100	21
APOB	rs267599185	chr2:21013214	G	A	p.Arg1388Cys	2	6.82×10^{-5}	NR	VUS	LB	50	20.7
APOB	**NR**	**chr2:21006246**	**A**	**G**	**p.Ile3541Thr**	**2**	**6.82×10^{-5}**	**NR**	**NR**	**NR**	**50**	**23.3**
APOB	**NR**	**chr2:21008255**	**A**	**T**	**p.Asn2871Lys**	**1**	**3.41×10^{-5}**	**NR**	**NR**	**NR**	**100**	**22**
APOB	rs1440306074	chr2:21012016	A	T	p.Ser1618Thr	1	3.41×10^{-5}	NR	NR	NR	100	22.8
APOB	rs775231207	chr2:21012456	A	C	p.Leu1471Trp	7	2.30×10^{-4}	NR	NR	NR	57	21.8
APOB	rs1208454201	chr2:21012474	A	G	p.Met1465Thr	2	6.82×10^{-5}	NR	NR	NR	50	21
APOB	rs140877474	chr2:21012493	T	C	p.Ser1459Gly	2	6.82×10^{-5}	NR	VUS	NR	50	25.5
APOB	**NR**	**chr2:21015242**	**A**	**G**	**p.Phe1176Ser**	**1**	**3.41×10^{-5}**	**NR**	**NR**	**NR**	**100**	**32**
APOB	rs765952330	chr2:21015514	C	G	p.Gly1122Arg	2	6.82×10^{-5}	NR	NR	NR	50	25.2
APOB	rs781511068	chr2:21023005	G	A	p.Val881Ala	2	6.82×10^{-5}	NR	VUS	NR	50	27
APOB	rs13306190	chr2:21032408	G	A	p.Ala433Val	4	1.36×10^{-4}	NR	LB	NR	50	24.9
APOB	rs12713559	chr2:21006196	G	A	p.Arg3558Cys	4	1.36×10^{-4}	DM	LP/VUS	P/VUS	50	29.5
PCSK9	rs185392267	chr1:55043921	C	T	p.Arg96cys	3	1.02×10^{-4}	DM	P/ LP/VUS	P/VUS	100	24.1
PCSK9	rs373323910	chr1:55061437	C	T	p.Arg582*	2	6.82×10^{-5}	NR	LB	NR	50	34
APOE	**NR**	**chr19: 44909102**	**G**	**A**	**p.Arg269His**	**1**	**3.41×10^{-5}**	**NR**	**NR**	**NR**	**100**	**24.8**

*Alt, alternative allele; CADD, combined annotation dependent depletion; DM, disease-causing mutation; DM?, likely disease-causing mutation; FH, familial hypercholesterolemia; Het, heterozygous; HGMD, Human Gene Mutation Database; LP, likely pathogenic; LOVD, Leiden Open Variation Database; MAF, minor allele frequency; NR, not previously reported; P, pathogenic; Ref, reference allele; VUS, variant of uncertain significance; *HC, high cholesterol (refer to methods for details). Genomic positions are in reference to GRCh38 genome build, and protein positions are in reference to the NCBI sequence NP_000518.1. Bold indicates novel variants not reported in genetic variation databases.*

classified as unlikely FH were re-classified as probable FH after considering genetic data. The proportions of smokers, myocardial infractions and family history of heart disease was considerably higher in definite cases of FH, while age at diagnosis of HC was significantly lower than those with unlikely FH (**Table 4**). Next, we investigated differences in lipid profile measurements of FH definition categorized by the DLCN criteria based on phenotypic and genotypic data (**Figure 3**). We found that total cholesterol, LDL-C and triglyceride levels were significantly higher in definite, probable and possible FH cases compared to subjects with unlikely FH (**Figure 3**). Moreover, HDL-C levels were significantly lower in probable and possible FH groups compared to unlikely FH.

Estimating the Prevalence of Familial Hypercholesterolemia in Qatar

Of the total 13,677 subjects used in this study, we identified 109 as definite or probable cases of FH based on the fulfillment of the DLCN criteria (**Figure 3**). Based on this, the prevalence of FH in Qatar was therefore estimated at 0.8% (1 in 125). Notably, 39 subjects were diagnosed as definite cases of FH (DLCN score >8) based on genotypic mutation and phenotypic evidence, while additional 13 subjects showed a DLCN score >8 based on phenotype alone and did not carry any of the known *pathogenic* FH variants. Combined these definite FH cases accounted for 52 subjects and yielded an overall prevalence of 0.38% (~1 in 263) for definite FH (**Figure 4**). In contrast, the number of subjects with probable FH (DLCN score ranging between 6–8), who carried a known *pathogenic* mutation but showed no phenotypic evidence, was 6, while 51 subjects were classified as probable FH based on phenotype. These subjects accounted for approximately half of the total suspected FH cases in our cohort. Of note, the prevalence of *possibly pathogenic* variants was 1:351 and the overall prevalence of FH in Qatar would be considerably higher (~1 in 92) when these mutations are included in the estimation of the prevalence. However, due to the lack of evidence on the pathogenicity of these variants, further investigations are required to incorporate them in the assessment of population-based FH prevalence. In addition, we did not detect any homozygous FH or autosomal recessive mutations in our study cohort, indicating their low prevalence in our study population.

DISCUSSION

This study provides the first reliable estimate of the prevalence of FH in the Qatari population and presents a comprehensive survey of population-specific monogenic FH variants in a considerably large population-based cohort. Combining FH-related phenotypes with whole-genome sequence data revealed a prevalence of definite and probable FH cases in Qatar of ~1 in 125, which puts Qatari subjects at a higher risk of FH than the global average of 1:250, previously determined from a meta-analysis of 19 published studies (Akioyamen et al., 2017), but at a moderately lower risk than neighboring countries in the Arabian peninsula (1:112) (Alhabib et al., 2021). The higher prevalence than global estimates could be attributed to the relatively

TABLE 4 | Characteristics of study subjects classified according to the Dutch Lipid Clinic Network (DLCN) criteria based on phenotypic and genotypic data.

Phenotypic trait(s)	Definite FH	Probable FH	Possible FH	Unlikely FH
Characteristic features				
n	52	57	627	12,942
Gender (males/females)	25/27	30/27	352/275	5,672/7,270
Age (years)	42.6 ± 13.2	46.5 ± 12.0*	46.6 ± 11.5*	39.7 ± 13.1
BMI (kg/m^2)	29.6 ± 5.7	29.6 ± 5.7	30.1 ± 5.4*	29.5 ± 6.1
Smoker (%)	16 (30.8%)*	10 (17.5%)	139 (22.2%)*	2,169 (16.8%)
Medical history				
Hypertension (%)	9 (17.3%)	23 (40.3%)*	160 (25.5%)*	1984 (15.3%)
Diabetes mellitus (%)	17 (32.7%)*	26 (45.6%)*	195 (31.1%)*	2,562 (19.8%)
Self-reported hypercholesterolemia (HC)	42 (80.8%)*	51 (89.4%)*	455 (72.6%)*	3,519 (27.2%)
Age at HC diagnosis (years)	31.8 ± 10.9*	38.5 ± 9.9	39.8 ± 10.3	40.6 ± 10.6
Cholesterol-lowering medication	31 (59.6%)*	42 (73.7%)*	269 (42.9%)*	1,668 (12.9%)
History of myocardial infarction (MI)	2 (3.8%)*	1 (1.7%)	31 (4.9%)*	35 (0.3%)
Age at MI (years)	29.5 ± 9.5	53	44.6 ± 8.0	49.5 ± 14.9
History of angina	1 (1.9%)	0 (0.0%)	20 (3.2%)*	24 (0.2%)
Paternal heart disease (%)	28 (53.8%)*	15 (26.3%)	162 (25.8%)*	2,755 (21.3%)
Maternal heart disease (%)	9 (17.3%)	11 (19.3%)	108 (17.2%)*	1,615 (12.5%)
DLCN Score	11.8 ± 2.9*	6.9 ± 1.0*	3.5 ± 0.7*	0.4 ± 0.6

†*Continuous traits are given as mean ± standard deviation from the mean. *Statistically significant (P < 0.05) compared to unlikely FH.*

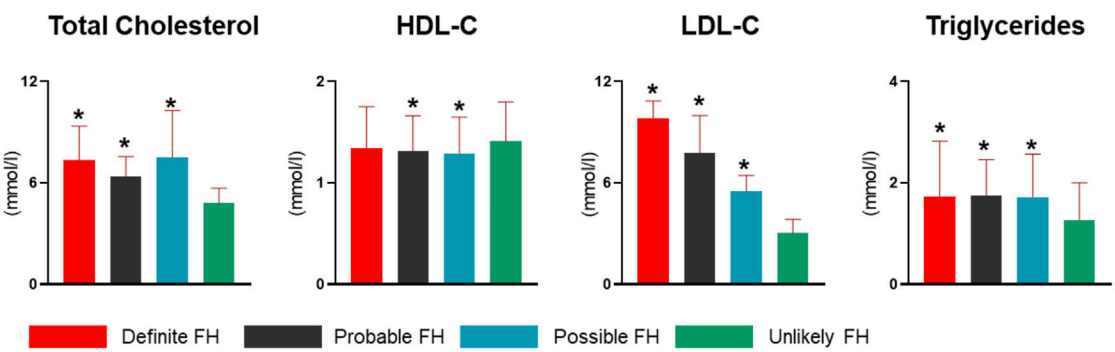

FIGURE 3 | Lipid profiles of FH definition groups in the study cohort. Study subjects were categorized into definite, probable, possible and unlikely FH cases based on DLCN criteria, and using phenotype and genotype data. Bar charts represent levels of total cholesterol, HDL-C, LDL-C and triglycerides. LDL-C measurements were corrected for medication. Statistically significant (P < 0.05) differences among each FH definition compared to unlikely FH are denoted with an asterisk (*) in each plot.

consanguineous nature of the populations in the region and in Qatar, but the prevalence is considerably lower than those reported for populations with founder effects such as the African Ashkenazi and Lebanese (Seftel et al., 1989; Abifadel et al., 2009). In addition, we also did not detect any cases of HoFH, indicating its rare prevalence in Qatar.

We identified 17 mutations, characterized in the literature as *pathogenic*, out of which 16 were located in *LDLR*. Notably, 6 of these variants in *LDLR* were previously reported in ~6000 subjects from Qatar but without any phenotypic associations with FH (Elfatih et al., 2021). In contrast, some of these 16 variants we identified have been directly associated with FH phenotypes in other populations. The p.Asp90Gly mutation in *LDLR* was reported in phenotypic FH patients from Western Australia who were screened for *LDLR*, *APOB* and *PCSK9* mutations (Hooper et al., 2012), while p.Glu31Lys has been reported in Asian Indian FH patients (Setia et al., 2020) and p.Phe282Leu was observed in Czech probands suspected to have FH (Tichý et al., 2012). Additionally, data from 6 studies investigating the influence of genotypes on response to PCSK9-targeting monoclonal antibody alirocumab also reported *LDLR* pGly382Val among the mutations identified in 898 HC patients (Defesche et al., 2004). While these mutations have been linked with FH, some of these variants are associated with severe disease complications related to FH. For instance, Cui *et al.* reported *LDLR* p.Arg81Cys mutation in patients with premature myocardial infarction (Cui et al., 2019).

Subjects carrying any of the known *pathogenic* mutations and with the presentation of FH-related phenotypes were identified as definite FH according to the DLCN. Notably, the definite FH identified in our study comprised a significantly higher proportion of subjects with self-reported HC and more importantly, a higher proportion of myocardial infarction and those diagnosed with HC at a younger age compared to subjects classified as unlikely FH. Moreover, a family history of heart disease was also considerably higher in

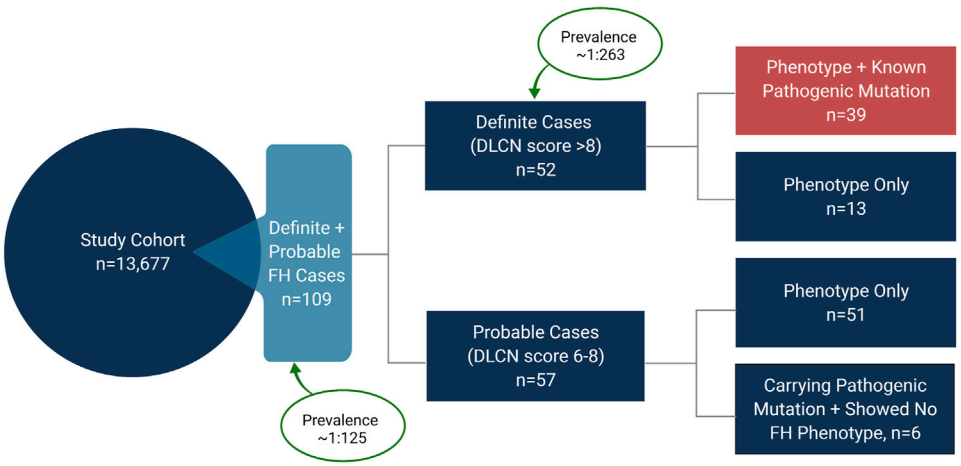

FIGURE 4 | Prevalence of familial hypercholesterolemia (FH) in Qatar. The study cohort comprised 13,677 individuals. 109 subjects were identified as definite and probable cases of FH based on the Dutch Lipid Clinic Network (DLCN) criteria, yielding a prevalence of ~1 in 125 in Qatar. 52 subjects were identified as definite cases of FH, indicating a prevalence of ~1 in 263 for definite FH. Of the 52 definite cases, 39 subjects carried a known *pathogenic* mutation in *LDLR* or *PCSK9* and showed FH-related phenotypes. 57 subjects were identified as probable cases of FH with 51 subjects showing FH-related phenotypes (DLCN score 6–8), while 6 subjects carried a known *pathogenic* mutation (DLCN score 8), but their LDL-C levels were below the threshold of 4.0 mmol/L defined by DLCN criteria.

definite FH cases. These clinical manifestations and prerequisites for FH classifications supported their classification into definite cases of FH. Considering the genotypic aberrations, the most frequently occurring variant was rs1064793799 found in 13 Qatari FH subjects, suspected to cause abnormal gene splicing. Single base changes occurring near or within introns can lead to intron retention, exon skipping, or activation of cryptic splice sites (Bourbon et al., 2009). Notably, an extreme reduction in *LDLR* expression, recorded in two families of Arab descent, was attributed to the activation of a cryptic splice acceptor site in *LDLR* due to a single substitution mutation (Shawar et al., 2012), however, this mutation was not detected in our study. In contrast, the majority of the mutations we detected in *LDLR* corresponded to missense mutations, which may be detected via PCR-based genotyping protocols for FH detection. For instance, Pandey et al. (2016) validated the detection of mutations in *APOB* and *LDLR*, by PCR, including p.Asp492Asn, also detected in our cohort. We also detected a *pathogenic* variant in *PCSK9* (p.Gly516Val) in two subjects with definite FH. This variant was detected in South African FH patients and was found to be pathogenic by functional studies (Huijgen et al., 2021).

Our study highlighted the importance of genetic testing to confirm FH diagnosis since only 19 subjects were classified as definite FH based on phenotype data while additional 33 subjects were considered definite FH when genetic testing was taken into consideration.

We also detected several *possibly pathogenic* variants in *LDLR, APOB, PCSK9* and *APOE* in our study subjects which were not previously confirmed in the literature as disease-causing mutations. These variants, however, were predicted to be among the top 1% of most deleterious mutations in the human genome and showed high penetrance for HC in our study. These variants could be presented as novel variants related to FH, however, further functional investigations are warranted for their validation. Notably, all variants detected in *APOB* were point mutations that could lead to defects in lipid hemostasis. Point mutations in *APOB* have been reported to cause FH by affecting its affinity for the LDL-R, which causes disruptions in LDL clearance via LDL-R-mediated internalization (Sharifi et al., 2017). These mutations differ from truncation mutations in *APOB* associated with hypobetalipoproteinemia. However, further functional investigations are warranted to confirm their pathogenicity in FH. Notably, five of the *possibly pathogenic* variants were novel and appear to be predominant in the Qatari population. Novel Qatari-predominant loci have been identified for many clinically relevant traits in a recent GWAS study of the QBB study participants (Thareja et al., 2021). A gene-based burden test for the *pathogenic* and *possibly pathogenic* variants in *LDLR*, *PCSK9* and *APOB* confirmed the association of these variants with HC. However, the gene-based burden test was not significant for *APOE* possibly because of the small number of *pathogenic* variants detected in our cohort. Overall, our findings reiterate that mutations in *LDLR* are the most common cause of FH in the Qatari population.

In this study, a substantially large cohort of 13,677 was used to achieve a reliable estimate for the prevalence of definite and probable cases of FH in the Qatari population, which was approximated to 1 in 125. The prevalence of subjects with *possibly pathogenic* variants was 1:351 and if future functional studies confirm the pathogenicity of these variants, the overall prevalence of FH will be 1:92 which is higher than our original estimate of 1:125 based on known *pathogenic* variants. We did not detect any cases of HoFH in our study cohort which indicates that HoFH is extremely rare in Qatar, as estimated in many European populations, but a

larger sample size would be required to accurately estimate the prevalence of HoFH. However, one key limitation of our data collection was the lack of phenotypic data related to xanthomas and arcus cornealis for subjects or their first-degree relatives in our cohort. In addition, we did not investigate structural variants in FH-related genes, however, FH is predominantly caused by point mutations and a small fraction of FH cases are attributed to structural variants in FH-related genes. Overall, we detected 17 known *pathogenic* mutations in FH subjects and identified further 18 *possibly pathogenic* variants as novel candidates for FH. However, further functional investigations are warranted to investigate their pathogenicity. Overall, the clinical translation of FH-related variants reported herein may be explored further to design FH diagnostic tools.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The raw whole-genome sequence data from Qatar Biobank are protected and are not available due to data privacy laws. Access to QBB/QGP phenotype and whole-genome sequence data can be obtained through an ISO-certified protocol, which involves submitting a project request at https://www.qatarbiobank.org.qa/research/how-apply, subject to approval by the Institutional Review Board of the QBB. Requests to access these datasets should be directed to https://www.qatarbiobank.org.qa/research/how-apply.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Qatar Biobank, Hamad Bin Khalifa University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ID: data curation, formal analysis, investigation, and writing—original draft. YA-S: investigation and methodology. ST: writing—review and editing and visualization. NQ: writing—review and editing. SM, MA, AJ, and JA: resources. OMEA: conceptualization, funding acquisition, formal analysis, supervision, investigation, and writing—review and editing.

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Do Biobank Recall Studies Matter? Long-Term Follow-Up of Research Participants With Familial Hypercholesterolemia

*Miriam Nurm[1,2], Anu Reigo[1], Margit Nõukas[1,3], Liis Leitsalu[1], Tiit Nikopensius[1], Marili Palover[1,3], Tarmo Annilo[1], Maris Alver[1,4], Aet Saar[5,6], Toomas Marandi[5,6], Tiia Ainla[5,6], Andres Metspalu[1,3], Tõnu Esko[1] and Neeme Tõnisson[1,7]**

[1]Institute of Genomics, University of Tartu, Tartu, Estonia, [2]Institute of Technology, University of Tartu, Tartu, Estonia, [3]Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia, [4]Department of Genetic Medicine and Development, University of Geneva, Geneva, Switzerland, [5]Department of Cardiology, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia, [6]Cardiology Centre, North Estonia Medical Centre, Tallinn, Estonia, [7]Genetics and Personalized Medicine Clinic, Tartu University Hospital, Tartu, Estonia

Recall-by-genotype (RbG) studies conducted with population-based biobank data remain urgently needed, and follow-up RbG studies, which add substance to this research approach, remain solitary. In such studies, potentially disease-related genotypes are identified and individuals with those genotypes are recalled for consultation to gather more detailed clinical phenotypic information and explain to them the meaning of their genetic findings. Familial hypercholesterolemia (FH) is among the most common autosomal-dominant single-gene disorders, with a global prevalence of 1 in 500 (Nordestgaard et al., Eur. Heart J., 2013, 34 (45), 3478–3490). Untreated FH leads to lifelong elevated LDL cholesterol levels, which can cause ischemic heart disease, with potentially fatal consequences at a relatively early age. In most cases, the pathogenesis of FH is based on a defect in one of three LDL receptor-related genes–*APOB*, *LDLR*, and *PCSK9*. We present our first long-term follow-up RbG study of FH, conducted within the Estonian Biobank (34 recalled participants from a pilot RbG study and 291 controls harboring the same *APOB*, *LDLR*, and *PCSK9* variants that were included in the pilot study). The participants' electronic health record data (FH-related diagnoses, lipid-lowering treatment prescriptions) and pharmacogenomic risk of developing statin-induced myopathy were assessed. A survey was administered to recalled participants to discern the impact of the knowledge of their genetic findings on their lives 4–6 years later. Significant differences in FH diagnoses and lipid-lowering treatment prescriptions were found between the recalled participants and controls (34 and 291 participants respectively). Our study highlights the need for more consistent lipid-lowering treatment adherence checkups and encourage more follow-up RbG studies to be performed.

Keywords: familial hypercholesterolemia (FH), lipid lowering treatment, recall-by-genotype, participant survey, biobank to practice, long-term survey, pharmacogenomic risk assessment

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****Correspondence:***
Neeme Tõnisson
neeme.tonisson@ut.ee

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Abbreviations: ATC/DDD, Anatomical Therapeutic Chemical/Defined Daily Dose; EHR, electronic health record; EstBB, Estonian Biobank; FH, familial hypercholesterolemia; ICD-10, International Classification of Diseases (10th Revision); LDL-C, low-density lipoprotein cholesterol; LLT, lipid-lowering treatment; RbG, recall-by-genotype; SNP, single nucleotide polymorphism.

INTRODUCTION

Recall-by-genotype (RbG) studies have proven value as population-based biobank studies conducted with a "genotype-first" approach. Individuals harboring high-risk genetic variants are first identified by genomic data and then stratified based on clinical phenotypic information and electronic health record (EHR) data, with the identification of groups in need of medical attention. Such studies have been performed successfully at the Estonian Biobank (EstBB) (Leitsalu et al., 2016; Alver et al., 2018; Leitsalu et al., 2020) and elsewhere (Haukkala et al., 2013; Stessman et al., 2014; Ormondroyd et al., 2020), and have been shown to benefit those carrying deleterious genetic variants. Although RbG studies are gaining more traction in the scientific community (Corbin et al., 2018), long-term follow-up studies of this type remain scarce due to the novelty of the concept.

The RbG approach is most beneficial when applied to the investigation of disorders that are present in sufficient frequency in the study population and for which actionable treatment options are available. Familial hypercholesterolemia (FH) is among the most common known autosomal-dominant single-gene disorders, with a prevalence of 1 in 500 globally and 1 in 200 in Northern European populations (Nordestgaard et al., 2013; Benn et al., 2016). In most cases, the pathogenesis of FH is caused by a defect in one of the three low-density lipoprotein receptor-related genes: *LDLR*, *APOB*, and *PCSK9* (Berberich and Hegele, 2019).

Despite its high rate of occurrence worldwide and readily available treatment (Gidding et al., 2015), FH is often underdiagnosed and undertreated; many patients with this disease receive suboptimal or delayed treatment, without the necessary attention given to genetic factors that may influence its course. Thus, treatment goals remain inadequate for long periods and, without genetic diagnosis, may not follow established guidelines (Cuchel et al., 2014). When left untreated, FH leads to the lifelong elevation of low-density lipoprotein cholesterol (LDL-C) levels, which can cause ischemic heart disease, potentially resulting in early death (Nordestgaard et al., 2013; Gidding et al., 2015). Moreover, lipid-lowering treatment (LLT) adherence tends to be inadequate among FH patients, despite its proven efficacy (Casula et al., 2016; Langslet et al., 2021). This reflects a further need for continuous FH education and follow-through.

Provided that the genotype data is available, primary prevention of FH could also start from pre-symptomatic genetics-first screening applied on a general population-wide biobank, and then be directed to families via cascade screening. To increase effectiveness, universal blood lipid measurements at a pre-defined (and still reasonably early) age should complement the strategy for those not yet reached via family members (Groselj et al., 2022).

The objectives of this follow-up study were to assess the long-term impacts of an RbG FH study (Alver et al., 2018) by surveying recalled participants 4–6 years after the initial return of their results at the EstBB, and to analyze FH-related diagnostic and treatment adherence data retrieved from the healthcare system from a period of up to 18 years encompassing the study period. The findings provide a longer-term perspective on the RbG study approach and the Estonian healthcare systems' handling of such information.

MATERIALS AND METHODS

The original study from which this follow-up study derived was conducted in 2016–2018 with the objectives of identifying EstBB participants with FH-associated gene variants based on sequencing data, and recalling carriers for biochemical analyses and medical examination to detect early FH manifestations (Alver et al., 2018). Additionally, family members were recruited through cascade screening, resulting in a final cohort of 41 confirmed FH variant carriers. All participants received feedback at the end of their visits, with explanation of their genetic findings and final diagnoses and recommendations for further treatment plans.

Cohort Overview

The EstBB currently has more than 200,000 participants (about 20% of Estonia's adult population) for whom multilevel molecular and phenotype data have been collected. These data are not only used by the scientific community, but also have potential for practical population-level healthcare applications. When joining the biobank, all participants provided broad written consent, allowing the EstBB to re-contact them and update their data through EHR and national health registry linkage (Leitsalu et al., 2020).

In the pilot RbG study (Alver et al., 2018), high-coverage sequencing data (available for 4,776 individuals) and Sanger sequencing were used to identify and confirm FH-associated variant carriers (n = 51). Thirty-four of these recalled individuals who continued to be EstBB participants were included in this follow-up study. Using genotype and imputed data (available for 201,146 EstBB participants), we identified carriers with similar genetic backgrounds (based on 14 genetic variants considered in the pilot study; Alver et al., 2018) to form a control group (n = 291) of individuals not yet recalled for the disclosure of their carrier status (**Supplementary Figure S1**). Clinical histories from linked EHRs were available for individuals in both groups; the workflow of the study can be seen in **Figure 1**.

Genotyping and Imputation

Genome and exome sequencing data for the primary study cohort (n = 4,776), including custom quality-control and variant-annotation pipeline outputs (Alver et al., 2018), were retrieved. Genotyping of the EstBB samples was performed using Illumina global screening arrays (v. 1.0 and 2.0; Illumina Inc., San Diego, CA, United States) at the Core Genotyping Lab of the Institute of Genomics, University of Tartu, Estonia. Quality control criteria

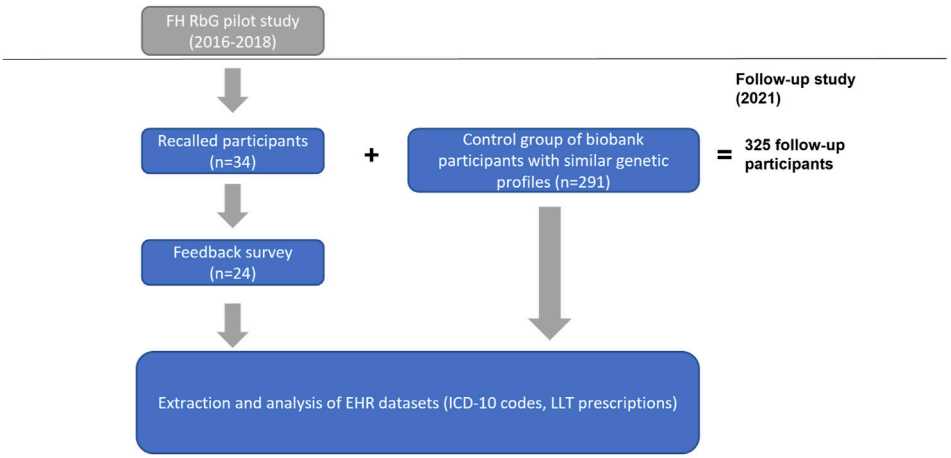

FIGURE 1 | The workflow of the study. The initial cohort of recalled participants originated from the FH RbG pilot study conducted by Alver et al. in 2016–2018. Recalled participants who had also signed up as biobank participants by Fall 2021 were eligible to participate in the follow-up.

for the exclusion of individual data from genotype-based analyses were call rate < 95% and sex mismatch between genotype and phenotype data. Before imputation, the variants were filtered by call rate <95%, Hardy-Weinberg equilibrium $p < 1 \times 10^{-4}$ (for autosomal variants), and minor allele frequency <1%. The Eagle software (v. 2.3; Loh et al., 2016) was applied for prephasing and the Beagle software (v. 28Sep18.793; Browning and Browning, 2007) was used for imputation based on the Estonian population-specific reference panel built from 2,297 genome sequencing samples (Mitt et al., 2017). We screened the available direct SNP genotyping and imputed data for 11 FH-associated variants considered by Alver et al. (2018). Priority was given to datasets in which individuals' carrier status was identified from both directly genotyped and imputed data (n = 225). A subset of individuals in whom the variants of interest were identified either from directly genotyped or imputed data (n = 66) were added for validation. Sanger sequencing confirmed FH-associated variants in a total of 291 persons.

EHR Data Linkage

EHRs covering a period of up to 18 years (2004–2022) were retrieved for all study participants as described by Leitsalu et al. (2015). All biobank participants had consented to make their EHRs available for scientific research. The participants' general information, ICD-10 diagnosis codes, and drug prescription data (ATC/DDD codes, dosage, and purchase) were obtained from the EHRs and used for analysis.

Participant Survey

Of the 34 participants recalled in the pilot RbG study, 32 were recontacted by mail and asked to fill out a questionnaire (**Supplementary Data 1**) 4–6 years after their initial recall. The remaining two recalled participants were excluded from the survey because of outdated contact information and death in 2018, respectively. The participants were given 14 days to return the questionnaire; in the event of nonresponse, they were contacted by telephone. Further nonresponse was taken to indicate that participants had declined to take part in the follow-up study.

The questionnaire consisted of 40 items regarding the perceived usefulness of the genetic feedback that participants had received (including their understanding of the information), the impact that knowledge of their genetic findings had had on their lives (including adherence to their treatment plans), the participants' assessment of the healthcare system's efficiency in integrating this information, and whether they had experienced potential FH complications after their feedback visits. Item responses were converted to a numeric scale ranging from 1–5, with values <3 signifying negative attitudes and those >3 signifying positive attitudes toward the item statements. The survey was developed in Estonian and contained questions inspired by published survey instruments (Marteau and Bekker, 1992) and analogous studies (Brehaut et al., 2003).

LLT Adherence

To visualize LLT adherence, a Gantt chart was created in Python using the plotly[1] library (v. 5.6.0, 2015; Plotly Technologies Inc., Montreal, QC, Canada) displaying the periods of LLT use from 2004 to March 2022 among recalled participants with LLT prescriptions. These periods were defined from the date of drug purchase, recorded in the drug prescription registry, to the end date of prescription consumption, calculated as the number of packages multiplied by the number of pills and added to the purchase date as the number of days (thus assuming that participants consumed one tablet per day). This formula was adjusted as needed on a case-by-

[1]https://plot.ly

TABLE 1 | FH-associated genetic variants detected in the follow-up cohort. Participants with *LDLR* p.Val436Ala (rs779732323) and *PCSK9* p.Ala103Ser (novel) were assigned to the control group because they declined visitation offered by Alver et al. (2018) in 2016–2018. *One individual had both *APOB* p.Arg3527Gln (rs5742904) and *LDLR* p.His250Arg (rs1256668310) variants. SNV, single nucleotide variant; rs, dbSNP reference number; RbG, recall by genotype; GS, genome sequencing; ES, exome sequencing.

Gene RefSeq Protein ID	SNP	RbG participants	Controls	Sum	Alver et al. (2018) (GS/ES)
APOB* *NP_000375*		**22**	**176**	**198**	
	p.Arg3527Gln (rs5742904)	20	176	196	11
	p.Gly861Glu (rs1663664782)	1	0	1	
	p.Cys4217Alafs3* (novel)	1	0	1	
LDLR* *NP_000518*		**11**	**112**	**123**	
	p.His250Arg (rs1256668310)	1	22	23	2
	p.Leu401His (rs121908038)	1	2	3	2
	p.Ala431Ser (rs28942079)	1	13	14	1
	p.Arg633His (rs754536745)	1	0	1	1
	p.Cys329Tyr (rs761954844)	3	72	75	5
	p.Arg215Cys (rs764042910)	2	1	3	1
	p.Gly396Ala (rs766474188)	1	0	1	1
	p.Arg115Cys (rs774723292)	1	0	1	1
	p.Val436Ala (rs779732323)	0	2	2	1
PCSK9 *NP_777596*		**1**	**4**	**5**	
	p.Arg357Cys (rs148562777)	1	3	4	1
	p.Ala103Ser (novel)	0	1	1	

case basis; for example, when a participant purchased several prescriptions on the same day, the start dates for subsequent packages were adjusted based on the consumption end dates for previous packages. Consistent LLT use was defined as use without a gap >6 months.

Pharmacogenomics

EstBB participants' genotype data were translated into pharmacogenomic risk phenotypes for 11 clinically important pharmacogenes using a pipeline developed by Reisberg et al. (2019). In the current study, results for the *SLCO1B1* gene were used to assess the risk of statin-induced side effects.

Pharmacogenomic profiles were created for the 27 recalled LLT users and included the following information: age, sex, FH-associated genetic variant, LLT adherence, previous FH-related medical history (ICD-10 codes), statin-adjusted (divided by 0.8 and 0.7, respectively) total cholesterol and LDL-C values measured during primary feedback visits, statin-induced myopathy risk, and an overview of survey responses about treatment plan adherence and health (**Supplementary Table S1**). LLT adherence was represented graphically using Python with the matplotlib library (v. 3.3.2; Hunter, 2007). Differences between the recalled and control groups were examined with Langsrud[2]'s two-tailed Fisher's exact test calculator, which uses Agresti (1992) as a reference.

The protocol (and further amendments) for this study was approved by the Ethics Review Committee on Human Research of the University of Tartu and Estonian Committee on Bioethics and Human Research (approval no 1.1-12/3015).

[2]https://langsrud.com/fisher.htm

RESULTS

Cohort Overview

In total, this study included 325 EstBB participants harboring FH-associated variants [34 recalled participants from the original RbG study (Alver et al., 2018) and 291 non-recalled controls]. A summary of the FH-associated variants identified is provided in **Table 1**. Of the participants, 60.9% (n = 198) were carriers of an *APOB* variant, 42.3% (n = 123) harbored an *LDLR* variant, and 1.7% (n = 5) were carriers of a *PCSK9* variant. The FH-associated variants identified most frequently in the follow-up cohort were p.Arg3527Gln (rs5742904) in the *APOB* gene and p.Cys329Tyr (rs761954844) in the *LDLR* gene. Three rare FH-associated variants not described by Alver et al. (2018) were identified from sequencing data in this follow-up cohort. The general characteristics of the recalled and non-recalled groups were comparable (**Table 2**).

Clinical Profiles of Recalled and Non-Recalled FH Variant Carriers

The most relevant FH-associated findings from participants' pharmacogenomic profiles are summarized in **Table 3**. The recalled cohort had significantly more diagnoses of lipoprotein metabolism disorders (ICD-10 E78*) and pure hypercholesterolemia (ICD-10 E78.0; the same code used for FH in clinical practice) than did the non-recalled group (94.1%

TABLE 2 | Cohort characteristics. RbG, recall-by-genotype; BMI, body mass index.

	RbG participants (n = 34)	Controls (n = 291)
Gender–female (%)	18 (52.9%)	186 (63.9%)
Age (range)	Median 49.5 (29–84)	Median 49 (21–100)
BMI (range)	Mean 25.5 (18.2–44.3)	Mean 26.3 (17.2–50.8)
Smoking (%)		
Never	19 (55.9%)	138 (47.4%)
Former	7 (20.6%)	70 (24.1%)
Current	8 (23.5%)	67 (23.0%)
Unknown	0	16 (5.5%)

vs. 67.0%, $p < 0.001$ and 82.4% vs. 46.0%, $p < 0.001$, respectively). Furthermore, the recalled group contained significantly more LLT users than did the non-recalled group (79.4% vs. 53.3%, $p < 0.005$). Statin-induced myopathy risks were similar in the recalled and non-recalled groups (higher than normal, 29.4% and 28.5%, respectively; much higher than normal, 2.9% and 2.4%, respectively).

LLT Adherence

The majority (59.3%) of participants continued LLT with the medications first prescribed to them. Atorvastatin was prescribed at least once to 70.4%, rosuvastatin to 63.0%, and medication combinations (rosuvastatin + fenofibrate or rosuvastatin + ezetimibe) to 14.8% of all participants (**Figure 2**).

The LLT users were allocated to three groups characterized by:

1) poor overall adherence (participants #7450029-#7450024 in the order listed in **Figure 2**; n = 11);
2) consistent LLT use at the time of the feedback visit or initiation shortly thereafter (participants #7450006-#7450019; n = 13); and
3) consistent LLT use for ≥2 years before the feedback visit, but termination for various reasons (participants #7450003-#7450004; n = 4).

More detailed data on LLT use are provided in **Supplementary Table S1**.

Survey Results

The survey was returned by 24 (75%) recipients. Two participants contacted the EstBB to decline to participate in the survey, one because of a bad personal experience with statin-related side effects and another for unknown reasons.

The respondents' assessment of the feedback received was overwhelmingly positive. On average, they agreed that "it was the right decision" to attend the feedback visit (4.96/5), that they "wished to have been informed earlier about the genetic finding and the potential health risks" (4.29/5), and that they would "make the same choice if [they] had to do it over again" (5/5; **Figure 3**). The respondents largely agreed that the knowledge of their genetic finding did not cause them distress ("I am able to cope with having this genetic finding in my family," 4.92/5). Average scores for the negative statements "I regret my choice" and "the choice did me a lot of harm" were 1.17/5 and 1.04/5, respectively. The respondents did not definitively agree or disagree with statements about access to healthcare and the improvement of their treatment and/or condition.

Regarding aspects of the healthcare system in respect to their genetic findings, the respondents rated the consistency of follow-up the lowest (3.71/5), followed by access to healthcare (3.96/5). Access to medication was rated the highest (4.38/5), followed by the clarity of recommendations (4.13/5; **Figure 4**).

Regarding potential complications related to FH, the majority of participants indicated only that high cholesterol levels had been detected after their feedback visits (**Figure 5**). Most respondents reported that no other potential complication (e.g., myocardial infarction, stroke, arrhythmia, chest pain after strenuous exercise, vertigo, balance problems, or cardiovascular disease) had occurred.

Most (92%) participants responded "yes" or "yes, partly" to the question about whether they were following the treatment plans developed with their doctors; 4% responded "yes, but not in the last 6 months" and another 4% reported that they were not following their treatment plans (**Figure 6**). Nearly two thirds (63%) of respondents reported that they had made at least some changes in their diet after receiving genetic feedback ("likely agree", 50%; "agree", 13%). "Disagree," "likely disagree," and "unsure" responses to this question made up 21%, 4%, and 8% of the total, respectively. Four percent of the respondents did not answer this question

TABLE 3 | Principal FH-related findings. The statin risk warning is applicable only for simvastatin, atorvastatin, and rosuvastatin. *ICD-10 codes were reduced to three characters, with each code counted only once for each individual.

	RbG participants n = 34	Controls n = 291	p-value (<0.005)
All diagnoses* (excluding Z codes)	34 people with 1,413 diagnosis codes–on average 41.6 per person	291 people with 11,857 diagnosis codes–on average 40.7 per person	
Participants with E78 diagnosis code (including all subsets)	32 (94.1%)	195 (67.0%)	<0.001
Participants with E78.0 Pure hypercholesterolemia diagnosis code	28 (82.4%)	134 (46.0%)	<0.001
Users of any lipid lowering medication	27 (79.4%)	155 (53.3%)	<0.005
Statin side effect (myopathy) risk assessment according to genotype			
Normal risk	23 (67.6%)	201 (69.1%)	
Higher risk	10 (29.4%)	83 (28.5%)	
Much higher risk	1 (2.9%)	7 (2.4%)	

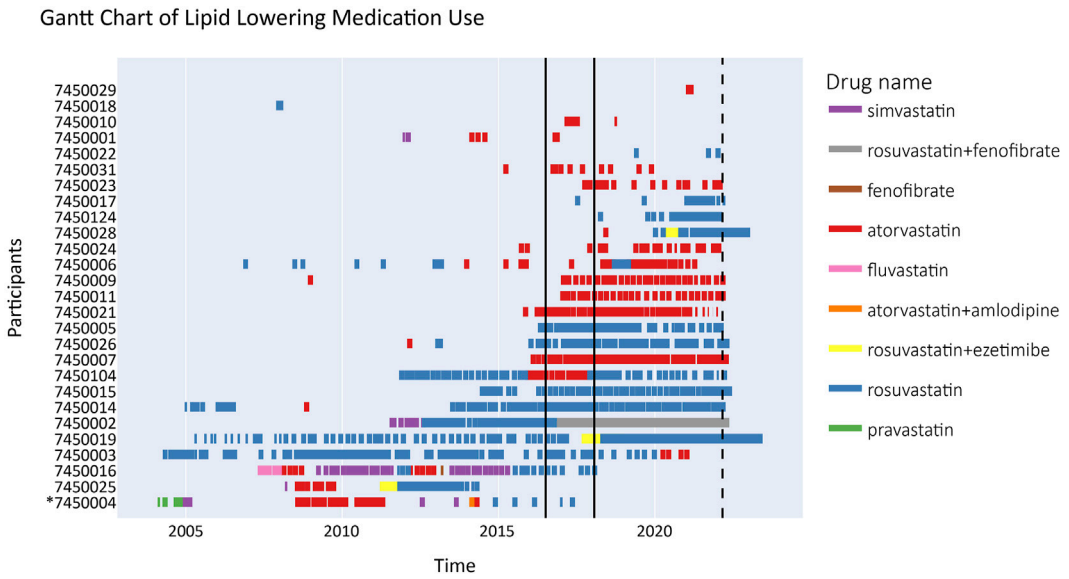

FIGURE 2 | Gantt chart depicting lipid lowering treatment for recalled participants from 2004 until 2022 (n = 27). The time period between the two solid black lines signifies the period of RbG visits (2016–2018) in the Alver et al. study. The dashed line signifies the end of the drug prescription registry follow-up. The lines crossing the end of follow-up indicate purchase of a significant stock of medication in advance. Drug name here indicates the active substance of the prescribed LLT, not the brand name. Participant #7450004 passed away in 2018 (denoted by *).

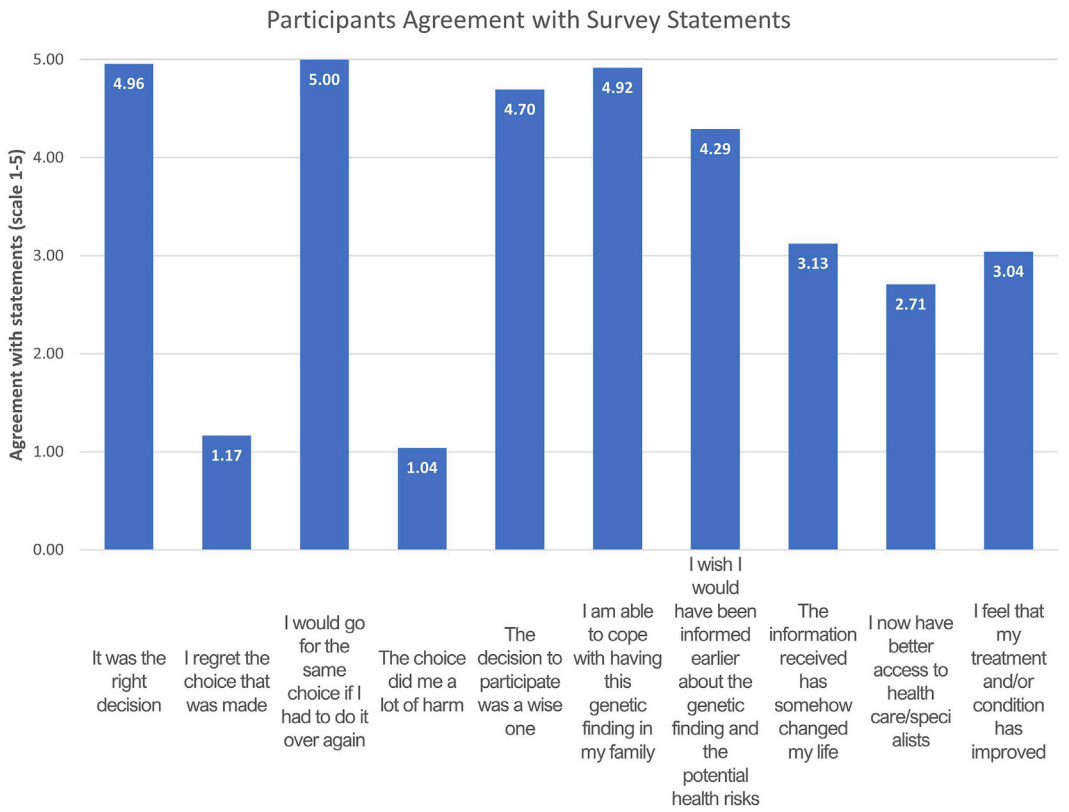

FIGURE 3 | Mean values for answers given to the first portion of the RbG feedback survey where the participants were asked to rate their agreement with the following statements (n = 24). The answers were converted to numeric values according to the scale: “agree”-5, “slightly agree”-4, “unsure”-3, “slightly disagree”-2. “disagree”-1. Mean values above three signify agreement with the specific statement.

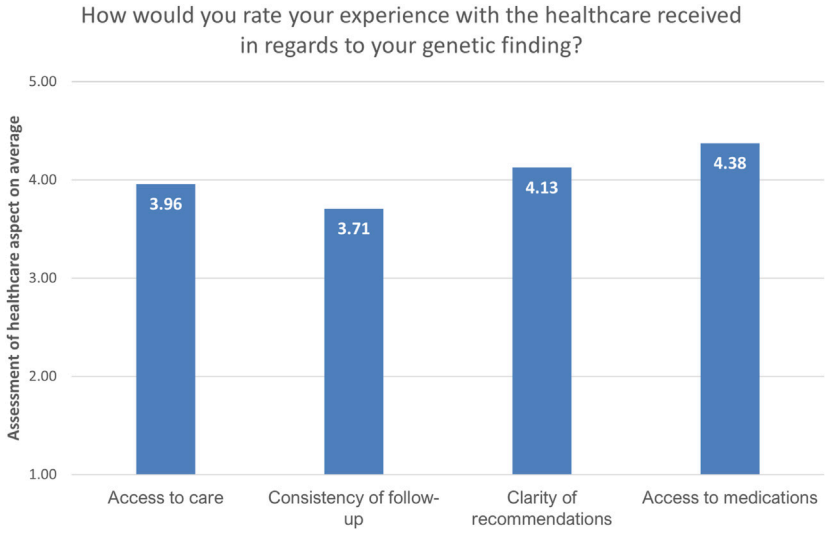

FIGURE 4 | Overview of the assessment of the current state of the healthcare system by recalled participants (n = 24). The answers are presented as mean values of provided answers converted to numeric values: "very good"-5, "good"-4, "unsure"-3, "satisfactory"-2, "unsatisfactory"-1. Mean values above three signify satisfaction with the specific healthcare aspect.

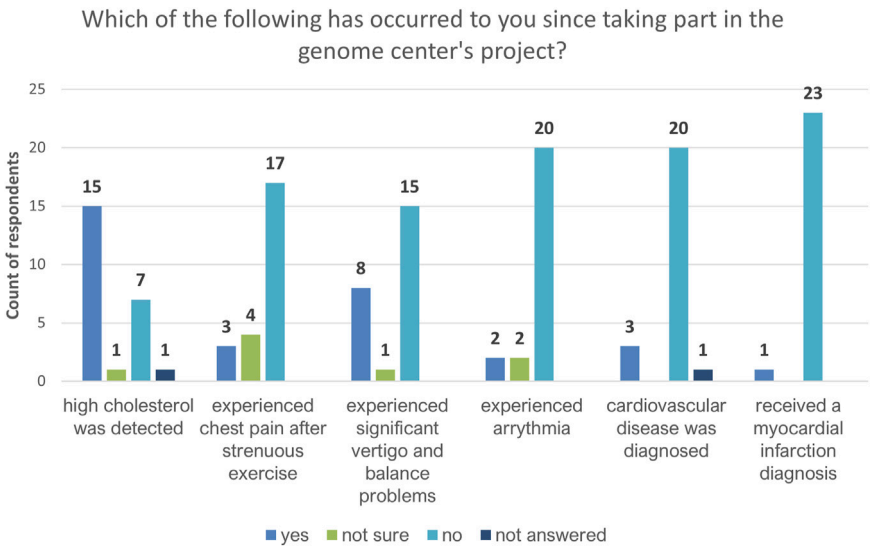

FIGURE 5 | Feedback on health issues potentially related to hypercholesterolemia as reported by recalled participants (n = 24).

(**Figure 7**). Responses to questions about changes in participants' smoking habits and physical activity levels, and with whom they had shared information about their carrier status, are summarized in **Supplementary Figures S2–S4**.

DISCUSSION

In this follow-up study, we investigated the impact of the RbG study approach, examining participants' assessments several years after their genetic findings had been disclosed to them and comparing their characteristics with those of an unrecalled group with similar genetic profiles. Overall, general phenotypic characteristics (i.e., height, weight, body mass index, age, and sex) were comparable in the recalled and non-recalled groups, indicating that the major general confounding factors had little impact on the study results and that the greatest effect on outcomes stemmed from participants' knowledge of their carrier status.

Similar to the findings reported by Alver et al. (2018), the FH-associated variants identified most frequently in the follow-up cohort were p.Arg3527Gln (rs5742904) in the *APOB* gene and

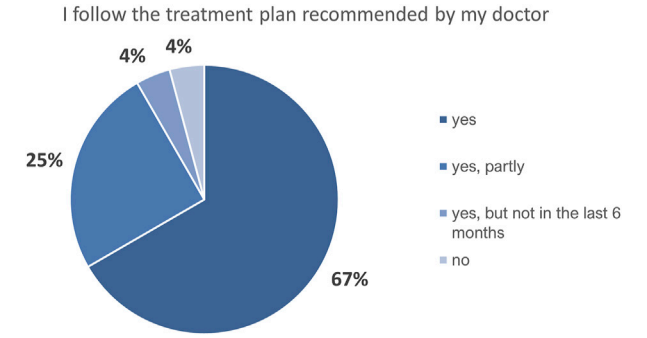

FIGURE 6 | Recalled participants' self-reported assessment of their adherence to the treatment plan proposed by their doctor (n = 24).

p.Cys329Tyr (rs761954844) in the *LDLR* gene. A possible explanation for the high proportion of *APOB* variants in our cohort could be that rare *LDLR* variants may be technically underrepresented in our genotyped and imputed dataset. To our knowledge, the Alver et al. (2018) study is the only available published overview of FH genetic variants in the Estonian population. No comprehensive overview of the FH-associated genetic variants in Estonian clinical cases has been published.

Significantly larger proportions of the recalled cohort than the control group were diagnosed with pure hypercholesterolemia (ICD-10 code E78.0) and E78* in general, and were prescribed LLTs. Consistent with the conclusions of Alver et al. (2018), these findings indicate that FH remains underdiagnosed and undertreated in Estonia. Despite the recalled participants' relatively low assessment of follow-up consistency and access to healthcare, these findings also indicate that the feedback visits improved their visibility in the Estonian healthcare system and access to FH treatment compared with the control group, whose disease severity should match that of the recalled cohort. Participants in the recalled cohort also reported very few potential FH complications such as myocardial infarction and stroke, which may be attributable to their earlier receipt of hypercholesterolemia-related diagnoses and statin prescription, when disease progression could still be stalled. However, further investigations are necessary to confirm this hypothesis.

The widespread use of atorvastatin among study participants is not surprising, as it was the best-selling drug in the early 2000s (Kogawa et al., 2019) and remained one of the most popular lipid-lowering medications in more recent years (Pijlman et al., 2010; Casula et al., 2016). In addition, rosuvastatin was among the most popular LLT medications in the study carried out by Casula et al. (2016).

Treatment adherence varied among the small cohort of LLT-using recalled participants; the largest proportion of these participants began using lipid-lowering medications consistently during or shortly after their feedback visits, but another group of nearly the same size had poor overall LLT adherence. The small sample of LLT users may have resulted in greater variance than is likely present in the general population. However, given the generally poor LLT adherence also reported in other studies (Phan et al., 2014; Casula et al., 2016), and several of our participants' clear improvement in LLT adherence (e.g., #7450009) or consistent LLT use right after the feedback visit (e.g., #7450011), it appears reasonable to speculate that the EstBB intervention had a positive impact on LLT adherence, the full scale of which remains to be captured with larger cohorts.

The third group of LLT users had periods of consistent (≥2 years) adherence prior to the feedback visits, but stopped LLT entirely before 2022. This group contains participants who changed statins at least once; all except one had tried at least three different forms of LLT. Treatment termination was recorded for one participant who died in 2018. Other known reasons for LLT discontinuation were subsequent pregnancies and breastfeeding for a young female participant (despite remarkably high total cholesterol and LDL-C levels in 2016), and myopathy-related side effects that persisted despite several changes in medication over the years reported by another participant. This participant's pharmacogenomic profile did indicate a higher risk of myopathy as a statin use side effect.

Overall, about 30% of all participants in this study were at greater than normal risk of developing myopathy as a side effect of statin use; about 3% of participants had genotypes corresponding even to a much greater than normal risk. As myopathy is the most predominant statin-related adverse effect and a major reason for poor LLT adherence (Mach et al., 2018), our results suggest the role of pharmacogenomic predisposition and highlight the value of pharmacogenomic profiling for the improvement of LLT adherence.

The survey response rate in this study was 75%, reflecting EstBB contributors' persisting willingness to cooperate with biobank inquiries, even several years after their feedback visits. This willingness stems partially from participants' interest in general physical health and awareness of their carrier status, and positive attitudes toward and gratitude for the disclosure of

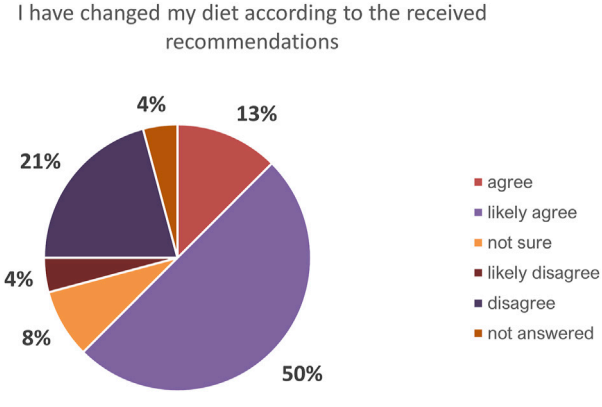

FIGURE 7 | Overview of the feedback to question "How have you changed your habits after receiving information on the genetic variant you carry?" in regard to changes in diet.

their genetic findings; many participants in the present study indicated that they would have liked to have had knowledge of their genetic findings earlier and most indicated that the choice to receive genetic feedback was good.

When asked to evaluate different aspects of the Estonian healthcare system in the light of their genetic findings, the participants rated their access to medication the highest and indicated that there was room for improvement in the clarity of recommendations, access to healthcare, and follow-up consistency. The participants' satisfaction with access to medication suggests that the cost or poor supply of medications is not a substantial barrier for patients adhering to their treatment plans in Estonia. Although our study did not estimate the cost-effectiveness of the RbG method, cost-efficiency calculations regarding a variety of genetic prevention services are likely to be performed in the course of the national personalized medicine initiative in Estonia.

Most survey respondents stated that they at least partly followed the treatment plans prescribed by their doctors. Although the importance of LLT in the management of FH is non-negligible (Cuchel et al., 2014), treatment plans for this small cohort did not necessarily involve LLT alone or at all, as dietary changes and various supplements were tried for some time before or together with LLT prescription when patients' medical histories and clinical characteristics allowed. Accordingly, 63% of the respondents indicated that they had or likely had made changes to their diets according to their doctors' recommendations. These results, combined with responses indicating a poor understanding of the importance of LLT in general, may explain several participants' indication that they did follow their treatment plans while having poor LLT adherence.

Granted, there are limitations to our study. The small sample size (and particularly low number of recalled participants that were available to participate in the survey) may lead to a higher variance in results than would be present in a larger cohort.

Additionally, lipid profiles were not available for all non-recalled participants of the control group. As such, we could not confirm the presence of hypercholesterolemia per se in these individuals ourselves but relied on ICD-10 codes (E78* and subsets) and rates of LLT prescription as proxies. Conversely, while the assembled linkage dataset (EHRs, medical prescription registry, etc.) covering a period of approximately 18 years was available for all the participants, an existing deviation from a normal lipid profile, if already diagnosed as a medical problem, would have been detectable. Therefore, by studying this cohort (more than 300 individuals with a confirmed molecular diagnosis of pathogenic or likely pathogenic FH variants) we have reached a turning point to change the situation of FH being underdiagnosed and undertreated in Estonia, and the time to implement nationwide healthcare actions has come.

Based on the current study results, we conclude that future FH screening by genetic testing should be performed more liberally compared to the current clinical practice. Cascade screening would be essential when a new FH case is confirmed. In countries already having large population biobanks and related legislation (including permissive consent forms signed by participants) in place, a large part of the population may receive genome-wide data that could be combined with the main current healthcare strategies. Genetics-first approach with selected additions from usual EHR data builds an empowered rationale for directed pre-screening. We foresee this kind of strategy as a part of personalized medicine or precision prevention. This could enhance the detection of common actionable monogenic disorders (such as FH) much earlier and thereby increase the effectiveness of the prevention activities as well as follow-up of the individuals at risk.

This study demonstrated that the FH RbG study had significant positive impacts on EstBB participants; on the recognition of hereditary dyslipidemia and prescription of clinically indicated LLT for FH-associated variant carriers in the Estonian healthcare system. The importance of recall was further underlined by the participants' overwhelmingly positive assessment of the utility of the feedback visits. Additionally, the study provided valuable insight into long-term (2004–2022) LLT adherence, suggesting that pharmacogenetic factors (i.e., elevated myopathy risk) play an important role in the generally poor observed adherence. Our results provide a basis for larger-scale RbG studies and add evidence that intervention studies in actionable disorders would be justified in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Estonian Committee on Bioethics and Human Research. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MNu and NT were responsible for the study design and carrying out the study overall and its design. AR provided guidance throughout and advised the interpretation of clinical aspects. MNõ and TN were responsible for variant discovery and validation with Sanger sequencing, TAn aided in variant evaluation. LL was responsible for creating the questionnaire and aided in the analysis of the survey results. MP, MA, AS, TM, TAi, AM, and TE contributed to the study by collecting data, performing primary analyses and conducting genetic feedback visits in the course of the RbG pilot study. MNu wrote the first draft of the manuscript. AR, MNõ, LL, MA, and MNu wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.936131/full#supplementary-material

Supplementary Data Sheet S1 | The questionnaire used in recalled participants' survey in 2021.

Supplementary Table S1 | The pharmacogenomic profiles of 27 lipid-lowering treatment using formerly recalled participants.

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EDITED BY
Hayato Tada,
Kanazawa University, Japan

REVIEWED BY
Masatsune Ogura,
Eastern Chiba Medical Center, Japan

*CORRESPONDENCE
Kjetil Retterstøl,
kjetil.retterstol@medisin.uio.no

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The risk of various types of cardiovascular diseases in mutation positive familial hypercholesterolemia; a review

Anders Hovland[1], Liv J. Mundal[2], Marit B. Veierød[3], Kirsten B. Holven[4,5], Martin Prøven Bogsrud[6], Grethe S. Tell[7,8], Trond P. Leren[6] and Kjetil Retterstøl[2,4]*

[1]Nordland Heart Center, Bodø, Norway, [2]The Lipid Clinic, Oslo University Hospital, Oslo, Norway, [3]Oslo Centre for Biostatistics and Epidemiology, Department of Biostatistics, University of Oslo, Oslo, Norway, [4]Department of Nutrition, University of Oslo, Oslo, Norway, [5]National Advisory Unit on Familial Hypercholesterolemia, Oslo University Hospital, Oslo, Norway, [6]Unit for Cardiac and Cardiovascular Genetics, Oslo University Hospital, Oslo, Norway, [7]Department of Global Public Health and Primary Care, University of Bergen, Bergen, Norway, [8]Division of Mental, Bergen, Norway

Familial hypercholesterolemia (FH) is a common, inherited disease characterized by high levels of low-density lipoprotein Cholesterol (LDL-C) from birth. Any diseases associated with increased LDL-C levels including atherosclerotic cardiovascular diseases (ASCVDs) would be expected to be overrepresented among FH patients. There are several clinical scoring systems aiming to diagnose FH, however; most individuals who meet the clinical criteria for a FH diagnosis do not have a mutation causing FH. In this review, we aim to summarize the literature on the risk for the various forms of ASCVD in subjects with a proven FH-mutation (FH+). We searched for studies on FH+ and cardiovascular diseases and also included our and other groups published papers on FH + on a wide range of cardiovascular and other diseases of the heart and vessels. FH + patients are at a markedly increased risk of a broad range of ASCVD. Acute myocardial infarction (AMI) is the most common in absolute numbers, but also aortic valve stenosis is by far associated with the highest excess risk. Per thousand patients, we observed 3.6 incident AMI per year compared to 1.9 incident aortic valve stenosis, however, standardized incidence ratio (SIR) for incident AMI was 2.3 compared to 7.9 for incident aortic valve stenosis. Further, occurrence of ischemic stroke seems not to be associated with increased risk in FH+. Clinicians should be aware of the excess risk of almost all kind of ASCVD in FH+, and the neutral risk of stroke need to be studied further in FH + patients.

KEYWORDS

familial hypecholesterolemia, ASCVD, LDL, cholesterol, statin (HMG-CoA reductase inhibitor), aortic valve stenosis, stroke, myocardial infaction

Introduction

Atherosclerotic cardiovascular diseases (ASCVDs) comprising coronary heart disease, cerebrovascular disease, peripheral artery disease and aortic disease are common diseases affecting a huge number of patients worldwide (Virani et al., 2021). Low-density lipoprotein cholesterol (LDL-C) is an important risk factor for ASCVD, and familial hypercholesterolemia (FH) is a common, inherited disease (Hu et al., 2020) characterized by high levels of LDL-C from birth. FH could be considered a model disease for studying the pathophysiological effect of LDL-C (Domanski et al., 2020). Therefore, any diseases associated with increased LDL-C levels would be expected to be overrepresented among FH patients.

A recent editorial by Khera and Hegele asked the seemingly unnecessary question—"What Is Familial Hypercholesterolemia, and Why Does It Matter?". Different definitions of FH lead to different groups of patients being identified as having FH (Khera and Hegele, 2020).There are several clinical scoring systems aiming to diagnose FH including the MedPed Criteria (MedPedProgram, 2005), the Simon Broome Register criteria (Group, 1991) and the modified Dutch Lipid Clinic Network score (Betteridge, 2000). These scoring algorithms for FH are useful for identifying individuals at high risk of ASCVD, but it has become evident that most individuals who meet the clinical criteria for probable or definite FH diagnosis do not have a mutation causing FH (Lee et al., 2019). However, those with a pathogenic FH mutation causing high LDL-C levels align with the classical concept of FH as a monogenic autosomal genetic disease (Khera and Hegele, 2020). Trinder et al. have clearly shown that mutation-positive FH patients, have increased risk of cardiovascular disease when compared to those with polygenic hypercholesterolemia (Trinder et al., 2019). Different criteria for FH create a knowledge gap for uniform risk assessment. Our aim with this article is to describe the risks associated with patients with a confirmed genetically verified pathological FH mutation.

In this review, we aim to summarize the literature on the risk for the various forms of ASCVD in subjects with a genetically proven FH-mutation (FH+).

In the present European guidelines for management of dyslipidemias, FH individuals are categorized as individuals at high-risk of disease by default, and in primary prevention treatment goals are at least a 50% reduction from baseline in LDL-C down to <1.8 mmol/L or <1.4 mmol/L depending on the global risk burden (Mach et al., 2019). However, very few patients with FH + reach treatment target even on maximal treatment with statins and ezetimibe and the risk of ASCVD remain high in FH + compared to controls (Arnesen et al., 2020). Use of PCSK9-inhibitors in addition to statins and ezetimibe can bring LDL-C down in many FH + patients, but its use is limited by high cost (Attipoe-Dorcoo et al., 2021). Also newer treatment options have been introduced including evinacumab, a monoclonal antibody inhibiting the angiopoetin-like 3, a hormone regulating the lipoprotein lipase (Raal et al., 2020).

Then we also included our and other groups published papers on FH + on a wide range of cardiovascular and other diseases of the heart and vessels including; coronary heart disease, cerebrovascular disease, peripheral artery disease, aortic disease, ASCVD mortality, heart failure and atrial fibrillation and aortic valve stenosis.

Materials and methods

We searched PubMed using the search string "[(familial hypercholesterolemia AND cvd AND mutation)" or "(familial hypercholesterolemia AND cvd AND genotyped)"]. No limitation for search period was used. Date of search was 1 July 2021. We identified 132 papers that were reviewed by two authors (KR and AH) to identify the inclusion criteria which were 1) a confirmed pathogenic FH mutation in more than 90% of the study population, 2) heterozygous FH only, not homozygous, 3) CVD outcomes clearly reported, 4) English language, 5) studies reporting on quantitative data, not qualitative studies, and not single case reports. Review articles and letters to the editor were also excluded. Nine articles on CVD in mutation positive FH were included and discussed below. The references of these articles were then reviewed to identify additional studies and we also performed hand searches of papers in our collections and the reference lists of extensive review articles.

Then we also included our and other groups' published papers on FH + on a wide range of cardiovascular and other diseases of the heart and vessels including; coronary heart disease, cerebrovascular disease, peripheral artery disease, aortic disease, ASCVD mortality, heart failure and atrial fibrillation and aortic valve stenosis. Lastly, we explore the spectrum of ASCVD in mutations positive FH patients, utilizing data from the Norwegian Register of FH+. In 2021, this register reported to have identified 264 different mutations to cause FH, of which 94.0% is in the *LDL-receptor* gene. Two mutations in the *APOB* gene (5.4%) and three mutations in the *PCSK9* gene (0.6%) have been found to cause FH.

Familial hypercholesterolemia-mutation and cardiovascular disease in general

(Table 1) Receptor negative FH, in which there are no detectable LDL-receptor activity, was compared to receptor

TABLE 1 CVD in mutation FH + patients.

Group	N =	Main finding
Brorholt-Petersen	62	LDL receptor activity did not affect IMT or coronary calcium
Umans-Eckenhausen	608	LDL receptor type only partially contribute to future CVD risk
Koeijvoets	593	LDL receptor type in children affects CVD risk in the parents
Civeira	951	Tendon xanthomas were associated with CVD risk
Sosōka	555	LDL particle size was associated with CVD risk
Besseling	14 283	LDL-C levels >8.0 mmol/L increased the risk of CVD
Paquette	725	Genetic risk scores further predict CVD risk in FH patients
Fantino	725	*ANKS1A* genotype predicts cardiovascular events in patients with FH
Gallo	1624	A positive calcium score by CT predicts risk of future CVD
Mundal	5538	25% were hospitalized due to CVD in a 15-year period
Bogsrud	599	FH patients with high Lp(a) had twice the risk of CHD
Pavanello	350	Increased Lp(a) increased risk of previous CVD
SAFEHEART	2752	FH patients had more than 3-fold angina pectoris compared to controls
Mundal	4273	FH patients 25–49 had a higher excess risk of AMI both (women and men)
Svendsen	4871	FH patients have increased mortality and recurrent AMI after their first AMI
SAFEHEART	2752	FH patients had 3.1 fold AMI compared to controls

N = denotes number FH + patients.
LDL: Low-Density Lipoprotein.
IMT: intima media thickness.

defective mutations, in which there are some LDL-receptor activity, and no difference in atherosclerotic disease assessed by measurement of intima-media thickness with carotid ultrasound and coronary calcification by CT spiral scan was found (Brorholt-Petersen et al., 2002).

When FH + patients was compared to unaffected relatives, the LDL receptor mutation type only partially contributed to the risk of future CVD (Umans-Eckenhausen et al., 2002).

When genotype-phenotype interactions were studied in FH + children the CVD risk in the parents was dependent on type of mutation (b).

In FH + patients, the presence of tendon xantomas was associated with increased risk of CVD (Civeira et al., 2005).

Small, dense LDL-particles in FH + patients were more prominent in patients with CVD (Soška et al., 2012)

Patients with severe FH + defined as LDL-C >8.0 mmol/L had increased risk of CVD with an adjusted hazard rate of 1.25 (Besseling et al., 2014) compared to those with lower LDL-C.

Using genetic risk scores in FH + patients, further enhanced prediction of CVD (Paquette et al., 2017). Also, recently, the same group has documented that rs17609940 variant of the *ANKS1A* gene is associated with increase in CVD events in FH + patients (Fantino et al., 2021).

Very recently, data from the prospective French and Spanish FH registries were combined, and 1624 patients with both FH+ and a coronary artery calcium score were included, and they established a high risk of future ACVD if the calcium score was above 100 (Gallo et al., 2021).

Familial hypercholesterolemia-mutation and coronary artery disease

Stable coronary artery disease

(Table 1) In a prospective registry study of FH + patients, 1411 patients were hospitalized for a CVD related diagnosis in a 15-year period, and ischemic heart disease was the most common diagnoses at admission reported in 90% of the hospitalizations (Mundal et al., 2016). Lipoprotein(a) is an important risk factor for ASCVD, so also in FH+; in FH patients with low lipoprotein(a), 7.8% had angina pectoris, while in the high lipoprotein(a) group, 16.7% had angina pectoris (Bogsrud et al., 2019). Similar results were also reported by Pavenello et al. from two cohorts of FH patients that previous episodes of CVD were associated with higher levels of lipoprotein(a) (Pavanello et al., 2019).

The prospective SAFEHEART registry study including FH + patients (average age 44, 46% males) compared to unaffected relatives (average age 40, 47% males) found a 3.4-fold increased prevalence of angina pectoris in the FH group (Pérez de Isla et al., 2016). This study also demonstrated that lipoprotein(a) is a risk factor for CVD in mutation positive FH patients (Alonso et al., 2014).

Acute coronary syndromes

In FH + patients without prior AMI, 99 incident AMI was observed during 2001–2009 in Norway, which is 3.58 AMI per thousand patients per year with an average age of 56.2 (13.4) at

the time of the first event (Mundal et al., 2018). Importantly, the standardized incidence ratios (SIRs) with 95% CIs for AMI (95% CIs) were highest in the young age group 25–39 years; 7.5 (3.7–14.9) in men and 13.6 (5.1–36.2) in women, and decreased down to close to one at age 79–79 years (Mundal et al., 2018). This study population was later expanded and studied during 2001–2017 to identify 232 patients with an incident AMI which is 2.98 AMI per thousand patients per year in the expanded cohort (Svendsen et al., 2021). The SAFEHEART registry reported a 3.1-fold increased prevalence of AMI in the FH + group compared to controls (Pérez de Isla et al., 2016).

In 103 patients with ACS with a mean age of 55 with LDL-C> 4.1 mmol/L, 87% men, and offered these patients genetic testing and found that the prevalence of genetically confirmed FH was 9% in this group (Amor-Salamanca et al., 2017). This was also confirmed in a study where 130 young patients (<45 years) with LDL-C levels >4.0 mmol/L with acute MI, were offered genetic testing (Bogsrud et al., 2020). Of these, fifty-two patients were genetically tested and 11 was identified with a positive FH diagnosis, constituting a prevalence of 8.5%, in accordance with the results from Amor-Salamanca et al.

By means of cascade testing FH + patients were identified and offered statin therapy and compared to a matched control group (10:1). The median follow-up time was 21 years, and a coronary event (AMI, PCI, CABG) was found in 23% of the FH patients and in 4% of the control population (Kjærgaard et al., 2017).

FH + patients have increased risk of coronary artery disease, but an unanswered question is whether the excess risk of other types of ASCVD is increased to a similar degree? To compare the risk of various types of CVD it is an advantage to study incidence in the same cohort during the same period. We here present risk of various types of ASCVD studied in all FH + patients registered in Norway either at 31 Dec 2009 or at 31 April 2014 and discuss these data together with updated relevant literature in the field.

Familial hypercholesterolemia and cerebrovascular disease

In 3166 FH + patients studied during more than 18 500 person years there was no increased risk of cerebrovascular disease or ischemic stroke (Hovland et al., 2019). A recent study confirmed the finding of no association between FH+ and stroke adjusted for cumulative statin exposure in the FH + group (Svendsen et al., 2022). Similarly, in the prospective SAFEHEART registry of FH + patients there was no increase in any atherosclerotic cerebrovascular disease (Pérez de Isla et al., 2016). Kjærgård et al. studying 118 FH + patients compared to 102 non-mutation carrying relatives with a median follow-up time of 21 years found the occurrence of stroke in 6% of the subjects in the FH group compared to 5% in the control group (Kjærgaard et al., 2017). In the Copenhagen General Population Study the cumulative incidence of ischemic stroke was similar in 185 FH + individuals compared to 106227 individuals with no FH mutation (Beheshti et al., 2018). Also, we have not found any association between FH+ and dementia (Mundal et al., 2022).

A Mendelian randomization study has demonstrated that people with lifelong low levels of PCSK9 and LDL-C had lower risk of coronary heart disease but with no effect on ischemic stroke (Hopewell et al., 2018). Taken together, the pathophysiological relation between LDL-C and ischemic strokes in FH + patients appears to be unclear at present.

Familial hypercholesterolemia and peripheral artery disease

In 3162 FH + patients compared to the general Norwegian population, we found that the risk of peripheral artery disease was nearly tripled in the FH group compared to controls in both women and men (Mundal et al., 2020).

Pereira et al. also demonstrated an increased risk of peripheral artery disease in FH (90% FH+) patients with a mean age of 51 years compared to normolipidemic persons, with a prevalence of peripheral artery disease assessed by ankle-brachial index of 17.3 vs 2.3% respectively, the patients were older in the FH group, and there was more diabetes in the FH group, however there was a higher prevalence of smokers in the control group (Pereira et al., 2015).

In a study investigating presence of comorbidities at time of death among 79 mainly FH + patients, 39% of the patients had peripheral artery disease (diseases of arteries, arterioles and capillaries) at time of death at mean 60 years of age (Krogh et al., 2016).

The SAFEHEART registry with 2752 FH + patients with a mean age of 44 years upon enrollment was compared to 993 unaffected relatives showed 39 cases of any peripheral artery disease in the FH group compared to two cases in the control group, and 14 cases of peripheral artery revascularization in the FH group, compared to none in the control group (Pérez de Isla et al., 2016).

Indeed, these studies suggest that the risk of peripheral artery disease is increased in FH, and accordingly, increased LDL-C burden during life should be considered a risk factor for this kind of ASCVD.

Familial hypercholesterolemia and aortic disease

We have recently demonstrated an increased risk of aortic disease including abdominal aortic aneurisms in FH + men, but not in women (Mundal et al., 2021). LDL-C is thought to be a risk factor for aortic aneurisms, and a Mendelian randomized trial

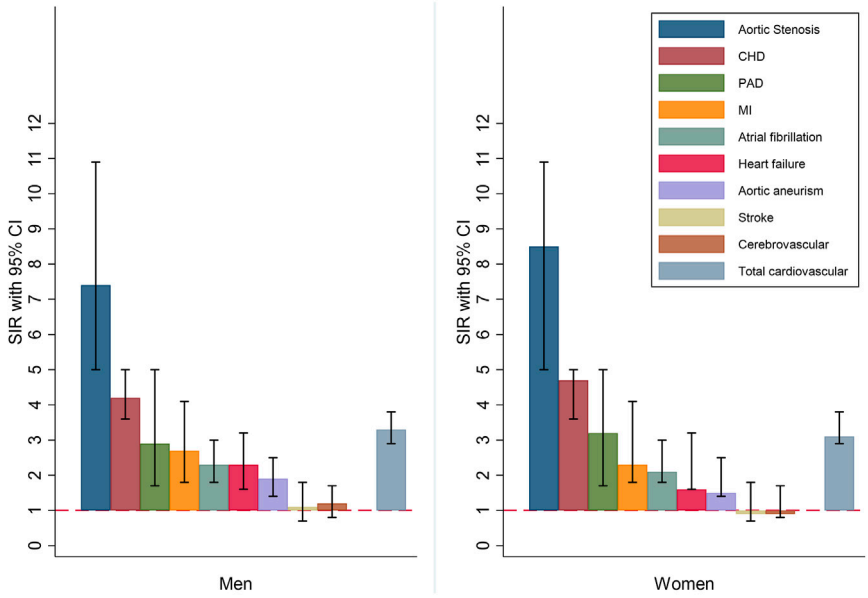

FIGURE 1
Standardized incidence ratios (SIRs) for different types of cardiovascular disease in men and women. CHD: Coronary Heart disease; PAD: Peripheral artery disease; MI: Myocardial infarction.

suggests that predicted LDL-C increase the risk of aortic aneurisms (Allara et al., 2019). In 79 patients mainly mutation positive FH patients, 14% of the patients had presence of aortic aneurysm at time of death at mean 60 years of age (Krogh et al., 2016).

Cardiovascular disease mortality

In a cohort of 4688 FH + patients, risk of CVD death was increased compared to the rest of the Norwegian population in all age groups below 70 years (Mundal et al., 2014). These data were later extended and in 5518 FH + patients in whom standardized mortality ratios was significantly higher in FH patients compared to matched controls, particularly in the young, and for those aged 20–39 years, the risk of cardiovascular disease death during hospitalization was increased 12-fold (Mundal et al., 2017). In another cohort, 93% of FH + had established CVD at the time of death, and 69% of them had experienced myocardial infarction (Krogh et al., 2016).

Other cardiovascular diseases

Heart failure and atrial fibrillation

Increased risk of heart failure was reported in 4273 Norwegian FH + patients compared to the general Norwegian population, and the highest excess risk was observed in the younger age group 25–49 years, and most of the heart failure patients had previous coronary artery disease (Hovland et al., 2017). We also studied the risk of atrial fibrillation in FH patients and found a doubling of atrial fibrillation and atrial flutter among the FH patients compared to the controls (Hovland et al., 2017). Krogh et al. showed that 19% of mainly FH + patients had presence of atrial fibrillation whereas 35% had heart failure at time of death at mean 60 years of age (Krogh et al., 2016).

Coronary heart diseases including myocardial infarction is a common disease in patients with familial hypercholesterolemia and therefore the increased risk of heart failure and atrial fibrillation could be expected as coronary heart disease is a common risk factor for both.

Aortic valve stenosis

LDL-C seems to be important in the early phase of aortic valve stenosis build up (Peeters et al., 2018), however, LDL-C lowering therapy has been futile in halting its progress (Zhao et al., 2016). We found 55 cases of a first-time diagnosis of aortic valve stenosis in 3161 FH + patients in a prospective registry from 2001 during 2009 which constitutes 1.93 incident cases per thousand patients per year. Compared to the Norwegian population and adjusted for

age and sex, SIR was 7.9 (95% CI, 6.1–10.4) (Mundal et al., 2019). Perez de Isla et al. have confirmed our findings in the SAFEHEART prospective cohort of 3712 FH + patients compared to non-affected relatives in which the odds ratio (95% CI) for having an aortic valve replacement was 5.71 (1.8–18.4) after a mean follow-up of 7.5 years (Pérez de Isla et al., 2021).

In a study of homozygous FH, Alonso et al. showed that patients with null mutations had higher levels of LDL-C and more aortic valve stenosis than patients with a receptor defective mutation, indicating that the level of LDL-C is important for progression of this disease (Alonso et al., 2016).

The association of increased risk of aortic valve stenosis in FH+ is quite clear despite those previous studies of LDL-C lowering in aortic valve stenosis being negative. Most of the studies on aortic valve stenosis have been performed relatively late in the disease, and early intervention is less studied. However, this could be further explored in FH patients. New treatment principles including PCSK9-inhibitors, inclisiran and evinacumab facilitate LDL-C- lowering in FH patients to even lower levels than we have achieved earlier and might be tested out with respect to aortic valve stenosis progression. Data suggest that high lipoprotein(a) values in particular increase the risk of aortic stenosis (Mach et al., 2019). This might be particularly important for FH + patients, because a 2-3 doubling of an already high risk will constitute a significant difference in the absolute risk. Thus, specific lowering of lipoprotein(a) using new antisense oligonucleotides treatment in the future may become a particularly important option for FH + patients with high lipoprotein(a) levels, when such drugs are eventually approved.

The spectrum of atherosclerotic cardiovascular disease in familial hypercholesterolemia

Regarding the increased risk of different kinds of ASCVD in patients with FH + there seems to be a wide range in the increased risk induction of the different diseases (Figure 1 and Supplementary Figure S1 and Supplementary Tables S1–S3). As an example we found that the excess risk of aortic stenosis and coronary heart disease is quite high (Mundal et al., 2016; Mundal et al., 2019), whereas there is no excess risk in risk of cerebrovascular disease and stroke (Hovland et al., 2019).

FH + patients are at a markedly increased risk of a broad range of ASCVD. AMI is the most common in absolute numbers, however, aortic valve stenosis is by far associated with the highest excess risk. Per thousand patients, we observed 3.6 incident AMI per year compared to 1.9 incident aortic valve stenosis, however, SIR for incident AMI was 2.3 compared to 7.9 for incident aortic valve stenosis. Further, occurrence of ischemic stroke seems not to be associated with increased risk in FH+.

It should be mentioned that most of the data on FH + are collected in Caucasian persons, and it remains to be studied whether this applies to all races.

Familial hypercholesterolemia–implications for the clinician

Clinicians should be aware of the excess risk of almost all kind of ASCVD in FH+, and the neutral risk of stroke need to be studied further in FH + patients. Clinicians should suspect an FH diagnosis in patients with high levels of LDL-C (>5.0 mmol/L in adults), and a family history of premature ASCVD. Fewer FH patients have xanthoma now compared to earlier times, as many are treated with lipid lowering drugs, however without knowing of the FH diagnosis. Further, as the number of treated FH patients increases, there will be less ASCVD in the parent generation which weakens the accuracy of the clinical scoring systems for FH. In emergency rooms and coronary care units, FH patients may present with early onset coronary artery disease, and an FH diagnosis should be suspected in young patients with high LDL-C. In suspected FH, genetic counselling and testing should then be offered to establish treatment and to perform cascade testing if a mutation is demonstrated. Early detection and correct treatment are paramount in reducing the risk of ASCVD among FH + patients.

Author contributions

Concept and design: KR, LM, and AH. Acquisition, analysis, or interpretation of data: AH, LM, KH, TL, MB, GT, MV, and KR. Drafting of the manuscript: AH and KR Critical revision of the manuscript: AH, LM, KH, TL, MB, GT, MV,and KR. Drafting of the manuscript: AH and KR Statistical analysis: GT and MV Obtained funding: KR Administrative, technical, or material support: LM, KH, TL, MB, GT, MV, and KR Supervision: MV, GT, AH, and KR.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.1072108/full#supplementary-material

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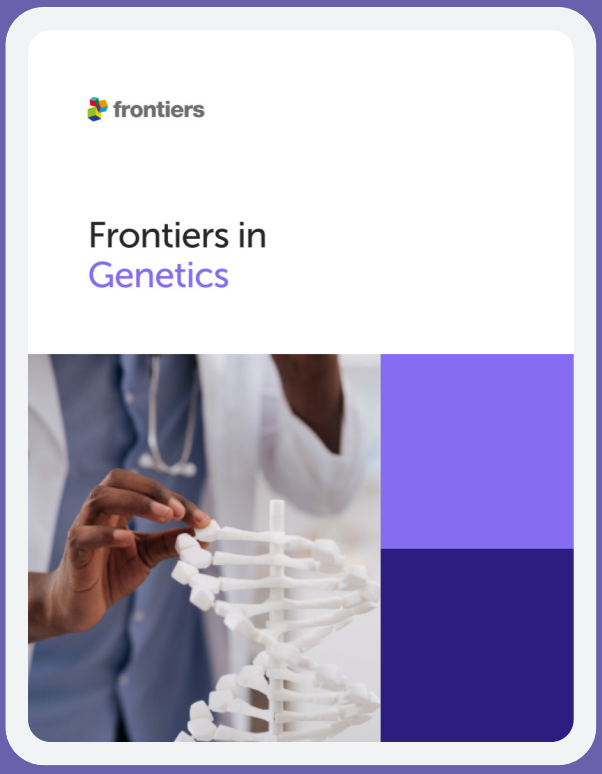

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