GENETICS OF FAMILIAL HYPERCHOLESTEROLEMIA: NEW INSIGHT

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GENETICS OF FAMILIAL HYPERCHOLESTEROLEMIA: NEW INSIGHT

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Editorial: Genetics of Familial Hypercholesterolemia: New Insight

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Editorial on the Research Topic

Genetics of Familial Hypercholesterolemia: New Insight

The Research Topic "Genetics Familial Hypercholesterolemia: New Insight" attracted over 100 authors from 16 countries to submit manuscripts on topical genetic research in familial hypercholesterolemia (FH). Encouragingly, over fifty percent of the manuscripts were from low- and middle-income countries researchers from very different backgrounds. This achieved our goal of bringing together researchers from worldwide. It also reaffirmed conclusions from Vrablik et al. review article: "The coordinated international efforts should increase the chances of achieving the principal goal—to identify, diagnose and provide treatment for all FH patients early enough." The strength of this review topic was the submission of manuscripts from researchers at different stages of their research portfolio. For example, Dušková et al. analyzed in detail pathogenic variants in the low-density lipoprotein gene and their effects on protein localization, function and expression of genes associate endoplasmic reticulum representing advanced molecular biology approaches. At the same time Vasilyev et al. were working with basic molecular biology questions and they concluded that "major achievements in the genetic investigation of the molecular features of FH in Russia are yet to come."

Unfortunately, FH still remains vastly underdiagnosed disease. As an example, is the study by Ramaswami et al. (2019) that was carried out in UK pediatric population. This study demonstrated that <550 children with a confirmed diagnosis of heterozygous FH (HeFH) were being managed in the health care system. This was despite an estimated prevalence of 50,000 children under the age of 18 years with HeFH in the UK based on a population prevalence of 1 in 250. Once diagnosed, it is essential to enable long-term follow-up of these patients and collating longitudinal data on the management and safety of disease modifying therapies such as statins and newer emerging treatments. This would be possible with both national and international HeFH registries. These registries could also potentially help genotype-phenotype correlations related to different HeFH pathogenic mutations in the *LDLR, APOB*, or *PCSK9* genes. This could also apply to genes associated to HeFH diagnosis, and which are differentially expressed compared to non-FH controls Udhaya Kumar et al..

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Truong et al. described the Vietnam Familial Registry (VINAFH) and reported that the VINAFH registry have had a favorable effect on government legislative policies. This highlighted the importance of registries in managing and providing evidence based supportive information for HeFH populations in low- and middle-income countries, and indeed we believe that national and international registries are a key part of management and follow up of both adults and children with HeFH globally. The advantage of HeFH registries would apply especially to large Asian countries where the HeFH diagnosis rates are low Huang and Charng and where HeFH awareness is also still low amongst health care professionals.

In addition to clinically suspected cases of HeFH who are mutation-negative for *LDLR, APOB,* or PCSK9 mutations, there are patients with severe hypercholesterolemia who have an accumulation of common small-effect LDL cholesterol raising alleles (Talmud et al., 2013). These patients are defined as "polygenic hypercholesterolemia." Jarauta et al. highlighted the importance of newer genetic methods to diagnose "polygenic hypercholesterolemia" early and to ensure initiation of a lipid lowering therapy in a timely manner. However, the genetic criterion for the diagnosis of "polygenic hypercholesterolemia" remains a challenge. Genetic testing for monogenic HeFH could potentially also help identify "polygenic hypercholesterolemia" but this option is not utilized effectively Kamar et al..

Recently, Futema et al. (2021) demonstrated that both children and adults with HeFH caused by pathogenic *LDLR* mutation have a higher LDL cholesterol concentration compared to HeFH caused by *APOB* mutation.

Healthy diet and lifestyle and early statin treatment are cornerstones in HeFH treatment (Vuorio et al., 2019; Rodríguez-Borjabad et al., 2021). Statin treatment has been shown to be safe and effective in children with HeFH (Humphries et al., 2018; Vuorio et al., 2019). However, many children with HeFH are not on appropriate lipid-lowering treatments (Ramaswami et al., 2020), including our European patients. It has been speculated that the widespread use of genetic testing has been crucial for the increased statin use in Norway, especially in young children with HeFH aged 10–19 years compared to their Scandinavian neighbors with less prevalent genetic testing (Svendsen et al., 2021). Despite this, the prevalence of patients with HeFH among patients with premature acute myocardial infarction (AMI) remains significantly higher compared to the general population in Norway (Bogsrud et al., 2020). The most likely explanation is the increased lifelong cholesterol burden prior to their AMI, and an increased LDL cholesterol concentration at the time of AMI. Similar results were also confirmed by Moradi et al. in Iran. It has been also shown that mortality and the risk of recurrent AMI is increased in HeFH compared with non-FH controls (Svendsen et al., 2020). A poor prognosis after the first AMI in HeFH patients was also confirmed in this Research Topic by Arnesen et al..

PCSK9 inhibitors represent a relatively new class of medication that targets the unique mechanism of action of *PCSK9* Guo et al.. The effectiveness and safety of PCSK9 inhibitor was shown recently in pediatric HeFH (Santos et al., 2020). In this 24-week study, the mean LDL cholesterol lowering was −44.5% and −6.2% in children with HeFH on evolocumab, a PCSK9 inhibitor, and a control group, respectively. Inclisiran (Hovingh et al., 2020) highlights the effective use of such adjunctive therapies that potentially help achieve appropriate target LDL cholesterol concentration and better clinical outcomes. Oommen et al. described that over half of the HeFH causing LDLR mutations resulted in protein misfolding, defective transport and trafficking, with the misfolded proteins being retained in the endoplasmic reticulum (ER). The authors postulate drugs modulating proteostasis in the ER as therapeutic alternatives for patients who have persistently elevated LDL cholesterol despite optimization of conventional lipid lowering therapies.

Homozygous form of FH (HoFH) is a rare disease with a prevalence ∼1:200,000–30,000 (Sjouke et al., 2015). HoFH is a serious disease due to very aggressive atherosclerosis progression. Without early interventions, HoFH causes AMI in childhood (Vallejo-Vaz et al., 2015). In the current Research Topic, Marusic et al. showed that HoFH patients represent a clinically heterogenous group. In some cases, the HoFH phenotype may overlap with HeFH, and hence genetic testing is paramount. The authors highlight the lack of therapeutic options for HoFH patients in less developed countries. Mlinaric et al. reported an interesting case of liver transplantation (LT) in a HoFH child with progressive atherosclerosis despite early LDL-apheresis. The authors suggest LT as a feasible option especially in HoFH unresponsive to lipid lowering therapies and/or LDL-apheresis. The authors also suggest the benefits of an international registry for HoFH and LT. The International Registry on Lipoprotein Apheresis in Children with HoFH could serve as an example (Luirink et al., 2020).

The most recent challenge related to FH is the COVID-19 pandemic (Vuorio et al., 2021a). It can be estimated that with a HeFH prevalence of 1 in 250, ∼440,000 HeFH patients may have had SARS-Cov-2 infection by February 2021. In severely ill COVID-19 patients, HeFH could potentially be overrepresented due to endothelial dysfunction and atherosclerotic cardiovascular disease as risk factors (Vuorio et al., 2020). A lifelong elevated LDL cholesterol concentration is often associated with an increased lipoprotein(a) Lp(a), with endothelial dysfunction starting in childhood. In a meta-analysis carried among hospitalized COVID-19 patients, AMI was present in 3.3% (95% CI 0.3–8.5) of the cases (Kunutsor and Laukkanen, 2020). A meta-analysis of COVID-19 studies revealed that statin use decreases not only the mortality but also the severity of SARS-Cov-2 infection significantly (OR 0.51, 95% CI 0.41–0.64) (Onorato et al., 2021). Therefore, among patients with HeFH, an effective lowering of LDL cholesterol concentration is essential to improve endothelial dysfunction (Iqbal et al., 2020; Vuorio and Kovanen, 2020) and potentially reduce COVID-19 risk. PSCSK9 inhibitors could also be considered as an adjunctive therapy to effectively lowering both LDL cholesterol concentration and Lp(a), with also potential antiviral properties (Iqbal et al., 2020; Vuorio and Kovanen, 2021). An effective lowering of LDL cholesterol concentration, especially in older patients with HeFH

is important to mitigate the higher risk of becoming critically ill with COVID-19 (Vuorio et al., 2021b). Currently, there is a great need to collect comprehensive epidemiologic data with the aid of international collaboration among the centers on the clinical course and outcomes of FH patients who have contracted COVID-19 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.

REFERENCES

Bogsrud, M. P., Øyri, L., Halvorsen S, Atar, D., Leren, T. P., and Holven, K. B. (2020). Prevalence of genetically verified familial hypercholesterolemia among young (<45 years) Norwegian patients hospitalized with acute myocardial infarction. *J. Clin. Lipidol.* 14, 339–345. doi: 10.1016/j.jacl.2020.04.002

Futema, M., Ramaswami, U., Tichy, L., Bogsrud, M. P., Holven, K. B., Roeters van Lennep, J., et al. (2021). Comparison of the mutation spectrum and association with pre and post treatment lipid measures of children with heterozygous familial hypercholesterolaemia (FH) from eight European countries. *Atherosclerosis* 319, 108–117. doi: 10.1016/j.atherosclerosis.2021.01.008

Hovingh, G. K., Lepor, N. E., Kallend, D., Stoekenbroek, R. M., Wijngaard, P. L. J., and Raal, F. J. (2020). Inclisiran durably lowers low-density lipoprotein cholesterol and proprotein convertase subtilisin/kexin type 9 expression in homozygous familial hypercholesterolemia: the ORION-2 Pilot study. *Circulation* 141, 1829–1831. doi: 10.1161/CIRCULATIONAHA.119.044431

Humphries, S. E., Cooper, J., Dale, P., Ramaswami, U., F. H., and Paediatric Register Steering Group (2018). The UK Paediatric familial hypercholesterolaemia register: statin-related safety and 1-year growth data. *J. Clin. Lipidol.* 12, 25–32. doi: 10.1016/j.jacl.2017.11.005

Iqbal, Z., Ho, J. H., Adam, S., France, M., Syed, A., and Neely, D. (2020). Managing hyperlipidaemia in patients with COVID-19 and during its pandemic: an expert panel position statement from HEART UK. *Atherosclerosis* 313, 126–136. doi: 10.1016/j.atherosclerosis.2020.09.008

Kunutsor, S. K., and Laukkanen, J. A. (2020). Incidence of venous and arterial thromboembolic complications in COVID-19: a systematic review and meta-analysis. *Thromb. Res.* 196, 27–30. doi: 10.1016/j.thromres.2020.08.022

Luirink, I. K., Hutten, B. A., Greber-Platzer, S., Kolovou, G. D., Dann, E. J., de Ferranti, S. D., et al. (2020). Practice of lipoprotein apheresis and short-term efficacy in children with homozygous familial hypercholesterolemia: data from an international registry. *Atherosclerosis* 299, 24–31. doi: 10.1016/j.atherosclerosis.2020.01.031

Onorato, D., Pucci, M., Carpene, G., Henry, B. M., Sanchis-Gomar, F., and Lippi, G. (2021). Protective effects of statins administration in European and North American patients infected with COVID-19: a meta-analysis. *Sem. Thromb. Hemos.* doi: 10.1055/s-0040-1722307

Ramaswami, U., Futema, M., Bogsrud, M. P., Holven, K. B., Roeters van Lennep, J., Wiegman, A., et al. (2020). Comparison of the characteristics at diagnosis and treatment of children with heterozygous familial hypercholesterolaemia (FH) from eight European countries. *Atherosclerosis* 292, 178–187. doi: 10.1016/j.atherosclerosis.2019.11.012

Ramaswami, U., Humphries, S. E., Priestley-Barnham, L., Green, P., Wald, D. S., Capps, N., et al. (2019). Current management of children and young people with heterozygous familial hypercholesterolaemia - HEART UK statement of care. *Atherosclerosis* 290, 1–8. doi: 10.1016/j.atherosclerosis.2019.09.005

Rodríguez-Borjabad, C., Narveud, I., Christensen, J. J., Ulven, S. M., Malo, A. I., Ibarretxe, D., et al. (2021). Dietary intake and lipid levels in Norwegian and Spanish children with familial hypercholesterolemia. *Nutr. Metab. Cardiovasc. Dis.* 31, 1299–1307. doi: 10.1016/j.numecd.2020.12.002

Santos, R. D., Ruzza, A., Hovingh, G. K., Wiegman, A., Mach, F., Kurtz, C. E., et al. (2020). Evolocumab in pediatric heterozygous familial hypercholesterolemia. *NEJM* 383, 1317–1327. doi: 10.1056/NEJMoa2019910

Sjouke, B., Hovingh, G. K., Kastelein, J. J., and Stefanutti, C. (2015). Homozygous autosomal dominant hypercholesterolaemia: prevalence, diagnosis, and current and future treatment perspectives. *Curr. Opin. Lipidol.* 26, 200–209. doi: 10.1097/MOL.0000000000000179

Svendsen, K., Krogh, H. W., Igland, J., Tell, G. S., Mundal, L. J., Holven, K. B., et al. (2020). 2.5-fold increased risk of recurrent acute myocardial infarction with familial hypercholesterolemia. *Atherosclerosis* 319, 28–34. doi: 10.1016/j.atherosclerosis.2020.12.019

Svendsen, K., Langslet, G., Krogh, H. W., Brinck, J., Klausen, I. C., Stenehjem, J. S., et al. (2021). Genetic testing is essential for initiating statin therapy in children with familial hypercholesterolemia: examples from Scandinavia. *Atherosclerosis* 316, 48–52. doi: 10.1016/j.atherosclerosis.2020.11.027

Talmud, P. J., Shah, S., Whittall, R., Futema, M., Howard, P., Cooper, J. A., et al. (2013). Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. *Lancet* 381, 1293–1301. doi: 10.1016/S0140-6736(12)62127-8

Vallejo-Vaz, A. J., Kondapally Seshasai, S. R., Cole, D., Hovingh, G. K., Kastelein, J. J., Mata, P., et al. (2015). Familial hypercholesterolaemia: a global call to arms. *Atherosclerosis* 243, 257–259. doi: 10.1016/j.atherosclerosis.2015.09.021

Vuorio, A., and Kovanen, P. T. (2020). Prevention of endothelial dysfunction and thrombotic events in COVID-19 patients with familial hypercholesterolemia. *J. Clin. Lipidol.* 14, 617–618. doi: 10.1016/j.jacl.2020.06.006

Vuorio, A., and Kovanen, P. T. (2021). PCSK9 inhibitors for COVID-19: an opportunity to enhance the antiviral action of interferon in patients with hypercholesterolaemia. *J. Int. Med.* 289, 749–751. doi: 10.1111/joim.13210

Vuorio, A., Kovanen, P. T., and Strandberg, T. E. (2021b). Older familial hypercholesterolemia patients with COVID-19. *Gerontology* 18, 1–3. doi: 10.1159/000514447

Vuorio, A., Kuoppala, J., Kovanen, P. T., Humphries, S. E., Tonstad, S., Wiegman, A., et al. (2019). Statins for children with familial hypercholesterolemia. *Cochrane Database Syst. Rev.* 2019:CD006401. doi: 10.1002/14651858.CD006401.pub5

Vuorio, A., Raal, F., Kaste, M., and Kovanen, P. T. (2021a). Familial hypercholesterolaemia and COVID-19: a two-hit scenario for endothelial dysfunction amenable to treatment. *Atherosclerosis* 320, 53–60. doi: 10.1016/j.atherosclerosis.2021.01.021

Vuorio, A., Watts, G. F., and Kovanen, P. T. (2020). Familial hypercholesterolaemia and COVID-19: triggering of increased sustained cardiovascular risk. *J. Int. Med.* 287, 746–747. doi: 10.1111/joim.13070

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ORIGINAL RESEARCH
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Low Density Lipoprotein Receptor Variants in the Beta-Propeller Subdomain and Their Functional Impact

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Background: Pathogenic variants in the low density lipoprotein receptor gene are associated with familial hypercholesterolemia. Some of these variants can result in incorrect folding of the LDLR protein, which is then accumulated inside the cell and cannot fulfill its function to internalize LDL particles. We analyzed the functional impact of 10 LDLR variants localized in the beta-propeller of epidermal growth factor precursor homology domain. The experimental part of the work was complemented by a structural analysis on the basis of 3D LDLR protein structure.

Methods: T-Rex Chinese hamster ovary cells transfected with the human LDLR gene were used for live cell imaging microscopy, flow cytometry, and qRT-PCR analysis.

Results: Our results showed that the analyzed LDLR protein variants can be divided into three groups. (1) The variants buried inside the 3D protein structure expressing proteins accumulated in the endoplasmic reticulum (ER) with no or reduced plasma membrane localization and LDL particle internalization, and associated with an increased gene expression of ER-resident chaperones. (2) The variants localized on the surface of 3D protein structure with slightly reduced LDLR plasma membrane localization and LDL particle internalization, and associated with no increased mRNA level of ER-resident chaperones. (3) The variants localized on the surface of the 3D protein structure but expressing proteins with cell responses similar to the group 1.

Conclusion: All analyzed LDLR variants have been evaluated as pathogenic but with different effects on protein localization and function, and expression of genes associated with ER stress.

Keywords: low density lipoprotein receptor, live cell imaging microscopy, flow cytometry, functional analysis, ER stress

INTRODUCTION

Familial hypercholesterolemia (FH) is characterized by elevated low-density lipoprotein cholesterol levels, which lead to accelerated atherosclerosis and premature coronary heart disease. FH frequency in most populations is estimated to be 1:200–250 (Benn et al., 2012; Sjouke et al., 2015). FH is an autosomal dominant disease associated with pathogenic variants in the low density lipoprotein receptor gene (LDLR), the apolipoprotein B gene (APOB), or the proprotein convertase subtilisin/kexin type 9 gene (PCSK9) (Brown and Goldstein, 1986; Rader et al., 2003).

The LDLR gene encodes a protein of 860 amino acids comprising a 21 amino acid signal sequence at the N-terminus. This sequence is excised during protein translocation into the endoplasmic reticulum (ER) (Strom et al., 2011). LDLR is synthesized on ER membrane-bound ribosomes, folded and partially glycosylated within the ER lumen and finally matured in the Golgi complex, where glycosylation is completed. Approximately 45 min after synthesis, LDLR appears on the cell surface and mediates LDL particle uptake by receptor mediated endocytosis. The LDL particle is then released in the endosome, and the protein recycles, i.e., it returns to the cell membrane (Brown et al., 1997). The complex function of the LDL receptor is ensured by its specific functional domains: an N-terminal ligand-binding domain composed of seven ligand binding repeats (R1-R7); an epidermal growth factor precursor homology domain composed of two epidermal growth factor (EGF)-like modules (A and B), a six-bladed beta-propeller, and a third EGF-like module (C) connected to an O-linked sugar domain followed by a transmembrane domain and C-terminal cytoplasmic tail (Hobbs et al., 1990).

LDLR variants have different effects on the protein expression, maturation, localization, and function. If LDLR reaches its native conformation, it is released from ER through the protein secretory pathway. Accumulation of misfolded proteins in the ER lumen can trigger ER stress and unfolded protein response (UPR), which activates an adaptive response to restore ER homeostasis by (1) lowering protein synthesis and translocation into ER, (2) increasing molecular chaperone expression to enhance protein folding capacity in ER, and (3) inducing apoptosis when UPR fails to re-establish ER homeostasis (Ron and Walter, 2007; Minamino et al., 2010; Hetz, 2012; Hetz et al., 2015).

In this study, we examined the impact of 10 missense LDLR sequence variants localized in the beta-propeller subdomain on the protein localization and function. Further, we investigated mRNA levels of ER-resident chaperones CALR (calreticulin), HSPA5 (heat-shock 70-kD protein 5), HSP90B1 (heat-shock 90-kD protein beta 1); and ER-resident protein HERPUD1 (homocysteine- and ER stress-inducible protein ubiquitin-like domain-containing 1). CALR is responsible for folding of synthesized glycoproteins and quality control in the calnexin/calreticulin cycle (Hebert and Molinari, 2007; Michalak et al., 2009). HSPA5 interacts with the luminal domain of three ER transmembrane proteins (the activating transcription factor 6, the eukaryotic translation initiation factor 2-alpha kinase 3, and the endoplasmic reticulum-to-nucleus signaling 1), which act as proximal UPR sensors under non-stressed conditions. With the accumulation of unfolded and misfolded proteins, HSPA5 binds to these proteins and this activates the mentioned UPR sensors (Ye et al., 2000; Back et al., 2005). HSP90B1 participates in protein folding, interacts with other components of the ER protein folding machinery, stores ER calcium, and assists in targeting misfolded proteins for the ER associated degradation (ERAD) (Eletto et al., 2010; Marzec et al., 2012). HERPUD1 plays a protective role in ER stress-induced apoptosis (Chan et al., 2004). HERPUD1 interaction with other ERAD components, proteasomes, and ubiquitinated substrates (Schulze et al., 2005) makes it a good candidate to coordinate UPR and ERAD processes, which are interdependent (Travers et al., 2000).

The experimental results were complemented by *in silico* variant analysis on the basis of the human 3D LDLR protein structure.

MATERIALS AND METHODS

Selection of LDLR Variants

Ten LDLR variants located in the beta-propeller of epidermal growth factor precursor homology domain were investigated. Six of them were found in Czech FH patients and also described in other FH populations – p.Glu408Val, c.1223A > T (exon 9); p.Arg416Trp, c.1246C > T (exon 9); p.Val429Met, c.1285G > A (exon 9); p.Gly478Arg, c.1432G > A (exon 9); p.His583Arg, c.1748A > G (exon 12); and p.Ser610Cys, c.1829C > G (exon 12) (Tichy et al., 2012; Vrablik et al., 2017). The variant p.Tyr532Cys, c.1595A > G (exon 11) was identified only in one Czech FH patient (Tichy et al., 2017). The other three variants were selected either on the basis of 3D protein structural analysis [p.Leu555Pro, c.1664T > C (exon 11); p.His583Tyr, 1747C > T (exon 12)] or on the basis of published studies [p.Gly565Val, c.1694G > T (exon 11)] as a reference for variants associated with complete ER protein retention (Sorensen et al., 2006). The LRG_274 reference sequence was used for nomenclature of the LDLR sequence variants.

Site-Directed Mutagenesis and Transfection

The pcDNA4-LDLR-linker-EYFP plasmid (kindly provided from MA Kulseth, Norway) was used for T-Rex CHO (Chinese hamster ovary) cell line transfections (Life Technologies). The T-Rex system allows induction of a transfected gene expression by tetracycline. The EYFP (Enhanced Yellow Fluorescent Protein) tag, inserted at the C-terminus of LDLR cDNA and separated by a 10 amino acid linker, was used to visualize the protein in cells. The EYFP tag does not affect the LDLR biosynthesis pathway (Sorensen et al., 2006). The LDLR sequence variants were created by a site-directed mutagenesis (QuickChange Mutagenesis Lightning kit, Agilent Technologies) and verified by DNA sequencing. T-Rex CHO cells were transfected with plasmids carrying wild type (wt) or mutated LDLR cDNA by lipid-mediated transfection (Lipofectamine 2000, Life Technologies). CHO cell lines were grown in Ham′s F12 medium supplemented

with fetal bovine serum (10%, MP Biomedicals), L-glutamine (2 mM, Sigma-Aldrich), and blasticidin (10 μg/ml, Invitrogen) in 37°C and 5% CO_2. Stable CHO cell lines were generated using zeocin selection (600 μg/ml, Invitrogen).

Live Cell Imaging Microscopy of LDLR, ER, and Internalized LDL Particles

Live cell imaging microscopy was used to study LDLR localization on the plasma membrane and ER, and LDL particle uptake. ER was visualized using CellLight® ER-RFP, BacMam 2.0 Reagent (Life Technologies), which is a fusion construct of ER signal sequence of calreticulin and KDEL (ER retention signal) with a Red Fluorescent Protein (RFP) tag packaged in the insect Baculovirus. T-Rex CHO cells were seeded (5 × 103) into 8-well chambers for live cell imaging (Cellvis C8-1,5H-N, Bio-Port) and transduced with 2 μl of BacMam 2.0 Reagent. Twenty-four hours after transduction, LDLR expression was induced by adding tetracycline (1 μg/ml, Sigma-Aldrich) for 24 h. The nucleus of the cells was labeled with Hoechst 33342 (1:2000, Thermo Fisher Scientific) for 45 min at 37°C. Finally, the cells were washed with 1× PBS (Phosphate Buffered Saline) and observed in Live Imaging Solution (Life Technologies) with Live Imaging Antifade (1:100, Life Technologies) on Zeiss LSM 880 laser scanning confocal microscope at 37°C and 5% CO_2. The images were analyzed by the ZEN software. Samples for each LDLR protein variant were prepared at least three times.

To study the function of the LDL receptor, LDL particles fluorescently labeled with pHRodo-Red (0.5 mg/ml, Life Technologies) were added to the cells expressing LDLR after 24 h of tetracycline induction and incubated for 40 min. After the incubation time, the cells were prepared for live imaging microscopy and analyzed as described above. The pHRodo-Red label gives fluorescence only in acidic pH and thus reflects LDL particles internalized by the cell and present in endosomes or lysosomes.

Flow Cytometry of LDLR Cell Surface Expression and LDL Particle Internalization

Flow cytometry was used for determination of LDLR plasma membrane localization and its ability to internalize LDL particles. T-Rex CHO cells were seeded into 12-well plates (Nunc, Life Technologies), LDLR expression was induced by adding tetracycline (1 μg/ml, Sigma-Aldrich) for 24 h. To determine LDLR plasma membrane localization, monoclonal anti-hLDLR APC-conjugated Mouse IgG1 antibody (1μl/105 cells, R&D Systems) was added to the cells and incubated overnight at 37°C and 5% CO_2. For determination of LDL particle internalization, LDL particles fluorescently labeled with pHRodo-Red (0.5 mg/ml, Life Technologies) were added to the cells and incubated for 40 min. After the incubation times, the cells were detached using Accutase (Life Technologies), washed with 1× PBS and suspended in FACS buffer for flow cytometric analysis on BD FACS Verse (BD Systems). Sytox blue staining (0.2 μM, Life Technologies) was used to separate dead and live cells. For each sample, 104 of single cells were analyzed. The median of APC or pHRodo-Red fluorescence intensity obtained from cells expressing a LDLR variant was compared to that obtained from wt LDLR. For each LDLR variant, the results are presented as a mean of triplicate experiments ± standard deviation (S.D.) compared with the wt LDLR, which represents 100%. Statistical significance was determined using the Dunnett's test with 95% confidence interval ($p < 0.05$) in the GraphPad Prism statistic software.

Quantitative mRNA Analysis of ER-Resident Proteins

The mRNA levels of selected genes were analyzed by quantitative RT-PCR (qRT-PCR) in T-Rex CHO cell lines stably transfected with wt LDLR or mutant LDLR. The cells were harvested 24 h after tetracycline induction (1 μg/ml, Sigma-Aldrich) into RNA Protect Cell Reagent (Qiagen). The total RNA was isolated using RNeasy Plus Mini Kit with on-column gDNA removal (Qiagen) and additional DNaseI treatment. RNA integrity was measured with Agilent 2100 Bioanalyzer (Agilent Technologies). Reverse transcription (RT) was carried out with 2 μg RNA using High Capacity RNA to cDNA Kit (Applied Biosytems). The qRT-PCR was performed in 96 well MicroAmp Fast Optical Reaction Plates (Applied Biosystems) with 20× TaqMan Gene Expression Assays [Cg04421473_g1 (Calr), Cg04423734_g1 (Hspa5), Cg04548386_g1 (Hsp90b1), Cg04495044_m1 (Herpud1), Cg04424038_gH (Gapdh); Applied Biosystems] and 2× TaqMan Fast Advanced Master Mix (Applied Biosystems) on 7900HT Fast Real-Time PCR system (Applied Biosystems). The results of expression of target genes were normalized against Gapdh as a reference control with verified stable gene expression, mRNA from wt LDLR transfected cell line served as a calibrator. All qRT-PCR measurements were run in triplicates. The gene expression measurements from three biological replicates were performed and the results are presented as mean ± S.D. The levels of significance were determined using the Dunnett's test with 95% confidence interval ($p < 0.05$) in the GraphPad Prism statistic software.

Structural Analysis of LDLR Variants

We analyzed the structural effect of 10 variants based on the LDLR human X-ray structure determined at pH = 5.3 (3.7 Å resolution), which should represent conformation adopted in endosomes (PDB code: 1N7D) (Rudenko et al., 2002). In this conformation, ligand binding repeats R4 and R5 interact with beta-propeller while on the membrane at neutral pH, the repeats bind various lipoprotein particles. Similarly to the previous study (Reblova et al., 2015), we analyzed the wt amino acids (AAs) side chain contacts, i.e., direct H-bonds, salt bridges, and stacking interactions using the VMD program (Humphrey et al., 1996). In addition, we measured the buriedness of the wt AAs in the protein structure. The residues' solvent accessibility in the protein structure was calculated using the STRIDE program (Frishman and Argos, 1995) and divided by the total surface area of the residue (Chothia, 1976). This value corresponds to the relative accessible surface area (RSA). A residue was considered buried if RSA is ≤10%. Replacing a buried AA is more likely to be

associated with structural defects especially when volume, charge, and polarity change upon a sequence substitution, and thus we measured these parameters for buried residues. Volume change upon a missense variant was calculated (Zamyatnin, 1972), a change ≥20 Å3 associated with the large to small substitution was considered destabilizing. A charge change upon a variant was considered between charged and uncharged AAs and a polarity change was considered between nonpolar (Leu, Ile, Phe, Trp, Cys, Met, Val, Tyr), polar (Pro, Ala, Thr, Gly, Ser), and very polar (His, Arg, Gln, Lys, Asn, Glu, Asp) AAs. Further, we detected the replacement of wt proline and glycine residues in turns where they are key structural factors. In addition, substituting any AA to proline in alpha-helical and beta-sheet structures was considered destabilizing (Li et al., 1996).

RESULTS

Analyzed LDLR variants were evaluated for their occurrence in Human Gene Mutation Database (HGMD)[1], ClinVar[2], and Leiden Open Variation Database (LOVD)[3]. Using live cell imaging microscopy and flow cytometry, qualitative and quantitative analyses were performed evaluating selected variants in terms of protein localization (**Figures 1A**, **2A**) and its ability to internalize LDL particles (**Figures 1B**, **2B**). Untransfected T-Rex CHO cells served as a negative control in microscopic and flow cytometric analyses. Further, mRNA analysis of selected genes encoding ER-resident proteins were performed for analysis of possible association between the variant and ER stress activation (**Figure 3**). The experimental part of the work was complemented by a structural analysis of particular variants on the basis of 3D LDLR protein structure (**Figure 4** and **Table 1**).

p.Glu408Val

The variant has been described in HGMD (as disease causing), ClinVar (as likely pathogenic), LOVD (as probably affects function), and in six Czech unrelated patients but functional analysis has not been performed yet. The variant was associated with plasma membrane localization, without formation of protein clumps on ER (as seen in p.Gly565Val). No statistically significant changes of gene expression of ER resident proteins were observed. Flow cytometry revealed slightly reduced LDLR plasma membrane expression (74 ± 15% of wt) and LDL particle uptake (64 ± 11% of wt). The structural analysis showed a defect associated with the loss of a specific side chain contact.

p.Arg416Trp

The variant has been described in HGMD (as disease causing), ClinVar (as pathogenic and likely pathogenic), LOVD (as probably affects function), and in 31 Czech patients. The variant was associated with plasma membrane localization, without formation of protein clumps on ER. No statistically significant changes of gene expression of ER resident proteins were observed. Flow cytometry showed a mild decrease of LDLR plasma membrane localization (72 ± 1% of wt) and LDL particle internalization (78 ± 15% of wt). Structural analysis

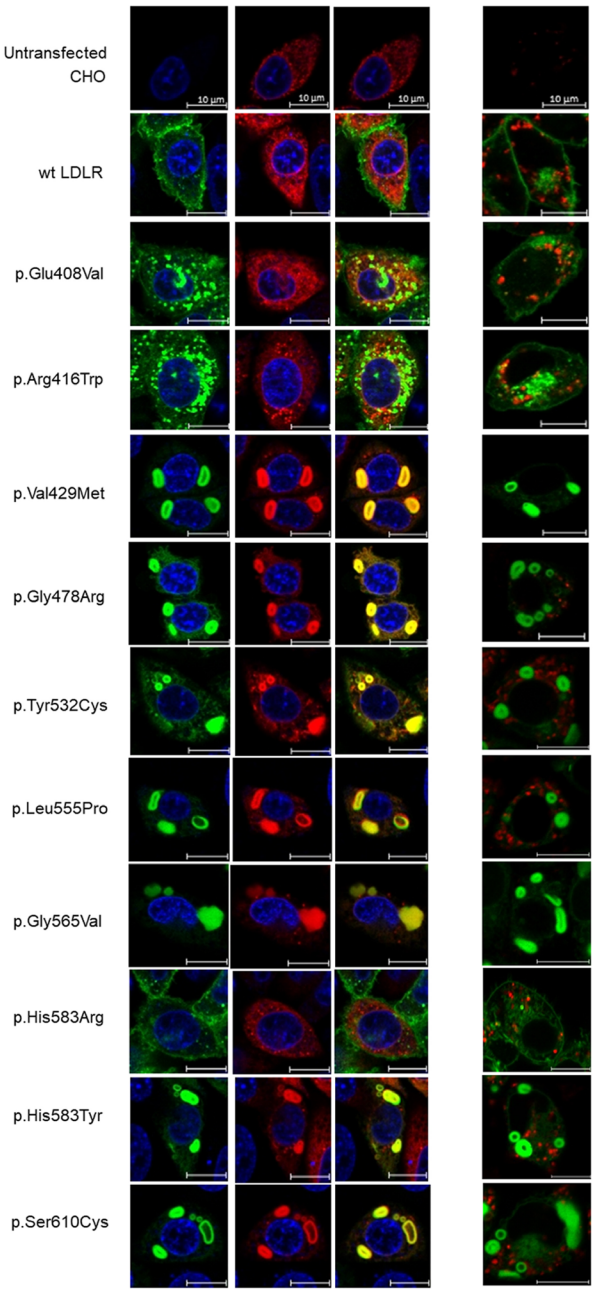

FIGURE 1 | Live cell imaging microscopy of LDLR and ER **(A)**. LDLR was visualized via EYFP (green), ER via RFP (red). The LDLR presence on ER was evaluated by EYFP/RFP fluorescence overlay (yellow). The cell nucleus was stained with Hoechst 33342 (blue). Live cell imaging microscopy of LDLR and internalized LDL particles **(B)**. LDLR was visualized via EYFP (green), LDL particles via pHRodo-Red (red). Representative cells are presented. Scale bar represents 10 µm.

[1]https://portal.biobase-international.com

[2]https://www.ncbi.nlm.nih.gov/clinvar/

[3]https://databases.lovd.nl/shared/genes/LDLR

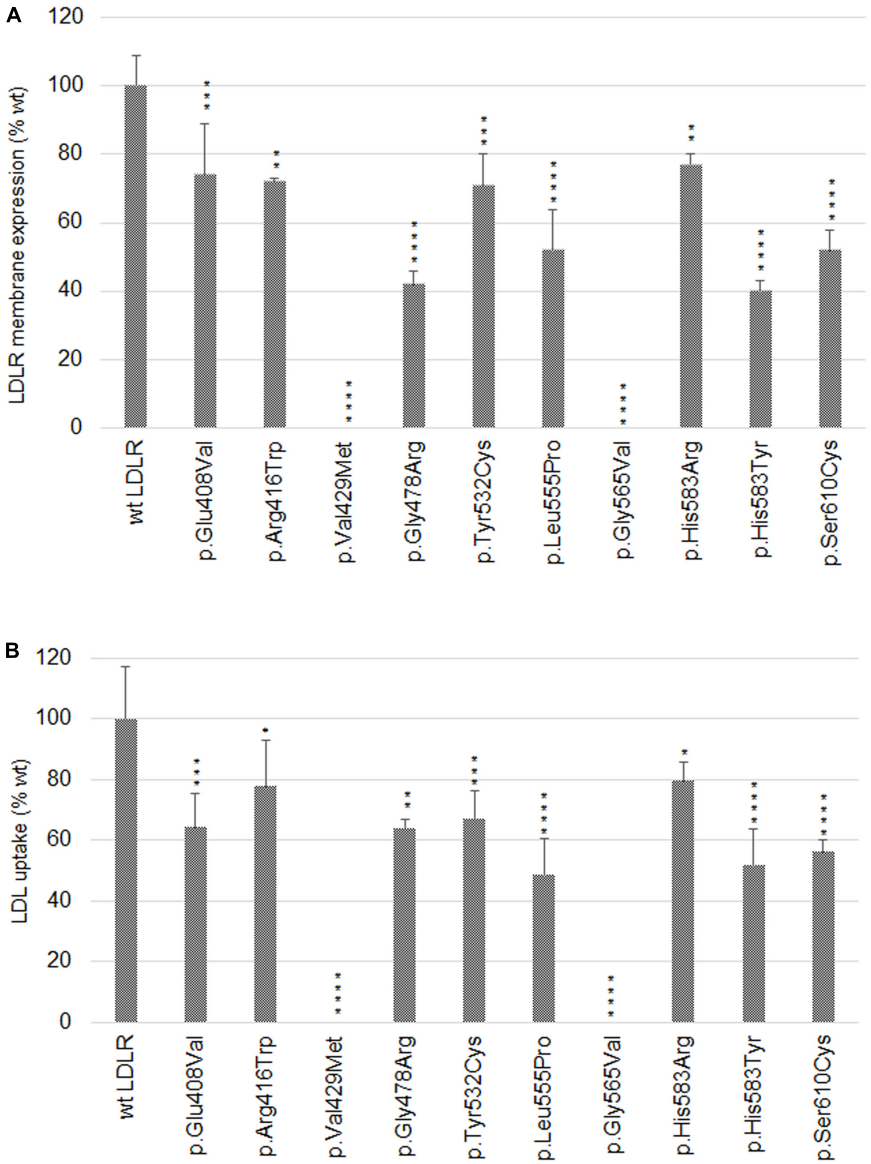

FIGURE 2 | Graphic evaluation of flow cytometry analyses of LDLR plasma membrane expression **(A)** and LDL particle internalization **(B)**. The values represent the mean of three independent experiments; error bars ± S.D. Statistical significance was determined using Dunnett's test. The statistical significance degree of difference: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

showed no defect. Functional analysis of p.Arg416Trp using flow cytometry was performed also in the study (Etxebarria et al., 2015). In this study, flow cytometry showed diminished LDLR plasma membrane localization (62 ± 5% of wt), LDLR–LDL binding activity (53 ± 13% of wt), and LDL particle uptake (59 ± 1% of wt).

p.Val429Met

The variant has been described in HGMD (as disease causing), ClinVar (as pathogenic and likely pathogenic), LOVD (as probably affects function), and in two Czech patients. The variant was associated with accumulation of the protein on ER in the form of clumps and with an increased expression of all analyzed ER resident proteins. Flow cytometry showed no LDLR on the plasma membrane and no LDL particle internalization. Structural analysis found a structural defect associated with the change of volume in buried AA.

p.Gly478Arg

The variant has been described in HGMD (as disease causing and uncertain significance), ClinVar (as pathogenic, likely pathogenic, and uncertain significance), LOVD (as probably affects function), and in one Czech patient. On the basis of our experiments, the variant has a deleterious effect on the protein

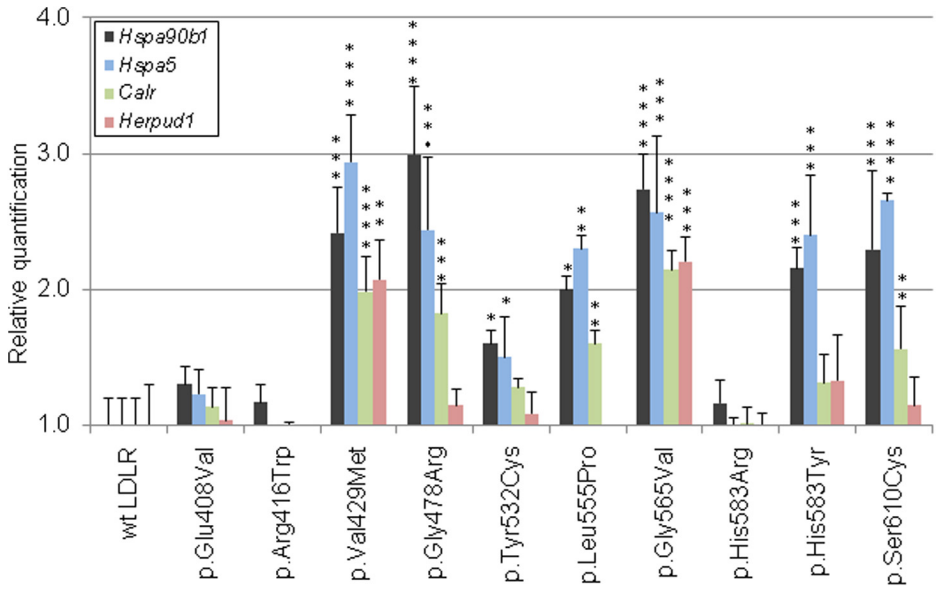

FIGURE 3 | Graphic evaluations of quantitative mRNA analyses of ER-resident proteins. The mRNA levels of Hsp90b1, Hspa5, Calr, and Herpud1 were determined by qRT-PCR. The values represent the mean of three independent experiments performed in triplicates; error bars ± S.D. The statistical significance was determined by the Dunnett's test. The statistical significance degree of difference: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

localization and function. It was associated with accumulation of the protein on ER in the form of clumps and with an increased expression of ER resident chaperones Hsp90b1, Hspa5, and Calr. Flow cytometry revealed a reduced LDLR plasma membrane expression (42 ± 4% of wt) and LDL particle internalization (64 ± 3% of wt). Structural analysis found a structural defect associated with the change of charge, polarity, and volume in buried AA.

p.Tyr532Cys

The variant was described only in the population of Czech FH patients (1 patient). Functional analysis showed that this variant has a deleterious effect on the protein. It was associated with accumulation of the protein on ER in the form of clumps and with an increased expression of ER resident chaperones Hsp90b1 and Hspa5. Flow cytometry showed a decreased protein plasma membrane localization (71 ± 9% of wt) and LDL particle internalization (67 ± 9% of wt). Structural analysis showed a structural defect associated with the loss of specific side chain contact.

p.Leu555Pro

The variant has been described in HGMD (as disease causing), ClinVar (as likely pathogenic), and LOVD (as probably affects function) but without performing a functional analysis so far. On the basis of our analysis, this variant has a deleterious effect on the protein localization and function. It was associated with accumulation of the protein on ER in the form of clumps and with an increased expression of ER resident chaperones Hsp90b1, Hspa5, and Calr. Flow cytometry showed a decreased protein localization on the plasma membrane (52 ± 12% of wt) and LDL particle internalization (49 ± 12% of wt). Structural analysis showed a structural defect associated with the Pro residue substitution.

p.Gly565Val

The variant has been described in HGMD (as disease causing), ClinVar (as pathogenic and likely pathogenic), and LOVD (as probably affects function). The variant was associated with a complete retention of the protein on ER in the form of clumps and with an increased expression of all analyzed ER resident proteins. Flow cytometry revealed no LDLR plasma membrane localization and no LDL particle internalization. Structural analysis showed a structural defect associated with the change of polarity and volume in buried AA and with the Gly residue substitution.

p.His583Arg

The variant has been described in HGMD (as disease causing), ClinVar (as likely pathogenic), LOVD (as probably affects function), and also in one Czech patients. The variant was associated with LDLR plasma membrane localization, no accumulation of the protein on ER in the form of clumps, and with no statistically significant changes of expression of ER resident proteins. Flow cytometry showed a slightly reduced LDLR plasma membrane expression and LDL particle uptake (77 ± 3% and 80 ± 6% of wt, respectively). For the p.His583Arg variant, we did not find any structural defect.

p.His583Tyr

The variant has been described in HGMD (as disease causing and uncertain significance), ClinVar (as pathogenic, likely

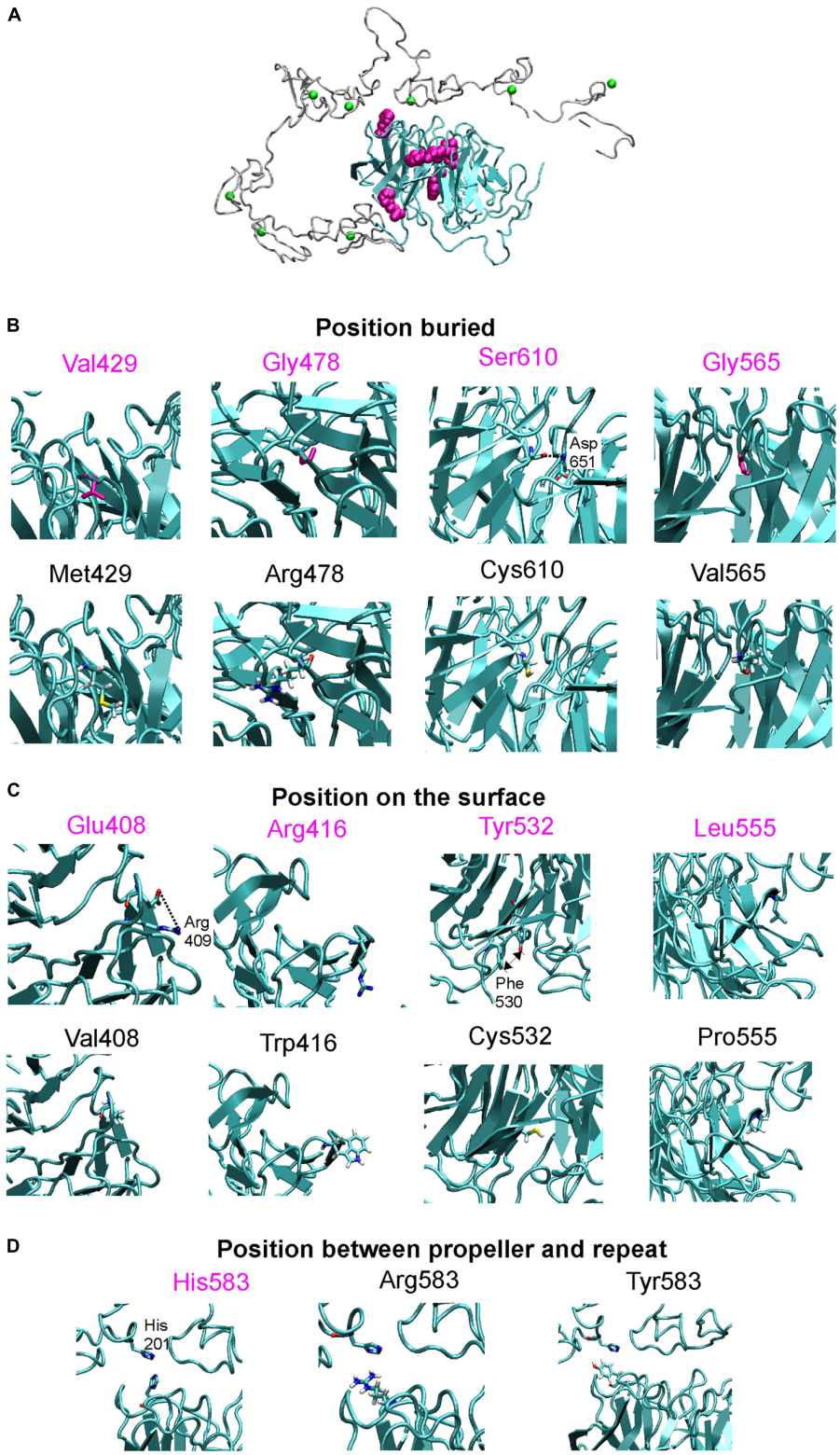

FIGURE 4 | (A) X-ray structure of LDLR with highlighted beta-propeller (in cyan) and studied amino acid positions (in magenta surface representation), Ca^{2+} ions bound in repeats are in green. Detailed view on wt and mutant structures which were divided according to amino acid positions into positions buried inside the protein **(B)**, positions on the protein surface **(C)**, and positions between the beta-propeller and the R5 repeat **(D)**. These views show interaction between studied amino acids and surroundings, note salt bridge between Glu408-Arg409, H-bonding between Ser610-Asp651, or stacking between Tyr532 and Phe530. Close contact between His583 from propeller and His201 from repeat 5 is also shown.

TABLE 1 | Summary of experimental and structural analysis results.

Protein variant	Contact via side chain	Buriedness (RSA)	Change of			Pro/Gly change	LCIM	FC (loc., % of wt)	FC (LDL int., % of wt)	Increased chaperone expression	Group
			Charge	Polarity	Volume						
wt	NA	NA	NA	NA	NA	NA	Membrane + ER	100 ± 9	100 ± 17	No	–
p.Glu408Val		26%					Membrane + ER	74 ± 15	64 ± 11	No	2
p.Arg416Trp		54%					Membrane + ER	72 ± 1	78 ± 15	No	2
p.Val429Met		6%					ER[a]	0	0	Yes	1
p.Gly478Arg		0%					ER[a]	42 ± 4	64 ± 3	Yes	1
p.Tyr532Cys		14%					ER[a]	71 ± 9	67 ± 9	Yes	3
p.Leu555Pro		18%					ER[a]	52 ± 12	49 ± 12	Yes	3
p.Gly565Val		0%					ER[a]	0	0	Yes	1
p.His583Arg		19%					Membrane + ER	77 ± 3	80 ± 6	No	2
p.His583Tyr		19%					Membrane + ER[a]	40 ± 3	52 ± 12	Yes	3
p.Ser610Cys		0%					ER[a]	52 ± 6	56 ± 4	Yes	1

The blue fields show detected structure features (see section "Materials and Methods" for definition of features). ER: endoplasmic reticulum; ER[a]: accumulation of LDLR on ER in the form of clumps; LCIM: live cell imaging microscopy; FC (loc.): LDLR plasma membrane localization determined by flow cytometry; FC (LDL int.): LDL particle internalization determined by flow cytometry; wt: wild-type; Group: three groups described in the study (see section "Discussion" for definition of groups).

pathogenic, and likely benign), and LOVD (as probably affects function). This variant has a deleterious effect on the protein localization and function on the basis of our analysis. It was associated with the accumulation of the protein on ER in the form of clumps and with an increased expression of ER resident chaperones Hsp90b1 and Hspa5. Flow cytometry analysis revealed a decreased LDLR protein localization on the plasma membrane (40 ± 3% of wt) and a decreased LDL particle internalization (52 ± 12% of wt). For the p.His583Tyr variant localized on the protein surface, we did not find any defect. However, His583 is localized on the interface between the beta-propeller and the ligand binding repeat R5, where it has a close contact (3.2 Å) with His211 from R5. Most probably, the variant p.His583Tyr, resulting in loss of charge, will have a more severe effect on the protein structure than p.His583Arg.

p.Ser610Cys

The variant has been described in HGMD (as disease causing), ClinVar (as pathogenic and likely pathogenic), LOVD (as probably affects function), and in five Czech FH patients. Functional analysis has not been performed yet. On the basis of our analysis this variant has a deleterious effect on the LDLR protein localization and function. It was associated with the accumulation of the protein on ER in the form of clumps and with an increased expression of ER resident chaperones Hsp90b1, Hspa5, and Calr. Flow cytometry revealed a decreased protein localization on the plasma membrane (52 ± 6% of wt) and a decreased LDL particle internalization (56 ± 4% of wt). Structural analysis showed a structural defect associated with the loss of a specific side chain contact and the change of polarity and volume in buried AAs.

The impact of missense variants was also analyzed using commonly used prediction programs such as MutationTaster, SIFT, and PolyPhen-2 (HumVar model). All variants were considered as disease causing and deleterious using MutationTaster and SIFT, respectively; and probably damaging using PolyPhen-2, except for p.Glu408Val and p.Val429Met that were evaluated as possibly damaging.

DISCUSSION

In this study, we focused on LDLR sequence variants localized in the six-bladed beta-propeller of epidermal growth factor precursor homology domain, and their effects on the LDLR protein localization, function, and on mRNA level of selected ER-resident proteins associated with ER stress and UPR. The experimental results were supplemented by the 3D protein structural analysis.

Our results show that the analyzed LDLR protein variants can be divided into three groups: (1) p.Val429Met, p.Gly478Arg, p.Gly565Val, and p.Ser610Cys. The variants are buried inside the 3D protein structure and replacing AAs change charge, polarity, and/or volume. Expression of proteins carrying the mentioned variants was associated with the ER protein accumulation in the form of clumps; significant increase in the expression of ER-resident chaperones Hsp90b1, Hspa5, and Calr; no or reduced LDLR plasma membrane localization (0%, 42 ± 4%, 0%, 52 ± 6% of wt, respectively); and no or reduced LDL particle internalization (0%, 55 ± 3%, 0%, 47 ± 4% of wt, respectively). (2) p.Glu408Val, p.Arg416Trp, and p.His583Arg. The variants are localized on the surface of 3D protein structure. Expression of the proteins was characterized by no ER protein accumulation in the form of clumps; no increased mRNA level of ER-resident chaperones; slightly reduced LDLR plasma membrane localization (74 ± 15%, 72 ± 1%, 77 ± 3% of wt, respectively) and

LDL particle internalization (64 ± 11%, 78 ± 15%, 80 ± 6% of wt, respectively). (3) p.Tyr532Cys, p.Leu555Pro, and p.His583Tyr. The variants are localized on the surface of the 3D protein structure but unlike the group 2, these were associated with ER protein accumulation in the form of clumps and increased mRNA level of ER-resident chaperones. Values of LDLR plasma membrane localization are 71 ± 9%, 52 ± 12%, 40 ± 3% of wt, respectively, and LDL particle internalization 67 ± 9%, 49 ± 12%, 52 ± 12% of wt, respectively. Apparently, structural defects of these variants are more severe than in variants of the group 2.

Based on the results of testing the suitability of monitoring mRNA expression changes of various ER stress related genes, Calr, HspaA5, Hsp90b1, and Herpud1 were selected as appropriate for qRT-PCR using TaqMan probes. The CALR, HSPA5, and HSP90B1 proteins belong to the most abundant ER chaperones (Araki and Nagata, 2012). Our qRT-PCR results indicated that transcript levels of all these genes were significantly increased in cells expressing the LDLR variants p.Val429Met, p.Gly478Arg, p.Leu555Pro, p.Gly565Val, and p.Ser610Cys; in case of Hspa5 and Hsp90b1 also in p.Tyr532Cys and p.His583Tyr. For all mentioned variants, LDLR was localized predominantly on ER in the form of clumps. HERPUD1 is a component of ERAD involved in ubiquitin-dependent degradation of misfolded ER proteins (Schulze et al., 2005). We have observed increased mRNA levels of Herpud1 in the variants p.Val429Met and p.Gly565Val. From all analyzed variants, p.Val429Met and p.Gly565Val demonstrated the most serious impact on the receptor localization and function.

Analyzing LDLR localization and function was performed using live cell imaging microscopy. This methodical approach replaced our earlier methods based on classical confocal laser scanning microscopy (CLSM) using fixed cells (Pavlouskova et al., 2016). It was proven that commonly used fixation and permeabilization agents can cause extraction or re-localization of membrane bound proteins in the cell, not reflecting the *in vivo* situation (Schnell et al., 2012). We observed similar results for both fixed and live cells. Further, we performed microscopy experiments analyzing LDL particle uptake. LDL particles were conjugated with Dil (1,10-Dioctadecyl-3, 3,3′,3′-Tetramethylindocarbocyanine Perchlorate, Molecular Probes) or pHRodo-Red in CLSM and live cell imaging microscopy, respectively. Results of these analyses were also similar – all variants except p.Val429Met and p.Gly565Val were able to internalize LDL particles to a diverse extent. For a more detailed evaluation, flow cytometry was used. The cut off value for determining whether a LDLR variant is considered a functional mutant by *in vitro* studies has not been established, but based on published studies, *in vitro* LDLR activity less than 70–80% of wt protein activity (either in the LDLR plasma membrane expression or LDL particle internalization) could classify a variant as pathogenic (Benito-Vicente et al., 2015). Based on this assumption, all analyzed variants are pathogenic but with distinct effects on induction of cellular responses at the level of protein localization, accumulation, function, and expression of genes associated with ER stress.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LD participated in the design of the study, performed live cell imaging microscopy, and together with JF confocal laser scanning microscopy. LN and TL performed the flow cytometry analyses. PZ performed the quantitative RT-PCR, KR 3D protein structural analyses. LT performed the selection of variants for functional and *in silico* studies. TF and LF supervised the entire project and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Araki, K., and Nagata, K. (2012). Protein folding and quality control in the ER. *Cold Spring Harb. Perspect. Biol.* 4:a015438. doi: 10.1101/cshperspect.a015438

Back, S. H., Schroder, M., Lee, K., Zhang, K., and Kaufman, R. J. (2005). ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods* 35, 395–416. doi: 10.1016/j.ymeth.2005.03.001

Benito-Vicente, A., Alves, A. C., Etxebarria, A., Medeiros, A. M., Martin, C., and Bourbon, M. (2015). The importance of an integrated analysis of clinical, molecular, and functional data for the genetic diagnosis of familial hypercholesterolemia. *Genet. Med.* 17, 980–988. doi: 10.1038/gim.2015.14

Benn, M., Watts, G. F., Tybjaerg-Hansen, A., and Nordestgaard, B. G. (2012). Familial hypercholesterolemia in the danish general population: prevalence, coronary artery disease, and cholesterol-lowering medication. *J. Clin. Endocrinol. Metab.* 97, 3956–3964. doi: 10.1210/jc.2012-1563

Brown, M. S., and Goldstein, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34–47. doi: 10.1126/science.3513311

Brown, M. S., Herz, J., and Goldstein, J. L. (1997). LDL-receptor structure. Calcium cages, acid baths and recycling receptors. *Nature* 388, 629–630. doi: 10.1038/41672

Chan, S. L., Fu, W., Zhang, P., Cheng, A., Lee, J., and Kokame, K. (2004). Herp stabilizes neuronal Ca2+ homeostasis and mitochondrial function during endoplasmic reticulum stress. *J. Biol. Chem.* 279, 28733–28743. doi: 10.1074/jbc.M404272200

Chothia, C. (1976). The nature of the accessible and buried surfaces in proteins. *J. Mol. Biol.* 105, 1–12. doi: 10.1016/0022-2836(76)90191-1

Eletto, D., Dersh, D., and Argon, Y. (2010). GRP94 in ER quality control and stress responses. *Semin. Cell Dev. Biol* . 21, 479–485. doi: 10.1016/j.semcdb.2010.03.004

Etxebarria, A., Benito-Vicente, A., Palacios, L., Stef, M., Cenarro, A., and Civeira, F. (2015). Functional characterization and classification of frequent low-density lipoprotein receptor variants. *Hum. Mutat.* 36, 129–141. doi: 10.1002/humu.22721

Frishman, D., and Argos, P. (1995). Knowledge-based protein secondary structure assignment. *Proteins* 23, 566–579. doi: 10.1002/prot.340230412

Hebert, D. N., and Molinari, M. (2007). In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiol. Rev.* 87, 1377–1408. doi: 10.1152/physrev.00050.2006

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* 13, 89–102. doi: 10.1038/nrm3270

Hetz, C., Chevet, E., and Oakes, S. A. (2015). Proteostasis control by the unfolded protein response. *Nat. Cell Biol.* 17, 829–838. doi: 10.1038/ncb3184

Hobbs, H. H., Russell, D. W., Brown, M. S., and Goldstein, J. L. (1990). The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu. Rev. Genet.* 24, 133–170. doi: 10.1146/annurev.ge.24.120190.001025

Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. *J Mol Graph* 14, 37–38. doi: 10.1016/0263-7855(96)00018-5

Li, S. C., Goto, N. K., Williams, K. A., and Deber, C. M. (1996). Alpha-helical, but not beta-sheet, propensity of proline is determined by peptide environment. *Proc. Natl. Acad. Sci. U.S.A.* 93, 6676–6681. doi: 10.1073/pnas.93.13.6676

Marzec, M., Eletto, D., and Argon, Y. (2012). GRP94: an HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. *Biochim. Biophys. Acta* 1823, 774–787. doi: 10.1016/j.bbamcr.2011.10.013

Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I., and Opas, M. (2009). Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem. J.* 417, 651–666. doi: 10.1042/BJ20081847

Minamino, T., Komuro, I., and Kitakaze, M. (2010). Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease. *Circ. Res.* 107, 1071–1082. doi: 10.1161/CIRCRESAHA.110.227819

Pavlouskova, J., Reblova, K., Tichy, L., Freiberger, T., and Fajkusova, L. (2016). Functional analysis of the p.(*Leu*15Pro) and p.(Gly20Arg) sequence changes in the signal sequence of LDL receptor. *Atherosclerosis* 250, 9–14. doi: 10.1016/j.atherosclerosis.2016.04.022

Rader, D. J., Cohen, J., and Hobbs, H. H. (2003). Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *J. Clin. Invest.* 111, 1795–1803. doi: 10.1172/JCI18925

Reblova, K., Kulhanek, P., and Fajkusova, L. (2015). Computational study of missense mutations in phenylalanine hydroxylase. *J. Mol. Model.* 21:70. doi: 10.1007/s00894-015-2620-6

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* 8, 519–529. doi: 10.1038/nrm2199

Rudenko, G., Henry, L., Henderson, K., Ichtchenko, K., Brown, M. S., and Goldstein, J. L. (2002). Structure of the LDL receptor extracellular domain at endosomal pH. *Science* 298, 2353–2358. doi: 10.1126/science.1078124

Schnell, U., Dijk, F., Sjollema, K. A., and Giepmans, B. N. (2012). Immunolabeling artifacts and the need for live-cell imaging. *Nat. Methods* 9, 152–158. doi: 10.1038/nmeth.1855

Schulze, A., Standera, S., Buerger, E., Kikkert, M., van Voorden, S., and Wiertz, E. (2005). The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J. Mol. Biol.* 354, 1021–1027. doi: 10.1016/j.jmb.2005.10.020

Sjouke, B., Kusters, D. M., Kindt, I., Besseling, J., Defesche, J. C., and Sijbrands, E. J. (2015). Homozygous autosomal dominant hypercholesterolaemia in the Netherlands: prevalence, genotype-phenotype relationship, and clinical outcome. *Eur. Heart J.* 36, 560–565. doi: 10.1093/eurheartj/ehu058

Sorensen, S., Ranheim, T., Bakken, K. S., Leren, T. P., and Kulseth, M. A. (2006). Retention of mutant low density lipoprotein receptor in endoplasmic reticulum (ER) leads to ER stress. *J. Biol. Chem.* 281, 468–476. doi: 10.1074/jbc.M507071200

Strom, T. B., Tveten, K., Holla, O. L., Cameron, J., Berge, K. E., and Leren, T. P. (2011). The cytoplasmic domain is not involved in directing Class 5 mutant LDL receptors to lysosomal degradation. *Biochem. Biophys. Res. Commun.* 408, 642–646. doi: 10.1016/j.bbrc.2011.04.077

Tichy, L., Fajkusova, L., Zapletalova, P., Schwarzova, L., Vrablik, M., and Freiberger, T. (2017). Molecular genetic background of an autosomal dominant hypercholesterolemia in the Czech Republic. *Physiol. Res.* 66(Suppl. 1), S47–S54. doi: 10.33549/physiolres.933587

Tichy, L., Freiberger, T., Zapletalova, P., Soska, V., Ravcukova, B., and Fajkusova, L. (2012). The molecular basis of familial hypercholesterolemia in the Czech Republic: spectrum of LDLR mutations and genotype-phenotype correlations. *Atherosclerosis* 223, 401–408. doi: 10.1016/j.atherosclerosis.2012.05.014

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101, 249–258. doi: 10.1016/s0092-8674(00)80835-1

Vrablik, M., Vaclova, M., Tichy, L., Soska, V., Blaha, V., and Fajkusova, L. (2017). Familial hypercholesterolemia in the Czech Republic: more than 17 years of systematic screening within the MedPed project. *Physiol. Res.* 66(Suppl. 1), S1–S9. doi: 10.33549/physiolres.933600

Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., and Prywes, R. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell.* 6, 1355–1364. doi: 10.1016/s1097-2765(00)00133-7

Zamyatnin, A. A. (1972). Protein volume in solution. *Prog. Biophys. Mol. Biol.* 24, 107–123. doi: 10.1016/0079-6107(72)90005-3

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CASE REPORT
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A Novel Nonsense Mutation of *ABCA8* in a Han-Chinese Family With ASCVD Leads to the Reduction of HDL-c Levels

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Arteriosclerotic cardiovascular disease (ASCVD) is one of the major causes of death worldwide and most commonly develops as a result of atherosclerosis (AS). As we all know, dyslipidemia is a leading pathogenic risk factor for ASCVD, which leads to cardiac ischemic injury and myocardial infarction. Dyslipidemias include hypercholesterolemia, hypertriglyceridemia, increased low-density lipoprotein cholesterol (LDL-c) and decreased high density lipoproteins cholesterol (HDL-c). Mutations of dyslipidemia related genes have been proved to be the crucial contributor to the development of AS and ASCVD. In this study, a Han-Chinese family with ASCVD was enrolled and the lipid testing discovered an obvious reduced levels of HDL-c in the affected members. We then performed whole exome sequencing to detect the candidate genes of the family. After data filtering, a novel heterozygous nonsense mutation (NM_007168: c.3460C>T; p.R1154X) of *ABCA8* was detected and validated to be co-separated in the family members by Sanger sequencing. Previous studies have proved that deleterious heterozygous *ABCA8* variants may disrupt cholesterol efflux and reduce HDL-c levels in humans and mice. This study may be the second report related to *ABCA8* mutations in patients with reduced levels of HDL-c. Our study not only contributed to the genetic counseling and prenatal genetic diagnosis of patients with ASCVD caused by reduced HDL-c levels, but also provided a new sight among ABCA8, cholesterol efflux and HDL-c levels.

Keywords: atherosclerosis, reduced HDL-c levels, cholesterol efflux, *ABCA8*, nonsense mutation

Abbreviations: ABCA1, ATP binding cassette subfamily A member 1; ABCA8, ATP binding cassette subfamily A member 8; AS, atherosclerosis; ASCVD, arteriosclerotic cardiovascular disease; HDL-c, high density lipoprotein; LDL-c, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

INTRODUCTION

Atherosclerosis is the major contributor of ischemic syndromes such as myocardial infarction or stroke, mainly resulted from plaque rupture and subsequent arterial blockade (Kobiyama and Ley, 2018). The incidence of AS is steadily rising along with an increasingly older population worldwide (Libby et al., 2016). The epidemiological survey shows that more than 20 million patients die from AS-related disorders worldwide every year (Herrington et al., 2016).

Previous studies have revealed that dyslipidemia was the predominant pathogenic factor of atherosclerotic plaque on the artery walls, which finally lead to AS and AS-related disorders (Hurtubise et al., 2016). And the main forms of dyslipidemia contains hypercholesterolemia, hypertriglyceridemia, and reduced high density lipoprotein (HDL-c). Dyslipidemia is a multi-factorial disease, which derives from complex interactions between genetic and environmental lesions. Many genes have been identified may be responsible for hypercholesterolemia and hypertriglyceridemia, for instance, *Low-Density Lipoprotein Receptor*, *Proprotein Convertase Subtilisin/Kexin type 9*, *Lipoprotein lipase*, *Apolipoprotein C2*, *Reticulon 3* and et al. (Surendran et al., 2012; Xiang et al., 2017, 2018). Low levels of HDL-c increase the risk of atherosclerotic cardiovascular disorder and shorten life expectancy. However, the underlying cause of reduced HDL-c values was still not clear. Several genes including ATP binding cassette subfamily A member 1 (ABCA1) and ATP binding cassette subfamily A member 8 (*ABCA8*) have been identified in patients with reduced HDL-c (Trigueros-Motos et al., 2017; Maranghi et al., 2019).

Here, we enrolled a Han-Chinese family with Coronary Heart Disease (ASCVD) (**Figure 1A**). Lipid testing revealed that the levels of HDL-c were overt decreased, while the levels of low-density lipoprotein cholesterol (LDL-c), total cholesterol (TC) and triglycerides (TG) were close to normal standards. Whole exome sequencing and Sanger sequencing were employed to detect the genetic lesion of the family.

CASE PRESENTATION

The proband (II-2), a 54-year-old lady, came to the hospital due to recurrent chest pain in last 2 years. Coronary angiography indicated approximately 60–80% stenosis of the anterior descending coronary artery (**Figure 1B**), ECG testing also suggested the patient suffered from CHD (**Figure 1C**). However, the lipid testing described the level of LDL-c (3.94 mmol/L; control: <3.12 mmol/L) and a distinctly reduced levels of HDL-c (0.41 mmol/L; control: 0.9–2.19 mmol/L) of the proband (**Table 1**). This discovery attracted our interest because most patients with ASCVD commonly presented high levels of LDL-c, TC, and TG (Hurtubise et al., 2016). We then investigated the family history of the proband (II-2), which indicated that her young sister (II-4) has been diagnosed as the occlusion of left iliac artery and her father (I-1) was died from myocardial infarction at 60-year-old. Lipid testing further described that both her son (III-1) and her young sister (II-4) showed an overt reduced levels of HDL-c and normal levels of LDL-c, TC, and TG (**Table 1**). And his young sister (II-4) also suffered from arterial plaque in the left lower limb. The blood pressure of the proband was 80–120 mmHg and the fasting blood-glucose was 5.2 mmol/L. The proband (II-2) accepted the treatment of percutaneous coronary intervention, the proband did not complain any uncomfortable after treatment. And breathing sound of the lungs was clearly, the heart rate was normal (**Figure 1D**), and the insertion site recovered well. The II-4 is accepting recovery treatment by exercise and diet control.

LABORATORY INVESTIGATIONS

We supposed that the low levels of HDL-c may be the leading cause of AS and ASCVD in this family. However, what's the genetic lesion underling the reduced levels of HDL-c in this family? We then isolated the genomic DNA of the proband and other family members (I-2, II-1, II-2, II-4, III-1, and III-2). Whole exome sequencing of the proband (II-2) was performed to detect the candidate gene of reduced levels of HDL-c.

In short, Exome capture and next-generation sequencing were conducted by Novogene Bioinformatics Institute (Beijing, China). One microgram of qualified genomic DNA from each person was captured by the Agilent's SureSelect Human All Exon kit V5 (Agilent Technologies, Inc., Santa Clara, CA, United States) and sequenced by Illumina Hiseq4000 (Illumina Inc., San Diego, CA, United States). Shortly, genomic DNA were randomly carved by Covaris S220 sonicator (Covaris, Inc., Woburn, MA, United States) (Fan et al., 2019a). Then the fragmented DNAs underwent three enzymatic steps: end repair, A-tailing and adapters ligation. The adapter-ligated DNA fragments were amplified with Herculase II fusion DNA polymerase (Agilent). Later, the exomes in the pre-capture libraries were captured by SureSelect capture library kit (Agilent) (Fan et al., 2019b). After DNA quality estimation, the captured DNA library was used for next-generation sequencing on Illumina Hiseq4000 platform (Fan et al., 2019a). Downstream processing was carried out by Genome Analysis Toolkit (GATK), Varscan2 and Picard, and variant calls were performed by the GATK Haplotype Caller (Fan et al., 2019a). Variant annotation referred to Ensemble release 82, and filtering was conducted by ANNOVAR Documentation.

The filtered non-synonymous SNPs or INDELs with an alternative allele frequency more than 1% in public databases were kicked before further analysis. The public databases contains the NHLBI Exome Sequencing Project Exome Variant Server (ESP6500), dbSNP144[1], the 1000 Genomes project[2], the ExAC database[3] and in-house exome databases of Novogene (2500 exomes) (Fan et al., 2019b). Then the filtered SNVs and

[1]http://www.ncbi.nlm.nih.gov/projects/SNP/index.html

[2]http://www.1000genomes.org/

[3]http://exac.broadinstitute.org

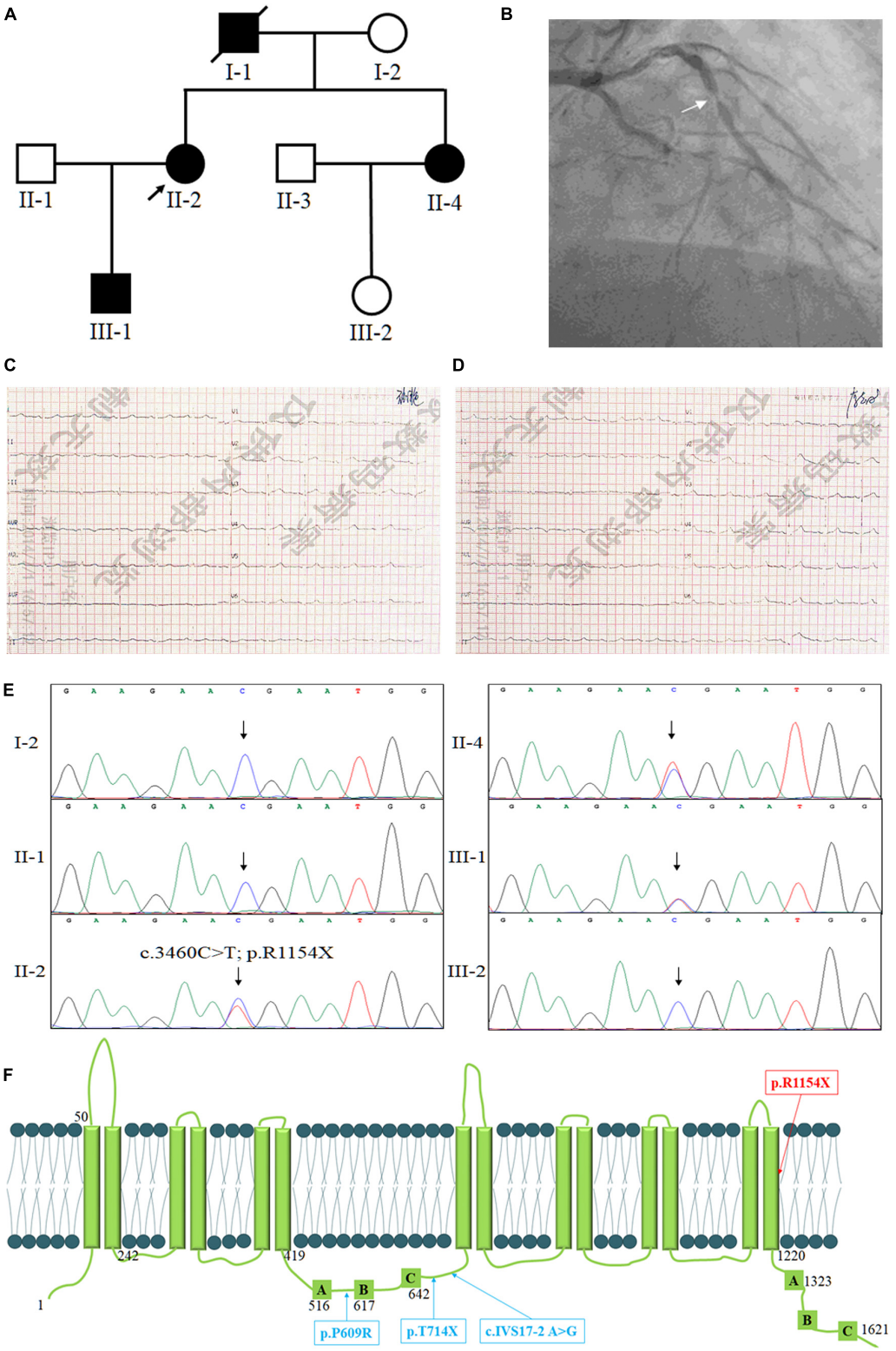

FIGURE 1 | The clinical and genetic information of the family. **(A)** Pedigree of the family with low levels of HDL-c. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; closed symbols, affected members; open symbols, unaffected members; arrow, proband. **(B)** The coronary angiography of the proband, the arrow shows the stenosis of the anterior descending coronary artery. The ECG testing of the proband before percutaneous coronary intervention **(C)** and after percutaneous coronary intervention **(D)**. **(E)** Sequencing results of the *ABCA8* mutation. Sequence chromatogram indicates a C to T transition of nucleotide 3460. **(F)** The structure of ABCA8 and the summary of reported mutations of *ABCA8*.

TABLE 1 | The lipid testing data of the family members.

Subjects	II-2 (proband)	I-2	II-1	II-3	II-4	III-1	III-2	Standard
TC (mmol/L)	5.20	5.84	4.51	3.40	4.98	4.75	3.22	2.86–5.98
TG (mmol/L)	1.07	1.28	1.14	1.09	1.05	1.30	1.17	0.22–1.21
LDL-c (mmol/L)	3.94	4.32	3.96	2.77	4.02	3.99	3.05	<3.12
HDL-c (mmol/L)	0.41	1.19	1.43	1.38	0.47	0.51	1.60	0.9–2.19
AS and/or CHD	Yes	No	No	No	Yes	No	No	N/A
BMI (kg/m^2)	22.48	23.55	22.49	23.71	22.20	24.07	24.34	18.5–23.9

TC, total cholesterol; TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; HDL-c, high density lipoprotein; AS, atherosclerosis; CHD, oronary heart disease; BMI, body mass index.

INDELs, predicted by SIFT[4], Polyphen2[5], and MutationTaster[6] to be damaging, were remained (Fan et al., 2019a).

After data filtering, a novel nonsense mutation (NM_007168: c.3460C>T; p.R1154X) of *ABCA8* was identified and validated by Sanger sequencing in the proband (**Figure 1E**). Previous studies have revealed that deleterious heterozygous *ABCA8* mutations may disrupt cholesterol efflux and reduce HDL-c levels in humans and mice (Trigueros-Motos et al., 2017; Sasaki et al., 2018). No other meaningful mutations related to lipid metabolism has been identified. Sanger sequencing further confirmed that only the affected individuals (II-2, II-4, and III-1) carried the novel nonsense mutation (NM_007168: c.3460C>T; p.R1154X) of *ABCA8* (**Figure 1F**). The novel mutation, resulting a truncated protein, was absent in the healthy members (I-2, II-1, and III-2) and 200 local people who were used as an internal control to exclude the SNP in local people (Fan et al., 2019a). Bioinformatics predicted that the newly identified mutation was deleterious and may disrupt the structure and function of ABCA8 (Schwarz et al., 2014). On the basis of ACMG guidelines (Richards et al., 2015), the novel variant meetings the following criteria from the ACMG guidelines: PVS1, PS3, and PM2.

DISCUSSION

As the extremely crucial transmembrane proteins, the ABC (ATP-binding cassette) transporters were encoded by 48 ABC transporter genes which were divided in to seven subfamilies named A–G in human (Kim et al., 2013; Hedditch et al., 2014). The ABC transporters are responsible for transferring substrates such as lipids, peptides, ions, carbohydrates, and vitamins across membranes by employing the energy from the hydrolysis of ATP (Trigueros-Motos et al., 2017; Sasaki et al., 2018). The subfamily A (ABCA) consists of 12 members in two subgroups: ABCA6-like and ABCA1-like transporter.

The subfamily A (ABCA) has 12 members with two subgroups, i.e., ABCA6-like and ABCA1-like transporter. The ABC1–4, 7, and 12 belongs to the ABCA1-like subgroup which play an important role in transporting cholesterol and phospholipids transport (Tsuruoka et al., 2002). However, less is known about the functional roles of the ABCA6-like subgroup transporters, i.e., ABCA5–6, 8–10 in humans, although several reports have described the tissue mRNA and protein expressions (Kim et al., 2013; Hedditch et al., 2014). ABCA8 is expressed in the brain, heart, small intestine, liver, lung, pancreas, prostate, spleen, testicle in human tissue (Bleasel et al., 2013; Demidenko et al., 2015; Gidding et al., 2015). However, less is known about its functional roles *in vivo*.

The human *ABCA8* gene encoding ATP-binding cassette-subfamily A, member 8 protein is located on chromosome 17q24.2, encoding 1621amino. ABCA8 contains 14 predicted transmembrane domains and 2 putative ATP-binding cassettes, but it lacks the common ABC transporter motif LSGGQ (Tsuruoka et al., 2002). At first, ABCA8 was classified into ABCA6-like transporters subgroup which was not responsible for lipid transporting in the ABCA family (Kaminski et al., 2001). However, ABCA8 was currently confirmed to associate with regulating cholesterol efflux and HDL-c levels in a similar fashion as the canonical cholesterol efflux proteins ABCA1 and adenosine triphosphate–binding cassette transporters G1 (Kim et al., 2008; Trigueros-Motos et al., 2017). Furthermore, ABCA8 has been suggested to associate with stimulating sphingomyelin production in oligodendrocytes (Bleasel et al., 2013; Kim et al., 2013). Meanwhile, the ABCA8 protein is reported to relate to ovarian cancer other than its role in anionic drugs transport across *Xenopus laevis* oocyte membranes (Hedditch et al., 2014; Liu et al., 2015). Here, we identified a novel nonsense mutation (NM_007168: c.3460C>T; p.R1154X) of *ABCA8* in a family with very low levels of HDL-c which further confirmed that deleterious heterozygous *ABCA8* mutations may disrupt cholesterol efflux and reduce HDL-c levels in humans. This discovery may be the second report related to *ABCA8* mutations in patients with reduced levels of HDL-c.

Commonly, increased LDL-c and TG were recognized as the crucial risk factors of ASCVD (Fernandez et al., 2015). Mutations of the pathogenic genes related to hypercholesterolemia and hypertriglyceridemia probably cause AS (Surendran et al., 2012; Xiang et al., 2017, 2018). Although reducing LDL-c levels has been proved to be an effective therapy, some patients still remain a high risk of ASCVD. This "residual risk" is majority result from elevated TG and low HDL-c levels (Fruchart et al., 2008). Our study, together with other researches, further revealed that the metabolism of HDL-c levels also played a significant role in ASCVD.

[4]http://sift.jcvi.org/

[5]http://genetics.bwh.harvard.edu/pph2/

[6]http://www.mutationtaster.org/

At present, only Trigueros-Motos et al. (2017) reported three deleterious heterozygous *ABCA8* mutations including p. P609R, c.IVS17-2 A>G and p. T741X in patients with reduced HDL-c levels (**Figure 1F**). In addition, the rs4148008 in *ABCA8* was reported to be significantly related to an average of 0.42 mg/dL HDL-c levels (Teslovich et al., 2010). In our study, the EXAC database contains three heterozygous carriers of the novel mutation, but we still believe that the novel mutation is the genetic factor of the family. Because the nonsense mutation is co-separated in the family members, bioinformatics analysis predict that this mutation is pathogenic and the mutation belongs to PVS1, PS3, and PM2 underling ACMG classification. It is reasonable when the novel mutation existed with an extremely lower MAF in EXAC database, since the reduced levels of HDL-c have a high risk of AS, but most symptoms of AS do not show up until a blockage occurs (Herrington et al., 2016; Kobiyama and Ley, 2018).

In mice, knockout *Abca8* may affect the efflux transporter for cholesterol and taurocholate. And the levels of HDL-c were significantly decreased in *Abca8* knockout mice. On the contrary, hepatic overexpression of human *ABCA8* in mice showed an obvious increased HDL-c in plasma (Trigueros-Motos et al., 2017). The phenotypes of human carried *ABCA8* mutations were consistent with the presentation in *Abca8* knockout mice. Here, the identified novel nonsense mutation (NM_007168: c.3460C>T; p.R1154X), resulting in the loss of function of ABCA8, also showed the same phenotypes with previous studies in human and *Abca8* knockout mice (Trigueros-Motos et al., 2017; Sasaki et al., 2018).

CONCLUSION

In conclusion, we enrolled a Han-Chinese family with ASCVD. Lipid testing indicated overt reduced levels of HDL-c. Whole exome sequencing and Sanger sequencing detected a nonsense mutation (NM_007168: c.3460C>T; p.R1154X) of *ABCA8* in the ASCVD patients and absent in the healthy members. This study may be the second report related to *ABCA8* mutations in patients with reduced levels of HDL-c. Our study not only contributed to the genetic counseling and prenatal genetic diagnosis of patients with ASCVD caused by reduced HDL-c levels, but also confirmed the genetic lesion of the family with reduced HDL-c levels which suggested the family members with the novel mutation pay attention to ASCVD and accept medical examination regularly. In addition, our study also provided a new sight among ABCA8, cholesterol efflux and HDL-c levels.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Second Xiangya Hospital of the Central South University Ethics Committee. 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 manuscript.

AUTHOR CONTRIBUTIONS

C-YW and L-LF performed genetic analysis. Y-QC enrolled the samples and clinical data. J-YJ and RD isolated the gDNA and performed PCR. C-YW, Y-QC, and L-LF wrote the manuscript. RX supported the study. All authors reviewed the manuscript.

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REFERENCES

Bleasel, J. M., Hsiao, J. H., Halliday, G. M., and Kim, W. S. (2013). Increased expression of ABCA8 in multiple system atrophy brain is associated with changes in pathogenic proteins. *J. Parkinsons Dis.* 3, 331–339. doi: 10.3233/jpd-130203

Demidenko, R., Razanauskas, D., Daniunaite, K., Lazutka, J. R., Jankevicius, F., and Jarmalaite, S. (2015). Frequent down-regulation of ABC transporter genes in prostate cancer. *BMC Cancer* 15:683. doi: 10.1186/s12885-015-1689-8

Fan, L. L., Chen, Y. Q., Huang, H., Yuan, Z. Z., Jin, J. Y., Hu, M., et al. (2019a). Exome sequencing identifies a novel nonsense mutation of ring finger protein 207 in a Chinese family with Long QT syndrome and syncope. *J. Hum. Genet.* 64, 233–238. doi: 10.1038/s10038-018-0549-1

Fan, L. L., Ding, D. B., Huang, H., Chen, Y. Q., Jin, J. Y., Xia, K., et al. (2019b). A de novo mutation of SMYD1 (p.F272L) is responsible for hypertrophic cardiomyopathy in a Chinese patient. *Clin. Chem. Lab. Med.* 57, 532–539. doi: 10.1515/cclm-2018-0578

Fernandez, R., Everett, B., Miranda, C., Rolley, J. X., Rajaratnam, R., and Davidson, P. M. (2015). Migratory implications for coronary heart disease risk prevention

in Asian Indians: evidence from the leading health indicators. *J. Cult. Divers.* 22, 30–38.

Fruchart, J. C., Sacks, F., Hermans, M. P., Assmann, G., Brown, W. V., Ceska, R., et al. (2008). The residual risk reduction initiative: a call to action to reduce residual vascular risk in patients with dyslipidemia. *Am. J. Cardiol.* 102, 1K–34K. doi: 10.1016/j.amjcard.2008.10.002

Gidding, S. S., Champagne, M. A., De Ferranti, S. D., Defesche, J., Ito, M. K., Knowles, J. W., et al. (2015). The agenda for familial hypercholesterolemia: a scientific statement from the American heart association. *Circulation* 132, 2167–2192. doi: 10.1161/cir.0000000000000297

Hedditch, E. L., Gao, B., Russell, A. J., Lu, Y., Emmanuel, C., Beesley, J., et al. (2014). ABCA transporter gene expression and poor outcome in epithelial ovarian cancer. *J. Natl. Cancer Inst.* 106:dju149.

Herrington, W., Lacey, B., Sherliker, P., Armitage, J., and Lewington, S. (2016). Epidemiology of atherosclerosis and the potential to reduce the global burden of Atherothrombotic disease. *Circ. Res.* 118, 535–546. doi: 10.1161/circresaha.115.307611

Hurtubise, J., Mclellan, K., Durr, K., Onasanya, O., Nwabuko, D., and Ndisang, J. F. (2016). The different facets of dyslipidemia and hypertension in atherosclerosis. *Curr. Atheroscler. Rep.* 18:82.

Kaminski, W. E., Wenzel, J. J., Piehler, A., Langmann, T., and Schmitz, G. (2001). ABCA6, a novel a subclass ABC transporter. *Biochem. Biophys. Res. Commun.* 285, 1295–1301. doi: 10.1006/bbrc.2001.5326

Kim, W. S., Hsiao, J. H., Bhatia, S., Glaros, E. N., Don, A. S., Tsuruoka, S., et al. (2013). ABCA8 stimulates sphingomyelin production in oligodendrocytes. *Biochem. J.* 452, 401–410. doi: 10.1042/bj20121764

Kim, W. S., Weickert, C. S., and Garner, B. (2008). Role of ATP-binding cassette transporters in brain lipid transport and neurological disease. *J. Neurochem.* 104, 1145–1166. doi: 10.1111/j.1471-4159.2007.05099.x

Kobiyama, K., and Ley, K. (2018). Atherosclerosis. *Circ. Res.* 123, 1118–1120.

Libby, P., Bornfeldt, K. E., and Tall, A. R. (2016). Atherosclerosis: successes, surprises, and future challenges. *Circ. Res.* 118, 531–534. doi: 10.1161/circresaha.116.308334

Liu, X., Gao, Y., Zhao, B., Li, X., Lu, Y., Zhang, J., et al. (2015). Discovery of microarray-identified genes associated with ovarian cancer progression. *Int. J. Oncol.* 46, 2467–2478. doi: 10.3892/ijo.2015.2971

Maranghi, M., Truglio, G., Gallo, A., Grieco, E., Verrienti, A., Montali, A., et al. (2019). A novel splicing mutation in the ABCA1 gene, causing Tangier disease and familial HDL deficiency in a large family. *Biochem. Biophys. Res. Commun.* 508, 487–493. doi: 10.1016/j.bbrc.2018.11.064

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., et al. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. *Genet. Med.* 17, 405–424.

Sasaki, K., Tachikawa, M., Uchida, Y., Hirano, S., Kadowaki, F., Watanabe, M., et al. (2018). ATP-Binding cassette transporter a subfamily 8 is a sinusoidal efflux transporter for cholesterol and taurocholate in mouse and human liver. *Mol. Pharm.* 15, 343–355. doi: 10.1021/acs.molpharmaceut.7b00679

Schwarz, J. M., Cooper, D. N., Schuelke, M., and Seelow, D. (2014). MutationTaster2: mutation prediction for the deep-sequencing age. *Nat. Methods* 11, 361–362. doi: 10.1038/nmeth.2890

Surendran, R. P., Visser, M. E., Heemelaar, S., Wang, J., Peter, J., Defesche, J. C., et al. (2012). Mutations in LPL, APOC2, APOA5, GPIHBP1 and LMF1 in patients with severe hypertriglyceridaemia. *J. Intern. Med.* 272, 185–196. doi: 10.1111/j.1365-2796.2012.02516.x

Teslovich, T. M., Musunuru, K., Smith, A. V., Edmondson, A. C., Stylianou, I. M., Koseki, M., et al. (2010). Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 466, 707–713.

Trigueros-Motos, L., Van Capelleveen, J. C., Torta, F., Castano, D., Zhang, L. H., Chai, E. C., et al. (2017). ABCA8 regulates cholesterol efflux and high-density lipoprotein cholesterol levels. *Arterioscler. Thromb. Vasc. Biol.* 37, 2147–2155.

Tsuruoka, S., Ishibashi, K., Yamamoto, H., Wakaumi, M., Suzuki, M., Schwartz, G. J., et al. (2002). Functional analysis of ABCA8, a new drug transporter. *Biochem. Biophys. Res. Commun.* 298, 41–45. doi: 10.1016/s0006-291x(02)02389-6

Xiang, R., Fan, L. L., Huang, H., Chen, Y. Q., He, W., Guo, S., et al. (2018). Increased reticulon 3 (RTN3) leads to obesity and hypertriglyceridemia by interacting with heat shock protein family A (Hsp70) member 5 (HSPA5). *Circulation* 138, 1828–1838. doi: 10.1161/circulationaha.117.030718

Xiang, R., Fan, L. L., Lin, M. J., Li, J. J., Shi, X. Y., Jin, J. Y., et al. (2017). The genetic spectrum of familial hypercholesterolemia in the central south region of China. *Atherosclerosis* 258, 84–88. doi: 10.1016/j.atherosclerosis.2017.02.007

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Analysis of Differentially Expressed Genes and Molecular Pathways in Familial Hypercholesterolemia Involved in Atherosclerosis: A Systematic and Bioinformatics Approach

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Background and Aims: Familial hypercholesterolemia (FH) is one of the major risk factor for the progression of atherosclerosis and coronary artery disease. This study focused on identifying the dysregulated molecular pathways and core genes that are differentially regulated in FH and to identify the possible genetic factors and potential underlying mechanisms that increase the risk to atherosclerosis in patients with FH.

Methods: The Affymetrix microarray dataset (GSE13985) from the GEO database and the GEO2R statistical tool were used to identify the differentially expressed genes (DEGs) from the white blood cells (WBCs) of five heterozygous FH patients and five healthy controls. The interaction between the DEGs was identified by applying the STRING tool and visualized using Cytoscape software. MCODE was used to determine the gene cluster in the interactive networks. The identified DEGs were subjected to the DAVID v6.8 webserver and ClueGo/CluePedia for functional annotation, such as gene ontology (GO) and enriched molecular pathway analysis of DEGs.

Results: We investigated the top 250 significant DEGs (p-value < 0.05; fold two change ≥ 1 or ≤ -1). The GO analysis of DEGs with significant differences revealed that they are involved in critical biological processes and molecular pathways, such as myeloid cell differentiation, peptidyl-lysine modification, signaling pathway of MyD88-dependent Toll-like receptor, and cell-cell adhesion. The analysis of enriched KEGG pathways revealed the association of the DEGs in ubiquitin-mediated proteolysis and cardiac muscle contraction. The genes involved in the molecular pathways were shown to be differentially regulated by either activating or inhibiting the genes that are essential for the canonical signaling pathways. Our study identified seven core genes (*UQCR11, UBE2N, ADD1, TLN1, IRAK3, LY96,* and *MAP3K1*) that are strongly linked to FH and lead to a higher risk of atherosclerosis.

Conclusion: We identified seven core genes that represent potential molecular biomarkers for the diagnosis of atherosclerosis and might serve as a platform for developing therapeutics against both FH and atherosclerosis. However, functional studies are further needed to validate their role in the pathogenesis of FH and atherosclerosis.

Keywords: familial hypercholesterolemia, atherosclerosis, coronary artery disease, functional enrichment analysis, expression profiling data, gene expression arrays

INTRODUCTION

Atherosclerosis is a chronic immune-inflammatory disease that is characterized by the progressive accumulation of lipids in the intimal space of the atrial walls, which results in such complications as chronic low-grade inflammation, endothelial dysfunction, and oxidative stress. A high level of low-density lipoprotein (LDL) in plasma induces atherosclerosis. In contrast, a decreasing level of LDL cholesterol is associated with a decreased frequency of severe cardiovascular events (Silverman et al., 2016). Elevated levels of blood cholesterol are caused by a group of genetic defects known as familial hypercholesterolemia (FH). FH is one of the known genetic causes of premature cardiovascular disease due to prolonged exposure to elevated LDL, with a prevalence of ∼1:220 being observed (Abul-Husn et al., 2016; Wald et al., 2016; Alhababi and Zayed, 2018). Recent studies estimated the prevalence of heterozygous FH (HeFH) to be significantly higher (1/220-250) than initially reported (1/500) (Hopkins et al., 2011; Genest et al., 2014; Perez-Calahorra et al., 2019). However, the homozygous FH (HoFH) prevalence has been estimated to be 1 in 300,000–1,000,000 (Austin et al., 2004; Sjouke et al., 2015; de Ferranti et al., 2016; Akioyamen et al., 2017; Alhababi and Zayed, 2018; Alonso et al., 2018). The prevalence of FH is higher due to founder effect that estimates to be upto 1 in 50–67 in some populations like Lebanese, Ashkenazi Jews, French Canadians, Finns, Afrikaners, and Tunisians (Leitersdorf et al., 1990; Nanchen et al., 2015; Amor-Salamanca et al., 2017; Alonso et al., 2018). In the past, the term FH was used to refer to defects in the LDL receptor (Goldberg et al., 2011). Among FH patients, the clinical phenotypes are distinctly versatile, even in patients who share the same disease-causing mutation. This finding suggests that FH is not a single disease but is a multifaceted syndrome (Hartgers et al., 2015; Di Resta and Ferrari, 2018; Masana et al., 2019). HeFH is mainly caused by mutations that occur in such genes as *LDLR*, less frequently, mutations in *APOB* and *PCSK9* genes can be found in patients with phenotypic FH (Soutar and Naoumova, 2007; Gidding et al., 2015). Several studies reported that HoFH causes considerable premature ASCVD, and would result in early death if left untreated (males are at 50% risk, and females are at 30% risk) (Slack, 1969; Stone et al., 1974; Joseph et al., 2001; Naoumova et al., 2004; Vuorio et al., 2014). For the management of patients above 75 years of age with clinically evident atherosclerotic cardiovascular disease (ASCVD), the ACC/AHA standards endorse a moderate intensity (but not a high-intensity) statin (Vuorio et al., 2013, 2017; Stone et al., 2014). Microarray technology is a robust procedure that is widely used to compare genes that are differentially expressed in patients with different diseases. This technology is also beneficial in understanding gene association, mapping, expression, and linkage studies (Russo et al., 2003). However, studies that investigated the white blood cells (WBCs) transcriptome of patients with FH versus healthy controls are limited. Therefore, this study aimed to identify differentially expressed genes (DEGs), protein-protein interactions, and dysregulated pathways that might be involved in an increased risk of atherosclerosis due to FH.

Abbreviations: BP, biological process; FDR, false discovery rate; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; MF, molecular function.

MATERIALS AND METHODS

Array Data Acquisition and Processing

The GEO database from NCBI[1] was used to access the GSE13985 dataset that contains expression profiles by array. The datasets from various experiments are deposited in this database and enable users to download the gene expression profiles stored in GEO (Barrett et al., 2013). To seek GEO datasets for related gene expression profiles, we used the keywords "Familial Hypercholesterolemia" and "Microarray" and "Homo sapiens." GSE13985 contains ten samples, including five patients with FH and five healthy control samples obtained with the help of platform GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Režen et al., 2008). The five FH patients and five controls were matched by age, BMI, sex, and smoking status. The FH patients were free of clinical ASCVD. The Blood samples were provided from the ten samples, and RNA was extracted to be used for the array analysis. The gene expression data were downloaded from the public database, and in this study, there were no animal or human experiments assisted by any of the authors.

Data Preprocessing and Identification of DEGs

With the help of powerful multiarray technology, the preliminary data from the dataset were made susceptible to the correction of background, quantile normalization, and log transition (Irizarry et al., 2003). Initial processing of the data involved altering specific gene symbols from probe IDs with the help of a Entrez's Gene ID converter (Alibés et al., 2007). When the same gene

[1] https://www.ncbi.nlm.nih.gov/geo/

contribution was observed in several samples, their mean value was determined and considered as the eventual level of gene expression. To examine the raw gene expression data, the online statistical tool GEO2R was utilized, and the tool incorporated the R/Bioconductor and Limma package v3.26.8 (Smyth, 2005; Barrett et al., 2013; Ritchie et al., 2015). The GEO2R inbuilt methods, such as *T*-test and Benjamini and Hochberg (false discovery rate), were used to calculate the *p*-value and FDR in order to determine the DEGs among patients with FH and controls (Aubert et al., 2004). We set the principal standards of $|\log(\text{fold change})| > 1$ and $p < 0.05$ to acquire DEGs that are significant from the dataset, whereas the upregulated DEGs were considered if the logFC ≥ 1 and logFC ≤ -1 for downregulated DEGs. The RStudio (v1.2.5019) and library Calibrate package were used to create the volcano plot. The subsequent DEGs were attained from a dataset, and further investigation was performed with selected DEGs. The heat map for the gene expression data was generated using a heat mapper webserver.[2] The inbuilt average linkage clustering method was used to compute the hierarchical clustering, and the Euclidean algorithm was used for computing the distance between rows and columns (Babicki et al., 2016). The flowchart diagram for this study is represented in **Figure 1**.

Establishment of PPI Networks and Module Analysis

We framed a PPI (protein-protein interaction) network by utilizing the STRING web-based tool (v11.0, [3]) to evaluate the relationship among the DEGs from the attained datasets (Szklarczyk et al., 2017, 2019). To eradicate the PPI interactions that are in consistent from the dataset, we fixed the cutoff standard to a confident interaction score $\geq$0.4. Therefore, we attained a strong PPI network. Then, we combined the outcomes from the STRING tool to Cytoscape software (v3.7.1, [4]) to conceptualize the PPI interactions among the statistically appropriate DEGs (Shannon et al., 2003). To recognize the intersected clusters from the attained PPI network, we exploited the Cytoscape plugin Molecular Complex Detection plugin (MCODE). The group (cluster) determining extremities were charted, such as Kappa score (K-core) fixed to five, Degree Cutoff fixed to two, Max. Depth fixed to 100, and Node score Cutoff fixed to 0.2, which constraints the cluster size for coexpressing networks (Bader and Hogue, 2003). Further, we utilized the GeneMANIA web server to perform the inter-relation analysis and predict the function of the identified seven potential DEGs (Warde-Farley et al., 2010; Franz et al., 2018).

DAVID and ClueGO Enrichment Analysis

For functional annotation of GO and analysis of KEGG pathway enrichment, we used the web-based DAVID v6.8 tool.[5] DAVID is a significant source for any functional evaluation of the high-throughput gene expression profiles (Huang et al., 2009a,b). The results from DAVID was further imported to GOplot in R Studio. The GOBubble and GOChord were used to visualize the functional enrichment of the top 250 DEGs, which facilitate the combination and integration of expression data with functional assessment results (Walter et al., 2015). For integrative analysis, we utilized both DAVID and ClueGO software to comprehensively observe the DEGs involved in the GO terms and pathways. The first DEGs from the GEO2R tool were exposed to ClueGO v2.5.5/CluePedia v1.5.5 to attain complete Gene Ontological terms (GO) and disease-related pathways from the DEG dataset. ClueGO syndicates KEGG or BioCarta pathways and GO, which delivers a fundamentally organized pathway network or GO from the DEG dataset (Bindea et al., 2009). Also, the study of molecular/biological function GO, and enrichment of pathways analysis was conducted for DEGs, and *p*-values < 0.05 were considered to be significant.

[2]http://heatmapper.ca/expression/

[3]http://www.string-db.org/

[4]http://www.cytoscape.org/

[5]https://david.ncifcrf.gov/

RESULTS

Data Acquisition and Identification of DEGs

The GSE13985 dataset contained the gene expression profiles of five patient samples with FH, and five control groups (atherosclerotic markers in human blood - a study in patients with familial hypercholesterolemia) were obtained from the GEO database (Režen et al., 2008). The GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) was used in this work. **Table 1** represents the original features of the patients and control samples involved in this study. By using the publicly available GEO2R tool, we identified the DEGs between FH patients and healthy controls according to the cutoff values of $|\log2FC| \geq 1.0$ and *p*-values < 0.05, which were calculated based on the inbuilt R/Bioconductor and limma package v3.26.8 from the GEO2R tool, the top 250 DEGs were accordingly identified. The R studio and library calibrate package was used to construct the volcano plot to compare the DEGs between FH patients and healthy controls. **Figure 2** represents the volcano plot, and the significant genes with satisfying values (*p*-value < 0.05, logFC $\geq$ 1, and logFC ≤ -1) are labeled and shown in green dots. The top 250 DEGs identified between both groups were subjected to a heat mapper web server to determine the expression level of the genes. As a result, clustering based on hierarchy and heat maps was generated and is depicted in **Figure 3**.

Establishment of PPI Networks and Module Analysis

The physical and functional associations among the proteins of DEGs of FH were assessed using the STRING tool. The minimum required interaction score was set to the confidence of 0.4. Simple tabular text output was generated from STRING. The interaction among the query proteins was further visualized using Cytoscape v3.7.1. **Figure 4** represents the network with 96 nodes and 134 edges of PPI. The nodes denote the number of proteins while edges and their interactions. The Cytoscape plugin MCODE v1.5.1 interpreted the closely interlinked regions

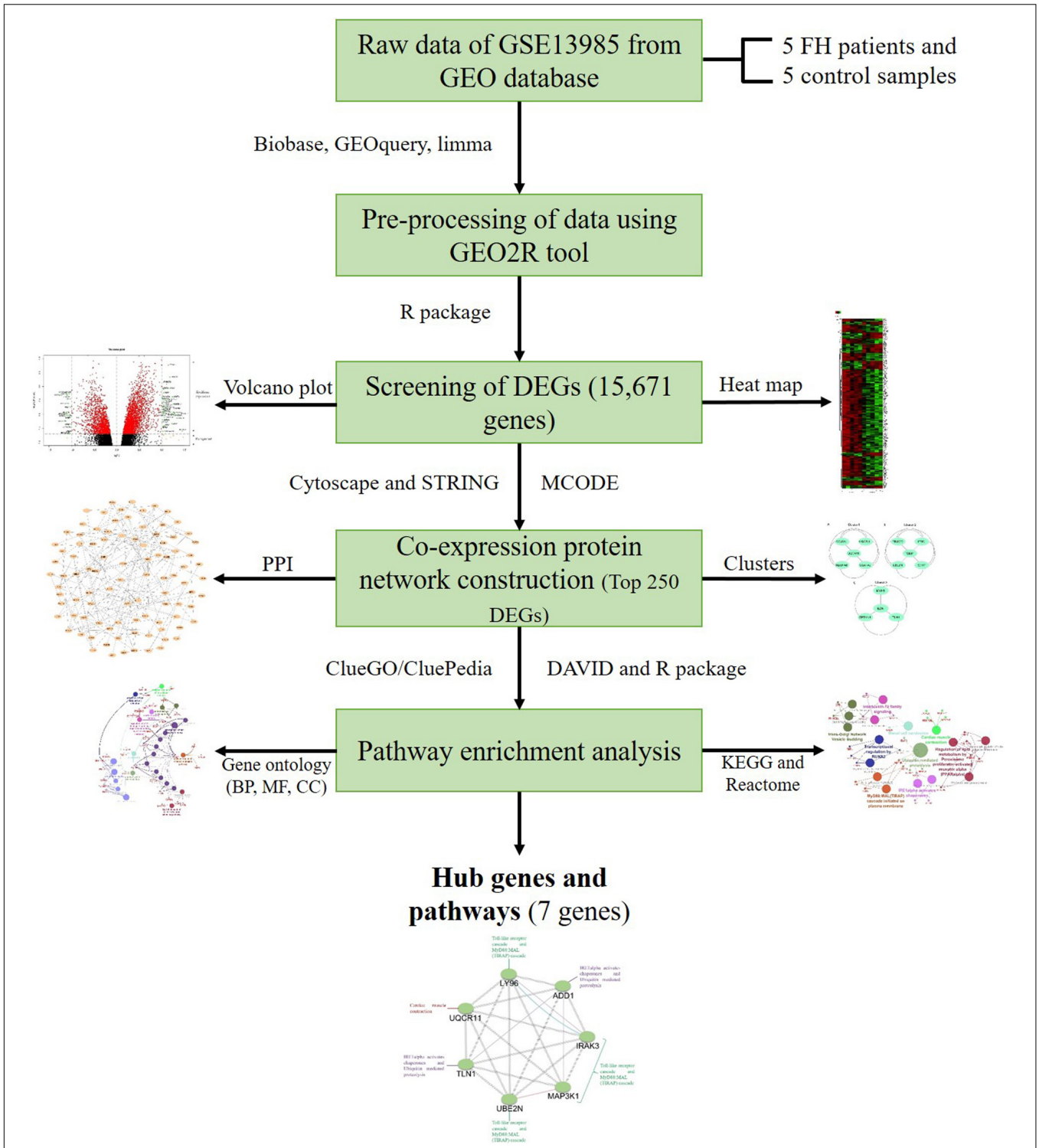

FIGURE 1 | Outline of the workflow and expression data analysis in the present study using bioinformatic strategies.

from the network of proteins in the form of clusters. The top three clusters that are significant from the PPI network with MCODE scores of 5, 5, and 4 were preferred. These clusters are represented graphically in **Figure 5**. Cluster 1 was derived from node *UQCR11* (**Figure 5A**). Cluster 2 and Cluster 3 were derived from nodes *TCEB1* and *EZR*, respectively (**Figures 5B,C**). The tabular column represents the detailed MCODE clusters of interlinked regions with their cluster

TABLE 1 | Information on patients and controls primary features in GSE13985 from the GEO database.

Group	Accession	Title	Organism	Gender	Age	Disease State	Tissue
Patient	GSM351336	Patient 1	Homo sapiens	Male	33 years	Heterozygous familial hypercholesterolemia	Blood
	GSM351337	Patient 2	Homo sapiens	Male	33 years	Heterozygous familial hypercholesterolemia	Blood
	GSM351338	Patient 3	Homo sapiens	Male	46 years	Heterozygous familial hypercholesterolemia	Blood
	GSM351339	Patient 4	Homo sapiens	Male	22 years	Heterozygous familial hypercholesterolemia	Blood
	GSM351340	Patient 5	Homo sapiens	Male	35 years	Heterozygous familial hypercholesterolemia	Blood
Control	GSM351341	Control 1	Homo sapiens	Male	35 years	None	Blood
	GSM351342	Control 2	Homo sapiens	Male	37 years	None	Blood
	GSM351343	Control 3	Homo sapiens	Male	22 years	None	Blood
	GSM351344	Control 4	Homo sapiens	Male	45 years	None	Blood
	GSM351345	Control 5	Homo sapiens	Male	33 years	None	Blood

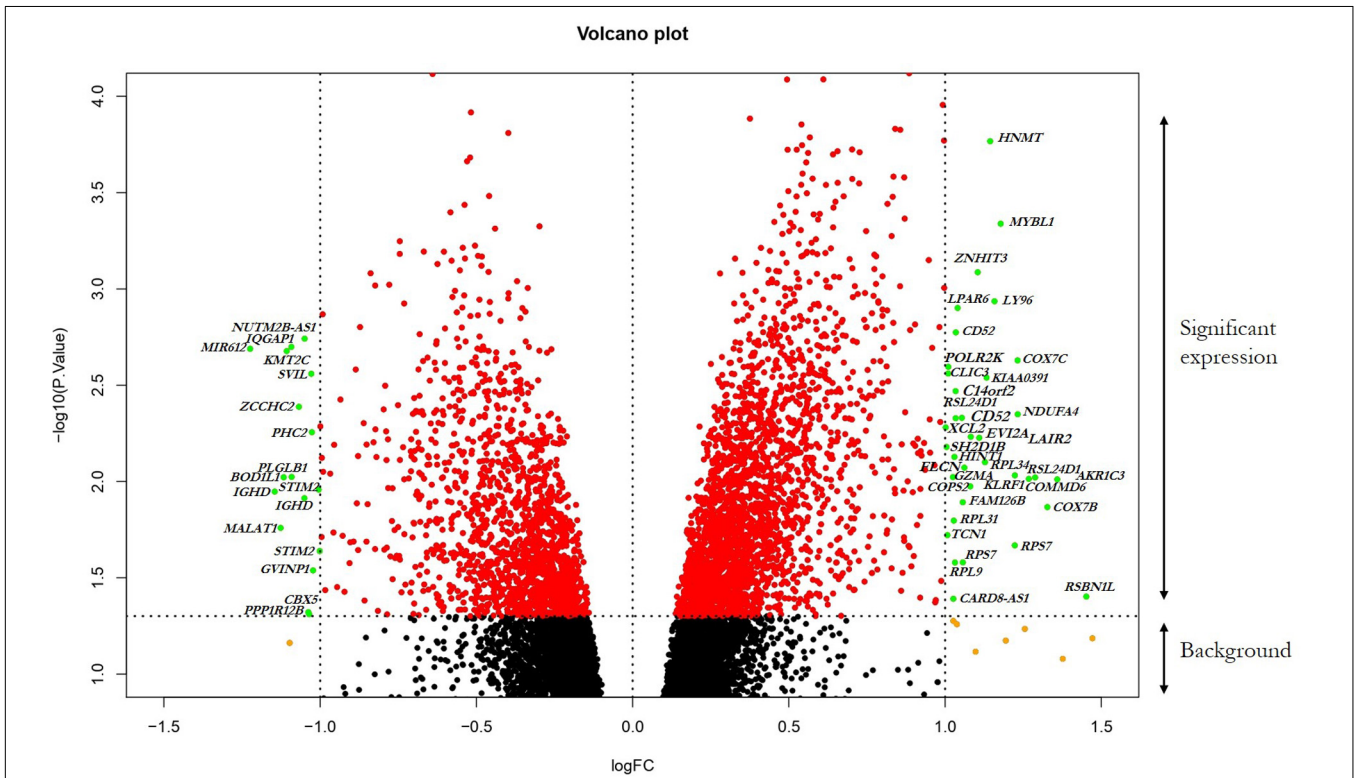

FIGURE 2 | Visualization of DEGs volcano plots using R studio. The plot compared the DEGs between FH patients and controls from the dataset. The representations are as follows: *x*-axis, logFC; *y*-axis, -log10 of a *p*-value. The *p*-values < 0.05 are in red dots, and logFC ≥ 1 and logFC ≤ -1 are in yellow dots; the significant DEGs with both satisfying values are in green dots and indicated with gene names. Black dots indicate the remaining genes present in the array that were not significantly changed. The genes that are upregulated in the array are on the right panel, and downregulated ones are on the left panel of the plot.

number, MCODE scores, node IDs, node numbers, and edge numbers (**Table 2**).

DAVID Enrichment Analysis

For the functional annotation of DEGs, the DAVID v6.8 online server was used. To ascertain the KEGG pathway-enriched genes and the potential GO (Gene Ontology) classification, terms approximating biological process, molecular functions, and signaling pathways concerning KEGG pathways were used. The modified Fisher exact *p*-value (EASE score) ≤ 0.05 and FDR < 0.05 are considered strongly enriched. By analyzing BP, we found that the DEGs from the complex PPI network were enriched in myeloid cell differentiation (GO: 0030099), intracellular transport (GO: 0046907), negative regulation of response to DNA damage stimulus (GO: 2001021), peptidyl-lysine modification (GO: 0018205), negative regulation of signal transduction by p53 class mediator (GO: 1901797), and MyD88-dependent toll-like receptor signaling pathway (GO: 0002755). The Gene Ontology MF analysis revealed the involvement of DEGs in antioxidant activity (GO: 0016209), p53 binding (GO: 0002039), thyroid hormone receptor binding (GO: 0046966), cadherin binding involved in cell-cell adhesion (GO: 0098641),

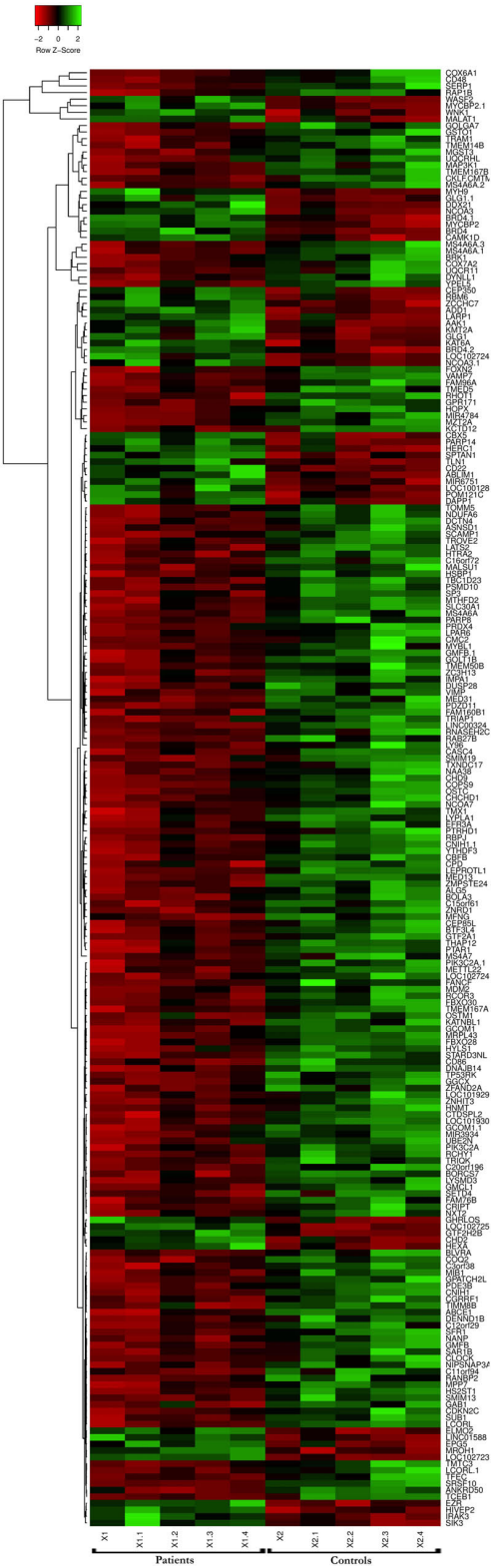

FIGURE 3 | Microarray heat map and clustering based on hierarchy for the top 250 gene expression data from the datasets. n = 250 DEGs. Upregulated genes are represented in red, and downregulated genes are represented in green.

transcription factor activity, and transcription factor binding (GO: 0000989). In addition, we utilized the DAVID online method to classify the DEGs entailed in the different biological pathways based on the KEGG reference database ($p < 0.05$; FDR < 0.05). The KEGG pathway enrichment analysis revealed the association of the DEGs in ubiquitin-mediated proteolysis (hsa04120) and cardiac muscle contraction (hsa04260). The annotated results for the following terms were tabulated (**Table 3**). The GOBubble plots were constructed for the BP and MF of the top 250 DEGs from the dataset (**Figures 6A,B**), whereas the enriched cellular components of the identified DEGs were plotted with GOChord (**Figure 6C**).

ClueGO/CluePedia Enrichment Analysis

The Cytoscape plugin ClueGO/CluePedia was used to study the functional enrichment of the DEGs from the dataset. ClueGo helped visualize the GO terms of the identified PPI complex network. The MF and BF terms of the GO functional enrichment analysis of the complex PPI network are depicted in **Figure 7**. The statistical options for ClueGO enrichment analysis were set based on a hypergeometric test that is two-sided with $p \leq 0.05$, Benjamini-Hochberg correction, and kappa score ≥ 0.4 as a primary criterion. The BF and MF of DEGs from the complex PPI network were predominantly enriched in the negative regulation of intracellular transport (GO: 0032387), endothelial cell development (GO: 0001885), scaffold protein binding (GO: 0097110), regulation of DNA damage response and signal transduction by p53 class mediator (GO: 0043516), p53 binding (GO: 0002039), peptidyl-lysine trimethylation (GO: 0018023), antioxidant activity (GO: 0016209), positive regulation of the Notch signaling pathway (GO: 0045747), and MyD88-dependent Toll-like receptor signaling pathway (GO: 0002755) (**Figure 7**). The KEGG and REACTOME pathway analysis from ClueGO showed that many DEGs were significantly enriched in cardiac muscle contraction (KEGG: 04260), regulation of lipid metabolism by peroxisome proliferator-activated receptor alpha (PPAR alpha) (R-HAS: 400206), transcriptional regulation by *RUNX3* (R-HAS: 8878159), ubiquitin-mediated proteolysis (KEGG: 04120), renal cell carcinoma (KEGG: 05211), MyD88: MAL (TIRAP) cascade initiated on plasma membrane (R-HAS: 166058), and IRE1 alpha-activated chaperones (R-HAS: 381070) (**Figure 8**). Taken together, the results from ClueGO enrichment clearly illustrate that the DEGs change the metabolic behavior of the signaling pathways and are closely linked to FH, contributing to the progression of such complications as coronary artery disease and cardiovascular disease, which may lead to atherosclerosis. Additionally, the dysregulated pathways identified by our bioinformatics enrichment analysis could play important roles in FH pathogenesis. However, functional validations are needed to test our bioinformatics findings.

DISCUSSION

In the present study, we investigated the DEGs between five patients with FH and five healthy controls with the GEO ID of GSE13985 (Režen et al., 2008). We examined a total of

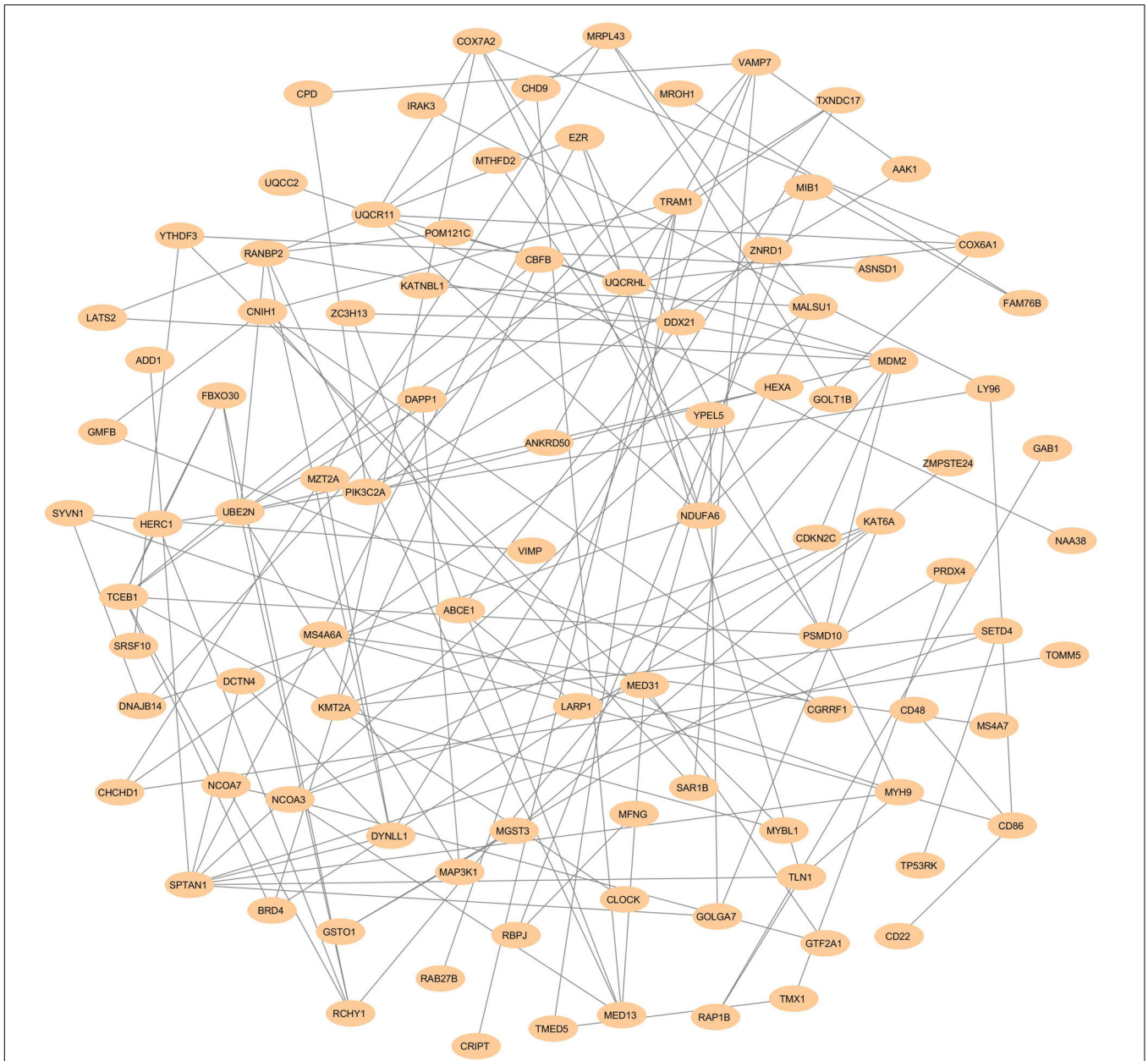

FIGURE 4 | PPI networks show the interaction of DEGs from the GSE13985 dataset. The nodes and edges are retrieved from the STRING tool and plotted using Cytoscape software. The nodes are displayed as Navajo white, while edges are shown as gray.

13,057 DEGs, and the top 250 significant DEGs were considered for further studies (**Supplementary Table S1**). We found the top three significant clusters that are distinguished by high scores and closely interconnected regions from the networks of PPI (**Figures 5A–C**). Screening the gene cluster could help in determining the crucial genes and their interactions and how they are associated with the pathogenesis and progression of atherosclerosis. The clusters retrieved from MCODE were often shown in the PPI network mechanisms, and representation of clusters is crucial for a functional and comprehensive understanding of network properties (Krogan et al., 2006; Rahman et al., 2013). The densely interconnected nodes and the less connected vertices of the PPI network were weighed using the core clustering coefficient of the MCODE plugin. After computation, an algorithm examined the weighted graph to isolate the densely connected regions, which is considered as clusters and represents the molecular complexes that formed with DEGs (Sharan et al., 2007). From **Table 2**, we found three seed nodes, namely, the *UQCR11*, *TCEB1*, and *EZR* genes that might have been involved in the differential regulation of the pathway.

To explore the involvement of the 250 identified DEGs in BP, MF, and molecular pathways of FH, we used the

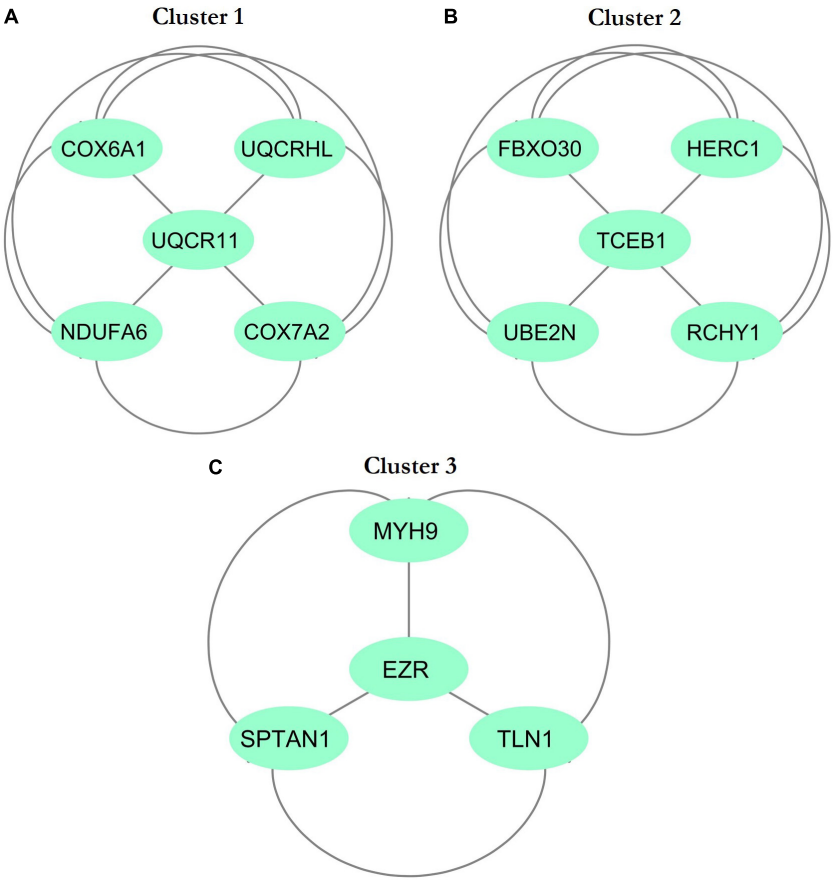

FIGURE 5 | Top three cluster subnetworks were identified from the PPI network with the help of Cytoscape using the MCODE plugin with a cluster score above three. The aquamarine (ellipse) color indicates the nodes, while the edges are in gray. **(A)** Cluster 1; **(B)** Cluster 2; **(C)** Cluster 3.

built GO and KEGG enrichment to determine the functional annotation of these genes. We found that these DEGs were primarily enriched in myeloid cell differentiation, intracellular transport, negative regulation of response to DNA damage stimulus, peptidyl-lysine modification, negative regulation of signal transduction by p53 class mediator, and the MyD88-dependent Toll-like receptor signaling pathway. The analysis of MF from GO showed that the DEGs were significantly enriched in antioxidant activity, p53 binding, thyroid hormone receptor binding, cadherin binding involved in cell-cell adhesion, and transcription factor activity. Similarly, the analysis of KEGG pathway enrichment showed that the DEGs are involved in ubiquitin-mediated proteolysis and cardiac muscle contraction (**Table 3**). Interestingly, MyD88-mediated signaling plays a prominent role in the development of human atherosclerosis and matrix degradation (Monaco et al., 2009). In line with this finding, Yu et al. (2014) found that MyD88-deficient myeloid cells are involved in the inhibition of macrophage recruitment to adipose tissue and result in atherosclerosis and diet-induced systemic inflammation (Yu et al., 2014). In this context, our study identified the DEGs involved in the MyD88 signaling pathway, such as *IRAK3* (interleukin 1 receptor-associated kinase 3), *LY96* (lymphocyte antigen 96), and *MAP3K1* (mitogen-activated protein kinase 1). Among these genes, *LY96* and *MAP3K1* were significantly downregulated in the FH patients, whereas *IRAK3* was upregulated compared to the healthy controls (**Supplementary Figure S1**). *IRAK3* prevents *IRAK1* and *IRAK4* from dissociating from *MyD88* and inhibits the formation of IRAK-TRAF6 complexes (Lyu et al., 2018; Yu and Feng, 2018). The increased expression of *IRAK3*, as shown in **Supplementary Figure S1A**, typically coveys the dysregulation of MyD88 and the TLR cascade via *IRAK3* expression. Similarly, the receptor complex resulting from the combination of *LY96* and Toll-like receptor 4 (*TLR4*) ectodomain mediates transduction

TABLE 2 | The most interlinked regions are clustered from the DEGs of GSE13985 dataset using MCODE.

Cluster	Score (Density x No. of nodes)	Nodes	Edges	Node IDs
1	5	5	10	*COX7A2, NDUFA6, UQCR11, UQCRHL, COX6A1*
2	5	5	10	*FBXO30, HERC1, TCEB1, UBE2N, RCHY1*
3	4	4	6	*EZR, SPTAN1, TLN1, MYH9*

TABLE 3 | Gene ontology (GO) terms such as biological process, molecular functions, and KEGG pathways of DEGs that are associated with familial hypercholesterolemia from DAVID.

Category	Term	Count	%	*p*-value	Fold enrichment	FDR
GOTERM_BP_FAT	GO:0030099~Myeloid cell differentiation	10	4.9	4.9E-3	3.1	8.4E0
GOTERM_BP_FAT	GO:0046907~Intracellular transport	24	11.8	2.3E-2	1.6	3.4E1
GOTERM_BP_FAT	GO:2001021~Negative regulation of response to DNA damage stimulus	4	2.0	1.3E-2	8.1	2.1E1
GOTERM_BP_FAT	GO:0018205~Peptidyl-lysine modification	10	4.9	1.4E-2	2.6	2.2E1
GOTERM_BP_FAT	GO:1901797~Negative regulation of signal transduction by p53 class mediator	3	1.5	2.8E-2	11.4	4.0E1
GOTERM_BP_FAT	GO:0002755~MyD88-dependent toll-like receptor signaling pathway	3	1.5	3.8E-2	9.7	5.0E1
GOTERM_MF_FAT	GO:0016209~Antioxidant activity	4	2.0	3.9E-2	5.3	4.3E1
GOTERM_MF_FAT	GO:0002039~p53 binding	5	2.5	3.4E-3	8.0	4.8E0
GOTERM_MF_FAT	GO:0046966~Thyroid hormone receptor binding	3	1.5	2.6E-2	11.9	3.1E1
GOTERM_MF_FAT	GO:0098641~Cadherin binding involved in cell-cell adhesion	8	3.9	1.8E-2	3.0	2.3E1
GOTERM_MF_FAT	GO:0000989~Transcription factor activity, transcription factor binding	14	6.9	4.1E-3	2.5	5.7E0
KEGG_PATHWAY	hsa04120:Ubiquitin mediated proteolysis	6	3.0	1.3E-2	4.2	1.4E1
KEGG_PATHWAY	hsa04260:Cardiac muscle contraction	4	2.0	4.2E-2	5.1	3.9E1

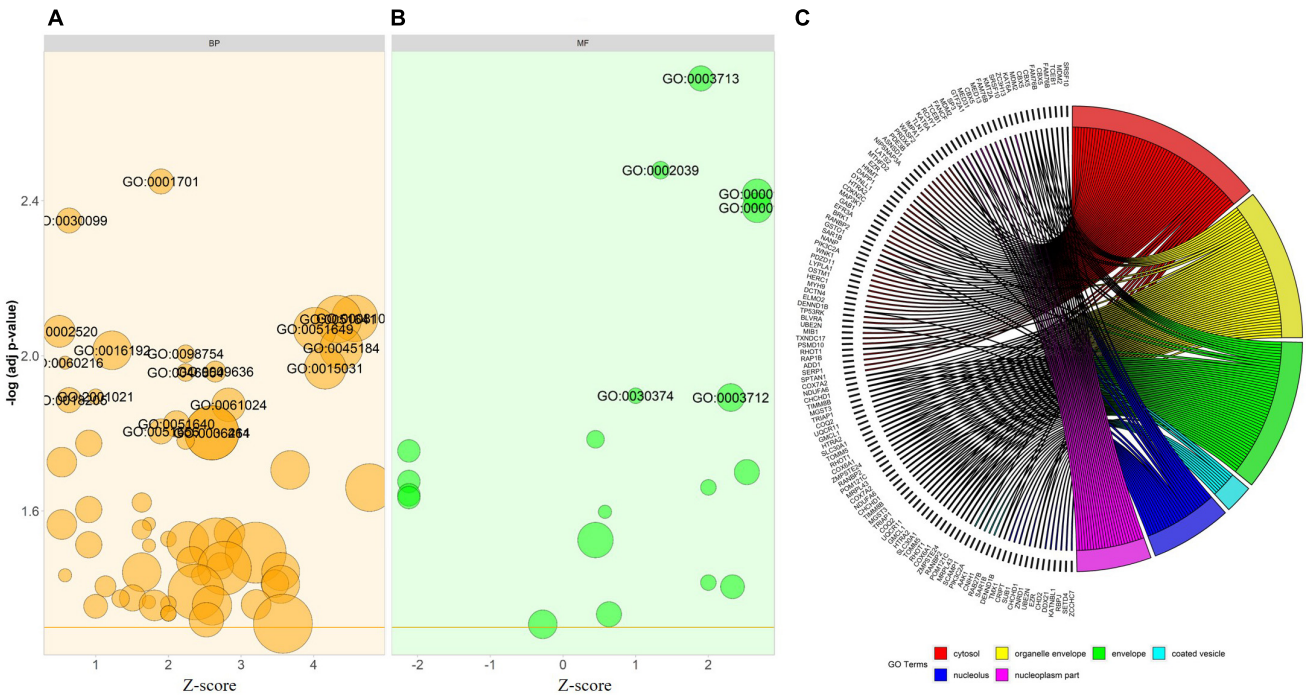

FIGURE 6 | Bubble plot depicts the **(A)** biological process (BP-in orange), and **(B)** molecular function (MF-in green) of the GO terms from DAVID. The –log *p*-value allocated to the y-axis and a *Z*-score to *x*-axis. The region of bubbles are significantly proportional to the number of DEGs in a given GO term. The threshold implies the *p*-value standardized to 0.05 (orange line in BP and MF). **(C)** The association between the selected DEGs; their respective cellular component (CC) and GO terms, along with the gene's log2FC are presented in GOChord plot. The plot's left half showed the DEGs that involved in different cellular components and the right half displays the GO terms of cellular components in various colors. The colored bands connected a gene to a specific term GO.

of the lipopolysaccharide (LPS) signal across the cell membrane (Gruber et al., 2004). A recent study found that *LY96* can bind to cholesterol (Choi et al., 2016), and *TLR4* activation involves agLDL, the predominant form of LDL found in atherosclerotic plaques (Singh et al., 2020). Thus, our examined DEGs are reliable with involvement in atherosclerosis-causing pathways.

To further refine the biological process, molecular functions, and pathways defined from the analysis of DAVID-GO terms (**Figure 6**), KEGG, and STRING, we implemented the ClueGO plugin from Cytoscape, an improved interpretation for biological terms, such as GO and KEGG pathway analysis/BioCarta, and constructed a functionally arranged network of terms GO/pathway. The plugin also helps to visualize the networks that are functionally grouped from more massive gene clusters (Bindea et al., 2009). To acquire a detailed picture of the DEGs involved in atherosclerosis, we utilized the ClueGO plugin to distinguish the molecular pathways that are differentially regulated and their

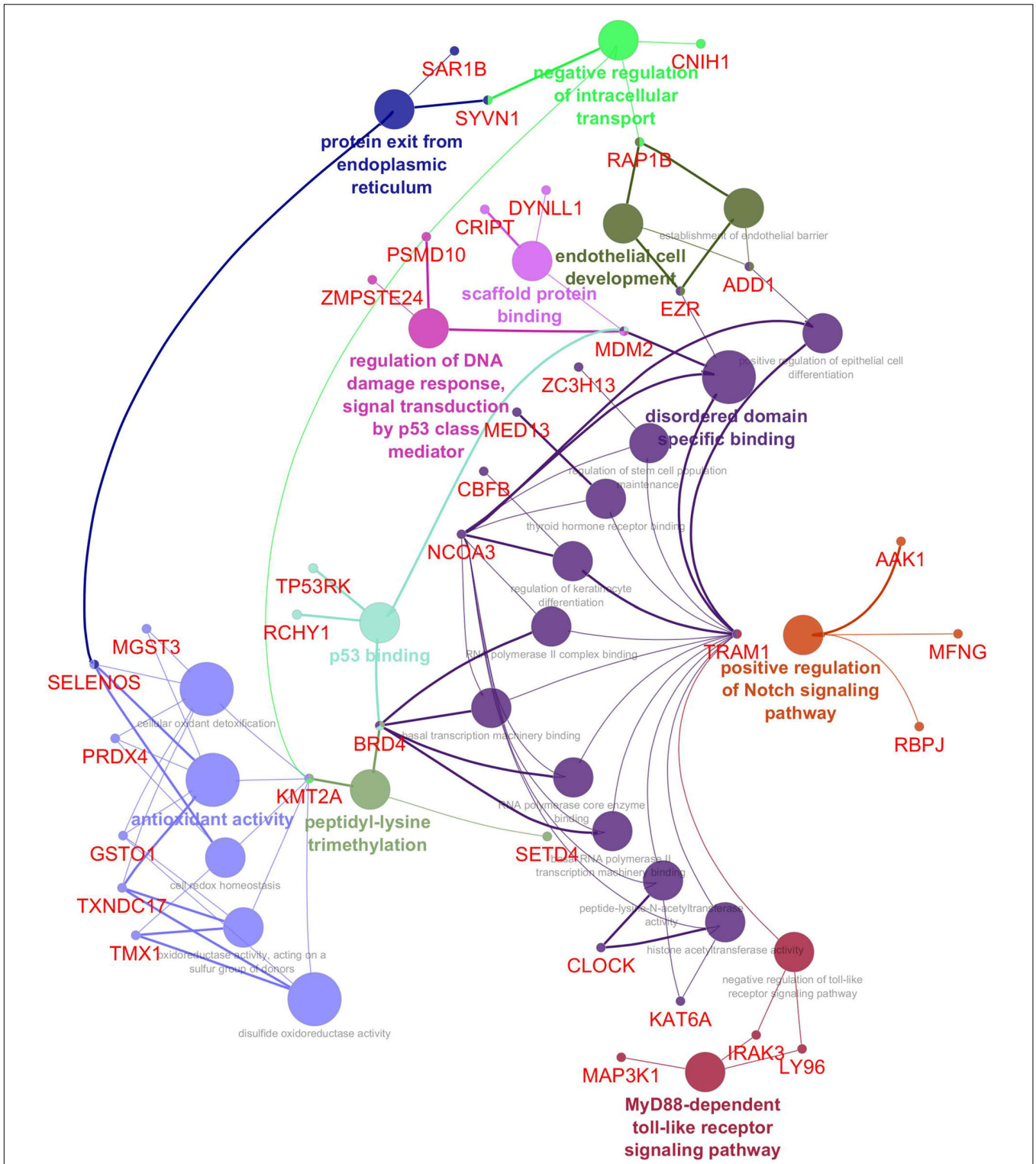

FIGURE 7 | Enrichment by Gene Ontology (GO) terms was visualized using the ClueGO/CluePedia plugin from Cytoscape. Vital molecular functions (MF) and biological processes (BP) involved in the DEGs are shown with the specific gene interactions. The MF and BP enrichment analyses are inferred from the 250 top DEGs PPI network. The connectivity of the GO terms network described by functional nodes and edges that are shared between the DEGs with a kappa score of 0.4. The enrichment shows only significant GO terms (p-value ≤ 0.05). The values of $p \leq 0.05$ indicate the node size. The node color code indicates the specific functional class that they are involved in. The color represents various molecular function and biological process involved in the enrichment analysis. The bold fonts indicate the most important functional GO terms that define the names of MF and BP of each group. The names of the DEGs involved in each group are displayed in red font.

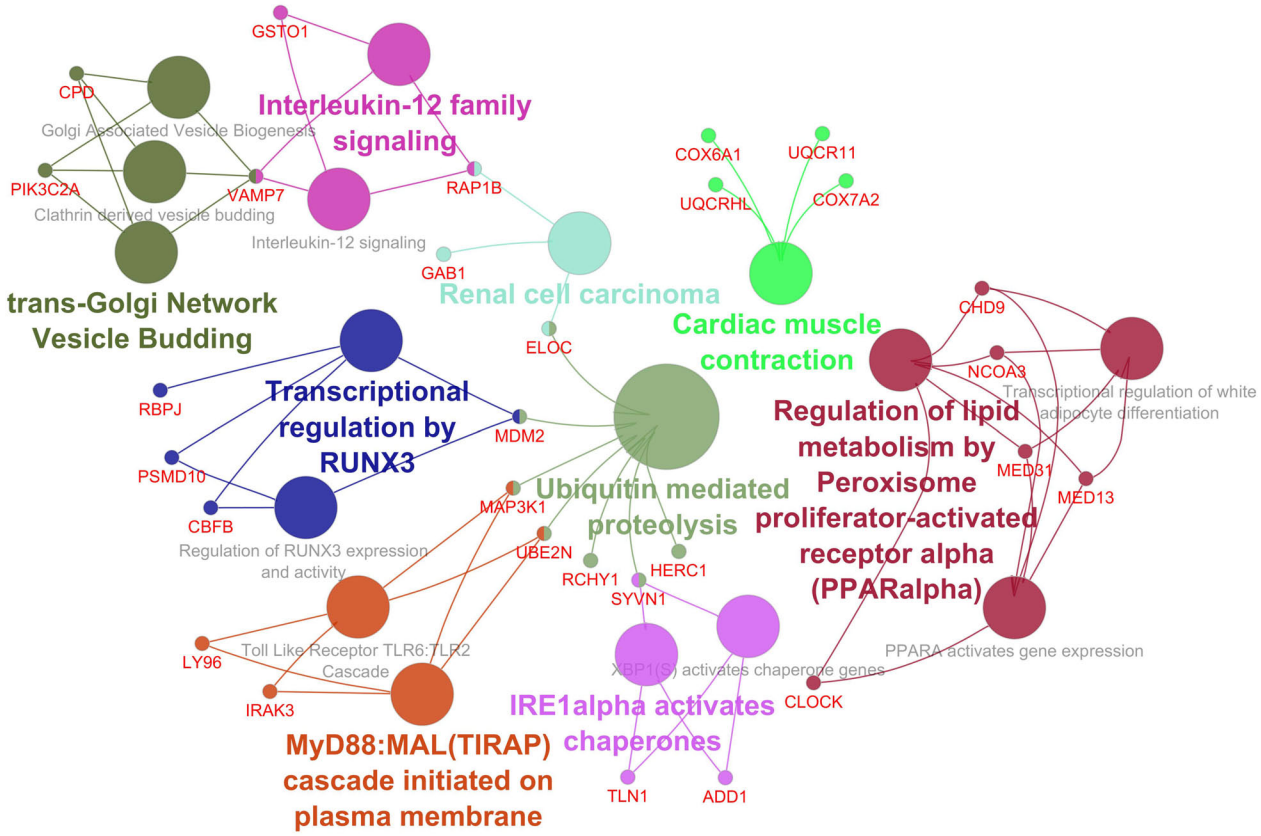

FIGURE 8 | Enrichment by pathway terms is visualized using the ClueGo/CluePedia plugin from Cytoscape. The plugin delivers a comprehensive enrichment analysis for DEGs, including KEGG and REACTOME pathways. The connectivity of the pathways in the network described by functional nodes and edges that are shared between the DEGs with a kappa score of 0.4. The enrichment shows only significant pathways (p-value ≤ 0.05). The values of $p \leq 0.05$ indicate the node size. The node color code indicates the specific functional class that they are involved in. The color represents various molecular pathways involved in the enrichment analysis of the identified DEGs. The bold fonts indicate the most critical functional pathways that define the names of the signaling pathway of each group. The names of the DEGs involved in each group are displayed in red font.

significant gene interactions depending on the p-values and kappa statistics.

Among the enriched biological process and molecular pathways, we identified that ubiquitin-mediated proteolysis, cardiac muscle contraction, MyD88-dependent Toll-like receptor signaling pathway, and IRE1 alpha-activated chaperones were considered dysregulated and are significant to atherosclerosis progression in FH patients based on kappa statistics and p-values (**Figures 7**, **8**). Additionally, we also identified the genes that are significant in the dysregulated molecular pathways and involved in FH progression. In line with this finding, *UQCR11, UBE2N, ADD1, TLN1, IRAK3, LY96*, and *MAP3K1* were found to be associated with the risk of Atherosclerosis in FH patients.

This study identified DEGs that are involved in ubiquitin-mediated proteolysis, IRE1 alpha-activated chaperones, and cardiac muscle contraction, such as *UQCR11, UBE2N, TLN1*, and *ADD1*, respectively. As shown in **Supplementary Figure S2**, a significant gene expression level was shown between the FH patients and healthy control samples. In this study, the expression levels of the *UQCR11* and *UBE2N* genes were significantly reduced, and the expression levels of the *TLN1* and *ADD1* genes were notably increased in the FH patients compared with the healthy controls. Our identified novel DEGs from the dataset indeed have high consistency with the risk of atherosclerosis and are involved in the dysregulation of such pathways as ubiquitin-mediated proteolysis, IRE1 alpha-activated chaperones, and cardiac muscle contraction. For instance, the transfer of ubiquitin from UBE2N to LDLR is required for its lysosomal degradation. The reduced gene expression level of *UBE2N* leads to skipping this event and might result in the accumulation of LDLR in the lysosome (Zhang et al., 2012).

Additionally, the polymorphism (p.G460W) present in ADD1 has reportedly contributed to the increased risk factor for coronary heart disease (Morrison et al., 2002). Our study clearly showed the increased expression of *ADD1* from the FH patient dataset, which could alter the casual signaling of IRE1 alpha-activated chaperones (**Supplementary Figure S2D**). *TLN1* functions as a molecular scaffolding protein and can contribute to the signaling of adhesion through its binding

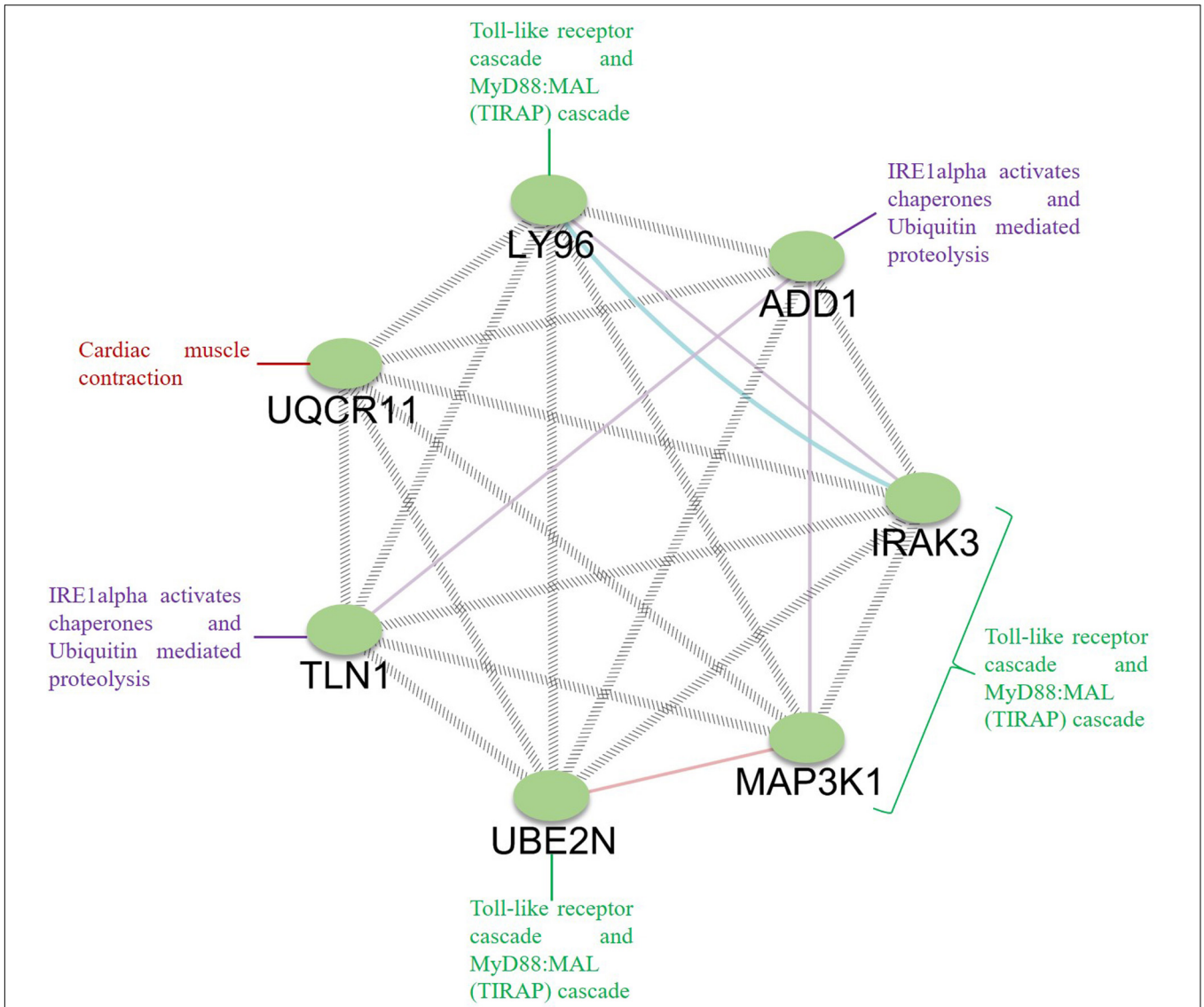

FIGURE 9 | Analysis of the interrelation between the identified core genes. GeneMANIA was used to map the network, and Cytoscape was used for network visualization. The genes *LY96, IRAK3, MAP3K1*, and *UBE2N* are involved in the Toll-like receptor cascade and MyD88:MAL (TIRAP) cascade; *ADD1* and *TLN1* are involved in ubiquitin-mediated proteolysis, IRE1alpha activates chaperones, and *UQCR11* is involved in cardiac muscle contraction.

partners, translating mechanical signals into chemical signals (Hytönen and Wehrle-Haller, 2014). The *TLN1* expression level in atherosclerotic plaques is significantly reduced, and it plays a central role in cell adhesion, indicating that tissue disintegration in atherosclerosis may be partly induced by *TLN1* downregulation, leading to cell-ECM interaction loosening and tissue reorganization (Essen et al., 2016). However, our dataset showed increased expression of *TLN1*, which might differentially regulate and activate IRE1alpha chaperones. Yet, recent research has identified that proatherogenic gene expression is regulated by *IRE1*, which includes several essential chemokines and cytokines (Tufanli et al., 2017).

To establish the interrelationship between the core genes *UQCR11*, *UBE2N*, *ADD1*, *TLN1*, *IRAK3*, *LY96*, and *MAP3K1*, this interrelationship will help in understanding the coexpression and molecular pathways that pertain to FH. It is essential to comprehend the interactions between the core genes since these genes dysregulate the general molecular pathways in FH patients (Kumar et al., 2019; Udhaya Kumar et al., 2020). The differentially expressed genes might be responsible for the clinical phenotypes of the patients and the progression of atherosclerosis. We found that the Toll-like receptor and MyD88:MAL (TIRAP) cascades are activated by such DEGs as *LY96, IRAK3, MAP3K1,* and *UBE2N* (Monaco et al., 2009; Yu et al., 2014). Similarly, *UQCR11* is directly involved in cardiac muscle contraction and has a physical interaction between *LY96, TLN1*, and *UBE2N*. The *TLN1* and *ADD1* genes directly associated with IRE1alpha-activated chaperones and

ubiquitin-mediated proteolysis (**Figure 9**). Taken together, our observed findings suggest that the involvement of core genes related to the risk of atherosclerosis might be a critical metric in ubiquitin-mediated proteolysis, Toll-like receptor, and MyD88: MAL (*TIRAP*) cascades and a beneficial tool for diagnosis and targeted therapy. In addition, the FH patients were free of clinical ASCVD, and the patient 2 and 5 did not receive lipid-lowering treatment, whereas the patient 1, 3, and 4 received lovastatin, simvastatin, and atorvastatin, respectively. This drug is also given to the patients as primary and secondary prevention that develops the risk of ASCVD and for those who already developed ASCVD, respectively (Taylor et al., 2013; Vuorio et al., 2013). With the help of a clinical diagnostic strategy, approximately 50% of patients are identified in FH, which is a cost-effective process. Indeed, a diagnosis by screening the cascades is a systematic and useful tool for diagnosing FH patients before the development of Atherosclerosis (Marks et al., 2002; Leren et al., 2008).

In general, FH can be progressed through many cellular and molecular mechanisms, and pathways. A recent study claimed that the *COX7B* is a potential novel gene target for FH treatment (Li et al., 2015); however, authors did not provide supportive evidence for metabolic network and pathway links between the Atherosclerosis and FH. Later, a similar study claimed that the *RPL17* and ribosome protein-related genes might increase the atherosclerotic risk. Although the study mentioned that these genes are downregulated in the FH patient's blood cells, they are significantly upregulated in the reported dataset. In addition, they claimed that cytochrome-c oxidase genes could contribute to atherosclerosis development, yet there was no substantial evidence provided (Wang and Zhao, 2016). The study conducted by Smolock et al. (2012) suggested that RPL17 acts as an inhibitor for vascular smooth muscle cells (VSMC) and might be a therapeutic target to limit the thickening of carotid intima (Smolock et al., 2012). However, increased expression of *RPL17* showed to inhibit the VSMC, reducing the progression of atherosclerosis, which could be a regular event that occurs in FH patients.

Interestingly, our functional enrichment analysis did not capture any biological/molecular functions related to inflammatory responses, as some patients with FH had shown inflammatory responses due to increased expression level of the molecules associated with tumor necrosis factor (TNF) (Fadini et al., 2014; Holven et al., 2014; Escate et al., 2018). The possible reason for this might be that the patients with FH in our study do not have any phenotypes characteristics to inflammation; consistent with the nature of FH, as it is a frequent disease (1/220-250) with a high variation in phenotypic expression associated with ASCVD (Alhababi and Zayed, 2018; Perez-Calahorra et al., 2019). Also, in era of personalized medicine, it is significant to identify the possible targeted therapy using advanced omics technologies (Prodan Žitnik et al., 2018).

Our study is the first to identify the association of core DEGs to the dysregulated pathways in FH patients. Our study recommends to screen WBCs from patients with FH to determine the metabolic and genetic factors that may help in identifying potential cardiovascular risks and might provide better diagnosis and improved therapy for the disease. The limitation of the present research is the small patient groups; therefore, investigating a larger sample size in different populations would help to confirm our data. The limitations of this study include; first, FH patients were free of clinical ASCVD and not possible to know if they have subclinical atherosclerosis by imaging. Second, the number is too small; therefore, it is important to study a larger cohort of patients that have clinical ASCVD that are compared with ones with no clinical ASCVD. Finally, no data were available for the LDL-C or TC of the FH patients in the GSE13985 dataset.

CONCLUSION

Most FH patients do not exhibit atherosclerotic symptoms in clinical diagnostic procedures. The findings using a transcriptome analysis from WBCs of FH patients and healthy controls to identify atherosclerotic markers are highly limited. Overall, our systematic interpretation demonstrated an essential role of DEGs and their essential role in the occurrence, development of FH, and increased risk of atherosclerosis. Our study identified a total of 250 genes that are differentially expressed and seven essential genes that are associated with FH patients compared to healthy controls. The study of expression from WBCs and the association with DEGs may help to elucidate the role played by these DEGs in FH progression and the development of atherosclerosis. Finally, we identified seven novel potential target genes (*UQCR11, UBE2N, ADD1, TLN1, IRAK3, LY96,* and *MAP3K1*), which might be valid targets for therapeutic development for FH, and might be used as diagnostic biomarkers for FH patients and prognostic indicators for atherosclerosis using WBCs from FH patients; however, functional studies are needed to validate their proposed role in FH and atherosclerosis.

DATA AVAILABILITY STATEMENT

The GEO database from NCBI (Gene Expression Omnibus database, https://www.ncbi.nlm.nih.gov/geo/) was used to access the GSE13985 dataset.

AUTHOR CONTRIBUTIONS

SU, DT, HZ, and CG were involved in the design of the study and the acquisition, analysis, and interpretation of the data. SU, DT, RB, SS, RM, MS, HZ, and CG were involved in the interpretation of the data and drafting the manuscript. CG and HZ supervised the entire study and were involved in study design, the acquisition, analysis, and understanding of the data, and drafting the manuscript. All authors contributed to the article 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.2020.00734/full#supplementary-material

REFERENCES

Abul-Husn, N. S., Manickam, K., Jones, L. K., Wright, E. A., Hartzel, D. N., Gonzaga-Jauregui, C., et al. (2016). Genetic identification of familial hypercholesterolemia within a single U.S. health care system. *Science* 354:aaf7000. doi: 10.1126/science.aaf7000

Akioyamen, L. E., Genest, J., Shan, S. D., Reel, R. L., Albaum, J. M., Chu, A., et al. (2017). Estimating the prevalence of heterozygous familial hypercholesterolaemia: a systematic review and meta-analysis. *BMJ Open* 7:e016461. doi: 10.1136/bmjopen-2017-016461

Alhababi, D., and Zayed, H. (2018). Spectrum of mutations of familial hypercholesterolemia in the 22 Arab countries. *Atherosclerosis* 279, 62–72. doi: 10.1016/j.atherosclerosis.2018.10.022

Alibés, A., Yankilevich, P., Cañada, A., and Díaz-Uriarte, R. (2007). IDconverter and IDClight: conversion and annotation of gene and protein IDs. *BMC Bioinformatics* 8:9. doi: 10.1186/1471-2105-8-9

Alonso, R., de Isla, L. P., Muñiz-Grijalvo, O., Diaz-Diaz, J. L., and Mata, P. (2018). Familial hypercholesterolaemia diagnosis and management. *J. Fam. Hypercholester. Diagn. Manag.* 13, 14–20.

Amor-Salamanca, A., Castillo, S., Gonzalez-Vioque, E., Dominguez, F., Quintana, L., Lluís-Ganella, C., et al. (2017). Genetically confirmed familial hypercholesterolemia in patients with acute coronary syndrome. *J. Am. Coll. Cardiol.* 70, 1732–1740. doi: 10.1016/j.jacc.2017.08.009

Aubert, J., Bar-Hen, A., Daudin, J. J., and Robin, S. (2004). Determination of the differentially expressed genes in microarray experiments using local FDR. *BMC Bioinformatics* 5:125. doi: 10.1186/1471-2105-5-125

Austin, M. A., Hutter, C. M., Zimmern, R. L., and Humphries, S. E. (2004). Genetic causes of monogenic heterozygous familial hypercholesterolemia: a huge prevalence review. *Am. J. Epidemiol.* 160, 407–420. doi: 10.1093/aje/kwh236

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., et al. (2016). Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* 44, W147–W153. doi: 10.1093/nar/gkw419

Bader, G. D., and Hogue, C. W. (2003). An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics* 4:2. doi: 10.1186/1471-2105-4-2

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., et al. (2013). NCBI GEO: archive for functional genomics data sets–update. *Nucleic Acids Res.* 41, D991–D995. doi: 10.1093/nar/gks1193

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., et al. (2009). ClueGO: a Cytoscape plugin to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 25, 1091–1093. doi: 10.1093/bioinformatics/btp101

Choi, S.-H., Kim, J., Gonen, A., Viriyakosol, S., and Miller, Y. I. (2016). MD-2 binds cholesterol. *Biochem. Biophys. Res. Commun.* 470, 877–880. doi: 10.1016/j.bbrc.2016.01.126

de Ferranti, S. D., Rodday, A. M., Mendelson, M. M., Wong, J. B., Leslie, L. K., and Sheldrick, R. C. (2016). Prevalence of Familial Hypercholesterolemia in the 1999 to 2012 United States National Health and nutrition examination surveys (NHANES). *Circulation* 133, 1067–1072. doi: 10.1161/CIRCULATIONAHA.115.018791

Di Resta, C., and Ferrari, M. (2018). Next generation sequencing: from research area to clinical practice. *EJIFCC* 29, 215–220.

Escate, R., Mata, P., Cepeda, J. M., Padreó, T., and Badimon, L. (2018). miR-505-3p controls chemokine receptor up-regulation in macrophages: role in familial hypercholesterolemia. *FASEB J.* 32, 601–612. doi: 10.1096/fj.201700476R

Essen, M. V., Rahikainen, R., Oksala, N., Raitoharju, E., Seppälä, I., Mennander, A., et al. (2016). Talin and vinculin are downregulated in atherosclerotic plaque; tampere Vascular Study. *Atherosclerosis* 255, 43–53. doi: 10.1016/j.atherosclerosis.2016.10.031

Fadini, G. P., Iori, E., Marescotti, M. C., de Kreutzenberg, S. V., and Avogaro, A. (2014). Insulin-induced glucose control improves HDL cholesterol levels but not reverse cholesterol transport in type 2 diabetic patients. *Atherosclerosis* 235, 415–417. doi: 10.1016/j.atherosclerosis.2014.05.942

Franz, M., Rodriguez, H., Lopes, C., Zuberi, K., Montojo, J., Bader, G. D., et al. (2018). GeneMANIA update 2018. *Nucleic Acids Res.* 46, W60–W64. doi: 10.1093/nar/gky311

Genest, J., Hegele, R. A., Bergeron, J., Brophy, J., Carpentier, A., Couture, P., et al. (2014). Canadian cardiovascular society position statement on familial hypercholesterolemia. *Can. J. Cardiol.* 30, 1471–1481. doi: 10.1016/j.cjca.2014.09.028

Gidding, S. S., Ann, C. M., de Ferranti, S. D., Defesche, J., Ito, M. K., Knowles, J. W., et al. (2015). The agenda for familial hypercholesterolemia. *Circulation* 132, 2167–2192. doi: 10.1161/CIR.0000000000000297

Goldberg, A. C., Hopkins, P. N., Toth, P. P., Ballantyne, C. M., Rader, D. J., Robinson, J. G., et al. (2011). Familial hypercholesterolemia: screening, diagnosis and management of pediatric and adult patients: clinical guidance from the national lipid association expert panel on familial hypercholesterolemia. *J. Clin. Lipidol.* 5, S1–S8. doi: 10.1016/j.jacl.2011.04.003

Gruber, A., Manèek, M., Wagner, H., Kirschning, C. J., and Jerala, R. (2004). Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. *J. Biol. Chem.* 279, 28475–28482. doi: 10.1074/jbc.M400993200

Hartgers, M. L., Ray, K. K., and Hovingh, G. K. (2015). New approaches in detection and treatment of familial hypercholesterolemia. *Curr. Cardiol. Rep.* 17:109. doi: 10.1007/s11886-015-0665-x

Holven, K. B., Narverud, I., Lindvig, H. W., Halvorsen, B., Langslet, G., Nenseter, M. S., et al. (2014). Subjects with familial hypercholesterolemia are characterized by an inflammatory phenotype despite long-term intensive cholesterol lowering treatment. *Atherosclerosis* 233, 561–567. doi: 10.1016/j.atherosclerosis.2014.01.022

Hopkins, P. N., Toth, P. P., Ballantyne, C. M., and Rader, D. J. (2011). Familial hypercholesterolemias: prevalence, genetics, diagnosis and screening recommendations from the national lipid association expert panel on familial hypercholesterolemia. *J. Clin. Lipidol.* 5, S9–S17. doi: 10.1016/j.jacl.2011.03.452

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37, 1–13. doi: 10.1093/nar/gkn923

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4, 44–57. doi: 10.1038/nprot.2008.211

Hytönen, V. P., and Wehrle-Haller, B. (2014). Protein conformation as a regulator of cell–matrix adhesion. *Phys. Chem. Chem. Phys.* 16, 6342–6357. doi: 10.1039/C3CP54884H

Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. (2003). Summaries of affymetrix GeneChip probe level data. *Nucleic Acids Res.* 31:e15. doi: 10.1093/nar/gng015

Joseph, L. G., Helen, H. H., and Michael, S. B. (2001). "Familial hypercholesterolemia," in *The Online Metabolic and Molecular Bases of Inherited Disease (OMMBID)*, eds D. L. Valle, S. Antonarakis, A. Ballabio, A. L. Beaudet, and G. A. Mitchell (New York, NY: McGraw-Hill), 2863–2913.

Krogan, N. J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., et al. (2006). Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* 440, 637–643. doi: 10.1038/nature04670

Kumar, S. U., Kumar, D. T., Siva, R., Doss, C. G. P., and Zayed, H. (2019). Integrative bioinformatics approaches to map potential novel genes and pathways involved in ovarian cancer. *Front. Bioeng. Biotechnol.* 7:391. doi: 10.3389/fbioe.2019.00391

Leitersdorf, E., Tobin, E. J., Davignon, J., and Hobbs, H. H. (1990). Common low-density lipoprotein receptor mutations in the French Canadian population. *J. Clin. Invest.* 85, 1014–1023. doi: 10.1172/JCI114531

Leren, T. P., Finborud, T. H., Manshaus, T. E., Ose, L., and Berge, K. E. (2008). Diagnosis of familial hypercholesterolemia in general practice using clinical diagnostic criteria or genetic testing as part of cascade genetic screening. *PHG* 11, 26–35. doi: 10.1159/000111637

Li, G., Wu, X.-J., Kong, X.-Q., Wang, L., and Jin, X. (2015). Cytochrome c oxidase subunit VIIb as a potential target in familial hypercholesterolemia by bioinformatical analysis. *Eur. Rev. Med. Pharmacol. Sci.* 19, 4139–4145.

Lyu, C., Zhang, Y., Gu, M., Huang, Y., Liu, G., Wang, C., et al. (2018). IRAK-M deficiency exacerbates ischemic neurovascular injuries in experimental stroke mice. *Front. Cell. Neurosci.* 12:504. doi: 10.3389/fncel.2018.00504

Marks, D., Wonderling, D., Thorogood, M., Lambert, H., Humphries, S. E., and Neil, H. A. W. (2002). Cost effectiveness analysis of different approaches of screening for familial hypercholesterolaemia. *BMJ* 324:1303. doi: 10.1136/bmj.324.7349.1303

Masana, L., Ibarretxe, D., Rodríguez-Borjabad, C., Plana, N., Valdivielso, P., Pedro-Botet, J., et al. (2019). Toward a new clinical classification of patients with familial hypercholesterolemia: one perspective from Spain. *Atherosclerosis* 287, 89–92. doi: 10.1016/j.atherosclerosis.2019.06.905

Monaco, C., Gregan, S. M., Navin, T. J., Foxwell, B. M. J., Davies, A. H., and Feldmann, M. (2009). Toll-like receptor-2 mediates inflammation and matrix degradation in human atherosclerosis. *Circulation* 120, 2462–2469. doi: 10.1161/CIRCULATIONAHA.109.851881

Morrison, A. C., Bray, M. S., Folsom, A. R., and Boerwinkle, E. (2002). ADD1 460W allele associated with cardiovascular disease in hypertensive individuals. *Hypertension* 39, 1053–1057. doi: 10.1161/01.HYP.0000019128.94483.3A

Nanchen, D., Gencer, B., Auer, R., Räber, L., Stefanini, G. G., Klingenberg, R., et al. (2015). Prevalence and management of familial hypercholesterolaemia in patients with acute coronary syndromes. *Eur. Heart J.* 36, 2438–2445. doi: 10.1093/eurheartj/ehv289

Naoumova, R. P., Thompson, G. R., and Soutar, A. K. (2004). Current management of severe homozygous hypercholesterolaemias. *Curr. Opin. Lipidol.* 15:413. doi: 10.1097/01.mol.0000137222.23784.2a

Perez-Calahorra, S., Laclaustra, M., Marco-Benedí, V., Lamiquiz-Moneo, I., Pedro-Botet, J., Plana, N., et al. (2019). Effect of lipid-lowering treatment in cardiovascular disease prevalence in familial hypercholesterolemia. *Atherosclerosis* 284, 245–252. doi: 10.1016/j.atherosclerosis.2019.02.003

Prodan Žitnik, I., Černe, D., Mancini, I., Simi, L., Pazzagli, M., Di Resta, C., et al. (2018). Personalized laboratory medicine: a patient-centered future approach. *Clin. Chem. Lab. Med.* 56, 1981–1991. doi: 10.1515/cclm-2018-0181

Rahman, K. M. T., Islam, M. F., Banik, R. S., Honi, U., Diba, F. S., Sumi, S. S., et al. (2013). Changes in protein interaction networks between normal and cancer conditions: total chaos or ordered disorder?. *Netw. Biol.* 3:15. doi: 10.1145/3064650.3064653

Režen, T., Cvikl, A., Brecelj, N., Rozman, D., Keber, I., and Fon Tacer, K. (2008). *Atherosclerotic Markers in Human Blood - A Study in Patients With Familial Hypercholesterolemia. GEO Accession Viewer*. Available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13985 (accessed November 10, 2019).

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., et al. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43:e47. doi: 10.1093/nar/gkv007

Russo, G., Zegar, C., and Giordano, A. (2003). Advantages and limitations of microarray technology in human cancer. *Oncogene* 22:6497. doi: 10.1038/sj.onc.1206865

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Sharan, R., Ulitsky, I., and Shamir, R. (2007). Network-based prediction of protein function. *Mol. Syst. Biol.* 3:88. doi: 10.1038/msb4100129

Silverman, M. G., Ference, B. A., Im, K., Wiviott, S. D., Giugliano, R. P., Grundy, S. M., et al. (2016). Association between lowering LDL-C and cardiovascular risk reduction among different therapeutic interventions: a systematic review and meta-analysis. *JAMA* 316, 1289–1297. doi: 10.1001/jama.2016.13985

Singh, R. K., Haka, A. S., Asmal, A., Barbosa-Lorenzi, V. C., Grosheva, I., Chin, H. F., et al. (2020). TLR4 (Toll-Like Receptor 4)-dependent signaling drives extracellular catabolism of LDL (Low-Density Lipoprotein) aggregates. *Arterioscler. Thromb. Vasc. Biol.* 40, 86–102. doi: 10.1161/ATVBAHA.119.313200

Sjouke, B., Kusters, D. M., Kindt, I., Besseling, J., Defesche, J. C., Sijbrands, E. J. G., et al. (2015). Homozygous autosomal dominant hypercholesterolaemia in the Netherlands: prevalence, genotype–phenotype relationship, and clinical outcome. *Eur. Heart J.* 36, 560–565. doi: 10.1093/eurheartj/ehu058

Slack, J. (1969). Risks of ischaemic heart-disease in familial hyperlipoproteinaemic states. *Lancet* 294, 1380–1382. doi: 10.1016/S0140-6736(69)90930-1

Smolock, E. M., Korshunov, V. A., Glazko, G., Qiu, X., Gerloff, J., and Berk, B. C. (2012). Ribosomal protein L17, RpL17, is an inhibitor of vascular smooth muscle growth and carotid intima formation. *Circulation* 126:2418. doi: 10.1161/CIRCULATIONAHA.112.125971

Smyth, G. K. (2005). "limma: linear models for microarray data," in *Bioinformatics and Computational Biology Solutions Using R and Bioconductor Statistics for Biology and Health*, eds R. Gentleman, V. J. Carey, W. Huber, R. A. Irizarry, and S. Dudoit (New York, NY: Springer), 397–420. doi: 10.1007/0-387-29362-0_23

Soutar, A. K., and Naoumova, R. P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat. Clin. Pract. Cardiovasc. Med.* 4, 214–225. doi: 10.1038/ncpcardio0836

Stone, N. J., Levy, R. I., Fredrickson, D. S., and Verter, J. (1974). Coronary artery disease in 116 kindred with familial type II hyperlipoproteinemia. *Circulation* 49, 476–488. doi: 10.1161/01.CIR.49.3.476

Stone, N. J., Robinson, J. G., Lichtenstein, A. H., Bairey Merz, C. N., Blum, C. B., Eckel, R. H., et al. (2014). 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the american college of cardiology/american heart association task force on practice guidelines. *J. Am. Coll. Cardiol.* 63, 2889–2934. doi: 10.1016/j.jacc.2013.11.002

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47, D607–D613. doi: 10.1093/nar/gky1131

Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., et al. (2017). The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res.* 45, D362–D368. doi: 10.1093/nar/gkw937

Taylor, F., Huffman, M. D., Macedo, A. F., Moore, T. H. M., Burke, M., Davey Smith, G., et al. (2013). Statins for the primary prevention of cardiovascular disease. *Cochrane Database Syst. Rev.* 1:CD004816. doi: 10.1002/14651858.CD004816.pub5

Tufanli, O., Telkoparan Akillilar, P., Acosta-Alvear, D., Kocaturk, B., Onat, U. I., Hamid, S. M., et al. (2017). Targeting IRE1 with small molecules counteracts progression of atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 114, E1395–E1404. doi: 10.1073/pnas.1621188114

Udhaya Kumar, S., Thirumal Kumar, D., Siva, R., George Priya Doss, C., Younes, S., Younes, N., et al. (2020). Dysregulation of signaling pathways due to differentially expressed genes from the B-cell transcriptomes of systemic lupus erythematosus patients – a bioinformatics approach. *Front. Bioeng. Biotechnol.* 8:276. doi: 10.3389/fbioe.2020.00276

Vuorio, A., Docherty, K. F., Humphries, S. E., Kuoppala, J., and Kovanen, P. T. (2013). Statin treatment of children with familial hypercholesterolemia – Trying to balance incomplete evidence of long-term safety and clinical accountability: are we approaching a consensus? *Atherosclerosis* 226, 315–320. doi: 10.1016/j.atherosclerosis.2012.10.032

Vuorio, A., Tikkanen, M. J., and Kovanen, P. T. (2014). Inhibition of hepatic microsomal triglyceride transfer protein – a novel therapeutic option for treatment of homozygous familial hypercholesterolemia. *Vasc. Health Risk Manag.* 10, 263–270. doi: 10.2147/VHRM.S36641

Vuorio, A., Watts, G. F., and Kovanen, P. T. (2017). Depicting new pharmacological strategies for familial hypercholesterolaemia involving lipoprotein (a). *Eur. Heart J.* 38, 3555–3559. doi: 10.1093/eurheartj/ehx546

Wald, D. S., Bestwick, J. P., Morris, J. K., Whyte, K., Jenkins, L., and Wald, N. J. (2016). Child–parent familial hypercholesterolemia screening in

primary care. *New Engl. J. Med.* 375, 1628–1637. doi: 10.1056/NEJMoa1602777

Walter, W., Sánchez-Cabo, F., and Ricote, M. (2015). GOplot: an R package for visually combining expression data with functional analysis. *Bioinformatics* 31, 2912–2914. doi: 10.1093/bioinformatics/btv300

Wang, H. X., and Zhao, Y. X. (2016). Prediction of genetic risk factors of atherosclerosis using various bioinformatic tools. *Genet. Mol. Res.* 15:gmr7347. doi: 10.4238/gmr.15027347

Warde-Farley, D., Donaldson, S. L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., et al. (2010). The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res.* 38, W214–W220. doi: 10.1093/nar/gkq537

Yu, L., and Feng, Z. (2018). The role of toll-like receptor signaling in the progression of heart failure. *Mediators Inflamm.* 2018, 1–15. doi: 10.1155/2018/9874109

Yu, M., Zhou, H., Zhao, J., Xiao, N., Roychowdhury, S., Schmitt, D., et al. (2014). MyD88-dependent interplay between myeloid and endothelial cells in the initiation and progression of obesity-associated inflammatory diseasesMyD88 in obesity-associated inflammatory diseases. *J. Exp. Med.* 211, 887–907. doi: 10.1084/jem.20131314

Zhang, L., Reue, K., Fong, L. G., Young, S. G., and Tontonoz, P. (2012). Feedback regulation of cholesterol uptake by the LXR–IDOL–LDLR axis. *Arterioscler. Thromb. Vasc. Biol.* 32, 2541–2546. doi: 10.1161/ATVBAHA.112.250571

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CASE REPORT
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Analysis of a Chinese Pedigree With Familial Chylomicronemia Syndrome Reveals Two Novel *LPL* Mutations by Whole-Exome Sequencing

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Familial chylomicronemia syndrome (FCS) is a rare monogenic autosomal recessive disease caused by loss-of-function mutations in genes involved in chylomicron breakdown through hydrolysis of triglycerides into free fatty acids. Patients are often diagnosed in early childhood with extremely high triglyceride levels and symptoms including abdominal pain, eruptive cutaneous xanthomata, hepatosplenomegaly, and significant cognitive, psychological, and social impairment. The most serious medical condition suffered by FCS patients is recurrent acute pancreatitis. Lipoprotein lipase (LPL) gene mutation accounts for majority of the known pathogenic mutations. Early diagnosis and strict low-fat diet are critical for successful management of the triglyceride concentration to lower the risk of pancreatitis. The true prevalence of FCS in China is unknown and here we report a Chinese female preterm neonate presented with an extremely high triglyceride level of 22.11 mmol/L on day 13 after birth. Clinical and laboratory workup including whole-exome sequencing revealed two novel compound heterozygous *LPL* mutations (c.406G > C and c.829G > C) that are co-segregated with her non-consanguineous parents, consistent with autosomal recessive inheritance. A diagnosis of FCS based on clinical, biochemical, and genetic ground was made to guide her management.

Keywords: Familial chylomicronemia syndrome, *LPL*, whole-exome sequencing, hypertriglyceridemia, missense mutation

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INTRODUCTION

Familial chylomicronemia syndrome (FCS, OMIM 238600) is characterized by very severe hypertriglyceridemia (Berglund et al., 2012; Hegele et al., 2014). Multiple symptoms may present starting from an early age, including episodic abdominal pain, eruptive cutaneous xanthomata, and hepatosplenomegaly (Feoli-Fonseca et al., 1998). The most severe and life-threatening complication of FCS is acute pancreatitis, which is shown to have a mortality rate of 5–30% in multiple studies (Guo et al., 2014; Gaudet et al., 2016). FCS shows a classic autosomal recessive inheritance pattern, and the prevalence in the United States population is approximately 1 in 1,000,000 (Hegele et al., 2014). FCS patients are deficient in the clearance of chylomicrons due to homozygous or compound

heterozygous loss-of-function mutations in genes that are involved in the catabolism of triglyceride (TG)-rich lipoproteins, including Lipoprotein lipase (*LPL*), *APOC2*, *GPIHBP1*, *APOA5*, and *LMF1*. *LPL* mutations account for 95% of all monogenic mutations associated with FCS (Murthy et al., 1996; Brahm and Hegele, 2015). Diagnosis of FCS is a complex process that involves clinical, biochemical, and genetic analysis (Beil et al., 1982). FCS may be underdiagnosed, and its true prevalence is underreported as specialized tests including *LPL* activity analysis and comprehensive genetic test may not be available when diagnosis is made (Davidson et al., 2017; Falko, 2018).

Herein, we report a Chinese female neonate with severe hypertriglyceridemia of 22.11 mmol/L. After clinical and laboratory workup, a genetic etiology was suspected and genetic testing was implemented with parents' consent.

CASE DESCRIPTION

The Chinese baby girl was born at 29 + 1 week by cesarean section weighing 1300 *g* to her non-consanguineous parents. She is the first child to a 25-year-old, G2P1 mother diagnosed with systemic lupus erythematosus (SLE) during her pregnancy. The family history was otherwise unremarkable. The parents' lipid profiles were largely within the normal range except for a slight increase of triglyceride for her mother (3.22 mmol/L). Apgar score was 9–10 at 1 min. After birth, she suffered from pneumonia and neonatal respiratory distress syndrome with symptoms including tachypnea, nasal flare, and grunting. She was admitted to NICU and received antibiotic treatment, symptomatic management, and total parenteral nutrition (TPN). She was transitioned to full gastrointestinal feeding on day 15 after birth. On day 13, her blood drawn for routine tests showed signs of chylomicronemia, and serum lipid profile was obtained and revealed severe hypertriglyceridemia (**Table 1** and **Supplementary Figure S1A**). Blood glucose level and thyroid, liver, and kidney function tests were normal. Studies of amino acids and carnitine metabolism were unremarkable. Her markedly elevated triglyceride level was suspected to have a genetic etiology and whole-exome sequencing (WES) was performed after genetic counseling and obtaining informed written consent from the parents. This study was approved by the Scientific Research Ethics Committee of Peking University Shenzhen Hospital [(2019) NO.058].

TABLE 1 | Patient's lipid profile on various diet.

Age (days)	Diet	TG	TC	HDL	LDL
		(mmol/L)			
13	Preterm Formula Milk (fat: 4.1 *g*/100 ml, MCT: 40%)	22.11	3.20	0.25	1.76
16	Formula Milk (fat: 3.4 *g*/100 ml)	15.25	3.90	0.28	2.03
21		21.00	3.75	0.25	1.85
25		9.40	2.80	0.31	1.47
33		10.83	2.99	0.30	1.55
36		13.57	2.06	0.19	0.97
40		13.13	2.15	0.21	0.99
49		14.03	2.13	0.20	0.93
67	Monogen Formula Milk (MCT: 84%)	2.86	/	/	/
90	Monogen Formula Milk: (MCT: 50%)	10.35	3.73	0.38	2.15
210	Monogen Formula Milk: (MCT: 50%)	7.38	6.86	2.26	0.29

TG, triglyceride (normal range: 0.20–2.31 mmol/L); TC, total cholesterol (normal range: 3.36–6.64 mmol/L); HDL, high-density lipoprotein (normal range: 0.83–1.96 mmol/L); LDL, low-density lipoprotein (2.07–3.1 mmol/L); and MCT, medium-chain triglyceride.

METHODS

Whole-Exome Sequencing and Variant Analysis

Genomic DNA was used for WES with SureSelect Human All Exon V6 array on the Illumina HiSeq X Ten platform with PE150 strategy according to the manufacturer's instructions (Chen et al., 2015). Sequences were aligned to the human reference genome UCSC/hg19 (Kent et al., 2002), and variants were called using the GATK Unified Genotyper with the default setting and annotated using a custom annotator (McKenna et al., 2010; DePristo et al., 2011; Auwera et al., 2013). Variants with minor allele frequency >1% were removed. Variants were then filtered into four categories: *de novo*, homozygous, and two parental compound heterozygous variants. Variants were further filtered according to GeneReviews (NCBI). *In silico* analysis of variants was performed with open access software, such as SIFT[1] and PolyPhen2[2]. Variant interpretation and prioritization were based on the clinical relevance of the gene and the pathogenicity of the variants using ACMG-AMP guidelines (Richards et al., 2015). The suspected variants were confirmed by Sanger sequencing for blood samples obtained from the patient as well as her parents.

RESULTS

Genetic Analysis by WES Revealed Two Novel Compound Heterozygous Mutations in *LPL* Gene

Variants on genes known to cause severe hypertriglyceridemia such as *LPL*, *APOC2*, *GPIHBP1*, *APOA5*, and *LMF1* were first selected and interpreted. Bioinformatic analysis revealed compound heterozygous missense mutations (c.406G > C p.A136P and c.829G > C p.D277H) in *LPL* gene. Both variants were neither found in Exome Aggregation Consortium (exac.broadinstitute.org) nor in 1000G[3]. A c.829G > A (p.A277N) transition was previously reported as a pathogenic mutation, causing a catalytically defective *LPL* protein (Ma et al., 1992). We performed sequence conservation analysis across different species, and results showed that both newly found missense mutations were highly conserved across species

[1]https://sift.bii.a-star.edu.sg/

[2]http://genetics.bwh.harvard.edu/pph2/index.shtml

[3]https://www.internationalgenome.org/1000-genomes-browsers

(*Mus musculus*, *Gallus gallus*, *Takifugu rubripes*, *Danio rerio*, and *Xenopus tropicalis*; **Figure 1B**). Molecular modeling of *LPL* protein with and without two-novel missense mutations using PyMOL software is shown in **Figure 1D** and suggested that both mutations would cause the protein to fold improperly. Furthermore, the two mutations were predicted to be deleterious by SIFT and "probably damaging" by PolyPhen, respectively. Sanger sequencing of the parents' DNA showed that they were heterozygous carriers, with the father being a heterozygous carrier of c.406G > C (p.A136P) mutation and the mother being a heterozygous carrier of c.829G > C (p.D277H) mutation (**Figures 1A,C**). Using the ACMG criteria for assessing pathogenicity, p.A136P and p.D277H were ranked as likely pathogenic.

Patient Management and Follow-Up

While in the hospital, the patient was switched from the preterm formula milk to term formula milk to lower her fat intake starting on day 15. Blood TG level decreased from 22.11 mmol/L on day 13 to 14.03 mmol/L on day 49 when she was discharged a day later. After discharge, the patient was advised to be fed on special Monogen formula

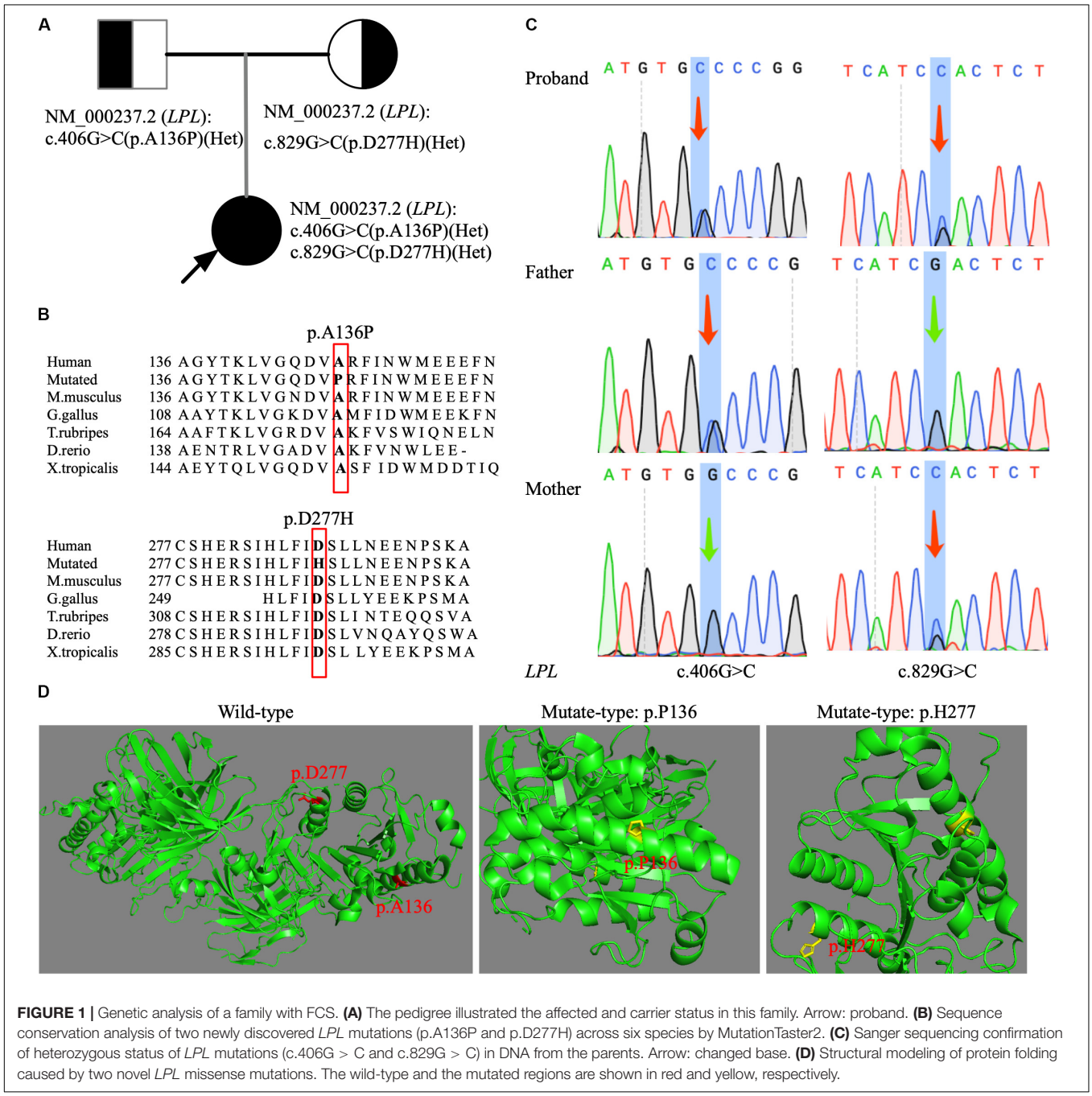

FIGURE 1 | Genetic analysis of a family with FCS. **(A)** The pedigree illustrated the affected and carrier status in this family. Arrow: proband. **(B)** Sequence conservation analysis of two newly discovered *LPL* mutations (p.A136P and p.D277H) across six species by MutationTaster2. **(C)** Sanger sequencing confirmation of heterozygous status of *LPL* mutations (c.406G > C and c.829G > C) in DNA from the parents. Arrow: changed base. **(D)** Structural modeling of protein folding caused by two novel *LPL* missense mutations. The wild-type and the mutated regions are shown in red and yellow, respectively.

milk with high medium-chain glycerides (MCT) content and to be followed up by clinicians periodically (**Table 1** and **Supplementary Figure S1**).

DISCUSSION

Chylomicronemia syndrome consists of two distinctive forms: rare monogenic early onset chylomicronemia and more commonly encountered polygenic chylomicronemia of adulthood (Berglund et al., 2012; Hegele et al., 2014). The nomenclatures "Familial *LPL* deficiency," "Type 1 hyperlipoproteinemia," or "Familial chylomicronemia syndrome (FCS)" all describe the monogenic form that shows an autosomal recessive inheritance pattern. FCS patients are deficient in chylomicrons clearance, thus suffering from a plethora of symptoms resulting from an extremely high plasma triglyceride level.

Early diagnosis of FCS is extremely important for proper implementation of management strategies that are individually targeted to reduce potential severe consequences of extremely high blood triglycerides, particularly acute, recurrent pancreatitis. Studies have shown that there is a close correlation between acute pancreatitis risk and plasma triglyceride levels, with 3–4% increase of acute pancreatitis risk with every 100 mg/dl increase of TG concentration (Murphy et al., 2013; Rashid et al., 2016). As FCS patients often present in early childhood, a delicate balance between ensuring developmental normalcy supported by sufficiency nutrition and reducing dietary fat intake is necessary for a successful long-term management strategy. FCS is frequently misdiagnosed and patients reported seeing on average at least five physicians before a correct diagnosis is made (Gelrud et al., 2017; Falko, 2018). Such diagnostic odyssey is common in rare disease diagnosis and poses significant psychological, lifestyle, and financial stress to patients and their family members.

Our case is unusual in that the patient was a 29-week preterm neonate with multiple medical complications and presented with extremely severe hypertriglyceridemia. After ruling out other possible causes of TG elevation, a suspicion of genetic etiology led to the decision to perform a comprehensive genetic analysis using WES, whose utility has been demonstrated in rare disease diagnosis (Niguidula et al., 2018). WES is an effective diagnostic tool in detecting point mutations, indels, and small copy number variations in the coding regions. However, WES cannot reliably detect mutations in the non-coding regions, low-level mosaicism, aneuploidy, trinucleotide repeat expansions, or chromosomal structural rearrangements. Studies have shown that WES has a diagnostic yield of around 25–30% in previously undiagnosed rare disease cases (Yang et al., 2013; Fitzgerald et al., 2015). We used WES and subsequent pedigree study to successfully identify two novel compound heterozygous *LPL* gene mutations, c.406G > C p.A136P and c.829G > C p.D277H, that were each passed down to the patient from her parents. To our knowledge, both mutations were previously unreported in the public database. They most likely result in a reduction in *LPL* catalytic activity rather than affecting protein synthesis and secretion by structural analysis (Ramasamy, 2016). So far, there has been no epidemiology study of the incidence and prevalence of FCS in China. Our report suggests that FCS can be readily diagnosed in the neonates with proper workup and accurate early diagnosis will be instrumental in setting a lipid-lowering goal to prevent serious complications such as recurrent acute pancreatitis and developmental issues.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Scientific Research Ethics Committee of Peking University Shenzhen Hospital [(2019) NO.058]. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the patient' parents for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YL contributed to the data collection, data interpretation, and genetic counseling. ZL contributed to the study design and writing of the manuscript. FZ and SZ contributed to the clinical data collection and data interpretation. WZ conceived of the study, participated in its design and coordination and revising the article critically for important intellectual content. All authors contributed to the article 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.2020.00741/full#supplementary-material

REFERENCES

Auwera, G. A., Carneiro, M. O., Hartl, C., Poplin, R., Angel, G., del Levy-Moonshine, A., et al. (2013). From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. *Curr. Protoc. Bioinform.* 11:43.

Beil, U., Grundy, S. M., Crouse, J. R., and Zech, L. (1982). Triglyceride and cholesterol metabolism in primary hypertriglyceridemia. *Arterioscler. Offic. J. Am. Heart Assoc., Inc.* 2, 44–57. doi: 10.1161/01.atv.2.1.44

Berglund, L., Brunzell, J. D., Goldberg, A. C., Goldberg, I. J., Sacks, F., and Murad, M. H. (2012). Evaluation and treatment of hypertriglyceridemia: an endocrine society clinical practice guideline. *J. Clin. Endocrinol. Metab.* 97, 2969–2989. doi: 10.1210/jc.2011-3213

Brahm, A. J., and Hegele, R. A. (2015). Chylomicronaemia-current diagnosis and future therapies. *Nat. Rev. Endocrinol.* 11, 352–362. doi: 10.1038/nrendo.2015.26

Chen, R., Im, H., and Snyder, M. (2015). Whole-exome enrichment with the agilent sureselect human all exon platform. *Cold Spring Harb. Protoc.* 2015:db.rot083659.

Davidson, M., Stevenson, M., Hsieh, A., Ahmad, Z., Crowson, C., and Witztum, J. L. (2017). The burden of familial chylomicronemia syndrome: interim results from the IN-FOCUS study. *Expert. Rev. Cardiovasc. Ther.* 15, 415–423. doi: 10.1080/14779072.2017.1311786

DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., et al. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* 43, 491–498.

Falko, J. M. (2018). Familial chylomicronemia syndrome: a clinical guide for endocrinologists. *Endocr. Pract.* 24, 756–763. doi: 10.4158/ep-2018-0157

Feoli-Fonseca, J. C., Lévy, E., Godard, M., and Lambert, M. (1998). Familial lipoprotein lipase deficiency in infancy: clinical, biochemical, and molecular study. *J. Pediatr.* 133, 417–423. doi: 10.1016/s0022-3476(98)70280-x

Fitzgerald, T. W., Gerety, S. S., Jones, W. D., Kogelenberg, M., Van King, D. A., McRae, J., et al. (2015). Large-scale discovery of novel genetic causes of developmental disorders. *Nature* 519, 223–228. doi: 10.1038/nature14135

Gaudet, D., Blom, D., Bruckert, E., Stroes, E., Kastelein, J., John, K., et al. (2016). Acute pancreatitis is highly prevalent and complications can be fatal in patients with familial chylomicronemia: results from a survey of lipidologist. *J. Clin. Lipidol.* 10, 680–681. doi: 10.1016/j.jacl.2016.03.048

Gelrud, A., Williams, K. R., Hsieh, A., Gwosdow, A. R., Gilstrap, A., and Brown, A. (2017). The burden of familial chylomicronemia syndrome from the patients' perspective. *Expert Rev. Cardiovasc. Ther.* 15, 879–887. doi: 10.1080/14779072.2017.1372193

Guo, Q., Li, M., Chen, Y., Hu, H., and Hu, W. (2014). Predictors for mortality following acute pancreatitis in children. *Pediatr. Surg. Intern.* 30, 1111–1115. doi: 10.1007/s00383-014-3595-6

Hegele, R. A., Ginsberg, H. N., Chapman, M. J., Nordestgaard, B. G., Kuivenhoven, J. A., Averna, M., et al. (2014). The polygenic nature of hypertriglyceridaemia: implications for definition, diagnosis, and management. *Lancet Diabetes Endocrinol.* 2, 655–666. doi: 10.1016/s2213-8587(13)70191-8

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., et al. (2002). The human genome browser at UCSC. *Genome Res.* 12, 996–1006. doi: 10.1101/gr.229102.

Ma, Y., Wilson, B. I., Bijvoet, S., Henderson, H. E., Cramb, E., Roederer, G., et al. (1992). A Missense mutation (Asp250→Asn) in Exon 6 of the human lipoprotein lipase gene causes chylomicronemia in patients of different ancestries. *Genomics* 13, 649–653. doi: 10.1016/0888-7543(92)90136-g

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The genome analysis toolkit: a mapreduce framework for analyzing next-generation dna sequencing data. *Genome Res.* 20, 1297–1303. doi: 10.1101/gr.107524.110

Murphy, M. J., Sheng, X., Macdonald, T. M., and Wei, L. (2013). Hypertriglyceridemia and acute pancreatitis. *JAMA Intern. Med.* 173, 162–164.

Murthy, V., Julien, P., and Gagné, C. (1996). Molecular pathobiology of the human lipoprotein lipase gene. *Pharmacol. Therapeut.* 70, 101–135. doi: 10.1016/0163-7258(96)00005-8

Niguidula, N., Alamillo, C., Shahmirzadi Mowlavi, L., Powis, Z., Cohen, J. S., and Farwell Hagman, K. D. (2018). Clinical whole-exome sequencing results impact medical management. *Mol. Genet. Genom. Med.* 6, 1068–1078. doi: 10.1002/mgg3.484

Ramasamy, I. (2016). Update on the molecular biology of dyslipidemias. *Clin. Chim. Acta* 454, 143–185. doi: 10.1016/j.cca.2015.10.033

Rashid, N., Sharma, P. P., Scott, R. D., Lin, K. J., and Toth, P. P. (2016). Severe hypertriglyceridemia and factors associated with acute pancreatitis in an integrated health care system. *J. Clin. Lipidol.* 10, 880–890. doi: 10.1016/j.jacl.2016.02.019

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., et al. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the american college of medical genetics and genomics and the association for molecular pathology. *Genet. Med.* 17, 405–423. doi: 10.1038/gim.2015.30

Yang, Y., Muzny, D. M., Reid, J. G., Bainbridge, M. N., Willis, A., Ward, P. A., et al. (2013). Clinical whole-exome sequencing for the diagnosis of mendelian disorders. *N. Engl. J. Med.* 369, 1502–1511.

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Genetic Diagnosis of Familial Hypercholesterolemia in Asia

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Familial hypercholesterolemia (FH) is a common genetic disease with an incidence of about 1 in 200–500 individuals. Genetic mutations markedly elevate low-density lipoprotein cholesterol and atherosclerotic cardiovascular disease (ASCVD) in FH patients. With advances in clinical diagnosis and genetic testing, more genetic mutations have been detected, including those in low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*), and so on. Globally, most FH patients remain undiagnosed, untreated, or inappropriately treated. Recently, there was a Global Call to Action by the Global Familial Hypercholesterolemia Community to reduce the health burden of FH. Asia, despite being the most populous continent with half of the global population, has low FH detection rates compared to Western countries. Therefore, we aimed to review the current status of FH genetic diagnosis in Asia to understand the gaps in FH diagnosis and management in this region.

Keywords: apolipoprotein B, Asia, diagnosis, familial hypercholesterolemia, gene, low-density lipoprotein receptor, proprotein convertase subtilisin/kexin type 9, mutation

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INTRODUCTION

Familial hypercholesterolemia (FH) is a common genetic disease with an incidence of about 1 in 200–500 individuals (Marks et al., 2003; Benn et al., 2012). Lipid metabolism changes in FH patients, including reduced low-density lipoprotein receptor (LDLR)-mediated low-density lipoprotein cholesterol (LDL-C) catabolism, impaired apolipoprotein B (APOB)-mediated LDL clearance, and increased proprotein convertase subtilisin/kexin type 9 (PCSK9) levels, mediate post-translational destruction of LDLRs (Chiou and Charng, 2016, Sturm et al., 2018). Genetic mutations in FH patients have been detected in *LDLR*, *APOB*, and *PCSK9*, and are either in a heterozygous (HeFH) or homozygous (HoFH) FH form (Chiou and Charng, 2016; Sturm et al., 2018, Di Taranto et al., 2020; Nawawi et al., 2020). These genetic mutations result in a marked elevation of LDL-C levels and atherosclerotic cardiovascular disease (ASCVD) if these patients are left underdiagnosed and undertreated (Nordestgaard et al., 2013; Gidding et al., 2015, Elkins and Fruh, 2019). For patients harboring FH-causing variants, the risk of ASCVD may be increased by 4.4- to 6.8- fold (Lee et al., 2019). Therefore, early diagnosis of FH is important.

However, globally, the majority of FH patients remain undiagnosed, untreated, or insufficiently treated (Nordestgaard et al., 2013; Khera et al., 2016, Cao et al., 2018; Wang et al., 2019). Based on clinical criteria, FH is not rare in the Asian population (Wang et al., 2019). However, around 42.5% to 62.5% of the FH patients remain underdiagnosed, according to definitions by the Dutch Lipid

Clinic Network (DLCN) and Simon Broome (SB). This finding supports the importance of genetic testing for the diagnosis of FH (Cao et al., 2018).

Furthermore, there are great advances in diagnostic technologies, such as next generation sequencing (NGS) (Di Resta and Ferrari, 2018), to improve the genetic diagnosis of FH. In the era of targeted and personalized medicine (Prodan Žitnik et al., 2018), it will be useful to identify different patient types to aid the selection of safer and more effective treatments.

Recently, there was a Global Call to Action by the Global Familial Hypercholesterolemia Community to reduce the health burden of FH (Wilemon et al., 2020). Asia is the most populous continent in the world, with more than half of the world's population. It is therefore important to better understand the current status of FH diagnosis and management in Asia. Here, we aimed to review the current status of FH genetic diagnosis in Asia.

FH DETECTION IN ASIA

There are two related studies on FH detection in Asia (Nordestgaard et al., 2013; Pang et al., 2019). Nordestgaard et al. (2013) reported the estimated number and percentage of individuals diagnosed with FH globally. Among roughly 200 countries/territories worldwide, data were only available in 22 countries/territories, including 3 in Asia. The data from Asian countries were derived from the second WHO consultation on FH in 1998. The estimated percentage of individuals diagnosed with FH was only 1% (n = 14,100) in Hong Kong, <1% (n = 46,300) in Taiwan, and <1% (n = 254,800) in Japan (Nordestgaard et al., 2013).

Another study in 2019 compared FH health care in 12 countries, including 9 countries selected in the Asia-Pacific region, and 2 in the Southern Hemisphere and United Kingdom (UK). In the United Kingdom, which was the benchmark, the estimated percentage of individuals diagnosed with FH was 10–20%. In non-Asian countries, the estimated percentage was 5.3% in South Africa, 4% in Australia, 2.9% in Brazil, and 2.5% in New Zealand. In Asian countries, however, the estimated percentage was only 3.8% in Taiwan, 2.2% in Hong Kong, 1.4% in Malaysia, 1% in Japan, <1% in China, <1% in the Philippines, and <1% in Vietnam. Although there is a continuous improvement in FH detection rates, compared to the 2013 data, there are still important gaps in comparison to other non-Asian countries and the United Kingdom (Pang et al., 2019).

AWARENESS AND KNOWLEDGE OF FH IN ASIA

The awareness and knowledge about FH have been surveyed among physicians across Asia (Pang et al., 2015, 2017). One anonymous internet-based survey involving 230 physicians was reported in 2015 (Pang et al., 2015). These physicians were selected from Japan, South Korea, and Taiwan. Only a small proportion of them was aware of the heritability (47%), prevalence (27%), risk of FH-related cardiovascular disease (13%), and FH specialists in their geographic area (35%) (Pang et al., 2015).

Another survey was done with 1,078 physicians and reported in 2017 (Pang et al., 2017). These physicians were from 10 different countries/regions, including Australia, Japan, Malaysia, South Korea, Philippines, Hong Kong, China, Vietnam, Taiwan, and the United Kingdom. Self-perceived FH familiarity was only 34% and there were no significant differences among most countries (except in Japan and China, where the value was lower) and the United Kingdom. Physicians from the Asia-Pacific region were significantly worse at selecting the correct FH descriptions (72%), compared to those from the United Kingdom (89%) (P = 0.001). FH guideline awareness was significantly lower in physicians from the Asia-Pacific region (35%), compared to those from the United Kingdom (61%) (P < 0.001). The awareness of lipid specialists for referral or medical advice was also significantly lower in physicians from the Asia-Pacific region (35%), compared to those from the United Kingdom (50%) (P = 0.003) (Pang et al., 2017).

These studies showed that the awareness, knowledge, and perception about FH were still low among Asian physicians, particularly in developing countries (Pang et al., 2015, 2017). More efforts are needed for improvement in these parameters, where extensive education and training programs could be of help.

GENETIC DIAGNOSIS OF FH IN ASIA

Taiwan

FH genetic studies in Taiwan are summarized in **Table 1**.

There are some studies that investigated the genetic mutations of FH patients and the development of genetic diagnostic methods in Taiwan (Charng et al., 2006; Yang et al., 2007, Chiou and Charng, 2010; Chiou et al., 2011, Chiou and Charng, 2012, 2017, Hsiung et al., 2018).

In a small population of 51 unrelated FH patients in Taiwan, *LDLR* and *APOB* mutations were screened using Sanger sequencing. Among these patients, 13 different functional mutations in *LDLR* and 1 in *APOB* were found in 21 patients (41.2%). For the *LDLR* mutations, there were 10 single-point missense mutations, 1 two-point mutation in the same allele, 1 non-sense mutation, and 1 frame-shift mutation. The study discovered 3 novel mutations, including 2 missense mutations [*LDLR* c.1592 T > A (p.M531K) and *LDLR* c.1597 T > C (p.W533R)] and 1 frame-shift mutation [*LDLR* c.1953,1954 del [TA] (p.M652Fs)] (Charng et al., 2006).

In another study, 87 FH patients from 30 unrelated Taiwanese families were screened for *LDLR*, *APOB*, and *PCSK9* mutations via Sanger sequencing. Genetic mutations were identified in 55 members (60%), including 6 previously reported *LDLR* mutations [*LDLR* c.383 G > A (p.C128Y), *LDLR* c.268 G > A (p.D90N), *LDLR* c.1216 C > T (p.R406W), *LDLR* c.1448 G > A (p.W483X), *LDLR* c.571 C > T (p.G191X), and *LDLR* c.1285 G > A (p.V429L)], 2 novel *LDLR* mutations [*LDLR* c.1954 del AT (p.M631Fs) and *LDLR* c.1586 + 5 G > C], 1 known [*APOB*

TABLE 1 | Genetic diagnosis of familial hypercholesterolemia in Taiwan.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
51	57 (26–84)	22 (43.1%)	Simon Broome criteria	Sanger sequencing for *LDLR* and *APOB* mutations	Thirteen different functional mutations in *LDLR* and one mutation in *APOB* were found in 21 patients (41.2%).	Charng et al., 2006
87	42 (14–70)	46 (52.9%)	Clinical diagnosed autosomal dominant hypercholesterolemia	Sanger sequencing for *LDLR*, *APOB*, and *PCSK9* mutations	Genetic mutations were identified in 55 members (60%) from 18 families.	Yang et al., 2007
102	44 (34–54)	40 (39.2%)	Simon Broome criteria	Sanger sequencing and MLPA	Frequency of large *LDLR* rearrangement was approximately 8% in Taiwanese patients with FH.	Chiou and Charng, 2010
208	NM	NM	Simon Broome criteria	Sanger sequencing and MLPA	*LDLR* mutations were found in 118 probands (56.7%), consisting of 61 different loci, and *APOB* c.10579 C > T (p.R3527W) mutations in 12 probands (5.8%).	Chiou and Charng, 2012
35 (first) and 125 (second)	42 (second)	46 (36.8%) (second)	Simon Broome criteria	A custom DNA resequencing array to detect mutations on 3 FH-causing genes (*LDLR*, *APOB*, and *PCSK9*) and 290 known insertion/deletion mutations on *LDLR*	The average microarray call rate was 98.45% and the agreement between microarray and capillary sequencing was 99.99%.	Chiou et al., 2011
120 (first) and 185 (second)	54 (second)	77 (41.6%) (second)	Dutch Lipid Clinic Network criteria	A custom Agena iPLEX assay to detect 68 point mutations on FH-causing genes	The detection sensitivity and specificity rates of Agena iPLEX were 92.5% and 100%, respectively, in the blind study.	Chiou and Charng, 2017
13 (first) and 28 (second)	NM	NM	Dutch Lipid Clinic Network score ≥ 5	NGS for *LDLR*, *APOB* and *PCSK9* genes	NGS correctly identify all the variants, including big duplications and deletions.	Hsiung et al., 2018

APOB, apolipoprotein B; DNA, deoxyribonucleic acid; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; MLPA, Multiplex ligation dependent probe analysis; NGS, next generation sequencing; NM, not mention; PCSK9, proprotein convertase subtilisin/kexin type 9; SNP, single nucleotide polymorphism.

c.10579 C > T (p.R3527W)], and 1 novel missense mutations [*APOB* c.10718 C > T (p.R3567W)] in the *APOB* gene. The study found no *PCSK9* gene mutations in this Taiwanese patient cohort (Yang et al., 2007).

One hundred two unrelated probands, who fulfilled the FH diagnostic criteria, were tested for mutations using Sanger sequence analysis and multiple ligation-dependent probe amplification (MLPA). Gene mutations were detected in 60 probands (58.8%), including *LDLR* mutations in 52 probands (51.0%) and the *APOB* c.10579 C > T (p.R3527W) mutation in 8 probands (7.8%). Among the 42 probands with mutations undetected by exome sequence analysis, 8 had abnormal MLPA patterns, including 2 with exon 6 to 18 deletions, 2 with exon 9 deletions, 1 with exon 6 to 8 deletions, 1 with exon 11 deletions, 1 with exon 3 to 5 duplications, and 1 with exon 7 to 12 duplications. This study found that the frequency of large *LDLR* rearrangements was approximately 8% in Taiwanese FH patients (Chiou and Charng, 2010).

In 208 Taiwanese patients with clinically diagnosed FH, common FH mutations were investigated using Sanger sequencing and MLPA. The top three most common mutations were *LDLR* c.986G > A (p.C329Y) (13.1%), c.1747C > T (p.H583Y) (10.8%), and *APOB* c.10579C > T (p.R3527W) (9.2%). Among these patients, 118 probands (56.7%) had *LDLR* mutations and 12 (5.8%) had the *APOB* c.10579 C > T (p.R3527W) mutation. Three novel mutations were discovered, including *LDLR* c.64 del G (p.A22Fs), *LDLR* c.1661 C > T (p.S554L), and *LDLR* c.2099 A > G (p.D700G). One haplotype [CAAGCCCCATGG/(dTA)n-112nt] was associated with *LDLR* c.1747 C > T (p.H583Y), and two were associated with *LDLR* c.986 G > A (p.C329Y). In comparison to southern Chinese patients, the same *LDLR* binding-domain pattern was associated with *APOB* c.10579 C > T (p.R3527W) in Taiwanese patients. The study found different common FH mutation-associated haplotypes and haplotype homologies in Taiwan and southern China, suggesting multiple historical migrations of Taiwanese and the presence of common ancestors in southern China (Chiou and Charng, 2012).

Some studies have investigated genetic diagnosis techniques in Taiwan. A custom DNA resequencing array was designed to detect mutations on all three FH-causing genes (*LDLR*, *APOB*, and *PCSK9*) and 290 known *LDLR* insertion/deletion mutations in the Taiwanese patients. In previously sequenced patients, the average call rate was 98.45% and the agreement between microarray and capillary sequencing was 99.99%. In screening patients blindly, the FH array detected at least 1 mutation in 77.5% of patients clinically diagnosed with definite FH and in 52.9% of patients with probable FH. The high-throughput FH resequencing array detected *LDLR*, *APOB*, and *PCSK9* with high efficiency and accuracy and identifies disease-causing mutations (Chiou et al., 2011).

A mass spectrometric assay (Agena iPLEX assay) was designed to simultaneously diagnose 68 point mutations in FH-causing

genes in Taiwanese patients. In the first part of the study, only 1 discrepancy was found between the mass spectrometry and Sanger sequencing data of 180 previously sequenced patients. In the second part, 62 probands with mutations were identified by both techniques out of 185 FH probands, with only 5 mutations detected by Sanger sequencing. The sensitivity and specificity of mass spectrometry were 92.5% and 100%, respectively. This study found great potential for this assay due to its low cost, speed (around 1 day), and flexibility for FH genetic screening in Taiwanese patients (Chiou and Charng, 2017).

Probes for NGS were designed to capture the whole *LDLR* gene and all coding sequences of *APOB* and *PCSK9* in Taiwanese patients. These probes correctly identified all the variants in 13 DNA samples, including 3 large duplications and 2 large deletions. In a new cohort of 28 unrelated FH patients with a Dutch Lipid Clinic Network score $\geq$ 5, they identified the causative variants in 21 unrelated probands. Five of them carried the novel splice site variant c.1186 + 2 T > G in *LDLR*. This study showed that this panel can comprehensively detect disease-causing variants in *LDLR*, *APOB*, and *PCSK9* in FH patients (Hsiung et al., 2018).

With the advances in molecular diagnostic methods, the detection rate of genetic mutations in clinically diagnosed FH has increased in Taiwan in recent years. However, there were still large proportions of FH patients that did not have genetic diagnoses. There were multiple *LDLR* and *APOB* mutations identified in Taiwan, including some novel mutations. However, *PCSK9* mutations, which had been reported in China and Japan, have not been identified in Taiwan.

China

FH genetic studies in China are summarized in **Table 2**.

In 105 young patients (age $\leq$ 35 years) with high LDL-C (LDL-C $\geq$ 3.4 mmol/L), genetic analysis was performed for 9 genes (*LDLR*, *APOB*, *PCSK9*, *APOE*, *STAP1*, *LIPA*, *LDLRAP1*, ABCG5 and ABCG8). Genetic mutations were detected in 40 patients (38.1%). This study found that the best LDL-C threshold was 4.56 mmol/L for genetically confirmed FH (Cao et al., 2018).

In a multi-center study in China, 285 unrelated index cases with clinical FH were analyzed by NGS for *LDLR*, *APOB*, and *PCSK9* mutations. Genetic mutations were detected in 148 cases (51.9%), including 84 (29.4%) in *LDLR*, 31 (10.9%) in *APOB*, and 4 (1.4%) in *PCSK9* genes. *LDLR* c.1448 G > A (p.W483X) was detected in 9 patients, which was the most frequent mutation. There were 8 novel mutations (7 *LDLR* and 1 *APOB*), which were considered as pathogenic by *in silico* analysis. There were 5 true *LDLR* homozygotes, 16 compound heterozygotes, and 13 double heterozygotes identified. The most severe phenotypes were noted in true *LDLR* homozygotes (Sun et al., 2018).

There was a review of 163 case reports about Chinese FH patients published before 2018. The mutation frequency was 82% in *LDLR*, 9% in *APOB*, and very rare in *PCSK9*. The study concluded that the epidemiological investigation of FH was not large-scale, FH recognition remained rudimentary, and guidelines for diagnosis and management of FH patients were incomplete in China (Peng et al., 2019).

In a previous systematic review of *LDLR* mutations in Chinese FH patients, a total of 74 studies were included. Among the 295 probands studied, 131 *LDLR* mutations were identified. Most of them were located in exon 4, and approximately 60% of them were missense mutations. There were 30 novel mutations and most of them were found pathogenic by *in silico* analysis. The major *LDLR* mutations were *LDLR* c.986 G > A (p.C329Y), *LDLR* c.1747 C > T (p.H583Y), and *LDLR* c.1879 G > A (p.A627T) (Jiang et al., 2015).

The majority of FH mutations found in Chinese were in *LDLR*, there was one in *APOB,* and were rare in *PCSK9*. The functionality of these novel mutations was predicted by *in silico* analysis. Functional testing or familial co-segregation analysis may be required to check the pathogenicity of these mutations.

Hong Kong

FH genetic studies in the Hong Kong are summarized in **Table 3**.

Hu et al. reported the epidemiologic features of 252 HeFH patients from the Chinese population in Hong Kong. For patients aged $\geq$ 18 years, xanthomata was noted in 40.6% of males and 54.8% of females, while corneal arcus was noted in 81.2% of males and 66.9% of females. The incidence of coronary artery disease (CAD) was 9.9% and 8.5% in males and females, respectively. This study found that age and the presence of xanthelasmata were associated with the risk of CAD (Hu et al., 2013, 2016).

In one genetic study from Hong Kong, DNA sequencing was performed for the promoter and coding regions of the 18 exons of the *LDLR* gene in 30 Chinese FH patients. Overall, *LDLR* mutations were detected in 21 patients (70.0%). There were 18 mutations in the promoter and 10 exons. Besides, there were 6 polymorphisms with genotypic distributions similar to those in the Japanese population but different from those in the Western populations. For the 9 clinically diagnosed FH patients with no detectable *LDLR* gene mutations, there was also no *APOB* R3500Q mutations detected (Mak et al., 1998).

In another genetic study from Hong Kong, a total of 94 unrelated probands with clinical FH received genetic testing for *LDLR*, *APOB*, and *PCSK9* by Sanger sequencing. Single-gene mutations were detected in 54 probands (57.4%), including 51 (54.2%) in *LDLR* and 3 (3.2%) in *APOB*. Heterozygous *LDLR* mutations were the most common. Compound heterozygous mutations were detected in five cases. Double heterozygous mutations were detected in three cases. A homozygous mutation in exon 10 of *LDLR* [c.1474G > A (p.D492N)] was detected in one case (Tan et al., 2018).

In a recent study, Chinese subjects with clinical FH received family based cascade screening and genetic testing. Among 96 index cases, 42 distinct mutations were identified in 64 cases (67%). The majority of mutations were in *LDLR*, and the three most common mutations were *LDLR* c.1241 T > G (p.L414R), *LDLR* c.1474G > A (p.D492N), and *LDLR* c.682G > A (p.E228X). Nine novel *LDLR* mutations were identified. *APOB* c.10579 C > T (p.R3527W) was identified in 5% of the total cases (Chan et al., 2019).

Japan

FH genetic studies in Japan are summarized in **Table 4**.

TABLE 2 | Genetic diagnosis of familial hypercholesterolemia in China.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
105	31.7	99 (94.3%)	Age ≤ 35 years and LDL-C ≥ 3.4 mmol/L	Genotyping for 9 genes, including *LDLR*, *APOB*, *PCSK9*, *APOE*, *STAP1*, *LIPA*, *LDLRAP1*, and *ABCG5*/8.	The prevalence of genetically confirmed FH was 38.1%.	Cao et al., 2018
285	49	173 (60.7%)	Dutch Lipid Clinic Network score ≥ 6	NGS for *LDLR*, *APOB* and *PCSK9* genes	The detection rate is 51.9% (148/285) with a total of 119 risk variants identified including 84 in *LDLR*, 31 in *APOB* and 4 in *PCSK9* gene.	Sun et al., 2018

APOB, apolipoprotein B; FH, familial hypercholesterolemia; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; NGS, next generation sequencing.

TABLE 3 | Genetic diagnosis of familial hypercholesterolemia in Hong Kong.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
30	42 (11–80	17 (56.7%)	(1) Fasting plasma total cholesterol levels >7.5 mmol/L in adults (>6.5 mmol/L in individuals younger than 16 years) and having normal fasting plasma triglyceride levels, and (2) presence of tendon xanthomata in proband or first degree relative, or other family members having raised LDL-C inherited in a dominant pattern.	DNA sequencing for the promoter and 18 coding exons of the *LDLR* gene	*LDLR* mutations were diagnosed in 21 patients (70.0%). For the 9 clinically diagnosed FH patients with no detectable *LDLR* gene mutations, there was also no *APOB* R3500Q mutation detected.	Mak et al., 1998
94	51.9	42 (44.7%)	Dutch Lipid Clinic Network criteria	Sanger sequencing for *LDLR*, *APOB*, or *PCSK9* genes	Single mutation was detected in 54 probands (57.4%), including 51 in *LDLR* and 3 in *APOB*.	Tan et al., 2018
96	51.7	43 (44.8%)	Clinical diagnosis of FH or severe hypercholesterolemia	Family based cascade screening incorporating genetic testing	Forty-two distinct mutations were identified in 67% of the index FH cases. The majority of causative mutations were in *LDLR*.	Chan et al., 2019

APOB, apolipoprotein B; DNA, deoxyribonucleic acid; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; MLPA.

In a previous report, 385 clinically diagnosed HeFH patients received genetic analysis for APOB 3500 mutations to estimate the frequency of familial defective apolipoprotein B-100 (FDB) in Japan. The study detected no APOB 3500 mutation in this cohort (Nohara et al., 1995).

In a study conducted in the Hokuriku district of Japan, 200 unrelated FH patients received genetic analysis for *LDLR* and *APOB* genes. *LDLR* mutations were detected in 125 patients (62.5%). There were 37 probable disease-causing mutations, 22 of which were novel. Common *LDLR* mutations included *LDLR* c. 2431 C > T (p.K811X) (19.5%), *LDLR* c. 2054 C > T (p.P685L) (6.0%), FH-Tonami-1 (6.0%), *LDLR* c.2141-3 C > A (5.5%), and FH-Tonami-2 (4.5%). *APOB* mutations were not detected in this cohort (Yu et al., 2002).

In another study conducted in the Hokuriku district of Japan, 25 clinical HoFH patients received genetic analysis reports. There were 15 true homozygotes and 10 compound heterozygotes for *LDLR* mutations. Five of these patients had *PCSK9* mutation [*PCSK9* c.94G > A (p.E32K)], including 2 true homozygotes and 3 compound heterozygotes (*LDLR* and *PCSK9*). *APOB* mutations were not detected in any FH patient. The study also confirmed a high incidence of HoFH (1/171,167) and HeFH (1/208) in the Hokuriku district of Japan (Mabuchi et al., 2011).

In 269 clinically diagnosed HeFH patients in Japan, genetic analyses were performed for *LDLR* and *PCSK9* gene mutations. Eleven PCSK9 variants were detected. The four most frequent *PCSK9* variants were c.63_64insCTG (p.L21_22insL), c.158 C > T (p.A53V), c.10 G > A (p.V4I), and c.94G > A (p.E32K). PCSK9 c.158 C > T (p.A53V) and c.63_64insCTG (p.L21_22insL) were in linkage disequilibrium with each other. Interestingly, the PCSK9 c.10 G > A (p.V4I) variant was linked to an increased risk of CAD in patients aged ≥ 30 years with *LDLR* mutations. The study suggested that *PCSK9* c.10 G > A (p.V4I) might affect *LDLR* metabolism and modify *LDLR* mutation phenotypes (Ohta et al., 2016).

Recently, 801 clinically diagnosed Japanese HeFH patients were analyzed for *LDLR* and *PCSK9* mutations. Among the 650 unrelated FH patients, *LDLR* pathogenic variants were identified in 296 patients (46%) and *PCSK9* pathogenic variants were identified in 51 patients (7.8%). *PCSK9* c.94G > A (p.E32K) was the most frequently detected pathogenic *PCSK9* variant in the Japanese FH population. The five most frequent *LDLR* pathogenic variants, found in about 17% of this FH cohort,

TABLE 4 | Genetic diagnosis of familial hypercholesterolemia in Japan.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
350	45	197 (56.3%)	Clinically diagnosed HeFH	Genetic analysis of *APOB* 3500 mutation	*APOB* 3500 mutation was not detected.	Nohara et al., 1995
200	45	90 (45.0%)	Clinically diagnosed HeFH	Denaturing gradient-gel electrophoresis, DNA sequencing and Southern blotting analysis	*LDLR* mutations were detected in 125 patients (62.5%). *APOB* mutation was not detected in this cohort.	Yu et al., 2002
25	32 (1–73)	12 (48.0%)	Clinically or genetically diagnosed HoFH	*LDLR* mutations were identified using the Invader assay method. Mutations in PCSK9 were detected by PCR-single-strand conformational polymorphism followed by direct sequence analysis.	There were 15 true homozygotes and 10 compound heterozygotes for *LDLR* mutations. Thirteen *LDLR* mutations were detected. Five of these patients had *PCSK9* mutation, including 2 patients with true homozygotes and 3 patients with compound heterozygotes. Three types of double heterozygotes for *LDLR* and *PCSK9* were detected.	Mabuchi et al., 2011
269	NM	NM	Clinically diagnosed HeFH	A direct sequence analysis for all 18 exons of *LDLR* and 12 exons of *PCSK9* gene	There were 11 *PCSK9* variants detected. *PCSK9* c.10 G > A (p.V4I) variant was linked to increased risk of coronary artery disease in patients aged ≥30 years and having *LDLR* mutations.	Ohta et al., 2016
801	NM	NM	The patients having at least two of the following factors: LDL-C ≥ 180 mg/dL, tendon/skin xanthomas, and familial history of FH or premature coronary artery disease within the second degree of kinship.	All coding regions and the exon-intron boundary sequence of the *LDLR* and *PCSK9* genes were examined.	Pathogenic variants in the *LDLR* and *PCSK9* genes were found in 296 (46%) and 51 (7.8%) of unrelated FH patients (n = 650), respectively.	Hori et al., 2019

APOB, apolipoprotein B; DNA, deoxyribonucleic acid; FH, familial hypercholesterolemia; HeFH, heterozygous familial hypercholesterolemia; HoFH, homozygous familial hypercholesterolemia; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; NM, not mention;PCSK9, proprotein convertase subtilisin/kexin type 9.

were identified: *LDLR* c.1845 + 2 T > C, c.1012T > A (p.C338S), c.1297G > C (p.D433H), c.1702C > G (p.L568V), and c.2431A > T (p.K811X). This study found that *LDLR* and *PCSK9* pathogenic variants were common in heterozygous Japanese FH patients (Hori et al., 2019).

APOB mutations, especially *APOB* c.10579 C > T (p.R3527W), were common mutations in Taiwan (9.2%) (Chiou and Charng, 2012) and China (10.9%) (Sun et al., 2018). However, it was not detected in Japan in the past (Nohara et al., 1995; Yu et al., 2002). The first Japanese case of *APOB* mutation, *APOB* c.10580 G > A (p.Arg3527Gln), was just reported recently (Hori et al., 2020). The origin of this *APOB* mutation was unknown and might be imported from other Asian countries. In contrast, *PCSK9* mutations, especially *PCSK9* c.94G > A (p.E32K), were quite prevalent in Japan (7.8%) (Hori et al., 2019), but have not been detected in Taiwan and were very rare in other Asian regions.

Korea

FH genetic studies in Korea are summarized in **Table 5**.

In 28 clinically diagnosed HeFH patients, genetic analysis was performed for *LDLR* gene by Southern blot hybridization. Three of them (10.7%) were diagnosed with two large *LDLR* deletion mutations (FH29 and FH110) (Chae et al., 1997).

In 80 clinically diagnosed FH patients, genetic analysis was performed to detect small structural rearrangements of the *LDLR* gene, which were undetected by southern blot hybridization. Three novel small deletions were identified (FH 2, FH 34 and FH 400) (Chae et al., 1999).

In 45 unrelated HeFH patients, genetic analysis was performed to assess the *LDLR* gene mutations by long-distance PCR. Two different large deletion mutations were identified (FH6 and FH 32) (Kim et al., 1999).

In 20 unrelated FH patients, genetic analysis was performed for the *LDLR* gene by single-strand conformation polymorphism. *LDLR* mutations were detected in five patients (25%). There were four novel point mutations, including one non-sense mutation and three missense mutations (Shin et al., 2000).

To study the role of the *LDLR* gene in polygenic hypercholesterolemia, a study was conducted in 244 members of 43 different pedigrees (15 normal and 28 FH pedigrees) and 245 individuals (187 normal and 58 FH) for 9 different restriction fragment length polymorphisms (RFLPs) (*Taq*I, *Stu*I, *Hinc*II, *Bst*EII, *Ava*II, *Pvu*II, MspIA, MspIB, and *Nco*I) and a sequence

TABLE 5 | Genetic diagnosis of familial hypercholesterolemia in Korea.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
28	NM	NM	(1) Plasma cholesterol levels > 300 mg/dl in adults and > 250 mg/dl in children, (2) a positive family history of hypercholesterolemia, Achilles tendon xanthomas, and coronary artery disease.	Southern blot hybridization with probes encompassing exons 1–18 of the *LDLR* gene	Two large deletion mutations of the *LDLR* gene were identified from three FH patients (10.7%).	Chae et al., 1997
80	NM	NM	(1) Plasma cholesterol levels > 300 mg/dl in adults and > 250 mg/dl in children, (2) a positive family history of hypercholesterolemia, Achilles tendon xanthomas, and coronary artery disease.	DNA sequencing for small structural rearrangements of *LDLR* gene	Three small deletions in exon 11 (FH 2, FH 34, and FH 400) were identified from three unrelated FH families.	Chae et al., 1999
45	NM	NM	LDL-C > 2.6 g/L, a positive family history of hypercholesterolemia or early coronary artery disease, and Achilles tendon xanthomas	Long-distance PCR for *LDLR* gene	Two large deletion mutations (FH6 and FH 32) were identified in four families	Kim et al., 1999
20	NM	NM	(1) Plasma cholesterol levels > 300 mg/dL in adults and > 250 mg/d: in children, (2) a positive history of hypercholesterolemia or early coronary artery disease, and Achilles tendon xanthomas.	DNA sequencing for *LDLR* gene by single-strand conformation	*LDLR* mutations were identified in 5 patients (25.0%).	Shin et al., 2000
489	NM	NM	244 members from 15 normal and 28 FH pedigrees 245 individuals, including 187 normal and 58 FH	Restriction fragment length polymorphisms	Among the 512 possible combinations for the nine polymorphic sites, there were 39 unique haplotypes detected. The four most common haplotypes accounted for 59.4% of those sampled. The haplotypes indicated marked linkage disequilibrium for these 10 sites and throughout the region containing the *LDLR* gene.	Chae et al., 2001
31	NM	NM	Total cholesterol >290 mg/dL (age > 40) or >220 mg/dL (age < 40), with tendon xanthoma and/or coronary heart disease, or with first-degree relatives who had hypercholesterolemia.	DNA sequencing for *LDLR* gene by single-strand conformation and by heteroduplex formation	Sixteen different mutations of the *LDLR* gene were identified in 25 unrelated Korean patients with HeFH (80.6%).	Kim et al., 2004
69	54	29 (42.0%)	Simon Broome criteria	Whole-exome sequencing	Genetic mutations were detected in 23 patients (33.3%).	Han et al., 2015
97	NM	NM	Unrelated patients older than 19 years who had (1) LDL-C > 190 mg/dL without lipid lowering agents and tendon xanthoma or (2) the same LDL-C levels and a family history of coronary artery disease or hypercholesterolemia	Whole exome sequencing, targeted exome sequencing, and Sanger sequencing	Putative pathogenic mutations in *LDLR*, *APOB*, or *PCSK9* genes were identified in 31 patients (32%).	Shin et al., 2015
97	54.1	38 (39.2%)	Unrelated patients older than 19 years who had (1) LDL-C > 190 mg/dL without lipid lowering agents and tendon xanthoma or (2) the same LDL-C levels and a family history of coronary artery disease or hypercholesterolemia	SNP	Mutation-negative FH patients had higher SNP scores than controls. SNP score analysis could be used for identification of polygenic cause of FH in Korean patients	Kwon et al., 2015
283	63.4	47 (16.6%)	Total cholesterol levels ≥ 290 mg/dL (7.5 mmol/L)	NGS	Seventeen different mutations in *LDLR*, *APOB*, and *PCSK9* genes were identified by NGS in 23 patients (8.1%). For the 110 subjects with a total cholesterol ≥ 310 mg/dL, 10 variants were identified in 10 patients (9.1%)	Kim et al., 2018

APOB, apolipoprotein B; DNA, deoxyribonucleic acid; FH, familial hypercholesterolemia; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; NGS, next generation sequencing; NM, not mention; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; SNP, single nucleotide polymorphism.

variation at Arg450. Frequencies of these polymorphisms were similar between FH patients and controls. Among the 512 possible combinations for the nine polymorphic sites, 39 unique haplotypes were detected. The four most common haplotypes accounted for 59.4% of the total patients sampled. The study found that the haplotypes indicated marked linkage disequilibrium for these 10 sites and throughout the region containing the *LDLR* gene (Chae et al., 2001).

In 31 clinically diagnosed HeFH patients, genetic analysis was performed for the *LDLR* gene by single-strand conformation and heteroduplex formation. *LDLR* gene mutations were detected in 25 patients (80.6%). There were sixteen different *LDLR* mutations. The study identified one mutation that had only been reported in a Korean FH patient, the in-frame 36-bp deletion (1591del36) in exon 11. Five novel mutations were identified, including *LDLR* c.311G > A (C104Y), c.661del17, c.1705insCTAG, c.2088C > A (C696X), and c.941-1G > A (Kim et al., 2004).

In 69 clinically diagnosed FH cases, whole-exome sequencing analysis was applied to identify genetic mutations in the 3 known FH-related genes (*LDLR*, *APOB*, and *PCSK9* genes). Genetic mutations were detected in 23 patients (33.3%). The most common genetic mutation was in the *LDLR* gene, which was identified in 19 patients (82.6%), included 2 copy number deletions and 17 mutations. There was also one *APOB* gene mutation and one *PCSK9* gene mutation. Two frameshift deletions in the *LDLR* gene and one *PCSK9* mutation were novel causative mutations. By co-segregation in their relatives, one copy number deletion and novel mutation were validated (Han et al., 2015).

One study enrolled patients with >190 mg/dL LDL-C and xanthoma or a family history of CAD or hypercholesterolemia. Among 97 patients, putative pathogenic mutations in *LDLR*, *APOB*, or *PCSK9* genes were identified in 31 patients (32%). When comparing the four clinical diagnosis criteria of FH (Simon Broome, Dutch, MEDPED, and Japanese), mutation detection rates were higher using the MEDPED criteria (67–75%) and lower in the Simon Broome or Dutch criteria (35%–37%). The study found that 225 mg/dL was the best LDL-C threshold for putative mutations in Korean FH patients (Shin et al., 2015).

The polygenic cause of FH in Korean patients was investigated in 97 FH patients and 2,274 controls from the Korean Health Examinee (HEXA) shared control study. These patients received genotyping for 12 single nucleotide polymorphisms (SNPs) used in prior studies to assess the polygenic causes of FH in Caucasian patients and 4 SNPs associated with LDL-C levels in East Asians. The study found that mutation-negative FH patients had higher SNP scores than controls. The study also showed that SNP score analysis could be used for the identification of the polygenic cause of FH in Korean patients (Kwon et al., 2015).

A total of 283 subjects with total cholesterol levels ≥ 290 mg/dL (7.5 mmol/L) were selected from the Namwon and Dong-gu Studies. Seventeen different mutations in *LDLR*, *APOB*, and *PCSK9* genes were identified by NGS in 23 patients (8.1%). For the 110 subjects with a total cholesterol ≥ 310 mg/dL, 10 variants were identified in 10 patients (9.1%). There were two novel *LDLR* variants, including *LDLR* c.2038 (p.L680V), and c.2201 (p.T734F) (Kim et al., 2018).

Malaysia

FH genetic studies in Malaysia are summarized in **Table 6**.

A total of 154 unrelated FH patients from Malaysia (Kelantan) were analyzed for *LDLR* gene mutations. Among these patients, 117 subjects (76.0%) had a total of 29 *LDLR* variants. The most frequent variant was *LDLR* c.1060 + 7 T > C (n = 18, 11.7%). The second most frequent variant was c.1411A > G (p.R471G) (n = 17, 11.0%) in *LDLR*. Eight of them [*LDLR* c.300 C > T (p.D100D), c.415G > C (p.D139H), c.1411A > G (p.R471G), c.1705 + 117 T > G, c.1186 + 41T > A, 1705 + 112C > G, Dup exon 12, and c.1966_2010del17 (p.W666Fs)] were reported for the first time. The study also found that patients with pathogenic mutations had higher LDL-C levels and a higher incidence of tendon xanthoma and cardiovascular diseases than those with non-pathogenic variants (Al-Khateeb et al., 2011).

A total of 140 clinically diagnosed autosomal dominant hypercholesterolemia (ADH) and 111 healthy control subjects were analyzed for selected SNPs in *LDLR*, *APOB*, *PCSK9*, and other genes. The ADH patients included Malays (60.0%), Indians (25.0%), and Chinese (15.0%). A total of 310 markers were examined, including 73 from *LDLR*, 130 from *APOB*, and 107 from *PCSK9*. Significant differences in allele frequency among Malaysians and other populations (European Whites, Han Chinese, Yoruba, and Gujarati Indians) were noted in 23 markers when compared to publicly available data. These markers included 2 on *LDLR*, 17 on *APOB*, and 4 on *PCSK9* (Alex et al., 2012).

In another study, 141 patients with clinically diagnosed FH and 111 unrelated control subjects were genetically analyzed via a high throughput microarray genotyping platform. Among the FH cases, 108 (76.60%) had at least one SNP that was associated with FH risk. Eleven of the 14 SNPs were significantly associated with an increased risk of FH, including 1 SNP in *LDLR*, 7 in *APOB*, and 3 in *PCSK9*. The study showed that *APOB* rs12720762 was associated with the highest risk of FH (odds ratio 14.78, $p < 0.001$) (Lye et al., 2013).

Two independent studies from the same team in Malaysia used SNPs for genetic analysis (Alex et al., 2012; Lye et al., 2013). Although they found SNPs with different allele frequencies between Malaysians, European Whites, Han Chinese, Yoruba, and Gujarati Indians, the functional significance of these SNPs requires further investigation.

Singapore

FH genetic studies in Singapore are summarized in **Table 7**.

In Singapore, 96 clinically suspected FH patients were genetically analyzed via NGS of 26 lipid-related genes. The cohort included Chinese (78.1%), Malays (13.5%), Indians (5.2%), and other ethnicities. A total of 50 patients (52.1%) had *LDLR* mutations and 4 (4.2%) had *APOB* mutations. No *PCSK9* mutations were detected in this cohort. There were 15 novel *LDLR* mutations. The study concluded that the mutation distribution was similar to other Asian countries, but the spectrum was different locally (Pek et al., 2018).

TABLE 6 | Genetic diagnosis of familial hypercholesterolemia in Malaysia.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
154	44.6	73 (47.4%)	Simon Broome criteria	The promoter region and exons 2–15 of the *LDLR* and MLPA for large rearrangement	Among these patients, 117 subjects (76.0%) had total 29 *LDLR* variants.	Al-Khateeb et al., 2011
140	47.0	73 (52.1%)	Dutch Lipid Clinic Network criteria	Selected SNPs of *LDLR*, *APOB*, *PCSK9*, and other genes	Significant differences in allele frequency among Malaysians and other populations (European Whites, Han Chinese, Yoruba and Gujarati Indians) were noted in 23 markers when comparing to publicly available data.	Alex et al., 2012
141	46.8	73 (51.8%)	Dutch Lipid Clinic Network criteria	A total of 1,536 SNPs by using high throughput microarray genotyping platform	Amongst the FH cases, 108 out of 141 (76.60%) have had at least one significant risk-associated SNP.	Lye et al., 2013

APOB, apolipoprotein B; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; MLPA, Multiplex ligation dependent probe analysis; SNP, single nucleotide polymorphism.

TABLE 7 | Genetic diagnosis of familial hypercholesterolemia in Singapore.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
96	33	77 (80.2%)	Simon Broome criteria or LDL-C > 4.9 mmol/L with unknown family history	NGS in 26 lipid-related genes	Among them, 50 patients (52.1%) had *LDLR* mutations and 4 patients (4.2%) had *APOB* mutations.	Pek et al., 2018

APOB, apolipoprotein B; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; NGS, next generation sequencing.

TABLE 8 | Genetic diagnosis of familial hypercholesterolemia in Philippines.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
60	55	21 (35.0%)	Dutch Lipid Clinic Network criteria	*LDLR* mutation identified by denaturing high performance liquid chromatography and DNA sequencing	Twelve patients (20%) had documented *LDLR* mutations and six of the mutations were considered novel.	Punzalan et al., 2005

DNA, deoxyribonucleic acid; LDLR, low-density lipoprotein receptor.

TABLE 9 | Genetic diagnosis of familial hypercholesterolemia in Sri Lankan.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
27	50 (24–73)	13 (48.1%)	Modified Simon Broome criteria or Dutch Lipid Clinic Network criteria	Sanger sequencing	Pathogenic missense mutations were detected only in 5 patients (18.5%).	Paththinige et al., 2018

TABLE 10 | Genetic diagnosis of familial hypercholesterolemia in India.

Case numbers	Age	Male	Diagnosis criteria	Genotyping	Main Findings	References
100	39 (3–74)	63 (63.0%)	Dutch Lipid Clinic Network criteria (adapted for the Indian population)	Sanger sequencing, MLPA, and NGS	Genetic mutations were identified in 47 cases (47.0%). Based on modified Dutch Lipid Clinic Network criteria, mutations were detected in 91.4% of definite FH cases, in 40% of probable FH cases, and in 18.8% of possible FH cases.	Setia et al., 2020

FH, familial hypercholesterolemia; MLPA, Multiplex ligation dependent probe analysis; NGS, next generation sequencing.

Philippines

FH genetic studies in Philippines are summarized in **Table 8**.

In the Philippines, 60 suspected FH patients were analyzed for *LDLR* mutations. *LDLR* mutations were detected in 12 patients (20%), and six novel mutations were identified. The study found that *LDLR* mutations were significantly associated with LDL-C levels, FH scores, and a family history of dyslipidemia (Punzalan et al., 2005).

In addition to *LDLR* mutations, further studies are still needed to identify relevant mutations in *APOB*, *PCSK9*, and other FH-related genes in this population.

Sri Lanka

FH genetic studies in Sri Lanka are summarized in **Table 9**.

In Sri Lanka, a total of 27 clinically diagnosed FH patients were analyzed for *LDLR* mutations using Sanger sequencing. Among these patients, pathogenic missense mutations were detected in only five (18.5%). Four patients were heterozygous for *LDLR* mutations, including *LDLR* c.682 G > C (p.E228Q) in 2 patients, *LDLR* c.1720C > A (p.R574S) in 1, and *LDLR* c.1855 T > A (p.F619L) in 1. One patient was compound heterozygous for *LDLR* c.2289 G > T (p.E763D) and *LDLR* c.1670 C > G (p.T557S). The study found that *LDLR* mutations were markedly low in this population (Paththinige et al., 2018).

This is the first report on *LDLR* mutations in this population. However, the detection rate of genetic mutations was low. Furthermore, the study did not screen for other FH mutations, including those in *APOB*, *PCSK9*, and *LDLRAP1*.

India

FH genetic studies in India are summarized in **Table 10**.

In one Indian study, a total of 100 potential FH cases were genetically analyzed using Sanger sequencing, MLPA, and NGS. Genetic mutations were identified in 47 cases (47.0%). Based on modified Dutch Lipid Clinic Network criteria, mutations were detected in 91.4% of definite FH cases, in 40% of probable FH cases, and in 18.8% of possible FH cases. A total of 38 pathogenic variants were identified, including 33 *LDLR* mutations, 3 *APOB* mutations, and 2 *PCSK9* mutations. There were ten novel pathogenic variants. Interestingly, a likely founder mutation in intron 10 (c.1587-1 G > A) of the *LDLR* gene was observed in 6 North Indian families. However, the conventional pathogenic variants in previously reported *LDLR*, *APOB*, and *PCSK9* mutations were not detected. This study found different genetic variants between the Indian population and Western populations (Setia et al., 2020).

In addition, there were other genetic studies as abstracts from India. However, we did not report them in this review article.

GENETIC DIFFERENCES OF FH BETWEEN DIFFERENT ETHNICITIES

Among the genetic studies in Asia, the detection rates of genetic mutations varied. The differences may be due to either the diagnostic methods used or the ethnicities sampled. With the advances in genetic diagnostic methods, detection rates will be further improved. Most of the genetic studies in Asia were confined to one ethnicity. Only a few studies enrolled FH patients with different ethnicities. Data from Singapore revealed a similar distribution of mutations as compared to other Asian countries (Pek et al., 2018). Data from Malaysia revealed significant differences in the allele frequency of markers for *LDLR*, *APOB*, and *PCSK9* genes compared to publicly available data from other populations (Alex et al., 2012). Until now, there is limited literature discussing the genetic differences in FH between Asian and non-Asian populations. Further studies are still needed to compare the genetic differences in FH between different ethnicities.

CONCLUSION

Although there has been a continuous improvement in FH detection rates, there are still important gaps to fill in Asian countries in comparison to other countries and the United Kingdom. FH awareness, knowledge, and perception remain low in Asian physicians, particularly in less economically developed countries. More efforts are required to close this gap, such as extensive education and training programs. Although there have been some FH genetic studies in the Asian population, each utilizing different techniques, further studies are required to understand the different genetic backgrounds of FH in Asia.

AUTHOR CONTRIBUTIONS

C-CH and M-JC contributed to conception and design of the study, manuscript revision, read, and approved the submitted version. C-CH wrote the first draft of the manuscript.

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REFERENCES

Alex, L., Chahil, J. K., Lye, S. H., Bagali, P., and Ler, L. W. (2012). Differences in allele frequencies of autosomal dominant hypercholesterolemia SNPs in the Malaysian population. *J. Hum. Genet.* 57, 358–362. doi: 10.1038/jhg.2012.34

Al-Khateeb, A., Zahri, M. K., Mohamed, M. S., Sasongko, T. H., Ibrahim, S., Yusof, Z., et al. (2011). Analysis of sequence variations in low-density lipoprotein receptor gene among Malaysian patients with familial hypercholesterolemia. *BMC Med. Genet.* 12:40. doi: 10.1186/1471-2350-12-40

Benn, M., Watts, G. F., Tybjaerg-Hansen, A., and Nordestgaard, B. G. (2012). Familial hypercholesterolemia in the danish general population: prevalence, coronary artery disease, and cholesterol-lowering medication. *J. Clin. Endocrinol. Metab.* 97, 3956–3964. doi: 10.1210/jc.2012-1563

Cao, Y. X., Wu, N. Q., Sun, D., Liu, H. H., Jin, J. L., Li, S., et al. (2018). Application of expanded genetic analysis in the diagnosis of familial hypercholesterolemia in patients with very early-onset coronary artery disease. *J. Transl. Med.* 16:345. doi: 10.1186/s12967-018-1737-7

Chae, J. J., Kim, S. H., Kim, U. K., Han, K. H., Kim, H. S., Kastner, D. L., et al. (1999). Three novel small deletion mutations of the LDL receptor gene in

Korean patients with familial hypercholesterolemia. *Clin. Genet.* 55, 325–331. doi: 10.1034/j.1399-0004.1999.550505.x

Chae, J. J., Kim, S. H., Kim, U. K., Hong, S. S., Kim, Y. S., Namkoong, Y., et al. (2001). Polymorphic DNA haplotypes at the human low-density lipoprotein receptor gene locus in Koreans. *Hum. Biol.* 73, 105–119. doi: 10.1353/hub.2001.0004

Chae, J. J., Park, Y. B., Kim, S. H., Hong, S. S., Song, G. J., Han, K. H., et al. (1997). Two partial deletion mutations involving the same Alu sequence within intron 8 of the LDL receptor gene in Korean patients with familial hypercholesterolemia. *Hum. Genet.* 99, 155–163. doi: 10.1007/s004390050331

Chan, M. L., Cheung, C. L., Lee, A. C., Yeung, C. Y., Siu, C. W., Leung, J. Y., et al. (2019). Genetic variations in familial hypercholesterolemia and cascade screening in East Asians. *Mol. Genet. Genomic. Med.* 7:e00520. doi: 10.1002/mgg3.520

Charng, M. J., Chiou, K. R., Chang, H. M., Cheng, H. M., Ye, Z. X., and Lin, S. J. (2006). Identification and characterization of novel low-density lipoprotein receptor mutations of familial hypercholesterolaemia patients in Taiwan. *Eur. J. Clin. Invest.* 36, 866–874. doi: 10.1111/j.1365-2362.2006.01735.x

Chiou, K. R., and Charng, M. J. (2010). Detection of mutations and large rearrangements of the low-density lipoprotein receptor gene in Taiwanese patients with familial hypercholesterolemia. *Am. J. Cardiol.* 105, 1752–1758. doi: 10.1016/j.amjcard.2010.01.356

Chiou, K. R., and Charng, M. J. (2012). Common mutations of familial hypercholesterolemia patients in Taiwan: characteristics and implications of migrations from southeast China. *Gene* 498, 100–106. doi: 10.1016/j.gene2012.01.092

Chiou, K. R., and Charng, M. J. (2016). Genetic diagnosis of familial hypercholesterolemia in Han Chinese. *J. Clin. Lipidol.* 10, 490–496. doi: 10.1016/j.jacl.2016.01.009

Chiou, K. R., and Charng, M. J. (2017). Detection of common sequence variations of familial hypercholesterolemia in Taiwan using DNA mass spectrometry. *J. Clin. Lipidol.* 11, 386–393. doi: 10.1016/j.jacl.2016.12.014

Chiou, K. R., Charng, M. J., and Chang, H. M. (2011). Array-based resequencing for mutations causing familial hypercholesterolemia. *Atherosclerosis* 216, 383–389. doi: 10.1016/j.atherosclerosis.2011.02.006

Di Resta, C., and Ferrari, M. (2018). Next generation sequencing: from research area to clinical practice. *EJIFCC* 29, 215–220.

Di Taranto, M. D., Giacobbe, C., and Fortunato, G. (2020). Familial hypercholesterolemia: a complex genetic disease with variable phenotypes. *Eur. J. Med. Genet.* 63:103831. doi: 10.1016/j.ejmg.2019.103831

Elkins, J. C., and Fruh, S. (2019). Early diagnosis and treatment of familial hypercholesterolemia. *Nurse Pract.* 44, 18–24. doi: 10.1097/01.NPR.0000552677.31028.57

Gidding, S. S., Champagne, M. A., de Ferranti, S. D., Defesche, J., Ito, M. K., Knowles, J. W., et al. (2015). The agenda for familial hypercholesterolemia: a scientific statement from the american heart association. *Circulation* 132, 2167–2192. doi: 10.1161/CIR.0000000000000297

Han, S. M., Hwang, B., Park, T. G., Kim, D. I., Rhee, M. Y., Lee, B. K., et al. (2015). Genetic testing of Korean familial hypercholesterolemia using whole-exome sequencing. *PLoS One* 10:e0126706. doi: 10.1371/journal.pone.0126706

Hori, M., Ohta, N., Takahashi, A., Masuda, H., Isoda, R., Yamamoto, S., et al. (2019). Impact of LDLR and PCSK9 pathogenic variants in Japanese heterozygous familial hypercholesterolemia patients. *Atherosclerosis* 289, 101–108. doi: 10.1016/j.atherosclerosis.2019.08.004

Hori, M., Takahashi, A., Son, C., Ogura, M., and Harada-Shiba, M. (2020). The first Japanese cases of familial hypercholesterolemia due to a known pathogenic *APOB* gene variant, c.10580 G>A: p.(Arg3527Gln). *J. Clin. Lipidol.* doi: 10.1016/j.jacl.2020.05.007 [Epub ahead of print].

Hsiung, Y. C., Lin, P. C., Chen, C. S., Tung, Y. C., Yang, W. S., Chen, P. L., et al. (2018). Identification of a novel LDLR disease-causing variant using capture-based next-generation sequencing screening of familial hypercholesterolemia patients in Taiwan. *Atherosclerosis* 277, 440–447. doi: 10.1016/j.atherosclerosis.2018.08.022

Hu, M., Hooper, A. J., Bockxmeer, F. M., Watts, G. F., Chan, J. C., and Tomlinson, B. (2016). Management of Familial Hypercholesterolemia in Hong Kong. *J. Atheroscler. Thromb.* 23, 520–531. doi: 10.5551/jat.34314

Hu, M., Lan, W., Lam, C. W., Mak, Y. T., Pang, C. P., and Tomlinson, B. (2013). Heterozygous familial hypercholesterolemia in Hong Kong Chinese. Study of 252 cases. *Int. J. Cardiol.* 167, 762–767. doi: 10.1016/j.ijcard.2012.03.048

Jiang, L., Sun, L. Y., Dai, Y. F., Yang, S. W., Zhang, F., and Wang, L. Y. (2015). The distribution and characteristics of LDL receptor mutations in China: a systematic review. *Sci. Rep.* 5:17272. doi: 10.1038/srep17272

Khera, A. V., Won, H. H., Peloso, G. M., Lawson, K. S., Bartz, T. M., Deng, X., et al. (2016). Diagnostic yield and clinical utility of sequencing familial hypercholesterolemia genes in patients with severe hypercholesterolemia. *J. Am. Coll. Cardiol.* 67, 2578–2589. doi: 10.1016/j.jacc.2016.03.520

Kim, H. N., Kweon, S. S., and Shin, M. H. (2018). Detection of familial hypercholesterolemia using next generation sequencing in two population-based cohorts. *Chonnam. Med. J.* 54, 31–35. doi: 10.4068/cmj.2018.54.1.31

Kim, J. H., Choi, H. K., Lee, H., Park, H. Y., Kim, J. H., Kim, J. W., et al. (2004). Novel and recurrent mutations of the LDL receptor gene in Korean patients with familial hypercholesterolemia. *Mol. Cells.* 18, 63–70.

Kim, S. H., Bae, J. H., Chae, J. J., Kim, U. K., Choe, S. J., Namkoong, Y., et al. (1999). Long-distance PCR-based screening for large rearrangements of the LDL receptor gene in Korean patients with familial hypercholesterolemia. *Clin. Chem.* 45, 1424–1430. doi: 10.1093/clinchem/45.9.1424

Kwon, M., Han, S. M., Kim, D. I., Rhee, M. Y., Lee, B. K., Ahn, Y. K., et al. (2015). Evaluation of polygenic cause in Korean patients with familial hypercholesterolemia - a study supported by korean society of lipidology and atherosclerosis. *Atherosclerosis* 242, 8–12. doi: 10.1016/j.atherosclerosis.2015.06.053

Lee, S., Akioyamen, L. E., Aljenedil, S., Rivière, J. B., Ruel, I., and Genest, J. (2019). Genetic testing for familial hypercholesterolemia: impact on diagnosis, treatment and cardiovascular risk. *Eur. J. Prev. Cardiol.* 26, 1262–1270. doi: 10.1177/2047487319829746

Lye, S. H., Chahil, J. K., Bagali, P., Alex, L., Vadivelu, J., Ahmad, W. A., et al. (2013). Genetic polymorphisms in LDLR, APOB, PCSK9 and other lipid related genes associated with familial hypercholesterolemia in Malaysia. *PLoS One* 8:e60729. doi: 10.1371/journal.pone.0060729

Mabuchi, H., Nohara, A., Noguchi, T., Kobayashi, J., Kawashiri, M. A., Tada, H., et al. (2011). Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan. *Atherosclerosis* 214, 404–407. doi: 10.1016/j.atherosclerosis.2010.11.005

Mak, Y. T., Pang, C. P., Tomlinson, B., Zhang, J., Chan, Y. S., Mak, T. W., et al. (1998). Mutations in the low-density lipoprotein receptor gene in Chinese familial hypercholesterolemia patients. *Arterioscler. Thromb. Vasc. Biol.* 18, 1600–1605. doi: 10.1161/01.atv.18.10.1600

Marks, D., Thorogood, M., Neil, H. A., and Humphries, S. E. (2003). A review on the diagnosis, natural history, and treatment of familial hypercholesterolaemia. *Atherosclerosis* 168, 1–14. doi: 10.1016/s0021-9150(02)00330-1

Nawawi, H. M., Chua, Y. A., and Watts, G. F. (2020). The brave new world of genetic testing in the management of the dyslipidaemias. *Curr. Opin. Cardiol.* 35, 226–233. doi: 10.1097/HCO.0000000000000721

Nohara, A., Yagi, K., Inazu, A., Kajinami, K., Koizumi, J., and Mabuchi, H. (1995). Absence of familial defective apolipoprotein B-100 in Japanese patients with familial hypercholesterolaemia. *Lancet* 345:1438. doi: 10.1016/s0140-6736(95)92627-5

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur. Heart J.* 34, 3478a–3490a. doi: 10.1093/eurheartj/eht273

Ohta, N., Hori, M., Takahashi, A., Ogura, M., Makino, H., Tamanaha, T., et al. (2016). Proprotein convertase subtilisin/kexin 9 V4I variant with LDLR mutations modifies the phenotype of familial hypercholesterolemia. *J. Clin. Lipidol.* 10, 547–555. doi: 10.1016/j.jacl.2015.12.024

Pang, J., Chan, D. C., Hu, M., Muir, L. A., Kwok, S., Charng, M. J., et al. (2019). Comparative aspects of the care of familial hypercholesterolemia in the "Ten Countries Study". *J. Clin. Lipidol.* 13, 287–300. doi: 10.1016/j.jacl.2019.01.009

Pang, J., Hu, M., Lin, J., Miida, T., Nawawi, H. M., Park, J. E., et al. (2017). An enquiry based on a standardised questionnaire into knowledge, awareness and preferences concerning the care of familial hypercholesterolaemia among

primary care physicians in the Asia-Pacific region: the "Ten Countries Study". *BMJ Open* 7:e017817. doi: 10.1136/bmjopen-2017-017817

Pang, J., Sullivan, D. R., Harada-Shiba, M., Ding, P. Y., Selvey, S., Ali, S., et al. (2015). Significant gaps in awareness of familial hypercholesterolemia among physicians in selected Asia-Pacific countries: a pilot study. *J. Clin. Lipidol.* 9, 42–48. doi: 10.1016/j.jacl.2014.09.011

Paththinige, C. S., Rajapakse, J. R. D. K., Constantine, G. R., Sem, K. P., Singaraja, R. R., Jayasekara, R. W., et al. (2018). Spectrum of low-density lipoprotein receptor (LDLR) mutations in a cohort of Sri Lankan patients with familial hypercholesterolemia - a preliminary report. *Lipids Health Dis.* 17:100. doi: 10.1186/s12944-018-0763-z

Pek, S. L. T., Dissanayake, S., Fong, J. C. W., Lin, M. X., Chan, E. Z. L., Tang, J. I., et al. (2018). Spectrum of mutations in index patients with familial hypercholesterolemia in Singapore: single center study. *Atherosclerosis* 269, 106–116. doi: 10.1016/j.atherosclerosis.2017.12.028

Peng, J., Wu, X., Wang, S., Zhang, S., Wang, X., Liu, Z., et al. (2019). Familial hypercholesterolemia in China half a century: a review of published literature. *Atheroscler. Suppl.* 36, 12–18. doi: 10.1016/j.atherosclerosissup.2019.01.003

Prodan Žitnik, I., Černe, D., Mancini, I., Simi, L., Pazzagli, M., Di Resta, M., et al. (2018). Personalized laboratory medicine: a patient-centered future approach. *Clin. Chem. Lab. Med.* 56, 1981–1991. doi: 10.1515/cclm-2018-0181

Punzalan, F. E., Sy, R. G., Santos, R. S., Cutiongco, E. M., Gosiengfiao, S., Fadriguilan, E., et al. (2005). Low density lipoprotein–receptor (LDL-R) gene mutations among Filipinos with familial hypercholesterolemia. *J. Atheroscler. Thromb.* 12, 276–283. doi: 10.5551/jat.12.276

Setia, N., Movva, S., Balakrishnan, P., Biji, I. K., Sawhney, J. P. S., Puri, R., et al. (2020). Genetic analysis of familial hypercholesterolemia in Asian Indians: a single-center study. *J. Clin. Lipidol.* 14, 35–45. doi: 10.1016/j.jacl.2019.12.010

Shin, D. G., Han, S. M., Kim, D. I., Rhee, M. Y., Lee, B. K., Ahn, Y. K., et al. (2015). Clinical features of familial hypercholesterolemia in Korea: predictors of pathogenic mutations and coronary artery disease - a study supported by the Korean Society of Lipidology and Atherosclerosis. *Atherosclerosis* 243, 53–58. doi: 10.1016/j.atherosclerosis.2015.08.033

Shin, J. A., Kim, S. H., Kim, U. K., Chae, J. J., Choe, S. J., Namkoong, Y., et al. (2000). Identification of four novel mutations of the low-density lipoprotein receptor gene in Korean patients with familial hypercholesterolemia. *Clin. Genet.* 57, 225–229. doi: 10.1034/j.1399-0004.2000.570309.x

Sturm, A. C., Knowles, J. W., Gidding, S. S., Ahmad, Z. S., Ahmed, C. D., Ballantyne, C. M., et al. (2018). Clinical genetic testing for familial hypercholesterolemia: JACC scientific expert panel. *J. Am. Coll. Cardiol.* 72, 662–680. doi: 10.1016/j.jacc.2018.05.044

Sun, D., Zhou, B. Y., Li, S., Sun, N. L., Hua, Q., Wu, S. L., et al. (2018). Genetic basis of index patients with familial hypercholesterolemia in Chinese population: mutation spectrum and genotype-phenotype correlation. *Lipids Health Dis.* 17:252. doi: 10.1186/s12944-018-0900-8

Tan, K., Cheung, C. L., Yeung, C. Y., Siu, D., Leung, J., and Pang, H. K. (2018). Genetic screening for familial hypercholesterolaemia in Hong Kong. *Hong Kong Med. J.* 3, 7–10.

Wang, Y., Li, Y., Liu, X., Tu, R., Zhang, H., Qian, X., et al. (2019). The prevalence and related factors of familial hypercholesterolemia in rural population of China using chinese modified dutch lipid clinic network definition. *BMC Public Health* 19:837. doi: 10.1186/s12889-019-7212-4

Wilemon, K. A., Patel, J., Aguilar-Salinas, C., Ahmed, C. D., Alkhnifsawi, M., Almahmeed, W., et al. (2020). Reducing the clinical and public health burden of familial hypercholesterolemia: a global call to action. *JAMA Cardiol.* [Epub ahead of print] doi: 10.1001/jamacardio.2019.5173,

Yang, K. C., Su, Y. N., Shew, J. Y., Yang, K. Y., Tseng, W. K., Wu, C. C., et al. (2007). LDLR and ApoB are major genetic causes of autosomal dominant hypercholesterolemia in a Taiwanese population. *J. Formos. Med. Assoc.* 106, 799–807. doi: 10.1016/S0929-6646(08)60044-3

Yu, W., Nohara, A., Higashikata, T., Lu, H., Inazu, A., and Mabuchi, H. (2002). Molecular genetic analysis of familial hypercholesterolemia: spectrum and regional difference of LDL receptor gene mutations in Japanese population. *Atherosclerosis* 165, 335–342. doi: 10.1016/s0021-9150(02)00249-6

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Genetics, Screening, and Treatment of Familial Hypercholesterolemia: Experience Gained From the Implementation of the Vietnam Familial Hypercholesterolemia Registry

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Familial hypercholesterolemia (FH) is underdiagnosed and undertreated in a majority of the low- and middle-income countries. FH registries could prove useful in bridging the knowledge gaps, supporting genetic and clinical research, and improving health-care planning and patient care. Here, we report the first usage experience of the Vietnam FH (VINAFH) Registry. The VINAFH Registry was established in 2016 as a long-term database for prospective cohorts. FH patients were detected based on the opportunistic and cascade screening. Diagnosis of FH was assessed using the Dutch Lipid Clinic Network criteria, plasma levels of low-density lipoprotein (LDL) cholesterol, and genetic testing. To date, a total of 130 patients with FH have been registered, with 48 index cases and 82 relatives. Of the 130 patients, 8 were homozygous FH patients and 38 were children. Of FH individuals, 46.7% was confirmed by genetic testing: 61 patients (96.8%) carried the *LDLR* mutation (c.681C > G, c.1427C > G, c.1187-?_2140 ± ?del, c.2529_2530delinsA), and two patients (3.2%) carried the *PCSK9* (protein convertase subtilisin/kexin type 9) mutation (c.42_43insTG). The c.2529_2530delinsA mutation detected in this study is novel and reported only in the Vietnamese population. However, only 53.8% of FH patients were followed up post diagnosis, and only 15.3% of these were approved for lipid-lowering therapy and specialized care. Notably, factors such as knowledge about FH in patients and/or guardians of FH children and support of primary care physicians affected patient participation with respect to treatment strategies and follow-up. Genetic identification, screening, and treatment of FH were feasible in Vietnam. The VINAFH Registry significantly contributed to the formation of the government agencies legislative acts that established the importance of FH as a socially and medically important disease requiring appropriate management strategies. Other low- and middle-income countries could, thus, use the VINAFH Registry model as a reference to establish programs for FH management according to the current status.

Keywords: familial hypercholesterolemia, VINAFH Registry, low- and middle-income country, genetics, screening, treatment

INTRODUCTION

Familial hypercholesterolemia (FH) is a common inherited disorder, affecting one in 250 individuals (de Ferranti et al., 2016; Akioyamen et al., 2017). This disease is characterized by elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) that facilitates the development of atherosclerosis, premature coronary artery disease (CAD), and mortality (Neil et al., 2004; Cuchel et al., 2014). FH involves mutations in the gene encoding LDL receptor (*LDLR*; 90% of reported FH–causing variants), gene encoding apo-lipoprotein B (*APOB*; 5–10%), and, rarely, gene encoding protein convertase subtilisin/kexin type 9 (*PCSK9*; 1%) (Sturm et al., 2018).

Early identification and lipid-lowering therapy play important roles in reducing the health burden for FH. Screening programs such as cascade screening, universal screening, and opportunistic screening are effective for early detection of FH (Kirke et al., 2012). Previously, the Dutch Lipid Clinic Network (DLCN) score was widely accepted for FH diagnosis (Vallejo-Vaz et al., 2018). The European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) recommend that lipid-lowering therapy should be started early and used optimally to achieve LDL-C goals (Mach et al., 2020). However, in reality, majority of FH patients remain underdiagnosed or undertreated. In most countries, less than 1% of FH patients are diagnosed (Nordestgaard et al., 2013). Furthermore, treatment for FH is still limited; for example, the CASCADE-FH Registry reported that only 48% of FH patients achieved LDL-C $<$ 100 mg/dl and 22% achieved LDL-C $<$ 70 mg/dl (Duell et al., 2019).

Because of these challenges, the EAS Familial Hypercholesterolemia Studies Collaboration (EAS-FHSC), the Familial Hypercholesterolemia Foundation, and the World Heart Federation initiated a global call to action for reducing the burden of disease and death due to FH (Vallejo-Vaz et al., 2015; Wilemon et al., 2020). Special attention was given to data registry as an important tool for providing knowledge and support management for FH. In fact, many developed countries have established FH registry. However, only a few low- and middle-income countries have established the same (Bamimore et al., 2015; Mehta et al., 2016; Vallejo-Vaz et al., 2018; Chen et al., 2019). Notably, Vietnam, a large and densely populated country in the Southeast Asia, has spurred rapid economic growth, greater than that of other low- and middle-income countries in the past years. With economic development, Vietnam has also achieved improvement in public health. However, changing lifestyles accompanying the economic growth lead to a double burden of disease; the burden of communicable disease remains, while the burden of non-communicable diseases, such as cardiovascular disease, is increasing (Nguyen and Hoang, 2018). Additionally, there exists a gap in the knowledge about cardiovascular disease, particularly FH. With a population at 97 million, we estimated 500,000 Vietnamese patients with FH, but most of them are underdiagnosed and undertreated (Vallejo-Vaz et al., 2018). This report describes the initial genetic characteristics, clinical characteristics, screening, diagnosis, and treatment of FH patients in Vietnam. We report the experiences gained from the implementation of the Vietnam Familial Hypercholesterolemia (VINAFH) Registry.

MATERIALS AND METHODS

On the basis of the success of our previous preliminary small-scope research on FH (Truong et al., 2018), we extended the study and established the VINAFH Registry as a long-term prospective cohort promoted by the Vietnam National Heart Institute (VNHI), Bach Mai Hospital – the largest hospital for cardiovascular disease in North Vietnam – and the national referral cardiovascular hospital. Implementation of the VINAFH Registry is presented in **Figure 1**. The VINAFH Registry was approved by the Council for Science of the VNHI, Bach Mai Hospital (No. 183/VTM-BVBM) and the Council for Science of the Ministry of Science and Technology of Vietnam (No. 828/GXNDGTD-BKHCN). The VINAFH Registry has the following three specific objectives: (1) to identify and enroll heterozygous and homozygous FH individuals in Vietnam; (2) to understand the clinical and genetic characteristics of FH individuals in Vietnam; and (3) to improve the management strategies for FH in Vietnam. Recruitment of FH individuals began in 2016 and is still ongoing. All the FH individuals included in this study provided informed consent. FH individuals younger than 18 years were enrolled only with the explicit consent of a parent or legal guardian.

Inclusion Criteria for Familial Hypercholesterolemia Individuals in the Vietnam Familial Hypercholesterolemia Registry

We enrolled phenotypic and/or genotypic FH individuals, including index cases and their relatives in the Registry. Index case was detected by opportunistic screening in patients with premature CAD and/or hypercholesterolemia. Cascade screening was performed to detect FH relatives of index case, as previously described (Truong et al., 2018).

Phenotypic Familial Hypercholesterolemia Individuals

In adults, phenotypic FH was confirmed based on the DLCN criteria (WHO Human Genetics Programme, 1998). Patients with DLCN scores of 3–5 (possible FH), 6–8 (probable FH), and $>$8 (definite FH) were enrolled. In case of children $<$ 18 years of age, phenotypic FH was diagnosed based on the presence of an LDL-C level consistent with FH in addition to a family history of premature CAD, and/or baseline high cholesterol in one parent, and/or presence of an FH-causing mutation (Wiegman et al., 2015). We also enrolled the likely FH phenotype relatives based on age- and gender-specific LDL-C cutoffs as described by Starr et al. (2008).

Genotypic Familial Hypercholesterolemia Individuals

Mutations in index cases were detected by massively parallel sequencing of FH genes (*LDLR*, *APOB*, and *PCSK9*) and multiplex ligation-dependent probe amplification (MLPA) of *LDLR*, as previously described (Hooper et al., 2012). Depending

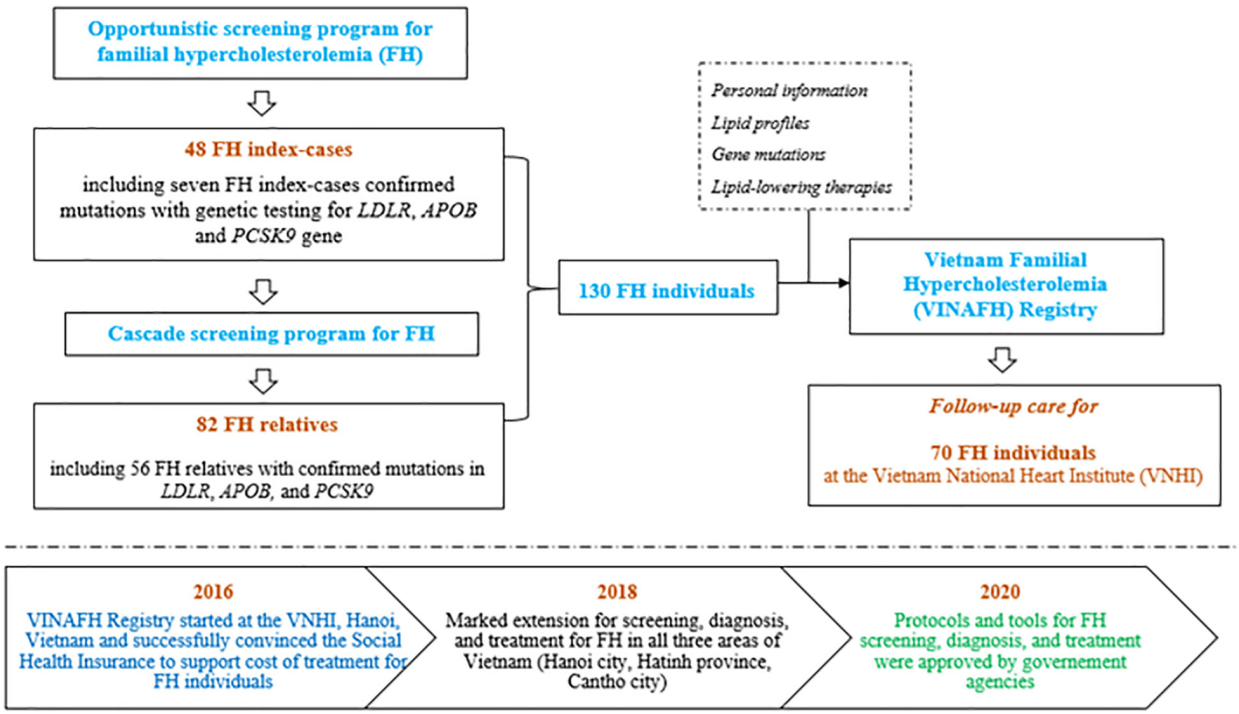

FIGURE 1 | Flow diagram of implementation strategy of the Vietnam Familial Hypercholesterolemia (VINAFH) Registry.

on the mutation present in the index case, genetic testing for relatives was performed by Sanger sequencing of the exon containing the family mutation, or by MLPA of *LDLR*. The ClinVar database, PolyPhen2, and MutationTaster were used to confirm the mutations. Genotypic FH individuals were classified as homozygous for FH (HoFH), carrying the same mutations in both alleles of FH genes, and heterozygous for FH (HeFH), carrying only one mutation in the alleles of FH genes.

Exclusion Criteria for Familial Hypercholesterolemia Individuals in the Vietnam Familial Hypercholesterolemia Registry

Individuals with known medical conditions other than FH that contribute to hyperlipidemia, such as hypothyroidism, nephrotic syndrome, cholestasis, and hypopituitarism, were excluded from the VINAFH Registry. Medical, family, and clinical history were recorded for all registered individuals: characteristics based on the DLCN criteria (WHO Human Genetics Programme, 1998), lipid profiles, and mutation characteristics. Furthermore, information regarding risk factors for cardiovascular disease, including age, sex, smoking, diabetes, hypertension, obesity, premature CAD, and ongoing/past lipid-lowering therapies, were also collected in this Registry.

Outcome Measures

Primary outcomes:

- Number of FH individuals identified.
- Genetic testing for FH-causing mutations (*LDLR*, *PCSK9*, and *APOB*).

Secondary outcomes:

- Number of FH individuals who received dietary and lipid-lowering therapy.
- Number of FH individuals who achieved LDL-C targets.
- Incidence of cardiovascular events (coronary, cerebral, or peripheral vascular diseases) and cardiovascular cause detected during annual follow-up.
- Contribution of the Registry in the formation of national health policies concerning the recognition of FH as an important disease with development of appropriate management strategies.

Statistical Analyses

Continuous variables are expressed as the arithmetic mean and standard deviation if normally distributed and as the median with inter-quartile range for non-normal distribution. Categorical variables are expressed as count and percentage. Normally distributed continuous variables between two groups were compared using the Student *t*-test for independent samples. Non-normally distributed continuous variables were compared using the Mann–Whitney *U* test. Proportions were compared by using the chi-squared test with continuity correction or Fisher's exact test, when appropriate. The analyses were performed with IBM SPSS 22.0 software. The results with *p* values below 0.05 were considered statistically significant.

RESULTS

Genetic Identification of Familial Hypercholesterolemia Individuals

A total of 130 FH individuals have been registered. Of these, 63 FH individuals (48.5%) underwent genetic testing, confirming the presence of mutations in the genes studied (*LDLR*, *APOB*, and *PCSK9*); eight individuals were HoFH and 55 were HeFH. Four *LDLR* mutations and one *PCSK9* mutation were identified in these individuals, while no pathogenic variants of *APOB* were identified. Mutations in *LDLR* were identified in 6/7 index cases (85.7%) in this Registry. We found novel mutations in *LDLR*, which has not been reported but are annotated in the ClinVar database: c.2529_2530delinsA, (p.Asp843Glufs*86). The variants and their distribution in the study cohort are given in **Table 1**.

Screening of Familial Hypercholesterolemia Individuals

We identified 48 FH index cases and 82 FH relatives. Clinical characteristics of FH individuals are given in **Table 2**. Notably, the mean ± standard deviation of age for FH diagnosis was 34.8 ± 1.95 years, with an earlier age at diagnosis in the relative group compared with that in the index-case group (28.6 ± 2.43 vs. 45.3 ± 2.66 years, respectively, $p < 0.001$).

Treatment of Familial Hypercholesterolemia Individuals

Post diagnosis, 53.8% (n = 70/130) of FH individuals continued to follow-up at the VNHI. All individuals were given standard treatment with dietary supplements containing plant stanols for controlling cardiovascular risks. Only 15.3% (n = 11/70) were given lipid-lowering therapy described in **Table 3**. Of these 11 individuals, five were HoFH while six were HeFH. After treatment with lipid-lowering therapies for 1 year, 83.3% (n = 5/6) of HeFH patients had LDL-C < 2.5 mmol/L, while the mean of plasma LDL-C of HoFH patients reduced from 17.5 ± 6.0 mmol/L at the time of diagnosis to 10.2 ± 4.1 mmol/L. No new cardiovascular events or mortalities were observed in these individuals. The lower incidence of lipid-lowering therapy was attributed to patient refusal. We recorded two important reasons for patient refusal to lipid-lowering therapy in case of individuals in the VINAFH Registry: lack of knowledge about the effects and side effects of lipid-lowering therapy and barriers due to treatment cost. Besides, FH individuals only agreed to lipid-lowering therapy after we contacted their primary care physicians and successfully convinced these physicians to join as collaborators in our network for FH management.

Impact of the Vietnam Familial Hypercholesterolemia Registry on Health Policy

On the basis of experiences gained from the implementation the VINAFH Registry, we created academic documents, including (1) screening and testing (diagnostic and genetic) protocols for FH; (2) guidance on genetic counseling for FH; (3) tools for FH screening, diagnosis, and treatment; and (4) management model for FH. All of them were evaluated, appraised, and approved for clinical practice in Vietnam by the Ministry of Science and Technology of Vietnam. Further, we successfully convinced the Social Health Insurance to cover treatment cost for FH individuals who participated in the VINAFH Registry and were followed up at the VNHI.

DISCUSSION

In our Registry, we registered a higher rate of FH individuals with confirmed mutations (48.5%, n = 63/130) than did few other countries (Nordestgaard and Benn, 2017), based on the support of national genetic experts and colleagues from Australia. We identified four different mutations in *LDLR* and one in *PCSK9*. The majority of FH individuals carried mutation in *LDLR* (85.7% of index cases and 96.8% in total), which is similar to that reported in previous studies (Soutar and Naoumova, 2007; Sjouke et al., 2015; Khera et al., 2016). We found that the exon mutations of *LDLR* were varied, which was also reported by a previous study in a Korean population (Lee, 2016). *LDLR* p.Asp227Glu (FH Afrikaner-1, FH Maine) missense variant, which occurs within repeat 5 of the ligand-binding domain of LDLR, has previously been identified (Kotze et al., 1993; Hooper et al., 2012; Sharifi et al., 2016). *LDLR* c.1187-?_2140 + ?del and *PCSK9* c.42_43insTG have also been identified in several cohorts of FH patients (Goldmann et al., 2010; Clinvar database, 2020). Notably, two *LDLR* mutations (p.Pro476Arg and p.Asp843Glufs*86) that we identified in Vietnamese patients have not been recorded in other ethnicities as confirmed by the Clinvar database (2020). We previously reported the identification of *LDLR* p.Pro476Arg missense variant, which occurs within the gene encoding the EGF spacer domain of the LDLR, in two Vietnamese families with two HoFH index cases and 11 HeFH relatives (Truong et al., 2018). Pro476 is conserved across species, and prediction algorithms PolyPhen2 and MutationTaster suggest that Pro476Arg is pathogenic. In the VINAFH Registry cohort, we identified the novel mutation, *LDLR* p.Asp843Glufs*86 frameshift variant, which occurs within the gene encoding the cytoplasmic domain of the LDLR; it was detected in a family with nine HeFH individuals. This frameshift variant results in the loss of 18 amino acids and addition of 86 amino acids at the C-terminal of the LDLR. In general, simultaneous occurrence of both the previously reported variants and the novel variant detected in this study suggest a broad spectrum of mutations and high heterogeneity of FH in the Vietnamese population, which is similar to that observed in other countries (Jiang et al., 2015; Fairoozy et al., 2017).

Massively parallel sequencing detects structural variations with high sensitivity and specificity. However, it is inefficient to detect large deletions/duplications. In contrast, MLPA is highly sensitive to detect these mutations. A previous report showed that in 19/377 (5%) patients with suspected FH, no mutation was found with massively parallel sequencing, whereas MLPA identified large deletions/duplications in *LDLR* (Taylor et al., 2009). Thus, besides combined massively parallel sequencing,

TABLE 1 | *LDLR* and *PCSK9* variants identified in the VINAFH Registry.

Gene	Exon	Variant	Predicted effect	Number of index case (*n* = 7)	Number of relative (*n* = 56)	Number of HoFH (*n* = 8)	Number of HeFH (*n* = 55)
LDLR	E.4	c.681C > G	p.Asp227Glu	2 (28.6%)	16 (28.5%)	2 (25%)	16 (29.1%)
LDLR	E.10	c.1427C > G	p.Pro476Arg	2 (28.6%)	11 (19.6%)	2 (25%)	11 (20%)
LDLR	E.9–E.14	c.1187-?_2140 ± ?del	–	1 (14.3%)	20 (35.7%)	4 (50%)	17 (30.1%)
*LDLR**	E.17	c.2529_2530delinsA	p.Asp843Glufs*86	1 (14.3%)	8 (14.3%)	0	9 (16.4%)
PCSK9	-	c.42_43insTG	p.Leu15fs	1 (14.3%)	1 (1.8%)	0	2 (3.6%)

*LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; FH, familial hypercholesterolemia; HoFH, homozygous familial hypercholesterolemia; HeFH, heterozygous familial hypercholesterolemia; VINAFH, Vietnam Familial Hypercholesterolemia. *Novel mutation that has not previously been reported and is absent from the ClinVar database.*

TABLE 2 | Baseline characteristics of FH individuals in the VINAFH Registry.

Characteristics	Total (*n* = 130)	Index cases FH (*n* = 48)	Relatives FH (*n* = 82)	*p* value
Age, year	34.8 ± 1.95 (30.9–38.6)	45.3 ± 2.66 (40.0–50.7)	28.6 ± 2.43 (23.8–33.4)	<0.001
Children less than 18 years old, *n* (%)	41 (31.5%)	5 (10.4%)	36 (43.6%)	<0.001
Male, *n* (%)	73 (56.2%)	34 (70.8%)	39 (47.6%)	0.01
PCAD, *n* (%)	33 (25.4%)	31 (64.6%)	2 (2.4%)	<0.001
Smoking, *n* (%)	29 (22.3%)	20 (41.7%)	9 (11%)	<0.001
Hypertension, *n* (%)	20 (15.4%)	10 (20.8%)	10 (12.2%)	0.188
Diabetes, *n* (%)	2 (1.5%)	0 (0%)	2 (2.4%)	0.531
Obesity, *n* (%)	18 (13.8%)	8 (16.7%)	10 (12.2%)	0.476
Xanthomas, *n* (%)	23 (17.7%)	16 (33.3%)	7 (8.5%)	<0.001
Arcus cornealis, *n* (%)	17 (13.1%)	13 (27.1%)	4 (4.9%)	0.001
TC, mmol/L	8.43 ± 0.28 (7.88–8.98)	9.83 ± 0.56 (8.69–10.96)	7.6 ± 0.25 (7.11–8.09)	<0.001
LDL-C, mmol/L	6.48 ± 0.26 (5.96–7.0)	7.63 ± 0.55 (6.52–8.74)	5.8 ± 0.23 (5.34–6.25)	0.001
HDL-C, mmol/L	1.25 ± 0.28 (1.2–1.31)	1.21 ± 0.05 (1.11–1.31)	1.28 ± 0.03 (1.21–1.35)	0.24
Triglyceride, mmol/L	1.87 ± 0.12 (1.62–2.11)	2.23 ± 0.24 (1.74–2.71)	1.66 ± 0.13 (1.39–1.92)	0.027

PCAD, premature coronary artery disease (≤55 years in men, ≤60 years in women); TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FH, familial hypercholesterolemia; VINAFH, Vietnam Familial Hypercholesterolemia.

TABLE 3 | Lipid-lowering therapy in 11 FH individuals.

Therapy	All (*n* = 11)	HoFH (*n* = 5)	HeFH (*n* = 6)
Statins (*n*,%)	11 (100%)	5 (100%)	6 (100%)
High-intensity statins* (*n*,%)	7 (63.6%)	5 (100%)	2 (33.3%)
Ezetimibe	4 (36.4%)	3 (60%)	1 (16.7%)
Plant stanols	3 (27.3%)	3 (60%)	0
Plasma exchange	2 (18.2%)	2 (40%)	0
Lipoprotein apheresis	0	0	0
PCSK9 inhibitors	0	0	0

*PCSK9, proprotein convertase subtilisin/kexin type 9; HoFH, homozygous familial hypercholesterolemia; HeFH, heterozygous familial hypercholesterolemia; FH, familial hypercholesterolemia. *Atorvastatin 40 or 80 mg and rosuvastatin 20 mg were defined as high-intensity statins.*

the MLPA method is useful for genetic testing in FH patients. Interestingly, our first index case with HoFH phenotype was also tested using massively parallel sequencing with targeted analysis of FH genes, but no mutations were detected. Therefore, the MLPA method was used to confirm this index case, and homozygotes for a deletion of exons 9 to 14 of *LDLR* were found. We also detected three HoFH relatives and 17 HeFH relatives carrying this mutation (Truong et al., 2018).

Screening is the first step to improve status of underdiagnosed and undertreated FH cases. In the VINAFH Registry, we combined opportunistic and cascade screening to increase the likelihood of detection. Opportunistic screening was the approach used to detect FH index cases in high-risk individuals such as those with premature CAD, hypercholesterolemia, xanthomas, and arcus cornealis (Nordestgaard et al., 2013). This approach was selected based on a high prevalence of FH in patients with premature CAD (Nanchen et al., 2015; Faggiano et al., 2018; Pirazzi et al., 2019). Elevated plasma total cholesterol and LDL-C levels are important clinical signs of FH. A previous study reported that 1.7% of individuals with LDL-C ≥ 190 mg/dl carried FH mutation (Khera et al., 2016). Moreover, xanthomas and arcus cornealis are also key signs of FH, especially in homozygotes (Bujo et al., 2004). Compared with previous preliminary small-scope report, including the first five FH index cases in Vietnam (Truong et al., 2018), the VINAFH Registry has significantly expanded the number of FH individuals, including 48 index cases detected by opportunistic screening. This provided evidence for the effectiveness of

opportunistic screening, especially in developing countries where FH is underdiagnosed.

Because FH is dominantly inherited, each new FH case could become an index case for cascade screening. Indeed, this is a highly effective method for detecting FH in family members and has been approved in many countries (Bell et al., 2015; Jannes et al., 2015; Rubio-Marin et al., 2018). In fact, we undertook cascade screening for FH in close relatives of index cases and detected 82 FH cases. On average, 14 new cases of FH were detected per HoFH index case (Truong et al., 2018). The VINAFH Registry revealed that FH relatives had a younger mean for age than FH index cases. Interestingly, 43.6% of FH relatives were children < 18 years old. Fortunately, only 2.4% of FH relatives had a history of premature CAD, which was lower than the FH index cases (64.6%). Early diagnosis and, thus, prevention or delaying the onset of atherosclerotic cardiovascular disease are the most important factors in the management of FH (Nordestgaard et al., 2013; Knowles et al., 2017; Mach et al., 2020).

The VINAFH Registry has also shown a high prevalence of FH individuals with cardiovascular risk factors including smoking, hypertension, and obesity. Like low- and middle-income countries, Vietnam also faces the increasing burden of non-communicable diseases (Bennett et al., 2018). In a national survey of risk factors for non-communicable diseases in 2015, prevalence of smoking, hypertension, and overweight/obesity was 55.7, 18.5, and 21.1%, respectively, in men and 1.73, 10.2, and 21.2%, respectively, in women (Bui et al., 2016). In FH management, controlling cardiovascular risk factors has been reported to be highly beneficial for reducing cardiovascular events and mortality (Akioyamen et al., 2019). Therefore, the prevention program for FH in Vietnam will focus on lifestyle modification education.

Considerable efforts have been taken for the management of FH in Vietnam, and 53.8% of FH individuals continued to follow-up post diagnosis. FH individuals were also educated in lifestyle modification. However, a large number of Vietnamese FH individuals were undertreated, which is also commonly observed in many countries (Nordestgaard et al., 2013). The VINAFH Registry showed that traditional lipid-lowering therapies, including statin, ezetimibe, plant stanols, and plasma exchange, were effective for reducing plasma LDL-C levels in FH individuals. As per the ESC/EAS guidelines, statin is recommended as the first-line therapy for FH, and high-intensity and maximal potent statin doses are preferred (Nordestgaard et al., 2013; Cuchel et al., 2014; Besseling et al., 2016; Mach et al., 2020). However, in the VINAFH Registry, prevalence of HeFH individuals prescribed with high-intensity statins was limited (33.3%), similar to previous reports in China (Auckle et al., 2017). In Japan, which is a developed country, only 19.2% of FH individuals were treated with high-intensity statins (Teramoto et al., 2018). Lipoprotein apheresis and PCSK9 inhibitors, which are currently presented as efficient methods of treatment of FH (Mach et al., 2020), are still not available in Vietnam. A previous survey by the EAS-FHSC showed that these lipid-lowering therapies are limited in most countries (Vallejo-Vaz et al., 2018).

Limited use of lipid-lowering therapies for FH individuals could be explained by the lack of knowledge and awareness about the disease. Our interviews with FH individuals or legal guardians of FH children noted confusion about the effects and side effects of long-term drug use. It should be noted that in the health-care system, patient-primary care physician relationship is of the utmost importance; everybody highly trusts their physician (O'Malley et al., 2004). Primary care physicians and cardiologists generally advice the patients regarding health and prescribe drugs on the basis of their knowledge. Therefore, updating the physicians' knowledge about FH is significant to obtain consent for the patient's treatment. However, Vietnamese physicians had a large deficit in FH knowledge and awareness (Pang et al., 2017). This emphasizes the critical importance of implementing education and awareness programs for both FH individuals and physicians. Ideally, physicians treating FH individual should collaborate for FH management.

As mentioned, many low- and middle-income countries suffer from a double disease burden, the backlog of common infections, and the emerging challenges of non-communicable diseases; moreover, their health resources are also limited. If the relevant information is not available to the government agencies, they may omit important diseases, such as FH, from the national health policy. Therefore, scientists play important roles for providing evidence accumulated though such registries and persuade government agencies to adjust the health policies. In our case, we conducted a preliminary small-scope research for FH, then extended it through the VINAFH Registry, and reported the updated results about FH status in Vietnamese individuals to the government agencies. Simultaneously, we convinced the Social Health Insurance to support cost of treatment for the registered FH individuals. In our experience, initial results of such a registry should provide data and documentation regarding evidence of the existence of the disease pathology, initial results of FH management, and protocols and tools for screening, diagnosis, and treatment of FH. Notably, including genetic information into the registry provides high-value scientific evidence that is a useful factor that increases the persuasiveness of the study for government agencies. Thus, the VINAFH Registry has significantly contributed to the formation of government agencies legislative acts, establishing FH as a socially and medically important disease with appropriate management strategies. It has also led to the deployment of a national screening and disease management program for FH in Vietnam in the future.

CONCLUSION

In conclusion, the VINAFH Registry is the first database on genetic screening and management of FH in the Vietnamese population. Moreover, we reported a novel variant in *LDLR* that were identified in our cohort. The likely occurrence of a complex of FH mutations suggests the need for a national FH genetic study. Based on the findings of this study with

respect to the treatment strategies for Vietnamese FH patients, we propose the need for awareness and educational programs about FH for patients and doctors, so as to increase the number of diagnosed and treated patients. The VINAFH Registry had an important contribution in the formation of government agencies legislative acts concerning the establishment of FH as a socially and medically important disease and development of appropriate management strategies. Low- and middle-income countries might refer to our Registry to establish similar programs for the management of FH on the basis of genetic testing combined with opportunistic and cascade screening. The management strategies for FH should be implemented in a step-by-step manner on the basis of the personal and financial resources available in these countries.

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 authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Council for Science of the VNHI, Bach Mai Hospital (No. 183/VTM-BVBM) and the Council for Science of the Ministry of Science and Technology of Vietnam (No. 828/GXNDGTD-BKHCN). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

T-HT and D-LD initiated the study, designed data collection tools, monitored data collection, cleaned and analyzed the data, and drafted and revised the manuscript. N-TK and M-NN monitored data collection, cleaned and analyzed the data, and revised the draft manuscript. T-TL and H-AL monitored data collection and revised the draft manuscript. All authors read and approved the final manuscript.

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REFERENCES

Akioyamen, L. E., Genest, J., Chu, A., Inibhunu, H., Ko, D. T., and Tu, J. V. (2019). Risk factors for cardiovascular disease in heterozygous familial hypercholesterolemia: a systematic review and meta-analysis. *J. Clin. Lipidol.* 13, 15–30. doi: 10.1016/j.jacl.2018.10.012

Akioyamen, L. E., Genest, J., Shan, S. D., Reel, R. L., Albaum, J. M., Chu, A., et al. (2017). Estimating the prevalence of heterozygous familial hypercholesterolaemia: a systematic review and meta-analysis. *BMJ Open* 7:e016461. doi: 10.1136/bmjopen-2017-016461

Auckle, R., Su, B., Li, H., Xu, S., Xie, M., Song, Y., et al. (2017). Familial hypercholesterolemia in Chinese patients with premature ST-segment-elevation myocardial infarction: prevalence, lipid management and 1-year follow-up. *PLoS One* 12:e0186815. doi: 10.1371/journal.pone.0186815

Bamimore, M. A., Zaid, A., Banerjee, Y., Al-Sarraf, A., Abifadel, M., Seidah, N. G., et al. (2015). Familial hypercholesterolemia mutations in the Middle Eastern and North African region: a need for a national registry. *J. Clin. Lipidol.* 9, 187–194. doi: 10.1016/j.jacl.2014.11.008

Bell, D. A., Pang, J., Burrows, S., Bates, T. R., van Bockxmeer, F. M., Hooper, A. J., et al. (2015). Effectiveness of genetic cascade screening for familial hypercholesterolaemia using a centrally co-ordinated clinical service: an Australian experience. *Atherosclerosis* 239, 93–100. doi: 10.1016/j.atherosclerosis.2014.12.036

Bennett, J. E., Stevens, G. A., Mathers, C. D., Bonita, R., Rehm, J., Kruk, M. E., et al. (2018). NCD Countdown 2030: worldwide trends in non-communicable disease mortality and progress towards sustainable development goal target 3.4. *Lancet* 392, 1072–1088. doi: 10.1016/S0140-6736(18)31992-5

Besseling, J., Hovingh, G. K., Huijgen, R., Kastelein, J. J. P., and Hutten, B. A. (2016). Statins in familial hypercholesterolemia: consequences for coronary artery disease and all-cause mortality. *J. the Am. Coll. Cardiol.* 68, 252–260. doi: 10.1016/j.jacc.2016.04.054

Bui, T. V., Blizzard, C. L., Luong, K. N., Truong Nle, V., Tran, B. Q., Otahal, P., et al. (2016). National survey of risk factors for non-communicable disease in Vietnam: prevalence estimates and an assessment of their validity. *BMC Public Health* 16:498. doi: 10.1186/s12889-016-3160-4

Bujo, H., Takahashi, K., Saito, Y., Maruyama, T., Yamashita, S., Matsuzawa, Y., et al. (2004). Clinical features of familial hypercholesterolemia in Japan in a database from 1996-1998 by the research committee of the ministry of health, labour and welfare of Japan. *J. Atheroscler. Thromb.* 11, 146–151. doi: 10.5551/jat.11.146

Chen, P., Chen, X., and Zhang, S. (2019). Current status of familial hypercholesterolemia in China: a need for patient FH registry systems. *Front. Physiol.* 10:280. doi: 10.3389/fphys.2019.00280

Clinvar database (2020). Available online at: https://www.ncbi.nlm.nih.gov/clinvar/ (accessed March 31, 2020).

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014). Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the consensus panel on familial hypercholesterolaemia of the european atherosclerosis society. *Eur. Heart J.* 35, 2146–2157. doi: 10.1093/eurheartj/ehu274

de Ferranti, S. D., Rodday, A. M., Mendelson, M. M., Wong, J. B., Leslie, L. K., and Sheldrick, R. C. (2016). Prevalence of familial hypercholesterolemia in the 1999 to 2012 United States national health and nutrition examination surveys (NHANES). *Circulation* 133, 1067–1072. doi: 10.1161/circulationaha.115.018791

Duell, P. B., Gidding, S. S., Andersen, R. L., Knickelbine, T., Anderson, L., Gianos, E., et al. (2019). Longitudinal low density lipoprotein cholesterol goal achievement and cardiovascular outcomes among adult patients with familial hypercholesterolemia: the CASCADE FH registry. *Atherosclerosis* 289, 85–93. doi: 10.1016/j.atherosclerosis.2019.08.007

Faggiano, P., Pirillo, A., Griffo, R., Ambrosetti, M., Pedretti, R., Scorcu, G., et al. (2018). Prevalence and management of familial hypercholesterolemia in patients with coronary artery disease: the heredity survey. *Intern. J. Cardiol.* 252, 193–198. doi: 10.1016/j.ijcard.2017.10.105

Fairoozy, R. H., Futema, M., Vakili, R., Abbaszadegan, M. R., Hosseini, S., Aminzadeh, M., et al. (2017). The genetic spectrum of familial hypercholesterolemia (FH) in the Iranian population. *Sci. Rep.* 7:17087. doi: 10.1038/s41598-017-17181-9

Goldmann, R., Tichy, L., Freiberger, T., Zapletalova, P., Letocha, O., Soska, V., et al. (2010). Genomic characterization of large rearrangements of the LDLR gene in Czech patients with familial hypercholesterolemia. *BMC Med. Genet.* 11:115. doi: 10.1186/1471-2350-11-115

Hooper, A. J., Nguyen, L. T., Burnett, J. R., Bates, T. R., Bell, D. A., Redgrave, T. G., et al. (2012). Genetic analysis of familial hypercholesterolaemia in Western Australia. *Atherosclerosis* 224, 430–434. doi: 10.1016/j.atherosclerosis.2012.07.030

Jannes, C. E., Santos, R. D., de Souza Silva, P. R., Turolla, L., Gagliardi, A. C., Marsiglia, J. D., et al. (2015). Familial hypercholesterolemia in Brazil: cascade screening program, clinical and genetic aspects. *Atherosclerosis* 238, 101–107. doi: 10.1016/j.atherosclerosis.2014.11.009

Jiang, L., Sun, L. Y., Dai, Y. F., Yang, S. W., Zhang, F., and Wang, L. Y. (2015). The distribution and characteristics of LDL receptor mutations in China: a systematic review. *Sci. Rep.* 5:17272. doi: 10.1038/srep17272

Khera, A. V., Won, H. H., Peloso, G. M., Lawson, K. S., Bartz, T. M., Deng, X., et al. (2016). Diagnostic yield and clinical utility of sequencing familial hypercholesterolemia genes in patients with severe hypercholesterolemia. *J. Am. Coll. Cardiol.* 67, 2578–2589. doi: 10.1016/j.jacc.2016.03.520

Kirke, A., Watts, G. F., and Emery, J. (2012). Detecting familial hypercholesterolaemia in general practice. *Austr. Fam. Phys.* 41, 965–968.

Knowles, J. W., Rader, D. J., and Khoury, M. J. (2017). Cascade screening for familial hypercholesterolemia and the use of genetic testing. *JAMA* 318, 381–382. doi: 10.1001/jama.2017.8543

Kotze, M. J., De Villiers, W. J., Steyn, K., Kriek, J. A., Marais, A. D., Langenhoven, E., et al. (1993). Phenotypic variation among familial hypercholesterolemics heterozygous for either one of two Afrikaner founder LDL receptor mutations. *Arterioscler. Thromb.* 13, 1460–1468. doi: 10.1161/01.atv.13.10.1460

Lee, S. H. (2016). Characteristics and vascular complications of familial hypercholesterolemia in Korea. *J. Atheroscler. Thromb.* 23, 532–538. doi: 10.5551/jat.34363

Mach, F., Baigent, C., Catapano, A. L., Koskinas, K. C., Casula, M., Badimon, L., et al. (2020). 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur. Heart J.* 41, 111–188. doi: 10.1093/eurheartj/ehz455

Mehta, R., Zubiran, R., Martagon, A. J., Vazquez-Cardenas, A., Segura-Kato, Y., Tusie-Luna, M. T., et al. (2016). The panorama of familial hypercholesterolemia in Latin America: a systematic review. *J. Lipid Res.* 57, 2115–2129. doi: 10.1194/jlr.R072231

Nanchen, D., Gencer, B., Auer, R., Raber, L., Stefanini, G. G., Klingenberg, R., et al. (2015). Prevalence and management of familial hypercholesterolaemia in patients with acute coronary syndromes. *Eur. Heart J.* 36, 2438–2445. doi: 10.1093/eurheartj/ehv289

Neil, H. A., Seagroatt, V., Betteridge, D. J., Cooper, M. P., Durrington, P. N., Miller, J. P., et al. (2004). Established and emerging coronary risk factors in patients with heterozygous familial hypercholesterolaemia. *Heart* 90, 1431–1437. doi: 10.1136/hrt.2003.022764

Nguyen, T. T., and Hoang, M. V. (2018). Non-communicable diseases, food and nutrition in Vietnam from 1975 to 2015: the burden and national response. *Asia Pac. J. Clin. Nutrit.* 27, 19–28. doi: 10.6133/apjcn.032017.13

Nordestgaard, B. G., and Benn, M. (2017). Genetic testing for familial hypercholesterolaemia is essential in individuals with high LDL cholesterol: who does it in the world? *Eur. Heart J.* 38, 1580–1583. doi: 10.1093/eurheartj/ehx136

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the european atherosclerosis society. *Eur. Heart J.* 34, 3478–3490. doi: 10.1093/eurheartj/eht273

O'Malley, A. S., Sheppard, V. B., Schwartz, M., and Mandelblatt, J. (2004). The role of trust in use of preventive services among low-income African-American women. *Prevent. Med.* 38, 777–785. doi: 10.1016/j.ypmed.2004.01.018

Pang, J., Hu, M., Lin, J., Miida, T., Nawawi, H. M., Park, J. E., et al. (2017). An enquiry based on a standardised questionnaire into knowledge, awareness and preferences concerning the care of familial hypercholesterolaemia among primary care physicians in the Asia-Pacific region: the ten countries Study. *BMJ Open* 7:e017817. doi: 10.1136/bmjopen-2017-017817

Pirazzi, C., Håkansson, L., Gustafsson, C., Omerovic, E., Wiklund, O., and Mancina, R. M. (2019). High prevalence of genetic determined familial hypercholesterolemia in premature coronary artery disease. *Appl. Clin. Genet.* 12, 71–78. doi: 10.2147/TACG.S202942

Rubio-Marin, P., Michan-Dona, A., Maraver-Delgado, J., Arroyo-Olivares, R., Barrado Varea, R., Perez de Isla, L., et al. (2018). Cascade screening program for familial hypercholesterolemia. *Endocrinol. Diabetes Nutr.* 65, 280–286. doi: 10.1016/j.endinu.2017.12.009

Sharifi, M., Walus-Miarka, M., Idzior-Walus, B., Malecki, M. T., Sanak, M., Whittall, R., et al. (2016). The genetic spectrum of familial hypercholesterolemia in south-eastern Poland. *Metab. Clin. Exper.* 65, 48–53. doi: 10.1016/j.metabol.2015.10.018

Sjouke, B., Kusters, D. M., Kindt, I., Besseling, J., Defesche, J. C., Sijbrands, E. J., et al. (2015). Homozygous autosomal dominant hypercholesterolaemia in the Netherlands: prevalence, genotype-phenotype relationship, and clinical outcome. *Eur. Heart J.* 36, 560–565. doi: 10.1093/eurheartj/ehu058

Soutar, A. K., and Naoumova, R. P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat. Clin. Pract. Cardiovasc. Med.* 4, 214–225. doi: 10.1038/ncpcardio0836

Starr, B., Hadfield, S. G., Hutten, B. A., Lansberg, P. J., Leren, T. P., Damgaard, D., et al. (2008). Development of sensitive and specific age- and gender-specific low-density lipoprotein cholesterol cutoffs for diagnosis of first-degree relatives with familial hypercholesterolaemia in cascade testing. *Clin. Chem. Lab. Med.* 46, 791–803. doi: 10.1515/cclm.2008.135

Sturm, A. C., Knowles, J. W., Gidding, S. S., Ahmad, Z. S., Ahmed, C. D., Ballantyne, C. M., et al. (2018). Clinical genetic testing for familial hypercholesterolemia: JACC scientific expert panel. *J. Am. Coll. Cardiol.* 72, 662–680. doi: 10.1016/j.jacc.2018.05.044

Taylor, A., Martin, B., Wang, D., Patel, K., Humphries, S. E., and Norbury, G. (2009). Multiplex ligation-dependent probe amplification analysis to screen for deletions and duplications of the LDLR gene in patients with familial hypercholesterolaemia. *Clin. Genet.* 76, 69–75. doi: 10.1111/j.1399-0004.2009.01168.x

Teramoto, T., Kai, T., Ozaki, A., Crawford, B., Arai, H., and Yamashita, S. (2018). Treatment patterns and lipid profile in patients with familial hypercholesterolemia in Japan. *J. Atheroscler. Thromb.* 25, 580–592. doi: 10.5551/jat.41483

Truong, T. H., Kim, N. T., Nguyen, M. N. T., Pang, J., Hooper, A. J., Watts, G. F., et al. (2018). Homozygous familial hypercholesterolaemia in Vietnam: case series, genetics and cascade testing of families. *Atherosclerosis* 277, 392–398. doi: 10.1016/j.atherosclerosis.2018.06.013

Vallejo-Vaz, A. J., De Marco, M., Stevens, C. A. T., Akram, A., Freiberger, T., Hovingh, G. K., et al. (2018). Overview of the current status of familial hypercholesterolaemia care in over 60 countries - the EAS familial hypercholesterolaemia studies collaboration (FHSC). *Atherosclerosis* 277, 234–255. doi: 10.1016/j.atherosclerosis.2018.08.051

Vallejo-Vaz, A. J., Kondapally Seshasai, S. R., Cole, D., Hovingh, G. K., Kastelein, J. J., Mata, P., et al. (2015). Familial hypercholesterolaemia: a global call to arms. *Atherosclerosis* 243, 257–259. doi: 10.1016/j.atherosclerosis.2015.09.021

WHO Human Genetics Programme (1998). *Familial Hypercholesterolaemia (ıı ıFH)ıı ı : Report of a Second WHO Consultation*. Geneva: WHO Human Genetics Programme.

Wiegman, A., Gidding, S. S., Watts, G. F., Chapman, M. J., Ginsberg, H. N., Cuchel, M., et al. (2015). Familial hypercholesterolaemia in children and adolescents: gaining decades of life by optimizing detection and treatment. *Eur. Heart J.* 36, 2425–2437. doi: 10.1093/eurheartj/ehv157

Wilemon, K. A., Patel, J., Aguilar-Salinas, C., Ahmed, C. D., Alkhnifsawi, M., Almahmeed, W., et al. (2020). Reducing the clinical and public health burden of familial hypercholesterolemia: a global call to action. *JAMA Cardiol.* 5, 217–229. doi: 10.1001/jamacardio.2019.5173

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frontiers in Genetics

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Genetic and Clinical Characteristics of Patients With Homozygous and Compound Heterozygous Familial Hypercholesterolemia From Three Different Populations: Case Series

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Homozygous familial hypercholesterolemia (HoFH) and compound heterozygous familial hypercholesterolemia (cHeFH) are rare disorders generated by disease-causing variants in both alleles of the *LDLR* or other familial hypercholesterolemia (FH)-related genes. HoFH and cHeFH are characterized by severely elevated low-density lipoprotein-cholesterol (LDL-C), frequently leading to early cardiovascular disease. We investigated the genetic and clinical characteristics of HoFH and cHeFH patients from the Slovenian FH registry and/or those who were previously diagnosed or managed at our institution (Slovenian, Pakhtun and Albanian ethnicity), where genetic testing is not available. Our study includes seven patients. Their median age at the time of clinical diagnosis was 6.3 years (2.9–12.9 years); 2/7 were females. Two patients were diagnosed through the universal FH screening and five patients were diagnosed due to the presence of xanthomas. All the mutations are present in *LDLR* gene: 7 different genotypes for HoFH (p.Cys167Leu, p.Asp178Asn, p.Cys243Tyr, p.Gly549Asp, p.Cys27Trp, p.Ile585Thr and p.Val797Met) and p.Gly549Asp/p.Gln384Pro genotype for cHeFH patient. The median initial level of LDL-C was 17.0 mmol/L [655 mg/dL] (range 7.6–21.6 mmol/L). The HoFH/cHeFH patients are clinically and genetically very diverse. The clinical criteria (as Simon Broome criteria) might be applicable already in children to raise suspicion of FH but in some cases fail to distinguish heterozygous FH and HoFH/cHeFH patients. However, genetic testing is helpful in confirming the diagnosis, also for a prompt awareness, better compliance to treatment and family screening.

Keywords: familial hypercholesterolaemia, FH, homozygous, compound heterozygous, LDLR gene

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INTRODUCTION

Familial Hypercholesterolemia (FH) is an autosomal dominant genetic disorder, characterized by elevated total cholesterol (TC) and LDL-cholesterol (LDL-C) levels, usually accompanied by clinical characteristics and an early onset of cardiovascular disease (CVD), with a variable severity depending on the causative mutation (Moorjani et al., 1993). The prevalence of homozygous FH is unknown because of underdiagnosis and of a wide spectrum of phenotypes overlapping with heterozygous FH (HeFH), but is estimated in 1:200,000–300,000 (Sjouke et al., 2015; EAS Familial Hypercholesterolaemia Studies Collaboration et al., 2018; Representatives of the Global Familial Hypercholesterolemia Community et al., 2020).

Homozygous FH can be classified as: a- Real homozygous (HoFH): when the same mutation affects both alleles of one of the major FH-related genes (*LDLR*, *APOB*, or *PCSK9*); b- Compound heterozygous (cHeFH): when different mutations located on different alleles affect one of the major FH-associated genes; c- Combined heterozygous: when different mutations affect two different FH-related genes (Masana et al., 2019).

Homozygous FH patients can develop xanthomas and progressive atherosclerosis already in early childhood. If untreated, they develop vascular lesions and CVD before the second decade of life and die before the end of the third decade (Watts et al., 2014; Vallejo-Vaz et al., 2015).

Slovenia has successfully implemented nationwide universal screening for FH in pre-school children, detecting also the HoFH/cHeFH patients (Klancar et al., 2015; Groselj et al., 2018). The implementation and optimization of the routine FH genetic testing enables us to offer it to other national and foreign centers.

We aimed to analyze characteristics of all patients with HoFH and cHeFH genetically diagnosed and/or managed at our center.

MATERIALS AND METHODS

In 2011, routine FH genetic diagnosis was introduced at the UMC – University Children's Hospital Ljubljana, which serves as the national accredited genetic testing facility for dyslipidemias (Klancar et al., 2015; Groselj et al., 2018).

The universal hypercholesterolemia screening in children is an obligatory part of the blood check-up at the programed visit in 5-year old children at the primary care pediatricians; if TC is elevated (over 6 mmol/L or, in case of positive family history, over 5 mmol/L), the child is referred to the tertiary center for the FH genetic screening (Groselj et al., 2018).

We also provide FH genetic testing to other national and international institutions. Recently, we have been receiving samples from Pakistan and Kosovo (where the major ethnic group is Albanian). The first is a multiracial nation with a very heterogeneous population and without geographical clustering, generally found in other populations (Ajmal et al., 2010; Ahmed et al., 2013). A few common/known mutations are identified but the rest are mainly new mutations (Khan et al., 2014). On the other hand, a study in 2009 of FH in the Albanian population showed a single common mutation in almost half of the probands, but no other recent studies have been published (Diakou et al., 2010). Both populations require efficient genetic testing methods and a laboratory strategy.

The principles of the Declaration of Helsinki were followed and the Slovenian National Medical Ethics Committee (NMEC) approved the study (#25/12/10, #63/07/13 and 0120-273/2019/9). The data was also obtained from the National registry of FH and rare dyslipidemias (NMEC #0120-14/2017/5). Informed consent was obtained from adult patients and parents or legal guardians of minors.

The underlying data is partly available in the Mendeley repository at https://data.mendeley.com/datasets/thpt9htws6/1. The prevalence of HoFH/cHeFH in the Slovenian cohort was calculated as the number of live-born children since the implementation of the universal FH screening program in 1995, divided by the number of the HoFH/cHeFH patients.

Genetic Analysis

By the beginning of 2020, around 1150 genetic analyses were performed on pediatric patients with hypercholesterolemia in our genetic laboratory. Genomic DNA was isolated from the patients whole blood samples using the FlexiGene isolation kit (Qiagen, Germany). Three different sequencing methods for FH gene detection were used over time: (1) targeted Sanger sequencing (n = 192) -LDLR gene and part of exon 26 of APOB gene-, (2) ADH MASTR v2 ready to use next-generation sequencing (NGS) based molecular assay (Multiplicom, Belgium) (n = 190)-for detection of the variants in coding regions of LDLR, PCSK9, APOE, part of exon 26 (c.10200 to c.11100) of I (Single Nucleotide Variants & Copy Number Variants), as well as 12 LDL-C raising SNPs for a comprehensive analysis-, and (3) xGen® Lockdown® NGS Probes (IDT, United States) (n = 652) -for an extended dyslipidemia panel (*APOB, LDLR, PCSK9, LDLRAP1*) and expanded dyslipidemia panel (*ABCA1, ABCG5, ABCG8, ALMS1, APOA1, APOA5, APOC2, APOC3, APOE, CREB3L3, GPIHBP1, LDLRAP1, LIPA, LMF1, LPL*)-. Samples were sequenced on MiSeq sequencer with MiSeq Reagent Kit (Illumina, United States) following the manufacturer's protocol including recommendations for quality control parameters. In all samples sequenced with NGS, more than 100-fold horizontal coverage of the regions of interest (ROI) was achieved. A disease-specific database was used for determining the residual activity information of the known variants (Benito-Vicente et al., 2018). All variants found by the NGS were confirmed by targeted Sanger DNA sequencing.

CASE SERIES

Patient 1 is a 13 years old boy of Pakhtun origin from Khyber Pakhtunkhwa Province of Pakistan. Two siblings of the patient had died prematurely at ages of 10 and 11 years; only after that the parents sought clinical help. He was clinically diagnosed with FH at the age of 11 because of xanthomas, corneal arcus and a TC level of 22.9 mmol/L [885 mg/dL]. The genetic testing showed three different mutations -p.Cys167Leu, p.Asp178Asn

and p.Cys243Tyr- in both alleles (HoFH). All family members, except one sister, presented the same mutations in one allele (HeFH). To date, no functional data have been reported about the LDLR activity for these three variants. The patient presented a normal CT coronary angiography and coronary angiogram. He has been treated with atorvastatin 40 mg and ezetimibe 10 mg; other therapeutic options are not available in Pakistan.

Patient 2 is a 14 years old male from Kosovo. Before the age of 3 years, his physician observed xanthomas on elbows and knees, suspecting FH. The lipid profile showed cholesterol levels more than 20 mmol/L (No more biochemical data is available from that country). The family history revealed hypercholesterolemia in both parents, brother and grandmother. He was initially treated in Kosovo with atorvastatin and underwent occasional LDL-apheresis, without any improvement. At 11 years of age, he was referred to Slovenia, confirming a homozygous mutation in the *LDLR*, with less than 2% of receptor activity. In the clinical center, the initial TC level was 15.8 mmol/L [610 mg/dL] and LDL-C level was 13.7 mmol/L [529 mg/dL]. At that moment he received atorvastatin 40 mg and ezetimibe 10 mg. The last carotid Ultrasound (US) at the age of 11 years revealed diffuse non-obstructive atherosclerosis in bilateral carotid arteries; the most significant plaque-type I-II in the right common carotid artery contributing to 30% stenosis. After that, he has continued with atorvastatin 80 mg, ezetimibe 10 mg and *PCSK9* inhibitors within a clinical trial, but was a non-responder to the later. Other therapeutic options are currently not available in Kosovo.

Patient 3, a 6 years old Slovenian girl (of Albanian origin), was recently diagnosed at the universal FH screening, with an initial TC level of 7.6 mmol/L [293 mg/dL]. She was referred to our tertiary care center, where the biochemical test was repeated and later the genetic test revealed an *LDLR* homozygous mutation with 15–30% of LDLR activity. She stays asymptomatic and no family history of CVD was found. Until now she had not been introduced to pharmacotherapy.

Patient 4 is a 18 years old Slovenian male (of Albanian origin), diagnosed during the universal FH screening with TC level of 9.0 mmol/L [348 mg/dL]. His parents and brother have hypercholesterolemia. The genetic testing confirmed a homozygous *LDLR* mutation with 15–30% of LDLR residual activity. The last carotid US at the age of 16 years had shown a borderline intima-media thickness (cITM) of 0.56 mm. He started with monotherapy of atorvastatin and later required dual therapy with higher doses of atorvastatin and ezetimibe. At the age of 16 he was enrolled in the clinical trial with *PCSK9* inhibitors. His latest TC and LDL-C levels were 6 mmol/L [232 mg/dL] and 4.5 mmol/L [174 mg/dL], respectively.

Patient 5 is a 31 years old Slovenian male with FH diagnosed in april 1993, at the age of 4, due to the presence of xanthomas. Both parents and sister have HeFH. His initial TC level was 24.8 mmol/L [959 mg/dL]. The genetic testing confirmed a homozygous mutation in *LDLR*. To date, no functional data were found about LDLR residual activity for this mutation. The last carotid US studies at the age of 30 years have shown a thickened intima-media (cITM 0.903 mm). He was treated with atorvastatin, LDL-apheresis and simvastatin to no effect. Finally, he required liver transplantation at the age of 16, with remarkable decrease of TC and LDL levels.

Patient 6 is a 43 years old Slovene female clinically diagnosed with hypercholesterolemia at the age of 12 because of a strong family history and an elevated TC (more than 19 mmol/L). Her father died at 53 years old because of myocardial infarction and her mother also presented with coronary heart disease after the age of 60. She had a bad compliance to the combined therapy to atorvastatin 40 mg and ezetimibe 10 mg. She was receiving LDL-apheresis for several years (data about frequency of treatment unavailable), discontinued it later because of the adverse effects (strong headache, dizziness, weight gain, cough). When she was 38 years old she suffered an acute coronary syndrome, whereas coronary angiography revealed stenosis of the left main coronary artery and distal right coronary artery. She was diagnosed with homozygous mutation in *LDLR*, with an unknown residual activity. She transiently received PCSK9 inhibitor and lomitapide, but had discontinued the treatments due to side effects.

Patient 7 is a 7 years old male from Kosovo. He was diagnosed with FH at the age of 6 after an abdominal pain event. At the examination, the physician discovered small xanthomas in the sacral region and the biochemical testing showed CT levels of 20.4 mmol/L [789 mg/dL]. His mother also presented hypercholesterolemia. The genetic analysis confirmed cHeFH; a variant in p.Gly549Asp, with less than 2% of LDLR residual activity, and another in p.Gln384Pro, with unknown residual activity. He has a normal carotid US. His current therapy consists of atorvastatin 80 mg and ezetimibe 10 mg and he is showing good response to the therapy. Other therapeutic options are not available in Kosovo.

Clinical and genetic features are summarized in **Table 1** and **Figure 1**. Biochemical values and therapy are plotted in **Figures 2A,B**.

RESULTS

Demographics

All patients had a family history of early CVD and/or hypercholesterolemia, except patient 3. At the time of the clinical diagnosis, the patient's median age was 6.3 (2.9–12.9) years. Five patients were symptomatic at the diagnosis (presence of xanthomas and/or corneal arcus), while two were asymptomatic detected through the FH screening program. Four patients were born in Slovenia: both patients born after the implementation of the universal FH screening in this country, were detected through the program; the older two HoFH patients were born prior to the program implementation. The pre-treatment median TC level was 17.2 mmol/L [665 mg/dL] (7.6–24.8 mmol/L) and median LDL-C level was 17.0 mmol/L [655 mg/dL] (7.6–21.6 mmol/L).

In the carotid US, an increased carotid artery intima-media thickness (cIMT) or atherosclerosis signs were found in cases 2, 4 and 5. One patient (case 6) with *angina pectoris* underwent coronarography, detecting stenosis of left and right coronary artery.

TABLE 1 | Phenotypic and genotypic features of our cohort of HoFH and cHeFH patients.

Patient		**1**	**2**	**3**	**4**	**5**	**6**	**7**
Age (years)		13.0	14.0	6.9	18.5	31.6	43.0	7.9
Gender		M	M	F	M	M	F	M
Ethnicity		Pakhtun	Albanian	Albanian	Albanian	Slovene	Slovene	Albanian
Genetic desorder	Zygosity	**homozygous**	**homozygous**	**homozygous**	**homozygous**	**homozygous**	**homozygous**	**compound heterozygous**
	FH-related gen	*LDLR*	*LDLR*	*LDLR*	*LDLR*	*LDLR*	*LDLR*	*LDLR*
	Genome mutation	*c.[500_501delinsTA; 532G > A; 728G > A]; c.[500_501delinsTA; 532G > A; 728G > A]*	*c.[1646G > A]; c.[1646G > A]*	*c.[81C > G]; c.[81C > G]*	*c.[81C > G]; c.[81C > G]*	*c.[1773T > C]; c.[1773T > C]*	*c.[2389G > A]; c.[2389G > A]*	*c.[1151A > C]; c.[1646G > A]*
	Exon	*4,4,5*	*11*	*2*	*2*	*12*	*17*	*8,11*
	Protein mutation	*Cys167Leu; Asp178Asn; Cys243Tyr*	*p.Gly549Asp*	*p.Cys27Trp*	*p.Cys27Trp*	*P.Ile585Thr*	*p.Val797Met*	*p.Gly549Asp; p.Gln384Pro*
	Residual LDLR activity	not determined	<2%	15–30%	15–30%	not determined	not determined	p.Gly549Asp < 2% p.Gln384Pro: not determined
Screening type		Other	Other	Universal	Universal	Other	Other	Other
Age of diagnosis (years)		11.1	2.9	6.3	5.5	4.3	12.9	7.3
Symtoms		Xanthomas	Xanthomas	Asymtomatic	Asymtomatic	Xanthomas	Angina pectoris, xanthomas	Xanthomas
Corneal arcus		Yes	No	No	No	No	No	no
Cardiovascular exams		CT coronary angiography and coronary angiogram: normal	Carotid US: diffuse non- obstructive atherosclerosis in both carotid arteries. In right CCA: plaque type I-II (30% stenosis)	No data	Carotid US: cIMT = 0,556 mm	Carotid US: cIMT = 0,903 mm	Coronarography: stenosis of left coronary artery, lower stenosis of right coronary artery	Carotid US: Normal
TC (mmol/l)	First	22.9	15.8	7.6	9.0	24.8	17.2	20.4
	Last	17.5	18.2	7.0	6.0	7.6	16.2	11
	↓	23.4%	−15.2%	7.9%	33.3%	69.4%	5.8%	46.1%
LDL-C (mmol/l)	First	20.5	13.7	No data	7.6	21.6	15.4	18.5
	Last	16.0	17.2	5.5	4.5	6.3	14.4	10.1
	↓	22.0%	−25.5%	–	40.8%	70.8%	6.5%	45.4%

(Continued)

TABLE 1 | Continued

Patient	1	2	3	4	5	6	7
Pharmacotherapy	Atorvastatin 40 mg + Ezetimibe 10 mg	Atorvastatin 80 mg + LDL-apheresis + Ezetimibe 10 mg + *PCSK9* inhibitor	No	Atorvastatin 40 mg + Ezetimibe 10 mg + *PCSK9* inhibitor	Atorvastatin 40mg/simvastatin 30 mg + LDL-apheresis + Liver trasplantation (2004)	Atorvastatin 40 mg + Ezetimibe 10 mg ± LDL-apheresis ± *PCSK9* inhibitor ± Lomitapide	Atorvastatin 80 mg + Ezetimibe 10 mg
Family with hypercholesterolemia	Both parents and brother	Father and grandmother	No	Both parents and brother	Both parents and sister	Both parents and sister	Mother and uncle
Family history of other CVD	Grandfather died of CVD and uncle has had 3 stents. Deceased brother and sister	Grandmother	Not reported	Not reported	Not reported	Father died of MI at 53 years old and mother has CHD	Uncle has CVD with cardiac intervention by-pass at 40 years old

M, male; F, female; CT, computed tomography; US, ultrasound; CCA, common carotid artery; cITM, carotid intima-media thickness; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; CVD, cardiovascular disease; MI, myocardial infarction; CHD, coronary heart disease.

Genotypes

Genetic evaluation confirmed mutations in *LDLR* gene in all of the patients (OMIM #143890). Six patients were confirmed as HoFH and one as cHeFH. The HoFH patients present the mutations p.Gly549Asp, p.Cys27Trp, p.Ile585Thr, p.Val797Met, p.Asp178Asn, p.Cys243Tyr, and p.Cys167Leu (the last three variants are present in each allele of patient 1). Case 7 presents the variants p.Gly549Asp and p.Gln384Pro in each allele (cHeFH) (**Table 1**). The cascade children-parent testing was performed for patients number 1, 2, 4 and 5, confirming all their parents and some of the siblings are carriers of disease-causing variants (**Figure 1**). The genetic testing for family members of patients 3, 6 and 7 were unavailable.

Treatment

As a response to treatment, the median TC and LDL-C decreased by 28 and 31%, respectively. The treatment response is summarized in **Figures 2A,B** and specified in the Mendeley database[1]. Six patients were treated with statins, five with ezetimibe, three with *PCSK9* inhibitors and one with lomitapide. Three patients received LDL-apheresis. One patient had liver transplantation at the age of 16 with excellent response. It is worth noting the distinguished response presented in the cHeFH patient (46%), compared to the mean response in HoFH patients.

DISCUSSION

Homozygous FH is an ultra-rare disease, but newer data show it to be more frequent as previously assumed. Sjouke et al. (2014) calculated the prevalence of HoFH and cHeFH in the Netherlands population ranges from 1/371,608 to 1/407,863. The prevalence in Slovenia was estimated at around 1/256,340, calculated by dividing the number of tested children with the number of confirmed HoFH/cHeFH cases in that period.

Up to date, there is no international unanimity on FH detection strategies; some guidelines recommend the cascade screening as the most cost-effective strategy (Santos et al., 2016). Others, like the US National Lipid Association (Goldberg et al., 2011) and American Academy of Pediatrics (Expert Panel on Integrated Guidelines for Cardiovascular Health, and Risk Reduction in Children, and Adolescents, 2011) recommend universal screening. Thus, each country adopts a screening method based on local scientific societies or experts (Ibarretxe et al., 2018; Umans-Eckenhausen et al., 2001; Representatives of the Global Familial Hypercholesterolemia Community et al., 2020). For example, in Sjouke's report in the Netherlands, 36 patients (73%) were diagnosed by referrals from pediatricians because of the presence of symptoms, while 13 patients (27%) were detected through cascade screening (Sjouke et al., 2014). Slovenia is the only country with implemented nationwide universal FH screening in pre-school children (with routinely implemented genetic FH diagnostics), detecting also the last two of our HoFH patients (the first

[1]http://dx.doi.org/10.17632/thpt9htws6.1

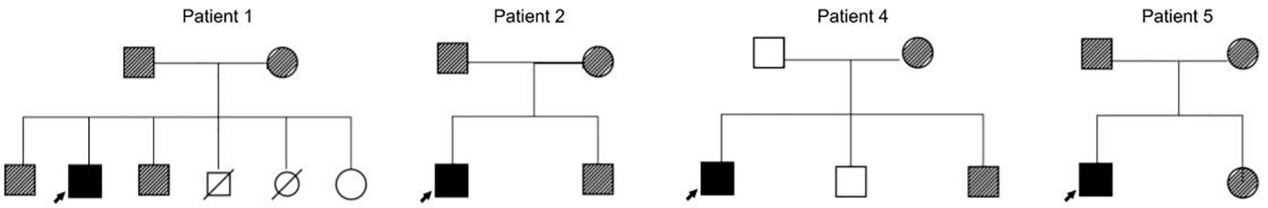

FIGURE 1 | Pedigree charts from patients 1, 2, 4, and 5. : Heterozygous FH. : Homozygous FH.

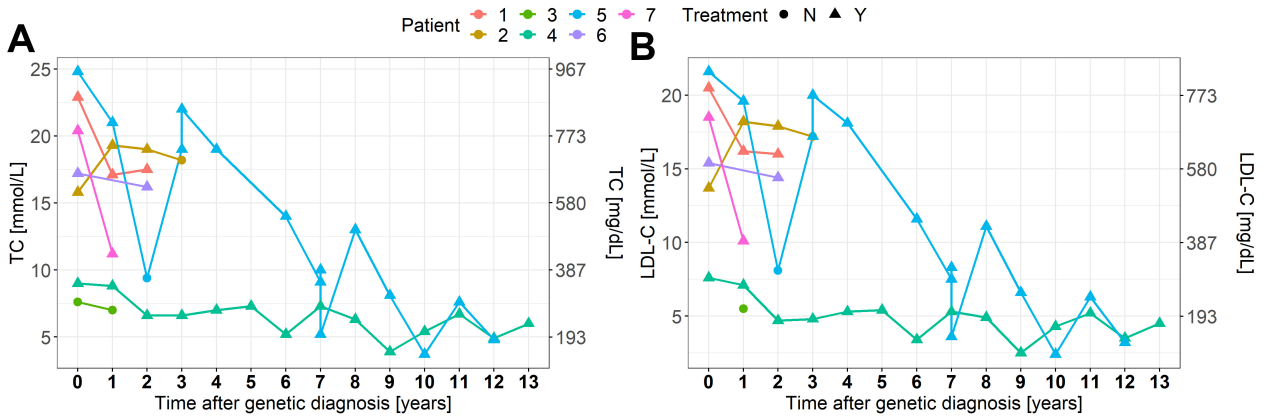

FIGURE 2 | (A,B) TC and LDL-C levels in response to therapy. N, no; Y, yes.

two were diagnosed prior to the program implementation) (Klancar et al., 2015; Groselj et al., 2018). However, beyond the initial screening method, almost all of the literature agrees on the importance of genetic testing. Recognition of a pathogenic FH mutation guides the cascade screening in the family, as well as the incorporation of genetic testing into cascade screening improves the detection rate for FH (Knowles et al., 2017). Furthermore, between HoFH and cHeFH patients some more subtle differences in the genotype-phenotype correlations or even regarding the response to therapies might exist due to inter-allele interactions and possibility that the phenotype is determined by the allele leading to the higher residual activity (as generally in inborn errors of metabolism) (Groselj et al., 2012).

We reported six patients with HoFH and one with cHeFH. All of them (100%) have mutations in *LDLR*, while other European reports show a prevalence of 91% in Netherlands (Sjouke et al., 2014), 75% in France (Bruckert et al., 2017), and 71% in Spain (Alonso et al., 2016). Our cohort's mean age at the time of clinical diagnosis was 7.2 years old, ranging from 2.9 to 12.9 years. In other European studies the mean age at the time of molecular diagnosis were: in Austria and France 6.6 and 7.5 years, respectively, in Belgium 2.45 years, while in Netherlands it was 28.2 years (49 cases included, 0–68 years old).

All the patients meet the FH Dutch diagnostic criteria for definite FH, except patient 3 and 4, who had a possible and probable FH, respectively, both detected by universal FH screening (Klancar et al., 2015; Groselj et al., 2018); all patients were genetically diagnosed for FH. Four patients presented xanthomas, whereas in France (Bruckert et al., 2017), Austria (Widhalm et al., 2017), and Belgium (Sanna et al., 2016) almost all of the patients had skin stigmata at the diagnosis.

The pre-treatment mean LDL-C level of our patients was 16.2 mmol/L [627 mg/dL] (but with quite wide range 7.6–21.6 mmol/L). Other European reports mostly showed similar initial LDL-C levels: Netherlands 12.9 mmol/L [498 mg/dL]; Belgium 19.6 mmol/L [757 mg/dL]; Austria 16.5 mmol/L [638 mg/dL] and France 13.2 mmol/L [510 mg/dL] (Sjouke et al., 2014; Sanna et al., 2016; Bruckert et al., 2017; Widhalm et al., 2017).

Regarding CVD, in our cohort four patients showed atherosclerotic signs (plaques in the carotid arteries, increased cITM or coronary stenosis) and one patient (case 6) also presented *angina pectoris*. In the United Kingdom, Thompson et al. (2015) reported that the most frequent complications in these patients were coronary heart disease and aortic stenosis.

Homozygous FH is usually very difficult to manage and the medical treatment often combines several cholesterol-lowering drugs (Cuchel et al., 2014). Initially, statins with ezetimibe are introduced and in responsive patients also *PCSK9* inhibitors, but frequently do not result in a satisfactory reductions of either TC or LDL-C levels, especially in moderate and severe HoFH patients, which have the highest risk of CVD (Kolansky et al., 2008; Rajendran et al., 2013). For

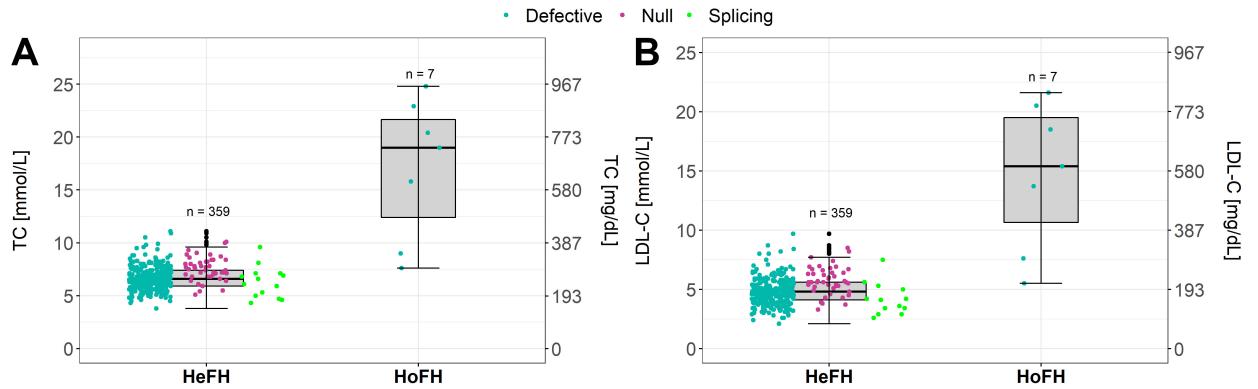

FIGURE 3 | (A,B) Variability in HoFH (real HoFH and cHeFH) from HeFH patients in CT and LDL-C levels.

over 30 years, LDL-apheresis has been used, becoming a mainstay in the management of the disease and it is currently considered the most safe and effective treatment (Julius, 2017). If LDL-apheresis is not successful, liver transplantation can be an alternative, considering also as a successful therapy in other metabolic liver diseases (McKiernan, 2017). In the Brussels cohort published by Sanna, the median reduction in TC in response to pharmacological treatment was 47% (Sanna et al., 2016). In our cohort, we observed a median decrease of TC levels in response to the combined treatment by 28% (range from a reduction of 69% to an increase of 15% besides the therapy). A huge variability in the treatment response is observed among our patients (**Figures 2A,B**). We could recognize the differences depending on the approach to therapies and on patient adherence, such as cases 2 and 6. In developing countries, the access to modern diagnostic and therapeutic methods is still limited, resulting in patients failure in reaching treatment goals and also in inadequate management of CVD (Representatives of the Global Familial Hypercholesterolemia Community et al., 2020).

Groselj et al. (2018) reported that around 45% of participants referred from the Slovenian universal FH screening presented a disease-causing genetic variant for FH, mostly heterozygous. Our institution could compare the TC and LDL-C levels of HeFH patients with the HoFH/cHeFH patients (**Figures 3A,B**). In concordance with the literature (Santos et al., 2016; Sturm et al., 2018; Berberich and Hegele, 2019), our cohort shows to be clinically and genetically very diverse, overlapping with HeFH phenotypes. The clinical criteria (as Simon Broome criteria) might be applicable already in children to raise suspicion of FH but in some cases fail to distinguish HeFH and HoFH/cHeFH patients. As reported previously, consideration of a diagnosis of HoFH/cHeFH should not be limited to those with very high LDL-C levels (Raal et al., 2016). Other factors besides Mendelian inheritance also play a role in the FH: polygenic variants, gene-environment interactions and non-mendelian mechanisms, such as epigenetic (Hooper et al., 2018; Berberich and Hegele, 2019).

Because of the retrospective design, our report has some limitations in data interpretation and not all the data of individual patients or their relatives were available to be included.

CONCLUSION

HoFH/cHeFH patients are clinically heterogeneous, possibly even overlapping with HeFH patients, highlighting the importance of establishing the genetic diagnosis. In addition, public policies are needed to improve early detection, family screening, adequate therapies, and appropriate follow-up. HoFH/cHeFH patients in developing countries frequently lack even basic access to diagnostics, management and adequate therapeutic options, leading to the inequality of outcomes. This should be better addressed at the global level.

PATIENTS PERSPECTIVE

Nowadays, in Slovenia patients have good access to genetic diagnosis, early detection (universal FH screening) and the current methods of treatment, which is not the case in Kosovo and in Pakistan.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the manuscript/supplementary material.

ETHICS STATEMENT

Written consent was obtained from the participants to conduct clinical examination, conducting clinical and laboratory investigations, including genetic studies and publishing the patient's data in scientific journals.

AUTHOR CONTRIBUTIONS

UG and TM conceptualized and designed the study, performed the clinical work, carried out the initial analyses, drafted the initial manuscript, and reviewed and revised the manuscript. US, MM, MC, JK, FS, SS, IK, and VK performed the clinical work, designed the data collection instruments, and reviewed and revised the manuscript. TB and KT coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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REFERENCES

Ahmed, W., Whittall, R., Riaz, M., Ajmal, M., Sadeque, A., Ayub, H., et al. (2013). The genetic spectrum of familial hypercholesterolemia in Pakistan. *Clin. Chim. Acta* 421, 219–225. doi: 10.1016/j.cca.2013.03.017

Ajmal, M., Ahmed, W., Sadeque, A., Benish Ali, S. H., Bokhari, S. H., Ahmed, N., et al. (2010). Identification of a recurrent insertion mutation in the LDLR gene in a Pakistani family with autosomal dominant hypercholesterolemia. *Mol. Biol. Rep.* 37, 3869–3875. doi: 10.1007/s11033-010-0043-0

Alonso, R., Diaz-Diaz, J. L., Arrieta, F., Fuentes-Jiménez, F., De Andres, R., Saenz, P., et al. (2016). Clinical and molecular characteristics of homozygous familial hypercholesterolemia patients: insights from SAFEHEART registry. *J. Clin. Lipidol.* 10, 953–961. doi: 10.1016/j.jacl.2016.04.006

Benito-Vicente, A., Uribe, K. B., Jebari, S., Galicia-Garcia, U., Ostolaza, H., and Martin, C. (2018). Validation of LDLr activity as a tool to improve genetic diagnosis of familial hypercholesterolemia: a retrospective on functional characterization of LDLr variants. *Int. J. Mol. Sci* 19:1676. doi: 10.3390/ijms19061676

Berberich, A. J., and Hegele, R. A. (2019). The complex molecular genetics of familial hypercholesterolaemia. *Nat. Rev. Cardiol.* 16, 9–20. doi: 10.1038/s41569-018-0052-6

Bruckert, E., Kalmykova, O., Bittar, R., Carreau, V., Béliard, S., Saheb, S., et al. (2017). Long-term outcome in 53 patients with homozygous familial hypercholesterolaemia in a single centre in France. *Atherosclerosis* 257, 130–137. doi: 10.1016/j.atherosclerosis.2017.01.015

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014). Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the consensus panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. *Eur. Heart J.* 35, 2146–2157. doi: 10.1093/eurheartj/ehu274

Diakou, M., Miltiadous, G., Xenophontos, S., Cariolou, M., and Heta, N. (2010). Characterization of low density lipoprotein receptor (LDLR) gene mutations in Albania. *Arch. Med. Sci.* 6, 198–200. doi: 10.5114/aoms.2010.13894

EAS Familial Hypercholesterolaemia Studies Collaboration, Vallejo-Vaz, A. J., De Marco, M., Stevens, C. A., Akram, A., Freiberger, T., et al. (2018). Overview of the current status of familial hypercholesterolaemia care in over 60 countries - The EAS Familial Hypercholesterolaemia Studies Collaboration (FHSC). *Atherosclerosis* 277, 234–255.

Expert Panel on Integrated Guidelines for Cardiovascular Health, and Risk Reduction in Children, and Adolescents (2011). Expert panel on integrated guidelines for cardiovascular health and risk reduction in children and adolescents. Summary report. *Pediatrics* 128, 213–256.

Goldberg, A. C., Hopkins, P. N., Toth, P. P., Ballantyne, C. M., Rader, D. J., Robinson, J. G., et al. (2011). Familial hypercholesterolemia: screening, diagnosis and management of pediatric and adult patients: clinical guidance from the national lipid association expert panel on familial hypercholesterolemia. *J. Clin. Lipidol.* 5, 1–8. doi: 10.1016/j.jacl.2011.04.003

Groselj, U., Kovac, J., Sustar, U., Mlinaric, M., Fras, Z., Trebusak Podkrajsek, K., et al. (2018). Universal screening for familial hypercholesterolemia in children: the Slovenian model and literature review. *Atherosclerosis* 277, 383–391. doi: 10.1016/j.atherosclerosis.2018.06.858

Groselj, U., Zerjav Tansek, M., Kovac, J., Hovnik, T., Trebusak Podkrajsek, K., and Battelino, T. (2012). Five novel mutations and two large deletions in a population analysis of the phenylalanine hydroxylase gene. *Mol. Genet. Metab.* 106, 142–148. doi: 10.1016/j.ymgme.2012.03.015

Hooper, A. J., Burnett, J. R., Bell, D. A., and Watts, G. F. (2018). The present and the future of genetic testing in familial. hypercholesterolemia: opportunities and caveats. *Curr. Atherosclerosis Rep.* 20:31. doi: 10.1007/s11883-018-0731-0

Ibarretxe, D., Rodríguez-Borjabad, C., Feliu, A., Bilbao, J. A., Masana, L., Plana, N., et al. (2018). Detecting familial hypercholesterolemia earlier in life by actively searching for affected children: the DECOPIN project. *Atherosclerosis* 278, 210–216. doi: 10.1016/j.atherosclerosis.2018.09.039

Julius, U. (2017). History of lipidology and lipoprotein apheresis. *Atheroscler. Suppl.* 30, 1–8. doi: 10.1016/j.atherosclerosissup.2017.05.034

Khan, S. P., Ghani, R., and Yaqub, Z. (2014). Single step PCR for the identification of Low Density Lipoprotein Receptor (LDL-R) gene mutations. *Pak. J. Med. Sci.* 30, 830–833. doi: 10.12669/pjms.304.4711

Klancar, G., Groselj, U., Kovac, J., Bratanic, N., Bratina, N., Trebusak Podkrajsek, K., et al. (2015). Universal screening for familial hypercholesterolemia in children. *JACC* 66, 1250–1257.

Knowles, J. W., Rader, D. J., and Khoury, M. J. (2017). Cascade screening for familial hypercholesterolemia and the use of genetic testing. *JAMA* 318, 381–382. doi: 10.1001/jama.2017.8543

Kolansky, D. M., Cuchel, M., Clark, B. J., Paridon, S., McCrindle, B. W., Wiegers, S. E., et al. (2008). Longitudinal evaluation and assessment of cardiovascular disease in patients with homozygous familial hypercholesterolemia. *Am. J. Cardiol.* 102, 1438–1443. doi: 10.1016/j.amjcard.2008.07.035

Masana, L., Ibarretxe, D., Rodríguez-Borjabad, C., Plana, N., Valdivielso, P., Pedro-Botet, J., et al. (2019). Toward a new clinical classification of patients with familial hypercholesterolemia: one perspective from Spain. *Atherosclerosis* 287, 89–92. doi: 10.1016/j.atherosclerosis.2019.06.905

McKiernan, P. J. (2017). Recent advances in liver transplantation for metabolic disease. *J. Inherit. Metab. Dis.* 40, 491–495. doi: 10.1007/s10545-017-0020-z

Moorjani, S., Roy, M., Torres, A., Bétard, C., Gagné, C., Lambert, M., et al. (1993). Mutations of low-density-lipoprotein-receptor gene, variation in plasma cholesterol, and expression of coronary heart disease in homozygous familial hypercholesterolaemia. *Lancet* 341, 1303–1306. doi: 10.1016/0140-6736(93)90815-x

Raal, F. J., Sjouke, B., Hovingh, K., and Isaac, B. J. (2016). Phenotype diversity among patients with homozygous familial hypercholesterolemia: a cohort study. *Atherosclerosis* 248, 238–244. doi: 10.1016/j.atherosclerosis.2016.03.009

Rajendran, R., Srinivasa, K. H., Rangan, K., Hegde, M., and Ahmed, N. (2013). Supravalvular aortic stenosis in a patient with homozygous familial hypercholesterolaemia. *Eur. Heart J. Cardiovasc. Imaging* 14:1023. doi: 10.1093/ehjci/jet072

Representatives of the Global Familial Hypercholesterolemia Community, Wilemon, K. A., Patel, J., Aguilar-Salinas, C., Ahmed, C. D., Alkhnifsawi, M., et al. (2020). Reducing the clinical and public health burden of familial hypercholesterolemia: a global call to action. *JAMA Cardiol.* 5, 217–229.

Sanna, C., Stephenne, X., Revencu, N., Smets, F., Sassolas, A., Di Filippo, M., et al. (2016). Homozygous familial hypercholesterolemia in childhood: genotype-phenotype description, established therapies and perspectives. *Atherosclerosis* 247, 97–104. doi: 10.1016/j.atherosclerosis.2016.02.009

Santos, R. D., Gidding, S. S., Hegele, R. A., Cuchel, M. A., Barter, P. J., Watts, G. F., et al. (2016). Defining severe familial hypercholesterolaemia and the implications for clinical management: a consensus statement from the international atherosclerosis society severe familial hypercholesterolemia panel. *Lancet Diab. Endocrinol.* 4, 850–861. doi: 10.1016/S2213-8587(16)30041-9

Sjouke, B., Hovingh, G. K., Kastelein, J. J., and Stefanutti, C. (2015). Homozygous autosomal dominant hypercholesterolaemia: prevalence, diagnosis, and current and future treatment perspectives. *Curr. Opin. Lipidol.* 26, 200–209. doi: 10.1097/mol.0000000000000179

Sjouke, B., Kusters, D. M., Kindt, I., Besseling, J., Defesche, J. C., Sijbrands, E. J., et al. (2014). Homozygous autosomal dominant hypercholesterolaemia in The Netherlands: prevalence, genotype-phenotype relationship, and clinical outcome. *Eur. Heart J.* 36, 560–565. doi: 10.1093/eurheartj/ehu058

Sturm, A. C., Knowles, J. W., Gidding, S. S., Ahmad, Z. S., Ahmed, C. D., Ballantyne, C. M., et al. (2018). Clinical genetic testing for familial hypercholesterolemia JACC scientific expert panel. *JACC* 72, 662–680.

Thompson, G., Seed, M., Naoumova, R. P., Neuwirth, C., Walji, S., Aitman, T. J., et al. (2015). Improved cardiovascular outcomes following temporal advances in lipid-lowering therapy in a genetically-characterised cohort of familial hypercholesterolaemia homozygotes. *Atherosclerosis* 243, 328–333. doi: 10.1016/j.atherosclerosis.2015.09.029

Umans-Eckenhausen, M. A., Defesche, J. C., Sijbrands, E. J., Scheerder, R. L., and Kastelein, J. J. (2001). Review of first 5 years of screening for familial hypercholesterolaemia in the Netherlands. *Lancet* 357, 165–168. doi: 10.1016/s0140-6736(00)03587-x

Vallejo-Vaz, A. J., Kondapally Seshasai, S. R., Cole, D., Hovingh, G. K., Kastelein, J. J., Mata, P., et al. (2015). Familial hypercholesterolaemia: a global call to arms. *Atherosclerosis* 243, 257–259.

Watts, G. F., Gidding, S., Wierzbicki, A. S., Toth, P. P., Alonso, R., Virgil Brown, W., et al. (2014). Integrated guidance on the care of familial hypercholesterolaemia from the international FH foundation: executive summary. *J. Atheroscler. Thromb.* 21, 368–374.

Widhalm, K., Benke, I. M., Fritz, M., Geiger, H., Helk, O., Fritsch, M., et al. (2017). Homozygous familial hypercholesterolemia: summarized case reports. *Atherosclerosis* 257, 86–89. doi: 10.1016/j.atherosclerosis.2017.01.002

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REVIEW
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Proteostasis Regulation in the Endoplasmic Reticulum: An Emerging Theme in the Molecular Pathology and Therapeutic Management of Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is an autosomal genetic disease characterized by high serum low-density lipoprotein (LDL) content leading to premature coronary artery disease. The main genetic and molecular causes of FH are mutations in low-density lipoprotein receptor gene (*LDLR*) resulting in the non-clearance of LDL from the blood by hepatocytes and consequently the formation of plaques. LDLR is synthesized and glycosylated in the endoplasmic reticulum (ER) and then transported to the plasma membrane via Golgi. It is estimated that more than 50% of reported FH-causing mutations in LDLR result in misfolded proteins that are transport-defective and hence retained in ER. ER accumulation of misfolded proteins causes ER-stress and activates unfolded protein response (UPR). UPR aids protein folding, blocks further protein synthesis, and eliminates misfolded proteins via ER-associated degradation (ERAD) to alleviate ER stress. Various studies demonstrated that ER-retained LDLR mutants are subjected to ERAD. Interestingly, chemical chaperones and genetic or pharmacological inhibition of ERAD have been reported to rescue the transport defective mutant LDLR alleles from ERAD and restore their ER-Golgi transport resulting in the expression of functional plasma membrane LDLR. This suggests the possibility of pharmacological modulation of proteostasis in the ER as a therapeutic strategy for FH. In this review, we picture a detailed analysis of UPR and the ERAD processes activated by ER-retained LDLR mutants associated with FH. In addition, we discuss and critically evaluate the potential role of chemical chaperones and ERAD modulators in the therapeutic management of FH.

Keywords: ERAD pathway, cholesterol, familial hypercholesterolemia, Class II mutations, lipid metabolism, LDLR, ER stress, unfolded protein response

Abbreviations: CAD, coronary artery disease; CMV, cytomegalovirus; ERAD, endoplasmic reticulum associated degradation; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; LDLR, low-density lipoprotein receptor; UPR, unfolded protein response.

INTRODUCTION

Familial Hypercholesterolemia

Familial hypercholesterolemia (FH) is a genetic disorder that results in altered lipid metabolism and consequently leading to elevated levels of plasma low-density lipoprotein cholesterol (LDL-C) (Soutar and Naoumova, 2007). Clinically, FH is characterized by increased levels of LDL-C, tendon xanthomas, corneal arcus, and premature coronary artery diseases (CAD) such as atherosclerosis (Müller, 1938; Kawaguchi et al., 1999; Kitahara et al., 2019). Mutations in the low-density lipoprotein receptor gene (*LDLR*) account for more than 80% of monogenic FH (Brown et al., 1986) (FHCL1, OMIM#143890). Monogenic FH can also be caused by mutations in other genes including *APOB* (FHCL2, OMIM#144010) (Innerarity et al., 1990), *PCSK9* (FHCL3, OMIM#603776) (Abifadel et al., 2003), and *LDLRAP1* (FHCL4, OMIM# 603813) (Garcia et al., 2001). FH can exist in both heterozygous and homozygous forms with homozygous FH (HoFH) patients at far greater risk of developing CAD in their first decade of life (Alonso et al., 2014). The clinical manifestations of a homozygous patient suffering from FH begin in the first decade of their life including abnormal cholesterol storage which results in the appearance of cutaneous xanthomas and the appearance of tendon xanthomas particularly in the joints and fingers. Another late symptom is the manifestation of xanthelasmata as well as corneal arcus. Also, coronary manifestations in HoFH appear in their second and third decades (Klose et al., 2014) though fatal myocardial infarctions (MIs) are possible even in early childhood (Wiegman et al., 2015). On the other hand, the clinical manifestations in heterozygous FH patients are possible from early adulthood onward and premature CAD in the second or third decade of life. Sometimes symptoms may remain clinically hidden (Klose et al., 2014). If left untreated, approximately 50% heterozygous males and 15% females have a fatal MI by the age of 60 (Henderson et al., 2016). In recent studies, it has been shown that the prevalence of heterozygous FH has increased and affects between 1:200 or 1:300 in most populations (Nordestgaard et al., 2013).

Cholesterol is an essential component of membranes and serves as a precursor for steroid molecules such as hormones, bile acids and vitamin D. Cellular cholesterol requirement is met either by *de novo* intracellular synthesis or by uptake of dietary cholesterol (Goldstein and Brown, 1990). Receptor-mediated endocytosis of cholesterol mediated by LDLR, unraveled by the seminal work of Brown and Goldstein, is the main pathway for cellular uptake of exogenous cholesterol (Brown et al., 1986). On the cell membrane, the LDLR receptors are localized to clathrin-coated pits and when the LDL-bound cholesterol attaches to the receptor, the complex is internalized and fuse with early sorting endosomes. There the receptor dissociates from the lipid and recycles back to the cell-surface repeating this cycle every 10 min (Brown et al., 1986). The LDL particles are eventually delivered via endosomal trafficking to the lysosomes for degradation and the cholesterol is released within the cell. Excess cellular cholesterol is esterified and stored in lipid droplets in the endoplasmic reticulum (ER) (Ikonen, 2008). Cellular cholesterol homeostasis is a tightly regulated process and the ER plays a crucial role in cholesterol sensing, regulation, and synthesis (Röhrl and Stangl, 2018). The ER is also the site of synthesis of many membrane proteins including that of LDLR which is in turn subject to feedback regulation by intracellular cholesterol levels. The review aims to present how LDLR mutants implicated in FH deregulates ER homeostasis and also explores the possibilities of targeting ER-proteostasis machinery for therapeutic management of FH.

Low-Density Lipoprotein Receptor (LDLR): Gene, Protein Structure, and Function

The low-density lipoprotein receptor (LDLR) is the prototype receptor of a group of structurally and functionally similar cell surface receptors. LDLR is encoded by the *LDLR* gene located on chromosome 19p13.1-13.3. It spans ∼45 kb and comprises 18 exons that are translated into 860 amino acids including a signal sequence of 21 amino acids which is cleaved during translocation into the ER (Francke et al., 1984) (**Figure 1A**). Each exon or group of exons constitutes a particular domain in the LDLR (**Figure 1A**) (Gent and Braakman, 2004). There are five LDLR domains and each domain mediates a specific function (Klee and Zimmermann, 2019) which are: a ligand-binding domain (LBD), an epidermal growth factor (EGF) homology domain, an *O*-linked sugar region, a membrane-spanning domain and a *C*-terminal cytoplasmic tail domain (Gent and Braakman, 2004) (**Figure 1B**).

The LBD is made up of 292 amino acids and consists of seven cysteine-rich ligand-binding repeats (LRs) each composed of 40 amino acid residues (Yamamoto et al., 1984; Südhof et al., 1985; Fass et al., 1997). Six cysteine residues along with a group of negatively charged amino acids in the LR sequence interact with positively charged residues on the APOB and APOE molecules to mediate the recognition and binding of LDL-C to LDLR (Bradley and Gianturco, 1986). The second domain is the EGF precursor domain which is composed of 400 amino acids and contributes to the dissociation of the LDL particles from the LDLR-LDL complex in the endosome at a low pH (Davis et al., 1987; Rudenko et al., 2002). The EGF precursor domain is composed of three EGF-like repeats, EGF-like 1, 2, and 3 each consisting of ∼40 amino acids. A domain of six YWTD motifs known as β-propeller domain occurs between repeats 2 and 3 (Springer, 1998; Jeon et al., 2001) (**Figure 1B**).

The *O*-linked sugars domain plays a major role in the post-translational modification of LDLR. This domain is encoded by exon 15 and is composed of 48 amino acids consisting of 18 threonine and cysteine residues that act as attachment sites for *O*-linked sugar chains. The membrane-spanning domain is responsible for LDLR integration and attachment to the cell membrane (Russell et al., 1984; Yamamoto et al., 1984; Südhof et al., 1985). Endocytosis of LDLR-LDL complex is mediated by the 5th domain in the LDLR which is the cytoplasmic tail encoded by exon 17 and exon 18 (Goldstein and Brown, 2009).

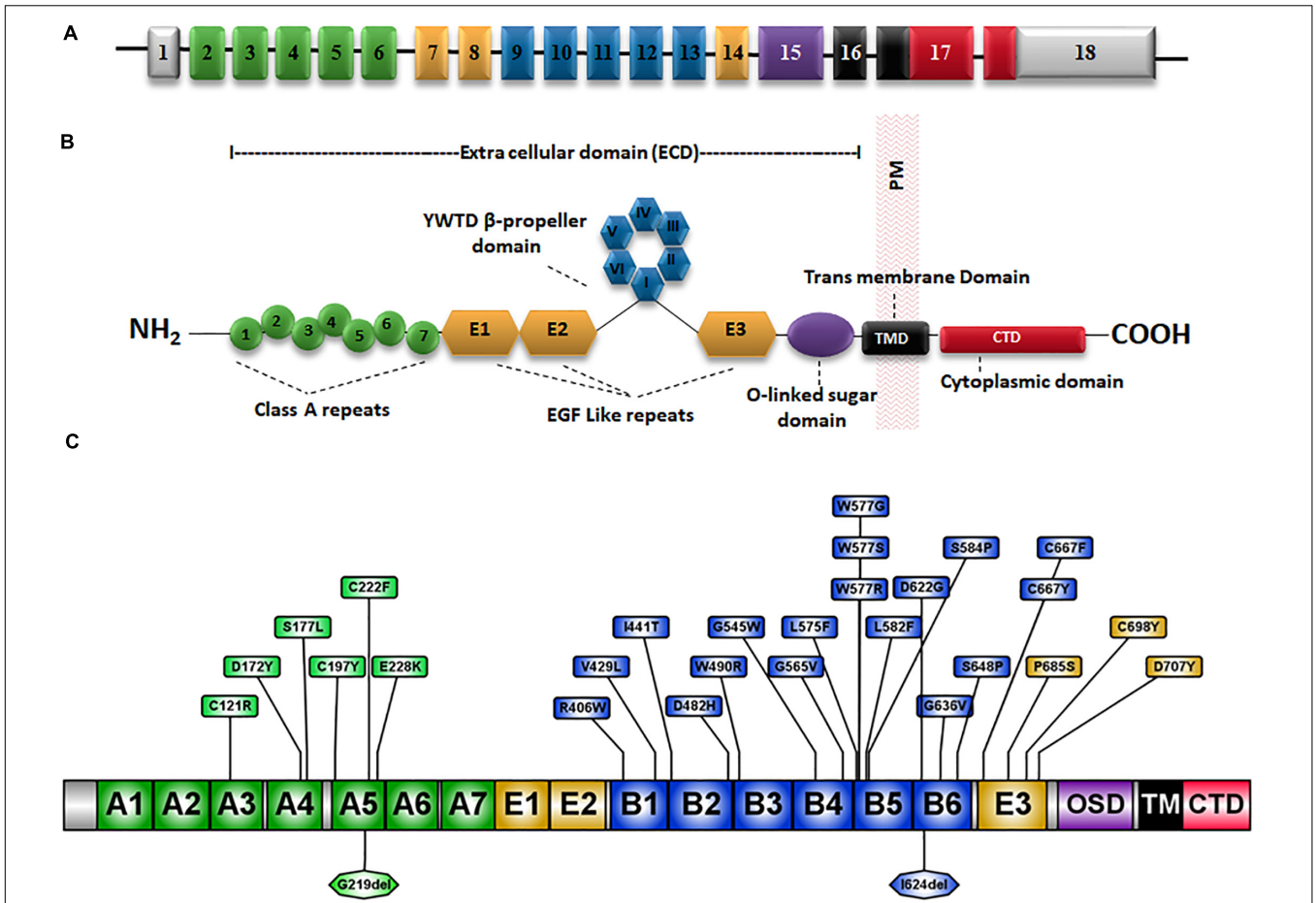

FIGURE 1 | Schematic representation of the *LDLR* gene and protein structure. **(A)** The 18 exons of *LDLR* are numbered and exons coding for different domains of the LDLR protein are represented by different colors. **(B)** The LDLR protein has an extracellular domain (ECD), a membrane-spanning domain (TMD) and a cytoplasmic *C*-terminal domain. The ECD consists of a ligand-binding domain (LBD), an epidermal growth factor (EGF) homology domain and an *O*-linked sugar region. The EGF homology domain is composed of three EGF-like repeats 1–3 and a β-propeller domain of six YWTD motifs occurs between repeats 2 and 3. **(C)** Schematic diagram showing the amino acid positions of well known class II mutations (**Table 2**) and the substitutions are marked in boxes. OSD, *O*-linked sugar domain.

LDLR Mutations-Types and Classifications

A total of 2,299 variants have been reported in association with FH in the Human Gene Mutation Database (HGMD) (Stenson et al., 2017), ranging between missense mutations, nonsense mutations, deletions, insertions or duplications. Based on functional consequences, LDLR mutations have been classified into five major classes (Hobbs et al., 1992), as described in detail in **Table 1**. Briefly, the functional impact of each classes are, Class I: Defects in synthesis of LDLR mainly due to null alleles; Class II: Impaired trafficking of the LDLR to Golgi compartments and cell surface, due to improper folding and complete or partial retention in the ER (2A and 2B, respectively); Class III: Deficient in binding to ligands; Class IV: Impaired clustering and endocytosis of ligand-bound receptors; Class V: Interferes with the cell-surface recycling of internalized LDLR due to defects in dissociation of ligand from the receptor, subsequently leading to the degradation of the receptor in the lysosome (Beglova et al., 2004; Van Hoof et al., 2005). Class VI is a new functional class of LDLR variants where the LDLR is properly synthesized by the ER and Golgi apparatus but fail to undergo basolateral sorting in polarized cells (Koivisto et al., 2001). Additional novel functional classes are emerging with increasing functional data (Susan-Resiga et al., 2017) and most recently a novel class of LDLR variants inducing ectodomain cleavage of the LDL receptor in the ER has been suggested (Strøm et al., 2014, 2017).

Around 50% of reported LDLR mutations are Class II mutants which are implicated to be transport-defective (Varret and Rabès, 2012). At present, there are 895 missense mutations reported in the HGMD (Stenson et al., 2017), occurring at 451 amino acid codons distributed across the whole length of the protein (**Supplementary Figure S1**). However, only limited information is available on the functional classes of these variants (Benito-Vicente et al., 2018). A compilation of functionally validated Class II mutations from the published literature is presented in **Table 2**.

TABLE 1 | Classes of LDLR variants.

LDLR variant classes	Type of variants	Protein/functional impact
Class I	• Early stop codons • Mutations in the promoter regions • Splicing aberrations • Large exonic deletions	Synthesis defective: Defects in LDLR protein synthesis
Class II: • Class II A • Class II B	• Missense mutations in the cysteine-rich domains • In-frame deletions/duplications • Protein truncating mutations	Transport defective: Defects in LDLR folding, maturation and transport in the secretory pathway Class IIA: Completely retained in the ER due to folding defects Class II B: Transport-competent but ER-retained due to slower processing
Class III	• Point mutations clustering in the ligand binding domain	Binding defective: Transport-competent but defective in binding to LDL
Class IV	• Mutations in the 4th and 5th domains • Complete deletion of those LDLR domains	Clustering and endocytosis defective: Impair with the clustering of ligand-bound LDLR in clathrin coated pits and endocytosis of LDLR-LDL complex
Class V	• Deletions in the EGF precursor domain	Dissociation and recycling defective: The LDLR-LDL complex is successfully internalized in the cell, but dissociation of the LDLR from the LDL does not happen leading to the degradation of LDLR along with LDL in the lysosomes (Beglova et al., 2004; Van Hoof et al., 2005)

A schematic representation of the position of occurrence of the reported variants is shown in **Figure 1C**. Unlike the other classes of mutants that interfere with a specific function of the receptor, class II mutations cause global conformation defects leading to their retention in the ER, potentially overwhelming the cellular proteostasis machinery in addition to impaired cholesterol homeostasis (Gent and Braakman, 2004).

MECHANISMS OF PROTEIN QUALITY CONTROL AND PROTEOSTASIS REGULATION IN THE ER

In eukaryotes, an estimated one-third of all newly synthesized proteins enter the ER to undergo post-translational modifications and achieve their three-dimensional native conformation, before reaching their proper cellular destination (Brodsky and Skach, 2011). However, protein folding is an inherently error-prone process and only a fraction of all produced proteins reaches a native conformation. Multiple stringent quality control mechanisms operates in the ER to ensure that only properly folded proteins are transported out of the ER and protein homeostasis or "proteostasis" is maintained (Sun and Brodsky, 2019). Many membrane and secretory proteins that fail to conform to the ER quality control (ERQC) are dislocated into the cytosol and degraded by the proteasome by a process termed as ER-associated degradation (ERAD) (Vembar and Brodsky, 2008; Ruggiano et al., 2014; Sun and Brodsky, 2019). Misfolded proteins can still retain their function and premature ERAD of mutant misfolded proteins is accounted for the cellular pathogenesis of several congenital disorders (Ward et al., 1995; Hume et al., 2009; Ali et al., 2011; Al-Kindi et al., 2014; Kizhakkedath et al., 2014, 2019; John et al., 2015). Sometimes the quality control mechanisms fail to recognize folding-incompetent forms which leads to the accumulation of folding-intermediates in the ER, causing ER stress. The cells respond to ER stress by initiating the unfolded protein response (UPR), an integrated stress response program, that aims to increase cell's folding capacity, accelerate clearance of unfolded proteins by ERAD, and restore protein homeostasis in the cell (Karagöz et al., 2019). Unresolved ER stress may lead to cell death (Karagöz et al., 2019). The ER-retained LDLR class II mutants have been reported to be degraded through a proteasome-mediated pathway (Li et al., 2004) and have been shown to activate ER-stress pathways (Sørensen et al., 2006).

Major Components of ERAD

ER-associated degradation is a collective term for a succession of events that starts with substrate recognition, followed by chaperone-assisted translocation to the cytosol and culminates in degradation by the ubiquitin-proteasome system (UPS) (Brodsky and Skach, 2011; Sun and Brodsky, 2019). Though complex, the fundamental ERAD machinery is conserved in eukaryotes from yeast to mammals (Brodsky and Skach, 2011; Sun and Brodsky, 2019). The folding of nascent polypeptides entering the ER is assisted by a chaperone system comprising of classical ER chaperones, lectin chaperones and protein disulfide isomerases (PDIs) (Braakman and Hebert, 2013). Classical chaperones belonging to the heat shock proteins (HSPs) family are GRP78/BiP (Hsp70), GRP94 (Hsp90), and J-proteins (Hsp40) (Braakman and Hebert, 2013). GRP78 recognizes and binds to misfolded proteins with exposed hydrophobic residues and helps in interaction other HSP chaperones and PDIs (Ni and Lee, 2007). *N*-linked glycosylation of Asn-X-Ser/Thr motif is an important post-translational modification that help nascent proteins to remain soluble and prevent aggregation by masking the hydrophobic stretches in the protein (Aebi et al., 2010). *N*-glycosylation involves the attachment of a preassembled

TABLE 2 | List of all functionally characterized Class II LDLR variants.

LDLR (NM_000527.4; NP_000518.1) class II variants with functional evidence	Variant class	References	Population frequency (gnomAD)	dbSNP ID
c.361T > C(p.C121R)	Class II	Guo et al. (2019)	N/A	rs879254492
c.514G > T(p.D172Y)	Class IIB	Jeenduang et al. (2010)	N/A	rs879254554
c.530C > T(p.S177L)	Class IIB	Li et al. (2004)	1.59E-05	rs121908026
c.590G > A(p.C197Y)	Class IIB	Li et al. (2004)	3.19E-05	rs376459828
c.665G > T(p.C222F)	Class IIB	Wang et al. (2014)	N/A	rs730882086
c.682G > A(p.E228K)	Class IIA	Li et al. (2004)	1.61E-05	rs121908029
c.1216C > T(p.R406W)	Class IIB or V	Benito-Vicente et al. (2015)	1.77E-05	rs121908043
c.1285G > C(p.V429L)	Class IIA	Etxebarria et al. (2014)	N/A	rs28942078
c.1322T > C(p.I441T)	Class IIA	Benito-Vicente et al. (2015)	N/A	rs879254862
c.1444G > C(p.D482H)	Class II	Kizhakkedath et al. (2019)	N/A	rs139624145
c.1468T > C(p.W490R)	Class IIA	Etxebarria et al. (2014)	N/A	rs730880130
c.1633G > T(p.G545W)	Class IIA	Benito-Vicente et al. (2015)	N/A	rs879254965
c.1694G > T(p.G565V)	Class II	Esser and Russell (1988)	N/A	rs28942082
c.1723C > T(p.L575F)	Class II	Jiang et al. (2016)	3.98E-06	rs1205480064
c.1729T > G(p.W577G)	Class IIA	Etxebarria et al. (2015)	N/A	rs879255000
c.1729T > C(p.W577R)	Class II	Schaefer et al. (2012)	N/A	rs879255000
c.1730G > C(p.W577S)	Class II	Holst et al. (2001)	7.95E-06	rs138947766
c.1744C > T(p.L582F)	Class II	Jiang et al. (2016)	N/A	rs1131692216
c.1750T > C(p.Ser584Pro)	Class IIA	Galicia-Garcia et al. (2020)	N/A	rs879255010
c.1775G > A(p.Gly592Glu)	Class IIB	Susan-Resiga et al. (2017)	5.66E-05	rs137929307
c.1865A > G(p.Asp622Gly)	Class IIA	Galicia-Garcia et al. (2020)	N/A	rs879255060
c.1907G > T(p.G636V)	Class IIB	Wang et al. (2014)	N/A	N/A
c.1942T > C(p.S648P)	Class IIB	Etxebarria et al. (2014)	N/A	rs879255079
c.2000G > T(p.C667F)	Class II	Kizhakkedath et al. (2019)	N/A	rs28942083
c.2000G > A(p.C667Y)	Class IIA	Li et al. (2004)	3.98E-06	rs28942083
c.2053C > T(p.P685S)	Class IIB	Etxebarria et al. (2014)	N/A	rs2569548
c.2093 G > A(p.Cys698Tyr)	Class IIA	Galicia-Garcia et al. (2020)	N/A	rs879255136
c.2119 G > T (p.Asp707Tyr)	Class IIA	Galicia-Garcia et al. (2020)	N/A	rs879255142
c.654_656delTGG (p.Gly219del)[a]	Class II	Omer et al. (2017)	2.79E-05	rs121908027
c.1871_1873delTCA (p.Ile624del)[a]	Class II	Etxebarria et al. (2015)	N/A	rs879255062
c.1878delA (p.Ala627Profs*38)[b]	Class II	Banerjee et al. (2019)	N/A	rs1057516134
c.2043C > A (p.Cys681Ter)[b]	Class II	Banerjee et al. (2019)	7.96E-06	rs121908031
c.2399_2403delTCTTCinsGGGT (p.Val800Glyfs*129)[b]	Class II	Etxebarria et al. (2015)	N/A	rs879255198
c.1885_1889delTTCAGinsGATCATCAACC (p.Phe629_Ser630delinsAspHisGlnPro)[c]	Class II	Shu et al. (2017)	N/A	N/A

[a] *In-frame amino acid deletions.* [b] *Protein truncating variant.* [c] *Complex deletion-insertion.*

carbohydrate, comprised of three glucoses, nine mannoses, and two *N*-acetyl glucosamines ($Glc_3Man_9GlcNAc_2$), to the Asn residue (Aebi et al., 2010).

Enzymatic deglucosylation of the *N*-glycan to $Glc_1Man_9GlcNAc_2$ by glucosidases I (GI) and 2 (GII), makes it a high-affinity ligand for lectin chaperones such as calnexin (CNX) and calreticulin (CRT). Binding of CNX/CRT to glycoproteins facilitates their retention in ER, prevention of aggregation and recruitment of PDIs such as ERp57 (PDIA3) (Zapun et al., 1998; Lamriben et al., 2016). Removal of the final glucose by GII prevents further binding CNX/CRT and if folded, the substrates progress toward ER exit sites. Unfolded proteins undergo further rounds of reglucosylation by UDP-glucose/glycoprotein glucosyltransferase (UGGT) and are reverted to CNX/CRT for folding. If folded the glycoproteins eventually exit the cycle (Lamriben et al., 2016) and terminally misfolded proteins are released from this cycle and diverted to ERAD for disposal. An example of glycoprotein folding is that of LDLR which is represented in **Figure 2**.

Demannosylation by the ER-resident mannosidases such as ER mannosidase 1 (ERMan1) and ER-degradation enhancing mannosidase-like proteins (EDEM1, 2, and 3) results in *N*-glycans with deglucosylated, demannosylated forms (Man5–Man7) that are incompatible with UGGT-mediated reglucosylation (Shenkman and Lederkremer, 2019). The deglycosylated and demannosylated misfolded proteins are selectively captured by the mannose-specific lectins (OS9 and XTP-3B) for their further delivery to the

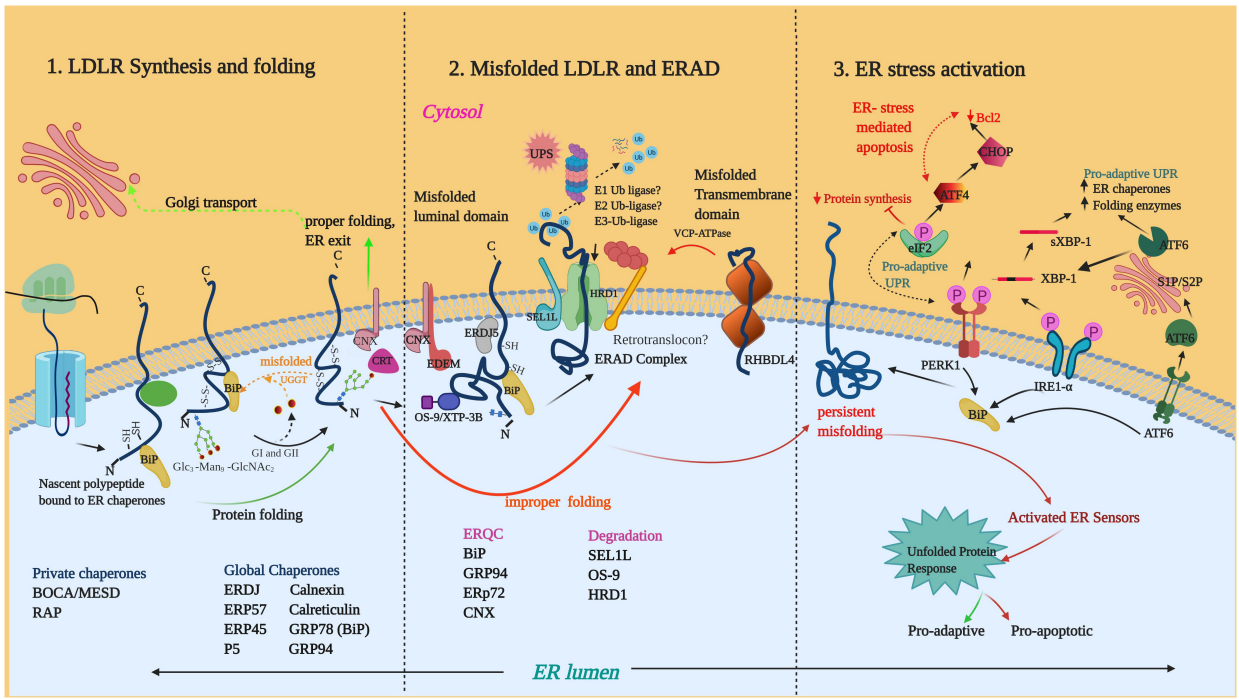

FIGURE 2 | LDLR folding, misfolding and activation of UPR: **(1)** The nascent LDLR is co-translationally inserted into the ER membrane and the LDLR ectodomain undergoes folding in the ER lumen with the assistance of several global and private chaperones as listed in the figure. **(2)** Misfolded proteins such as Class II mutants engage in prolonged interaction with the chaperone system. BiP, GRP94, ERP72 are ERQC factors implicated in LDLR retention. Terminally misfolded proteins are extracted from the chaperone system and delivered to membrane-embedded ERAD complex for degradation by the ubiquitin-proteasome system. So far, the components known to be involved in LDLR-ERAD are OS9, SEL1L and HRD1. RHBDL4 is a metalloprotease involved in the ERQC of ERAD-M candidates of LDLR. **(3)** Accumulation of misfolded LDLR induces ER stress and activates the UPR proteins IRE1, PERK, and ATF6. Phosphorylation of eIF2α by PERK leads to the attenuation of protein translation. Activated IRE1α induces splicing of the long XBP1 mRNA to form XBP1s mRNA which encodes XBP1s protein. Activated ATF6 is cleaved in the Golgi to form the active ATF6 N-terminal fragment. XBP1s and ATF6 are transcription factors that target the transcriptional induction of UPR target genes. Unresolved ER-stress turn-on proapoptotic pathways through the PERK-arm of the UPR. Illustration created with Biorender.com.

ER degradation machinery (Olzmann et al., 2013) (**Figure 2**). Non-glycosylated misfolded proteins are also targeted for ERAD and it is believed that features other than glycan trimming may contribute to their recognition (Okuda-Shimizu and Hendershot, 2007). Non-glycosylated proteins can be directly recruited by BiP (GRP78) and J-domain proteins to ERAD complex, bypassing the CNX pathway (Ushioda et al., 2013).

Once selected, the ERAD substrates are delivered to the cytosol for ubiquitination by E3-ubiquitin ligases and proteolytic degradation by the UPS. In yeast, specialized ERAD pathways exist to degrade misfolded proteins with defects exposed in the ER luminal (ERAD-L), transmembrane (ERAD-M), and cytosolic domains (ERAD-C) (Carvalho et al., 2006). An emerging body of evidence suggests that mammalian ERAD does not follow rigid rules for substrate engagement and an array of E3 ligases cooperates to complete the ERAD processing of substrates with diverse topologies (Christianson et al., 2011; Olzmann et al., 2013). Two well-known mammalian E3 ubiquitin ligases are polytopic RING domain ubiquitin ligases, HMG-CoA reductase degradation protein 1 (HRD1/SYVN1) and gp78/autocrine motility factor receptor (AMFR) (Fang et al., 2001; Nadav et al., 2003). E3 ligases such as RMA1 (RNF5), TRC8, TEB4 (MARCH IV) have been reported to be involved in the degradation of a limited number of ERAD clients (Olzmann et al., 2013).

Since the proteins targeted for degradation have diverse structures and topologies, distinct combinations of adaptors that recognize these features are recruited by the E3-ubiquitin ligases. In mammals, the transmembrane (TM) protein SEL1L works in conjunction with the HRD1 E3 ligase and is necessary to deliver the ERAD substrates from ER lectins (OS9, XTP) to HRD1 (Christianson et al., 2008; Hosokawa et al., 2008). Depletion of SEL1L has been reported to destabilize HRD1 and prevent the degradation of misfolded lumenal/TM proteins (Christianson et al., 2008; Horimoto et al., 2013; Bianchini et al., 2014; Kizhakkedath et al., 2018). Other mammalian ER adapters are ERLINSs, INSIGs and F-box proteins (Olzmann et al., 2013).

Derlin family of proteins DER1, 2, and 3 have been proposed to play a role in substrate dislocation through association with HRD1/SEL1L (Lilley and Ploegh, 2005). During dislocation, the disulfide bonds are reduced by oxidoreductase enzymes like ERFAD and ERDJ5 (Smith et al., 2011) and partially unfolded by rhomboid pseudoproteases such as Derlins, UBCA2 and UBXD8 (Olzmann et al., 2013). The

dislocation process is powered by the cytosolic valosin containing protein (VCP)/p97 ATPase (Guerriero and Brodsky, 2012). During dislocation, an E1 ubiquitin ligase enzyme activates ubiquitin and an ubiquitin-conjugating enzyme (E2 ligase) in conjunction with a ubiquitin ligase (E3) then transfer ubiquitin to the substrate (Christianson and Ye, 2014). UBA1 is a well characterized E1 ligase enzyme in humans and initially believed to be the only subtype of E1 ligases. Examples of mammalian E2 ligases are UBE2J1, UBE2J2 and UBE2G2. The ubiquitin-tagged substrates are then delivered to degradation by the 26S proteasome in the cytosol with or without the help of small cytosolic heat-shock chaperones (Christianson and Ye, 2014) (**Figure 2**).

Quality Control of Membrane Proteins With Defective Transmembrane Domains

The quality control of TM proteins containing defects in their membrane-spanning domain appear to be distinct from that of ERAD-L and ERAD-C substrates, since luminal or cytosolic factors do not have access to the domain location. An intramembrane rhomboid protease, RHBDL4 has been shown to cleave TM-anchors of unstable single-membrane spanning or polytopic membrane proteins in an ubiquitin-dependent manner and divert them to the UPS coupled to VCP/p97 ATPase (**Figure 2**) (Fleig et al., 2012). Recent studies have shown that the ERAD-M substrates containing less hydrophobic TM-domains get translocated entirely to the ER lumen leading to recognition by BiP and degradation through the canonical ERAD-L pathway (Feige and Hendershot, 2013). Another study proposed that degradation of an ERAD-M substrate containing an unassembled TMD was dependent upon ubiquitination on cytoplasmic lysine residues and occurs through a specific ERAD pathway that is mechanistically distinct from that which mediates degradation of membrane proteins with luminal folding defects (Briant et al., 2015).

Non-canonical ERAD Pathways

While ERAD is highly efficient in handling a variety of misfolded proteins, some membrane and soluble proteins form aggregates and place constraints on ER retrotranslocation machinery. These aggregates are diverted to the lysosome for degradation via alternative pathways collectively termed as ER-to-lysosome–associated degradation (ERLAD) (Houck et al., 2014; Fregno and Molinari, 2019; De Leonibus et al., 2019). ERLAD include (i) ER-phagy, (ii) microautophagy, and (iii) vesicular transport (De Leonibus et al., 2019; Fregno and Molinari, 2019). In ER-phagy, ER fragments are engulfed by a double membrane LC3-decorated autophagosome that fuses with the lysosome. Microautophagy is an ER autophagy where misfolded proteins segregated on ER exit sites (ERES) coated with LC3 and COPII, are directly engulfed by lysosomal invagination or protrusion. Vesicular transport is mediated by single membrane ER-derived vesicles that bud from the ER and fuse with endolysosomes decorated with LC3 (De Leonibus et al., 2019). In ER-phagy, membrane-embedded LC3-binding receptors regulate the delivery of ER-subdomains to lysosome. In mammals the ER-phagy receptors include FAM134B, RTN3, SEC62, CCPG1, ATL3, and TEX264 (Khaminets et al., 2015).

ERAD PROCESSING OF LDLR MUTANTS

Canonical Role of ERAD in the Regulation of Sterol Synthesis

Other than performing as a quality control checkpoint, ERAD plays a quintessential role in providing protein quantity control as well in response to environmental demands (Hegde and Ploegh, 2010; Printsev et al., 2017). Cholesterol metabolism and homeostasis are tightly regulated processes and ubiquitin-dependent protein degradation is involved in transcriptional regulation, the synthesis, efflux and uptake of cholesterol (Sharpe et al., 2014). At the transcriptional level, cholesterol metabolism is regulated by the opposing actions of two transcription factors, namely sterol regulatory element-binding proteins (SREBPs) and the liver X receptors (LXRs) (Sharpe et al., 2014). Under low cellular cholesterol levels, SREBPs are involved in the transcriptional induction of genes required for *de novo* biosynthesis of cholesterol and LDLR for the uptake of cholesterol (Innerarity et al., 1990). Under elevated cellular cholesterol levels, LXRs induce genes involved in cholesterol efflux pathways and degradation of LDLR (Nadav et al., 2003).

The most widely known example of quantity control by ERAD is the post-translational feedback-regulation of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), a rate-limiting enzyme in the mevalonate pathway which produces cholesterol and other isoprenoids (DeBose-Boyd, 2008). The accumulation of sterols in ER membranes triggers the binding of HMGCR to ER-membrane proteins INSIG1 and INSIG2 which in turn recruit ubiquitin ligases GP78, TRC8, and RNF145 (Jo et al., 2011; Menzies et al., 2018). Ubiquitinated reductase is then extracted by VCP ATPase and delivered to the proteasome (Jo et al., 2011). Squalene monooxygenase/Epoxidase (SQLE) is another rate-limiting enzyme in the mevalonate pathway downstream of HMGCR and recent studies have shown that another ER-resident E3 ligase, MARCH6 is involved in the ubiquitin-proteasome degradation of SQLE (Loregger et al., 2015). MARCH6 is postulated to play a multifaceted role in cholesterol homeostasis as an endogenous negative modulator of SREBP and HMGCR (Loregger et al., 2015). The E3 ligases FBW7 and RNF20 are involved in the ubiquitin-dependent regulation of SREBPs (Sundqvist et al., 2005; Lee et al., 2014; Sharpe et al., 2014). Inhibition of cholesterol synthesis activates SREBP and transcriptional upregulation of LDLR.

Other than transcriptional regulation by SREBP, LDLR is post-translationally regulated by ubiquitin-dependent degradation mediated by the E3 ligase- inducible degrader of the LDLR (IDOL) (Zelcer et al., 2009). IDOL is transcriptionally controlled by LXRs and appear to preferentially ubiquitinate the cytoplasmic tails of plasma-membrane localized LDLR and mediate lysosomal rather than proteasomal degradation of the receptor (Zelcer et al., 2009). Interestingly, IDOL was found to be capable of regulating the ER located LDLR forms also, since a

Class II LDLR mutant G546D was demonstrated to be degraded by IDOL by a lysosomal pathway (Zelcer et al., 2009).

Molecular Players in the LDLR Folding Pathway

The LDLR receptor family has a modular organization consisting of LDL-repeats, EGF-like repeats with β-propeller, a single TM domain, and a small cytosolic tail (**Figure 1B**) (Gent and Braakman, 2004). Even though the different domains are organized from NH_2-to-COOH terminus and the folding is co-translational, the nascent LDLR polypeptide is demonstrated to fold rapidly into compact structures by forming non-native disulfide bonds linking distant domains of the receptor (Jansens et al., 2002; Gent and Braakman, 2004). The non-native disulfides are later isomerized and native short-range disulfide bridges are formed with high efficiency and rarely lead to aggregate formation. The high-efficiency folding of LDLR requires the assistance of several general and private chaperones (Garcia et al., 2001). The HSP chaperone GRP78 and PDI family member ERDJ5 have been reported to be involved in the folding of LDLR (Gent and Braakman, 2004; Oka et al., 2013).

ERDJ5 (DNAJC10) is an ER-localized oxidoreductase containing J domain and thioredoxin domains important for its disulfide exchange activity (Oka et al., 2013). ERDJ5 is known to participate in the degradation pathway of misfolded proteins by reducing the disulfide bonds prior to retrotranslocation (Ushioda et al., 2008). ERDJ5 has been proposed to take part in the processing of non-native disulfide bonds in LDLR which is required for the native disulfide formation and proper folding (Oka et al., 2013). Another member of the PDI family of oxidoreductases, ERP57 is involved in the native disulfide bond formation of substrates in the ER and functions closely with both CNX and CRT (Jessop et al., 2007). ERP57 is indicated to be important for the isomerization of non-native disulfide bonds in LDLR (Jessop et al., 2007). Other PDI family members such as P5 and ERP45 are also reported to exhibit substrate specificity toward LDLR (Jessop et al., 2009).

In addition to the aforementioned chaperones, several private chaperones are involved in LDLR folding. The LBD of LDLR family members require the assistance of the receptor associated protein (RAP) for maturation, which prevents premature interaction of the domain with its ligands in the same compartment (Herz and Marschang, 2003). The BOCA/MESD family of chaperones is shown to be specifically required for the folding of the β-propeller domain that is contained within the EGF precursor homology region of LDLR (Culi et al., 2004). Calcium has been shown to an absolute requirement for LDLR folding in the ER and lack of calcium, even at very early folding stages, was reported to result in irreversible misfolding of the wild-type protein (Pena et al., 2010). A detailed depiction of LDLR folding is presented in **Figure 2**.

Proteostasis Components Involved in the Degradation of LDLR Class II Mutants

It was demonstrated that different Class II mutants of LDLR affecting the LBD (S156L, C176Y, and E207K) and EGF domain (C646Y) were retained in the ER and degraded by a proteasome-dependent pathway in cell lines stably expressing the mutants (Li et al., 2004). One of the first ER factors discovered to be involved in the retention of mutant LDLR was the molecular chaperone GRP78/BiP (Jørgensen et al., 2000). In human liver cells overexpressing the wild-type and mutant receptors (W556S and C646Y), GRP78 strongly interacts with mutant LDLR whereas GRP78-wild type LDLR interaction is weak, suggesting a key role for this chaperone in ER-retention/quality control of class II LDLR mutants (Jørgensen et al., 2000). However, the overexpression of GRP78 was not capable of rescuing the mutants from ER retention. Nevertheless, abundant GRP78 reduces the processing time of newly synthesized wild type LDLR, suggesting GRP78 is critical in protein maturation of wild type LDLR. Other chaperones GRP94, ERP72 (PDIA4), and CNX have also been found to associate with class II mutants (G544V) but not with wild type LDLR (Sørensen et al., 2006). Recently we have also reported that three LDLR class II variants were found to be associated with ER chaperones: GRP78 (BiP), GRP94, the lectin chaperone CNX (Kizhakkedath et al., 2019). In cells overexpressing the G544V mutant, the ER-retention of the mutant was shown to induce ER-stress and activation of UPR as evidenced by the upregulation of mRNAs for GRP78, GRP94, ERP72, attributed to the activity of ER sensors IRE1 and PERK (Sørensen et al., 2006). Apart from its chaperoning activity, GRP94 also has a very specific role in the maturation and stability of wild-type LDLR, as it was shown to protect LDLR from PCSK-mediated degradation (Poirier et al., 2015).

Very little information is available on the ERAD components involved in the substrate recognition, retrotranslocation and degradation of LDLR mutants. Recent results from our lab indicate that the LDLR mutants interact with HRD1 and its partners SEL1L and OS9 (Kizhakkedath et al., 2019). Our results also demonstrated that proteasomal inhibition leads to stabilization of the ER-retained mutants, but had no effect on their folding. Further, inhibitors of ER mannosidase 1 also had a stabilizing effect on the mutants (Kizhakkedath et al., 2019). ER-retained variants of VLDLR, another LDLR family member, were also found to be degraded by the HRD1-SEL1L mediated proteasomal degradation (Kizhakkedath et al., 2018). Unlike VLDLR mutants, the ER retained LDLR mutants were not observed to be aggregation-prone, though overexpression of mutants caused ER stress (Kizhakkedath et al., 2019). The ERAD adaptor protein SEL1L is reported to also play an ERAD independent role in the maturation and processing of lipoprotein lipase (LPL) and hepatic lipid metabolism (Sha et al., 2014). The cell-surface rescue of an ER-retained LDLR mutant was demonstrated to be possible by the use of a chemical chaperone 4-phenylbutyrate (4-PBA) (Tveten et al., 2007). It was later revealed that 4-PBA targets COPII protein and reduces the stringency of ER-retention of misfolded substrates (Ma et al., 2017). It was suggested that stringent ER retention of misfolded substrates requires the efficient packaging of p24-family of proteins via the B site of the COPII coat and 4-PBA competes with p24 and reduces this stringency (Ma et al., 2017). The available information about the ERAD of misfolded LDLR class II mutants are limited and more detailed investigations utilizing cellular

models derived from FH patients or model systems expressing physiological levels of LDLR mutants are still required to enhance our understanding of the specificities. However, it is likely that many of the ER factors involved in the folding pathway and physiological quantity control of LDLR participate in some of these processes (**Figure 2**).

The role of non-canonical ERAD pathways in the degradation of LDLR class II mutants have not been explored to our knowledge. We have reported previously that a small fraction of ER-retained VLDLR missense mutants are aggregation-prone and may undergo an autophagy-related process for degradation (Kizhakkedath et al., 2018). It is probable that aggregation-prone class II LDLR mutants also might undergo non-canonical ERAD.

A Possible Role of ERAD Components Implicated in Misfolded Membrane Proteins in the ERAD of LDLR Mutants

The mechanisms by which the mutations affecting the TM domain of LDLR cause FH are only emerging. It was reported that a mutation affecting the TM domain of the LDLR (G805R), undergoes ectodomain cleavage by a metalloproteinase in the ER and results in lower LDLR levels at the cell surface (Strøm et al., 2014). The ER-resident rhomboid protease RHBDL4 is proposed as a likely candidate for this metalloproteinase. The cleaved ectodomain however does not undergo proteasomal degradation, instead, appear to pass through the secretory pathway and eventually get secreted to the extracellular space (Strøm et al., 2014). Subsequent studies revealed that many mutations affecting the TM domain of LDLR interfere with membrane-insertion of LDLR are subjected to diverse processes such as metalloproteinase cleavage, complete extracellular secretion or rapid degradation at the cell surface (Strøm et al., 2015). The underlying mechanisms of low cell-surface expression of some of these mutants were elusive (Strøm et al., 2017) and it has been proposed that the mutations affecting the TM domain of LDLR must therefore be considered to be a separate class.

DEREGULATION OF ER HOMEOSTASIS AND ACTIVATION OF UPR BY LDLR MISSENSE MUTANTS

Accumulation of unfolded proteins in ER activates a battery of cellular stress responses, altogether called as UPR. The UPR aims to restore the normal ER-homeostasis, however, if the stress is severe and irreversible, then UPR switches to apoptosis. Sørensen et al. (2006) for the first time reported that overexpression of LDLR mutants causes ER-stress and elicit UPR (**Figure 2**). Recent studies from our lab have also confirmed that LDLR mutants retained in ER results in the activation of UPR (Kizhakkedath et al., 2019). However, quite surprisingly, studies focusing on ER-stress, especially the link between ER-stress activation and cellular signaling process that modulate cell fate are missing. These studies are significantly relevant in understanding the molecular pathology of FH where liver damage due to cell death is a critical factor. The following is a detailed picture of the research that has been done pertaining to ER-stress in cell line models expressing mutant LDLR. We also briefly discuss the contradicting findings in stem cell model of FH where ER-stress is not activated when mutant LDLR is expressed.

UPR: An Overview

Endoplasmic reticulum serves as a site for protein synthesis, folding as well as the internal cellular calcium reservoir and hence plays a critical role in cell physiology. Disruption of ER homeostasis due to derailed calcium physiology; redox imbalance; accumulation of misfolded proteins causes ER-stress (Almanza et al., 2019). Cells respond to ER-stress by activating components of counter stress response mechanisms together named UPR. UPR predominantly involves the shutdown of protein translation to reduce further protein load in ER, transcriptional upregulation of ER chaperones to assist protein folding and retrotranslocation of irreversibly misfolded proteins via ERAD. UPR is initially aimed to alleviate ER-stress and regain the normal ER-physiology. However, if the stress persists and the damage is irreversible, then the initial adaptive UPR switches to ER stress-induced apoptosis (Szegezdi et al., 2006; Almanza et al., 2019). UPR is initiated by three major ER stress sensors; PERK, IRE1, and ATF6. The three ER stress sensors are maintained inactive in resting cells by binding to ER chaperone BiP. During ER stress, BiP, which has more affinity to misfolded proteins detaches from the ER sensors and causes the activation of the latter. The concerted cellular response to ER stress is largely mediated by these sensors (Almanza et al., 2019).

Activated PERK phosphorylates the eukaryotic initiation factor 2 alpha (eIF2 alpha) (Schröder and Kaufman, 2005). Phosphorylation of eIF2 blocks cap-dependent translation and thereby reduce further protein load in the ER. Interestingly, certain mRNAs such as ATF4 which possess internal ribosome entry sites at 5′ at their untranslated regions can bypass the PERK-eIF2 alpha pathway mediated translational block. ATF4 up-regulates the expression of ER chaperones as well as induces CHOP, a pro-apoptotic transcriptional factor that induce apoptosis by repressing the anti-apoptotic protein Bcl-2. Initial PERK activation mounts a pro-survival adaptive UPR, however, persistent activation of PERK due to unresolved ER-stress leads to ATF4 mediated transcriptional induction of CHOP which switches initial adaptive UPR to ER-stress mediated apoptosis. ATF6 is cleaved by two Golgi resident proteases named site-1 and site-2 to generate an active transcriptional factor which induces the expression of ER chaperones and folding enzymes. Apart from the ER chaperones, ATF6 induces the upregulation of XBP1 mRNA, which is further processed by splicing into a smaller mRNA (XBP1s) by the ribonuclease activity of IRE-1, another ER stress sensor. Similar to ATF-6, the protein encoded by XBP1s is an active transcription factor which induces the expression of ER chaperones, folding enzymes and ERAD components (**Figure 2**).

Activation of UPR is aimed to resolve the stress in ER and bring back the normal ER homeostasis. Notably, major targets of all three ER stress sensors are directed to mitigate any harmful consequences of ER stress. However, when stress is overwhelming and beyond the capacity of ER adaptive stress machinery then the initial adaptive UPR switches to ER-stress activated apoptosis.

Persistent activation of three major ER sensors results from unresolvable ER-stress which activates ER-apoptotic signaling principally mediated by pro-apoptotic proteins involving JNK, CHOP and BCL-2 family proteins (Szegezdi et al., 2006). ER-stress induced apoptosis has been implicated as a contributing factor in the pathophysiology of cardiovascular diseases and liver fibrosis (Maiers and Malhi, 2019), two major disease conditions manifested in FH patients.

ER-Stress and Activation of UPR in Cell Line Models Expressing LDLR Mutants

Surprisingly, only few studies pertaining to the association of ER-stress with the molecular pathology of FH have been reported (Jørgensen et al., 2000; Sørensen et al., 2006; Kizhakkedath et al., 2019) in contrast to the fact that around 50% of LDLR mutations implicated in FH are class II mutants that are retained in ER due to misfolding. Two decades back, an interesting study by Jorgensen et al. demonstrated ERQC system play a critical role in the proteostasis of class 2 mutant LDLR proteins (Jørgensen et al., 2000). However, it was Sørensen et al. who established for the first time in 2006 that ER-retained LDLR mutants activates ER-stress (Sørensen et al., 2006). Detailed investigations on the activation of UPR by LDLR mutants revealed transcriptional induction of ER chaperones as well as the activation of three UPR sensors (Sørensen et al., 2006).

Our lab has recently reported that two missense LDLR mutants D482H and C667F associated with FH were misfolded and retained in ER (Kizhakkedath et al., 2019). Further analysis of ER-stress markers in cells expressing the aforesaid mutants pointed activation of UPR. Similar to the previous reports (Jørgensen et al., 2000; Sørensen et al., 2006), we also found ER-retained LDLR mutants strongly interact with GRP78 and other ER chaperones suggesting critical role of these chaperones in ER retention and further ERAD processing of LDLR mutants. Interestingly, our studies disclosed ER-retained LDLR mutants remained soluble in ER lumen which indicates UPR mediated induction of ER chaperones successfully chaperone the mutants and block protein aggregation. However, despite the induction of ER chaperones by UPR, mutant LDLR were not folded and transported to the cell surface. It is well known that ER stress diminish the ERAD capacity of ER and UPR mediated transcriptional induction of ER chaperones is required to sustain the ERAD machinery (Travers et al., 2000). The inferences from ER-stress studies in cell line models overexpressing LDLR mutants suggest that the activation of UPR augment ERAD process where cells can eliminate the unfolded LDLR mutants via ERAD and thereby mitigate toxic ER stress which otherwise activates cell death.

Another important aspect to consider is the role of ER-synthesized sterol regulators such as proprotein convertase subtilisin/kexin type 9 (PCSK9) in the ER physiology of class II LDLR mutant expressing cells. Both PCSK9 and LDLR are transcriptionally upregulated by SREBP2, an ER-resident transcriptional factor that binds to sterol regulatory elements in the promoter regions of sterol inducible genes including LDLR and PCSK9 (Maxwell et al., 2003). PCSK9 is expressed as a pro-form which is autocatalytically processed in ER and the active form is secreted (Seidah et al., 2003). Interestingly, PCSK9 targets surface expressing LDLR for degradation and negatively modulates the latter's function. Decreased LDLR surface expression and increased serum LDL levels have been reported in FH patients with gain-of-function mutation in PCSK9 (Abifadel et al., 2003; Cameron et al., 2006). In contrast, the African population harboring loss-of-function PCSK9 mutants (unprocessed) have been reported to have lesser occurrence of cardiovascular diseases due to increased expression of surface LDLR and reduced serum LDL levels (Cohen et al., 2005). The aforementioned findings had also raised important questions on how cells manage the ER accumulation of unprocessed PCSK9 mutant pro forms. Does this cause ER-stress? Also, it had been quite intriguing how LDLR and PCSK9 co-exist in the same secretory pathway despite the former being a target of the latter. Hence it was widely speculated that PCSK9 would have interacting protein partners in ER. It was until 2015, Poirier et al. (2015) demonstrated that GRP94, an ER chaperone specifically interacts with PCSK9 and blocks its interaction with LDLR. Interestingly, it was later identified that GRP94 chaperones PCSK mutant pro-forms in ER and alleviate the potential toxic ER-stress (Lebeau et al., 2018). The underlying mechanism was further delineated to GRP94 interaction with mutant PCSK9 which prevents the latter's binding to GRP78, an ER luminal chaperone that signals proteotoxic stress in ER to major ER stress sensors (Lebeau et al., 2018). In a way, contradicting to the blocker role of GRP94 in PCSK9-LDLR interactions, PCSK9 is also reported to act as a chaperone for LDLR. In ER, PCSK9 binds to LDLR and aids the transport of the latter (Stroøm et al., 2014). Interestingly, binding to LDLR augments the autocatalytic processing of PCSK9. However, it is important to note that neither the PCSK9 mutants chaperone LDLR nor the class II LDLR mutants are chaperoned by PCSK9. In fact, reduced levels of PCSK9 has been reported in FH patients harboring class II LDLR mutants (Cameron et al., 2012). It is yet to be determined how GRP94 modulates PCSK9-LDLR or PCSK9-class II mutant LDLR interactions. It is also worthwhile to investigate the efficiency of PCSK9 processing in hepatocytes homozygous for class II mutant LDLR. The other cardinal question is whether class II mutant LDLR causes PCSK9 pro-form accumulation and consequent ER-stress which further compound the already de-regulated ER homeostasis in FH patients. It remains largely unknown whether the mutual dependence of these functionally antagonizing proteins (PCSK9 and LDLR) contribute to ER physiology and cholesterol homeostasis in FH patients.

Interestingly, ER stress induced by pharmacological ER stress inducers appear to inhibit the secretion of PCSK9 due to their retention in the ER by GRP94 (Lebeau et al., 2017). This is an interesting finding since LDLR class II mutants are known to induce ER-stress and it remains to be found whether PCSK9 is retained and non-functional in this context. Chemical chaperones or pharmacological chaperones (PCs) have been identified as a promising new strategy to re-instate ER-Golgi-cell surface transport of ER-retained mutant proteins including class II LDLR mutants that retain their original biological function to

some extent (a detailed review is performed in the coming section "Therapeutic Potential of Pharmacological Chaperones (PCs) and Proteostasis Regulators (PRs) in the Disease Management of FH"). Does PCSK9 remains ER-retained and non-functional when chemical chaperones are used to target LDLR mutants? The absence of functional PCSK9 might be an added advantage as it increases the number of cell surface LDLRs re-instated by the intervention of PCs. Taken together, the activation of ER stress by LDLR mutants and the inhibition of PCSK9 secretion by ER-stress activation can be well exploited for therapeutic management of FH. However, it has to be determined, to what extent ER stress is activated in cells of FH subjects, whether it is in the adaptive range where cellular UPR is equipped to manage the constant levels of ER stress or a severe irreversible ER stress where adaptive UPR response switches to apoptosis.

Also, it is quite important to note that in protein conformation diseases such as alpha-1 antitrypsin deficiency, ER-stress induced apoptosis plays a critical role in the molecular pathology associated with liver failure (Lawless et al., 2004). Adding further, ER-stress has been implicated in the pathology of various diseases such as diabetes, cystic fibrosis and neurodegenerative diseases (Almanza et al., 2019). It remains to be identified whether ER-stress play any role in the pathogenesis of class II mutants associated with FH. In order to establish a link between ER-stress and FH pathology, lymphocytes and fibroblast from FH patients expressing class two LDLR mutants have to be studied for ER-stress activation. It is known that cellular consequence to misfolded proteins retained in ER varies depending on the: mutations, tissue types, between physiological conditions of the same patient (Kim, 1998). It has to be identified whether ER-stress response to various LDLR mutations has any role in the phenotypic variation between FH patients. It is also an informed presumption that UPR activated in cells expressing mutant LDLR aids cell survival by eliminating the misfolded via ERAD. However, this has to be proved in cells from FH patients.

ER-Stress Studies in Induced Pluripotent Stem Cells (iPSCs) Model for FH Expressing Class II Mutant LDLR

Induced Pluripotent Stem Cell (iPSCs) have been developed by reprogramming fibroblasts from a FH patient carrying a homozygous three-base pair deletion in LDLR exon 4. The mutation results in ER-retention of LDLR and hence comes under class II mutation (Omer et al., 2017). Apart from being a clinically relevant model, class II iPSCs also shows potential for stem cell-based therapy for FH. Genome editing mediated by CRISPR-Cas9 tool successfully corrected the mutation and rescued LDLR function. An interesting recent study by the same group illustrated that FH class II iPSCs and hepatocytes derived from these iPSCs elicit no ER-stress response when LDLR mutants are induced by statins (Omer et al., 2020). Statins are drugs extensively used to reduce serum LDL-cholesterol. They inhibit the enzyme HMG-CoA reductase involved in the synthesis of mevalonate from which the body makes sterols including cholesterol. Statins are known to up-regulate the expression of LDLR. Adding further, the study reveals statin mediated induction of LDLR is higher in FH class II iPSCs compared to the CRISPR corrected ones (Omer et al., 2020). However, the induced mutant LDLR which is trapped in ER elicits no ER-stress response. The report surprisingly contradicts other studies including from our lab where UPR is activated upon the expression of mutant LDLRs (Sørensen et al., 2006; Kizhakkedath et al., 2019). Although ER-stress biology of stem cells is yet to be fully understood, iPSCs are capable of activating UPR in response to pharmacological inducers of ER stress such as tunicamycin. Statins do inhibit UPR in some models, however, lipoprotein deficient serum, which is also known to induce LDLR expression, fails to activate UPR in FH class II iPSCs (Omer et al., 2020). The absence of UPR in response to mutant LDLR accumulation in ER is intriguing and one elementary clarification is that, the amount of induced LDLR mutants falls below the threshold to induce any considerable ER-stress response. It is also possible that the particular mutant used in this study (Omer et al., 2020) is only partially retained in the ER as one can infer from the data where there is a significant presence of the mature form of LDLR in response to statin treatment. The other point to be considered is the efficient removal of mutant LDLR by ERAD and therefore less likely to induce any considerable amount of ER stress. Studies involving analysis of glycosylation status and protein turn over kinetics would clarify whether the accumulated LDLR is completely or partially retained in ER. In cell line overexpressing models, LDLR mutants are expressed from CMV promoters and each cell carries more than one copy of the plasmid and consequently, the protein is expressed in enough quantity to mount an ER-stress response. A comparative protein expression study involving cell line overexpression models and FH class II iPSCs is required to ascertain the above-said assumption.

THERAPEUTIC POTENTIAL OF PHARMACOLOGICAL CHAPERONES (PCs) AND PROTEOSTASIS REGULATORS (PRs) IN THE DISEASE MANAGEMENT OF FH

Research underpinning the molecular pathology of FH from various labs including our group have demonstrated that class II mutations in LDLR cause misfolding, ER retention and consequent protein degradation via ERAD (Sørensen et al., 2006; Kizhakkedath et al., 2019). As abundantly mentioned in this review, ER protein quality control systems (ERQC) maintain proteostasis by facilitating protein folding and eliminates misfolded proteins via ERAD. Sophisticated ERQC comprises ER chaperones as well as the protein components of UPR, ERAD and cytosolic proteasomal degradation machinery. Decades-long research in ERQCs led to the development of various proteostasis regulators (PRs) and PCs that either positively or negatively modulates ERQC components (Gámez et al., 2018). Currently, the first-line therapy for FH include statins which either exert their lipid-lowering effect through the inhibition of HMG-CoA reductase or via SREBP activation, which in turn induces LDLR expression. However, SREBP2 also induces the expression of

PCSK9, which targets LDLR for lysosomal degradation (Dubuc et al., 2004). Therefore, novel targets for the modulation of LDLR expression and function are increasingly being sought as a supplementation therapy with statins.

Multiple reports are available evidencing the successful application of PCs and PRs in various protein conformation diseases (Mu et al., 2008; Mohamed et al., 2017; Gámez et al., 2018). This suggests that PCs and PRs are promising candidates in the clinical management of FH. It is interesting to note that the idea of using PCs or PRs emerged from an early observation where ΔF508 CFTR, a single phenylalanine deletion mutant found in more than 85% of cystic fibrosis alleles was functional when expressed in *Xenopus* oocyte grown at room temperature (Drumm et al., 1991). Normally, ΔF508 CFTR is misfolded and retained in ER followed by degradation via ERAD. The functional correction of ΔF508 CFTR at low temperature was later established *in vitro* in cell lines (Denning et al., 1992). This suggests that restoration of protein transport and functionality of ER-retained mutants can be achieved by modulating proteostasis.

Proteostasis regulators are very often small molecule modulators of protein homeostasis (Balch et al., 2008). PRs predominantly act by manipulating the cellular stress response pathways including UPR. PRs have been proved to rescue misfolded proteins from ER retention either by modulating ERAD or by enhancing the expression of ER/cytosolic chaperones. PRs such as celastrol, curcumin, and HSP90 inhibitors induce the expression of cytosolic chaperones, Kifunensine and Eeyarestatin I inhibit ERAD, thapsigargin modulates calcium signaling and activates UPR (Wang et al., 2011; Gámez et al., 2018).

Unlike PRs, PCs by itself act on the target misfolded proteins and tilt the equilibrium toward the folding state (Ringe and Petsko, 2009). Interestingly, PCs that have been successfully developed for misfolded enzymes are their substrate variants. Substrate binding sites or active binding sites of enzymes are generally formed by more than one domain and therefore PCs bind and aid interaction between protein domains and thereby assist correct folding (Ringe and Petsko, 2009; Gámez et al., 2018). Natural co-factors and ligands are also being used as PCs for various protein conformation diseases. PCs are often protein-specific and sometimes mutation specific. However, cases have been reported where the same PCs are found effective for various mutants of the same protein (Conn and Janovick, 2009). Adding further, the combined application of PRs and PCs can be more effective as PRs increases the cellular chaperonic capability/QCS whereas PCs increases the availability of active folded missense proteins (Gámez et al., 2018).

Potential of PRs and PCs in Treating FH

The 4-phenyl butyric acid (4-PBA), a low molecular weight bipolar fatty acid derivative appears to rescue ER-retained transport defective class II mutant LDLR G544V in cell line overexpressing model. Although, 4-PBA mediates the rescue of only 30% of mutant LDLR expressed, the rescued LDLR mutant is expressed on the cell surface and capable of LDL binding and internalization compared to wild type (Tveten et al., 2007). Interestingly, the rescue effect is mutation-specific as other class II LDLR mutants are not rescued by 4-PBA. Recently, it has been demonstrated that 4-PBA act on the COPII machinery to promote the ER exit of the G544V mutant (Ma et al., 2017). 4-PBA has also been shown to mitigate ER-stress in animal models of neurodegenerative disorders and type 2 diabetes (Özcan et al., 2006; Bondulich et al., 2016). However, despite the promising outcome from the 4-PBA studies, no further studies have been done at preclinical level. It is also notable that very limited studies have been performed regarding the potential of established PRs and PCs in functionally rescuing the class II LDLR mutants.

Our lab has been studying proteostasis regulation of various missense mutants implicated in various genetic diseases including FH. We have demonstrated that the genetic ablation of ERAD components leads to the stabilization of ER-retained VLDLR missense mutants (Kizhakkedath et al., 2018). However, we are yet to demonstrate whether the rescued mutants are functional. Pharmacological inhibitors of ERAD such as Kifunensine and Eeyarestatin I are found to functionally rescue missense mutants associated with lysosomal storage diseases (Wang et al., 2011). Kifunensine inhibits Mannosidase which is a critical component in the recognition of misfolded proteins that are marked for ERAD. Eeyarestatin I blocks the extraction of ubiquitinated proteins from the ER membrane by inhibiting p97 ATPase activity (Wang et al., 2011). Even though we have demonstrated the stabilization of LDLR missense mutants in cell lines (Kizhakkedath et al., 2019), it is yet to be studied whether the aforementioned small molecule ERAD inhibitors have any impacts on the functional rescue of LDLR mutants.

The UPS is the end component of ERAD where the retrotranslocated misfolded proteins are finally processed. We and others have shown that proteasome inhibitors such as MG132 aid protein transport and significantly improve the protein function of missense mutants which otherwise retained in ER and subsequently subjected to ERAD (Wilke et al., 2012; Kizhakkedath et al., 2018). It is quite intriguing how inhibition of proteasome rescues misfolded proteins from ER retention and subsequent ERAD. As reported earlier (Pirkkala et al., 2000), inhibition of proteasome causes perturbation in proteostasis which elicit proteotoxic stress response with transcriptional induction of chaperones. The increase in chaperone reservoir aid protein folding and thus probably explains the partial rescue of ER-retained misfolded proteins and consequent transport to its destined locations. From our studies and other published reports, one can presume that partial functional rescue of ER retained mutant proteins by ERAD blockers and proteasome inhibitors are either mediated by the increased expression of chaperones due to perturbance in proteostasis or the increased boarding of misfolded proteins in ER due to the blocked ERAD. The increased duration of misfolded proteins in the ER lumen might increase their chances to get transported out to Golgi. It would be interesting to investigate these aspects. The exact mechanism by which the inhibition of ERAD or proteasome leads to functional rescue of misfolded proteins is yet to be studied in depth. Proteasome inhibitors such MG132 acts on multiple pathways, the synergistic effect of which has been shown to be protective during acute

myocardial ischemia (Yu and Kem, 2010). The cardioprotective mechanisms mediated by MG132 were proposed to be through degradation of IkB (inhibitory kB), GRK-2 (G-protein-receptor kinase 2), ARC (apoptosis repressor with caspase recruitment domain), and also by induction of HSP (Yu and Kem, 2010). In HepG2 cells, other than proteasomal inhibition, MG132 has been shown to enhance LDL uptake by upregulating LDLR mRNA expression through a PKC-dependent pathway. An unexpected effect of MG132 was the suppression of PCSK9 expression, which aided in decreased LDLR degradation and enhanced LDL uptake (Yan et al., 2014). Curcumin is a plant-derived natural polyphenolic compound which has been demonstrated to induce HSPs and reported to have anti-inflammatory, antioxidant properties, in addition to preventing protein-aggregation (Maiti et al., 2014). Curcumin has been recently reported to produce a hypocholesterolemic effect by enhancing the cell-surface expression of LDLR and LDL uptake through downregulation of PCSK9 gene expression in HepG2 cells (Tai et al., 2014). The effect of curcumin on the rescue of mutant LDLR misfolding has not been investigated so far and can be explored. As mentioned above, only a few studies have been performed on the potential roles of PCs and PRs in the rescue of class II LDLR mutants. More investigations involving already established PRs and PCs that show rescue potential in other protein conformation diseases might improve the chances of finding effective PCs/PRs for FH class II mutant LDLRs.

Potential Challenges in Using PRs and PCs as Drugs for FH

Even though, the cell line-based studies are encouraging, targeting normal cellular process such as ERAD and proteasome has deleterious effects. One has to be cautious of the fact that majority of the information on the molecular pathology of class II LDLR mutants come from cell line-based overexpression studies. The cell line-based data has to be validated with studies involving tissues from FH patients. Low efficiency also poses a problem while considering PCs/PRs as therapeutic agents. Cell line-based study shows only 30% functional recovery of rescued LDLR by 4-PBA, an extensively studied chemical chaperone for various mutants associated with protein conformational diseases. Pre-clinical studies show that application of PCs result in variable increase in the activity of mutant enzymes associated with lysosomal storage diseases (Parenti et al., 2015). Some mutations respond well and others not at all (Parenti et al., 2015). Hence, the efficiency of PCs, in general, is debatable. Therefore, it is a long way ahead to determine whether restoration of mutant LDLR activity by 4-PBA is beneficial to FH patients.

It is quite interesting to note that 4-PBA mediated rescue of LDLR mutants is mutation specific. The authors diligently prove that the other mutations in the same domain of the protein which are also class II mutations are not rescued by 4-PBA (Tveten et al., 2007). One can reasonably assume that mutations that grossly affect the protein stability or core LDL binding site may not be rescued by PCs. However, regardless of various limitations, PCs and PRs have the potential to be considered as therapeutic agents for FH patients with class II LDLR mutations.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we have outlined the impact of class II LDLR mutants on ER-proteostasis and how it can be modulated for the therapeutic management of FH. Conventional lipid-lowering drugs are effective in maintaining LDL-C levels in heterozygous FH patients since these patients have residual LDLR function due to the presence of a normal copy of the *LDLR* gene. In homozygous FH patients where a functional LDLR is lacking, the current LDL-C lowering drugs have minimal effect (Wilemon et al., 2020). A growing body of evidence suggests that ER-proteostasis can be modulated for therapeutic purposes to treat various protein conformation diseases including class II FH. LDLR is the founding member of the LDL receptor family whose members share structural and functional domains. The exquisite structure of these class of proteins requires a specialized array of private and global quality control factors, many of which remain to be unraveled. Despite the significant development in ERAD research, only limited information is available on the factors responsible for the recognition, ER-retention and degradation of defective LDLRs. Notably, we are yet to find out whether non-canonical ERAD pathways such as ER-phagy and microautophagy are involved in the disposal of misfolded mutant LDLRs. Cell line models of class II mutants associated with FH indicate a role for ER-stress and UPR activation in the pathogenesis. More studies using fibroblasts from heterozygous and homozygous FH patients with diverse Class II LDLR mutations will clarify this aspect and lay foundations for designing therapies focused on adaptive UPR and folding-rescue of these class of mutants. A growing body of evidence suggests that PCs and PRs have the potential to augment conventional therapies for FH. Though the benefits have to be carefully weighed against manipulating the natural processes of ERAD and proteostasis, PRs have the potential to be considered for therapeutic management of FH. Cholesterol is a constituent of the ER membrane and is regulated by ER. Therefore, mechanisms aimed at restoring ER homeostasis are likely to influence cholesterol homeostasis (Fu et al., 2012). Additional investigations on the influence of PRs on cholesterol metabolism independent of proteostasis would also provide insights into novel pathways of LDLR regulation.

AUTHOR CONTRIBUTIONS

DO, PK, and AJ drafted different sections of the manuscript. PK and BA compiled and edited the manuscript. PK and DV created the figures. DO, PK, DV, AJ, and BA critically reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Abifadel, M., Varret, M., Rabès, J. P., Allard, D., Ouguerram, K., Devillers, M., et al. (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat. Genet.* 34, 154–156. doi: 10.1038/ng1161

Aebi, M., Bernasconi, R., Clerc, S., and Molinari, M. (2010). N-glycan structures: recognition and processing in the ER. *Trends Biochem. Sci.* 35, 74–82. doi: 10.1016/j.tibs.2009.10.001

Ali, B. R., Ben-Rebeh, I., John, A., Akawi, N. A., Milhem, R. M., Al-Shehhi, N. A., et al. (2011). Endoplasmic reticulum quality control is involved in the mechanism of endoglin-mediated hereditary haemorrhagic telangiectasia. *PLoS One* 6:e26206. doi: 10.1371/journal.pone.0026206

Al-Kindi, A., Kizhakkedath, P., Xu, H., John, A., Sayegh, A. A. A., Ganesh, A., et al. (2014). A novel mutation in DDR2 causing spondylo-meta-epiphyseal dysplasia with short limbs and abnormal calcifications (SMED-SL) results in defective intra-cellular trafficking. *BMC Med. Genet.* 15:42. doi: 10.1186/1471-2350-15-42

Almanza, A., Carlesso, A., Chintha, C., Creedican, S., Doultsinos, D., Leuzzi, B., et al. (2019). Endoplasmic reticulum stress signalling – from basic mechanisms to clinical applications. *FEBS J.* 286, 241–278. doi: 10.1111/febs.14608

Alonso, R., Andres, E., Mata, N., Fuentes-Jiménez, F., Badimón, L., López-Miranda, J., et al. (2014). Lipoprotein(a) levels in familial hypercholesterolemia: an important predictor of cardiovascular disease independent of the type of LDL receptor mutation. *J. Am. Coll. Cardiol.* 63, 1982–1989. doi: 10.1016/j.jacc.2014.01.063

Balch, W. E., Morimoto, R. I., Dillin, A., and Kelly, J. W. (2008). Adapting proteostasis for disease intervention. *Science* 319, 916–919.

Banerjee, P., Chan, K. C., Tarabocchia, M., Benito-Vicente, A., Alves, A. C., Uribe, K. B., et al. (2019). Functional analysis of LDLR (low-density lipoprotein receptor) variants in patient lymphocytes to assess the effect of evinacumab in homozygous familial hypercholesterolemia patients with a spectrum of LDLR activity. *Arterioscler. Thromb Vasc. Biol.* 39, 2248–2260. doi: 10.1161/ATVBAHA.119.313051

Beglova, N., Jeon, H., Fisher, C., and Blacklow, S. C. (2004). Cooperation between fixed and low pH-inducible interfaces controls lipoprotein release by the LDL receptor. *Mol. Cell* 16, 281–292. doi: 10.1016/j.molcel.2004.09.038

Benito-Vicente, A., Alves, A. C., Etxebarria, A., Medeiros, A. M., Martin, C., and Bourbon, M. (2015). The importance of an integrated analysis of clinical, molecular, and functional data for the genetic diagnosis of familial hypercholesterolemia. *Genet. Med.* 17, 980–988. doi: 10.1038/gim.2015.14

Benito-Vicente, A., Uribe, K. B., Jebari, S., Galicia-Garcia, U., Ostolaza, H., and Martin, C. (2018). Validation of LDLr activity as a tool to improve genetic diagnosis of familial hypercholesterolemia: a retrospective on functional characterization of LDLr variants. *Int. J. Mol. Sci.* 19:1676. doi: 10.3390/ijms19061676

Bianchini, E., Fanin, M., Mamchaoui, K., Betto, R., and Sandonà, D. (2014). Unveiling the degradative route of the V247M a-sarcoglycan mutant responsible for LGMD-2D. *Hum. Mol. Genet.* 23, 3746–3758. doi: 10.1093/hmg/ddu088

Bondulich, M. K., Guo, T., Meehan, C., Manion, J., Rodriguez Martin, T., Mitchell, J. C., et al. (2016). Tauopathy induced by low level expression of a human brain-derived tau fragment in mice is rescued by phenylbutyrate. *Brain* 139, 2290–2306. doi: 10.1093/brain/aww137

Braakman, I., and Hebert, D. N. (2013). Protein folding in the endoplasmic reticulum. *Cold Spring Harb. Perspect. Biol.* 5:a013201. doi: 10.1101/cshperspect.a013201

Bradley, W. A., and Gianturco, S. H. (1986). ApoE is necessary and sufficient for the binding of large triglyceride-rich lipoproteins to the LDL receptor; apoB is unnecessary. *J. Lipid Res.* 27, 40–48.

Briant, K., Koay, Y. H., Otsuka, Y., and Swanton, E. (2015). ERAD of proteins containing aberrant transmembrane domains requires ubiquitylation of cytoplasmic lysine residues. *J. Cell Sci.* 128, 4112–4125. doi: 10.1242/jcs.171215

Brodsky, J. L., and Skach, W. R. (2011). Protein folding and quality control in the endoplasmic reticulum: recent lessons from yeast and mammalian cell systems. *Curr. Opin. Cell Biol.* 23, 464–475. doi: 10.1016/j.ceb.2011.05.004

Brown, M. S., Goldstein, J. L., and Braakman, I. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34–47. doi: 10.1126/science.3513311

Cameron, J., Bogsrud, M. P., Tveten, K., Strom, T. B., Holven, K., Berge, K. E., et al. (2012). Serum levels of proprotein convertase subtilisin/kexin type 9 in subjects with familial hypercholesterolemia indicate that proprotein convertase subtilisin/kexin type 9 is cleared from plasma by low-density lipoprotein receptor-independent pathways. *Transl. Res.* 160, 125–130. doi: 10.1016/j.trsl.2012.01.010

Cameron, J., Holla, ØL., Ranheim, T., Kulseth, M. A., Berge, K. E., and Leren, T. P. (2006). Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. *Hum. Mol. Genet.* 15, 1551–1558. doi: 10.1093/hmg/ddl077

Carvalho, P., Goder, V., and Rapoport, T. A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126, 361–373. doi: 10.1016/j.cell.2006.05.043

Christianson, J. C., Olzmann, J. A., Shaler, T. A., Sowa, M. E., Bennett, E. J., Richter, C. M., et al. (2011). Defining human ERAD networks through an integrative mapping strategy. *Nat. Cell Biol.* 14, 93–105. doi: 10.1038/ncb2383

Christianson, J. C., Shaler, T. A., Tyler, R. E., and Kopito, R. R. (2008). OS-9 and GRP94 deliver mutant α1-antitrypsin to the Hrd1–SEL1L ubiquitin ligase complex for ERAD. *Nat. Cell Biol.* 10, 272–282. doi: 10.1038/ncb1689

Christianson, J. C., and Ye, Y. (2014). Cleaning up in the endoplasmic reticulum: ubiquitin in charge. *Nat. Struct. Mol. Biol.* 21, 325–335. doi: 10.1038/nsmb.2793

Cohen, J., Pertsemlidis, A., Kotowski, I. K., Graham, R., Garcia, C. K., and Hobbs, H. H. (2005). Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat. Genet.* 37, 161–165. doi: 10.1038/ng1509

Conn, P. M., and Janovick, J. A. (2009). Drug development and the cellular quality control system. *Trends Pharmacol. Sci.* 30, 228–233. doi: 10.1016/j.tips.2009.02.002

Culi, J., Springer, T. A., and Mann, R. S. (2004). Boca-dependent maturation of β-propeller/EGF modules in low-density lipoprotein receptor proteins. *EMBO J.* 23, 1372–1380. doi: 10.1038/sj.emboj.7600132

Davis, C. G., Goldstein, J. L., Südhof, T. C., Anderson, R. G. W., Russell, D. W., and Brown, M. S. (1987). Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature* 326, 760–765. doi: 10.1038/326760a0

De Leonibus, C., Cinque, L., and Settembre, C. (2019). Emerging lysosomal pathways for quality control at the endoplasmic reticulum. *FEBS Lett.* 593, 2319–2329. doi: 10.1002/1873-3468.13571

DeBose-Boyd, R. A. (2008). Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. *Cell Res.* 18, 609–621. doi: 10.1038/cr.2008.61

Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992). Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature* 358, 761–764. doi: 10.1038/358761a0

Drumm, M. L., Wilkinson, D. J., Smit, L. S., Worrell, R. T., Strong, T. V., Frizzell, R. A., et al. (1991). Chloride conductance expressed by ΔF508 and other mutant CFTRs in xenopus oocytes. *Science* 254, 1797–1799. doi: 10.1126/science.1722350

Dubuc, G., Chamberland, A., Wassef, H., Davignon, J., Seidah, N. G., Bernier, L., et al. (2004). Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial

hypercholesterolemia. *Arterioscler. Thromb Vasc. Biol.* 24, 1454–1459. doi: 10.1161/01.ATV.0000134621.14315.43

Esser, V., and Russell, D. W. (1988). Transport-deficient mutations in the low density lipoprotein receptor. Alterations in the cysteine-rich and cysteine-poor regions of the protein block intracellular transport. *J. Biol. Chem.* 263, 13276–13281.

Etxebarria, A., Benito-Vicente, A., Alves, A. C., Ostolaza, H., Bourbon, M., and Martin, C. (2014). Advantages and versatility of fluorescence-based methodology to characterize the functionality of LDLR and class mutation assignment. *PLoS One* 9:e112677. doi: 10.1371/journal.pone.0112677

Etxebarria, A., Benito-Vicente, A., Palacios, L., Stef, M., Cenarro, A., Civeira, F., et al. (2015). Functional characterization and classification of frequent low-density lipoprotein receptor variants. *Hum. Mutat.* 36, 129–141. doi: 10.1002/humu.22721

Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S., and Weissman, A. M. (2001). The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14422–14427. doi: 10.1073/pnas.251401598

Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997). Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. *Nature* 388, 691–693. doi: 10.1038/41798

Feige, M. J., and Hendershot, L. M. (2013). Quality control of integral membrane proteins by assembly-dependent membrane integration. *Mol. Cell* 51, 297–309. doi: 10.1016/j.molcel.2013.07.013

Fleig, L., Bergbold, N., Sahasrabudhe, P., Geiger, B., Kaltak, L., and Lemberg, M. K. (2012). Ubiquitin-dependent intramembrane rhomboid protease promotes ERAD of membrane proteins. *Mol. Cell* 47, 558–569. doi: 10.1016/j.molcel.2012.06.008

Francke, U., Brown, M. S., and Goldstein, J. L. (1984). Assignment of the human gene for the low density lipoprotein receptor to chromosome 19: synteny of a receptor, a ligand, and a genetic disease. *Proc. Natl. Acad. Sci. U.S.A.* 81, 2826–2830. doi: 10.1073/pnas.81.9.2826

Fregno, I., and Molinari, M. (2019). Proteasomal and lysosomal clearance of faulty secretory proteins: ER-associated degradation (ERAD) and ER-to-lysosome-associated degradation (ERLAD) pathways. *Crit. Rev. Biochem. Mol. Biol.* 54, 153–163. doi: 10.1080/10409238.2019.1610351

Fu, S., Watkins, S. M., and Hotamisligil, G. S. (2012). The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. *Cell Metab.* 15, 623–634. doi: 10.1016/j.cmet.2012.03.007

Galicia-Garcia, U., Benito-Vicente, A., Uribe, K. B., Jebari, S., Larrea-Sebal, A., Alonso-Estrada, R., et al. (2020). Mutation type classification and pathogenicity assignment of sixteen missense variants located in the EGF-precursor homology domain of the LDLR. *Sci. Rep.* 10:1727. doi: 10.1038/s41598-020-58734-9

Gámez, A., Yuste-Checa, P., Brasil, S., Briso-Montiano, A., Desviat, L. R., Ugarte, M., et al. (2018). Protein misfolding diseases: prospects of pharmacological treatment. *Clin. Genet.* 93, 450–458. doi: 10.1111/cge.13088

Garcia, C. K., Wilund, K., Arca, M., Zuliani, G., Fellin, R., Maioli, M., et al. (2001). Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. *Science* 292, 1394–1398. doi: 10.1126/science.1060458

Gent, J., and Braakman, I. (2004). Low-density lipoprotein receptor structure and folding. *Cell Mol. Life Sci.* 61, 2461–2470. doi: 10.1007/s00018-004-4090-3

Goldstein, J. L., and Brown, M. S. (1990). Regulation of the mevalonate pathway. *Nature* 343, 425–430. doi: 10.1038/343425a0

Goldstein, J. L., and Brown, M. S. (2009). The LDL receptor. *Arterioscler. Thromb Vasc. Biol.* 29, 431–438. doi: 10.1161/ATVBAHA.108.179564

Guerriero, C. J., and Brodsky, J. L. (2012). The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. *Physiol. Rev.* 92, 537–576. doi: 10.1152/physrev.00027.2011

Guo, J., Gao, Y., Li, X., He, Y., Zheng, X., Bi, J., et al. (2019). Systematic prediction of familial hypercholesterolemia caused by low-density lipoprotein receptor missense mutations. *Atherosclerosis* 281, 1–8. doi: 10.1016/j.atherosclerosis.2018.12.003

Hegde, R. S., and Ploegh, H. L. (2010). Quality and quantity control at the endoplasmic reticulum. *Curr. Opin. Cell Biol.* 22, 437–446. doi: 10.1016/j.ceb.2010.05.005

Henderson, R., O'Kane, M., McGilligan, V., and Watterson, S. (2016). The genetics and screening of familial hypercholesterolaemia. *J. Biomed. Sci.* 23:39. doi: 10.1186/s12929-016-0256-1

Herz, J., and Marschang, P. (2003). Coaxing the LDL receptor family into the fold. *Cell* 112, 289–292.

Hobbs, H. H., Brown, M. S., and Goldstein, J. L. (1992). Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum. Mutat.* 1, 445–466. doi: 10.1002/humu.1380010602

Holst, H. U., Dagnaes-Hansen, F., Corydon, T. J., Andreasen, P. H., Jørgensen, M. M., Kølvraa, S., et al. (2001). LDL receptor-GFP fusion proteins: new tools for the characterisation of disease-causing mutations in the LDL receptor gene. *Eur. J. Hum. Genet.* 9, 815–822. doi: 10.1038/sj.ejhg.5200718

Horimoto, S., Ninagawa, S., Okada, T., Koba, H., Sugimoto, T., Kamiya, Y., et al. (2013). The unfolded protein response transducer ATF6 represents a novel transmembrane-type endoplasmic reticulum-associated degradation substrate requiring both mannose trimming and SEL1L protein. *J. Biol. Chem.* 288, 31517–31527. doi: 10.1074/jbc.M113.476010

Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., and Nagata, K. (2008). Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1-SEL1L ubiquitin ligase complex and BiP. *J. Biol. Chem.* 283, 20914–20924. doi: 10.1074/jbc.M709336200

Houck, S. A., Ren, H. Y., Madden, V. J., Bonner, J. N., Conlin, M. P., Janovick, J. A., et al. (2014). Quality control autophagy degrades soluble ERAD-resistant conformers of the misfolded membrane protein GnRHR. *Mol. Cell* 54, 166–179. doi: 10.1016/j.molcel.2014.02.025

Hume, A. N., Buttgereit, J., Al-Awadhi, A. M., Al-Suwaidi, S. S., John, A., Bader, M., et al. (2009). Defective cellular trafficking of missense NPR-B mutants is the major mechanism underlying acromesomelic dysplasia-type Maroteaux. *Hum. Mol. Genet.* 18, 267–277. doi: 10.1093/hmg/ddn354

Ikonen, E. (2008). Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* 9, 125–138. doi: 10.1038/nrm2336

Innerarity, T. L., Mahley, R. W., Weisgraber, K. H., Bersot, T. P., Krauss, R. M., Vega, G. L., et al. (1990). Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. *J. Lipid Res.* 31, 1337–1349.

Jansens, A., Van Duijn, E., and Braakman, I. (2002). Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science* 298, 2401–2403. doi: 10.1126/science.1078376

Jeenduang, N., Ruangpracha, A., Promptmas, C., Pongrapeeporn, K. U. S., and Porntadavity, S. (2010). Two novel D151Y and M391T LDLR mutations causing LDLR transport defects in Thai patients with Familial hypercholesterolemia. *Clin. Chim. Acta* 411, 1656–1661. doi: 10.1016/j.cca.2010.06.021

Jeon, H., Meng, W., Takagi, J., Eck, M. J., Springer, T. A., and Blacklow, S. C. (2001). Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nat. Struct. Biol.* 8, 499–504. doi: 10.1038/88556

Jessop, C. E., Chakravarthi, S., Garbi, N., Hämmerling, G. J., Lovell, S., and Bulleid, N. J. (2007). ERp57 is essential for efficient folding of glycoproteins sharing common structural domains. *EMBO J.* 26, 28–40. doi: 10.1038/sj.emboj.7601505

Jessop, C. E., Watkins, R. H., Simmons, J. J., Tasab, M., and Bulleid, N. J. (2009). Protein disulphide isomerase family members show distinct substrate specificity: P5 is targeted to BiP client proteins. *J. Cell Sci.* 122, 4287–4295. doi: 10.1242/jcs.059154

Jiang, L., Wu, W. F., Sun, L. Y., Chen, P. P., Wang, W., Benito-Vicente, A., et al. (2016). The use of targeted exome sequencing in genetic diagnosis of young patients with severe hypercholesterolemia. *Sci. Rep.* 6:36823. doi: 10.1038/srep36823

Jo, Y., Lee, P. C. W., Sguigna, P. V., and DeBose-Boyd, R. A. (2011). Sterol-induced degradation of HMG CoA reductase depends on interplay of two Insigs and two ubiquitin ligases, gp78 and Trc8. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20503–20508. doi: 10.1073/pnas.1112831108

John, A., Kizhakkedath, P., Al-Gazali, L., and Ali, B. R. (2015). Defective cellular trafficking of the bone morphogenetic protein receptor type II by mutations underlying familial pulmonary arterial hypertension. *Gene* 561, 148–156. doi: 10.1016/j.gene.2015.02.038

Jørgensen, M. M., Jensen, O. N., Holst, H. U., Hansen, J. J., Corydon, T. J., Bross, P., et al. (2000). Grp78 is involved in retention of mutant low density lipoprotein

receptor protein in the endoplasmic reticulum. *J. Biol. Chem.* 275, 33861–33868. doi: 10.1074/jbc.M004663200

Karagöz, G. E., Acosta-Alvear, D., and Walter, P. (2019). The unfolded protein response: detecting and responding to fluctuations in the protein-folding capacity of the endoplasmic reticulum. *Cold Spring Harb. Perspect. Biol.* 11:a033886. doi: 10.1101/cshperspect.a033886

Kawaguchi, A., Miyatake, K., Yutani, C., Beppu, S., Tsushima, M., Yamamura, T., et al. (1999). Characteristic cardiovascular manifestation in homozygous and heterozygous familial hypercholesterolemia. *Am. Heart J.* 137, 410–418. doi: 10.1016/S0002-8703(99)70485-0

Khaminets, A., Heinrich, T., Mari, M., Grumati, P., Huebner, A. K., Akutsu, M., et al. (2015). Regulation of endoplasmic reticulum turnover by selective autophagy. *Nature* 522, 354–358. doi: 10.1038/nature14498

Kim, P. S. (1998). Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: disorders of protein trafficking and the role of ER molecular chaperones. *Endocr. Rev.* 19, 173–202. doi: 10.1210/er.19.2.173

Kitahara, H., Mori, N., Saito, Y., Nakayama, T., Fujimoto, Y., and Kobayashi, Y. (2019). Prevalence of Achilles tendon xanthoma and familial hypercholesterolemia in patients with coronary artery disease undergoing percutaneous coronary intervention. *Heart Vessels* 34, 1595–1599. doi: 10.1007/s00380-019-01400-6

Kizhakkedath, P., John, A., Al-Gazali, L., and Ali, B. R. (2018). Degradation routes of trafficking-defective VLDLR mutants associated with Dysequilibrium syndrome. *Sci. Rep.* 8:1583. doi: 10.1038/s41598-017-19053-8

Kizhakkedath, P., John, A., Al−Sawafi, B. K., Al−Gazali, L., and Ali, B. R. (2019). Endoplasmic reticulum quality control of LDLR variants associated with familial hypercholesterolemia. *FEBS Open Biol.* 9, 1994–2005. doi: 10.1002/2211-5463.12740

Kizhakkedath, P., Loregger, A., John, A., Bleijlevens, B., Al-Blooshi, A. S., Al-Hosani, A. H., et al. (2014). Impaired trafficking of the very low density lipoprotein receptor caused by missense mutations associated with dysequilibrium syndrome. *Biochim. Biophys. Acta Mol. Cell Res.* 1843, 2871–2877. doi: 10.1016/j.bbamcr.2014.08.013

Klee, E. W., and Zimmermann, M. T. (2019). Molecular modeling of LDLR aids interpretation of genomic variants. *J. Mol. Med.* 97, 533–540. doi: 10.1007/s00109-019-01755-3

Klose, G., Laufs, U., März, W., and Windler, E. (2014). Familial hypercholesterolemia: developments in diagnosis and treatment. *Dtsch. Arztebl. Int.* 111, 523–529. doi: 10.3238/arztebl.2014.0523

Koivisto, U. M., Hubbard, A. L., and Mellman, I. (2001). A novel cellular phenotype for familial hypercholesterolemia due to a defect in polarized targeting of LDL receptor. *Cell* 105, 575–585. doi: 10.1016/S0092-8674(01)00371-3

Lamriben, L., Graham, J. B., Adams, B. M., and Hebert, D. N. (2016). N-Glycan-based ER molecular chaperone and protein quality control system: the calnexin binding cycle. *Traffic* 17, 308–326. doi: 10.1111/tra.12358

Lawless, M. W., Greene, C. M., Mulgrew, A., Taggart, C. C., O'Neill, S. J., and McElvaney, N. G. (2004). Activation of endoplasmic reticulum-specific stress responses associated with the conformational disease Z α1-antitrypsin deficiency. *J. Immunol.* 172, 5722–5726. doi: 10.4049/jimmunol.172.9.5722

Lebeau, P., Al-Hashimi, A., Sood, S., Lhoták, Š, Yu, P., Gyulay, G., et al. (2017). Endoplasmic reticulum stress and Ca2+ depletion differentially modulate the sterol regulatory protein PCSK9 to control lipid metabolism. *J. Biol. Chem.* 292, 1510–1523. doi: 10.1074/jbc.M116.744235

Lebeau, P., Platko, K., Al-Hashimi, A. A., Byun, J. H., Lhoták, Š, Holzapfel, N., et al. (2018). Loss-of-function PCSK9 mutants evade the unfolded protein response sensor GRP78 and fail to induce endoplasmic reticulum stress when retained. *J. Biol. Chem.* 293, 7329–7343. doi: 10.1074/jbc.RA117.001049

Lee, J. H., Lee, G. Y., Jang, H., Choe, S. S., Koo, S. H., and Kim, J. B. (2014). Ring finger protein20 regulates hepatic lipid metabolism through protein kinase A-dependent sterol regulatory element binding protein1c degradation. *Hepatology* 60, 844–857. doi: 10.1002/hep.27011

Li, Y., Lu, W., Schwartz, A. L., and Bu, G. (2004). Degradation of the LDL receptor class 2 mutants is mediated by a proteasome-dependent pathway. *J. Lipid Res.* 45, 1084–1091. doi: 10.1194/jlr.M300482-JLR200

Lilley, B. N., and Ploegh, H. L. (2005). Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14296–14301. doi: 10.1073/pnas.0505014102

Loregger, A., Cook, E. C. L., Nelson, J. K., Moeton, M., Sharpe, L. J., Engberg, S., et al. (2015). A MARCH6 and IDOL E3 ubiquitin ligase circuit uncouples cholesterol synthesis from lipoprotein uptake in hepatocytes. *Mol. Cell Biol.* 36, 285–294. doi: 10.1128/mcb.00890-15

Ma, W., Goldberg, E., and Goldberg, J. (2017). ER retention is imposed by COPII protein sorting and attenuated by 4-phenylbutyrate. *eLife* 6:e26624. doi: 10.7554/eLife.26624

Maiers, J. L., and Malhi, H. (2019). Endoplasmic reticulum stress in metabolic liver diseases and hepatic fibrosis. *Semin. Liver Dis.* 39, 235–248. doi: 10.1055/s-0039-1681032

Maiti, P., Manna, J., Veleri, S., and Frautschy, S. (2014). Molecular chaperone dysfunction in neurodegenerative diseases and effects of curcumin. *Biomed. Res. Int.* 2014:495091. doi: 10.1155/2014/495091

Maxwell, K. N., Soccio, R. E., Duncan, E. M., Sehayek, E., and Breslow, J. L. (2003). Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J. Lipid Res.* 44, 2109–2119. doi: 10.1194/jlr.M300203-JLR200

Menzies, S. A., Volkmar, N., van den Boomen, D. J. H., Timms, R. T., Dickson, A. S., Nathan, J. A., et al. (2018). The sterol-responsive RNF145 E3 ubiquitin ligase mediates the degradation of HMG-CoA reductase together with gp78 and hrd1. *eLife* 7:e40009. doi: 10.7554/eLife.40009

Mohamed, F. E., Al-Gazali, L., Al-Jasmi, F., and Ali, B. R. (2017). Pharmaceutical chaperones and proteostasis regulators in the therapy of lysosomal storage disorders: current perspective and future promises. *Front. Pharmacol.* 8:448. doi: 10.3389/fphar.2017.00448

Mu, T.-W., Ong, D. S. T., Wang, Y.-J., Balch, W. E., Yates, J. R., Segatori, L., et al. (2008). Proteostasis regulators and pharmacologic chaperones synergize to correct protein misfolding diseases. *Cell* 134, 769–781. doi: 10.1016/j.cell.2008.06.037

Müller, C. (1938). Xanthomata, hypercholesterolemia, angina pectoris. *Acta Med. Scand.* 95, 75–84. doi: 10.1111/j.0954-6820.1938.tb19279.x

Nadav, E., Shmueli, A., Barr, H., Gonen, H., Ciechanover, A., and Reiss, Y. (2003). A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochem. Biophys. Res. Commun.* 303, 91–97. doi: 10.1016/S0006-291X(03)00279-1

Ni, M., and Lee, A. S. (2007). ER chaperones in mammalian development and human diseases. *FEBS Lett.* 581, 3641–3651. doi: 10.1016/j.febslet.2007.04.045

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease. *Eur. Heart J.* 34, 3478–3490a. doi: 10.1093/eurheartj/eht273

Oka, O. B. V., Pringle, M. A., Schopp, I. M., Braakman, I., and Bulleid, N. J. (2013). ERdj5 is the ER reductase that catalyzes the removal of non-native disulfides and correct folding of the LDL receptor. *Mol. Cell* 50, 793–804. doi: 10.1016/j.molcel.2013.05.014

Okuda-Shimizu, Y., and Hendershot, L. M. (2007). Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol. Cell* 28, 544–554. doi: 10.1016/j.molcel.2007.09.012

Olzmann, J. A., Kopito, R. R., and Christianson, J. C. (2013). The mammalian endoplasmic reticulum-associated degradation system. *Cold Spring Harb. Perspect. Biol.* 5:a013185. doi: 10.1101/cshperspect.a013185

Omer, L., Hindi, L., Militello, G., Stivers, K. B., Tien, K. C., and Boyd, N. L. (2020). Familial hypercholesterolemia class II low-density lipoprotein receptor response to statin treatment. *Dis. Model. Mech.* 13:dmm042911. doi: 10.1242/dmm.042911

Omer, L., Hudson, E. A., Zheng, S., Hoying, J. B., Shan, Y., and Boyd, N. L. (2017). CRISPR correction of a homozygous low-density lipoprotein receptor mutation in familial hypercholesterolemia induced pluripotent stem cells. *Hepatol. Commun.* 1, 886–898. doi: 10.1002/hep4.1110

Özcan, U., Yilmaz, E., Özcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., et al. (2006). Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 313, 1137–1140. doi: 10.1126/science.1128294

Parenti, G., Andria, G., and Valenzano, K. J. (2015). Pharmacological chaperone therapy: preclinical development, clinical translation, and prospects for the treatment of lysosomal storage disorders. *Mol. Ther.* 23, 1138–1148. doi: 10.1038/mt.2015.62

Pena, F., Jansens, A., Van Zadelhoff, G., and Braakman, I. (2010). Calcium as a crucial cofactor for low density lipoprotein receptor folding in the endoplasmic reticulum. *J. Biol. Chem.* 285, 8656–8664. doi: 10.1074/jbc.M110.105718

Pirkkala, L., Alastalo, T.-P., Zuo, X., Benjamin, I. J., and Sistonen, L. (2000). Disruption of heat shock factor 1 reveals an essential role in the ubiquitin proteolytic pathway. *Mol. Cell Biol.* 20, 2670–2675. doi: 10.1128/mcb.20.8.2670-2675.2000

Poirier, S., Mamarbachi, M., Chen, W. T., Lee, A. S. S., and Mayer, G. (2015). GRP94 regulates circulating cholesterol levels through blockade of PCSK9-induced LDLR degradation. *Cell Rep.* 13, 2064–2071. doi: 10.1016/j.celrep.2015.11.006

Printsev, I., Curiel, D., and Carraway, K. L. (2017). Membrane protein quantity control at the endoplasmic reticulum. *J. Membr. Biol.* 250, 379–392. doi: 10.1007/s00232-016-9931-0

Ringe, D., and Petsko, G. A. (2009). What are pharmacological chaperones and why are they interesting? *J. Biol.* 8:80. doi: 10.1186/jbiol186

Röhrl, C., and Stangl, H. (2018). Cholesterol metabolism—physiological regulation and pathophysiological deregulation by the endoplasmic reticulum. *Wiener Med. Wochenschrift* 168, 280–285. doi: 10.1007/s10354-018-0626-2

Rudenko, G., Henry, L., Henderson, K., Ichtchenko, K., Brown, M. S., Goldstein, J. L., et al. (2002). Structure of the LDL receptor extracellular domain at endosomal pH. *Science* 298, 2353–2358. doi: 10.1126/science.1078124

Ruggiano, A., Foresti, O., and Carvalho, P. (2014). ER-associated degradation: protein quality control and beyond. *J. Cell Biol.* 204, 869–879. doi: 10.1083/jcb.201312042

Russell, D. W., Schneider, W. J., Yamamoto, T., Luskey, K. L., Brown, M. S., and Goldstein, J. L. (1984). Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor. *Cell* 37, 577–585. doi: 10.1016/0092-8674(84)90388-X

Schaefer, J. R., Kurt, B., Sattler, A., Klaus, G., and Soufi, M. (2012). Pharmacogenetic aspects in familial hypercholesterolemia with the special focus on FHMarburg (FH p.W556R). *Clin. Res. Cardiol. Suppl.* 7, 2–6. doi: 10.1007/s11789-012-0041-y

Schröder, M., and Kaufman, R. J. (2005). The mammalian unfolded protein response. *Annu. Rev. Biochem.* 74, 739–789. doi: 10.1146/annurev.biochem.73.011303.074134

Seidah, N. G., Benjannet, S., Wickham, L., Marcinkiewicz, J., Bélanger Jasmin, S., Stifani, S., et al. (2003). The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 928–933. doi: 10.1073/pnas.0335507100

Sha, H., Sun, S., Francisco, A. B., Ehrhardt, N., Xue, Z., Liu, L., et al. (2014). The ER-associated degradation adaptor protein Sel1L regulates LPL secretion and lipid metabolism. *Cell Metab.* 20, 458–470. doi: 10.1016/j.cmet.2014.06.015

Sharpe, L. J., Cook, E. C. L., Zelcer, N., and Brown, A. J. (2014). The UPS and downs of cholesterol homeostasis. *Trends Biochem. Sci.* 39, 527–535. doi: 10.1016/j.tibs.2014.08.008

Shenkman, M., and Lederkremer, G. Z. (2019). Compartmentalization and selective tagging for disposal of misfolded glycoproteins. *Trends Biochem. Sci.* 44, 827–836. doi: 10.1016/j.tibs.2019.04.012

Shu, H., Chi, J., Li, J., Zhang, W., Lv, W., Wang, J., et al. (2017). A novel indel variant in LDLR responsible for familial hypercholesterolemia in a Chinese family. *PLoS One* 12:e0189316. doi: 10.1371/journal.pone.0189316

Smith, M. H., Ploegh, H. L., and Weissman, J. S. (2011). Road to ruin : targeting proteins for degradation in the endoplasmic reticulum E3 ubiquitin ligases : central organizers. *Science* 334, 1086–1090.

Sørensen, S., Ranheim, T., Bakken, K. S., Leren, T. P., and Kulseth, M. A. (2006). Retention of mutant low density lipoprotein receptor in endoplasmic reticulum (ER) leads to ER stress. *J. Biol. Chem.* 281, 468–476. doi: 10.1074/jbc.M507071200

Soutar, A. K., and Naoumova, R. P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat. Clin. Pract. Cardiovasc. Med.* 4, 214–225. doi: 10.1038/ncpcardio0836

Springer, T. A. (1998). An extracellular β-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. *J. Mol. Biol.* 283, 837–862. doi: 10.1006/jmbi.1998.2115

Stenson, P. D., Mort, M., Ball, E. V., Evans, K., Hayden, M., Heywood, S., et al. (2017). The human gene mutation database: towards a comprehensive repository of inherited mutation data for medical research, genetic diagnosis and next-generation sequencing studies. *Hum. Genet.* 136, 665–677. doi: 10.1007/s00439-017-1779-6

Strøm, T. B., Laerdahl, J. K., and Leren, T. P. (2015). Mutation p.L799R in the LDLR, which affects the transmembrane domain of the LDLR, prevents membrane insertion and causes secretion of the mutant LDLR. *Hum. Mol. Genet.* 24, 5836–5844. doi: 10.1093/hmg/ddv304

Strøm, T. B., Laerdahl, J. K., and Leren, T. P. (2017). Mutations affecting the transmembrane domain of the LDL receptor: impact of charged residues on the membrane insertion. *Hum. Mol. Genet.* 26, 1634–1642. doi: 10.1093/hmg/ddx068

Strøm, T. B., Tveten, K., Laerdahl, J. K., and Leren, T. P. (2014). Mutation G805R in the transmembrane domain of the LDL receptor gene causes familial hypercholesterolemia by inducing ectodomain cleavage of the LDL receptor in the endoplasmic reticulum. *FEBS Open Biol.* 4, 321–327. doi: 10.1016/j.fob.2014.03.007

Stroøm, T. B., Tveten, K., and Leren, T. P. (2014). Pcsk9 acts as a chaperone for the ldl receptor in the endoplasmic reticulum. *Biochem. J.* 457, 99–105. doi: 10.1042/BJ20130930

Südhof, T. C., Russell, D. W., Goldstein, J. L., Brown, M. S., Sanchez-Pescador, R., and Bell, G. I. (1985). Cassette of eight exons shared by genes for LDL receptor and EGF precursor. *Science* 228, 893–895. doi: 10.1126/science.3873704

Sun, Z., and Brodsky, J. L. (2019). Protein quality control in the secretory pathway. *J. Cell Biol.* 218, 3171–3187. doi: 10.1083/jcb.201906047

Sundqvist, A., Bengoechea-Alonso, M. T., Ye, X., Lukiyanchuk, V., Jin, J., Harper, J. W., et al. (2005). Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCFFbw7. *Cell Metab.* 1, 379–391. doi: 10.1016/j.cmet.2005.04.010

Susan-Resiga, D., Girard, E., Kiss, R. S., Essalmani, R., Hamelin, J., Asselin, M. C., et al. (2017). The proprotein convertase subtilisin/kexin type 9-resistant R410S low density lipoprotein receptor mutation: a novel mechanism causing familial hypercholesterolemia. *J. Biol. Chem.* 292, 1573–1590. doi: 10.1074/jbc.M116.769430

Szegezdi, E., Logue, S. E., Gorman, A. M., and Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* 7, 880–885. doi: 10.1038/sj.embor.7400779

Tai, M. H., Chen, P. K., Chen, P. Y., Wu, M. J., Ho, C. T., and Yen, J. H. (2014). Curcumin enhances cell-surface LDLR level and promotes LDL uptake through downregulation of PCSK9 gene expression in HepG2 cells. *Mol. Nutr. Food Res.* 58, 2133–2145. doi: 10.1002/mnfr.201400366

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101, 249–258. doi: 10.1016/S0092-8674(00)80835-1

Tveten, K., Holla, ØL., Ranheim, T., Berge, K. E., Leren, T. P., and Kulseth, M. A. (2007). 4-Phenylbutyrate restores the functionality of a misfolded mutant low-density lipoprotein receptor. *FEBS J.* 274, 1881–1893. doi: 10.1111/j.1742-4658.2007.05735.x

Ushioda, R., Hoseki, J., Araki, K., Jansen, G., Thomas, D. Y., and Nagata, K. (2008). ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. *Science* 321, 569–572. doi: 10.1126/science.1159293

Ushioda, R., Hoseki, J., and Nagata, K. (2013). Glycosylation-independent ERAD pathway serves as a backup system under ER stress. *Mol. Biol. Cell* 24, 3155–3163. doi: 10.1091/mbc.E13-03-0138

Van Hoof, D., Rodenburg, K. W., and Van der Horst, D. J. (2005). Intracellular fate of LDL receptor family members depends on the cooperation between their ligand-binding and EGF domains. *J. Cell Sci.* 118(Pt 6), 1309–1320. doi: 10.1242/jcs.01725

Varret, M., and Rabès, J.-P. (2012). Missense mutation in the LDLR gene: a wide spectrum in the severity of familial hypercholesterolemia. *Mutations Hum. Genet. Dis.* 55–74. doi: 10.5772/2912

Vembar, S. S., and Brodsky, J. L. (2008). One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* 9, 944–957. doi: 10.1038/nrm2546

Wang, F., Song, W., Brancati, G., and Segatori, L. (2011). Inhibition of endoplasmic reticulum-associated degradation rescues native folding in loss of function

protein misfolding diseases. *J. Biol. Chem.* 286, 43454–43464. doi: 10.1074/jbc.M111.274332

Wang, H., Xu, S., Sun, L., Pan, X., Yang, S., and Wang, L. (2014). Functional characterization of two low-density lipoprotein receptor gene mutations in two Chinese patients with familial hypercholesterolemia. *PLoS One* 9:e92703. doi: 10.1371/journal.pone.0092703

Ward, C. L., Omura, S., and Kopito, R. R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 83, 121–127. doi: 10.1016/0092-8674(95)90240-6

Wiegman, A., Gidding, S. S., Watts, G. F., Chapman, M. J., Ginsberg, H. N., Cuchel, M., et al. (2015). Familial hypercholesterolæmia in children and adolescents: gaining decades of life by optimizing detection and treatment. *Eur. Heart J.* 36, 2425–2437. doi: 10.1093/eurheartj/ehv157

Wilemon, K. A., Patel, J., Aguilar-Salinas, C., Ahmed, C. D., Alkhnifsawi, M., Almahmeed, W., et al. (2020). Reducing the clinical and public health burden of familial hypercholesterolemia: a global call to action. *JAMA Cardiol.* 5, 217–229. doi: 10.1001/jamacardio.2019.5173

Wilke, M., Bot, A., Jorna, H., Scholte, B. J., and de Jonge, H. R. (2012). Rescue of murine F508del CFTR activity in native intestine by low temperature and proteasome inhibitors. *PLoS One* 7:e52070. doi: 10.1371/journal.pone.0052070

Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., et al. (1984). The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 39, 27–38. doi: 10.1016/0092-8674(84)90188-0

Yan, H., Ma, Y. L., Gui, Y. Z., Wang, S. M., Wang, X. B., Gao, F., et al. (2014). MG132, a proteasome inhibitor, enhances LDL uptake in HepG2 cells in vitro by regulating LDLR and PCSK9 expression. *Acta Pharmacol. Sin.* 35, 994–1004. doi: 10.1038/aps.2014.52

Yu, X., and Kem, D. C. (2010). Proteasome inhibition during myocardial infarction. *Cardiovasc. Res.* 85, 312–320. doi: 10.1093/cvr/cvp309

Zapun, A., Darby, N. J., Tessier, D. C., Michalak, M., Bergeron, J. J. M., and Thomas, D. Y. (1998). Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. *J. Biol. Chem.* 273, 6009–6012. doi: 10.1074/jbc.273.11.6009

Zelcer, N., Hong, C., Boyadjian, R., and Tontonoz, P. (2009). LXR regulates cholesterol uptake through idol-dependent ubiquitination of the LDL receptor. *Science* 325, 100–104. doi: 10.1126/science.1168974

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REVIEW
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PCSK9 Variants in Familial Hypercholesterolemia: A Comprehensive Synopsis

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Autosomal dominant familial hypercholesterolemia (FH) affects approximately 1/250, individuals and potentially leads to elevated blood cholesterol and a significantly increased risk of atherosclerosis. Along with improvements in detection and the increased early diagnosis and treatment, the serious burden of FH on families and society has become increasingly apparent. Since FH is strongly associated with proprotein convertase subtilisin/kexin type 9 (*PCSK9*), increasing numbers of studies have focused on finding effective diagnostic and therapeutic methods based on *PCSK9*. At present, as *PCSK9* is one of the main pathogenic FH genes, its contribution to FH deserves more explorative research.

Keywords: gene, genetics, proprotein convertase subtilisin/kexin type 9, familial hypercholesterolemia, variant

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INTRODUCTION

A hereditary propensity for elevated serum levels of low-density lipoprotein cholesterol (LDL-C) that leading to cardiovascular disease (CVD) is typical FH and affects approximately 1 in 250 individuals. Currently recognized FH-inducing variants that lead to disease occur mostly in the apolipoprotein B (*APOB*), *PCSK9,* and LDL receptor (*LDLR*) genes. While most cases are caused by *LDLR* variants, they may also be caused by autosomal dominant variants of *PCSK9*, although less frequently (Defesche et al., 2017; Viigimaa et al., 2018; Sarraju and Knowles, 2019). PCSK9 encodes the proprotein convertase subtilisin/kexin type 9 protein which binds LDL and regulates the numbers of LDLR. As the third gene implicated in FH, *PCSK9* has been found to reduce the uptake of LDL-C in the liver by increasing the endosomal and lysosomal degradation of *LDLR* (Maxwell and Breslow, 2005; Stoekenbroek and Kastelein, 2018). Moreover, experimental studies indicate that *PCSK9* might independently accelerate atherosclerosis by enhancing inflammation,

Abbreviations: ADH, autosomal dominant hypercholesterolemia; APEX, arrayed primer extension; APOB, apolipoprotein B; CAD, coronary artery disease; Cas, CRISPR/CRISPR-associated; CETP, cholesterol ester transfer protein; COS, chitosan oligosaccharides; CRISPR, clustered regularly interspaced short palindromic repeats; CVD, cardiovascular disease; EGF-A, epidermal growth factor precursor homology domain A; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; GOF, gain-of-function; HDL, high density lipoprotein; HeFH, heterozygous FH; HINFP, histone nuclear factor P; HoFH, homozygous FH; HRM, high-resolution melting; LDL-C, low-density lipoprotein cholesterol; LDLR, LDL receptors; LOF, loss-of-function; LOX-1, lectin-like ox-LDL receptor-1; LR, ligand-binding; iPLEX, Multiplex MassARRAY Spectrometry; mRNA, messenger ribonucleic acid; mtDNA, mitochondrial DNA; mtROS, mitochondria-derived reactive oxygen species; NGS, next-generation sequencing; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; SCAP, SREBP cleavage-activating protein; SERBP, sterol regulatory element–binding protein; siRNA, small interfering RNA; STAP1, signal-transducing adaptor family member 1; TC, total cholesterol; WT, wild-type.

endothelial dysfunction, and hypertension (Urban et al., 2013). *PCSK9* variants implicated in autosomal dominant hypercholesterolemia (ADH) were first identified in 2003. At the time, they were believed to induce abnormal cholesterol metabolism through undefined mediators. Since then, *PCSK9* variants have been extensively investigated. Among these, loss-of-function (LOF) variants are associated with reduced LDL-C levels and coronary artery disease (CAD) risk, while gain-of-function (GOF) variants diminish *LDLR* levels, thereby inducing hypercholesterolemia (Abifadel et al., 2009). To prevent morbidity and mortality, it is crucial to diagnose FH early. Predominantly, laboratory tests or medical traits including family history are used in diagnosis, however, genetic screening of pathological variants might make definite diagnoses more accessible (Hovingh et al., 2013). To explore *PCSK9* variants in FH and their biological roles, we provide a detailed discussion and summary of novel *PCSK9* variants. We also discuss the important interplay between *PCSK9* and other molecules. Finally, this review sums up novel treatment strategies aimed at *PCSK9* in FH to pave the way for future investigative studies.

HISTORY OF *PCSK9* VARIANT DISCOVERIES

Seventeen years ago, through the sequencing of 12 exons of the *PCSK9* gene, Abifadel et al. (2003) opened the door to researching the role of the *PCSK9* gene in FH which suggested a novel mechanism of dyslipidemia. They identified a T to A substitution in exon 2 at nucleotide 625 resulting in a non-synonymous change at codon 127 of arginine for the conserved serine p.(S127R) and the substitution of T to C, resulting in an amino acid substitution of phenylalanine to leucine p.(F216L) among different families. In the following 5 years, many studies have been carried out to discover new variants in *PCSK9* (**Figure 1**). In addition, different populations have different types and frequencies of *PCSK9* variants, so we also provide a summary table of *PCSK9* variants with their population frequencies and LDL-C levels from previous studies (**Tables 1**, **2**).

Variant Sites Identified Within the First 6 Years

Variants Related to Elevated Levels of LDL-C

In 2004, Timms et al. (2004) recognized a single G→T nucleotide variant present on the K1173 haplotype variant resulting in the non-synonymous p.(D374Y). Leren identified an asparagine to lysine substitution at position 157, p.(N157K) (Leren, 2004). Benjannet et al. (2004) subsequently identified two further *PCSK9* variants occurring naturally in the gene pool, namely p.(R218S) and p.(R237W). In addition, Shioji et al. (2004) demonstrated the association between total cholesterol (TC) and LDL-C levels with exon 9/I474V or intron 1/C(-161)T polymorphisms.

In 2005, the amount of plasma LDL-C and the extent of atherosclerosis in coronary arteries were shown by Chen et al. (2005) to depend on a single nucleotide polymorphism resulting in p.(E670G). Subsequently, Allard et al. (2005) found four heterozygous missense variations resulting in changes in *PCSK9*, namely p.(R218S), p.(R357H), p.(R469W), and p.(A443T) in the coding region and intronic junctions of the *PCSK9* gene after analyzing 130 patients with ADH.

From 2006 to 2008, Pisciotta et al. (2006) sequenced multiple genes including *PCSK9* in two patients with heterozygous *LDLR* genes who were diagnosed with homozygous FH (HoFH). They identified one patient with the p.(R496W) variant from her mother and another one with the p.(N425S) variant likely from her deceased father. Patients with mutated *LDLR* were speculated to suffer worse pathological symptoms if they had an uncommon missense *PCSK9* variant. One year later, Bourbon et al. (2008) identified a p.(D374H) variant of *PCSK9* in 184 patients and 418 relatives in Portugal, and found that the number of confirmed FH patients increased through cascade screening. They recommended that if patients received appropriate treatment to restrain progress of premature CAD after early identification of FH, their life expectancy and quality of life could be improved. FH patients from New Zealand were observed to have two novel missense variants, namely p.(D129G) and p.(A168E) and together with two established variants from South Africa, namely p.(S127R) and p.(R237W), these gave a *PCSK9* variant total of four discovered by Homer et al. (2008) They found that the inhibition of *LDLR* mediated by *PCSK9* occurred independently of *PCSK9* release or autocatalytic destruction and speculated that *PCSK9* might play a role in cells. In addition, five novel *PCSK9* variants were found by Cameron et al. (2008), including p.(R215H), p.(G236S), p.(N354I), p.(A245T), and p.(R272Q), with p.(R215H) resulting in GOF and hypercholesterolemia. Since their effect on the internalization of LDL-C was similar to that of the wild-type (WT) *PCSK9*, it was demonstrated that p.(R272Q) and p.(A245T) were non-pathological aberrations which maintained normal *PCSK9* performance, though both variants were identified in a hypercholesterolemic group. After studying the promoter variant of the *PCSK9* gene, Blesa et al. (2008) identified a c.-332C > A variant in the region of the *PCSK9* gene promoter that increased transcription of PCSK9. In fact, the variant could result in a 2.5-fold increase in transcription compared to WT, thereby leading to ADH. At the end of the year, Abifadel et al. (2008) found familial combined hyperlipidemia present in two families, with variants of two leucines [designated p.(L15_L16ins2L) and p.(L21tri)] in family members who also had elevated LDL-C, thus suggesting an association.

Variants Associated With Decreased Levels of LDL-C

Cohen et al. (2005) investigated 128 people whose plasma LDL-C was low and identified two nonsense variants including p.(C679X) and p.(Y142X) through determining the coding region sequence of *PCSK9*. These had been recognized as LOF variants since 2005 as they had opposite effects compared to the GOF *PCSK9* variants. Subsequently, in 2006, while examining 38 people with hypocholesterolemia and 25 heterozygotes with hypercholesterolemia, all from different families, Berge et al. (2006) screened for *PCSK9* variants. They identified four variants including p.(R46L), p.(G106R), p.(R237W), and p.(N157K) among the two groups. In the same year, Kotowski et al. (2006) employed oligonucleotide hybridization on a

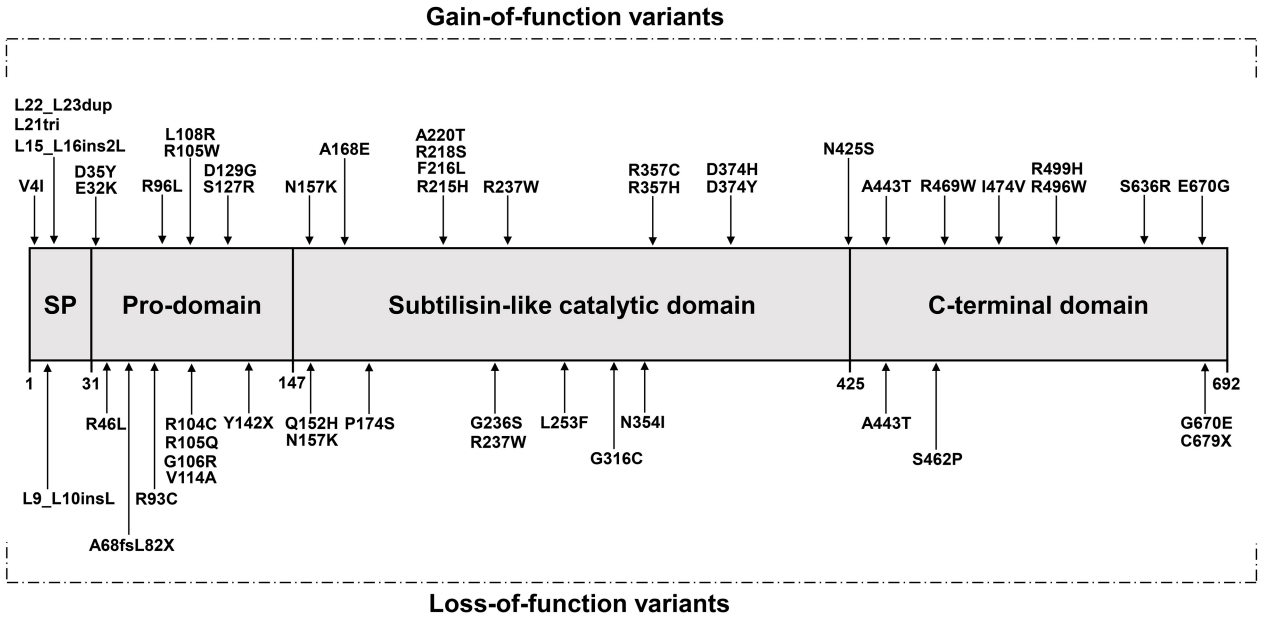

FIGURE 1 | The protein's domain structure of *PCSK9* and location of variants (adopted and redrawn from Maxwell and Breslow, 2005; Soutar and Naoumova, 2007) SP, Signal peptide.

TABLE 1 | Summary of GOF variants among different populations.

Researchers	*PCSK9* variants	Populations	Sample size	Variant frequency, %	LDL-C ranges, mmol/L
Leren (2004)	D374Y	Norwegian, FH	51	5.9	7.0–10.6
Allard et al. (2005)	R357H	French, FH	130	0.8	4.3–6.2
	R469W	French, FH	130	0.8	6.0–9.2
Humphries et al. (2006)	D374Y	British, FH	409	1.7	1.82–6.77
Taylor et al. (2007)	D374Y	British, FH	400	2.2	>4.9
Bourbon et al. (2008)	D374H	Portuguese, FH and relatives	602	0.5	4.9–9.4
Noguchi et al. (2010)	E32K	Japanese, FH	55	6.4	5.8–8.8
Abifadel et al. (2012)	D35Y	French, ADH	75	2.7	6.0
Mabuchi et al. (2014)	E32K	Japanese, FH	1,055	5.9	8.0–16.0
Ohta et al. (2016)	V4I	Japanese, FH	269	6.3	4.5–7.8
	E32K	Japanese, FH	269	6.3	4.5–7.8
	R496W	Japanese, FH	269	0.4	4.5–7.8
Xiang et al. (2017)	R96L	Chinese, FH	219	0.5	4.5–12.2
	R105W	Chinese, FH	219	0.5	4.5–12.2
Kaya et al. (2017)	D374Y	Turkish, FH	80	5.0	2.2–6.5
	R496W	Turkish, FH	80	8.7	2.0–9.8
Eroğlu et al. (2018)	D374Y	Turkish, dyslipidemia	200	7.0	3.7–8.7
	R496W	Turkish, dyslipidemia	200	6.5	3.7–8.7
Luirink et al. (2019)	A220T	Netherlander, FH	1,903	0.1	7.7–9.0

Normal range of LDL-C: Low-risk groups < 3mmol/L; High-risk groups < 1.8 mmol/L; FH groups < 1.4 mmol/L. (According to the 2019 ESC/EAS guidelines for the management of dyslipidaemias). PCSK9, proprotein convertase subtilisin/kexin type 9; LDL-C, low-density lipoprotein cholesterol; FH, familial hypercholesterolemia; ADH, autosomal dominant hypercholesterolemia.

chip and deoxyribonucleic acid (DNA) sequencing to identify p.(L253F), p.(A443T), and p.(R46L) missense mutants which were significantly correlated with diminished amounts of LDL-C. Moreover, after sequencing for variations in 403 Caucasians, Yue et al. (2006) identified a c.43_44insCTG mutant that mediated LDL-C level reductions in normal people. In 2007, Fasano et al. (2007) identified a novel variant in exon 1 (c.202delG) of a single nucleotide deletion from one heterozygous patient, that resulted in messenger ribonucleic acid (mRNA) generating peptide frameshift and truncation at p.(A68fsL82X). In the next year, Miyake et al. (2008) found 33 *PCSK9* gene sequence variants, with one at p.(R93C)

TABLE 2 | Summary of LOF variants among different populations.

Researchers	*PCSK9* variants	Population	Sample size	Variant frequency, %	LDL-C ranges, mmol/L
Cohen et al. (2006)	Y142X	ARIC study, general	3,363	0.8	1.7–3.7
	C679X	ARIC study, general	3,363	1.8	1.4–3.8
	R46L	ARIC study, general	9,524	3.2	2.2–3.9
Hooper et al. (2007)	C679X	African, general	653	3.7	1.3–1.9
Scartezini et al. (2007)	R46L	British, general	2,444	1.0*	1.8–4.0
Guella et al. (2010)	R46L	Italian, MI patients	1,880	1.0*	2.1–3.9
Chernogubova et al. (2012)	R46L	Swedish, general	5,722	1.9*	2.5–4.9
Saavedra et al. (2014)	R46L	Canadian, FH	582	3.0	5.8–7.7
Langsted et al. (2016)	R46L	CGPS study	103,083	1.3*	2.2–3.4
Mostaza et al. (2018)	R46L	Spanish, adults	1,188	2.9*	2.6–4.3
	R46L	Spanish, children and adolescents	1,933	3.2*	2.2–2.6

*Normal range of LDL-C: Low-risk groups < 3 mmol/L; High-risk groups < 1.8 mmol/L; FH groups < 1.4 mmol/L. (According to the 2019 ESC/EAS guidelines for the management of dyslipidaemias). PCSK9, proprotein convertase subtilisin/kexin type 9; LDL-C, low-density lipoprotein cholesterol; ARIC, Atherosclerosis Risk in Communities; MI, myocardial infarction; CGPS, Copenhagen General Population. *Represents the allele frequency which is different from the PCSK9 variant proportion of the population in the different references.*

which had a 0.051 R93C allele prevalence in low vs. high LDL-C groups, and was associated with low LDL-C levels. Meanwhile, the LOF variants p.(G236S) and p.(N354I) were described by Cameron et al. (2008). The p.(G236S) variant prevented *PCSK9* release from the endoplasmic reticulum (ER) while p.(N354I) resulted in failure of *PCSK9* to undergo autocatalytic cleavage.

Identification of Novel *PCSK9* Variants Associated With FH in the Last 10 Years

In the past 10 years, with the emergence of related studies on *PCSK9* inhibitors and other treatment of patients with statin-intolerant hyperlipidemia, including patients with FH, less attention has been paid to the analysis of *PCSK9* variants than previously (**Figure 1**).

With respect to LOF variants of *PCSK9*, Cameron et al. (2009) identified the novel variant p.(S462P) in exon 9 of the *PCSK9* gene and suggested that this variant, like the p.(G236S) and p.(N354I) variants, prevented normal C-terminal domain folding precluding release of the protein from the ER. Another LOF variant, the double-mutant p.(R104C)/p.(V114A), which improved the clearance rate of LDL-C (Cariou et al., 2009). Mayne et al. (2011) identified a p.(Q152H) substitution in a French-Canadian family which resulted in a 48% reduction in LDL-C concentration compared with non-carriers. Slimani et al. (2012) investigated *PCSK9* and *LDLR* variants in Tunisian FH families, identifying a new missense variation p.(P174S) that appeared to cause decreased levels of LDL-C with respect to the LDLR genotype in six family members. In Pakistan, FH family members who carried the p.(R105Q) variant had lower levels of total cholesterol suggesting that this variant might cause LOF (Ahmed et al., 2013). Through targeted next-generation sequencing (NGS), the variants p.(R93C) and p.(G670E) were identified by Lee et al. (2017) in nine patients with very low levels of LDL-C. In addition, one carrier of the heterozygous missense variant p.(G316C) that was associated with hypocholesterolemia and steatosis was found (Di Filippo et al., 2017).

Regarding *PCSK9* GOF variants, two novel variants were found in 75 patients with ADH and normal *APOB* and *LDLR* genes by Abifadel et al. (2012) namely p.(D35Y) and p.(L108R) substitutions. The variants were absent from individuals with normal cholesterol levels and were associated with the presence of ADH in families. The authors also assessed the quantitative and qualitative effects of these *PCSK9* variants on lipoprotein granules and their effect on the activity of cholesterol ester transfer protein (CETP). This was the first report of the impact of *PCSK9* variants on high density lipoprotein (HDL)-mediated cholesterol release from cells. Ohta et al. (2016) found that the p.(V4I) variant of *PCSK9* in 269 clinically diagnosed FH heterozygotes was related to a remarkably increased CAD prevalence accompanied by increasing levels of LDL-C, although the levels of serum lipids and the CAD prevalence between p.(V4I) carriers and non-carriers without *LDLR* variants remained similar. Di Taranto et al. (2017) demonstrated two GOF variants, p.(S636R) and p.(R357C), considered to cause FH. In their study, the p.(S636R) and p.(R357C) variants showed a lower binding capacity for WT *PCSK9* than *LDLR*. They also found a further two mutants with uncharacterized effects on FH disease progression. Elbitar et al. (2018) sequenced exons from 13 French FH families and discovered a *PCSK9* variant at p.(R96C) in a patient with a severe phenotype from a family with the p.(A3396T) *APOB* variant. They demonstrated that this was the first reported compound heterozygote having both *APOB* and *PCSK9* variants. In addition, Sánchez-Hernández et al. (2019) found a novel *PCSK9* variant, p.(R499H) of in two unrelated FH patients from Spain and Italy. This variant resulted in decreased expression of *LDLR* from *in vitro* functional assays. There were two related studies on *PCSK9* in FH children: DECOPIN and GoTCHA. In the DECOPIN project, Ibarretxe et al. (2018) identified 49 different variants including two in *PCSK9* parents and children with FH. In the child-to-parent study, p.(R496W) and p.(L22_L23dup) were found to be pathogenic in families. Luirink et al. (2019) analyzed a total of 1903 FH children with molecular assays in the GoTCHA study. They also conducted candidate gene sequencing in HoFH children whose LDL-C levels were above the lowest level

measured in pediatric patients with HoFH. In their study, a GOF variant of *PCSK9* p.(A220T) was found in two related patients.

In studies of the *PCSK9* variants in Asian populations, Noguchi et al. (2010) identified the p.(E32K) variant in Japanese people. In their study, the frequency of the p.(E32K) variant in clinical FH was significantly higher than that in a control group (6.42 vs. 1.71%). Although this GOF variant might have milder effects than the p.(D374Y) and *LDLR* variants, it could worsen lipid conditions in true homozygous or double heterozygous probands with *LDLR* variants. In China, Xiang et al. (2017) identified two *PCSK9* variants in FH patients including p.(R96L) and p.(R105W) from the central southern region of China. As GOF variants, these two variants might cause increased *LDLR* degradation, resulting in a decrease of LDL-C clearance, eventually giving rise to hypercholesterolemia. In their study, they also found another nine novel variants which included seven *LDLR* variants and two *APOB* variants.

MOLECULAR AND BIOLOGICAL MECHANISMS TO EXPLORE THE INFLUENCE OF *PCSK9*

PCSK9 belongs to the proprotein convertase family of serine proteases. Biological and gene analyses have shown that *PCSK9* is a vital regulator of *LDLR* proteins which in turn, regulate plasma LDL-C. Through indirectly causing the degradation of *LDLR*, *PCSK9* normally downregulates *LDLR* with LOF *PCSK9* variants resulting in low levels of LDL-C in the plasma (Soutar and Naoumova, 2007; Ding and Kullo, 2008; Schmidt et al., 2008; **Figure 2**).

Interactions Between *PCSK9* and Its Related Proteins

Influential Elements in *PCSK9* Transcription

Conversion of *PCSK9* is regulated by the sterol regulatory element-binding protein (SREBP) 2, a membrane transcription factor. In addition, transcription to enable *PCSK9* production is also regulated by SREBP 2 (Jeong et al., 2008; Wiciński et al., 2017). GOF variants in *PCSK9* have been found proven to lead to FH through reducing *LDLR* protein expression in the liver and decreasing the clearance of circulating cholesterol. Statin therapy is able to inhibit cholesterol biosynthesis followed by the activation of SREBP to increase the expression of *LDLR*. However, statins also induce the synthesis of *PCSK9*, leading to the degradation of *LDLR* (Rashid et al., 2005; Tall, 2006). Transcription of *PCSK9*, either basal or sterol-regulated, depends on the recognition of a motif in the promoter region by histone nuclear factor P (HINFP). Variants in this region prevent sterol-induced blockade and diminish promoter activity at basal levels in addition to abating promoter activation by SREBP 2 (Li and Liu, 2012). Transcription factors for SREBP depend on binding to the SREBP cleavage-activating protein (SCAP) in the ER membrane. Investigations of SCAP inhibition in a monkey model revealed that when SCAP small interfering RNA (siRNA) encapsulated in lipid nanoparticles is added, both LDL-C levels and *PCSK9* are significantly reduced (Murphy et al., 2017). Chitin oligosaccharides (COS) have antioxidant and anti-inflammatory activities and have been recently found to suppress *PCSK9* gene expression thereby decreasing the number of LDLRs on the cell surface. Thus, down-regulation of SREBP2 by COS decreases the expression of *PCSK9* (Yang et al., 2018).

Connection Between *PCSK9* and *LDLR*

PCSK9 binds to the epidermal growth factor precursor homology domain A (EGF-A) on extracellular *LDLR* domains which regulates hepatic *LDLR*s and leads to their degradation. Asp-374 on the surface of the subtilisin-like catalytic domain of *PCSK9* binds the *LDLR* EGF-A domain. Substitution of the Asp with Tyr (the p.(D374Y) GOF variant enhances *PCSK9* affinity for *LDLR*. This combination of *PCSK9* and *LDLR* may cause a conformational change in *LDLR* that prevents the recycling of *LDLR* from the plasma membrane, instead of leading the complex containing *PCSK9* and *LDLR* to degradation (Kwon et al., 2008; Cariou and Dijk, 2020; Martin et al., 2020). Effects on the folding of *PCSK9* potentially caused by LOF variants such as p.(Q152H), could trigger increased clearance of LDL-C in the circulation, due to the reduction of *LDLR* degradation mediated by *PCSK9* (Garvie et al., 2016). In tandem, researchers have found that the 314–355 *LDLR* EGF-A domain and the 153–421 *PCSK9* catalytic domain are involved in the interaction between *PCSK9* and *LDLR* that leads to decreased *LDLR* levels and LDL-C accumulation (Alghamdi et al., 2015). In a strategy using synthetic EGF-A analogs, it has been found that the peptide with the greatest potency enhanced *PCSK9* binding affinity compared with WT EGF-A (Schroeder et al., 2014). In addition, a study of the C-terminal domain of *PCSK9* created seven *de novo* mutants of *PCSK9* and investigated their affinity toward a calcium-independent mutant of the EGF-A domain. This showed that the p.(G517R), p.(V644R), and p.(V610R) mutants have descending abilities to prevent LDL-C growth in HepG2 cells (Geschwindner et al., 2015). Moreover, ligand-binding (LR) repeats of *LDLR* have been identified for *PCSK9*-mediated *LDLR* degradation and the p.(D203N) variant in the LR5 of full-length LDLR was found to significantly reduce *PCSK9* binding (Deng et al., 2019). Interestingly, in studies of post-translational modifications, *PCSK9* treatment can also result in ubiquitination of *LDLR*. Importantly, if the *LDLR* protein carried variants in its C-terminal ubiquitination sites, it was able to resist *PCSK9*-mediated degradation (Chen et al., 2011). Recently, it has been observed that phosphorylation may enhance the secretion of *PCSK9* from hepatocytes, thus maximizing *LDLR* degradation through both extracellular and intracellular pathways (Ben Djoudi Ouadda et al., 2019).

Possible Effects of *PCSK9* on Cellular Function

The lectin-like ox-LDL receptor-1 (LOX-1) has been shown to play a critical role in inflammatory diseases, including atherosclerosis, where LOX-1 and *PCSK9* positively influence each other's expression and it appears that mitochondria-derived reactive oxygen species (mtROS) may to be important initiators of *PCSK9* and LOX-1 expression (Ding et al., 2015). Interestingly,

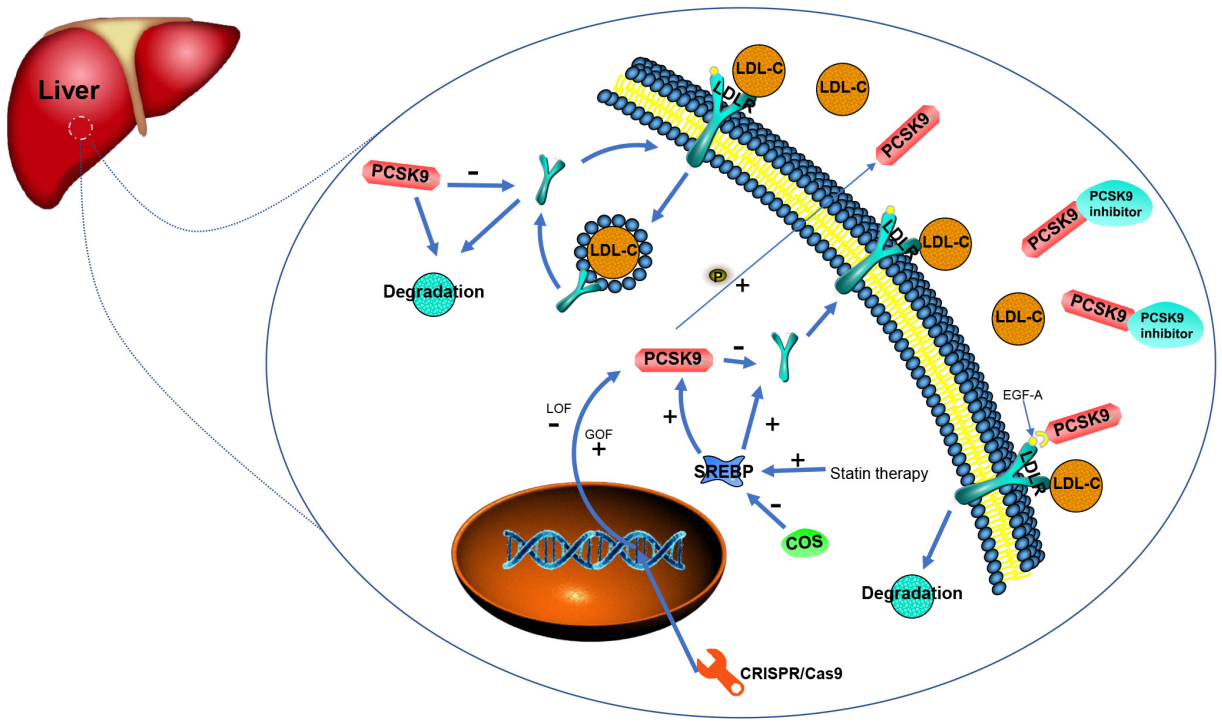

FIGURE 2 | Function and regulation related to *PCSK9* and existing/potential therapies for *PCSK9* inhibition (adopted and redrawn from Hovingh et al., 2013; German and Shapiro, 2020).

LOX-1 and *PCSK9* might be upregulated secondary to induction by ox-LDL in a concentration-dependent manner and ox-LDL-induced human umbilical vein endothelial cell death could be inhibited by *PCSK9* siRNA (Wu et al., 2012). Subsequently, in a study of vascular smooth muscle cell mitochondrial DNA (mtDNA) and (SMC)-derived *PCSK9* damage, it was found that in the presence of mtROS, there was a positive relationship between mtDNA damage and SMC-derived *PCSK9*. This interaction leads to cell damage, characterized by apoptosis (Ding et al., 2016). Meanwhile, endothelial cell apoptosis may be repressed through mitogen-activated protein kinase signaling in atherosclerosis by shRNA-*PCSK9* targeting of *PCSK9* (Li et al., 2017). Investigation of *PCSK*-induced autophagy mechanisms showed that *PCSK9* might be up-regulated in ischemic hearts, thus determining infarct size, cardiac function, and autophagy development through the activation of the ROS-related axis (Ding et al., 2018).

FROM DIAGNOSIS METHODS TO THERAPY STRATEGIES OF FH BASED ON *PCSK9*

Cascade Screening

Cascade screening is a vital procedure for identifying people at risk for inherited diseases. For some autosomal dominant diseases, such as FH, relatives can be identified for significant health-affecting interventions, thus significantly increasing life expectancy. Cascade screening is an evidence-based intervention that has been found to reduce cardiovascular morbidity and mortality in the FH population (Knowles et al., 2017). A series of population-based screening and research initiatives, represented by the Dutch Lipid Clinic Network in the Netherlands (DLCNC), have made tremendous progress in raising awareness and treatment of FH (O'Brien et al., 2014). When cascade screening was first introduced in the Netherlands, 2039 relatives of 237 FH cases were found to have FH, 39% of whom had already been receiving treatment. An Australian study also found that among 100 relatives of genetically diagnosed FH patients, 51.4% had pathogenic variants identified by cascade screening. In other recent cases, they have demonstrated other CAD risk factors and have already started using statins without prior diagnosis of FH (Bell et al., 2015; Henderson et al., 2016). In Asia, cascade screening is proving to be an efficient method for the diagnosis of FH in Vietnamese family members. After screening 107 relatives in five FH patients, 56 cases were diagnosed with FH, including three HoFH cases (Truong et al., 2018). Cascade screening is also important in children and adolescents. After analyzing 292 children with FH from 205 parents, researchers found that 20 percent of the parents and 49 percent of the maternal grandparents had an early history of CVD. Similarly, in a Slovenian study, nearly every child diagnosed with FH had a parent who was at high risk for FH. Thus, CVD is still the main cause of FH, and despite the absence of evidence on the long-term safety of drug therapy in childhood, genetic natural history

studies confirm the benefits of lifelong low LDL-C levels, so early initiation of cascade screening will facilitate early intervention in the next generation (Galema-Boers et al., 2015; Wiegman et al., 2015; Groselj et al., 2018).

Early detection and treatment of FH in individuals and families could help reduce the morbidity and mortality associated with FH. Indeed, the setting up of an FH database and registration system would be a critical measure to enhance the long-term outcomes of FH patients. Currently, in most countries, diagnosis and treatment models are not adequate (Singh and Bittner, 2015; Chen et al., 2019). However, the cost of DNA sequencing in patients has fallen considerably in the last few years and if the rate of progress continues, the current lack of detection and screening might change (Louter et al., 2017). Below, we list the essential and potential ways of FH detection and screening.

Innovative Ways of Detecting and Screening in FH

From Sanger Sequencing to NGS

Before NGS was widely applied, the genetic diagnosis of FH mainly relied on Sanger sequencing to identify variants in the *APOB* and *PCSK9* genes. After overcoming many of the scalability barriers faced by clinical laboratories using traditional Sanger methods when performing large-scale DNA sequencing according to the guideline and list of genes reported as incidental or secondary findings of the American College of Medical Genetics (ACMG), NGS has gradually been proved to be a reliable and practical molecular screening method for FH pathogenic genes and become one of the main techniques for *PCSK9* detection, while Sanger sequencing is mainly used as a verification method to assist the accuracy of NGS detection (Rehm et al., 2013; Rimbert et al., 2016; Kalia et al., 2017; Pek et al., 2018). Moreover, the guidelines for diagnostic NGS of the EuroGentest and European Society of Human Genetics have emphasized that, although NGS testing was still being explored and developed, NGS technology offered potential overall benefits for the diagnosis of patients' diseases (Matthijs et al., 2016). As NGS are increasingly used for routine FH diagnosis, FH-related variants may be identified exponentially, so detection of disease-related variants in FH patients is critical for early intervention to reduce the risk of CVD. In a study of a British cohort, compared with multiplex polymerase chain reaction and oligonucleotide arrays, the NGS method has shown great analytical performance with approximately 89–100% concordance to other methods (Reiman et al., 2016; Iacocca et al., 2018). What is more, in the first reported NGS test for variants in clinically suspected FH patients in Singapore, the percentage of detected variants was similar to that of western countries, and although no *PCSK9* variants were found, it indicated that NGS technology covering all exons of *LDLR* might be a better strategy (Pek et al., 2018).

In the 2020 technical standard of ACMG, it is recommended that the laboratory must consider the effectiveness of NGS analysis and augment NGS testing with ancillary assays (Bean et al., 2020). In a study of NGS as a potential method for diagnosing FH, NGS-based testing has been shown to involve lower cost and less labor than traditional sequencing genetic testing. This may provide a way to increase the genetic diagnosis of the current low proportion of FH (Norsworthy et al., 2014). With the continuous development of NGS technology, new NGS-based detection is also gradually being applied to FH screening. In a single target NGS panel study, this new NGS is found to be an effective variant detection method, which can better help to understand the phenotype of FH, and is expected to become a personalized diagnosis method for dyslipidemia (Marmontel et al., 2018). In addition, in the first study of capture-based NGS, this NGS method covering the entire *LDLR* genome region has improved the efficiency of structural variation detection. This method is expected to comprehensively detect the pathogenic variants of *LDLR*, *APOB,* and *PCSK9* in FH patients (Hsiung et al., 2018).

iPLEX Test

To relieve the shortage of intensive procedures which have complicated genetic diagnoses, Wright et al. (2008) developed the Multiplex MassARRAY Spectrometry (iPLEX) and identified 56 variants in a number of genes, including one variant in *PCKS9,* in DNA samples from 92 FH patients. From this study, it is clear that, while the FH iPLEX test is aimed at screening for FH variants in large-scale targeted populations, it is also suitable for population screening. The Agena iPLEX designed by Chiou and Charng (2017). has been found to have higher specificity and sensitivity to FH gene screening, compared with the traditional diagnostic Sanger sequencing procedure, in detecting DNA from 120 FH patients with defined molecular causes.

HRM Method

In contrast to existing detection methods for genetic abnormalities in FH patients, a high-resolution melting (HRM) analysis known as the polymerase chain reaction (PCR) with modifiable melting conditions, has been proven to be more efficient compared to DNA sequencing. Firstly, it is more cost-effective and timesaving. Secondly, as a sensitive and robust technique, it is capable of detecting new sequence changes that would make sense in cascade screening of HF subjects (Liyanage et al., 2008; Whittall et al., 2010; Pećin et al., 2013). In a Turkish FH cohort using HRM analysis on isolated DNA, it was demonstrated that two *PCSK9* GOF variants [p.(D374Y) and p.(R496W)] in FH have a higher frequency and different courses of disease compared to other populations around the world (Kaya et al., 2017).

Chips Technology

Given the large number of patients with suspected FH, molecular genetic analysis of the entire genome is time-consuming. A new diagnostic tool in the form of a genotyping DNA microarray chip called FH chip based on arrayed primer extension (APEX), has been proposed to accelerate variant screening in Czech FH patients. In this study, researchers found that the validation phase of this FH chip had 100% sensitivity and 99.1% specificity. They suggested that the FH chip could be implemented for genotyping with features of rapidity, reproducibility, specificity, and economy (Dušková et al., 2011). The Belfast Genetics Laboratory aims to keep abreast of evolving technology by simple

and economic genetic testing. An FH biochip array protocol is followed by using samples analyzed for FH variants prior to incorporation into cascade screening. Study results estimate that NGS is five times more expensive than the cost of testing and reporting one sample through the FH Biochip technique due to the latter's high sensitivity and rapid detection ability (Martin et al., 2016).

Current Therapies for *PCSK9*-Associated FH

PCSK9 Inhibitors

Appropriate medical treatment is essential to effectively manage FH, including reducing cardiovascular risk and improving the prognosis of affected patients. The current drug treatments for FH mainly include statins, bile acid-binding resins, and cholesterol absorption inhibitors. Considering that the risk of cardiovascular events in FH patients is significantly increased, timely reduction of LDL-C is essential to reduce the risk of CVD. However, traditional drugs may have difficulty in achieving the goal of decreased blood lipid in FH patients, thus, *PCSK9* inhibitors with strong lipid-lowering effects have gradually become a new class of drugs for the treatment of FH (Fala, 2016; Papademetriou et al., 2018). In a meta-study, it was found that when evolucomab is given as a 420 mg monthly dose, LDL-C could be reduced by 54.71%, indicating that evolocumab might be a potential drug for FH patients (Eslami et al., 2017). It has also been found that alirocumab treatment is well-tolerated in heterozygous FH (HeFH) patients and could significantly reduce LDL-C by 12 and 24 weeks while evolocumab could effectively reduce the LDL-C levels in HoFH or severe HeFH patients over a median of 4.1 years (Ginsberg et al., 2019; Santos et al., 2020). In summary, *PCSK9* inhibitors can significantly reduce LDL-C, even in FH patients who have not yet achieved their LDL-C goals. Therefore, to ensure that FH patients can receive the *PCSK9* inhibitor drug therapies, it is necessary to increase the diagnosis rate and conduct family screening in certain populations (Ogura, 2018).

Inclisiran

In recent years, with the advent of siRNA, researchers have designed a new generation of drugs to combat *PCSK9* named Inclisiran. It can reduce the concentration of *PCSK9* in the body by interfering *PSCK9* gene expression in hepatocytes with a double-stranded short sequence of RNA, thereby reducing the degradation of *LDLR* and enhancing the ability of hepatocytes to eliminate LDL-C to reduce its levels (Dyrbuś et al., 2020). In 2017, Ray et al. (2017) found that Inclisiran could significantly reduce LDL-C after the first subcutaneous injection (>50%) and maintained this level for up to 1 year after the first injection. Compared with the placebo group, the Inclisiran group had no serious adverse reactions. In the subsequent phase III clinical study of Inclisiran, compared with placebo, LDL-C in Inclisiran group was significantly reduced, with the efficacy able to last for more than 18 months (Raal et al., 2020; Ray et al., 2020). The introduction of *PCSK9* inhibitors is a milestone in the treatment of FH, and Inclisiran provides a new lipid-lowering technology. The clinical trials of the Inclisiran series of drugs are expected to produce a revolutionary new lipid-lowering drug (German and Shapiro, 2020).

Development of CRISPR/Cas9 Therapy in *PCSK9*

In 2003, the characterization of *PCSK9*'s LDL-C regulatory functions resulted in a landmark paradigm shift in therapies for hypercholesterolemia (Seidah et al., 2019). There is reason to believe that it might soon be possible to achieve effective cholesterol management by permanently and selectively modifying the genome and inactivating the function of target genes with a single injection (Fazio and Tavori, 2014). Among the nine ways to realize *PCSK9* inhibition, clustered regularly interspaced short palindromic repeats (CRISPR) technology might have great potential (Mullard, 2017). Though the CRISPR/Cas9 technology offers flexibility for treating hyperlipidemia and is capable of changing the genome to permanently decrease cholesterol levels, more research is needed before their application in human subjects (Banerjee et al., 2016; Chadwick and Musunuru, 2017).

The Attempt and Exploration

Ding et al. (2014) use CRISPR/CRISPR-associated (Cas) technology to obtain a *PCSK9*-targeting CRISPR guide RNA and Cas 9 expression in murine livers with an adenovirus to effectively disrupt murine *PCSK9* genes, resulting in reduced levels of cholesterol and *PCSK9*, but elevated amounts of liver *LDLR*. This momentous finding in genome editing might have potential for the therapy and prevention of CVD in humans. Subsequently, severely diminished levels of blood *PCSK9* are seen in mice with humanized livers with almost 50% of highly specific human hepatocyte variants, thus demonstrating the safety and effectiveness of CRISPR-Cas9 in reducing human *PCSK9* levels (Wang et al., 2016). In addition, CRISPR/Cas9-mediated genome editing decreases *PCSK9* levels in both human and murine hypercholesterolemic models, which could be a valuable tool in the search for novel therapeutic approaches against hypercholesterolemia (Carreras et al., 2019).

The Development and Outlook

It is to get into mammalian cells without a vehicle. Additionally, gene editing using CRISPR/Cas9 technology, which is both sizeable and based mainly on DNA, mRNA, or protein, also poses a significant challenge. Although viral vectors have higher delivery efficiency and adeno-associated viral vectors have recognized efficiency in atherosclerosis research, their biosecurity issues hinder their wide application. Based on the nanocarrier-delivered CRISPR/Cas9 system, Zhang et al. (2019) use a triple targeting strategy to produce a LOF variant in the *PCSK9* gene and this strategy might be a potential target therapy for CVD without side effects (Jarrett et al., 2018). In addition, precise knock-in of specific nucleotide changes have proven to be inefficient in non-proliferating cells *in vivo*. Chadwick et al. (2017) therefore, used *PCSK9* base editing, which has the ability to generate alterations in genes without the need for breaks in double-stranded DNA. This demonstrates the ability to precisely

introduce therapeutically relevant nucleotide variants into the genome in somatic tissues in adult mammals.

RECENTLY DISCOVERED POTENTIAL GENETIC TARGETS RELATED TO FH

Presumptive Loci Related to FH

In order to identify ADH disease loci besides *PCSK9*, *LDLR* and *APOB*, on the basis of a genome-wide scan and linkage analysis, Marques-Pinheiro et al. (2010) report a large lineage from France and hypothesized the involvement of a fourth gene, named *HCHOLA4*, at 16q22.1. In their study, it is shown that other ADH genes do exist, while they also identify nine affected families with no linkage to the *HCHOLA4* locus nor the three known genes. Apart from *PCSK9*, *LDLR,* and *APOB*, it has been found that there is a significant relationship between ADH and rs965814 G allele mapping to 8q24.22 through genome-wide analysis on 15 family members (Cenarro et al., 2011).

The Controversial Relationship Between *STAP1* and FH

Exome sequencing was performed by Fouchier et al. (2014) along with parametric linkage analysis in a family with FH4 (ADH with unmutated genes for *PCSK9*, *APOB,* and *LDLR*) to identify a fourth ADH relevant locus, leading to the mapping of the ADH locus at 4p13. In addition, ADH associates with four signal-transducing adaptor family member 1 (*STAP1*) variants, including p.(E97D), p.(L69S), p.(I71T), and p.(D207N). Out of all the *STAP1* missense variants analyzed by bioinformatics analysis and available structural information in one man and his two siblings, p.(P176S) which might play a role of affecting cholesterol homeostasis has been associated with FH as the likely most damaging variant (Blanco-Vaca et al., 2018). However, after *STAP1* was analyzed in hypercholesterolemic patients from Germany, whose variants are negative in canonical FH genes, it was concluded that the positive predictive value of *STAP1* analysis would be comparably small and in order to characterize *STAP1* contributions to FH pathogenesis, its molecular interactions should be explored through *in vitro* functional studies (Danyel et al., 2019). Moreover, despite noting that FH patients carrying *STAP1* have lower LDL-C levels than non-carriers, Lamiquiz-Moneo et al. (2020) found no phenotypic penetrance of their genome when exploring potential associations between phenotype and *STAP1* variants. What is more, studies of mouse models and carriers of *STAP1* variants, indicate that *STAP1* might not be the FH or LDL-C modulating gene and should not be considered for FH genetic screening (Loaiza et al., 2020).

PERSPECTIVE

The unique mechanism of action of *PCSK9* and the identification of its genetic variants have brought a new therapeutic target to FH. Among the numerous variant sites, both GOF and LOF might become new breakthrough points of treatment. Since the use of *PCSK9* inhibitors has emerged outside conventional FH treatment strategies, their gradually increasing applications in clinical practice have not only brought good news to FH patients but also new hope for patients with hyperlipidemia that are at high risk of CAD. It is reasonable to believe that, with continuous research progress on *PCSK9* in FH, more therapeutic methods and diagnostic methods with superior accuracy, safety, and economy will be applied in FH patients. Interactions of *PCSK9* with various cellular components, based on its unique features and influence on the body, are also worthy of further research and discussion. Last, but not least, as a disease related to genetic variant, there are undoubtedly more undiscovered genetic loci related to FH that are worth exploring in addition to the existing FH genes.

AUTHOR CONTRIBUTIONS

QG and XF wrote this review and YZ revised it. All authors read and approved the final manuscript.

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REFERENCES

Abifadel, M., Bernier, L., Dubuc, G., Nuel, G., Rabès, J. P., Bonneau, J., et al. (2008). A PCSK9 variant and familial combined hyperlipidaemia. *J. Med. Genet.* 45, 780–786. doi: 10.1136/jmg.2008.059980

Abifadel, M., Guerin, M., Benjannet, S., Rabès, J. P., Le Goff, W., Julia, Z., et al. (2012). Identification and characterization of new gain-of-function mutations in the PCSK9 gene responsible for autosomal dominant hypercholesterolemia. *Atherosclerosis* 223, 394–400. doi: 10.1016/j.atherosclerosis.2012.04.006

Abifadel, M., Rabès, J. P., Devillers, M., Munnich, A., Erlich, D., Junien, C., et al. (2009). Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. *Hum. Mutat.* 30, 520–529. doi: 10.1002/humu.20882

Abifadel, M., Varret, M., Rabès, J. P., Allard, D., Ouguerram, K., Devillers, M., et al. (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat. Genet.* 34, 154–156. doi: 10.1038/ng1161

Ahmed, W., Whittall, R., Riaz, M., Ajmal, M., Sadeque, A., Ayub, H., et al. (2013). The genetic spectrum of familial hypercholesterolemia in Pakistan. *Clin. Chim. Acta.* 421, 219–225. doi: 10.1016/j.cca.2013.03.017

Alghamdi, R. H., O'Reilly, P., Lu, C., Gomes, J., Lagace, T. A., and Basak, A. (2015). LDL-R promoting activity of peptides derived from human PCSK9 catalytic domain (153-421): design, synthesis and biochemical evaluation. *Eur. J. Med. Chem.* 92, 890–907. doi: 10.1016/j.ejmech.2015.01.022

Allard, D., Amsellem, S., Abifadel, M., Trillard, M., Devillers, M., Luc, G., et al. (2005). Novel mutations of the PCSK9 gene cause variable phenotype of autosomal dominant hypercholesterolemia. *Hum. Mutat.* 26:497. doi: 10.1002/humu.9383

Banerjee, Y., Santos, R. D., Al-Rasadi, K., and Rizzo, M. (2016). Targeting PCSK9 for therapeutic gains: Have we addressed all the concerns? *Atherosclerosis* 248, 62–75. doi: 10.1016/j.atherosclerosis.2016.02.018

Bean, L. J. H., Funke, B., Carlston, C. M., Gannon, J. L., Kantarci, S., Krock, B. L., et al. (2020). Diagnostic gene sequencing panels: from design to report-a technical standard of the American College of Medical Genetics and Genomics (ACMG). *Genet. Med.* 22, 453–461. doi: 10.1038/s41436-019-0666-z

Bell, D. A., Pang, J., Burrows, S., Bates, T. R., van Bockxmeer, F. M., Hooper, A. J., et al. (2015). Effectiveness of genetic cascade screening for familial hypercholesterolaemia using a centrally co-ordinated clinical service: an Australian experience. *Atherosclerosis* 239, 93–100. doi: 10.1016/j.atherosclerosis.2014.12.036

Ben Djoudi Ouadda, A., Gauthier, M. S., Susan-Resiga, D., Girard, E., Essalmani, R., Black, M., et al. (2019). Ser-Phosphorylation of PCSK9 (Proprotein Convertase Subtilisin-Kexin 9) by Fam20C (Family With Sequence Similarity 20, Member C) Kinase Enhances Its Ability to Degrade the LDLR (Low-Density Lipoprotein Receptor). *Arterioscler. Thromb. Vasc. Biol.* 39, 1996–2013. doi: 10.1161/ATVBAHA.119.313247

Benjannet, S., Rhainds, D., Essalmani, R., Mayne, J., Wickham, L., Jin, W., et al. (2004). NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J. Biol. Chem.* 279, 48865–48875. doi: 10.1074/jbc.M409699200

Berge, K. E., Ose, L., and Leren, T. P. (2006). Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. *Arterioscler. Thromb. Vasc. Biol.* 26, 1094–1100. doi: 10.1161/01.ATV.0000204337.81286.1c

Blanco-Vaca, F., Martín-Campos, J. M., Pérez, A., and Fuentes-Prior, P. (2018). A rare STAP1 mutation incompletely associated with familial hypercholesterolemia. *Clin. Chim. Acta.* 487, 270–274. doi: 10.1016/j.cca.2018.10.014

Blesa, S., Vernia, S., Garcia-Garcia, A. B., Martinez-Hervas, S., Ivorra, C., Gonzalez-Albert, V., et al. (2008). A new PCSK9 gene promoter variant affects gene expression and causes autosomal dominant hypercholesterolemia. *J. Clin. Endocrinol. Metab.* 93, 3577–3583. doi: 10.1210/jc.2008-0269

Bourbon, M., Alves, A. C., Medeiros, A. M., Silva, S., and Soutar, A. K. (2008). Investigators of Portuguese FH Study. *Familial hypercholesterolaemia in Portugal. Atherosclerosis* 196, 633–642. doi: 10.1016/j.atherosclerosis.2007.07.019

Cameron, J., Holla, O. L., Laerdahl, J. K., Kulseth, M. A., Ranheim, T., Rognes, T., et al. (2008). Characterization of novel mutations in the catalytic domain of the PCSK9 gene. *J. Intern. Med.* 263, 420–431. doi: 10.1111/j.1365-2796.2007.01915.x

Cameron, J., Holla, ØL., Laerdahl, J. K., Kulseth, M. A., Berge, K. E., and Leren, T. P. (2009). Mutation S462P in the PCSK9 gene reduces secretion of mutant PCSK9 without affecting the autocatalytic cleavage. *Atherosclerosis* 203, 161–165. doi: 10.1016/j.atherosclerosis.2008.10.007

Cariou, B., and Dijk, W. (2020). EGF-A peptides: A promising strategy for PCSK9 inhibition. *Atherosclerosis* 292, 204–206. doi: 10.1016/j.atherosclerosis.2019.11.010

Cariou, B., Ouguerram, K., Zaïr, Y., Guerois, R., Langhi, C., Kourimate, S., et al. (2009). PCSK9 dominant negative mutant results in increased LDL catabolic rate and familial hypobetalipoproteinemia. *Arterioscler. Thromb. Vasc. Biol.* 29, 2191–2197. doi: 10.1161/ATVBAHA.109.194191

Carreras, A., Pane, L. S., Nitsch, R., Madeyski-Bengtson, K., Porritt, M., Akcakaya, P., et al. (2019). In vivo genome and base editing of a human PCSK9 knock-in hypercholesterolemic mouse model. *BMC Biol.* 17:4. doi: 10.1186/s12915-018-0624-2

Cenarro, A., García-Otín, A. L., Tejedor, M. T., Solanas, M., Jarauta, E., Junquera, C., et al. (2011). A presumptive new locus for autosomal dominant hypercholesterolemia mapping to 8q24.*22. Clin. Genet.* 79, 475–481. doi: 10.1111/j.1399-0004.2010.01485.x

Chadwick, A. C., and Musunuru, K. (2017). Treatment of Dyslipidemia Using CRISPR/Cas9 Genome Editing. *Curr. Atheroscler. Rep.* 19:32. doi: 10.1007/s11883-017-0668-8

Chadwick, A. C., Wang, X., and Musunuru, K. (2017). In Vivo Base Editing of PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9) as a Therapeutic Alternative to Genome Editing. *Arterioscler. Thromb. Vasc. Biol.* 37, 1741–1747. doi: 10.1161/ATVBAHA.117.309881

Chen, P., Chen, X., and Zhang, S. (2019). Current Status of Familial Hypercholesterolemia in China: A Need for Patient FH Registry Systems. *Front. Physiol.* 10:280. doi: 10.3389/fphys.2019.00280

Chen, S. N., Ballantyne, C. M., Gotto, A. M. Jr., Tan, Y., Willerson, J. T., and Marian, A. J. (2005). A common PCSK9 haplotype, encompassing the E670G coding single nucleotide polymorphism, is a novel genetic marker for plasma low-density lipoprotein cholesterol levels and severity of coronary atherosclerosis. *J. Am. Coll Cardiol.* 45, 1611–1619. doi: 10.1016/j.jacc.2005.01.051

Chen, Y., Wang, H., Yu, L., Yu, X., Qian, Y. W., Cao, G., et al. (2011). Role of ubiquitination in PCSK9-mediated low-density lipoprotein receptor degradation. *Biochem. Biophys. Res. Commun.* 415, 515–518. doi: 10.1016/j.bbrc.2011.10.110

Chernogubova, E., Strawbridge, R., Mahdessian, H., Mälarstig, A., Krapivner, S., Gigante, B., et al. (2012). Common and low-frequency genetic variants in the PCSK9 locus influence circulating PCSK9 levels. *Arterioscler. Thromb. Vasc. Biol.* 32, 1526–1534. doi: 10.1161/ATVBAHA.111.240549

Chiou, K. R., and Charng, M. J. (2017). Detection of common sequence variations of familial hypercholesterolemia in Taiwan using DNA mass spectrometry. *J. Clin. Lipidol.* 11, 386–393.e6. doi: 10.1016/j.jacl.2016.12.014

Cohen, J., Pertsemlidis, A., Kotowski, I. K., Graham, R., Garcia, C. K., and Hobbs, H. H. (2005). Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat. Genet.* 37, 161–165. doi: 10.1038/ng1509

Cohen, J. C., Boerwinkle, E., Mosley, T. H. Jr., and Hobbs, H. H. (2006). Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N. Engl. J. Med.* 354, 1264–1272. doi: 10.1056/NEJMoa054013

Danyel, M., Ott, C. E., Grenkowitz, T., Salewsky, B., Hicks, A. A., Fuchsberger, C., et al. (2019). Evaluation of the role of STAP1 in Familial Hypercholesterolemia. *Sci. Rep.* 9:11995. doi: 10.1038/s41598-019-48402-y

Defesche, J. C., Gidding, S. S., Harada-Shiba, M., Hegele, R. A., Santos, R. D., and Wierzbicki, A. S. (2017). Familial hypercholesterolaemia. *Nat. Rev. Dis. Primers.* 3;17093. doi: 10.1038/nrdp.2017.93

Deng, S. J., Alabi, A., Gu, H. M., Adijiang, A., Qin, S., and Zhang, D. W. (2019). Identification of amino acid residues in the ligand binding repeats of LDL receptor important for PCSK9 binding. *J. Lipid. Res.* 60, 516–527. doi: 10.1194/jlr.M089193

Di Filippo, M., Vokaer, B., and Seidah, N. G. (2017). A case of hypocholesterolemia and steatosis in a carrier of a PCSK9 loss-of-function mutation and polymorphisms predisposing to nonalcoholic fatty liver disease. *J. Clin. Lipidol.* 11, 1101–1105. doi: 10.1016/j.jacl.2017.06.005

Di Taranto, M. D., Benito-Vicente, A., Giacobbe, C., Uribe, K. B., Rubba, P., Etxebarria, A., et al. (2017). Identification and in vitro characterization of two new PCSK9 Gain of Function variants found in patients with Familial Hypercholesterolemia. *Sci. Rep.* 7:15282. doi: 10.1038/s41598-017-15543-x

Ding, K., and Kullo, I. J. (2008). Molecular population genetics of PCSK9: a signature of recent positive selection. *Pharmacogenet. Genom.* 18, 169–179. doi: 10.1097/FPC.0b013e3282f44d99

Ding, Q., Strong, A., Patel, K. M., Ng, S. L., Gosis, B. S., Regan, S. N., et al. (2014). Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ. Res.* 115, 488–492. doi: 10.1161/CIRCRESAHA.115.304351

Ding, Z., Liu, S., Wang, X., Deng, X., Fan, Y., Shahanawaz, J., et al. (2015). Cross-talk between LOX-1 and PCSK9 in vascular tissues. *Cardiovasc. Res.* 107, 556–567. doi: 10.1093/cvr/cvv178

Ding, Z., Liu, S., Wang, X., Mathur, P., Dai, Y., Theus, S., et al. (2016). Cross-Talk Between PCSK9 and Damaged mtDNA in Vascular Smooth Muscle Cells: Role in Apoptosis. *Antiox. Redox. Sign.* 25, 997–1008. doi: 10.1089/ars.2016.6631

Ding, Z., Wang, X., Liu, S., Shahanawaz, J., Theus, S., Fan, Y., et al. (2018). PCSK9 expression in the ischaemic heart and its relationship to infarct size, cardiac function, and development of autophagy. *Cardiovasc. Res.* 114, 1738–1751. doi: 10.1093/cvr/cvy128

Dušková, L., Kopečková, L., Jansová, E., Tichý, L., Freiberger, T., Zapletalová, P., et al. (2011). An APEX-based genotyping microarray for the screening of 168 mutations associated with familial hypercholesterolemia. *Atherosclerosis* 216, 139–145. doi: 10.1016/j.atherosclerosis.2011.01.023

Dyrbuś, K., Gąsior, M., Penson, P., Ray, K. K., and Banach, M. (2020). Inclisiran-New hope in the management of lipid disorders? *J. Clin. Lipidol.* 14, 16–27. doi: 10.1016/j.jacl.2019.11.001

Elbitar, S., Susan-Resiga, D., Ghaleb, Y., El Khoury, P., Peloso, G., Stitziel, N., et al. (2018). New Sequencing technologies help revealing unexpected mutations in Autosomal Dominant Hypercholesterolemia. *Sci. Rep.* 8:1943. doi: 10.1038/s41598-018-20281-9

Eroğlu, Z., Tetik Vardarlı, A., Düzgün, Z., Gündüz, C., Bozok Çetintaş, V., and Kayıkçıoğlu, M. (2018). Case-control study on PCSK9 R496W (rs374603772) and D374Y (rs137852912) mutations in Turkish patients with primary dyslipidemia. *Anatol. J. Cardiol.* 19, 334–340. doi: 10.14744/AnatolJCardiol.2018.86648

Eslami, S. M., Nikfar, S., Ghasemi, M., and Abdollahi, M. (2017). Does Evolocumab, as a PCSK9 Inhibitor, Ameliorate the Lipid Profile in Familial Hypercholesterolemia Patients? A Meta-Analysis of Randomized Controlled Trials. *J. Pharm. Pharm. Sci.* 20, 81–96. doi: 10.18433/J36C8N

Fala, L. (2016). Repatha (Evolocumab): Second PCSK9 Inhibitor Approved by the FDA for Patients with Familial Hypercholesterolemia. *Am. Health Drug Benefits* 9, 136–139.

Fasano, T., Cefalù, A. B., Di Leo, E., Noto, D., Pollaccia, D., Bocchi, L., et al. (2007). A novel loss of function mutation of PCSK9 gene in white subjects with low-plasma low-density lipoprotein cholesterol. *Arterioscler Thromb Vasc. Biol.* 27, 677–681. doi: 10.1161/01.ATV.0000255311.26383.2f

Fazio, S., and Tavori, H. (2014). Peeking into a cool future: genome editing to delete PCSK9 and control hypercholesterolemia in a single shot. *Circ. Res.* 115, 472–474. doi: 10.1161/CIRCRESAHA.114.304575

Fouchier, S. W., Dallinga-Thie, G. M., Meijers, J. C., Zelcer, N., Kastelein, J. J., Defesche, J. C., et al. (2014). Mutations in STAP1 are associated with autosomal dominant hypercholesterolemia. *Circ. Res.* 115, 552–555. doi: 10.1161/CIRCRESAHA.115.304660

Galema-Boers, J. M., Versmissen, J., Roeters van Lennep, H. W., Dusault-Wijkstra, J. E., Williams, M., Roeters, et al. (2015). Cascade screening of familial hypercholesterolemia must go on. *Atherosclerosis* 242, 415–417. doi: 10.1016/j.atherosclerosis.2015.07.020

Garvie, C. W., Fraley, C. V., Elowe, N. H., Culyba, E. K., Lemke, C. T., Hubbard, B. K., et al. (2016). Point mutations at the catalytic site of PCSK9 inhibit folding, autoprocessing, and interaction with the LDL receptor. *Protein Sci.* 25, 2018–2027. doi: 10.1002/pro.3019

German, C. A., and Shapiro, M. D. (2020). Small Interfering RNA Therapeutic Inclisiran: A New Approach to Targeting PCSK9. *BioDrugs* 34, 1–9. doi: 10.1007/s40259-019-00399-6

Geschwindner, S., Andersson, G. M., Beisel, H. G., Breuer, S., Hallberg, C., Kihlberg, B. M., et al. (2015). Characterisation of de novo mutations in the C-terminal domain of proprotein convertase subtilisin/kexin type 9. *Protein Eng. Des. Sel.* 28, 117–125. doi: 10.1093/protein/gzv008

Ginsberg, H. N., Tuomilehto, J., Hovingh, G. K., Cariou, B., Santos, R. D., Brown, A. S., et al. (2019). Impact of Age on the Efficacy and Safety of Alirocumab in Patients with Heterozygous Familial Hypercholesterolemia. *Cardiovasc. Drugs Ther.* 33, 69–76. doi: 10.1007/s10557-019-06852-6

Groselj, U., Kovac, J., Sustar, U., Mlinaric, M., Fras, Z., Podkrajsek, K. T., et al. (2018). Universal screening for familial hypercholesterolemia in children: The Slovenian model and literature review. *Atherosclerosis* 277, 383–391. doi: 10.1016/j.atherosclerosis.2018.06.858

Guella, I., Asselta, R., Ardissino, D., Merlini, P. A., Peyvandi, F., Kathiresan, S., et al. (2010). Effects of PCSK9 genetic variants on plasma LDL cholesterol levels and risk of premature myocardial infarction in the Italian population. *J. Lipid. Res.* 51, 3342–3349. doi: 10.1194/jlr.M010009

Henderson, R., O'Kane, M., McGilligan, V., and Watterson, S. (2016). The genetics and screening of familial hypercholesterolaemia. *J. Biomed. Sci.* 23:39. doi: 10.1186/s12929-016-0256-1

Homer, V. M., Marais, A. D., Charlton, F., Laurie, A. D., Hurndell, N., Scott, R., et al. (2008). Identification and characterization of two non-secreted PCSK9 mutants associated with familial hypercholesterolemia in cohorts from New Zealand and South Africa. *Atherosclerosis* 196, 659–666. doi: 10.1016/j.atherosclerosis.2007.07.022

Hooper, A. J., Marais, A. D., Tanyanyiwa, D. M., and Burnett, J. R. (2007). The C679X mutation in PCSK9 is present and lowers blood cholesterol in a Southern African population. *Atherosclerosis* 193, 445–448. doi: 10.1016/j.atherosclerosis.2006.08.039

Hovingh, G. K., Davidson, M. H., Kastelein, J. J., and O'Connor, A. M. (2013). Diagnosis and treatment of familial hypercholesterolaemia. *Eur. Heart J.* 34, 962–971. doi: 10.1093/eurheartj/eht015

Hsiung, Y. C., Lin, P. C., Chen, C. S., Tung, Y. C., Yang, W. S., Chen, P. L., et al. (2018). Identification of a novel LDLR disease-causing variant using capture-based next-generation sequencing screening of familial hypercholesterolemia patients in Taiwan. *Atherosclerosis* 277, 440-447. doi: 10.1016/j.atherosclerosis.2018.08.022

Humphries, S. E., Whittall, R. A., Hubbart, C. S., Maplebeck, S., Cooper, J. A., Soutar, A. K., et al. (2006). Genetic causes of familial hypercholesterolaemia in patients in the UK: relation to plasma lipid levels and coronary heart disease risk. *J. Med. Genet.* 43, 943–949. doi: 10.1136/jmg.2006.038356

Iacocca, M. A., Chora, J. R., Carrié, A., Freiberger, T., Leigh, S. E., Defesche, J. C., et al. (2018). ClinVar database of global familial hypercholesterolemia-associated DNA variants. *Hum. Mutat.* 39, 1631–1640. doi: 10.1002/humu.23634

Ibarretxe, D., Rodríguez-Borjabad, C., Feliu, A., Bilbao, J., Masana, L., and Plana, N. (2018). Detecting familial hypercholesterolemia earlier in life by actively searching for affected children: The DECOPIN project. *Atherosclerosis* 278, 210–216. doi: 10.1016/j.atherosclerosis.2018.09.039

Jarrett, K. E., Lee, C., De Giorgi, M., Hurley, A., Gillard, B. K., Doerfler, A. M., et al. (2018). Somatic Editing of Ldlr With Adeno-Associated Viral-CRISPR Is an Efficient Tool for Atherosclerosis Research. *Arterioscler Thromb Vasc. Biol.* 38, 1997–2006. doi: 10.1161/ATVBAHA.118.311221

Jeong, H. J., Lee, H. S., Kim, K. S., Kim, Y. K., Yoon, D., and Park, S. W. (2008). Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. *J. Lipid. Res.* 49, 399–409. doi: 10.1194/jlr.M700443-JLR200

Kalia, S. S., Adelman, K., Bale, S. J., Chung, W. K., Eng, C., Evans, J. P., et al. (2017). Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. *Genet. Med.* 19, 249–255. doi: 10.1038/gim.2016.190

Kaya, E., Kayıkçıoğlu, M., Tetik Vardarlı, A., Eroğlu, Z., Payzın, S., and Can, L. (2017). PCSK 9 gain-of-function mutations (R496W and D374Y) and clinical cardiovascular characteristics in a cohort of Turkish patients with familial hypercholesterolemia. *Anatol. J. Cardiol.* 18, 266–272. doi: 10.14744/AnatolJCardiol.2017.7654

Knowles, J. W., Rader, D. J., and Khoury, M. J. (2017). Cascade Screening for Familial Hypercholesterolemia and the Use of Genetic Testing. *JAMA* 318, 381–382. doi: 10.1001/jama.2017.8543

Kotowski, I. K., Pertsemlidis, A., Luke, A., Cooper, R. S., Vega, G. L., Cohen, J. C., et al. (2006). A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. *Am. J. Hum. Genet.* 78, 410–422. doi: 10.1086/500615

Kwon, H. J., Lagace, T. A., McNutt, M. C., Horton, J. D., and Deisenhofer, J. (2008). Molecular basis for LDL receptor recognition by PCSK9. *Proc. Natl. Acad. Sci. U S A.* 105, 1820–1825. doi: 10.1073/pnas.0712064105

Lamiquiz-Moneo, I., Restrepo-Córdoba, M. A., Mateo-Gallego, R., Bea, A. M., Del Pino Alberiche-Ruano, M., García-Pavía, P., et al. (2020). Predicted pathogenic mutations in STAP1 are not associated with clinically defined familial hypercholesterolemia. *Atherosclerosis* 292, 143–151. doi: 10.1016/j.atherosclerosis.2019.11.025

Langsted, A., Nordestgaard, B. G., Benn, M., Tybjærg-Hansen, A., and Kamstrup, P. R. (2016). PCSK9 R46L Loss-of-Function Mutation Reduces Lipoprotein(a), LDL Cholesterol, and Risk of Aortic Valve Stenosis. *J. Clin. Endocrinol. Metab.* 101, 3281–3287. doi: 10.1210/jc.2016-1206

Lee, C. J., Lee, Y., Park, S., Kang, S. M., Jang, Y., Lee, J. H., et al. (2017). Rare and common variants of APOB and PCSK9 in Korean patients with extremely low low-density lipoprotein-cholesterol levels. *PLoS One.* 12:e0186446. doi: 10.1371/journal.pone.0186446

Leren, T. P. (2004). Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. *Clin. Genet.* 65, 419–422. doi: 10.1111/j.0009-9163.2004.0238.x

Li, H., and Liu, J. (2012). The novel function of HINFP as a co-activator in sterol-regulated transcription of PCSK9 in HepG2 cells. *Biochem. J.* 443, 757–768. doi: 10.1042/BJ20111645

Li, J., Liang, X., Wang, Y., Xu, Z., and Li, G. (2017). Investigation of highly expressed PCSK9 in atherosclerotic plaques and ox-LDL-induced endothelial cell apoptosis. *Mol. Med. Rep.* 16, 1817–1825. doi: 10.3892/mmr.2017.6803

Liyanage, K. E., Hooper, A. J., Defesche, J. C., Burnett, J. R., and van Bockxmeer, F. M. (2008). High-resolution melting analysis for detection of familial ligand-defective apolipoprotein B-100 mutations. *Ann. Clin. Biochem.* 45(Pt 2), 170–176. doi: 10.1258/acb.2007.007077

Loaiza, N., Hartgers, M. L., Reeskamp, L. F., Balder, J. W., Rimbert, A., Bazioti, V., et al. (2020). Taking One Step Back in Familial Hypercholesterolemia: STAP1 Does Not Alter Plasma LDL (Low-Density Lipoprotein) Cholesterol in Mice and Humans. *Arterioscler Thromb Vasc. Biol.* 40, 973–985. doi: 10.1161/ATVBAHA.119.313470

Louter, L., Defesche, J., and Roeters van Lennep, J. (2017). Cascade screening for familial hypercholesterolemia: Practical consequences. *Atheroscler Suppl.* 30, 77–85. doi: 10.1016/j.atherosclerosissup.2017.05.019

Luirink, I. K., Braamskamp, M. J. A. M., Wiegman, A., Hartgers, M. L., Sjouke, B., Defesche, J. C., et al. (2019). The clinical and molecular diversity of homozygous familial hypercholesterolemia in children: Results from the GeneTics of clinical homozygous hypercholesterolemia (GoTCHA) study. *J. Clin. Lipidol.* 13, 272–278. doi: 10.1016/j.jacl.2018.12.003

Mabuchi, H., Nohara, A., Noguchi, T., Kobayashi, J., Kawashiri, M. A., Inoue, T., et al. (2014). Genotypic and phenotypic features in homozygous familial hypercholesterolemia caused by proprotein convertase subtilisin/kexin type 9 (PCSK9) gain-of-function mutation. *Atherosclerosis* 236, 54–61. doi: 10.1016/j.atherosclerosis.2014.06.005

Marmontel, O., Charrière, S., Simonet, T., Bonnet, V., Dumont, S., Mahl, M., et al. (2018). Single, short in-del, and copy number variations detection in monogenic dyslipidemia using a next-generation sequencing strategy. *Clin. Genet.* 94, 132–140. doi: 10.1111/cge.13250

Marques-Pinheiro, A., Marduel, M., Rabčs, J. P., Devillers, M., Villéger, L., Allard, D., et al. (2010). A fourth locus for autosomal dominant hypercholesterolemia maps at 16q22.1. *Eur. J. Hum. Genet.* 18, 1236–1242. doi: 10.1038/ejhg.2010.94

Martin, R., Latten, M., Hart, P., Murray, H., Bailie, D. A., Crockard, M., et al. (2016). Genetic diagnosis of familial hypercholesterolaemia using a rapid biochip array assay for 40 common LDLR, APOB and PCSK9 mutations. *Atherosclerosis* 254, 8–13. doi: 10.1016/j.atherosclerosis.2016.09.061

Martin, W. R., Lightstone, F. C., and Cheng, F. (2020). In Silico Insights into Protein-protein Interaction Disruptive Mutations in the PCSK9-LDLR complex. *Int. J. Mol. Sci.* 21:E1550. doi: 10.3390/ijms21051550

Matthijs, G., Souche, E., Alders, M., Corveleyn, A., Eck, S., Feenstra, I., et al. (2016). Guidelines for diagnostic next-generation sequencing. *Eur. J. Hum. Genet.* 24, 2–5. doi: 10.1038/ejhg.2015.226

Maxwell, K. N., and Breslow, J. L. (2005). Proprotein convertase subtilisin kexin 9: the third locus implicated in autosomal dominant hypercholesterolemia. *Curr. Opin. Lipidol.* 16, 167–172. doi: 10.1097/01.mol.0000162321.31925.a3

Mayne, J., Dewpura, T., Raymond, A., Bernier, L., Cousins, M., Ooi, T. C., et al. (2011). Novel loss-of-function PCSK9 variant is associated with low plasma LDL cholesterol in a French-Canadian family and with impaired processing and secretion in cell culture. *Clin. Chem.* 57, 1415–1423. doi: 10.1373/clinchem.2011.165191

Miyake, Y., Kimura, R., Kokubo, Y., Okayama, A., Tomoike, H., Yamamura, T., et al. (2008). Genetic variants in PCSK9 in the Japanese population: rare genetic variants in PCSK9 might collectively contribute to plasma LDL cholesterol levels in the general population. *Atherosclerosis* 196, 29–36. doi: 10.1016/j.atherosclerosis.2006.12.035

Mostaza, J. M., Lahoz, C., Salinero-Fort, M. A., de Dios, O., Castillo, E., González-Alegre, T., et al. (2018). R46L polymorphism in the PCSK9 gene: Relationship to lipid levels, subclinical vascular disease, and erectile dysfunction. *J. Clin. Lipidol.* 12, 1039.e–1046.e. doi: 10.1016/j.jacl.2018.04.004

Mullard, A. (2017). Nine paths to PCSK9 inhibition. *Nat. Rev. Drug. Discov.* 16, 299–301. doi: 10.1038/nrd.2017.83

Murphy, B. A., Tadin-Strapps, M., Jensen, K., Mogg, R., Liaw, A., Herath, K., et al. (2017). siRNA-mediated inhibition of SREBP cleavage-activating protein reduces dyslipidemia in spontaneously dysmetabolic rhesus monkeys. *Metabolism* 71, 202–212. doi: 10.1016/j.metabol.2017.02.015

Noguchi, T., Katsuda, S., Kawashiri, M. A., Tada, H., Nohara, A., Inazu, A., et al. (2010). The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolaemia by increasing PCSK9 function and concentration in the circulation. *Atherosclerosis* 210, 166–172. doi: 10.1016/j.atherosclerosis.2009.11.018

Norsworthy, P. J., Vandrovcova, J., Thomas, E. R., Campbell, A., Kerr, S. M., Biggs, J., et al. (2014). Targeted genetic testing for familial hypercholesterolaemia using next generation sequencing: a population-based study. *BMC Med. Genet.* 15:70. doi: 10.1186/1471-2350-15-70

O'Brien, E. C., Roe, M. T., Fraulo, E. S., Peterson, E. D., Ballantyne, C. M., Genest, J., et al. (2014). Rationale and design of the familial hypercholesterolemia foundation CAscade SCreening for Awareness and DEtection of Familial Hypercholesterolemia registry. *Am. Heart J.* 167, 342.e–349.e. doi: 10.1016/j.ahj.2013.12.008

Ogura, M. (2018). PCSK9 inhibition in the management of familial hypercholesterolemia. *J. Cardiol.* 71, 1–7. doi: 10.1016/j.jjcc.2017.07.002

Ohta, N., Hori, M., Takahashi, A., Ogura, M., Makino, H., Tamanaha, T., et al. (2016). Proprotein convertase subtilisin/kexin 9 V4I variant with LDLR mutations modifies the phenotype of familial hypercholesterolemia. *J Clin. Lipidol.* 10, 547.e–555.e. doi: 10.1016/j.jacl.2015.12.024

Papademetriou, V., Stavropoulos, K., Papadopoulos, C., Koutsampasopoulos, K., Dimitriadis, K., and Tsioufis, K. (2018). Role of PCSK9 Inhibitors in High Risk Patients with Dyslipidemia: Focus on Familial Hypercholesterolemia. *Curr. Pharm. Des.* 24, 3647–3653. doi: 10.2174/1381612824666181010124651

Pećin, I., Whittall, R., Futema, M., Sertić, J., Reiner, Z., Leigh, S. E., et al. (2013). Mutation detection in Croatian patients with familial hypercholesterolemia. *Ann. Hum. Genet.* 77, 22–30. doi: 10.1111/j.1469-1809.2012.00735.x

Pek, S. L. T., Dissanayake, S., Fong, J. C. W., Lin, M. X., Chan, E. Z. L., Tang, J. I., et al. (2018). Spectrum of mutations in index patients with familial hypercholesterolemia in Singapore: Single center study. *Atherosclerosis* 269, 106-116. doi: 10.1016/j.atherosclerosis.2017.12.028

Pisciotta, L., Priore Oliva, C., Cefalù, A. B., Noto, D., Bellocchio, A., Fresa, R., et al. (2006). Additive effect of mutations in LDLR and PCSK9 genes on the phenotype of familial hypercholesterolemia. *Atherosclerosis* 186, 433–440. doi: 10.1016/j.atherosclerosis.2005.08.015

Raal, F. J., Kallend, D., Ray, K. K., Turner, T., Koenig, W., Wright, R. S., et al. (2020). Inclisiran for the Treatment of Heterozygous Familial Hypercholesterolemia. *N. Engl. J. Med.* 382, 1520–1530. doi: 10.1056/NEJMoa1913805

Rashid, S., Curtis, D. E., Garuti, R., Anderson, N. N., Bashmakov, Y., Ho, Y. K., et al. (2005). Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proc. Natl. Acad. Sci. U S A.* 102, 5374–5379. doi: 10.1073/pnas.0501652102

Ray, K. K., Landmesser, U., Leiter, L. A., Kallend, D., Dufour, R., Karakas, M., et al. (2017). Inclisiran in Patients at High Cardiovascular Risk with Elevated LDL Cholesterol. *N. Engl. J. Med.* 376, 1430–1440. doi: 10.1056/NEJMoa1615758

Ray, K. K., Wright, R. S., Kallend, D., Koenig, W., Leiter, L. A., Raal, F. J., et al. (2020). Two Phase 3 Trials of Inclisiran in Patients with Elevated LDL Cholesterol. *N. Engl. J. Med.* 382, 1507–1519. doi: 10.1056/NEJMoa1912387

Rehm, H. L., Bale, S. J., Bayrak-Toydemir, P., Berg, J. S., Brown, K. K., Deignan, J. L., et al. (2013). ACMG clinical laboratory standards for next-generation sequencing. *Genet. Med.* 15, 733–747. doi: 10.1038/gim.2013.92

Reiman, A., Pandey, S., Lloyd, K. L., Dyer, N., Khan, M., Crockard, M., et al. (2016). Molecular testing for familial hypercholesterolaemia-associated mutations in a UK-based cohort: development of an NGS-based method and comparison with multiplex polymerase chain reaction and oligonucleotide arrays. *Ann. Clin. Biochem.* 53, 654–662. doi: 10.1177/0004563216629170

Rimbert, A., Pichelin, M., Lecointe, S., Marrec, M., Le Scouarnec, S., Barrak, E., et al. (2016). Identification of novel APOB mutations by targeted next-generation sequencing for the molecular diagnosis of familial hypobetalipoproteinemia. *Atherosclerosis* 250, 52-56. doi: 10.1016/j.atherosclerosis.2016.04.010

Saavedra, Y. G., Dufour, R., Davignon, J., and Baass, A. (2014). PCSK9 R46L, lower LDL, and cardiovascular disease risk in familial hypercholesterolemia: a cross-sectional cohort study. *Arterioscler Thromb Vasc. Biol.* 34, 2700–2705. doi: 10.1161/ATVBAHA.114.304406

Sánchez-Hernández, R. M., Di Taranto, M. D., Benito-Vicente, A., Uribe, K. B., Lamiquiz-Moneo, I., Larrea-Sebal, A., et al. (2019). The Arg499His gain-of-function mutation in the C-terminal domain of PCSK9. *Atherosclerosis* 289, 162–172. doi: 10.1016/j.atherosclerosis.2019.08.020

Santos, R. D., Stein, E. A., Hovingh, G. K., Blom, D. J., Soran, H., Watts, G. F., et al. (2020). Long-Term Evolocumab in Patients With Familial

Hypercholesterolemia. *J. Am. Coll. Cardiol.* 75, 565–574. doi: 10.1016/j.jacc.2019.12.020

Sarraju, A., and Knowles, J. W. (2019). Genetic Testing and Risk Scores: Impact on Familial Hypercholesterolemia. *Front. Cardiovasc. Med.* 6:5. doi: 10.3389/fcvm.2019.00005

Scartezini, M., Hubbart, C., Whittall, R. A., Cooper, J. A., Neil, A. H., and Humphries, S. E. (2007). The PCSK9 gene R46L variant is associated with lower plasma lipid levels and cardiovascular risk in healthy U.K. *men. Clin. Sci.* 113, 435–441. doi: 10.1042/CS20070150

Schmidt, R. J., Zhang, Y., Zhao, Y., Qian, Y. W., Wang, H., Lin, A., et al. (2008). A novel splicing variant of proprotein convertase subtilisin/kexin type 9. *DNA Cell. Biol.* 27, 183–189. doi: 10.1089/dna.2007.0667

Schroeder, C. I., Swedberg, J. E., Withka, J. M., Rosengren, K. J., Akcan, M., Clayton, D. J., et al. (2014). Design and synthesis of truncated EGF-A peptides that restore LDL-R recycling in the presence of PCSK9 in vitro. *Chem. Biol.* 21, 284–294. doi: 10.1016/j.chembiol.2013.11.014

Seidah, N. G., Prat, A., Pirillo, A., Catapano, A. L., and Norata, G. D. (2019). Novel strategies to target proprotein convertase subtilisin kexin 9: beyond monoclonal antibodies. *Cardiovasc. Res.* 115, 510–518. doi: 10.1093/cvr/cvz003

Shioji, K., Mannami, T., Kokubo, Y., Inamoto, N., Takagi, S., Goto, Y., et al. (2004). Genetic variants in PCSK9 affect the cholesterol level in Japanese. *J. Hum. Genet.* 49, 109–114. doi: 10.1007/s10038-003-0114-3

Singh, S., and Bittner, V. (2015). Familial hypercholesterolemia–epidemiology, diagnosis, and screening. *Curr. Atheroscler. Rep.* 17:482. doi: 10.1007/s11883-014-0482-5

Slimani, A., Jelassi, A., Jguirim, I., Najah, M., Rebhi, L., Omezzine, A., et al. (2012). Effect of mutations in LDLR and PCSK9 genes on phenotypic variability in Tunisian familial hypercholesterolemia patients. *Atherosclerosis* 222, 158–166. doi: 10.1016/j.atherosclerosis.2012.02.018

Soutar, A. K., and Naoumova, R. P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat. Clin. Pract. Cardiovasc. Med.* 4, 214–225. doi: 10.1038/ncpcardio0836

Stoekenbroek, R. M., and Kastelein, J. J. P. (2018). Proprotein convertase subtilisin/kexin type 9: from genetics to clinical trials. *Curr. Opin. Cardiol.* 33, 269–275. doi: 10.1097/HCO.0000000000000517

Tall, A. R. (2006). Protease variants, LDL, and coronary heart disease. *N. Engl. J. Med.* 354, 1310–1312. doi: 10.1056/NEJMe068026

Taylor, A., Tabrah, S., Wang, D., Sozen, M., Duxbury, N., Whittall, R., et al. (2007). Multiplex ARMS analysis to detect 13 common mutations in familial hypercholesterolaemia. *Clin. Genet.* 71, 561–568. doi: 10.1111/j.1399-0004.2007.00807.x

Timms, K. M., Wagner, S., Samuels, M. E., Forbey, K., Goldfine, H., Jammulapati, S., et al. (2004). A mutation in PCSK9 causing autosomal-dominant hypercholesterolemia in a Utah pedigree. *Hum. Genet.* 114, 349–353. doi: 10.1007/s00439-003-1071-9

Truong, T. H., Kim, N. T., Nguyen, M. N. T., Pang, J., Hooper, A. J., Watts, G. F., et al. (2018). Homozygous familial hypercholesterolaemia in Vietnam: Case series, genetics and cascade testing of families. *Atherosclerosis* 277, 392–398. doi: 10.1016/j.atherosclerosis.2018.06.013

Urban, D., Pöss, J., Böhm, M., and Laufs, U. (2013). Targeting the proprotein convertase subtilisin/kexin type 9 for the treatment of dyslipidemia and atherosclerosis. *J. Am. Coll. Cardiol.* 62, 1401–1408. doi: 10.1016/j.jacc.2013.07.056

Viigimaa, M., Heinsar, S., Lovic, D., Katsimardou, A., Piperidou, A., and Duishvili, D. (2018). New Horizons in the Pathogenesis, Pathophysiology and Treatment of Familial Hypercholesterolaemia. *Curr. Pharm. Des.* 24, 3599–3604. doi: 10.2174/1381612824666181009105305

Wang, X., Raghavan, A., Chen, T., Qiao, L., Zhang, Y., Ding, Q., et al. (2016). CRISPR-Cas9 Targeting of PCSK9 in Human Hepatocytes In Vivo-Brief Report. *Arterioscler Thromb. Vasc. Biol.* 36, 783–786. doi: 10.1161/ATVBAHA.116.307227

Whittall, R. A., Scartezini, M., Li, K., Hubbart, C., Reiner, Z., Abraha, A., et al. (2010). Development of a high-resolution melting method for mutation detection in familial hypercholesterolaemia patients. *Ann. Clin. Biochem.* 47(Pt 1), 44–55. doi: 10.1258/acb.2009.009076

Wiciński, M., Żak, J., Malinowski, B., Popek, G., and Grześk, G. (2017). PCSK9 signaling pathways and their potential importance in clinical practice. *EPMA J.* 8, 391–402. doi: 10.1007/s13167-017-0106-6

Wiegman, A., Gidding, S. S., Watts, G. F., Chapman, M. J., Ginsberg, H. N., Cuchel, M., et al. (2015). Familial hypercholesterolaemia in children and adolescents: gaining decades of life by optimizing detection and treatment. *Eur. Heart J.* 36, 2425–2437. doi: 10.1093/eurheartj/ehv157

Wright, W. T., Heggarty, S. V., Young, I. S., Nicholls, D. P., Whittall, R., Humphries, S. E., et al. (2008). Multiplex MassARRAY spectrometry (iPLEX) produces a fast and economical test for 56 familial hypercholesterolaemia-causing mutations. *Clin. Genet.* 74, 463–468. doi: 10.1111/j.1399-0004.2008.01071.x

Wu, C. Y., Tang, Z. H., Jiang, L., Li, X. F., Jiang, Z. S., and Liu, L. S. (2012). PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9-caspase3 pathway. *Mol. Cell Biochem.* 359, 347–358. doi: 10.1007/s11010-011-1028-6

Xiang, R., Fan, L. L., Lin, M. J., Li, J. J., Shi, X. Y., Jin, J. Y., et al. (2017). The genetic spectrum of familial hypercholesterolemia in the central south region of China. *Atherosclerosis* 258, 84–88. doi: 10.1016/j.atherosclerosis.2017.02.007

Yang, X., Zhang, J., Chen, L., Wu, Q., and Yu, C. (2018). Chitosan oligosaccharides enhance lipid droplets via down-regulation of PCSK9 gene expression in HepG2 cells. *Exp. Cell Res.* 366, 152–160. doi: 10.1016/j.yexcr.2018.03.013

Yue, P., Averna, M., Lin, X., and Schonfeld, G. (2006). The c.*43*_44insCTG variation in PCSK9 is associated with low plasma LDL-cholesterol in a Caucasian population. *Hum. Mutat.* 27, 460–466. doi: 10.1002/humu.20316

Zhang, L., Wang, L., Xie, Y., Wang, P., Deng, S., Qin, A., et al. (2019). Triple-Targeting Delivery of CRISPR/Cas9 To Reduce the Risk of Cardiovascular Diseases. *Angew. Chem. Int. Ed. Engl.* 58, 12404–12408. doi: 10.1002/anie.201903618

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REVIEW
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Genetics of Familial Hypercholesterolemia: New Insights

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Familial hypercholesterolemia (FH) is one of the most common monogenic diseases, leading to an increased risk of premature atherosclerosis and its cardiovascular complications due to its effect on plasma cholesterol levels. Variants of three genes (*LDL-R*, *APOB* and *PCSK9*) are the major causes of FH, but in some probands, the FH phenotype is associated with variants of other genes. Alternatively, the typical clinical picture of FH can result from the accumulation of common cholesterol-increasing alleles (polygenic FH). Although the Czech Republic is one of the most successful countries with respect to FH detection, approximately 80% of FH patients remain undiagnosed. The opportunities for international collaboration and experience sharing within international programs (e.g., EAS FHSC, ScreenPro FH, etc.) will improve the detection of FH patients in the future and enable even more accessible and accurate genetic diagnostics.

Keywords: familial hypercholesterolemia, gene score, epidemiology, variants, polygenic FH

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HISTORICAL INTRODUCTION

Variants of the genes causing familial hypercholesterolemia (FH) have recently been shown to be important risk factors leading to premature atherosclerotic cardiovascular disease (ASCVD) and premature death. However, these straightforward associations have not always been considered unambiguously evidenced and have at times been rejected.

A small but interesting study by Sijbrands et al. (2001) suggested that mortality in subjects with FH (three large pedigrees, carriers of the Val408Met variant, a total of 412 subjects through 8 generations) significantly varied over time. In the nineteenth century, the mortality of FH subjects was lower than that in the general population. The peak mortality in FH patients (twofold increased risk of premature CVD death) was reached by the first half of the last century followed by a decreasing trend thereafter.

To a certain extent, this observation can be explained by the fact that in the 19th century, life expectancy was much lower and mortality causes were dominated by infections (Zaffiri et al., 2012). In contrast, in the twenty-first century, non-communicable diseases top mortality statistics as the most frequent causes of death[1], and it has been shown that plasma cholesterol plays an important

[1]https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death

role in protection against bacterial infection. One of the most robust pieces of evidence indicates an increased risk of severe infections (sepsis) in subjects with low LDL-C (Guirgis et al., 2016).

Nonetheless, with the current progress in the treatment of infections (antibiotics), a sedentary lifestyle together with abundant caloric intake lead to an environment (for review see Loscalso, 2004; Hubacek, 2009) in which FH causing variants show their detrimental effects, increasing the risk of ASCVD.

FAMILIAL HYPERCHOLESTEROLEMIA

Definition of Familial Hypercholesterolemia

Familial hypercholesterolemia (FH; OMIM ID-143890) is an autosomal dominant inherited disease, the cause of which is most often a variant in the gene for the LDL receptor (*LDLR*), less often a variant in the gene for its ligand, apolipoprotein B 100 (*APOB*). Rarely, a specific gain-of-function type of variant in the subtilisin-kexin type 9 proprotein convertase gene (*PCSK9*) may be the cause. The phenotypic expression of FH can also be caused by variants in the LDL-receptor adapter protein (*LDLRAP*) gene with a recessive inheritance. As a result of the abovementioned variants, the removal of low-density lipoproteins (LDL) from the blood is significantly slowed down in hepatocytes. Consequently, the level of LDL cholesterol (LDL-C) (and thus total cholesterol as well) increases very substantially: in patients with heterozygous FH, total cholesterol is above 8 mmol/l (usually approximately 9–12 mmol/l) and LDL-C is above 5 mmol/l (usually 6–9 mmol/l) but may be even higher (Cuchel et al., 2014). Both HDL cholesterol and triglyceride levels are usually normal, but hypertriglyceridemia does not rule out FH. The rare homozygous form of FH presents with LDL-C levels above 13 mmol/l without therapy or persistent LDL-C elevations above 8 mmol/l with statin therapy (Cuchel et al., 2014). However, blood lipid levels can have considerable variability, depending especially on the type of variant but also on lifestyle or other associated diseases, which makes it more difficult to diagnose FH, suggesting that the disease should always be considered. It has been well established that very high cumulative LDL-C levels significantly accelerate the development of atherosclerosis, and because patients with FH are exposed to markedly increased cholesterol levels for most of their lives, cardiovascular disease (especially myocardial infarction) may manifest at a very early age (in the first decade in untreated homozygotes, after the second decade in severe FH heterozygotes). More than 1,700 different variants have been described in the LDL receptor gene that affect the structure and function of the LDL receptor in various ways; consequently, the level of LDL-C in patients with FH caused by a variant in this gene can have considerable variability (Leigh et al., 2017). In contrast, only a few variants are described in the apolipoprotein B gene, with the vast majority of patients having a single type of variant and thus relatively uniform LDL-C levels (and on average lower than in the LDL-receptor gene). A causal variant in the gene for PCSK9, which may lead to a clinical picture of FH, has so far been identified in only one proband in the Czech Republic and is therefore a rare cause of FH. The practical conclusion of the dominant genetic transmission of FH is the fact that a patient with confirmed FH always has at least one parent with the same disease, and their offspring or siblings have a 50% risk of inheriting FH. Therefore, the so-called cascade screening in families, in which genetic testing of a known variant is performed in relatives of an already diagnosed patient, appears to be a suitable method for searching for people with FH.

Epidemiology of FH

The classic work of Goldstein et al. (1982) from the 1990s reported a 1:500 prevalence of heterozygous FH. However, a 2012 Danish study of a large sample of more than 69,000 people showed a much higher prevalence, approximately 1:250, of heterozygous FH; similar data have been replicated in other European populations (Benn et al., 2012). It can be assumed that the Czech population will have the same disease frequency as other white populations. These new findings indicate that FH is the most common congenital metabolic disorder in humans. The homozygous form of FH is rare. Its incidence has so far been estimated at 1:1,000,000; the abovementioned recent studies estimate the prevalence of homozygous FH at 1: 160,000–1:300,000 (Hu et al., 2020).

Methodological Considerations in FH Genetic Testing

A large array of methods has been used for analysis in patients with the FH phenotype to identify underlying genetic defects to date. All of them have their own advantages and disadvantages. The cost of genetic testing has dramatically fallen over the past decade due to major advances in sequencing technology, especially the introduction of next-generation sequencing (NGS).

This technology offers the possibility of sequencing the whole human genome or exome (Farhan and Hegele, 2014) in a relatively short time and produces a large amount of genetic data. Given the vast amount of data generated, it is critical to have an integrated and validated bioinformatics pipeline, which assembles millions of overlapping small sequenced fragments into a string of large contiguous sequence information. Not all the data are useful for routine diagnostics, of course, especially regarding the focused diagnosis of a specific condition such as FH. This fact led to the development of a gene panel that produces more relevant data with an enhanced likelihood of detecting potentially clinically useful variants (Hegele et al., 2015).

This technology can also be used for the analysis of a high number of SNPs, which have been described in connection with the construction of population-specific/unspecific polygenic genetic risk scores (Paquette et al., 2017; Futema et al., 2018; Rader and Sheth, 2019; Sharifi et al., 2019). Gene panels currently dominate FH molecular diagnostics and can be either customized on demand by individual labs or offered as designed commercial kits (Johansen et al., 2014).

Of course, there are some limitations of NGS technology. Middle-range insertions and deletions containing reads were unable to be mapped with earlier versions of alignment software, while recent updates have overcome those issues by

extending the range of mapping quality values. The initial limit was dramatically increased to approximately 90 bp. The next limitation is the existence of pseudogenes. Due to high genomic sequence homology with genes of interest, it is very difficult or impossible to distinguish between reads originating from genes and their pseudogenes. Other limitations also exist, but these two are the most important.

Another methodological issue is that analysis of large intragenic rearrangements, which can be causal in many cases. These events can account for between 2 and 20% of all positive cases in some populations (Marduel et al., 2010; Pirillo et al., 2017; Tichý et al., 2017; Sun et al., 2018). Although multiplex ligation-dependent probe amplification is still the gold standard for the identification of large intragenic rearrangements, the analysis of copy number variations (CNVs) from NGS data can also achieve very good results (Marmontel et al., 2018) and has been gradually introduced into standard diagnostic schemes in routine labs. The opportunity to obtain information about the genomic sequence of many genes and about the potential presence of rearrangements in these genes at the same time in one step is the main advantage of the NGS approach.

Another strategy to detect CNVs is array comparative genomic hybridization (aCGH) (Ahmad and Iqbal, 2012), which evaluates patterns of intensities on single nucleotide polymorphism (SNP) microarrays to detect differences in allele dosages over a wide stretch of DNA. However, this method requires complex infrastructure and analytical tools for SNP microarrays. aCGH has relatively limited use in germinal genetics, and due to the special equipment needed, it is not common in standard diagnostic labs.

Frequent Genetic Causes of FH

Most cases of FH are caused by defects in the gene for LDL receptor (*LDLR*) or for apolipoprotein B-100 (*APOB*) and rarely by variants in the gene for PCSK9 that lead to its overproduction (gain-of-function variants). Other variants in genes causing the FH phenotype have been described sporadically: for example, gene variations at *STAP1* or *APOE* loci (Santos et al., 2017). The phenotype of homozygous FH can also be caused by variants in the *LDLRAP1* gene, which are associated with an autosomal recessive form of the disease. Only a partial correlation between the genotype and phenotype has been reported in FH subjects. Higher LDL-C and a more severe phenotype are associated with so-called "null variants" in the *LDLR* gene, which lead to a decrease in LDL receptor activity below 2% of normal, while in the so-called "defective variants", LDL receptor activity remains between 2 and 70% of normal function.

As mentioned above, pathogenic variants in the *LDLR* gene are the most common cause of FH. In most populations there is a very wide spectrum of LDLR variants. As we have published previously (Tichý et al., 2012), only 3 most common Czech pathogenic *LDLR* variants (**Table 1**) are relatively common in neighboring countries as well. Similar situation can be documented when reviewing the available literature worldwide (Bertolini et al., 2013; Komarova et al., 2013; Bañares et al., 2017; Alhababi and Zayed, 2018; Pek et al., 2018; Sun et al., 2018).

TABLE 1 | The most frequent pathogenic variants in the *LDLR* gene and their frequencies detected in Czech Republic.

Variant at cDNA level	Variant at protein level	Type of variant	Location	Frequency (%)
c.1775G > A	p.(Gly592Glu)	Missense	Exon 12	16.77
c.798T > A	p.(Asp266Glu)	Missense	Exon 5	14.29
c.1061A > C	p.(Asp354Ala)	Missense	Exon 8	3.90
c.626G > A	p.(Cys209Tyr)	Missense	Exon 4	3.55
c.1246C > T	p.(Arg416Trp)	Missense	Exon 9	2.84
c.67 + 3968_940 + 296dup	Exon2_6dup	Gross duplication		2.48
c.1186 + 700_2141 −545del	Exon9_14del	Gross deletion		2.13
c.1567G > A	p.(Val523Met)	Missense	Exon 10	1.60
c.662A > G	p.(Asp221Gly)	Missense	Exon 4	1.51
c.1474G > A	p.(Asp492Asn)	Missense	Exon 10	1.51

Frequency is calculated as a percentage of the particular variant of all pathogenic variants in the LDLR gene found in the Czech population.

Ten *LDLR* gene variants presented in **Table 1** account for approximately 50% of all pathogenic variants in the Czech Republic. Except for the variant p.(Arg3527Gln) in the *APOB* gene, no other pathogenic variants within the less frequent FH-causing genes have been shown to be prevalent in this population. Some of the common causal variants in these genes are presented in **Table 2**.

Identification of a causal variant in one of the genes responsible for the development of FH confirms the diagnosis of FH and thus a lifelong elevation of LDL-C. Most importantly, identification of the variant is crucial for successful cascade screening, which enables unambiguous confirmation or exclusion of FH in the proband's family members. Moreover, identification of the variant in the family increases compliance of family members to undergo the examination, which is supported by findings from the Czech national database. In families with a known causal variant, the number of FH patients per family is on average 1.77, while in families without this information, it is only 1.18 (Vrablik et al., 2018). In the Czech Republic, we have found 226 different pathogenic variants—the p.(Arg3527Gln) variant in the *APOB* gene, 2 variants in the *PCSK9* gene and 223 variants in the *LDLR* gene. More information regarding the genetic analysis of the Czech MedPed cohort can be found in the articles by Tichý et al. (2012, 2017).

Recent community studies from the United States underline the benefits of genetic diagnostics, as these have shown that identification of a variant is an independent predictor of a manifest atherosclerotic cardiovascular disease in hypercholesterolemic patients (Khera et al., 2016). It is important to note that a negative result in the genetic examination does not completely rule out the possibility of FH. Such a result can be caused by low sensitivity of the methods used, the position of the variant being outside the analyzed part of the gene or a defect located in another gene. Additionally, some patients with a clinical diagnosis of FH can have polygenic hypercholesterolemia, as discussed below.

TABLE 2 | Frequent pathogenic gene variants responsible for the development of FH (apart from the *LDLR* gene).

Gene	OMIM	Most frequent genetic mutations
APOB	107730	p.(Arg3527Gln) (Fernández-Higuero et al., 2015); p.(Arg3527Trp) (Yang et al., 2007)
PCSK9	607786	p.(Ser127Arg) (Abifadel et al., 2003); p.(Asp374Tyr) (Timms et al., 2004; Leren, 2004, Naoumova et al., 2005)
LDLRAP1	605747	p.(Ala145Serfs*26) (Arca et al., 2002); p.(Trp22*) (Garcia et al., 2001)
LIPA	613497	p.(Leu200Pro) (Anderson et al., 1994); p.(Gly266*) (Aslanidis et al., 1996)
ABCG5/ABCG8	605459/605460	ABCG8 p.(Trp361*) (Berge et al., 2000); ABCG5 p.(Arg408*) (Lee et al., 2001)

Recently, the diagnostic possibilities have improved significantly with the use of NGS. In a recent study using a whole-exome sequencing approach, the causal variant was discovered in 20% of patients with a definite clinical diagnosis of FH in whom previous DNA analysis found no variant (Futema et al., 2014). On the other hand, targeted NGS in a cohort of hypercholesterolemic patients in a primary care setting revealed an FH-causing variant in only 2% of the individuals examined (Norsworthy et al., 2014). Thus, appropriate selection of patients for genetic analysis remains a crucial step in FH identification.

Occasional Variants in "Non-traditional Genes"

Typically, novel, rare FH-associated variants have been discovered through pedigree studies. This was the case for the identification of *CYP27A1* (cytochrome P450, subfamily XXVIIA, polypeptide 1), *LIPA* (lysosomal lipase A), *LIPC* (hepatic lipase), *LIPG* (endothelial lipase), *CYP7A1* (cytochrome P450 family 7 subfamily A member 1), *PNPLA5* (patatin-like phospholipase domain containing 5) and some other gene variants responsible for the FH phenotype in particular families (Lange et al., 2014; Al-Allaf et al., 2015; Pirillo et al., 2017; Corral et al., 2018; Mikhailova et al., 2019; **Table 3**).

"False" Familial Hypercholesterolemia Cases

The seemingly simple picture of FH is further complicated by the fact that not all cases described as FH are correctly classified and that they do not fulfill the universally recognized criteria of the disease. Sometimes, these situations are reported as FH phenocopies (Page et al., 2020).

A typical example of such a misclassification is represented by variants of the *ABCG5/G8* transporter genes. In fact, these variants cause sitosterolaemia (Berge et al., 2000; Hubacek et al., 2001), a disease with an autosomal recessive mode of inheritance, where high plasma levels of cholesterol in fact represent a severe elevation of plant sterol plasma concentration. Common enzymatic assays do not discriminate between cholesterol and plant sterols; thus, sitosterolaemia is frequently confused with FH (Moghadasian et al., 2002). Although sitosterolaemia was originally thought to occur in the general population with a frequency of approximately 1:1,000,000, a recent study (Brinton et al., 2018) suggested a prevalence of approximately 1:2,000. This finding is of utmost importance, as 5–10% of patients with the FH phenotype may actually be affected by sitosterolaemia. "Reclassification" of these FH cases accordingly would have important therapeutic implications as the therapy of choice of the latter condition is a cholesterol absorption blocker (ezetimibe) and not a statin.

Similarly, variants of the LDL receptor adaptor protein 1 gene [*LDLRAP1*, originally named the ARH (autosomal recessive hypercholesterolemia) gene] (Garcia et al., 2001) should formally not be considered FH-causing, as the mode of inheritance is recessive. However, this condition cannot be clinically distinguished from the homozygous form of autosomal dominant FH, as its consequences are similar to those of the "classical" form of the disease.

Finally, severe FH-like hypercholesterolemia might infrequently be mimicked by very high plasma levels of lipoprotein (a) [Lp(a)] (Zlatohlavek et al., 2008; Langsted et al., 2016). When LDL-C concentration is measured, it always comprises the amount of cholesterol carried within Lp(a) particles. Such a situation would be connected to statin resistance, as Lp(a) levels cannot be reduced with common lipid-lowering therapy. Moreover, high Lp(a) concentrations in the context of elevated LDL-C represent an additional factor aggravating atherothrombotic risk. Thus, all patients with the FH phenotype must be screened for Lp(a) levels as well (Langsted et al., 2016).

Polygenic Familial Hypercholesterolemia (Pseudo-FH)

The term "polygenic familial hypercholesterolemia" was probably first mentioned by Talmud et al. (2013), although simultaneous analyses of more genes to describe the polygenic nature of hypercholesterolemia are much older (for example, Pedersen and Berg, 1990; Poledne et al., 1994).

The last decade has witnessed an intensive scientific debate triggered by the fact that the majority of subjects with probable or definite FH based on clinical and biochemical criteria cannot be confirmed genetically (e.g., do not have any detectable variation in *LDL-R*, *APOB,* and *PCSK9*). Efforts to identify novel causal genes using the results of genome-wide association studies have been typically unsuccessful at the population level, albeit some rare cases of FH caused by rare variants in different genes have been described (see information above).

Many potential candidate genes, however, have failed to be confirmed as FH-causing genes. For example, sortilin (*SORT-1*) has been documented to play an important role in LDL particle internalization, and common variants of this gene represent an important and highly significant determinant of plasma cholesterol values at the population level. Nevertheless, no *SORT-1* variants have been detected in almost 900 FH patients

TABLE 3 | Overview of genetic causes of the FH phenotype.

Gene	Protein	Protein function	Mode of inheritance	Resulting effect
LDLR	Low-density lipoprotein receptor	Cell surface receptor that plays an important role in cholesterol homeostasis, transporting cholesterol through cell surface	AD	↓ binding affinity of LDL-receptor protein
APOB	Apolipoprotein B	Major protein of LDL particle, ligand for LDL receptor	AD	↓ binding affinity of APOB to LDL-receptor
PCSK9	Proprotein-convertase subtilisin kexin 9	Protease that plays a role in LDL-receptor degradation	AD	↑ LDL-receptor protein degradation
STAP1	Signal-transducing adaptor protein-1	Unknown	AD	Unknown/incomplete association with hypercholesterolemia
LDLRAP1	Low-density lipoprotein receptor adaptor protein 1	Adaptor protein that interacts with cytoplasmatic tail of LDL receptor, promoting LDL particle internalization	AR	LDL-receptor dysfunction
LIPA	Lysosomal acid lipase alias cholesterol esters lipase	Hydrolysis of cholesterol esters or triglycerides	AR	APOB overproduction, upregulation of HMGCoA reductase
ABCG5/ABCG8	Sterolin-1 and 2	Transporters required for secretion of cholesterol into bile	AR	↑ absorption of plant sterols
PNPLA5	Patatin-like phospholipase domain-containing protein 5	Influencing adipocyte differentiation, triglyceride hydrolysis	AR	Possibly lipolysis impairment

AR, autosomal recessive; AD, autosomal dominant; HMGCoA, hydroxy-methyl glutaryl coenzyme A.

negatively tested for variants at *LDL-R*, *APOB,* or *PCSK9* loci (Tveten et al., 2012).

It is hypothesized that (at least some) FH variant-negative patients are in fact carriers of a high number of commonly present genetic variants associated with increased plasma cholesterol values, thus having polygenic FH.

Variant negative FH cases are sometimes referred to as "pseudo-FH" or "polygenic FH" patients.

In 2013, Talmud and co-workers proposed 12 common single nucleotide variants of the *APOE*, *SORT1*, *LDLR*, *APOB, PCSK9, HFE, ABCG8, NYNRIN*, *MYLIP*, *SLC-22* and *ST3GAL4* genes identified through the Global Lipids Genetics Consortium (GLGC; definition at http://lipidgenetics.org), which could be useful for the detection of pseudo-FH subjects. The authors concluded that FH-causing variant-negative "pseudo-FH" patients have a significantly higher mean weighted LDL-C genetic score than the general population.

Later, the 12-SNP gene score was reduced to a 6-SNP gene score (Futema et al., 2015). This 6-SNP LDL-C score has been found to be increased in variant-negative FH patients of Israeli origin with respect to controls from the general population. This further confirms that a significant accumulation of common gene variants of small effect can lead to severe hypercholesterolemia that might not be distinguished from autosomal dominant FH (Durst et al., 2017).

All the abovementioned models suppose simple additive effects of genetic and environmental effects. However, due to gene-gene or gene-environment interactions, the final cholesterol values can be much higher (however, also much lower) than expected from these models (Ritchie, 2015). Currently, this is primarily a hypothesis, but there are examples of gene-environment (Hubacek et al., 2003; Shirts et al., 2012; Kim et al., 2013) and gene-gene interactions (Hubacek et al., 2008; Ma et al., 2012; Grave et al., 2016) modifying plasma cholesterol levels at the general population level; thus, there is no reason that such effects would not work for FH patients. To date, no results have been published for FH patient populations, although some studies (Gaspar and Gaspar, 2019) focused on variable FH penetration indirectly support this model.

Additional Common Gene Variants as the Basis for Novel Polygenic FH Scores

It could be speculated that the gene score can also comprise SNPs occurring within the genes known to cause monogenic forms of FH. Such an approach is plausible, as the products of these genes determine important pathways of cholesterol absorption and metabolism. The putative genes that could be included in this extended gene score include, for example, the genes for BRAP (BRCA-1 associated protein), CETP (cholesterol ester transfer protein), FADS (fatty acid desaturase) and PPP1R3B (protein phosphatase 1, regulatory subunit 3B). Interestingly, they were suggested by the same authors who presented the reduced 6-SNP-based score of polygenic FH (Futema et al., 2015).

The accumulation of common cholesterol-increasing alleles could lead to a condition mimicking and/or worsening a coexisting monogenic form of FH. On the other hand, the possibility of "camouflaging" the FH phenotype by the accumulation of common alleles associated with lower plasma cholesterol levels can occur in the FH population as well, despite there being no literature on the topic so far.

ETHNICITY/POPULATION-SPECIFIC SNPs

One could speculate about the utility of a single universal gene score, especially as during the last decade we have witnessed increased interest in the implementation of the

principles of personalized medicine (Currie and Delles, 2018). The impact of GWAS-detected, lipid trait-associated SNPs could significantly differ in different populations (Hubacek et al., 2017, 2019). For example, in the Japanese population (Tada et al., 2018), variants within the *ABO* gene have been identified as significantly modifying plasma lipoprotein metabolism, while "the usual suspects" (genes for *SORT-1*, *LDLR* and *HMGCR*) showed rather negligible effects. Importantly, identical variants could have different effects on plasma lipids and the frequencies of genetic variants can differ between different ethnicities (Han et al., 2019); thus, a different gene score should be introduced for populations of different ethnic backgrounds.

Given that hundreds of SNPs are probably significantly associated with plasma cholesterol levels, we assume that the gene score needs to be more complex and probably includes dozens of individual SNPs.

TREATMENT CONSIDERATIONS

The treatment of FH patients must be comprehensive and always include non-pharmacological approaches (promoted from childhood—in this case with greater success than later age) and (combination) pharmacotherapy. Patients with FH in childhood and adolescence, as well as women of childbearing potential and women during pregnancy, require a special approach to treatment. A healthy diet and physical activity alone in patients with FH never lead to sufficient changes in the lipoprotein profile, and pharmacotherapy remains essential. Nevertheless, a healthy diet with adequate (as high as possible) physical activity has a positive effect on all known risk factors for atherosclerosis and, most likely, those that have not yet been identified. Pharmacotherapy for patients with FH is based on highly effective statins with a long half-life allowing administration at any time of the day and thus favorably affecting patient adherence. We can titrate the treatment to the maximum dose or at least to a high intensity (atorvastatin 40–80 mg, rosuvastatin 20-40 mg), which is a procedure necessary in patients with partial statin intolerance (Vrablik et al., 2014). Monotherapy with a high-intensity statin usually reduces LDL-C by 50%. Once the maximum tolerated dose of a statin is not sufficient to reach the LDL-cholesterol goal, a combination of statin + ezetimibe should be introduced into the treatment of FH in the next step. Ezetimibe can also be added to combinations for patients who cannot tolerate high doses of statins. Given the relatively lower efficacy of ezetimibe monotherapy (due to the compensatory increase in endogenous cholesterol production in the liver with cholesterol absorption blockage), we always try to guide patients to at least a small dose of statins (e.g., 5 mg atorvastatin or rosuvastatin daily or an alternative dosing). The addition of ezetimibe to a statin reduces LDL-C levels by an additional 20–25%. The use of resins (bile acid sequestrants) is limited mostly due to their poor tolerance; they are used mainly in pediatric patients with FH. On the other hand, the population of patients with FH represents a target group in which we continue to use resins as part of a comprehensive effort to maximize the LDL-C reducing effect. To date, the latest additions to the family of lipid-lowering drugs are monoclonal antibodies against PCSK9. PCSK9 is a protein involved in both intracellular and extracellular regulation of LDL cholesterol receptor expression. One of the functions of PCSK9 is the formation of a complex of PCSK9 with the LDL receptor and its internalization in the endosome. Binding of PCSK9 to the LDL receptor in the cell prevents the normal course of receptor recycling and re-exposure to the plasma membrane. Instead, the LDL receptor-PCSK9 complex is transferred intracellularly to the lysosome, where it undergoes degradation. The number of LDL receptors on the cell surface is thus reduced depending on the presence of PCSK9. Anti-PCSK9 antibodies are capable of increasing LDL receptor expression and ultimately lowering LDL-C levels by up to twenty percent (Ogura, 2018). Two agents, alirocumab and evolocumab, have been introduced into clinical practice.

Lipoprotein apheresis should be considered a therapeutic option for patients with severe hypercholesterolemia who have persistently elevated LDL-C levels despite optimized and intensive drug therapy (Bambauer et al., 2012). It is an extracorporeal elimination technique that removes LDL particles but usually also some other atherogenic lipoproteins, such as Lp(a) or triglyceride-rich lipoproteins, from the circulation. The main indications for lipoprotein apheresis are a diagnosis of homozygous FH, severe heterozygous FH poorly responding to standard therapy, and patients with Lp(a) increase resistant to pharmacotherapy (Blaha et al., 2017a). Lipoprotein apheresis is also a potent therapeutic player that impacts inflammation and related mediators. A large body of evidence on this is available (Blaha et al., 2017b; Stefanutti and Zenti, 2018).

FUTURE DIRECTIONS

FH variants leading to very high plasma cholesterol levels are not necessarily associated with premature atherosclerosis and mortality (Williams et al., 1986). Interestingly, even Brown and Goldstein (1983) in their pioneering works mentioned the lack of association between plasma cholesterol values in FH patients and the prognosis of the disease.

Development of genetic testing has enabled better discrimination between "classical" FH and other forms of hypercholesterolemia, as well as improvement in our understanding of the pathophysiology of the disease. As elegantly summarized in a consensus statement published by Sturm et al. (2018), genetic testing in FH:

- provides a definitive molecular diagnosis of FH
- provides prognostic and risk stratification information and improves outcomes
- facilitates family-based cascade testing
- allows for precision during genetic counseling
- has implications for therapeutic choices in FH
- has value to the pediatric FH patient population.

However, with developments in the genetic diagnosis and the availability of high-throughput technologies, many "innocent" genetic variants have been identified in the genes recognized as causally linked to FH. Thus, based on the consensus of the American College of Medical Genetics (ACMG), the ClinVar initiative has been established to determine the likely pathogenicity of variants in *LDLR/APOB/PCSK9* genes reported in patients with clinical FH (Richards et al., 2015; Iacocca et al., 2018). Classifications include "definitely not", "likely not pathogenic," "variants of unknown significance" (VUS), "likely pathogenic" and "definitely pathogenic." While more than 70% of the 2314 published *LDLR* variants are classified as "likely" or "definitely pathogenic," only 10% of the *APOB* and 13% of *PCSK9* variants are classified as such (Iacocca et al., 2018).

The next primary goal in the management of FH is to increase medical community awareness of FH and the active search for patients; this should lead to an increase in the number of diagnosed and well-managed patients and, even more importantly, a substantial increase in the number of examined members of affected families. Many initiatives focusing on FH detection have been launched recently. In Australia, Asia-Pacific countries and South America, the "Ten Countries Study" was successfully conducted by Watts et al. (2016). Another rapidly developing FH project creating a platform for mutual interaction of FH patients and health care professionals, "The FH Foundation", has been developing since 2011 in the United States (O'Brien et al., 2014). In Europe, the "FH Studies Collaboration" project led by K. Ray and supported by the European Atherosclerosis Society (EAS) has evolved into a multinational project aimed at providing consolidated data on FH worldwide together with the creation of a universal database platform for data collection (Vallejo-Vaz et al., 2015). The ScreenProFH project, endorsed by the International Atherosclerosis Society and embedded in the FHSC initiative, helps to enhance FH screening activities in the Central, Eastern and Southern European region as well as Central Asia and is described in detail in a separate article (Ceska et al., 2017). The Czech MedPed project is actively participating in and/or collaborating with all these international activities. Undoubtedly, these coordinated international efforts should increase the chances of achieving the principal goal—to identify, diagnose and provide treatment for all FH patients early enough to prevent the development of atherosclerotic vascular complications and avoid unnecessary premature death.

AUTHOR CONTRIBUTIONS

MV and JAH contributed substantially to the concept. LT, MS, MV, JAH, TF, and VB were involved in interpretation of the data. All authors were involved in drafting of the manuscript, provided critical revisions for important intellectual content, approved the final version submitted for publication, and agreed to be accountable for all aspects of the work.

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REFERENCES

Abifadel, M., Varret, M., Rabès, J. P., Allard, D., Ouguerram, K., Devillers, M., et al. (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat. Genet.* 34, 154–156. doi: 10.1038/ng1161

Ahmad, A., and Iqbal, M. A. (2012). Significance of genome-wide analysis of copy number alterations and UPD in myelodysplastic syndromes using combined CGH - SNP arrays. *Curr. Med. Chem.* 19, 3739-3747. doi: 10.2174/092986712801661121

Al-Allaf, F. A., Athar, M., Abduljaleel, Z., Taher, M. M., Khan, W., Ba-Hammam, F. A., et al. (2015). Next generation sequencing to identify novel genetic variants causative of autosomal dominant familial hypercholesterolemia associated with increased risk of coronary heart disease. *Gene* 565, 76-84. doi: 10.1016/j.gene.2015.03.064

Alhababi, D., and Zayed, H. (2018). Spectrum of mutations of familial hypercholesterolemia in the 22 Arab countries. *Atherosclerosis* 279, 62–72. doi: 10.1016/j.atherosclerosis.2018.10.022

Anderson, R. A., Byrum, R. S., Coates, P. M., and Sando, G. N. (1994). Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2718–2722. doi: 10.1073/pnas.91.7.2718

Arca, M., Zuliani, G., Wilund, K., Campagna, F., Fellin, R., Bertolini, S., et al. (2002). Autosomal recessive hypercholesterolaemia in Sardinia, Italy, and mutations in ARH: a clinical and molecular genetic analysis. *Lancet.* 359, 841–847. doi: 10.1016/S0140-6736(02)07955-2

Aslanidis, C., Ries, S., Fehringer, P., Büchler, C., Klima, H., and Schmitz, G. (1996). Genetic and biochemical evidence that CESD and Wolman disease are distinguished by residual lysosomal acid lipase activity. *Genomics* 33, 85–93. doi: 10.1006/geno.1996.0162

Bambauer, R., Bambauer, C., Lehmann, B., Latza, R., and Schiel, R. (2012). LDL-apheresis: technical and clinical aspects. *Sci. World J.* 2012:314283.

Bañares, V. G., Corral, P., Medeiros, A. M., Araujo, M. B., Lozada, A., Bustamante, J., et al. (2017). Preliminary spectrum of genetic variants in familial hypercholesterolemia in Argentina. *J. Clin. Lipidol.* 11, 524–531. doi: 10.1016/j.jacl.2017.02.007

Benn, M., Watts, G. F., Tybjaerg-Hansen, A., and Nordestgaard, B. G. (2012). Familial hypercholesterolemia in the danish general population: prevalence, coronary artery disease, and cholesterol-lowering medication. *J. Clin. Endocrinol. Metab.* 97, 3956-3964. doi: 10.1210/jc.2012-1563

Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., et al. (2000). Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 290, 1771-1775. doi: 10.1126/science.290.5497.1771

Bertolini, S., Pisciotta, L., Rabacchi, C., Cefalù, A. B., Noto, D., Fasano, T., et al. (2013). Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy. *Atherosclerosis* 227, 342–348. doi: 10.1016/j.atherosclerosis.2013.01.007

Blaha, V., Blaha, M., Lanska, M., Solichova, D., Kujovska Krcmova, L., Havel, E., et al. (2017a). Lipoprotein apheresis in the treatment of dyslipidaemia - the Czech Republic experience. *Physiol. Res.* 66, S91–S100.

Blaha, V., Blaha, M., Solichova, D., Kujovska Krcmova, M., Lanska, M., Havel, E., et al. (2017b). Antioxidant defense system in familial hypercholesterolemia and the effects of lipoprotein apheresis. *Atheroscl. Suppl.* 30, 159–165.

Brinton, E. A., Hopkins, P. N., Hegele, R. A., Geller, A. S., Polisecki, E. Y., Diffenderfer, M. R., et al. (2018). The association between hypercholesterolemia and sitosterolemia, and report of a sitosterolemia kindred. *J. Clin. Lipidol.* 12, 152-161. doi: 10.1016/j.jacl.2017.10.013

Brown, M. S., and Goldstein, J. L. (1983). Lipoprotein metabolism in the macrophage: implications in cholesterol deposition in atherosclerosis0. *Annu. Rev. Biochem.* 52, 223–261.

Ceska, R., Freiberger, T., Vaclova, M., Aleksicova, T., Votavova, L., and Vrablik, M. (2017). ScreenPro FH: from the Czech MedPed to international collaboration. ScreenPro FH is a participating project of the EAS-FHCS. *Physiol Res* 66(Suppl. 1), S85-S90. doi: 10.33549/physiolres.933599

Corral, P., Geller, A. S., Polisecki, E. Y., Lopez, G. I., Bañares, V. G., Cacciagiu, L., et al. (2018). Unusual genetic variants associated with hypercholesterolemia in Argentina. *Atherosclerosis* 277, 256-261. doi: 10.1016/j.atherosclerosis.2018.06.009

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014). Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the consensus panel on familial hypercholesterolaemia of the european atherosclerosis society. *Eur. Heart J.* 35, 2146–2157. doi: 10.1093/eurheartj/ehu274

Currie, G., and Delles, C. (2018). Precision medicine and personalized medicine in cardiovascular disease. *Adv. Exp. Med. Biol.* 1065, 589-605. doi: 10.1007/978-3-319-77932-4_36

Durst, R., Ibe, U. K., Shpitzen, S., Schurr, D., Eliav, O., Futema, M., et al. (2017). Molecular genetics of familial hypercholesterolemia in Israel-revisited. *Atherosclerosis* 257, 55–63. doi: 10.1016/j.atherosclerosis.2016.12.021

Farhan, S. M., and Hegele, R. A. (2014). Exome sequencing: new insights into lipoprotein disorders. *Curr. Cardiol. Rep.* 16:507. doi: 10.1007/s11886-014-0507-2

Fernández-Higuero, J. A., Etxebarria, A., Benito-Vicente, A., Alves, A. C., Arrondo, J. L., Ostolaza, H., et al. (2015). Structural analysis of APOB variants, p.(Arg3527Gln), p.(Arg1164Thr) and p.(Gln4494del), causing Familial Hypercholesterolaemia provides novel insights into variant pathogenicity. *Sci. Rep.* 5:18184. doi: 10.1038/srep18184

Futema, M., Bourbon, M., Williams, M., and Humphries, S. E. (2018). Clinical utility of the polygenic LDL-C SNP score in familial hypercholesterolemia. *Atherosclerosis* 277, 457-463. doi: 10.1016/j.atherosclerosis.2018.06.006

Futema, M., Plagnol, V., Li, K., Whittall, R. A., Neil, H. A., Seed, M., et al. (2014). Whole exome sequencing of familial hypercholesterolaemia patients negative for LDLR/APOB/PCSK9 mutations. *J. Med. Genet.* 51, 537–544. doi: 10.1136/jmedgenet-2014-102405

Futema, M., Shah, S., Cooper, J. A., Li, K., Whittall, R. A., Sharifi, M., et al. (2015). Refinement of variant selection for the LDL cholesterol genetic risk score in the diagnosis of the polygenic form of clinical familial hypercholesterolemia and replication in samples from 6 countries. *Clin. Chem.* 61, 231–238. doi: 10.1373/clinchem.2014.231365

Garcia, C. K., Wiklund, K., Arca, M., Zuliani, G., Fellin, R., Maioli, M., et al. (2001). Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. *Science* 292, 1394-1398. doi: 10.1126/science.1060458

Gaspar, I. M., and Gaspar, A. (2019). Variable expression and penetrance in Portuguese families with Familial Hypercholesterolemia with mild phenotype. *Atheroscl. Suppl.* 36, 28–30. doi: 10.1016/j.atherosclerosissup.2019.01.006

Goldstein, J. L., Kottke, B. A., and Brown, M. S. (1982). Biochemical genetics of LDL receptor mutations in familial hypercholesterolemia. *Prog. Clin. Biol. Res.* 103(Pt. B), 161–176.

Grave, N., Tovo-Rodrigues, L., da Silveira, J., Rovaris, D. L., Dal Bosco, S. M., Contini, V., et al. (2016). A vitamin D pathway gene-gene interaction affects low-density lipoprotein cholesterol levels. *J. Nutr. Biochem.* 38, 12-17. doi: 10.1016/j.jnutbio.2016.08.002

Guirgis, F. W., Donnelly, J. P., Dodani, S., Howard, G., Safford, M. M., Levitan, E. B., et al. (2016). Cholesterol levels and long-term rates of community-acquired sepsis. *Crit Care*. 20:408. doi: 10.1186/s13054-016-1579-8

Han, S., Hwang, M. Y., Yoon, K., Kim, Y. K., Kim, Y. J., Kim, B. J., et al. (2019). Exome chip-driven association study of lipidemia in >14,000 Koreans and evaluation of genetic effect on identified variants between different ethnic groups. *Genet. Epidemiol.* 43, 617–628. doi: 10.1002/gepi.22208

Hegele, R. A., Ban, M. R., Cao, H., McIntyre, A. D., Robinson, J. F., and Wang, J. (2015). Targeted next-generation sequencing in monogenic dyslipidemias. *Curr. Opin. Lipidol.* 26, 103-113. doi: 10.1097/MOL.0000000000000163

Hu, P., Dharmayat, K. I., Stevens, C. A. T., Sharabiani, M. T. A., Jones, R. S., Watts, G. F., et al. (2020). Prevalence of Familial Hypercholesterolemia Among the general population and patients with atherosclerotic cardiovascular disease: a systematic review and meta-analysis. *Circulation* 141, 1742–1759. doi: 10.1161/CIRCULATIONAHA.119.044795

Hubacek, J. A. (2009). Eat less and exercise more - is it really enough to knock down the obesity pandemia ? *Physiol. Res.* 58(Suppl. 1), S1-S6.

Hubacek, J. A., Adamkova, V., Lanska, V., and Dlouha, D. (2017). Polygenic hypercholesterolemia: examples of GWAS results and their replication in the Czech-Slavonic population. *Physiol. Res.* 66(Suppl. 1), S101-S111. doi: 10.33549/physiolres.933580

Hubacek, J. A., Berge, K. E., Cohen, J. C., and Hobbs, H. H. (2001). Mutations in ATP-cassette binding proteins G5 (ABCG5) and G8 (ABCG8) causing sitosterolemia. *Hum. Mutat.* 18, 359-360. doi: 10.1002/humu.1206

Hubacek, J. A., Dlouha, D., Adamkova, V., Schwarzova, L., Lanska, V., Ceska, R., et al. (2019). The gene score for predicting hypertriglyceridemia: new insights from a Czech case-control study. *Mol. Diagn. Ther.* 23, 555-562.

Hubacek, J. A., Lanska, V., Skodova, Z., Adamkova, V., and Poledne, R. (2008). Sex-specific interaction between APOE and APOA5 variants and determination of plasma lipid levels. *Eur. J. Hum. Genet.* 16, 135-138. doi: 10.1038/sj.ejhg.5201941

Hubacek, J. A., Pitha, J., Skodova, Z., Poledne, R., Lanska, V., Waterworth, D. M., et al. (2003). Polymorphisms in CYP-7A1, not APOE, influence the change in plasma lipids in response to population dietary change in an 8 year follow-up; results from the Czech MONICA study. *Clin. Biochem.* 36, 263-267. doi: 10.1016/s0009-9120(03)00025-0

Iacocca, M. A., Chora, J. R., Carrie, A., Freiberger, T., Leigh, S. E., Defesche, J. C., et al. (2018). ClinVar database of global familial hypercholesterolemia-associated DNA variants. *Hum. Mutat.* 39, 1631–1640.

Johansen, C. T., Dubé, J. B., Loyzer, M. N., MacDonald, A., Carter, D. E., McIntyre, A. D., et al. (2014). LipidSeq: a next-generation clinical resequencing panel for monogenic dyslipidemias. *J. Lipid. Res.* 55, 765-772. doi: 10.1194/jlr.D045963

Khera, A. V., Won, H. H., Peloso, G. M., Lawson, K. S., Bartz, T. M., Deng, X., et al. (2016). Diagnostic yield and clinical utility of sequencing familial hypercholesterolemia genes in patients with severe hypercholesterolemia. *J. Am. Coll. Cardiol.* 67, 2578–2589. doi: 10.1016/j.jacc.2016.03.520

Kim, D. S., Burt, A. A., Ranchalis, J. E., Jarvik, E. R., Rosenthal, E. A., Hatsukami, T. S., et al. (2013). Novel gene-by-environment interactions: APOB and NPC1L1 variants affect the relationship between dietary and total plasma cholesterol. *J. Lipid. Res.* 54, 1512-1520. doi: 10.1194/jlr.P035238

Komarova, T. Y., Korneva, V. A., Kuznetsova, T. Y., Golovina, A. S., Vasilyev, V. B., and Mandelshtam, M. Y. (2013). Familial hypercholesterolemia mutations in Petrozavodsk: no similarity to St. Petersburg mutation spectrum. *BMC Med. Genet.* 14:128. doi: 10.1186/1471-2350-14-128

Lange, L. A., Hu, Y., Zhang, H., Xue, C., Schmidt, E. M., Tang, Z. Z., et al. (2014). Whole-exome sequencing identifies rare and low-frequency coding variants associated with LDL cholesterol. *Am. J. Hum. Genet.* 94, 233–245. doi: 10.1016/j.ajhg.2014.01.010

Langsted, A., Kamstrup, P. R., Benn, M., Tybjaerg-Hansen, A., and Nordestgaard, B. G. (2016). High lipoprotein(a) as a possible cause of clinical familial hypercholesterolaemia: a prospective cohort study. *Lancet Diab. Endocrinol.* 4, 577–587. doi: 10.1016/S2213-8587(16)30042-0

Lee, M. H., Lu, K., Hazard, S., Yu, H., Shulenin, S., Hidaka, H., et al. (2001). Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat. Genet.* 27, 79–83. doi: 10.1038/83799

Leigh, S., Futema, M., Whittall, R., Taylor-Beadling, A., Williams, M., denDunnen, J. T., et al. (2017). The UCL low-density lipoprotein receptor gene variant database: pathogenicity update. *J. Med. Genetics* 54, 217–223. doi: 10.1136/jmedgenet-2016-104054

Leren, T. P. (2004). Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. *Clin. Genet*. 65, 419–422. doi: 10.1111/j.0009-9163.2004.0238.x

Loscalso, J. (2004). *Molecular Mechanisms of Atherosclerosis*. Boca Raton, FL: CRC press.

Ma, L., Brautbar, A., Boerwinkle, E., Sing, C. F., Clark, A. G., and Keinan, A. (2012). Knowledge-driven analysis identifies a gene-gene interaction affecting high-density lipoprotein cholesterol levels in multi-ethnic populations. *PLoS Genet*. 8:e1002714. doi: 10.1371/journal.pgen.1002714

Marduel, M., Carrié, A., Sassolas, A., Devillers, M., Carreau, V., Di Filippo, M., et al. (2010). Molecular spectrum of autosomal dominant hypercholesterolemia in France. *Hum. Mutat.* 31, E1811-E1824. doi: 10.1002/humu.21348

Marmontel, O., Charrière, S., Simonet, T., Bonnet, V., Dumont, S., Mahl, M., et al. (2018). Single, short in-del, and copy number variations detection in monogenic dyslipidemia using a next-generation sequencing strategy. *Clin. Genet.* 94, 132-140. doi: 10.1111/cge.13250

Mikhailova, S., Ivanoshchuk, D., Timoshchenko, O., and Shakhtshneider, E. (2019). Genes potentially associated with familial hypercholesterolemia. *Biomolecules* 9:807. doi: 10.3390/biom9120807

Moghadasian, M. H., Frohlich, J. J., and Scudamore, C. H. (2002). Specificity of the commonly used enzymatic assay for plasma cholesterol determination. *J. Clin. Pathol.* 55, 859-861. doi: 10.1136/jcp.55.11.859

Naoumova, R. P., Tosi, I., Patel, D., Neuwirth, C., Horswell, S. D., Marais, A. D., et al. (2005). Severe hypercholesterolemia in four British families with the D374Y mutation in the PCSK9 gene: long-term follow-up and treatment response. *Arterioscl. Thromb. Vasc. Biol.* 25, 2654–2660. doi: 10.1161/01.ATV.0000190668.94752.ab

Norsworthy, P. J., Vandrovcova, J., Thomas, E. R., Campbell, A., Kerr, S. M., Biggs, J., et al. (2014). Targeted genetic testing for familial hypercholesterolaemia using next generation sequencing: a population-based study. *BMC Med. Genet.* 15:70. doi: 10.1186/1471-2350-15-70

O'Brien, E. C., Roe, M. T., Fraulo, E. S., Peterson, E. D., Ballantyne, C. M., Genest, J., et al. (2014). Rationale and design of the familial hypercholesterolemia foundation CAscade SCreening for Awareness and DEtection of Familial Hypercholesterolemia registry. *Am. Heart J.* 167, 342-349. doi: 10.1016/j.ahj.2013.12.008

Ogura, M. (2018). PCSK9 inhibition in the management of familial hypercholesterolemia. *J. Cardiol.* 71, 1–7. doi: 10.1016/j.jjcc.2017.07.002

Page, M. M., Bell, D. A., and Watts, G. F. (2020). Widening the spectrum of genetic testing in familial hypercholesterolaemia: will it translate into better patient, and population outcomes? *Clin. Genet.* 97, 543–555.

Paquette, M., Chong, M., Thériault, S., Dufour, R., Paré, G., and Baass, A. (2017). Polygenic risk score predicts prevalence of cardiovascular disease in patients with familial hypercholesterolemia. *J. Clin. Lipidol.* 11, 725.e5-732.e5. doi: 10.1016/j.jacl.2017.03.019

Pedersen, J. C., and Berg, K. (1990). gene interaction between the low-density lipoprotein receptor and apolipoprotein E loci affects lipid levels. *Clin. Genet.* 38, 287-294. doi: 10.1111/j.1399-0004.1990.tb03583.x

Pek, S. L. T., Dissanayake, S., Fong, J. C. W., Lin, M. X., Chan, E. Z. L., Tang, J. I., et al. (2018). Spectrum of mutations in index patients with familial hypercholesterolemia in Singapore: single center study. *Atherosclerosis* 269, 106–116. doi: 10.1016/j.atherosclerosis.2017.12.028

Pirillo, A., Garlaschelli, K., Arca, M., Averna, M., Bertolini, S., Calandra, S., et al. (2017). Spectrum of mutations in Italian patients with familial hypercholesterolemia: new results from the LIPIGEN study. *Atheroscl. Suppl.* 29, 17-24. doi: 10.1016/j.atherosclerosissup.2017.07.002

Poledne, R., Hubacek, J., Pisa, Z., Pistulkova, H., and Valenta, Z. (1994). Genetic markers in hypercholesterolemic and normocholesterolemic Czech children. *Clin. Genet.* 46, 88-91. doi: 10.1111/j.1399-0004.1994.tb04208.x

Rader, D. J., and Sheth, S. (2019). Polygenic risk scores in familial hypercholesterolemia. *J. Am. Coll. Cardiol.* 74, 523-525. doi: 10.1016/j.jacc.2019.06.006

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., et al. (2015). Standards and guidelines for theinterpretation of sequence variants: a joint consensus recommendation of the american college of medical genetics and genomics and the association for molecular pathology. *Genet.Med.* 17, 405–424.

Ritchie, M. D. (2015). Finding the epistasis needles in the genome-wide haystack. *Methods Mol. Biol.* 1253, 19–33. doi: 10.1007/978-1-4939-2155-3_2

Santos, R. D., Bourbon, M., Alonso, R., Cuevas, A., Vasques-Cardenas, N. A., Pereira, A. C., et al. (2017). Clinical and molecular aspects of familial hypercholesterolemia in Ibero-American countries. *J. Clin. Lipidol.* 11, 160–166. doi: 10.1016/j.jacl.2016.11.004

Sharifi, M., Futema, M., Nair, D., and Humphries, S. E. (2019). Polygenic hypercholesterolemia and cardiovascular disease risk. *Curr. Cardiol. Rep.* 21:43. doi: 10.1007/s11886-019-1130-z

Shirts, B. H., Howard, M. T., Hasstedt, S. J., Nanjee, M. N., Knight, S., Carlquist, J. F., et al. (2012). Vitamin D dependent effects of APOA5 polymorphisms on HDL cholesterol. *Atherosclerosis* 222, 167-174. doi: 10.1016/j.atherosclerosis.2012.02.030

Sijbrands, E. J., Westendorp, R. G., Defesche, J. C., de Meier, P. H., Smelt, A. H., and Kastelein, J. J. (2001). Mortality over two centuries in large pedigree with familial hypercholesterolaemia: family tree mortality study. *BMJ* 322, 1019-1023. doi: 10.1136/bmj.322.7293.1019

Stefanutti, C., and Zenti, M. G. (2018). Lipoprotein apheresis and PCSK9-Inhibitors. impact on atherogenic lipoproteins and anti-inflammatory mediators in familial Hypercholesterolaemia. *Curr. Pharm. Des*. 24, 3634–3637.

Sturm, A. C., Knowles, J. W., Gidding, S. S., Ahmad, Z. S., Ahmed, C. D., Ballantyne, C. M., et al. (2018). Clinical genetic testing for familial hypercholesterolemia: JACC scientific expert panel. *J. Am. Coll. Cardiol.* 72, 662–680. doi: 10.1016/j.jacc.2018.05.044

Sun, D., Zhou, B. Y., Li, S., Sun, N. L., Hua, Q., Wu, S. L., et al. (2018). Genetic basis of index patients with familial hypercholesterolemia in Chinese population: mutation spectrum and genotype-phenotype correlation. *Lipids Health Dis.* 17:252. doi: 10.1186/s12944-018-0900-8

Tada, H., Kawashiri, M. A., Nomura, A., Teramoto, R., Hosomichi, K., Nohara, A., et al. (2018). Oligogenic familial hypercholesterolemia, LDL cholesterol, and coronary artery disease. *J. Clin. Lipidol.* 12, 1436-1444. doi: 10.1016/j.jacl.2018.08.006

Talmud, P. J., Shah, S., Whittall, R., Futema, M., Howard, P., Cooper, J. A., et al. (2013). Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. *Lancet* 381, 1293-1301. doi: 10.1016/S0140-6736(12)62127-8

Tichý, L., Fajkusova, L., Zapletalova, P., Schwarzova, L., Vrablík, M., and Freiberger, T. (2017). Molecular genetic background of an autosomal dominant hypercholesterolemia in the Czech Republic. *Physiol. Res.* 66(Suppl. 1), S47-S54. doi: 10.33549/physiolres.933587

Tichý, L., Freiberger, T., Zapletalová, P., Soška, V., Ravčuková, B., and Fajkusová, L. (2012). The molecular basis of familial hypercholesterolemia in the Czech Republic: spectrum of LDLR mutations and genotype-phenotype correlations. *Atherosclerosis* 223, 401–408. doi: 10.1016/j.atherosclerosis.2012.05.014

Timms, K. M., Wagner, S., Samuels, M. E., Forbey, K., Goldfine, H., Jammulapati, S., et al. (2004). A mutation in PCSK9 causing autosomal-dominant hypercholesterolemia in a Utah pedigree. *Hum. Genet*. 114, 349–353. doi: 10.1007/s00439-003-1071-9

Tveten, K., Strøm, T. B., Cameron, J., Berge, K. E., and Leren, T. P. (2012). Mutations in the SORT1 gene are unlikely to cause autosomal dominant hypercholesterolemia. *Atherosclerosis* 225, 370-375. doi: 10.1016/j.atherosclerosis.2012.10.026

Vallejo-Vaz, A. J., Kondapally Seshasai, S. R., Cole, D., Hovingh, G. K., Kastelein, J. J., Mata, P., et al. (2015). Familial hypercholesterolaemia: A global call to arms. *Atherosclerosis* 243, 257-259. doi: 10.1016/j.atherosclerosis.2015.09.021

Vrablik, M., Raslova, K., Vohnout, B., Blaha, V., Satny, M., Kyselak, O., et al. (2018). Real-life LDL-C treatment goals achievement in patients with heterozygous familial hypercholesterolemia in the Czech Republic and Slovakia: results of the PLANET registry. *Atherosclerosis* 277, 355-361. doi: 10.1016/j.atherosclerosis.2018.08.008

Vrablik, M., Zlatohlavek, L., Stulc, T., Adamkova, V., Prusikova, M., Schwarzova, L., et al. (2014). Statin-associated myopathy: from genetic predisposition to clinical management. *Physiol. Res.* 63(Suppl. 3), S327-S334.

Watts, G. F., Ding, P. Y., George, P., Hagger, M. S., Hu, M., Lin, J., et al. (2016). Translational research for improving the care of familial hypercholesterolemia: the "ten countries study" and beyond. *J. Atheroscler. Thromb.* 23, 891–900. doi: 10.5551/jat.35949

Williams, R. R., Hasstedt, S. J., Wilson, D. E., Ash, K. O., Yanowitz, F. F., Reiber, G. E., et al. (1986). Evidence that men with familial hypercholesterolemia can avoid early coronary death. An analysis of 77 gene carriers in four Utah pedigrees. *JAMA* 255, 219–224.

Yang, K. C., Su, Y. N., Shew, J. Y., Yang, K. Y., Tseng, W. K., Wu, C. C., et al. (2007). LDLR and ApoB are major genetic causes of autosomal dominant hypercholesterolemia in a Taiwanese population. *J. Formos. Med. Assoc.* 106, 799–807. doi: 10.1016/S0929-6646(08)60044-3

Zaffiri, L., Gardner, J., and Toledo-Pereyra, L. H. (2012). History of antibiotics. From salvarsan to cephalosporins. *J. Invest. Surg.* 25, 67-77. doi: 10.3109/08941939.2012.664099

Zlatohlavek, L., Zídkova, K., Vrablík, M., Haas, T., Prusíkova, M., Svobodova, H., et al. (2008). Lipoprotein(a) and its position among other risk factors of atherosclerosis. *Physiol. Res.* 57, 777–783.

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CASE REPORT
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Case Report: Liver Transplantation in Homozygous Familial Hypercholesterolemia (HoFH)—Long-Term Follow-Up of a Patient and Literature Review

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Homozygous familial hypercholesterolemia (HoFH) is a rare inherited metabolic disorder, frequently leading to an early cardiovascular death if not adequately treated. Since standard medications usually fail to reduce LDL-cholesterol (LDL-C) levels satisfactorily, LDL-apheresis is a mainstay of managing HoFH patients but, at the same time, very burdensome and suboptimally effective. Liver transplantation (LT) has been previously shown to be a promising alternative. We report on a 14 year-long follow-up after LT in a HoFH patient. At the age of 4, the patient was referred to our institution because of the gradually increasing number of xanthomas on the knees, elbows, buttocks, and later the homozygous mutation c.1754T>C (p.Ile585Thr) on the LDL-receptor gene was confirmed. Despite subsequent intensive treatment with the combination of diet, statins, bile acid sequestrant, probucol, and LDL-apheresis, the patient developed valvular aortic stenosis and aortic regurgitation by 12 years. At 16 years, the patient successfully underwent deceased-donor orthotopic LT. Nine years post-LT, we found total regression of the cutaneous xanthomas and atherosclerotic plaques and with normal endothelial function. Fourteen years post-LT, his clinical condition remained stable, but LDL-C levels have progressively risen. In addition, a systematic review of the literature and guidelines on the LT for HoFH patients was performed. Six of the 17 identified guidelines did not take LT as a treatment option in consideration at all. But still the majority of guidelines suggest LT as an exceptional therapeutic option or as the last resort option when all the other treatment options are inadequate or not tolerated. Most of the observed patients had some kind of cardiovascular disease before the LT. In 76% of LT, the cardiovascular burden did not progress after LT. According to our experience and in several other reported cases, the LDL-C levels are slowly increasing over time post LT. Most of the follow-up data were short termed; only a few case reports have followed patients for 10 or more years after LT.

LT is a feasible therapeutic option for HoFH patients, reversing atherosclerotic changes uncontrollable by conservative therapy, thus importantly improving the HoFH patient's prognosis and quality of life.

Keywords: homozygous familial hypercholesterolemia, HoFH, fh, LDL-apheresis, liver transplantation, Slovenia, review

INTRODUCTION

Familial hypercholesterolemia (FH), the most common autosomal dominant condition, is due to the defective LDL-receptor leading to a decreased clearance of LDL-cholesterol (LDL-C) from plasma. In consequence, there is 2–3-fold elevation in the levels of total cholesterol (TC) and LDL-C after birth (1). Although the heterozygous FH (HeFH) is common (1/200–1/500), homozygous FH (HoFH) is a rare disease, affecting only four to six people in a million (2). Patients with HoFH can develop cutaneous and tendon xanthomas, arcus cornealis, and progressive generalized atherosclerosis in their early childhood. If untreated, patients with HoFH develop vascular lesions and cardiovascular disease (CVD) before the second decade of life and die before the end of the third decade of life (3, 4). Slovenia is, according to the available data, the only country with implemented nationwide universal screening for FH in preschool children, detecting both HeFH and HoFH patients (5–8).

HoFH is very difficult to manage. The medical treatment combines several cholesterol-lowering drugs used in other hypercholesterolemias. Initially, statins with ezetimibe are introduced and, in responsive patients, also PCSK9 inhibitors. Frequently, they do not result in satisfactory reductions in either TC or LDL-C levels, especially in moderate and severe HoFH patients with the highest CVD risk (9, 10). For over 30 years, LDL-apheresis is used, becoming a mainstay in the management of HoFH. It is currently considered the only safe and effective treatment for HoFH (11, 12). If LDL-apheresis is not successful, liver transplantation (LT) can be an alternative, also considering that LT is shown to be a successful treatment option in other metabolic liver diseases (13).

We aimed to report on a 14 year-long follow-up after LT in a HoFH patient at our center. In addition, a systematic review of the literature on LT for HoFH patients was performed.

METHODS

We collected all the available clinical information of a now 31 year-old male patient, who was followed at the Department of Endocrinology, Diabetes, and Metabolism of the University Children's Hospital Ljubljana, UMC Ljubljana, Slovenia from the age of 4. FH in the patient was detected incidentally by the dermatologist before implementing the Slovenian universal screening program (5–8). The medical records were collected and entered into the national FH registry database with informed consent from the patient. Genetic analysis was performed after obtaining informed consent as a part of a prospective study on clinical and genetic characteristics of FH patients approved by the National Medical Ethics Committee [the genetic analysis was explained previously in detail by Klančar et al. (6)].

For the systematic review, two approaches were applied. For the case reports on LT as a treatment for FH patients, the PubMed database was used. The following search terms were used: "liver transplantation" (AND) ["familial hypercholesterolemia" (OR) "homozygous hypercholesterolemia" (OR) "familial hyperlipidemia"]. We found 111 research articles. By reading all the abstracts and titles, we excluded 93 articles that did not meet the following conditions: (1) only articles in English and articles published after 1998 were used, but no limits were made on the country of research; (2) only articles that were fully accessible were included; (3) only the articles on humans and not on animals nor cell models were used; (4) only articles where the recipient of the liver transplant has had FH were used; and (5) only articles with follow-ups longer than 1 month were included. All the available articles that met the criteria were read in full-text form. In addition, other case reports were found through the articles' reference list. In the end, 23 articles were included. A systematic review of the clinical guidelines on LT was based on the search procedure used by Migliara et al. (14). In the end, we have found 17 guidelines.

CASE DESCRIPTION

A 4 year-old male patient was referred to our institution by a dermatologist because of the gradually increasing number of xanthomas on the knees, elbows, and buttocks that first appeared when he was 3 years old. He was born after an uneventful pregnancy and delivery as the second child of apparently non-consanguineous parents. The family history for premature CVD was negative. His early development was normal. Extremely high levels of TC (24.8 mmol/L; 928 mg/dl), LDL-C (21.6 mmol/L; 834 mg/dl), and triglycerides (TGC) (5.0 mmol/L; 443 mg/dl) and low levels of HDL-cholesterol (HDL-C) (0.3 mmol/L; 11.6 mg/dl) were measured at the first exam. Elevated levels of TC and LDL-C were also found in his parents, sister, and daughter (afterward) (**Table 1**). The clinical and biochemical picture suggested the diagnosis of HoFH, which was later confirmed genetically; a previously described homozygous mutation c.1754T>C (p.Ile585Thr) was found in the patient, while both his parents, his sister, and daughter were confirmed to be heterozygotes for the same mutation. Despite treatment

Abbreviations: FH, familial hypercholesterolemia; HoFH, homozygous familial hypercholesterolemia; HeFH, heterozygous familial hypercholesterolemia; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TC, total cholesterol; Tgc, triglycerides; LT, liver transplantation; CVD, cardiovascular disease; RHI, reactive hyperemia index; PWV, pulse wave velocity; IMT, intima-media thickness; BMI, body mass index.

TABLE 1 | Plasma levels of total, LDL, HDL cholesterol, and Lp(a) in patient, parents, and sister at the same time point (in 2012 and PWV values in 2016).

	Patient (23 years[a])	Mother (42 years[a])	Father (49 years[a])	Sister(25 years[a])	Daughter (3 years[c])
FH defect	Homozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Therapy	Liver transplantation	None	None	None	None
TC (mmol/L)	5.5 (24.8c[c])	8.2	11.0	10.4	6.9
TC (mg/dl)	213 (959c[c])	317	425	402	267
LDL (mmol/L)	3.8 (21.6c[c])	6.0	8.9	7.7	5.1
LDL (mg/dl)	147 (835c[c])	232	344	298	197
HDL (mmol/L)	(0.3[c])	1.5	1.3	1.5	1.4
HDL (mg/dl)	42.5 (11.6c[c])	58	50	58	54
Tg (mmol/L)	1.4 (5[c])	1.5	1.5	2.6	1.0
Tg (mg/dl)		133	133	230	88
Lp(a) (mg/L)	111	<93.1	1260	687	323
cIMT (mm)	Left 0.6	Left 1.4	Left 0.7	Left 0.5–0.6	NS
	Right 0.6	Right 1.4	Right 0.7	Right 0.6	
Carotid stenosis	No	Yes	No	No	NS
PWV (m/s)[b]	4.6 ± 0.2	7.4 ± 0.7	8.9 ± 0.6	4.9 ± 0.2	NS

In 2019 his daughter was referred to our center by cascade screening.

[a]*Age in 2012.*

[b]*Measured in 2016.*

[c]*Measurements at the first referral.*

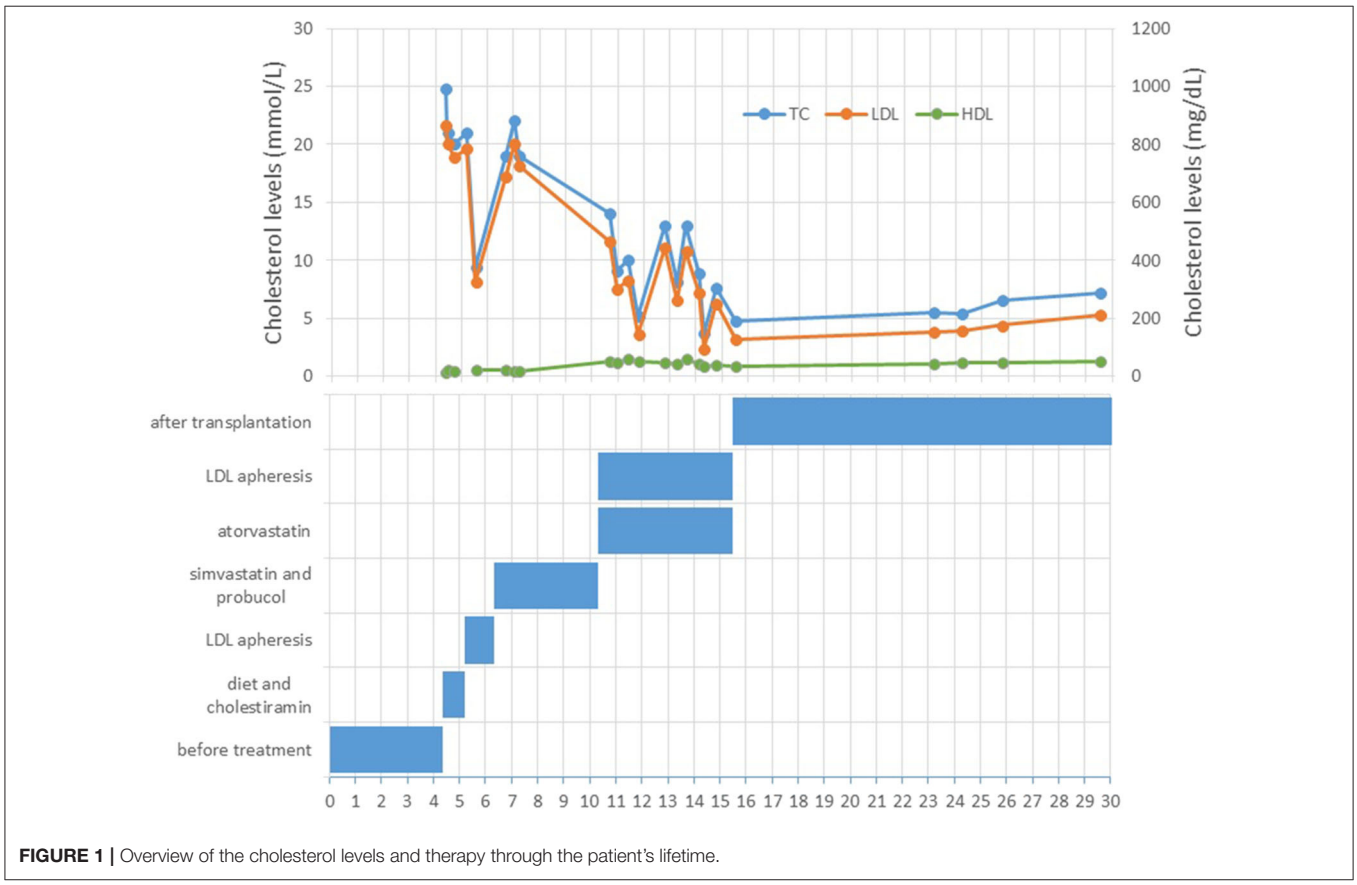

FIGURE 1 | Overview of the cholesterol levels and therapy through the patient's lifetime.

with a highly restrictive diet, cholestyramine, statins, and LDL-apheresis (the exact timeline is described in **Figure 1**, **Table 2**), xanthomas extended progressively. At 7 years, increased IMT of the common carotid artery was measured with ultrasound. At 12 years, echocardiographically valvular aortic stenosis and aortic regurgitation were observed. Levels of LDL-C at the time of LDL-apheresis ranged from 2.4 to 11.6 mmol/L (92.8–449 mg/dl) in between treatments. Lp(a) levels ranged from 756 mg/L at the beginning to 135 mg/L after the last treatment. He refused to have apheresis for 3 years, but with psychological support, he continued.

Due to progressive worsening conditions (valvular aortic stenosis and aortic regurgitation), the patient underwent deceased-donor orthotopic LT when he was 16 years old. There were no intraoperative or postoperative complications. The patient's immunosuppression regimen first consisted of

TABLE 2 | Timeline of clinical event.

Age	Description
3	Xanthomas were found by the dermatologist
4	First examination at the tertiary clinic (total cholesterol: 2.8 mmol/L, 959 mg/dl) a diet and cholestyriamine were prescribed
5	Biweekly LDL-apheresis was started, xanthomas extended
6	The appearance of psychological problems, treatment with probucol and simvastatin, progression of xanthomas
7	Increased cIMT was first observed
9.5	Psychological support was introduced, started again with LDL-apheresis
12	A new systolic heart murmur was found, valvular aortic stenosis and aortic regurgitation were observed
16	Ortopic liver transplantation
16.5	Steroids after transplantation where gradually tapered, xanthomas had regressed, TC was in the normal range
17.5	TC was at the lowest value 4.4 mmol/L (170 mg/dl)
18	An episode of acute rejection developed, successfully treated with steroids. At the end of the treatment, TC was 5.1 mmol/L (197 mg/dl)
25	Cutaneous xanthomas completely resolved, complete regression of aortic stenosis
28	Pulse wave velocity was in the normal range (4.6 ± 0.2 m/s)
29	cIMT was 0.903 mm, TC was 7.2 mmol/L (278 mg/dL), statins were prescribed again

tacrolimus, methylprednisolone, and ursodeoxycholic acid, but the steroid was gradually tapered. Six months after the LT, the patient's xanthomas had regressed, and the TC levels have fallen to 5.2 mmol/L (201 mg/dl). A year and a half after the LT, the TC level was at the lowest value [4.4 mmol/L (170 mg/dl)]. Two years after the LT, an episode of acute rejection developed, which was successfully treated with steroids, and the TC level was measured at 5.1 mmol/L (197 mg/dl).

Eight years after the LT, the patient who was then 24 years old was doing well. The patient had been on maintaining tacrolimus therapy. His cutaneous xanthomas completely resolved. An echocardiogram showed complete regression of aortic stenosis, and his IMT was in the normal range (0.6 mm on both common carotid arteries). His TC and LDL-C levels were 5.4 and 3.9 mmol/L, respectively (209 and 151 mg/dl), and Lp(a) levels were 111 mg/L. His LDL-C levels were lower than the LDL-C levels measured on the same occasion in his parents and sister (**Table 1**). Twelve years after the LT, the PWV velocity was measured with ultrasound, and the values were in the normal range (4.6 ± 0.2 m/s) The RHI was 2.46.

Afterward, the patient was not responding to the invitations from our clinic for almost 5 years (at that time, he was only seeing his gastroenterologist) and returned for a follow-up visit only in the year 2018, almost 14 years after the LT. The total regression of xanthomas was evident at the last visit. At that point, he was almost 30 years old, employed, and had a child who was scheduled for a later visit at our lipid clinic and was confirmed to be heterozygote for the same mutation. His work was mostly sedentary, and he was not very physically active. In addition, he started smoking. He was still taking tacrolimus, but no antilipemic treatment was prescribed to him after the LT. His TC and LDL-C levels were 7.2 mmol/L (278 mg/dl) and 5.3 mmol/L (205 mg/dl), respectively. In addition, an elevated ApoB level was present (1.48 g/L), while his Lp(a) level was normal (145 mg/L). Furthermore, hsCRP was 2.11 mg/L. His carotid IMT level had increased to 0.90 mm on both carotid arteries (abnormal result for his age) (15). He was normotensive (97/67 mmHg), and his BMI was 18 kg/m^2. Liver enzyme levels were in the normal range. The HbA1c level was 4.8%, and the fasting insulin level was 4.3 mE/L. The glomerular filtration rate was above 90 ml/min. At the last visit to our lipid clinic, the patient was recommended to start statin treatment (rosuvastatin, 10 mg) and was transitioned to the adult lipid clinic, where his first visit was scheduled in early 2019. At that visit, his TC and LDL-C levels were 7.36 mmol/L (284.6 mg/dl) and 5.9 mmol/L (228.15 mg/dl), respectively.

DISCUSSION

HoFH frequently leads to early cardiovascular death if not adequately treated. However, the standard therapy with medications and LDL-apheresis at least in more severe cases often fail to address difficult clinical situation satisfactorily (16–18). The management of patients with HoFH represents a medical challenge despite the approval of new lipid-lowering agents (i.e., mipomersen, lomitapide, PCSK9 inhibitors) (19). An individualized approach in the management of the disease is of great importance (20). Diagnosis of FH and its early treatment is recommended in all guidelines (10, 21–23).

Lipid-lowering drug therapy is recommended for the treatment of HoFH in all age groups (24). LDL-apheresis has been used and progressively became a mainstay in the management of HoFH. In line with current guidelines, treatment should be started as soon as possible, ideally by age 5 and not later than 8 years. However, this and the frequency of treatment represent a compromise between access to centers, the severity of the disease, and the patient's choice and/or compliance (21, 25, 26). Currently, LDL-apheresis is recommended at weekly or biweekly intervals with concurrent administration of maximal doses of lipid-lowering agents in exceptional circumstances such as pregnancy; more frequent treatment without statins may be considered (4, 27, 28).

Despite the lack of randomized studies, there is clinical evidence that long-term lipoprotein apheresis can contribute to plaque regression and/or stabilization, slow coronary atherosclerosis progression, and improved prognosis (29). Thompson et al. have concluded in a 50 year follow-up study that improved treatment and treatment to lower TC levels have a better prognosis (30, 31). Combining apheresis with additional drug therapy to slow down the rapid rebound of LDL-C, which follows each procedure, and to keep the LDL-C level as low as possible for as long as possible is also essential (32, 33). A more recent study has shown that achieving a mean LDL-C level of 4.2 mmol/L (162.4 mg/dl) by weekly apheresis plus statin/ezetimibe therapy failed to prevent the progression of the aortic, coronary, and carotid disease (28, 34). The most recent statement on target

levels for both HoFH and HeFH, which advocates lowering LDL-C to <3.5 mmol/L (135 mg/dl) in children and to <2.5 mmol/L (97 mg/dl) in adults, or even <1.8 mmol/L (70 mg/dl) in those at the highest risk can seldom be achieved in homozygotes with existing apheresis/drug therapy regimens (2, 10, 24, 35).

When our patient was 5 years old, biweekly LDL-apheresis first without and then with statins was used. Despite the early treatment initiation, he has developed valvular aortic stenosis. One week after LDL-apheresis, LDL-C levels were up to 10 mmol/L (386.7 mg/dl).

Therefore, LT was considered as a therapeutic option (19, 36), as it was also considered in 23 case reports and case series (19, 36–57), with a total of 90 patients with FH who have undergone LT. Data are presented in detail in **Supplement Table 1**. Of the 90 patients found, 47 were female. Most of them had HoFH (77.8%) (genetic or clinical diagnosis). Eight patients were just diagnosed with FH. Most of the patients had some kind of CVD before the LT, some having coronary stenting or dilatation (22 patients) or heart valve replacement (4 patients) before LT. Four patients also received a heart transplant in the same surgical procedure, and one also received a kidney transplant at the same time as LT. Although a successful therapeutic strategy (41, 42, 46, 47), there are obvious disadvantages, including the risk of post-transplantation surgical complications and mortality, the risk of acute or chronic organ rejection, and the need for life-long treatment with immunosuppressive therapy (58, 59). In most case studies (63.3%), the cardiovascular disease burden after LT was comparable to the one before LT. In 24.4%, however, the progression of CVD was observed, and in 12.62%, regression was observed (19, 36–57). The latter was also the case in our patient. Cardiovascular complications and disease progression were observed in 11 patients. Of them, six died because of heart failure after LT (19, 36, 38–40, 42, 45, 52). Another two patients died because of septicemia (19, 36). Interestingly, there were no deaths in the five patients with combined heart and liver transplantation (41, 43, 51, 53, 55). One patient lived for 20 years with a combined heart–liver transplant with an absence of CVD (41).

Corticosteroids, cyclosporine, tacrolimus, and sirolimus, the main treatments after transplantation, are all associated with elevated cholesterol levels (60). Cyclosporine interferes with the binding of LDL-C to LDL receptors and also interferes with bile acid synthesis acting on the enzyme 26 hydroxylases. Tacrolimus has similar but lesser effects on lipid metabolism, then cyclosporine (37). In addition, metabolic syndrome can develop after transplantation (19). In our patient, this was not the case. After successful LT, the cardiovascular complications regressed, endothelial dysfunction was not detected, and IMT was reversed.

In the gathered case reports and our case report interestingly in the short term, a decrease in TC levels after LT was seen,

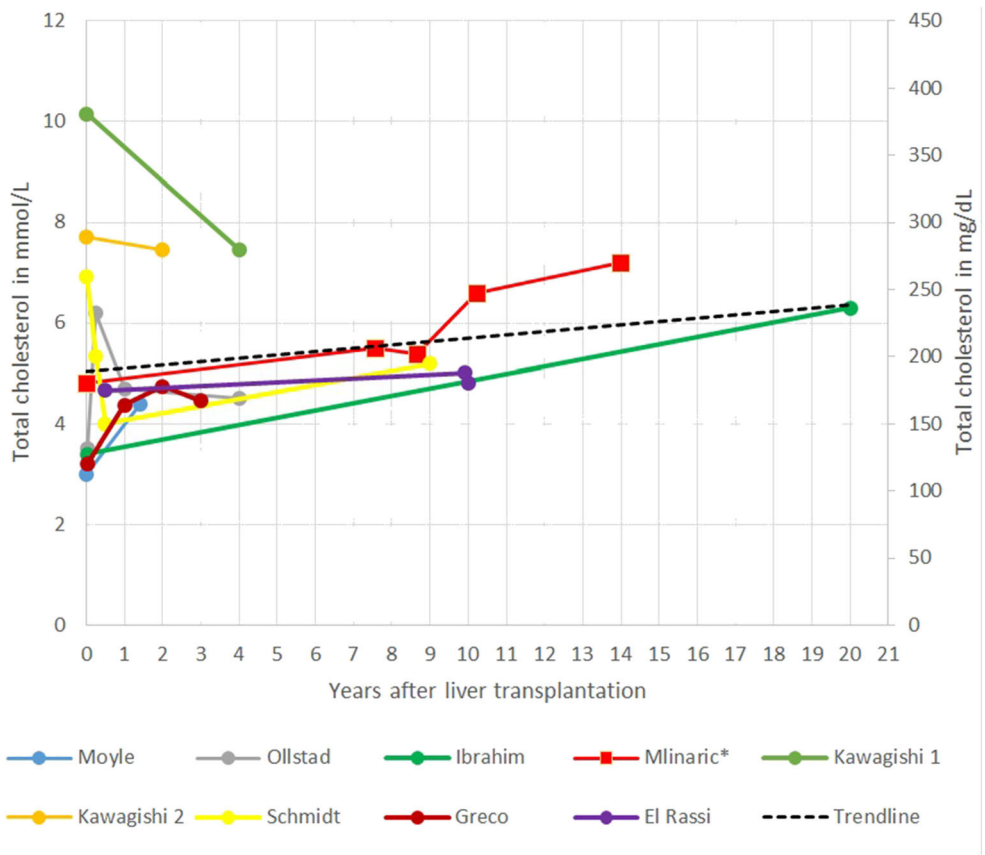

FIGURE 2 | Representation of total cholesterol (TC) after liver transplantation (LT) in different case reports.

reaching the lowest level in the first years after LT. The main limitation of systematic review of the case reports is that most of the follow-up data were short termed; only a few case reports have followed patients for 10 or more years after LT. The longest report is 20 years (21, 22). Afterward, TC seems to increase steadily, in some patients, even despite taking cholesterol-lowering drugs (**Figure 2**) (51). Ibrahim et al. (41) report of an increase in TC levels from 3.4 mmol/L (132 mg/dl) to 6.3 mmol/L (244 mg/dl) 20 years after LT. An unhealthy lifestyle (excessive dietary intake of cholesterol and saturated fats), diabetes, obesity, proteinuria, age, genetic predisposition, and medications are known risk factors for elevated cholesterol levels in patients after LT for different defects (37). Some of them are also present in our patient.

Out of the 16 guidelines (2, 4, 21, 22, 27, 61–71) proposed by professional societies and one by Raal et al. (10), 6 of the 17 did not take LT as a treatment option in consideration at all (2, 22, 64, 68, 69). In the guidelines that do mention LT, the majority suggests that it as an exceptional therapeutic option or as the last resort option when all the other treatment options are inadequate or not tolerated (4, 10, 22, 61–63, 65). The International FH foundation states that the LT decision should be in partnership with the patient and/or their relatives (4). In the article by Raal et al. (10), the LT is an acceptable option only for the treatment of HoFH patients that are unresponsive to conventional lipid-lowering therapy and possibly before the onset of significant CVD. However, if the CVD is discovered in preoperative cardiac investigations, the FH Australasia Network consensus group suggests that coronary artery bypass surgery and/or aortic valve replacement should also be considered (62). The same is proposed by the International FH group (4), also suggesting that a combined heart and LT should be considered according to the clinical context. EAS recommends that we should also have in mind the disadvantages of LT (i.e., the need for life-long immunosuppressive therapy, the paucity of donors, and possible surgical complications) (21).

LT is, therefore, especially indicated for patients with HoFH that do not otherwise respond to maximal medical therapy (4, 61–63, 65), but it is not a feasible option for all HoFH patients. However, LT has substantiated the development of other novel therapeutic approaches for patients with severe FH (e.g., liver-direct gene delivery, stem cell transplantation) (67).

The decision should be made with the patient and his relatives in an appropriate setting, and all the benefits or potential harms of the transplantation and of declining LT should be explained (22).

CONCLUSIONS

LT is a feasible therapeutic option, especially in patients with HoFH with progressive atherosclerotic disease that cannot be sufficiently controlled by medications and/or LDL-apheresis. In our HoFH patient, the long-term outcome after the LT was considered highly favorable, reversing the already severely progressed atherosclerosis before the procedure, despite the relatively early detection and intensive treatment with LDL-apheresis.

Replacing the liver where most of the LDL-C metabolism occurs represents a way of somatic gene therapy for HoFH, a frequently fatal inborn metabolic disorder. On the other hand, LT exposes the patient to considerable other clinical risks and burdens associated with the transplantation (e.g., the need for life-long immunosuppressive therapy, the paucity of donors, and possible surgical complications) (21). According to our experience and from the case reports, the LDL-C levels are slowly increasing years after the LT due to an unhealthy lifestyle, tacrolimus, or other therapy (37) and maybe because of LT organ regeneration with cells stemming from the patient. CVD and death of myocardial infarction are also reported in HoFH patients after LT (19, 39). LT might not represent the definite treatment of HoFH. Thus, after LT, the patients need to be further regularly followed at the lipid clinic (and also by the hepatologist), but at least in our patient, the LT vastly improved his quality of life (10, 19). The data indicate that early transplantation may be favorable and that actively preventing septicemia might be improving outcomes.

Finally, early detection programs and the clinical availability of novel therapeutic strategies are urgently needed to address the needs of all HoFH patients. Developing specific guidelines and international patient registries on LT for HoFH might be beneficial, following the example of the research group, who have made the International Registry on Lipoprotein Apheresis in Children with Homozygous Familial Hypercholesterolemia (72).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. 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

MM collected the data and prepared the manuscript. NB and UG followed and treated the patient, collected the data, and prepared the first draft of the case report. VD made the first suspicion of the disease and referred him early for further evaluation and treatment and reviewed the manuscript. AS performed follow-up tests and reviewed the manuscript. MC, UG, and TB supervised the work and helped writing the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.567895/full#supplementary-material

REFERENCES

1. Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In: Scriver CR, editor. *The Metabolic and Molecular Bases of Inherited Disease, 8th Edn*. New York, NY: McGraw-Hill (2001). p. 2863–2913.
2. Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur Heart J*. (2020) 41:111–88. doi: 10.15829/1560-4071-2020-3826
3. Vallejo-Vaz AJ, Kondapally Seshasai SR, Cole D, Hovingh GK, Kastelein JJ, Mata P, et al. Familial hypercholesterolaemia: a global call to arms. *Atherosclerosis*. (2015) 243:257–9. doi: 10.1016/j.atherosclerosis.2015.09.021
4. Watts GF, Gidding S, Wierzbicki AS, Toth PP, Alonso R, Brown WV, et al. Integrated guidance on the care of familial hypercholesterolaemia from the international FH foundation: executive summary. *J Atheroscler Thromb*. (2014) 21:368–74. doi: 10.1016/j.ijcard.2013.11.025
5. Groselj U, Kovac J, Sustar U, Mlinaric M, Fras Z, Podkrajsek KT, et al. Universal screening for familial hypercholesterolemia in children: the Slovenian model and literature review. *Atherosclerosis*. (2018) 277:383–91. doi: 10.1016/j.atherosclerosis.2018.06.858
6. Klančar G, Grošelj U, Kovač J, Bratanič N, Bratina N, Trebušak Podkrajšek K, et al. Universal screening for familial hypercholesterolemia in children. *J Am Coll Cardiol*. (2015) 66:1250–7. doi: 10.1016/j.jacc.2015.07.017
7. Sedej K, Kotnik P, Avbelj Stefanija M, Grošelj U, Širca Campa A, Lusa L, et al. Decreased prevalence of hypercholesterolaemia and stabilization of obesity trends in 5-year-old children: possible effects of changed public health policies. *Eur J Endocrinol*. (2014) 170:293–300. doi: 10.1530/EJE-13-0566
8. Vallejo-Vaz AJ, De Marco M, Stevens CAT, Akram A, Freiberger T, Hovingh GK, EAS Familial Hypercholesterolaemia Studies Collaboration. Overview of the current status of familial hypercholesterolaemia care in over 60 countries - The EAS Familial Hypercholesterolaemia Studies Collaboration (FHSC). *Atherosclerosis*. (2018) 277:234–55. doi: 10.1016/j.atherosclerosis.2018.08.051
9. Perez de Isla L, Alonso R, Watts GF, Mata N, Saltijeral Cerezo A, Muñiz O, et al. Attainment of LDL-cholesterol treatment goals in patients with familial hypercholesterolemia: 5-year safeheart registry follow-up. *J Am Coll Cardiol*. (2016) 67:1278–85. doi: 10.1016/j.jacc.2016.01.008
10. Raal FJ, Hovingh GK, Catapano AL. Familial hypercholesterolemia treatments: guidelines and new therapies. *Atherosclerosis*. (2018) 277:483–92. doi: 10.1016/j.atherosclerosis.2018.06.859
11. Drouin-Chartier JP, Tremblay AJ, Bergeron J, Lamarche B, Couture P. High serum triglyceride concentrations in patients with homozygous familial hypercholesterolemia attenuate the efficacy of lipoprotein apheresis by dextran sulfate adsorption. *Atherosclerosis*. (2018) 270:26–32. doi: 10.1016/j.atherosclerosis.2018.01.005
12. Julius U. History of lipidology and lipoprotein apheresis. *Atherosclerosis*. (2017) 30:1–8. doi: 10.1016/j.atherosclerosissup.2017.05.034
13. Mc Kiernan PJ. Recent advances in liver transplantation for metabolic disease. *J Inherit Metab Dis*. (2017) 40:491–5. doi: 10.1007/s10545-017-0020-z
14. Migliara G, Baccolini V, Rosso A, D'Andrea E, Massimi A, Villari P, et al. Familial hypercholesterolemia: a systematic review of guidelines on genetic testing and patient management. *Front Public Health*. (2017) 5:252. doi: 10.3389/fpubh.2017.00252
15. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Cardiology and the European Society of Hypertension. *J Hypertens*. (2018) 36:1953–2014. doi: 10.1097/HJH.0000000000001940
16. Rajendran R, Srinivasa KH, Rangan K, Hegde M, Ahmed N. Supra-valvular aortic stenosis in a patient with homozygous familial hypercholesterolaemia. *Eur Heart J Cardiovasc Imaging*. (2013) 14:1023. doi: 10.1093/ehjci/jet072
17. Kolansky DM, Cuchel M, Clark BJ, Paridon S, McCrindle BW, Wiegers SE, et al. Longitudinal evaluation and assessment of cardiovascular disease in patients with homozygous familial hypercholesterolemia. *Am J Cardiol*. (2018) 102:1438–43. doi: 10.1016/j.amjcard.2008.07.035
18. Raal FJ, Santos RD. Homozygous familial hypercholesterolemia: current perspectives on diagnosis and treatment. *Atherosclerosis*. (2012) 223:262–8. doi: 10.1016/j.atherosclerosis.2012.02.019
19. Martinez M, Brodlie S, Griesemer A, Kato T, Harren P, Gordon B, et al. Effects of liver transplantation on lipids and cardiovascular disease in children with homozygous familial hypercholesterolemia. *Am J Cardiol*. (2016) 118:504–10. doi: 10.1016/j.amjcard.2016.05.042
20. Farnier M, Civeira F, Descamps O, FH expert working group. How to implement clinical guidelines to optimise familial hypercholesterolaemia diagnosis and treatment. *Atheroscler Suppl*. (2017) 26:25–35. doi: 10.1016/S1567-5688(17)30022-3
21. Catapano AL, Graham I, De Backer G, Wiklund O, Chapman MJ, Drexel H, et al. 2016 ESC/EAS guidelines for the management of dyslipidaemias. *Atherosclerosis*. (2016) 253:281–344. doi: 10.1016/j.atherosclerosis.2016.08.018
22. National Institute for Health and Care Excellence. *Familial Hypercholesterolaemia: Identification and Management. Guidance and Guidelines. National Institute for Health and Care Excellence*. (2017). Available online at: https://www.nice.org.uk/guidance/cg71 (accessed May 18, 2020).
23. Daniels SR, Gidding SS, de Ferranti SD, National Lipid Association Expert Panel on Familial Hypercholesterolemia. Pediatric aspects of familial hypercholesterolemias: recommendations from the national lipid association expert panel on familial hypercholesterolemia. *J Clin Lipidol*. (2011) 5:30–7. doi: 10.1016/j.jacl.2011.03.453
24. Hovingh GK, Goldberg AC, Moriarty PM. Managing the challenging homozygous familial hypercholesterolemia patient: academic insights and practical approaches for a severe dyslipidemia, a National Lipid Association Masters Summit. *J Clin Lipidol*. (2017) 11:602–16. doi: 10.1016/j.jacl.2017.03.008
25. Luirink IK, Hutten BA, Wiegman A. Optimizing treatment of familial hypercholesterolemia in children and adolescents. *Curr Cardiol Rep*. (2015) 17:629. doi: 10.1007/s11886-015-0629-1
26. Beliard S, Gallo A, Duchêne E, Carrié A, Bittar R, Chapman MJ, et al. Lipoprotein-apheresis in familial hypercholesterolemia: long-term patient compliance in a French cohort. *Atherosclerosis*. (2018) 277:66–71. doi: 10.1016/j.atherosclerosis.2018.08.007
27. Gidding SS, Champagne MA, de Ferranti SD, Defesche J, Ito MK, Knowles JW, et al. The agenda for familial hypercholesterolemia. a scientific statement

from the American Heart Association. *Circulation*. (2015) 132:2167–92. doi: 10.1161/CIR.0000000000000297
28. Stefanutti C, Julius U, Watts GF, Harada-Shiba M, Cossu M, Schettler VJ, et al. Toward an international consensus-Integrating lipoprotein apheresis and new lipid-lowering drugs. *J Clin Lipidol*. (2017) 11:858–71. doi: 10.1016/j.jacl.2017.04.114
29. Schuff-Werner P, Fenger S, Kohlschein P. Role of lipid apheresis in changing times. *Clin Res Cardiol Suppl*. (2012) 7:7–14. doi: 10.1007/s11789-012-0049-3
30. Thompson GR, Seed M, Naoumova RP, Neuwirth C, Walji S, Aitman TJ, et al. Improved cardiovascular outcomes following temporal advances in lipid-lowering therapy in a genetically-characterised cohort of familial hypercholesterolaemia homozygotes. *Atherosclerosis*. (2015) 243:328–33. doi: 10.1016/j.atherosclerosis.2015.09.029
31. Omer L, Hudson EA, Zheng S, Hoying JB, Shan Y, Boyd NL. CRISPR correction of a homozygous low-density lipoprotein receptor mutation in familial hypercholesterolemia induced pluripotent stem cells. *Hepatol Commun*. (2017) 1:886–98. doi: 10.1002/hep4.1110
32. Thompson GR. The evidence-base for the efficacy of lipoprotein apheresis in combating cardiovascular disease. *Atheroscler Suppl*. (2013) 14:67–70. doi: 10.1016/j.atherosclerosissup.2012.10.001
33. Stefanutti C, Julius U. Lipoprotein apheresis: state of the art and novelties. *Atheroscler Suppl*. (2013) 14:19–27. doi: 10.1016/j.atherosclerosissup.2012.10.021
34. Græsdal A, Bogsrud MP, Holven KB, Nenseter MS, Narverud I, Langslet G, et al. Apheresis in homozygous familial hypercholesterolemia: the results of a follow-up of all Norwegian patients with homozygous familial hypercholesterolemia. *J Clin Lipidol*. (2012) 6:331–9. doi: 10.1016/j.jacl.2012.03.004
35. Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease. Consensus Statement of the European Atherosclerosis Society. *Eur Heart J*. (2013) 34:3478–90. doi: 10.1093/eurheartj/eht273
36. Mansoorian M, Kazemi K, Nikeghbalian S, Shamsaeefar A, Mokhtari M, Dehghani SM, et al. Liver transplantation as a definitive treatment for familial hypercholesterolemia: a series of 36 cases. *Pediatr Transplant*. (2015) 19:605–11. doi: 10.1111/petr.12562
37. Akdur A, Kirnap M, Ayvazoglu Soy EH, Ozcay F, Moray G, Arslan G, et al. Unusual indications for a liver transplant: a single-center experience. *Exp Clin Transplant*. (2017) 15:128–32. doi: 10.6002/ect.mesot2016.P11
38. Alim A, Tokat Y, Erdogan Y, Gokkaya Z, Dayangac M, Yuzer Y, et al. Liver transplantation for homozygote familial hypercholesterolemia: the only curative treatment. *Pediatr Transplant*. (2016) 20:1060–4. doi: 10.1111/petr.12763
39. Greco M, Robinson JD, Eltayeb O, Benuck I. Progressive aortic stenosis in homozygous familial hypercholesterolemia after liver transplant. *Pediatrics*. (2016) 138:e20160740. doi: 10.1542/peds.2016-0740
40. El-Rassi I, Chehab G, Saliba Z, Alawe A, Jebara V. Fatal cardiac atherosclerosis in a child 10 years after liver transplantation: a case report and a review. *J Clin Lipidol*. (2011) 5:329–32. doi: 10.1016/j.jacl.2011.05.002
41. Ibrahim M, El-Hamamsy I, Barbir M, Yacoub MH. Translational lessons from a case of combined heart and liver transplantation for familial hypercholesterolemia 20 years post-operatively. *J Cardiovasc Transl Res*. (2012) 5:351–8. doi: 10.1007/s12265-011-9311-1
42. Palacio CH, Harring TR, Nguyen NT, Goss JA, O'Mahony CA. Homozygous familial hypercholesterolemia: case series and review of the literature. *Case Rep Transplant*. (2011) 2011:154908. doi: 10.1155/2011/154908
43. Alkofer BJ, Chiche L, Khayat A, Deshayes JP, Lepage A, Saloux E, et al. Liver transplant combined with heart transplant in severe heterozygous hypercholesterolemia: report of the first case and review of the literature. *Transplant Proc*. (2005) 37:2250–2. doi: 10.1016/j.transproceed.2005.03.037
44. Khalifeh M, Faraj W, Heaton N, Rela M, Sharara AI. Successful living-related liver transplantation for familial hypercholesterolemia in the Middle East. *Transpl Int*. (2005) 17:735–9. doi: 10.1007/s00147-004-0791-7
45. Shrotri M, Fernando BS, Sudhindran S, Delriviere L, Watson CJ, Gibbs P, et al. Long-term outcome of liver transplantation for familial hypercholesterolemia. *Transplant Proc*. (2003) 35:381–2. doi: 10.1016/S0041-1345(02)03910-6
46. Küçükkartallar T, Yankol Y, Kanmaz T, Topaloglu S, Acarli K, Kalayoglu M. Liver transplantation as a treatment option for three siblings with homozygous familial hypercholesterolemia. *Pediatr Transplant*. (2011) 15:281–4. doi: 10.1111/j.1399-3046.2010.01469.x
47. Maiorana A, Nobili V, Calandra S, Francalanci P, Bernabei S, El Hachem M, et al. Preemptive liver transplantation in a child with familial hypercholesterolemia. *Pediatr Transplant*. (2011) 15:25–9. doi: 10.1111/j.1399-3046.2010.01383.x
48. Kawagishi N, Satoh K, Akamatsu Y, Sekiguchi S, Ishigaki Y, Oikawa S, et al. Long-term outcome after living donor liver transplantation for two cases of homozygous familial hypercholesterolemia from a heterozygous donor. *J Atheroscler Thromb*. (2007) 14:94–8. doi: 10.5551/jat.14.94
49. Schmidt HH, Tietge UJ, Buettner J, Barg-Hock H, Offner G, Schweitzer S, et al. Liver transplantation in a subject with familial hypercholesterolemia carrying the homozygous p.W577R LDL-receptor gene mutation. *Clin Transplant*. (2008) 22:180–4. doi: 10.1111/j.1399-0012.2007.00764.x
50. López-Santamaria M, Migliazza L, Gamez M, Murcia J, Diaz-Gonzalez M, Camarena C, et al. Liver transplantation in patients with homozygotic familial hypercholesterolemia previously treated by end-to-side portocaval shunt and ileal bypass. *J Pediatr Surg*. (2000) 35:630–3. doi: 10.1053/jpsu.2000.0350630
51. Offstad J, Schrumpf E, Geiran O, Søreide O, Simonsen S. Plasma exchange and heart-liver transplantation in a patient with homozygous familial hypercholesterolemia. *Clin Transplant*. (2011) 15:432–6. doi: 10.1034/j.1399-0012.2001.150612.x
52. Popescu I, Simionescu M, Tulbure D, Sima A, Catana C, Niculescu L, et al. Homozygous familial hypercholesterolemia: specific indication for domino liver transplantation. *Transplantation*. (2003) 76:1345–50. doi: 10.1097/01.TP.0000093996.96158.44
53. van Heyningen C. Progressive vascular disease in homozygous familial hypercholesterolaemia despite apheresis and heart and liver transplantation. *Br J Diabetes Vasc Dis*. (2011) 11:270–2. doi: 10.1177/1474651411422652
54. Moyle M, Tate B. Homozygous familial hypercholesterolaemia presenting with cutaneous xanthomas: response to liver transplantation. *Australas J Dermatol*. (2004) 45:226–8. doi: 10.1111/j.1440-0960.2004.00103.x
55. Ahualli L, Stewart-Harris A, Bastianelli G, Radlovachki D, Bartolomé A, Trigo PL, et al. Combined cardiohepatic transplantation due to severe heterozygous familiar hypercholesteremia type II: first case in Argentina–a case report. *Transplant Proc*. (2007) 39:2449–53. doi: 10.1016/j.transproceed.2007.07.068
56. Gulsoy Kirnap N, Kirnap M, Bascil Tutuncu N, Moray G, Haberal M. The curative treatment of familial hypercholesterolemia: liver transplantation. *Clin Transplant*. (2019) 33:e13730. doi: 10.1111/ctr.13730
57. Cephus CE, Qureshi AM, Sexson Tejtel SK, Alam M, Moodie DS. Coronary artery disease in a child with homozygous familial hypercholesterolemia: regression after liver transplantation. *J Clin Lipidol*. (2019) 13:880–6. doi: 10.1016/j.jacl.2019.09.007
58. Malatack JJ. Liver transplantation as treatment for familial homozygous hypercholesterolemia: too early or too late. *Pediatr Transplant*. (2011) 15:123–5. doi: 10.1111/j.1399-3046.2010.01458.x
59. Al-Ashwal A, Alnouri F, Sabbour H, Al-Mahfouz A, Al-Sayed N, Razzaghy-Azar M, et al. Identification and treatment of patients with homozygous familial hypercholesterolaemia: information and recommendations from a middle east advisory panel. *Curr Vasc Pharmacol*. (2015) 13:759–70. doi: 10.2174/1570161113666150827125040
60. Agarwal A, Prasad GV. Post-transplant dyslipidemia: mechanisms, diagnosis and management. *World J Transplant*. (2016) 6:125–34. doi: 10.5500/wjt.v6.i1.125
61. Goldberg AC, Hopkins PN, Toth PP, Ballantyne CM, Rader DJ, Robinson JG, et al. Familial hypercholesterolemia: screening, diagnosis and management of pediatric and adult patients: clinical guidance from the national lipid association expert panel on familial hypercholesterolemia. *J Clin Lipidol*. (2011) 5:1–8. doi: 10.1016/j.jacl.2011.04.003
62. Watts GF, Sullivan DR, Poplawski N, van Bockxmeer F, Hamilton-Craig I, Clifton PM, et al. Familial hypercholesterolaemia: a model of care for Australasia. *Atheroscler Suppl*. (2011) 12:221–63. doi: 10.1016/j.atherosclerosissup.2011.06.001
63. Descamps OS, Tenoutasse S, Stephenne X, Gies I, Beauloye V, Lebrethon MC, et al. Management of familial hypercholesterolemia in children and young adults: consensus paper developed by a

panel of lipidologists, cardiologists, paediatricians, nutritionists, gastroenterologists, general practitioners and a patient organization. *Atherosclerosis*. (2011) 218:272–80. doi: 10.1016/j.atherosclerosis.2011.06.016

64. Village EG. Expert panel on integrated guidelines for cardiovascular health and risk reduction in children and adolescents: summary report. *Pediatrics*. (2011) 128:213–56. doi: 10.1542/peds.2009-2107C
65. France M, Rees A, Datta D, Thompson G, Capps N, Ferns G, et al. HEART UK statement on the management of homozygous familial hypercholesterolaemia in the United Kingdom. *Atherosclerosis*. (2016) 255:128–39. doi: 10.1016/j.atherosclerosis.2016.10.017
66. The Cardiac Society of Australia and New Zealand. *Diagnosis and Management of Familial Hypercholesterolaemia – Position Statement*. (2016). Available online at: https://www.csanz.edu.au/wp-content/uploads/2017/07/Familial-Hypercholesterolaemia_ratified_-25-Nov-2016.pdf (accessed May 18, 2020).
67. Santos RD, Gidding SS, Hegele RA, Cuchel MA, Barter PJ, Watts GF, et al. Defining severe familial hypercholesterolaemia and the implications for clinical management: a consensus statement from the International Atherosclerosis Society Severe Familial Hypercholesterolemia Panel. *Lancet Diabetes Endocrinol*. (2016) 4:850–61. doi: 10.1016/S2213-8587(16)30041-9
68. Volpe M, Volpe R, Gallo G, Presta V, Tocci G, Folco E, et al. 2017 Position paper of the Italian society for cardiovascular prevention (SIPREC) for an updated clinical management of hypercholesterolemia and cardiovascular risk: executive document. *High Blood Press Cardiovasc Prev*. (2017) 24:313–29. doi: 10.1007/s40292-017-0211-6
69. Li YH, Ueng KC, Jeng JS, Charng MJ, Lin TH, Chien KL, et al. 2017 Taiwan lipid guidelines for high risk patients. *J Formos Med Assoc*. (2017) 116:217–48. doi: 10.1016/j.jfma.2016.11.013
70. Jellinger PS, Handelsman Y, Rosenblit PD, Bloomgarden ZT, Fonseca VA, Garber AJ, et al. American Association Of Clinical Endocrinologists and American College Of Endocrinology Guidelines for management of dyslipidemia and prevention of cardiovascular disease – executive summary. *Endocr Pract*. (2017) 23:479–97. doi: 10.4158/EP171764.APPGL
71. Harada-Shiba M, Ohta T, Ohtake A, Ogura M, Dobashi K, Nohara A, et al. Guidance for pediatric familial hypercholesterolemia 2017. *J Atheroscler Thromb*. (2018) 25:539–53. doi: 10.5551/jat.CR002
72. Luirink IK, Hutten BA, Greber-Platzer S, Kolovou GD, Dann EJ, de Ferranti SD, et al. Practice of lipoprotein apheresis and short-term efficacy in children with homozygous familial hypercholesterolemia: data from an international registry. *Atherosclerosis*. (2020) 299:24–31. doi: 10.1016/j.atherosclerosis.2020.01.031

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REVIEW
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Genetics of Hypercholesterolemia: Comparison Between Familial Hypercholesterolemia and Hypercholesterolemia Nonrelated to LDL Receptor

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Severe hypercholesterolemia (HC) is defined as an elevation of total cholesterol (TC) due to the increase in LDL cholesterol (LDL-C) >95th percentile or 190 mg/dl. The high values of LDL-C, especially when it is maintained over time, is considered a risk factor for the development of atherosclerotic cardiovascular disease (ASCVD), mostly expressed as ischemic heart disease (IHD). One of the best characterized forms of severe HC, familial hypercholesterolemia (FH), is caused by the presence of a major variant in one gene (*LDLR*, *APOB*, *PCSK9*, or *ApoE*), with an autosomal codominant pattern of inheritance, causing an extreme elevation of LDL-C and early IHD. Nevertheless, an important proportion of serious HC cases, denominated polygenic hypercholesterolemia (PH), may be attributed to the small additive effect of a number of single nucleotide variants (SNVs), located along the whole genome. The diagnosis, prevalence, and cardiovascular risk associated with PH has not been fully established at the moment. Cascade screening to detect a specific genetic defect is advised in all first- and second-degree relatives of subjects with FH. Conversely, in the rest of cases of HC, it is only advised to screen high values of LDL-C in first-degree relatives since there is not a consensus for the genetic diagnosis of PH. FH is associated with the highest cardiovascular risk, followed by PH and other forms of HC. Early detection and initiation of high-intensity lipid-lowering treatment is proposed in all subjects with severe HC for the primary prevention of ASCVD, with an objective of LDL-C <100 mg/dl or a decrease of at least 50%. A more aggressive reduction in LDL-C is necessary in HC subjects who associate personal history of ASCVD or other cardiovascular risk factors.

Keywords: primary hypercholesterolemia, familial hypercholesterolemia, polygenic hypercholesterolemia, cardiovascular disease, LDL-cholesterol, atherosclerosis

INTRODUCTION

Severe primary hypercholesterolemia (HC) is a disorder of lipid metabolism, clinically characterized by an elevation of LDL cholesterol (LDL-C) >190 mg/dl and/or total cholesterol (TC) >95th percentile or >300 mg/dl, with normal values of triglycerides (TGs). Despite the great efforts and health plans carried out to improve the detection and clinical management of HC subjects, that population still remains underdiagnosed and undertreated (Nordestgaard et al., 2013; Representatives of the Global Familial Hypercholesterolemia Community et al., 2020). Classically, severe HC constitutes an inherited trait, frequently associated with high cardiovascular risk due to lifelong exposure to elevated cholesterol levels, causing ischemic heart disease (IHD) as the main clinical manifestation (Civeira and International Panel on Management of Familial Hypercholesterolemia, 2004; Mach et al., 2020). One of the best known cause of HC is familial hypercholesterolemia (FH)# 143890, a genetic disorder with an autosomal codominant inheritance pattern, due to a monogenic defect in LDL receptor (*LDLR*), apolipoprotein B (*APOB*), Proprotein convertase subtilisin/kexin type 9 (*PCSK9*), or apolipoprotein E (*APOE*) genes, involved in the LDL receptor endocytic and recycling pathways.

However, a deeper knowledge about genomics has made it possible to detect genetic variations related to a specific trait. One of the most extended methods, genome-wide association studies (GWAS), allows to detect hundreds of single variations in a nucleotide (SNVs) in a unique subject, located throughout the genome. Some of these SNVs may be associated with differences in LDL-C serum values and IHD. Afterwards, it makes possible to compare the same unbiased genome screens of unrelated individuals and appropriately matched controls. When a number of these SNVs cluster in the same subject, it has been set out as a cause of primary HC (Teslovich et al., 2010), being labeled as polygenic hypercholesterolemia (PH) (Talmud et al., 2013). Nonetheless, there still remains a proportion of HC subjects in whom no significant increase is detected on SNVs related to LDL-C with respect to the general population. Hitherto, the prevalence, diagnosis, and clinical management of subjects with PH have not been set up. The aim of this review was to describe the clinical profile, inheritance pattern, and treatment of subjects with PH, as well as the main difference between monogenic and non-monogenic origen of HC.

Abbreviations: ASCVD, atherosclerotic cardiovascular disease; DLCN, Dutch Lipid Clinic Network; FH, familial hypercholesterolemia; GWAS, genome-wide association study; HC, hypercholesterolemia; IHD, ischemic heart disease; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-HDL cholesterol; PH, polygenic hypercholesterolemia; TC, total cholesterol; TG, triglycerides; TX, tendinous xanthoma; SNV, single nucleotide variant.

PRIMARY HYPERCHOLESTEROLEMIA AS A DRIVING FACTOR FOR THE DEVELOPMENT OF CARDIOVASCULAR DISEASE

Classically, severe HC has been established as a causal risk factor of atherosclerotic cardiovascular disease (ASCVD) (Ross, 1999; Nordestgaard et al., 2013). However, a positive correlation between TC and ASCVD has been observed in men and women, as a continuous variable from TC values >180 mg/dl, whereas a very low incidence of ASCVD has been observed in those with TC below that value (Stamler et al., 1986; Nagasawa et al., 2012; Kwon et al., 2019; Maihofer et al., 2020). Similarly, the treatment with hypolipemiant drugs maintained over the years has demonstrated a significant decrease in IHD and cardiovascular mortality in subjects with primary HC (Besseling et al., 2014; Humphries et al., 2018; Perez-Calahorra et al., 2019).

Recent meta-analyses of prospective cohort studies, Mendelian randomization studies, and randomized controlled trials, including more than 2 million of participants, allowed to establish a log-linear, dose-dependent association between TC, non-HDL cholesterol (non-HDL-C) and LDL-C, and the risk of IHD and mortality (**Figure 1**). All of them are surrogate markers of apolipoprotein B (ApoB), an apoprotein that transports the majority of cholesterol in the blood stream. From among them all, LDL-C is the best correlated to ApoB, since it contains up to 90% of all apoB detected, while in non-HDL-C and TC, a bigger amount of TG and other lipoparticles is included (Ference et al., 2017).

All these studies offered the possibility of establishing a temporal association between LDL-C exposure and IHD. However, Mendelian randomization studies go a step further introducing a lifelong randomization scheme by the genetic variants associated with serum LDL-C values (Ference et al., 2012; Holmes et al., 2015) (**Figure 1**). The GWAS and custom genotyping arrays, carried out in populations from European, American, and African ancestry, have allowed to detect a number of SNV linked up with the catabolism and overproduction of LDL particles as well as the prevalence of IHD (Willer et al., 2013). Each of these variants is approximately inherited randomly at the time of conception in a process referred as "Mendelian randomization" (Ference, 2015). If there is no association with any other variant in other gene-modifying LDL-C, that genetic variant should provide and estimation of the effect on LDL-C levels. It is possible to establish a relation between that genetic variant and the risk of ASCVD, analogously to a long-term clinical trial. These studies have demonstrated that only variants in genes that modify LDL-C, but not in another traits of lipid profile, are associated with a lower risk of IHD. Finally, randomized control trials show the reduction in ASCVD incidence by reducing LDL-C. In a classical meta-analysis including 170,000 subjects, a decrease of 1 mmol (38 mg/dl) of LDL-C by statins was associated with a 22% reduction in the risk of major cardiovascular events over a median of 5 years of treatment. The magnitude of this reduction was independent of baseline LDL-C and any type of hypolipemiant

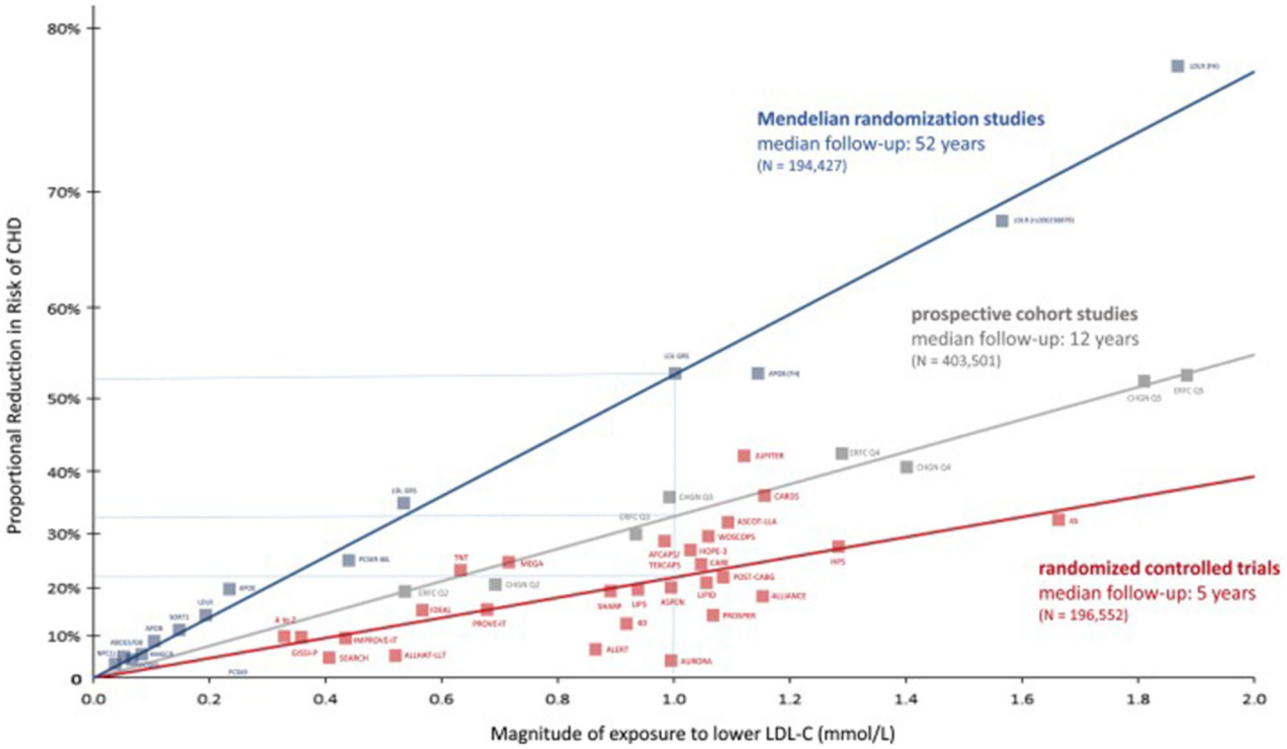


FIGURE 1 | Reproduced with permission from Ference et al. (2017). Log-linear association per unit change in low-density lipoprotein cholesterol (LDL-C) and the risk of cardiovascular disease as reported in meta-analyses of Mendelian randomization studies, prospective epidemiological cohort studies, and randomized clinical trials.

drug used, and its results are remarkably consistent in different subgroups of patients studied [(Cholesterol Treatment Trialists' (CTT) Collaborators et al., 2008; Wang et al., 2020)] (**Figure 1**).

The comparison of data from these three sources has enabled to draw a figure relating the magnitude of LDL-c exposition and the proportional reduction in risk of coronary heart disease observed in the three types of studies. Interestingly, with the same magnitude of lowering LDL-C, the biggest effect was observed in Mendelian randomized studies, emphasizing not only the importance of the serum values of LDL-C but also the time of exposure, expressed as the LDL burden for atherosclerosis (**Figure 1**) (Ference et al., 2017). High serum concentration of LDL particles maintained over the time implies a high burden of ApoB, which can cross the endothelial barrier and interact with other wall components, leading to the thickening of the arterial walls and the development of atherosclerotic plaques. Some of the mechanisms implied as vascular inflammation is related to the pathological internalization of apoB-containing particles by macrophages, especially when LDL particles become oxidized or are otherwise modified (Borén et al., 2020). Those damaged plaques may evolve to be broken, especially when it contains a necrotic core and macrophage accumulation full of LDL particles (Ross, 1999). Recent findings have correlated the descent in LDL-C with a decrease in atherosclerotic plaque volume and ASCVD (Forbes et al., 2016).

GENETICS OF PRIMARY HYPERCHOLESTEROLEMIA: FROM MAJOR VARIANTS IN A UNIQUE GEN TO THE ADDITIVE EFFECTS OF SINGLE NUCLEOTIDE VARIANTS IN MULTIPLE GENES

A new approach to genetic diagnosis of primary HC has been developed: from searching for new monogenic variations with large effect on disease status, to the additive effect of several small variants with little pathogenic effect on several genes related to lipids metabolism in the same subject (Berberich and Hegele, 2019). The traditional definition of severe HC corresponds to a monogenic disease in which one copy of a variant allele produces the disease phenotype. The first cases of HC associated with a familial pattern, tendinous xanthomas (TX), and early mortality were reported in the 1930s by Carl Müller, based on the findings of 17 families with xanthomata and early IHD in Norway (Ose, 2002). It was not until the 1970s that the identification of a variant of *LDLR* causing lack of affinity of LDL particle to the LDL receptor enabled Goldstein and Brown to establish the pathogenesis of FH (Goldstein and Brown, 1974). Until now, more than 2,000 causative variants of FH have been described, the majority (80%) located in *LDLR* and the rest in *APOB* and *PCSK9* genes. The effects of that allelic variants

encompass any of the stages of receptor-mediated endocytosis of LDL particles. The effect of large *LDLR* variants can be sorted out in two categories: no protein synthesis or synthesis of a totally non-functional receptor, whereas large *APOB* variants affect the receptor-binding domain of ApoB. Finally, *PCSK9* variants with a gain of function increase the LDL-receptor recycling process, decreasing its half-life and its availability in the cell surface. Since all of them correspond to pathogenic variants that are expressed with autosomal codominant inheritance pattern, they have been grouped as causes of FH (Awan et al., 2013; Cenarro et al., 2016; Chora et al., 2018). Recently, the p.(Leu167) polymorphism in *APOE* gene has been associated with *LDLR* downregulation, raising LDL plasma levels, with clear familial segregation and the presence of TX, accounting for 3.1% subjects with PH and negative variants *in LDLR*, *APOB*, and *PCSK9* genes (Awan et al., 2013; Cenarro et al., 2016). In an attempt to find other new genes causing PH phenotype, a variant in signal transducer adaptor family member 1 (*STAP1*) gene causing FH phenotype was described in a large Dutch family (Fouchier et al., 2014). However, its role in FH seems to have been discarded recently (Hegele et al., 2020; Lamiquiz-Moneo et al., 2020).

Regarding PH, the first definition was provided by Talmud et al. (2013). From 27 SNVs related to LDL-C serum values, they built a score by the 12 more predictive of PH, observing a maximum difference of 44 mg/dL in LDL-C concentration between subjects in the highest and the lowest decile. In the same study, 52% of HC subjects non-carriers of large variants causing FH had a score within deciles 7–10 of SNVs distribution. Similar findings were reproduced in populations from Wales and Belgium (Talmud et al., 2013).

Afterwards, the same authors simplify the diagnosis of PH, removing the least frequent or least predictive SNVs, leaving only 6 from the initial 12, obtaining similar yields in the diagnosis of PH. These polymorphisms were located in genes involved in different pathways of LDL particles metabolism such as *APOB*, *LDLR*, and *APOE* genes. Others, as *ABCG5/8* (ATP-binding cassette, subfamily G, member 5/8), modulate cholesterol production by sterols hyperabsorption (Baila-Rueda et al., 2016); meanwhile, *CELSR2* (cadherin, EGF LAG 7-pass G-type receptor 2) is related to cells signaling, although its function with respect to LDL-C metabolism is unknown (Paththinige et al., 2017). In a group of 1,158 probands with HC and a family history of premature ASCVD, the polygenic score built with those SNVs was able to diagnose 36% of subjects with PH when the cutoff value was 75th percentile in SNVs distribution. These results were reproduced in seven cohorts of HC subjects from different countries (Futema et al., 2015). However, when we tried to replicate the same experiment in our population, no differences in its prevalence between HC and normolipemic members of the same family were observed, explaining the 6.9% of LDL-C value in HC subjects (Lamiquiz-Moneo et al., 2017). Another large study carried out in 5,415 subjects who belonged to the general population included nine SNVs from genes related to LDL-C and HDL-C metabolism (*LDLR*, *APOB*, *APOE*, and *ABCG5/8* among others), observing a maximum difference of 72 mg/dl between the homozygote classes of SNVs. Despite the fact that this score was an independent risk factor for ASCVD, the genotype did not improve ASCVD prediction with respect to classical cardiovascular risk factors (Kathiresan et al., 2008).

More recently, a deeper coverage of whole genome sequencing analyses by next-generation sequency technology has enabled to detect monogenic variants, in addition to hundreds of SNVs, gene copy numbers, and genomic rearrangements from the various types of DNA-sequencing and microarray data, related to lipid metabolism and/or ASCVD (Natarajan et al., 2018). Nevertheless, no new variants related to LDL-C have been detected. These SNVs have been located in genes encoding structural components of lipoproteins, lipoprotein receptors and related proteins, enzymes, lipid transporters, lipid transfer proteins, and activators or inhibitors of some protein function and gene transcription. However, some of them are within or in the vicinity of genes that are not known to be involved in lipid metabolism. Besides, over 90% of these SNVs are located outside the coding regions, hence will be missed in routine exome-sequencing techniques (Paththinige et al., 2017). Each polymorphism is associated with small but reproducible increase in LDL-C levels, explaining the impact of each one between 0.1 and 2.5% of LDL-C serum values (Kathiresan et al., 2008; Willer et al., 2013; Lamiquiz-Moneo et al., 2017; Trinder et al., 2020).

Because polygenic LDL-C *loci* are scattered throughout the genome and segregate independently during meiosis, most individuals have an overall balance between LDL-C-raising and LDL-C-lowering alleles. Rare individuals at the high extreme of polygenic scores have inherited a preponderance of LDL-C-raising alleles (**Figure 2**) (Berberich and Hegele, 2019) and frequently clustered and inherited in the same family by mechanisms not described hitherto (**Table 1**) (Jarauta et al., 2016; Berberich and Hegele, 2019). Furthermore, these SNVs seem to contribute to the severity of HC and increased IHD in some FH subjects that exhibit higher LDL-C levels with respect to FH without a polygenic inheritance (Talmud et al., 2013; Ghaleb et al., 2018; Trinder et al., 2019). Nowadays, the diagnosis of PH varies depending on the number and type of SNVs included and the percentage cutoff point chosen for the diagnosis, without any standard definition for PH accepted at the moment (**Table 2**). Thus, the identification of a specific genetic pathological variant is not a necessary condition for the diagnosis of a genetic HC (Berberich and Hegele, 2019).

ARE POLYGENIC HYPERCHOLESTEROLEMIA AND FAMILIAL HYPERCHOLESTEROLEMIA CLINICALLY THE SAME OR DIFFERENT? COMPARISON OF CRITERIA FOR CLINICAL DIAGNOSIS, LIPID PROFILE, AND CARDIOVASCULAR DISEASE

Recent data delivered a higher prevalence of FH than previously documented, with a general pooled estimation in 1:250 (0.4%) (Benn et al., 2016; Akioyamen et al., 2017) accounting about 1.7–5.6% of subjects with severe HC (Benn et al., 2012; Khera et al., 2016; Trinder et al., 2020). On the contrary, other

forms of HC comprise a larger proportion of individuals in both population-based approach studies or large samples from different genetic backgrounds: 13.7% of 1.18 million of adults in the United States (Patel et al., 2019), about 10% of 48,781 participants in the UK Biobank cohort study (Trinder et al., 2020), up to 7% of 67,019 subjects from the Danish general population study (Benn et al., 2012) and 6.8% of 20,485 ASCVD-free subjects belonging to 12 cohorts from different countries (Khera et al., 2016) (**Table 2**).

Conversely, when starting from ASCVD cases, the clinical diagnosis of FH increases significantly, being reported in 6.7% patients suffering from first myocardial infarction (Mortensen et al., 2016). Besides, in case of premature myocardial infarction, differences in FH and PH prevalence disappeared. In a study including 626 HC patients younger than 55 years old with a personal history of IHD, FH prevalence was doubled than PH (43.9 vs. 21.4%), with the highest cardiovascular risk for FH subjects with an added polygenic trait for LDL-C. In addition, PH was not associated with an increased cardiovascular risk with respect to those HC subjects with no associated genetic trait for LDL-C (Trinder et al., 2019). On the contrary, in a study including 2,041 subjects with premature onset of myocardial infarction, prevalence of PH was 10 times higher than those with FH, showing a similar risk for IHD between PH and FH subjects. However, in that case, the diagnosis of PH was not based on LDL-C serum values, rather on the 95th percentile of 6.6 million of common DNA variants associated with myocardial infarction detected by deep coverage whole exome sequencing (Khera et al., 2018) (**Table 2**).

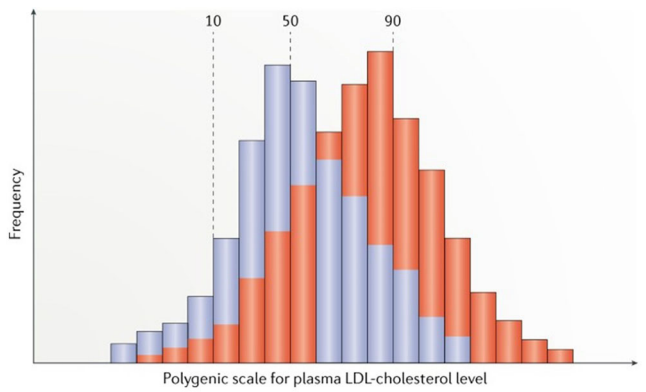


FIGURE 2 | Reproduced with permission from Berberich and Hegele (2019). Scheme of distributions for polygenic risk scores for LDL-cholesterol (LDL-C) levels in the general normolipidemic population (blue) and in clinically ascertained patients with suspected familial hypercholesterolemia (FH) but no monogenic variant in genes causing FH (red). Scores are calculated from single nucleotide polymorphism genotypes, by simply tallying trait-raising alleles, or scores can be further weighted according to effect sizes for the alleles reported in genome-wide association studies.

The main clinical characteristics of FH as the extreme elevation of LDL-C, premature ASCVD, the presence of TX, and family history of severe HC and premature ASCVD have been collected in several scores as predictors of FH, since genetic diagnosis is not affordable in all cases (Representatives of the Global Familial Hypercholesterolemia Community et al., 2020). The most popular, the Dutch Lipid Clinic Network (DLCN) score, classifies subjects as "unlikely FH" (score < 3), "probable FH" (score $= 3$–5), "possible FH" (score $= 6$–8), and "definite FH" (score > 8) (**Table 3**). Quite similarly, the Simon Broome Score labels subjects as "definite FH" if LDL-C is above 190 mg/dl and they have TX and "possible FH" for those with familial history of premature IHD besides the elevation of LDL-C. In both of them, a higher score or the presence of TX increased significantly the probability of FH diagnosis (Civeira et al., 2008b; Palacios et al., 2012; Benn et al., 2016; Trinder et al., 2019).

TX constitutes nearly a pathognomonic sign of FH, increasing the odds ratio for the diagnosis of FH (**Table 3**). Nonetheless, it might be detected in some cases of sytosterolemia, an autosomal recessive form of HC caused by defects on *ABCG5* and *ABCG8* genes, which increases plasma sterols absorption (Bastida et al., 2019). TX are composed of an extravascular deposit of

TABLE 1 | Heritage pattern analysis and heritability by family-based association test in HC families non-related to FH (Jarauta et al., 2016).

Dependent Variable	Dominant model (number of informative trios = 55)			Recessive model (number of informative trios = 23)			Covariates
	P-value (FBAT)	Power (FBAT)	Heritability	*P*-value (FBAT)	Power (FBAT)	Heritability	
Total cholesterol	3.92E-14	0.999	0.389*	0.001	0.934	0.059*	Sex, age, BMI, *APOE*
Triglycerides	0.114	0.250	0.028	3.79E-06	0.999	0.178*	Sex, age, BMI, *APOE*
HDL cholesterol	0.600	0.855	0.118	0.047	0.124	−0.012*	Sex, age, LDLc, BMI, *APOE*
LDL cholesterol	2.80E-14	0.999	0.322*	0.005	0.674	0.035*	Sex, age, HDLc, BMI, *APOE*

BMI, body mass index; FBAT, family-based association tests; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; non-FH-GH, non-familial hypercholesterolemia-genetic hypercholesterolemia.

**Statistically significant model. Variables with significant univariate association with the lipid profile were included as covariates.*

TABLE 2 | Principal studies including data about prevalence of familial hypercholesterolemia and polygenic hypercholesterolemia.

Study first author, year	*N*	Clinical characteristics	Time follow-up (years)	Number and genes with SNVs included for diagnosis of PH	Prevalence of PH (%), % of score	Prevalence of FH (%)	ASCVD risk FH	ASCVD risk associated to PH
Kathiresan et al. (2008)	5,414	Cardiovascular cohort Malmö Diet and Cancer Study	10.6	*9 (APOB, PCSK9 LDLR; CETP LIPC LPL, APOE, HMGCR, ABCA1)*	26.2 (> fourth quartile)	NA	NA	1,63 hazard ratio
Willer et al. (2013)	1,158	PrH in proband plus family history of premature myocardial infarction	NA	*6 (CELSR2, APOB, ABCG5/8 LDLR and APOE)*	36 (>fourth quartile)	351 (33,3)	NA	NA
Khera et al. (2016)	26,025	5,540 coronary artery disease	NA	NA	NA	1.9	22	6 (HC subjects non-FH)
Khera et al. (2018)	2,081	Early onset of myocardial infarction	NA	*6,6 $\times$ 10^6	17.3 (>95th decile)	1.7 (*LDLR truncation, frameshift, splicing)*	3.8	3.7
Benn et al. (2012)	69,016	Danish community-based population	NA	NA	6.9 (definitive, probable or possible FH)	0.2	10.3	NA
Benn et al. (2016)	98,098	Copenhagen general population Study	36	NA	7.2	0.46	5.3 *LDLR* carriers 1.8 *APOB* carriers	NA
Natarajan et al. (2018)	16,324	From 4 ancestries**	NA	2 $\times$ 10^6-SNV LDL-C polygenic score	23% of HC (>95th decile)	2% of HC	NA	NA
Patel et al. (2019)	1.18 $\times$ 10^6	Geisinger Health System patients	NA	NA	13.7	0.15 (definitive FH)	NA	1.52
Trinder et al. (2019)	626	British Columbia FH patients according to DLCN, <55 yr. and myocardial infarction	7.2	28	21.4 (>80th percentile)	43.9 (frameshift novel and no sense in LDLR and APOB mutations; LDLR variants < 1% and pathogenic)	1.97	1.39
Trinder et al. (2020)	48,718	UK biobank	7.2	223	4.9 (>95th percentile)	0.57	1.93	1.29

**Variants drawn were related to LDL-C and myocardial infarction trait.*

***Subjects with lipid profile available from Framingham Heart Study (FHS), Old Order Amish (OOA), Jackson Heart Study (JHS), Multi-Ethnic Study of Atherosclerosis (MESA), FINRISK Study (FIN), and Estonian Biobank (EST).*

In case that PH score study was not available, data referred as PH express the prevalence of primary hypercholesterolemia (LDL-C >95th percentile) non-related to FH. Type of event evaluated were myocardial infarction, ischemic stroke, and death from coronary heart disease. "Definite," "probable," or "possible FH" was defined by the Dutch Lipid Clinic network score (>8, 6–8, 5–7, respectively). PH, polygenic hypercholesterolemia; FH, familial hypercholesterolemia; ASCVD, atherosclerotic cardiovascular disease; SNV, single nucleotide variation; NA, non-available; NS, non-significant.

APOB, apolipoprotein B; APOE, apolipoprotein E; CELSR2, cadherin; CETP, cholesteryl ester transfer protein; EGF LAG 7-pass G-type receptor 2; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDLR, low-density lipoprotein receptor; LIPC, hepatic lipase; LPL, lipoprotein lipase; PCSK9, proprotein convertase subtilisin/kexin type 9.

TABLE 3 | Dutch Lipid Clinic Network score criteria for diagnosis of heterozygous familial hypercholesterolemia: odds ratio of every item for the diagnosis of FH.

	Points	Odds ratio for FH (Civeira et al., 2008b) *N* = 825	Odds ratio for FH, (Benn et al., 2016) Danish General population *N* = 98,098	Odds ratio for FH, (Palacios et al., 2012) Hypercholesterolemic population *N* = 5,430	Odds ratio for FH %(Trinder et al., 2019) HC subjects <55 yr + Myocardial infarction
-First degree relative with known premature coronary heart disease or	1	NA	1.3(0.9–2.0)*	NA	NA
-First degree relative with known LDL-C >95th percentile by age and gender for country	1	NA	5.2(3.8–7.1)*	NA	NA
-First degree relative with tendon xanthoma and/or corneal arcus or	2	7.8	NA	NA	NA
-Child(ren) <18 years with LDL-C > 96th percentile by age and gender for country	2	NA	NA	NA	NA
-Subject has premature coronary heart disease	2	NA	3.2(1.8–5.6)*	NA	NA
-Subject has premature cerebral or peripheral vascular disease	1	NA	0.8(0.3–1.9)*	NA	NA
Tendon Xanthoma	6	3.7	NA	NA	NA
Corneal arcus in a person <45 years	4	2.6	NA	NA	NA
LDL-C> 325 mg/dL	8	NA	138 (60–318)†	NA	NA
LDL-C> 251–325 mg/dL	5	NA	53(35–80)†	NA	NA
LDL-C>191–250 mg/dL	3	NA	53(35–80)†	Na	NA
LDL-C> 155–190 mg/dL	1	NA	25(19–34)†	NA	NA
Definite FH: DLCN >8		NA	24$	53,9$	74.3
Possible FH: DLCN 6–8		NA	6$	30,7$	37.4
Probable FH: DLCN 3–5			1,2$	23,9$	11.8
Unlike FH: DLCN <3			0,07$	16,4$	NA

Table built with data from Civeira et al. (2008b), Palacios et al. (2012), Benn et al. (2016), and Trinder et al. (2019).

LDL-C denotes low density cholesterol, DLCN denotes Dutch Lipid Clinic Network. Premature coronary heart disease was considered <55 years, men; <60 years, women.

**Odds ratios for each criterion are risk of carrying a variant in individuals fulfilling the specific criteria versus those not fulfilling the same criteria used as reference group.*

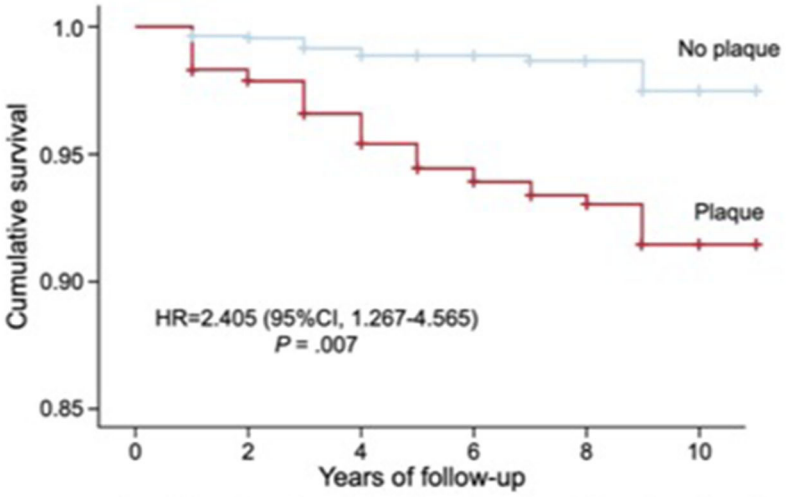
† *Odds ratios in groups by low-density lipoprotein (LDL)-cholesterol levels are risk of carrying a variant in genes causing FH in individuals with an LDL-cholesterol level above the threshold compared those below.*

$ *Percentage of subjects with pathogenic variant causing FH according to DLCN category.*

LDL-C and non-cholesterol sterols (Baila-Rueda et al., 2018), resembling the lipid composition of vascular atheromatous plaques (Vermeer et al., 1982). The presence of TX has been associated with early IHD independently of the genetic variant causing FH (Civeira et al., 2005). However, the prevalence of TX in FH subjects is only about 40% (Civeira et al., 2008b), increasing up to 68% when sonographic evaluation is used for the diagnosis (Junyent et al., 2005). A lower prevalence of TX has been observed in the last decades due to long-term intensive lipid-lowering therapy and the early diagnosis of FH (Benn et al., 2012; Bea et al., 2017; Berberich and Hegele, 2019). Consequently, the absence of TX defines most of the subjects with HC as "probable FH" or "possible FH" (Patel et al., 2019), decreasing the sensitivity of DLCN and Simon Broome scores for the diagnosis of FH (Cuchel et al., 2014; Trinder et al., 2020).

The choice of a high value of LDL-C, above 99th percentile (>250 mg/dl), and normal values of TG is highly sensitive but not too specific for the diagnosis of FH (Civeira and International Panel on Management of Familial Hypercholesterolemia, 2004; Benn et al., 2016; Trinder et al., 2019). In fact, a significant overlap between LDL-C values in FH and PH subjects has been observed (**Figure 3**) (Nordestgaard et al., 2013; Trinder et al., 2020). Besides, most population registers are based on lipid profile and personal history of ASCVD, whereas a familial history of HC or ASCVD is not available (Benn et al., 2016; Khera et al., 2016; Trinder et al., 2020). All these make more difficult to rule out the diagnosis of FH from a clinical perspective.

Despite all of the above, FH cannot be discarded in all cases when TGs are elevated. In 143 subjects with a clinical diagnosis of familial combined hyperlipidemia, 19.6% carried a variant in *LDLR* causing FH. This corresponds to non-diabetic subjects with lower waist circumference and higher LDL-C serum, without a significant difference in TG serum values (Civeira et al., 2008a). In the same vein, a recent study including 49 families collected from 49 probands with severe HC and TG <90th percentile ruled out a monogenic cause; up to 25% of family members with dyslipemia had serum values of TG above 90th percentile. In addition, a polygenic nature of the disease and the influence of environmental factors were observed, which are expressed by the lipid profile variability and the coexistence of different heritage patterns for TC, LDL-C, and TG in the same family (**Table 1**) (Jarauta et al., 2016).



Years of follow-up, no.	0	1	2	3	4	5	6	7	8	9	10
Participants with no plaque, no.	1117	1097	1062	932	785	669	552	413	250	79	3
Cumulative events, no.	-	4	5	9	12	12	12	13	13	16	16
Participants with plaque, no.	661	650	624	562	479	393	334	261	175	42	3
Cumulative events, no.	-	11	14	22	29	34	36	38	39	42	42

FIGURE 3 | Prevalence of subclinical atherosclerosis measured by carotid plaques detected by ultrasound and incidence of ASCVD in a population of 1,771 subjects with primary hypercholesterolemia. Kaplan–Meier cumulative survival curves for patients with and without arteriosclerotic plaque in carotid arteries. 95% CI, 95% confidence interval; HR, hazard ratio adjusted by history of cardiovascular disease, presence of carotid plaque, age, and sex.

IHD disease is the most frequent clinical expression of ASCVD in both FH and PH patients (Benn et al., 2016; Khera et al., 2018; Trinder et al., 2020). Nevertheless, cardiovascular risk may vary within subjects with severe HC. In patients clinically diagnosed as FH followed during the last 30 years, those with "definite FH" according to Simon Broome criteria showed 2.4-fold excess of coronary mortality, whereas the same excess risk was 1.7 in "probable FH." The excess risk continued being higher in subjects with previous IHD and a genetic diagnosis of FH despite the decrease in cardiovascular risk in the general population and the rest of the HC population during the same period of follow-up. Surprisingly, in the same study, the reduction in mortality in women over three decades was less important than the one observed in men despite the same efficacy observed on lowering LDL-C in men and women treated with statins [(Cholesterol Treatment Trialists' (CTT) Collaborators et al., 2008)]. This fact raises the question of whether these "definite FH" women are being treated as rigorously as their male counterparts (Humphries et al., 2018).

Likewise, those subjects with PH present more frequently ASCVD in other locations as cerebrovascular or peripheral arteries and at older ages than FH. This fact has been related to the lower mortality and the higher prevalence of other cardiovascular risk factors observed in PH subjects (Benn et al., 2012; Perez-Calahorra et al., 2019). Other forms of HC out of the scope of this review, such as familial combined hyperlipidemia or hypercholesterolemia secondary to the elevation of lipoprotein (a), increase the risk of ASCVD with regard to the general population (Langsted et al., 2016; Berberich and Hegele, 2019; Luijten et al., 2019).

APPROACH FOR THE SCREENING AND MANAGEMENT OF PRIMARY HYPERCHOLESTEROLEMIA

The clinical significance of PH is as much as FH, since the prevalence of severe HC and polygenic origin of disease is more frequent in the general population (Khera et al., 2018; Berberich and Hegele, 2019; Trinder et al., 2020). Despite the fact that it may lead to misclassification bias for FH, the necessity of an inclusive strategy has been considered. On the basis of LDL-C serum values >90th percentile or >190 mg/dl in the adulthood, it is possible to identify most patients with higher risk for FH and ASCVD (**Figure 4**). The early diagnosis and treatment of severe HC should be considered a key point to prevent ASCVD in these subjects (Nordestgaard et al., 2013; Humphries et al., 2018; Perez-Calahorra et al., 2019). The higher IHD risk in FH population, especially at early ages, makes it necessary to carry out genetic diagnosis by cascade screening in patients clinically identified as "possible or definite FH" by DLCN score (Benn et al., 2012; Palacios et al., 2012; Humphries et al., 2018; Trinder et al., 2019). Nonetheless, a genetic diagnosis of PH is not available. In addition to the difference in the

predictive SNVs depending on the study, its prevalence is not well-correlated to the presence of severe HC in all cases (Lamiquiz-Moneo et al., 2017).

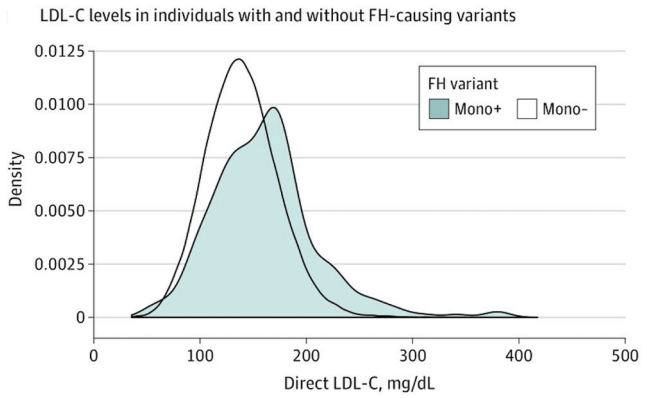


FIGURE 4 | Reproduced with permission from Trinder et al. (2020). Distribution of LDL-C levels (to convert to millimoles per liter, multiply by 0.0259) at enrollment between individuals with an FH-associated variant (mono+) and those without an FH-associated variant (mono–).

The prevalence of subclinical atherosclerosis may be a useful method to detect those HC subjects whose ASCVD risk may be increased. However, only a few studies comparing subclinical atherosclerosis in FH and HC subjects are available so far. The Coronary Artery Calcium Score was a discriminative measurement of subclinical atherosclerosis for FH, showing higher atherosclerotic burden for FH than other HC patients (Sharifi et al., 2017). In a recent study, after a follow-up of 3.7 years, a 3.3-fold-higher risk of ASCVD for FH was observed when the calcium Agastont score was >100 with respect to those with a calcium Agastont score = 0 (Miname et al., 2019). Besides, the detection of atherosclerotic plaques in the carotid arteries by ultrasound increased 2.4-fold the risk of ASCVD in 1,778 subjects with HC, after a follow-up of 6 years (Bea et al., 2017) (**Figure 5**).

There is neither a case–control study to compare the effects of lipid-lowering drugs vs. placebo for the treatment of FH subjects nor any specific risk equations for ASCVD in this population (Nordestgaard et al., 2013). However, statins of moderate–high intensity, ezetimibe, and *PCSK9* monoclonal antibodies have shown a drop in the incidence of IHD and mortality in subjects with FH or severe HC (Versmissen et al., 2008; Karatasakis et al., 2017; Giugliano et al., 2018).

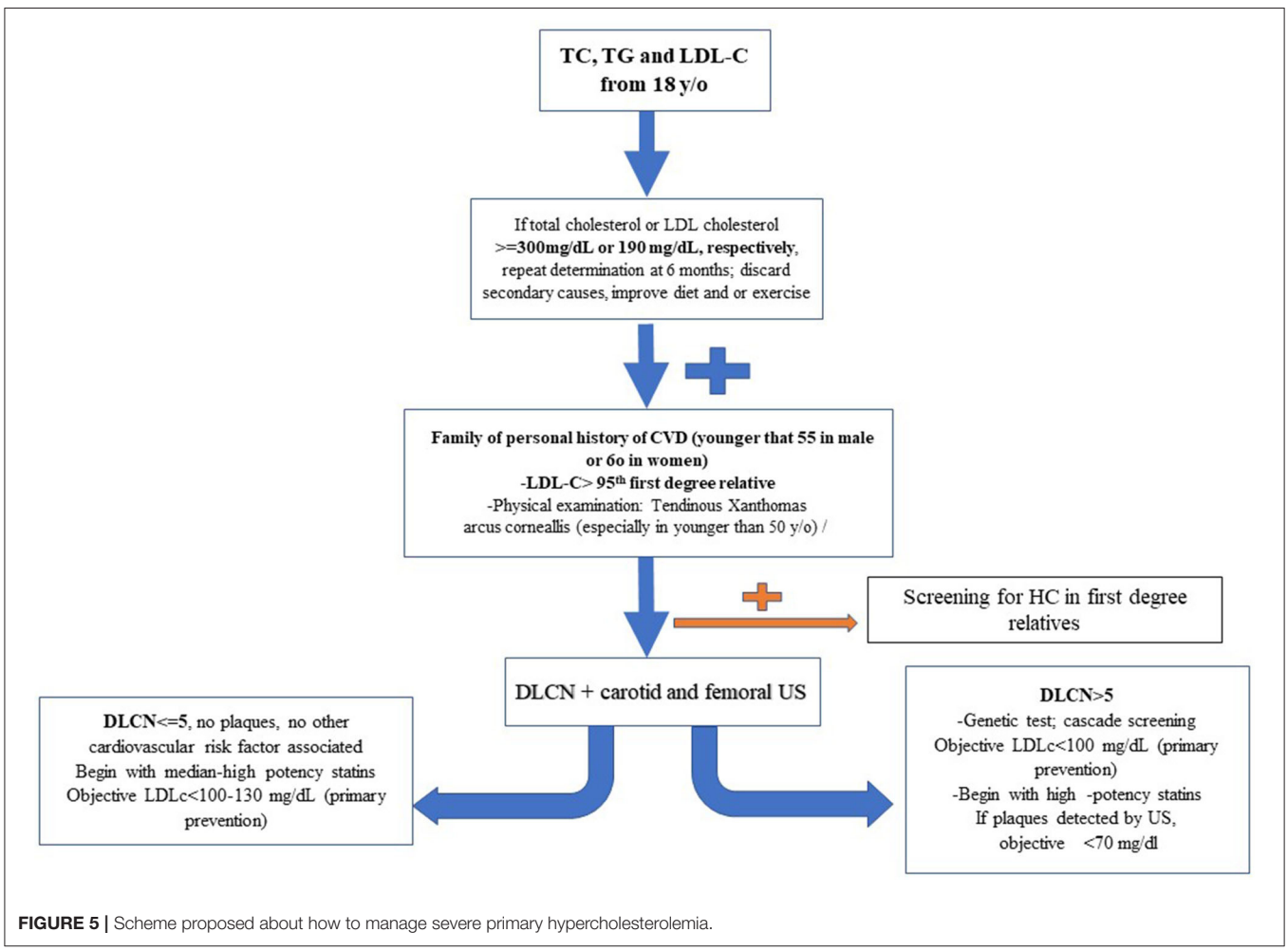


FIGURE 5 | Scheme proposed about how to manage severe primary hypercholesterolemia.

Clinical management of PH and FH in primary prevention does not differ from each other, since, until recently, the diagnosis of severe HC has been based on lipid profile and history of IHD. Consequently, there are few data for FH and PH separately. Nonetheless, a 10 times lower odds for CVD has been observed in HC subjects exposed to continuous lipid-lowering treatment than in naive patients (Perez-Calahorra et al., 2019). In another large study including patients with "definite FH" diagnosis previously treated with statins and non-personal history of IHD, cardiovascular risk was not greater than the general population after 20 years of follow-up. It underlines the clinical utility of identifying subjects with FH before they have developed IHD and ensuring they receive intensive lipid-lowering therapy (Humphries et al., 2018). In FH children from 10 years old and above, early treatment with moderate or high intensity statins has reported a reduction in LDL-C between 28 and 54% with no associated secondary effects (Ramaswami et al., 2020). The addition of ezetimibe to statins for the treatment of HC has been shown equally effective in decreasing LDL-C in FH children and adults (Kastelein et al., 2008; Kusters et al., 2015).

Moreover, a different response to high-intensity statins between FH and PH subjects has been observed. A higher response to statins was observed in subjects with no genetic diagnosis of FH than those with defective or null alleles, being the last ones whom the objective of LDL-C <100 mg/dl is less frequently achieved (47.4, 27.1, and 47.4%, respectively, $p = 0.02$) (Santos et al., 2014). More recently, in a study of treatment with high-intensity statins carried out in FH subjects, a significant reduction in LDL-C was observed in the p.(Leu167del) carriers on *APOE* (−52.1%) with respect to *LDLR* carriers (−39.7%) ($p = 0.040$) (Bea et al., 2019).

The decrease of LDL-C <100 mg/dl or at least 50% of the basal LDL-C concentration has been advocated by observational studies as the objective for primary prevention in populations with severe HC (Nordestgaard et al., 2013; Mach et al., 2020). However, this target is not attained for the greatest proportion of HC and FH populations (Perez-Calahorra et al., 2019; Pérez de Isla et al., 2019). Furthermore, <50% of these subjects, particularly young ones, are in chronic treatment with a high-intensity statin (Benn et al., 2012; Bucholz et al., 2018; Kotseva et al., 2019; Patel et al., 2019). The addition of other cardiovascular risk factors may modulate the intensity of the treatment in the rest of subjects with moderate HC (Mach et al., 2020). There is neither a minimum level of LDL-C below which benefit has not been observed nor has been observed a higher incidence of secondary effects associated with lower concentrations of LDL-C. PCSK9 inhibitors, the most potent lipid-lowering treatment available so far, reach an extra reduction of 50–60% on LDL-C with regard to conventional treatment. It has been proben to be effective to get LDL-C objectives in more than 50% of FH subjects (Kastelein et al., 2015; Santos et al., 2020), reducing the risk of cardiovascular events proportionally in the same grade than statins (Karatasakis et al., 2017; Giugliano et al., 2018) (**Figure 1**). Besides, PCSK9 inhibitors have proved to regress atherosclerotic plaques in coronary arteries with respect to conventional treatment in subjects with LDL-C ≤100 mg/dl (Nicholls et al., 2016; Bea et al., 2017). Nowadays, due to the high price of PSCK9 inhibitors, its use has only been established in FH subjects with a very high risk or personal history of ASCVD (Ascaso et al., 2019). Recent data suggest that only 23% of FH subjects in treatment with moderate- to high-intensity statins with or without ezetimibe reach LDL-C <100 mg/dl, whereas only 12% of those with a personal history of ASCVD had LDL-C <70 mg/dl. Nevertheless, it is estimated that only 17% of FH subjects may be eligible for treatment with PCSK9 inhibitors, according to current European guidelines (Masana et al., 2017).

DISCUSSION

In the last years, high-quality researches have been developed trying to find out new genes causing primary HC. However, no new genes other than *PCSK9* and *APOE* have been added to the list of those causing FH, accounting only for a small proportion of these subjects (Nordestgaard et al., 2013; Ghaleb et al., 2018). At the same time, a deeper knowledge about genetics related to lipid metabolism has allowed to detect the effect of small nucleotide variants related to LDL-C metabolism as a new pathogenic mechanism causing primary HC (Willer et al., 2013; Khera et al., 2016; Berberich and Hegele, 2019 (**Figure 2**). Despite the description of a polygenic trait (Willer et al., 2013), its effect has not been replicated homogeneously in a significant proportion of subjects with severe HC, and a consensus with respect to the diagnosis of PH has not been released so far.

There has been observed a higher cardiovascular risk in FH with respect to PH subjects, especially at early ages (Khera et al., 2016; Trinder et al., 2019). Moreover, the increased cardiovascular risk associated with PH is mostly observed in adulthood, when other cardiovascular risk factors occur in the same subject, underlining the effect of the environment or behavior on the phenotypic expression of genetic traits (Humphries et al., 2018; Perez-Calahorra et al., 2019; Trinder et al., 2020).

Despite the above facts, a large proportion of subjects with FH remains underdiagnosed and undertreated. New strategies are required to improve the diagnosis and treatment of HC subjects: cascade screening on first- and second-degree relatives of an FH proband or a more inclusive one by LDL-C measurement of first-degree relatives from any HC proband. Early diagnosis and treatment with moderate- to high-intensity statins result in the best approach to primary prevention disease in HC subjects, independently of the genetic background. Indeed, the use of high-intensity statins for 12 years allowed to avoid 90% of ASCVD in a large FH population (Perez-Calahorra et al., 2019). Only a small proportion of HC subjects as those with familial history of ASCVD or more than one risk factor associated may require a more aggressive treatment for primary prevention of ASCVD (Ascaso et al., 2019; Mach et al., 2020).

CONCLUSION

Severe HC is a clinical condition that constitutes an important public health issue. Besides the genetic diagnosis of FH, new

genetic methods have allowed to detect the additive effect of several variants in a unique nucleotide as a cause of PH. Nonetheless, a unique genetic criterion for the diagnosis of PH has not been established so far. Both FH and PH are associated with increased risk of ASCVD. The early diagnosis of severe HC in addition to a familial history of dyslipidemia and/or premature ASCVD is the starting point to detect those forms of HC more related to a genetic defect and with increased risk of ASCVD. Early treatment with high-intensity statins is mandatory for primary prevention of ASCVD in all subjects with severe HC.

AUTHOR CONTRIBUTIONS

EJ drafted the manuscript. IL-M, VM-B, and AB-S revised the manuscript. EJ, AB-S, and IL-M contributed to the acquisition and interpretation of data. EJ designed the work. All authors contributed to the manuscript revision, read, and approved the submitted version.

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REFERENCES

Akioyamen, L. E., Genest, J., Shan, S. D., Reel, R. L., Albaum, J. M., Chu, A., et al. (2017). Estimating the prevalence of heterozygous familial hypercholesterolaemia: a systematic review and meta-analysis. *BMJ Open* 7:e016461. doi: 10.1136/bmjopen-2017-016461

Ascaso, J. F., Civeira, F., Guijarro, C., López Miranda, J., Masana, L., Mostaza, J. M., et al. (2019). Indications of PCSK9 inhibitors in clinical practice. Recommendations of the Spanish Sociey of Arteriosclerosis (SEA), 2019. *Clin. Investig. Arterioscler.* 31, 128–139. doi: 10.1016/j.arteri.2019.04.002

Awan, Z., Choi, H. Y., Stitziel, N., Ruel, I., Bamimore, M. A., Husa, R., et al. (2013). APOE p.Leu167del mutation in familial hypercholesterolemia. *Atherosclerosis* 231, 218–222. doi: 10.1016/j.atherosclerosis.2013.09.007

Baila-Rueda, L., Lamiquiz-Moneo, I., Jarauta, E., Mateo-Gallego, R., Perez-Calahorra, S., Marco-Benedí, V., et al. (2018). Association between non-cholesterol sterol concentrations and Achilles tendon thickness in patients with genetic familial hypercholesterolemia. *J. Transl. Med.* 16:6. doi: 10.1186/s12967-018-1380-3

Baila-Rueda, L., Pérez-Ruiz, M. R., Jarauta, E., Tejedor, M. T., Mateo-Gallego, R., Lamiquiz-Moneo, I., et al. (2016). Cosegregation of serum cholesterol with cholesterol intestinal absorption markers in families with primary hypercholesterolemia without mutations in LDLR, APOB, PCSK9 and APOE genes. *Atheroscler.* 246, 202–207. doi: 10.1016/j.atherosclerosis.2016.01.005

Bastida, J. M., Girós, M. L., Benito, R., Janusz, K., Hernández-Rivas, J. M., and González-Porras, J. R. (2019). Sitosterolemia: diagnosis, metabolic and hematological abnormalities, cardiovascular disease and management. *Curr. Med. Chem.* 26, 6766–6775. doi: 10.2174/0929867325666180705145900

Bea, A. M., Civeira, F., Jarauta, E., Lamiquiz-Moneo, I., Pérez-Calahorra, S., Marco-Benedí, V., et al. (2017). Association between the presence of carotid artery plaque and cardiovascular events in patients with genetic hypercholesterolemia. *Rev. Esp. Cardiol.* 70, 551–558. doi: 10.1016/j.recesp.2016.10.018

Bea, A. M., Lamiquiz-Moneo, I., Marco-Benedí, V., Mateo-Gallego, R., Pérez-Calahorra, S., Jarauta, E., et al. (2019). Lipid-lowering response in subjects with the p.(Leu167del) mutation in the APOE gene. *Atherosclerosis* 282, 143–147. doi: 10.1016/j.atherosclerosis.2019.01.024

Benn, M., Watts, G. F., Tybjærg-Hansen, A., and Nordestgaard, B. G. (2016). Mutations causative of familial hypercholesterolaemia: screening of 98 098 individuals from the Copenhagen General Population Study estimated a prevalence of 1 in 217. *Eur. Heart J.* 37, 1384–1394. doi: 10.1093/eurheartj/ehw028

Benn, M., Watts, G. F., Tybjaerg-Hansen, A., and Nordestgaard, B. G. (2012). Familial hypercholesterolemia in the danish general population: prevalence, coronary artery disease, and cholesterol-lowering medication. *J. Clin. Endocrinol. Metab.* 97, 3956–3964. doi: 10.1210/jc.2012-1563

Berberich, A. J., and Hegele, R. A. (2019). The complex molecular genetics of familial hypercholesterolaemia. *Nat. Rev. Cardiol.* 16, 9–20. doi: 10.1038/s41569-018-0052-6

Besseling, J., Kindt, I., Hof, M., Kastelein, J. J. P., Hutten, B. A., and Hovingh, G. K. (2014). Severe heterozygous familial hypercholesterolemia and risk for cardiovascular disease: a study of a cohort of 14,000 mutation carriers. *Atheroscler.* 233, 219–223. doi: 10.1016/j.atherosclerosis.2013.12.020

Borén, J., Chapman, M. J., Krauss, R. M., Packard, C. J., Bentzon, J. F., Binder, C. J., et al. (2020). Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.* 41:ehz962. doi: 10.1093/eurheartj/ehz962

Bucholz, E. M., Rodday, A. M., Kolor, K., Khoury, M. J., and de Ferranti, S. D. (2018). Prevalence and predictors of cholesterol screening, awareness, and statin treatment among us adults with familial hypercholesterolemia or other forms of severe dyslipidemia (1999–2014). *Circ.* 137, 2218–2230. doi: 10.1161/CIRCULATIONAHA.117.032321

Cenarro, A., Etxebarria, A., de Castro-Orós, I., Stef, M., Bea, A. M., Palacios, L., et al. (2016). The p.Leu167del mutation in APOE gene causes autosomal dominant hypercholesterolemia by down-regulation of LDL receptor expression in hepatocytes. *J. Clin. Endocrinol. Metab.* 101, 2113–2121. doi: 10.1210/jc.2015-3874

Cholesterol Treatment Trialists' (CTT) Collaborators, Kearney, P. M., Blackwell, L., Collins, R., Keech, A., Simes, J., et al. (2008). Efficacy of cholesterol-lowering therapy in 18,686 people with diabetes in 14 randomised trials of statins: a meta-analysis. *Lancet* 371, 117–125. doi: 10.1016/S0140-6736(08)60104-X

Chora, J. R., Medeiros, A. M., Alves, A. C., and Bourbon, M. (2018). Analysis of publicly available LDLR, APOB, and PCSK9 variants associated with familial hypercholesterolemia: application of ACMG guidelines and implications for familial hypercholesterolemia diagnosis. *Genet. Med.* 20, 591–598. doi: 10.1038/gim.2017.151

Civeira, F., Castillo, S., Alonso, R., Meriño-Ibarra, E., Cenarro, A., Artied, M., et al. (2005). Tendon xanthomas in familial hypercholesterolemia are associated with cardiovascular risk independently of the low-density lipoprotein receptor gene mutation. *Arterioscler. Thromb. Vasc. Biol.* 25, 1960–1965. doi: 10.1161/01.ATV.0000177811.14176.2b

Civeira, F., and International Panel on Management of Familial Hypercholesterolemia (2004). Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia. *Atherosclerosis* 173, 55–68. doi: 10.1016/j.atherosclerosis.2003.11.010

Civeira, F., Jarauta, E., Cenarro, A., García-Otín, A. L., Tejedor, D., Zambón, D., et al. (2008a). Frequency of low-density lipoprotein receptor gene

mutations in patients with a clinical diagnosis of familial combined hyperlipidemia in a clinical setting. *J. Am. Coll. Cardiol.* 52, 1546–1553. doi: 10.1016/j.jacc.2008.06.050

Civeira, F., Ros, E., Jarauta, E., Plana, N., Zambon, D., Puzo, J., et al. (2008b). Comparison of genetic versus clinical diagnosis in familial hypercholesterolemia. *Am. J. Cardiol.* 102, 1187–1193. doi: 10.1016/j.amjcard.2008.06.056

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014). Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. *Eur. Heart J.* 35, 2146–2157. doi: 10.1093/eurheartj/ehu274

Ference, B. A. (2015). Mendelian randomization studies: using naturally randomized genetic data to fill evidence gaps. *Curr. Opin. Lipidol.* 26, 566–571. doi: 10.1097/MOL.0000000000000247

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., et al. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.* 38, 2459–2472. doi: 10.1093/eurheartj/ehx144

Ference, B. A., Yoo, W., Alesh, I., Mahajan, N., Mirowska, K. K., Mewada, A., et al. (2012). Effect of long-term exposure to lower low-density lipoprotein cholesterol beginning early in life on the risk of coronary heart disease. *J. Am. Coll. Cardiol.* 60, 2631–2639. doi: 10.1016/j.jacc.2012.09.017

Forbes, C., Quek, R. G. W., Deshpande, S., Worthy, G., Ross, J., Kleijnen, J., et al. (2016). Relationship between changes in coronary atherosclerotic plaque burden measured by intravascular ultrasound and cardiovascular disease outcomes: a systematic literature review. *Curr. Med. Res. Opin.* 32, 1143–1150. doi: 10.1185/03007995.2016.1162775

Fouchier, S. W., Dallinga-Thie, G. M., Meijers, J. C. M., Zelcer, N., Kastelein, J. J. P., Defesche, J. C., et al. (2014). Mutations in STAP1 are associated with autosomal dominant hypercholesterolemia. *Circ. Res.* 115, 552–555. doi: 10.1161/CIRCRESAHA.115.304660

Futema, M., Shah, S., Cooper, J. A., Li, K., Whittall, R. A., Sharifi, M., et al. (2015). Refinement of variant selection for the LDL cholesterol genetic risk score in the diagnosis of the polygenic form of clinical familial hypercholesterolemia and replication in samples from 6 countries. *Clin. Chem.* 61, 231–238. doi: 10.1373/clinchem.2014.231365

Ghaleb, Y., Elbitar, S., El Khoury, P., Bruckert, E., Carreau, V., Carrié, A., et al. (2018). Usefulness of the genetic risk score to identify phenocopies in families with familial hypercholesterolemia? *Eur. J. Hum. Genet.* 26, 570–578. doi: 10.1038/s41431-017-0078-y

Giugliano, R. P., Cannon, C. P., Blazing, M. A., Nicolau, J. C., and Corbalán, R., Špinar, J., et al. (2018). Benefit of adding ezetimibe to statin therapy on cardiovascular outcomes and safety in patients with versus without diabetes mellitus: results from IMPROVE-IT (Improved Reduction of Outcomes: Vytorin Efficacy International Trial). *Circulation* 137, 1571–1582. doi: 10.1161/CIRCULATIONAHA.117.030950

Goldstein, J. L., and Brown, M. S. (1974). Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* 249, 5153–5162.

Hegele, R. A., Knowles, J. W., and Horton, J. D. (2020). Delisting *STAP1*: the rise and fall of a putative hypercholesterolemia gene. *ATVB* 40, 847–849. doi: 10.1161/ATVBAHA.120.314006

Holmes, M. V., Asselbergs, F. W., Palmer, T. M., Drenos, F., Lanktree, M. B., Nelson, C. P., et al. (2015). Mendelian randomization of blood lipids for coronary heart disease. *Eur. Heart J.* 36, 539–550. doi: 10.1093/eurheartj/eht571

Humphries, S. E., Cooper, J. A., Seed, M., Capps, N., Durrington, P. N., Jones, B., et al. (2018). Coronary heart disease mortality in treated familial hypercholesterolaemia: Update of the UK Simon Broome FH register. *Atherosclerosis* 274, 41–46. doi: 10.1016/j.atherosclerosis.2018.04.040

Jarauta, E., Pérez-Ruiz, M. R., Pérez-Calahorra, S., Mateo-Gallego, R., Cenarro, A., Cofán, M., et al. (2016). Lipid phenotype and heritage pattern in families with genetic hypercholesterolemia not related to LDLR, APOB, PCSK9, or APOE. *J. Clin. Lipidol.* 10, 1397–1405.e2. doi: 10.1016/j.jacl.2016.09.011

Junyent, M., Gilabert, R., Zambón, D., Núñez, I., Vela, M., Civeira, F., et al. (2005). The use of Achilles tendon sonography to distinguish familial hypercholesterolemia from other genetic dyslipidemias. *Arterioscler. Thromb. Vasc. Biol.* 25, 2203–2208. doi: 10.1161/01.ATV.0000183888.48105.d1

Karatasakis, A., Danek, B. A., Karacsonyi, J., Rangan, B. V., Roesle, M. K., Knickelbine, T., et al. (2017). Effect of PCSK9 inhibitors on clinical outcomes in patients with hypercholesterolemia: a meta-analysis of 35 randomized controlled trials. *J Am Heart Assoc.* 6:e006910. doi: 10.1161/JAHA.117.006910

Kastelein, J. J. P., Akdim, F., Stroes, E. S. G., Zwinderman, A. H., Bots, M. L., Stalenhoef, A. F. H., et al. (2008). Simvastatin with or without ezetimibe in familial hypercholesterolemia. *N. Engl. J. Med.* 358, 1431–1443. doi: 10.1056/NEJMoa0800742

Kastelein, J. J. P., Ginsberg, H. N., Langslet, G., Hovingh, G. K., Ceska, R., Dufour, R., et al. (2015). ODYSSEY FH I and FH II: 78 week results with alirocumab treatment in 735 patients with heterozygous familial hypercholesterolaemia. *Eur. Heart J.* 36, 2996–3003. doi: 10.1093/eurheartj/ehv370

Kathiresan, S., Melander, O., Anevski, D., Guiducci, C., Burtt, N. P., Roos, C., et al. (2008). Polymorphisms associated with cholesterol and risk of cardiovascular events. *N. Engl. J. Med.* 358, 1240–1249. doi: 10.1056/NEJMoa0706728

Khera, A. V., Chaffin, M., Zekavat, S. M., Collins, R. L., Roselli, C., Natarajan, P., et al. (2018). Whole genome sequencing to characterize monogenic and polygenic contributions in patients hospitalized with early-onset myocardial infarction. *Circulation* 139, 1593–1602. doi: 10.1161/CIRCULATIONAHA.118.035658

Khera, A. V., Won, H.-H., Peloso, G. M., Lawson, K. S., Bartz, T. M., Deng, X., et al. (2016). Diagnostic yield and clinical utility of sequencing familial hypercholesterolemia genes in patients with severe hypercholesterolemia. *J. Am. Coll. Cardiol.* 67, 2578–2589. doi: 10.1016/j.jacc.2016.03.520

Kotseva, K., De Backer, G., De Bacquer, D., Rydén, L., Hoes, A., Grobbee, D., et al. (2019). Lifestyle and impact on cardiovascular risk factor control in coronary patients across 27 countries: Results from the European Society of Cardiology ESC-EORP EUROASPIRE V registry. *Eur. J. Prev. Cardiol.* 26, 824–835. doi: 10.1177/2047487318825350

Kusters, D. M., Caceres, M., Coll, M., Cuffie, C., Gagné, C., Jacobson, M. S., et al. (2015). Efficacy and safety of ezetimibe monotherapy in children with heterozygous familial or nonfamilial hypercholesterolemia. *J. Pediatr.* 166, 1377–1384.e1–3. doi: 10.1016/j.jpeds.2015.02.043

Kwon, D., Yi, J.-J., Ohrr, H., and Yi, S.-W. (2019). Total cholesterol and mortality from ischemic heart disease and overall cardiovascular disease in Korean adults. *Medicine* 98:e17013. doi: 10.1097/MD.0000000000017013

Lamiquiz-Moneo, I., Pérez-Ruiz, M. R., Jarauta, E., Tejedor, M. T., Bea, A. M., Mateo-Gallego, R., et al. (2017). Single nucleotide variants associated with polygenic hypercholesterolemia in families diagnosed clinically with familial hypercholesterolemia. *Rev. Esp. Cardiol.* 71, 351–356. doi: 10.1016/j.rec.2017.07.010

Lamiquiz-Moneo, I., Restrepo-Córdoba, M. A., Mateo-Gallego, R., Bea, A. M., Del Pino Alberiche-Ruano, M., García-Pavía, P., et al. (2020). Predicted pathogenic mutations in STAP1 are not associated with clinically defined familial hypercholesterolemia. *Atherosclerosis* 292, 143–151. doi: 10.1016/j.atherosclerosis.2019.11.025

Langsted, A., Kamstrup, P. R., Benn, M., Tybjærg-Hansen, A., and Nordestgaard, B. G. (2016). High lipoprotein(a) as a possible cause of clinical familial hypercholesterolaemia: a prospective cohort study. *Lancet Diabetes Endocrinol.* 4, 577–587. doi: 10.1016/S2213-8587(16)30042-0

Luijten, J., van Greevenbroek, M. M. J., Schaper, N. C., Meex, S. J. R., van der Steen, C., Meijer, L. J., et al. (2019). Incidence of cardiovascular disease in familial combined hyperlipidemia: a 15-year follow-up study. *Atherosclerosis* 280, 1–6. doi: 10.1016/j.atherosclerosis.2018.11.013

Mach, F., Baigent, C., Catapano, A. L., Koskinas, K. C., Casula, M., Badimon, L., et al. (2020). 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur. Heart J.* 41, 111–188. doi: 10.1093/eurheartj/ehz455

Maihofer, A. X., Shadyab, A. H., Wild, R. A., and LaCroix, A. Z. (2020). Associations between serum levels of cholesterol and survival to age 90 in postmenopausal women. *J. Am. Geriatr. Soc.* 68, 288–296. doi: 10.1111/jgs.16306

Masana, L., Plana, N., Pérez-Calahorra, S., Ibarretxe, D., Lamiquiz-Moneo, I., Pedro-Botet, J., et al. (2017). How many familial hypercholesterolemia

patients are eligible for PCSK9 inhibition? *Atheroscler.* 262, 107–112. doi: 10.1016/j.atherosclerosis.2017.05.013

Miname, M. H., Bittencourt, M. S., Moraes, S. R., Alves, R. I. M., Silva, P. R. S., Jannes, C. E., et al. (2019). Coronary artery calcium and cardiovascular events in patients with familial hypercholesterolemia receiving standard lipid-lowering therapy. *JACC Cardiovasc. Imaging* 12, 1797–1804. doi: 10.1016/j.jcmg.2018.09.019

Mortensen, M. B., Kulenovic, I., Klausen, I. C., and Falk, E. (2016). Familial hypercholesterolemia among unselected contemporary patients presenting with first myocardial infarction: prevalence, risk factor burden, and impact on age at presentation. *J Clin Lipidol.* 10, 1145–1152.e1. doi: 10.1016/j.jacl.2016.06.002

Nagasawa, S., Okamura, T., Iso, H., Tamakoshi, A., Yamada, M., Watanabe, M., et al. (2012). Relation between serum total cholesterol level and cardiovascular disease stratified by sex and age group: a pooled analysis of 65 594 individuals from 10 cohort studies in Japan. *J. Am. Heart Assoc.* 1:e001974. doi: 10.1161/JAHA.112.001974

Natarajan, P., Peloso, G. M., Zekavat, S. M., Montasser, M., Ganna, A., Chaffin, M., et al. (2018). Deep-coverage whole genome sequences and blood lipids among 16,324 individuals. *Nat. Commun.* 9:3391. doi: 10.1038/s41467-018-05747-8

Nicholls, S. J., Puri, R., Anderson, T., Ballantyne, C. M., Cho, L., Kastelein, J. J. P., et al. (2016). Effect of evolocumab on progression of coronary disease in statin-treated patients: the glagov randomized clinical trial. *JAMA* 316, 2373–2384. doi: 10.1001/jama.2016.16951

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur. Heart J.* 34, 3478–3490a. doi: 10.1093/eurheartj/eht273

Ose, L. (2002). Müller-Harbitz' sykdom–familiaer hyperkolesterolemi [Müller-Harbitz disease–familial hypercholesterolemia]. *Tidsskr. Nor. Laegeforen.* 122, 924–925.

Palacios, L., Grandoso, L., Cuevas, N., Olano-Martín, E., Martinez, A., Tejedor, D., et al. (2012). Molecular characterization of familial hypercholesterolemia in Spain. *Atherosclerosis* 221, 137–142. doi: 10.1016/j.atherosclerosis.2011.12.021

Patel, P., Hu, Y., Kolinovsky, A., Geng, Z., Ruhl, J., Krishnamurthy, S., et al. (2019). Hidden burden of electronic health record-identified familial hypercholesterolemia: clinical outcomes and cost of medical care. *J. Am. Heart Assoc.* 8:e011822. doi: 10.1161/JAHA.118.011822

Paththinige, C. S., Sirisena, N. D., and Dissanayake, V. (2017). Genetic determinants of inherited susceptibility to hypercholesterolemia - a comprehensive literature review. *Lipids Health Dis.* 16:103. doi: 10.1186/s12944-017-0488-4

Pérez de Isla, L., Arroyo-Olivares, R., Muñiz-Grijalvo, O., Diaz-Díaz, J. L., Zamb?n, D., Fuentes, F., et al. (2019). Long-term effect of 2 intensive statin regimens on treatment and incidence of cardiovascular events in familial hypercholesterolemia: The SAFEHEART study. *J. Clin. Lipidol.* 13, 989–996. doi: 10.1016/j.jacl.2019.10.005

Perez-Calahorra, S., Laclaustra, M., Marco-Bened,í, V., Lamiquiz-Moneo, I., Pedro-Botet, J., Plana, N., et al. (2019). Effect of lipid-lowering treatment in cardiovascular disease prevalence in familial hypercholesterolemia. *Atherosclerosis* 284, 245–252. doi: 10.1016/j.atherosclerosis.2019.02.003

Ramaswami, U., Futema, M., Bogsrud, M. P., Holven, K. B., Roeters van Lennep, J., Wiegman, A., et al. (2020). Comparison of the characteristics at diagnosis and treatment of children with heterozygous familial hypercholesterolaemia (FH) from eight European countries. *Atherosclerosis* 292, 178–187. doi: 10.1016/j.atherosclerosis.2019.11.012

Representatives of the Global Familial Hypercholesterolemia Community, Wilemon, K. A., Patel, J., Aguilar-Salinas, C., Ahmed, C. D., Alkhnifsawi, M., et al. (2020). Reducing the clinical and public health burden of familial hypercholesterolemia: a global call to action. *JAMA Cardiol.* 5:217. doi: 10.1001/jamacardio.2019.5173

Ross, R. (1999). Atherosclerosis–an inflammatory disease. *N. Engl. J. Med.* 340, 115–126. doi: 10.1056/NEJM199901143400207

Santos, P. C. J. L., Morgan, A. C., Jannes, C. E., Turolla, L., Krieger, J. E., Santos, R. D., et al. (2014). Presence and type of low density lipoprotein receptor (LDLR) mutation influences the lipid profile and response to lipid-lowering therapy in Brazilian patients with heterozygous familial hypercholesterolemia. *Atherosclerosis* 233, 206–210. doi: 10.1016/j.atherosclerosis.2013.12.028

Santos, R. D., Stein, E. A., Hovingh, G. K., Blom, D. J., Soran, H., Watts, G. F., et al. (2020). Long-term evolocumab in patients with familial hypercholesterolemia. *J. Am. Coll. Cardiol.* 75, 565–574. doi: 10.1016/j.jacc.2019.12.020

Sharifi, M., Higginson, E., Bos, S., Gallivan, A., Harvey, D., Li, K. W., et al. (2017). Greater preclinical atherosclerosis in treated monogenic familial hypercholesterolemia vs. polygenic hypercholesterolemia. *Atherosclerosis* 263, 405–411. doi: 10.1016/j.atherosclerosis.2017.05.015

Stamler, J., Wentworth, D., and Neaton, J. D. (1986). Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). *JAMA* 256, 2823–2828.

Talmud, P. J., Shah, S., Whittall, R., Futema, M., Howard, P., Cooper, J. A., et al. (2013). Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. *Lancet* 381, 1293–1301. doi: 10.1016/S0140-6736(12)62127-8

Teslovich, T. M., Musunuru, K., Smith, A. V., Edmondson, A. C., Stylianou, I. M., Koseki, M., et al. (2010). Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 466, 707–713. doi: 10.1038/nature09270

Trinder, M., Francis, G. A., and Brunham, L. R. (2020). Association of monogenic vs polygenic hypercholesterolemia with risk of atherosclerotic cardiovascular disease. *JAMA Cardiol.* 5, 390–399. doi: 10.1001/jamacardio.2019.5954

Trinder, M., Li, X., DeCastro, M. L., Cermakova, L., Sadananda, S., Jackson, L. M., et al. (2019). Risk of premature atherosclerotic disease in patients with monogenic versus polygenic familial hypercholesterolemia. *J. Am. Coll. Cardiol.* 74, 512–522. doi: 10.1016/j.jacc.2019.05.043

Vermeer, B. J., Mateysen, A. A., van Gent, C. M., van Sabben, R. M., and Emeis, J. J. (1982). The lipid composition and localization of free and esterified cholesterol in different types of xanthomas. *J. Invest. Dermatol.* 78, 305–308. doi: 10.1111/1523-1747.ep12507376

Versmissen, J., Oosterveer, D. M., Yazdanpanah, M., Defesche, J. C., Basart, D. C. G., Liem, A. H., et al. (2008). Efficacy of statins in familial hypercholesterolaemia: a long term cohort study. *BMJ* 337, a2423–a2423. doi: 10.1136/bmj.a2423

Wang, N., Fulcher, J., Abeysuriya, N., Park, L., Kumar, S., Di Tanna, G. L., et al. (2020). Intensive LDL cholesterol-lowering treatment beyond current recommendations for the prevention of major vascular events: a systematic review and meta-analysis of randomised trials including 327 037 participants. *Lancet Diabetes Endocrinol.* 8, 36–49. doi: 10.1016/S2213-8587(19)30388-2

Willer, C. J., Schmidt, E. M., Sengupta, S., Peloso, G. M., Gustafsson, S., Kanoni, S., et al. (2013). Discovery and refinement of loci associated with lipid levels. *Nat. Genet.* 45, 1274–1283. doi: 10.1038/ng.2797

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REVIEW
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Familial Hypercholesterolemia in Russia: Three Decades of Genetic Studies

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The first studies of familial hypercholesterolemia (FH) in Russia go back to late 1980-ies. For more than 10 years the research in this field was carried out in Saint-Petersburg, the megapolis in the North-West Russia. Studies were focused on the search for causative mutations in low-density lipoprotein receptor gene (*LDLR*). Gradually the research was spread to Petrozavodsk in Karelia and in the XXI century two more centers contributed in investigations of genetics of FH, i.e., in Moscow and Novosibirsk. The best studied is the spectrum of mutations in *LDLR*, though genetic abnormalities in *APOB* and *PCSK9* genes were also considered. Despite that some 40% mutations in *LDLR* found in Saint-Petersburg and Moscow are referred to as specific for Russian population, and this proportion is even higher in Karelia (ca. 70%), rapid introduction of NGS and intensifying genetic research all over the world result in continuous decrease of these numbers as "Slavic" mutations become documented in other countries. The samplings of genetically characterized patients in Russia were relatively small, which makes difficult to specify major mutations reflecting the national specificity of FH. Moreover, the majority of studies accomplished so far did not explore possible associations of certain mutations with ethnic origin of patients. By now the only exception is the study of Karelian population showing the absence of typical Finnish mutations in the region that borders on Finland. It can be concluded that the important primary research partly characterizing the mutation spectrum in FH patients both in the European and Siberian parts of Russia has been done. However, it seems likely that the most interesting and comprehensive genetic studies of FH in Russia, concerning various mutations in different genes and the variety of ethnic groups in this multi-national country, are still to be undertaken.

Keywords: familial hypercholesterolemia, mutation spectrum, genetic studies in Russia, PCSK9 gene, low-density lipoprotein receptor gene, APOB gene

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INTRODUCTION

At present the term "familial hypercholesterolemia" (FH) has been used to describe different genetic conditions. Traditionally FH is used to designate the monogenic forms of the disease (Defesche et al., 2017), others have proposed also to include polygenic disorders causing elevated plasma cholesterol (Masana et al., 2019). Any application of the term implies the specific elevation of

blood plasma low-density lipoprotein (LDL), which increases the risk of cardiovascular incidents (Sarraju and Knowles, 2019).

For example, recent paper has categorized FH into four types (Masana et al., 2019): (1) monogenic heterozygous FH, where patients carry functionally significant mutations in a single allele of the *LDLR* (GeneID: 3949, GenBank NM_000527.4), *APOB* (GeneID: 338, GenBank NM_000384.3), or *PCSK9* (Gene ID: 255738, GenBank NM_174936.4) genes; (2) monogenic homozygous FH, caused either by dominant mutations in *LDLR*, *APOB*, or *PCSK9*, or recessive mutations in the *ARH* (*LDLRAP1*) gene (Gene ID: 100286472, GenBank NM_015627.3), with both alleles affected; (3) polygenic FH, featuring clear manifestations of the clinical phenotype, despite patients having no mutations in genes associated with classical FH, but differing from non-familial cases of multifactorial hypercholesterolemia; and (4) FH combined with hypertriglyceridemia, which includes a subgroup of patients with familial combined hypercholesterolemia (FCH), who satisfy the criteria for FH, but also exhibit hypertriglyceridemia. This classification has primary significance for the choice of therapeutic approach (Masana et al., 2019). This review concerns only monogenic forms of FH as interpreted by Defesche et al. (2017), since genetic testing in Russia was carried out exclusively for these types of the disease. Generally, knowledge of the lipid profile of the patient and their family history of hypercholesterolemia is sufficient for diagnosis of FH. Although not mandatory, genetic testing is helpful for confirming diagnoses (Defesche et al., 2017). Such testing is challenging, given the substantial heterogeneity of the disease. In monogenic FH, causative mutations are most frequently found in the LDL receptor gene, *LDLR*, and patients with such mutations comprise up to 80% of all FH cases. More than 2,000 different mutations in *LDLR* have been documented worldwide, the majority of which are rare, except in populations with strong founder effects. The great majority (50–60%) of nucleotide substitutions in *LDLR* are missense mutations, further increasing the difficulty of DNA-based diagnosis of heterozygous forms of FH, since for each genetic alteration discovered the causal role in the development of hypercholesterolemia must be demonstrated (Mandelshtam, 2011).

Mutations in *APOB* causing FH vary in frequency among populations, and account for roughly 5–10% of all FH cases globally, although the contribution of these mutations may be somewhat higher in Central Europe (Germany, Austria, and Switzerland) (Andersen et al., 2016). Mutations in *PCSK9* and *LDLRAP1* resulting in FH are rare; specifically, mutations in *PCSK9* are responsible for a maximum of 1% of all FH cases (Mandelshtam and Vasilyev, 2008; Defesche et al., 2017). Some mutations in the sterolin genes, *ABCG5/ABCG8*, which usually cause sitosterolemia, can also lead to FH in compound heterozygotes, while mutations in other genes, e.g., *APOE*, *STAP1*, and *LIPA*, are extremely rare in FH.

This review presents data on mutations identified in genes causing heterozygous FH in Russia. Polymorphisms in *LDLR* and *APOB* in the Russian population as a whole, but not in families with verified FH, are not discussed.

FREQUENCY OF FAMILIAL HYPERCHOLESTEROLEMIA IN RUSSIA

Familial hypercholesterolemia is the most frequent inherited disease in humans (Defesche et al., 2017). The frequency of heterozygous FH is estimated as 1 case per 500 examined individuals, while the homozygous form occurs in 1 in 1,000,000 (Goldstein et al., 2001); however, studies using standard diagnostic criteria have been conducted in a number of countries, revealing FH frequencies approximately twice as high in outbred populations (1:219–1:300). The corresponding figure for homozygous mutations was 1:300,000 (Defesche et al., 2017; Sarraju and Knowles, 2019). Frequencies can be much higher in populations with evident founder effects.

No attempts were made to evaluate FH frequency in Russia for many years. Present estimates show that the number of patients heterozygous for mutations causing FH among the approximately 144.5 million population of Russia varies between 287,000 (Karpov et al., 2015) and 1,300,000 (Ershova et al., 2017), while approximately 150–300 people have the homozygous form of FH (Karpov et al., 2015). This wide range of values results from differences in the criteria used to diagnose FH and in patient selection criteria. In some studies the number of patients with a family history of hypercholesterolemia were calculated, while other protocols considered the number of individuals with high cholesterol levels within the whole population screened. This reflects the absence of a consensus on the meaning of the term "familial hypercholesterolemia" (Masana et al., 2019).

Almost every estimate of FH frequency has been based on clinical, rather than genetic, criteria for FH diagnosis. A population study in West Siberia, conducted under the auspices of The Epidemiology of Cardiovascular Risk Factors and Diseases in Regions of the Russian Federation Study (ESSE-RF), is a very recent example of evaluation of the frequency of heterozygous FH in Russia (Ershova et al., 2017). That study included 1,630 and 1,622 individuals from the Tyumen and Kemerovo regions, respectively, with an age range of 24–65 years. The overall frequency of definite FH, diagnosed using the criteria of the Dutch Lipid Clinic Network (DLCN), was 0.24% (1 per 407 persons examined), while that of probable FH was 0.68% (1 per 148 examined); hence the total frequency of patients with definite or probable FH was 0.92% (1 per 108 examined). In that study, 40% of patients with definite or probable FH had coronary artery disease (CAD), while only 23% of patients received statin therapy. The odds ratios for development of CAD and myocardial infarction in the overall group of patients with definite or probable FH were, 3.71 (95% confidence interval (CI): 1.58–8.72; $p = 0.003$) and 4.06 (95% CI: 0.89–18.55; $p = 0.070$), respectively, relative to patients unlikely to have FH. The authors concluded that the frequency of FH in Russia may be significantly higher than previously considered, and that only a small proportion of patients with FH receive a correct diagnosis and adequate treatment. The high occurrence of FH in Russia reported by Ershova et al. (2017) may arise from the sampling, i.e., the group of patients with probable FH might contain not only patients with the disease in the direct sense of the term, but include individuals with polygenic inheritance as well. Therefore

it is hard to compare these data with reports of other authors cited by Defesche et al. (2017).

The high risk of coronary atherosclerosis among patients with FH and the modes of preventive treatment currently practiced highlight the need to develop genetic investigations to elucidate the specific molecular features of FH in Russia.

STUDIES OF FH GENETICS IN RUSSIA

The LDLR Gene Mutation Spectrum in Saint-Petersburg

Molecular genetic research into FH in Russia was launched in 1987 at the Institute of Experimental Medicine in Saint-Petersburg. The work began by constructing molecular probes to search for large-scale rearrangements in the *LDLR* gene and to analyze its restriction fragment length polymorphism (RFLP) haplotypes. To meet this aim, a series of plasmids, based on the pLDLR-3 vector containing the full-length cDNA encoding LDL receptor, were constructed (Yamamoto et al., 1984). Sub-cloning of *LDLR* fragments was required, since the 3′-untranslated region of the mRNA contained Alu repeats, which had to be eliminated to identify sequences unique to *LDLR*. Southern hybridization experiments using samples from 50 patients with verified clinical diagnoses of FH revealed a 5 kb deletion, encompassing exons 4–6 of *LDLR* segregating in a family with FH (Mandelshtam et al., 1993). These data demonstrate that large-scale rearrangements within *LDLR* are a rare cause of FH in Russia, while the majority of alterations are point mutations, which cannot be detected by Southern hybridization. These findings are consistent with current notions of the global distribution of mutations in *LDLR*; that is, that partial deletions comprise 8–10% of all genetic abnormalities identified at this locus (Bourbon et al., 2017; Defesche et al., 2017).

A second large deletion in *LDLR* was identified in the Russian population by geneticists in Moscow after the introduction of targeted sequencing (Averkova et al., 2018). As Southern blot hybridization is a time-consuming and relatively inefficient method for mutation screening, no more attempts to use this approach to identify large-scale rearrangements at the *LDLR* locus have been undertaken in Russia.

At the very beginning of the study of FH in Saint-Petersburg, it became clear that various *LDLR* RFLP haplotypes segregate with the clinical phenotype, which is indicative of the variety of mutations, the molecular heterogeneity of the disease, and the absence of profound founder effects (Mandel'shtam et al., 1995). Further research into the mutation spectrum at *LDLR* confirmed those findings (Zakharova et al., 2005, 2007). Early studies of FH genetics in Saint-Petersburg provided evidence of varying degrees of atherogenic manifestations within a separate family carrying the same genetic defect, leading to increased plasma cholesterol concentration. This observation implies the participation of other endogenous and exogenous factors in generating the biochemical and clinical manifestations associated with FH (Lipovetsky et al., 1996).

A new stage of the study of FH in Russia began with the introduction of PCR into routine practice. The first experiments with PCR in Saint-Petersburg were conducted in the Nuclear Physics Institute by E. I. Schwartz. Rapid dissemination of PCR soon allowed its use to study *LDLR*. The longest exon, exon 4 (Südhof et al., 1985), became the first target of searches for point mutations. Exon 4 was considered most likely to contain mutations, as it encodes the longest amino acid stretch in the functionally important ligand-binding domain of the LDLR protein. A set of primers designed by Hobbs et al. (1992) was used to search for mutations and mutant species were selected for manual sequencing by screening using single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989a,b), visualized by silver-staining of DNA in polyacrylamide gels (Bassam et al., 1991).

The first point mutations in *LDLR* identified in Saint-Petersburg were c.478T > G p.Cys160Gly (initially designated C139G) and c.444T > G p.Cys148Trp (initially termed as C127W) (**Supplementary Table 1**). Those alterations had never previously been described in other world populations, hence they were termed "Slavic" mutations (Chakir K. H. et al., 1998), although the ethnicity of the patients had not been verified, except that they reported themselves of Russian origin. Interestingly, p.Cys148Trp has never since been detected in other Russian families. In contrast, p.Cys160Gly has subsequently been detected several times; for example, in another family from Saint-Petersburg (unrelated to the first), and in probands from Novosibirsk (Mandelshtam and Maslennikov, 2001) and Moscow (Meshkov et al., 2004, 2009). Subsequently, it became clear that p.Cys160Gly is the most frequent mutation found among ethnic Russian populations. No other "Slavic" mutations were detected in four unrelated families (**Supplementary Table 1**). Later, collaborative research conducted by the Laboratory of Human Molecular Genetics, headed by E. I. Schwartz, and the Department of Molecular Genetics of the Institute of Experimental Medicine, headed by V. I. Gaitskhoki, identified several other mutations in *LDLR* using the same screening methods (Chakir K. et al., 1998; Mandelshtam et al., 1998; Zakharova et al., 2001; Tatishcheva et al., 2001; **Supplementary Table 1**).

The introduction of highly sensitive automated SSCP analysis in DNA gel analyzers (e.g., the "ALFExpress-2" from Amersham), as well as automated fluorescent DNA sequencing, stimulated further progress in the study of the molecular basis of FH in Saint-Petersburg. Consequently, systematic investigations documented 33 mutations in *LDLR*, 18 of which had never been described elsewhere in the world (**Supplementary Table 1**; Zakharova et al., 2005, 2007; Mandelshtam, 2011). These findings demonstrated the high variability of the *LDLR* gene and supported the virtually complete absence of a founder effect in the Saint-Petersburg population (Zakharova et al., 2005, 2007). Mutations were discovered in 59% (44 of 74) of probands in Saint-Petersburg with verified FH in whom all exons of *LDLR* were completely investigated, including alterations that (in our opinion) likely caused the disease in 55% of families with FH (41 of 74); however, the most interesting result was not finding novel and/or known mutations in families considered separately, which was expected in the poorly explored Russian population, but detection of mutations that were ethnically specific. Two mutations typical

of neighboring Finland were found in Saint-Petersburg: a seven-nucleotide deletion, c.925-931del (FH North Karelia) and c.1202T > A p.Leu401His (FH Pori) (Zakharova et al., 2005). The variant widely detected in Eastern Finland, FH North Karelia (Koivisto et al., 1992), was detected in a Finnish family from Petrozavodsk (Komarova et al., 2013a,b,c). Use of allele-specific PCR did not allow detection of the 9.5 kb *LDLR* deletion prevalent in Finland, FH Helsinki c.2311 + 245_c.*807del p.771-860delins55aa), in either the Saint-Petersburg population, or among patients with FH in Karelia (Komarova et al., 2013a).

Mutations typical of the Finnish population appeared to be rare, and were found in only a few families, which can be explained by the absence of ethnic Finns among the patient sample. The absence of FH Helsinki among Russians resident in Saint-Petersburg, alongside Finns, is not surprising, since miscegenation has been prevented by linguistic and religious differences, especially in the times of the Russian Empire; hence, Russians and Karelians practicing Orthodox religion seldom married Finnish Lutherans. Notably, the Finnish population differs significantly from those in neighboring countries, as mutation spectra associated with other hereditary diseases, such as phenylketonuria and familial breast cancer, among others, are commonly specific for that ethnic group. Hence, it is predictable that typically Finnish mutations in *LDLR* are rare in North-West Russia.

The situation among the Ashkenazi Jewish subpopulation in Saint-Petersburg appears very different. The common c.654_656del p.Gly219del LDLR pathogenic variant was detected (Mandelshtam et al., 1998) in 7 of 22 (32%) families with FH. This mutation had been previously described as the allele FH-Lithuania (Meiner et al., 1991) and is known mostly under its initial designation, G197del or c.652_654delGGT in the literature (**Supplementary Table 1**). This trinucleotide deletion was previously identified in 35% of Ashkenazi with FH in Israel, of which 64% (16 of 25) originated from Lithuania (Meiner et al., 1991). An even higher frequency (8 of 10 families) of FH-Lithuania was observed in the Jewish community of the South African Republic, which formed as a result of mass-scale immigration of Jews from Lithuania at the turn of the 20th century (Meiner et al., 1991).

The FH-Lithuania mutation was studied in 78 Ashkenazi probands from Israel, Russia, South Africa, the Netherlands, and the United States (Durst et al., 2001). All patients confirmed that their families originated from Lithuania and carried the mutation on a chromosome with a common haplotype. Analysis of flanking DNA markers allowed the mutation to be dated to the early 14th century, which coincides with the settling of Jews in Lithuania (1338 A.D.) and with the years of rapid growth of the Jewish population in Eastern Europe (Durst et al., 2001). The high frequency of p.Gly219del among Ashkenazi Jews has been proposed to be the result of a founder effect, rather than natural selection (Durst et al., 2001; Risch et al., 2003).

The Jewish community of Saint-Petersburg essentially consists of Ashkenazi and originates mostly from the western regions of the Russian Empire, Lithuania, and Poland (Mandelshtam et al., 1998). The FH-Lithuania mutation has been found in 14 patients from Saint-Petersburg and in an Ashkenazi proband from Novosibirsk (Mandelshtam and Maslennikov, 2001). Other than individuals with Ashkenazi origin, the same mutation was found in two Saint-Petersburg families with no record of Jewish origin (described as c.651_653delTGG in Zakharova et al., 2005).

Patients homozygous for p.Gly219del are characterized by low residual LDL receptor activity (approximately 2%) (Hobbs et al., 1992), termed the receptor-negative phenotype. Biochemical analysis of patient samples from Saint-Petersburg clearly demonstrated that lipid profile aberrations are more pronounced in patients heterozygous for the "Lithuanian" mutation than in the group comprising other patients carrying various non-characterized mutations (Lipovetsky et al., 1998). Patients with FH-Lithuania also had more severe symptoms related to FH than those in the combined group. Further, they were resistant to treatment with fluvastatin (Lipovetsky et al., 1998). The FH-Lithuania mutation has not been found in Karelia to date (Komarova et al., 2013c; Korneva et al., 2017a) or in Moscow (Meshkov et al., 2009).

The LDLR Mutation Spectrum in Patients From Moscow With FH

Molecular genetic studies of FH in Moscow began in 2001 (Krapivner et al., 2001). Unlike researchers in Saint-Petersburg, their colleagues in Moscow chose a better set of primers to amplify the *LDLR* gene (Jensen et al., 1996), which permitted analysis of all the exon-intron boundaries, rather than only the sequences of exons and some of the invariant splicing sites detected by the primers introduced by Hobbs et al. (1992), which were used in Saint-Petersburg and Petrozavodsk.

A two-step protocol was applied to detect mutations, comprising exon screening by SSCP, where conformers were stained using ethidium bromide, fluorescent dye (Krapivner et al., 2001), or SYBR Gold (Meshkov et al., 2004, 2009), and automatic sequencing of selected samples. This approach was used to conduct molecular analysis of *LDLR* in 50 patients with heterozygous FH, facilitating detection of 21 potentially disease-causing mutations in 23 patients (46%), excluding several frequent polymorphisms. Only two mutations (c.1246C > T p.Arg416Trp and c.2389 + 5G > A) were detected in two families, with all of the other 19 mutations each present in a single family.

The mutation spectrum of *LDLR* in the Moscow cohort was rather peculiar. The published results indicated that 15 of 21 mutations (71%) were specific to Russia, with only 6 (29%) previously reported in other countries (Meshkov et al., 2009). By 2020 the spectrum of mutations in *LDLR* had extended, and our analysis shows that only 9 (43%) of all mutations previously described (Meshkov et al., 2009) remain specific for Russia, while the remaining 12 (57%) are shared with other populations. This conclusion is supported by analysis of previous data obtained from the Saint-Petersburg population (Zakharova et al., 2005, 2007; Mandelshtam, 2011). Indeed, calculations made in 2020 show that only 12, rather than 18, of 33 mutations studied (36%) are specific to Russia, while 21 of 33 mutations (64%) have been described elsewhere.

The results obtained in the two largest Russian cities provide evidence that some 40% of mutations in *LDLR* are specific

to Russia. Only two mutations (p.Cys160Gly and c.810G > A p.Cys270Ter) were common among the populations of Moscow and Saint-Petersburg (Zakharova et al., 2007; Meshkov et al., 2009). At present, DNA-based diagnosis of *LDLR* mutations in both Moscow and Saint-Petersburg is conducted by routine commercial analysis; therefore, no summary data on FH mutations are published.

New expectations of more efficient DNA-based diagnosis of FH have been raised by the introduction of targeted sequencing in routine practice. A recent study of 38 patients from Moscow with clinically diagnosed FH and acute coronary syndrome revealed genetic abnormalities in 24 individuals (63.2%) (Averkova et al., 2018); however, mutations in *LDLR* were found in only 10% of probands (four sequence variants, of which one (c.58G > A p.Gly20Arg) was experimentally demonstrated to be neutral). This result is at variance with data from previous studies conducted in Russia and elsewhere in the world, showing that patients with *LDLR* mutations constitute the great majority (usually 70–80%) of cases with a hereditary background (Defesche et al., 2017). This discrepancy likely arose from the distinctive method of sampling and inaccuracy of the clinical FH diagnosis, making the results obtained (Averkova et al., 2018) incomparable with earlier data (Meshkov et al., 2009).

When this review was ready for submission a report was published on a cohort of 52 probands with definite or probable diagnosis of FH according to DLCN criteria (Semenova et al., 2020). Targeted NGS was used to study that cohort consisting of individuals from various regions of Russia who had settled in Moscow. Genetic defects were revealed in 48% of index cases i.e., 24 probands with mutations in the *LDLR* gene and 2 with mutations in the *APOB* gene were identified. 22 mutations in the *LDLR* gene were identified as pathogenic or likely pathogenic. Of those six mutations (but not eight as stated by the authors) were first described. Six mutations (but not three as the authors wrote) had been found in Russia previously. That study allowed to indicate 16 novel *LDLR* mutations in Russia. The most common pathogenic variant in *LDLR* was c.1775G > A p.Gly571Glu found in four probands. It had been previously reported as a repeatedly occurring mutation in St. Petersburg (Zakharova et al., 2001, 2007) and in Novosibirsk (Voevoda et al., 2008), also found in a number of populations in the world. Despite using NGS the percentage of probands with pathogenic variants was low, which probably results from the inaccuracy of the clinical FH diagnosis.

LDLR Mutation Spectrum in Patients With FH From Petrozavodsk

In Petrozavodsk, the molecular basis of FH was studied using a similar approach to that applied in Saint-Petersburg. DNA was collected from 102 patients with definite FH, including 94 probands, according to DLCN criteria (Defesche et al., 2017). The presence of the c.10580G > A p.Arg3527Gln (R3500Q) mutation in *APOB*, which generates non-binding apolipoprotein B-100 (FDB, familial-defective apoB-100) was ruled out in all samples, using allele-specific PCR (Hansen et al., 1991). Patients with FH were also tested for the two major Finnish mutations: the 9.5 kb deletion FH Helsinki (Aalto-Setälä et al., 1988; Aalto-Setälä, 1988) and the 7-nucleotide deletion c.925_931delCCCATCA (known as FH-North Karelia) (Koivisto et al., 1992). The first of these two mutations was detected by allele-specific PCR (Kontula et al., 1992), while for the second heteroduplex analysis and detection of single-strand conformers were conducted in *LDLR* exon 6 amplified by PCR (Komarova et al., 2013a). The FH Helsinki mutation was not found, while FH-North Karelia was detected in a single proband among 80, who reported he was of Finnish origin (*ibid.*). Subsequently, Cy-5 tagged primers (Hobbs et al., 1992; Jensen et al., 1996) were used to amplify separate exons, and samples with abnormal mobility were automatically sequenced (Komarova et al., 2013b,c). This analysis resulted in discovery of 14 different mutations capable of causing a clinical phenotype and a number of frequent polymorphisms in the cohort of 52 patients with completely sequenced *LDLR* genes (**Supplementary Tables 1, 2**; Komarova et al., 2013b,c; Korneva et al., 2013, 2014, 2016, 2017a,b).

Of the 52 patients subjected to genetic analysis, 22 probands (42%) carried mutations. Mutations likely to cause the disease were discovered in 14 families (27%) (Korneva et al., 2017a). Only the p.Leu646Ile mutation was found in two probands (Komarova et al., 2013b,c), while all other variants were detected in single families with FH, but not in controls (Komarova et al., 2013c; Korneva et al., 2013).

Interestingly, the spectrum of *LDLR* mutations in Karelia had few similarities with that found in Saint-Petersburg; the one common variant was FH-North Karelia (Komarova et al., 2013a,b,c). Further, the c.1327T > C p.Trp443Arg mutation was present in both the Karelian and Moscow populations (Korneva et al., 2017a). Of 14 mutations reported in the latter publication only 4 had been found elsewhere in the world, while the remaining 10 (71%) were specific for Russia (*ibid.*). The small size of the cohort studied may partly account for these differences. In our opinion, these results highlight the genetic peculiarity of the Russian population, with respect to the spectrum of *LDLR* gene mutations found in patients with FH (Komarova et al., 2013b; Korneva et al., 2017a).

LDLR Mutation Spectrum in Patients With FH in Novosibirsk

Studies of FH accomplished in Novosibirsk to date were conducted in three stages. First, patients were screened for mutations discovered in Saint-Petersburg (Mandelshtam and Maslennikov, 2001) and families carrying p.Cys160Gly and p.Gly219del identified. Second, *LDLR* coding regions were sequenced in samples from 20 patients aged 45–49 years with the highest levels of serum cholesterol, disregarding family history of hypercholesterolemia (Voevoda et al., 2008). This approach facilitated detection of 7 novel and 12 previously described mutations in *LDLR* (**Supplementary Table 1**), however, 4 of the 7 novel mutations identified were silent, and one variant appeared to be a well-known neutral polymorphism. The remaining variants were not tested functionally and no *in silico* predictions were conducted. Further, as there was no familial analysis, the importance of the newly detected mutations for the

etiology of hypercholesterolemia remains unclear. Nevertheless, this research was meaningful, since it revealed a significant difference between the *LDLR* mutation spectrum in a population sample of patients with hypercholesterolemia and that among patients with FH (Voevoda et al., 2008).

In Novosibirsk, mutation screening of patients with hypercholesterolemia is currently conducted by targeted sequencing of several genes involved in the development of monogenic FH (i.e., *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1*). The most recent results demonstrate the efficiency of this approach (Shakhtshneider E. et al., 2019; Shakhtshneider E. V. et al., 2019), which facilitated examination of 42 patients with clinically diagnosed definite FH, resulting in detection of 12 different mutations in *LDLR* in 17 probands and two cases with a p.Arg3527Gln mutation in *APOB*. Thus, mutations were identified in 45% of families, of which 40% had changes in *LDLR* and 4.8% in *APOB*. No mutations associated with FH were found in *PCSK9*. Similarly, no homozygotes or compound-heterozygotes for mutations in *LDLRAP1* (ARH) were observed. Of the 12 mutations in *LDLR*, 10 were missense, one was localized in a splice donor site (Shakhtshneider et al., 2017a), and the other was a deletion, however, the mutations were not detailed in the abstract (Shakhtshneider E. V. et al., 2019).

Why Is It Challenging to Compare Research Carried out in Different Regions of Russia?

As described above, there have been a limited number of investigations into the mutation spectrum of FH in Russia. The studies that have been conducted differed noticeably in design, making comparisons among them difficult. The best characterized, with respect to the *LDLR* mutation spectrum, are the populations of Saint-Petersburg, Moscow, Petrozavodsk, and Novosibirsk. The clearest differences among those studies are as follows: (1) different methods of study cohort selection; (2) different primer sets; and (3) incomparable methods of mutation screening. Below, we consider each of these dissimilarities separately.

Differences in Patient Selection Criteria

In both Saint-Petersburg and Moscow, patients were primarily selected based on familial history of disease, whereas researchers in Novosibirsk used population sampling of individuals aged 45–49 years with high levels of serum cholesterol (Voevoda et al., 2008). Further, the following criteria were used for FH clinical diagnosis in Saint-Petersburg (Mandelshtam et al., 1993): (1) type IIa or type IIb hyperlipidemia, featuring high cholesterol, particularly LDL cholesterol; (2) several members of the proband's family have hypercholesterolemia or reported myocardial infarctions at an early age; (3) the patient has tendinous xanthomas and/or lipoid corneal arches. Only patients meeting at least two of these criteria were included in the study. In Petrozavodsk, patient selection and diagnosis of FH were largely based on the recommendations of the DLCN (Korneva et al., 2017a), while researchers in Moscow relied on MEDPED (The Make Early Diagnosis To Prevent Early Death) FH program or Simon Broom Register criteria (Meshkov et al., 2009).

Different Sets of Primers Used to Search for *LDLR* Gene Mutations

Different sets of primers were used to amplify *LDLR* gene exons and were not detailed in scientific publications. Only the coding region of the gene was studied in Novosibirsk by direct sequencing (Voevoda et al., 2008); hence, mutations in introns were not detected. The set of primers suggested by H. Hobbs et al. (1992) was used in Saint-Petersburg and Petrozavodsk to amplify the majority of *LDLR* amplicons. These primers facilitated full analysis of the gene exon sequences, however, only 12 of 34 exon-intron boundaries, which contain signals crucial for splicing, were covered by this primer set. An alternative pair of primers was used to amplify *LDLR* exon 3 alone (Jensen et al., 1996), facilitating detection of novel mutations in the splice donor site of intron 3, including the c.313 + 1G > A transition in two probands among 74 from Saint-Petersburg and a c.313 + 2G > T transversion in one proband of 52 from Petrozavodsk. Nevertheless, even with sequencing of the intron 3 boundaries, more than half the *LDLR* gene exon-intron interfaces were not covered in either the Saint-Petersburg or Petrozavodsk investigations. Researchers in Moscow used a more advantageous set of primers (Jensen et al., 1996; Meshkov et al., 2004, 2009), which allowed exploration of all exon-intron interfaces.

Notably, at least one study with an optimized *LDLR* mutation search strategy showed that up to 27% of patients can carry mutations that potentially disturb splicing; of 54 mutations identified by the authors, 13 were found in introns (Amsellem et al., 2002). That work is of great interest, although the contribution of intron mutations to the development of FH suggested by the authors should be treated with caution. Studies reported in this review usually did not explore *LDLR* introns deeply and so far only the results relating to the *LDLR* coding region can be directly compared in the various studies conducted in Russia.

Methods for Mutation Screening and Identification

The majority of methods used to look for mutations in *LDLR* in the Russian population aimed to identify point mutations, and short deletions and insertions, which can be detected by PCR and direct DNA sequencing. Less attention has been paid to searching for large-scale rearrangements at the *LDLR* locus. To date, such research in Russia has been limited to identification of two deletions. The first is a single 5 kb deletion that eliminates exons 4–6 of *LDLR*, found in two individuals of the same descent among 50 patients studied in Saint-Petersburg by Southern hybridization (Mandelshtam et al., 1993). The other is a deletion of 1,468 bp revealed by targeted sequencing of *LDLR* in Moscow (Averkova et al., 2018). Initial studies showed that large-scale rearrangements within the *LDLR* locus seldom cause FH in the population of Saint-Petersburg (Mandelshtam et al., 2004). Subsequently, research in Russia focused on other issues, despite the appearance of novel

methods for identification of deletions and exon duplications; for example, real-time PCR.

In ethnic isolates, such as French-speaking Canadians, deletions can constitute more than 50% of all mutations causing FH (e.g., the French Canadian-1 and French Canadian-5 mutations) (Hobbs et al., 1992). Meanwhile in ethnically heterogeneous populations, for example, English-speaking Canadians, large deletions are found in 5–7% cases (Langlois et al., 1988). Since analysis of large-scale rearrangements of the *LDLR* locus has only been conducted in two Russian studies, one cannot compare different regions and various ethnic groups in this country with respect to the frequency of such mutations.

Various methods of searching for mutations at the *LDLR* locus have been used in different studies. Most involved amplification of separate exons and adjacent introns, as well as exon screening by DNA conformational polymorphism analysis, but not direct sequencing of exons. To identify single-strand conformers, silver staining of gels was used following electrophoresis (Chakir K. H. et al., 1998; Chakir K. et al., 1998; Mandelshtam et al., 1998; Tatishcheva et al., 2001; Zakharova et al., 2001). Alternatively, DNA was stained in gels using the fluorescent dye, SYBR Gold ("Molecular Probes") (Krapivner et al., 2001; Meshkov et al., 2004, 2009). SSCP analysis was conducted using polyacrylamide, rather than mutation detection enhancement (MDE) gels. Analyses were often conducted at a single maintained temperature; for example, at 4°C (Meshkov et al., 2004) or 20°C (Tatishcheva et al., 2001; Zakharova et al., 2001). Some protocols did not require the addition of 10% glycerol to the polyacrylamide gels, diminishing the efficiency of SSCP analysis. Hence, even the most extensive research accomplished during the previous decade in Moscow, only detected mutations in 23 of 50 (46%) probands with FH (Meshkov et al., 2009). An efficiency of SSCP analysis for mutation screening comparable with direct sequencing can be achieved only when varied conditions and MDE gels are used (Jensen et al., 1996).

Automated fluorescent SSCP analysis noticeably increased the efficiency of the method, making it comparable with direct automated DNA sequencing. In our study (Zakharova et al., 2005) fluorescent SSCP analysis was used to reveal *LDLR* mutations in 56% (14 of 26) of patients, while direct sequencing of all exons allowed detection of those in 53% (10 of 19) of probands. Our results indicate the high efficiency of fluorescent SSCP analysis in screening for *LDLR* mutations. Subsequently, this approach was comprehensively applied in studies conducted in Petrozavodsk (Komarova et al., 2013c; Korneva et al., 2017a).

Mutations in the APOB Gene and Their Contribution to FH Etiology

Frequent mutations in exon 26 of *APOB* in a large sample of 730 patients from Moscow with FH (OMIM #143890) indicated that patients with such changes comprise approximately 2.6% of cases (Meshkov et al., 2009). Among all variants found in Moscow, only the p.Arg3527Gln (R3500Q) mutation is unambiguously associated with hypercholesterolemia, as it causes a deficiency of binding with the ApoB-100 receptor (FDB); it was found in 14 (1.9%) patients with FH (Meshkov et al., 2009).

A smaller scale study, including 111 patients with the heterozygous form of FH, identified five heterozygotes (4.5%) (Malyshev et al., 2007), with at least three families where patients carried p.Arg3527Gln originating from Central Russia. In four families, the carriers were ethnic Russians, and in one family the mutation was supposedly inherited from the Lithuanian mother (*ibid.*). This is an indication that p.Arg3527Gln, which is most frequent in Switzerland, Austria, and Germany (Hansen, 1998), is also disseminated in Eastern Europe. The p.Arg3527Gln mutation was found in only 2 of 42 patients (4.8%) on high throughput targeted sequencing of *APOB* in patients with verified FH in Novosibirsk (Shakhtshneider E. V. et al., 2019).

Allele-specific PCR, used in a purposeful search for R3500Q mutation in *APOB* among 74 probands with FH from Saint-Petersburg and 52 probands from Karelia, generated negative results (Komarova et al., 2013b,c; Korneva et al., 2017a). In comparison, p.Arg3527Gln was found in 6.6% of independently reported cases (25 of 378) of autosomal dominant hypercholesterolemia in Poland (Chmara et al., 2010). No clear link has been proven between other *APOB* mutations found in Moscow and FH development.

Mutations in PCSK9 and Their Contribution to FH Etiology

Globally, the contribution of *PCSK9* gene mutations does not exceed 1% of all FH cases (Defesche et al., 2017). Only gain-of-function mutations, which intensify receptor degradation, cause the autosomal-dominant form of FH, while loss-of-function mutations favor a decrease in cholesterol levels (Abifadel et al., 2009). A vivid example of the low frequency of such mutations is provided by a study of a French FH patient cohort, involving 1,358 probands from various regions (Marduel et al., 2010), where the authors found mutations in *PCSK9* in only 0.7% of probands.

Despite the rarity of *PCSK9* mutations, studies of their frequency were undertaken using targeted sequencing in Novosibirsk (Shakhtshneider et al., 2017b; Shakhtshneider E. et al., 2019; Shakhtshneider E. V. et al., 2019) and Moscow (Averkova et al., 2018). Different variants of the gene were found, which either favored hypocholesterolemia (c.137G > A p.Arg46Leu, rs11591147), or were of unclear function (Shakhtshneider et al., 2017b). To date, no single *PCSK9* variant capable of provoking FH has been detected in Russia (Shakhtshneider E. V. et al., 2019). The c.2009G > A p.Gly670Glu (rs505151) variant was regarded as possibly favoring FH development (Abifadel et al., 2009); however, in the Russian population this variant is found at equal frequencies among individuals with hyper- and normo-cholesterolemia (Astrakhova et al., 2016) and, therefore, cannot be considered a cause of FH. Concerning other variants of *PCSK9* discovered in Russian populations, no comparisons were made between patients with FH and the control group; hence, identification of *PCSK9* mutations associated with FH in Russia has yet to occur.

DISCUSSION

The percentage of FH patients with identified mutations causing the clinical phenotype in various regions of Russia is lower than that among other world populations; there are several reasons for this situation. First, the majority of studies in Russia were dedicated to searching for mutations in the *LDLR* gene, but not *APOB*, which can be responsible for approximately 10–15% of FH cases (Defesche et al., 2017). Second, screening for large-scale rearrangements in *LDLR* has not been comprehensive, and this type of alteration can cause 8–10% of FH cases in populations with no clear founder effect. Third, a number of studies focused primarily on the *LDLR* coding region, with no analysis of exon-intron boundaries or the promotor region (Voevoda et al., 2008). Finally, and probably the most important the patient samples included not only patients with definite FH, but also those with probable FH, as judged by the DLCN criteria (Defesche et al., 2017) that may be caused not by monogenic mutations.

The actual percentage of families with identified *LDLR* mutations in Russia may be even lower than reported in the literature cited, since the samples of patients with FH subjected to genetic testing were relatively small, not exceeding 100 probands. Genetic analysis of 50 patients with clinical FH revealed 21 causative mutations in 23 individuals (46%) (Meshkov et al., 2009). Overall, the percentage of *LDLR* mutations among 74 probands in Saint-Petersburg was 59% (44 of 74), of which 55% were causative (41 of 74) (Zakharova et al., 2005; Mandelshtam, 2011). Of 52 patients with clinical FH studied in Karelia, 22 probands (42%) carried pathogenic mutations in *LDLR* (Komarova et al., 2013c; Korneva et al., 2017a). Targeted sequencing of *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1* in 42 patients with verified FH from Novosibirsk revealed nucleotide substitutions in 45% of patients that would be expected to cause inadequate receptor function (Shakhtshneider E. et al., 2019; Shakhtshneider E. V. et al., 2019). In comparison, in the Netherlands, screening of *LDLR* and *APOB* in patients with an FH clinical phenotype revealed causative mutations in 77% of patients (Fouchier et al., 2005). High-resolution melting analysis allowed detection of such mutations in 17 of 28 (61%) patients with FH of Greek origin (Whittall et al., 2010). Further, a Turkish study revealed mutations causing FH in 37 of 56 (66%) alleles tested (Sözen et al., 2005), while a genetic study of FH in a French population detected mutations in 73.9% of patients with autosomal-dominant hypercholesterolemia (Marduel et al., 2010). Moreover, 6.6% of patients carried *APOB* mutations, and 0.7% had mutations of *PCSK9*. In only 19% of patients, no mutations associated with FH were detected (*ibid.*). An investigation of a large cohort of 1,018 Italian patients with autosomal-dominant hypercholesterolemia revealed mutations in 832 probands (82%), of whom 97.4% had mutations in *LDLR*, 2.2% in *APOB*, and 0.36% in *PCSK9* (Bertolini et al., 2013). A cohort of 378 patients with FH was studied in Poland, of which 234 (62%) carried variants of *LDLR* and *APOB*; the researchers found 99 *LDLR* sequence variants and one mutation (p.Arg3527Gln; R3500Q) in *APOB* (Chmara et al., 2010). Of the 99 *LDLR* variants, 71 were potentially pathogenic mutations (*ibid.*).

In Russia the majority of *LDLR* mutations were discovered in unique families. This can be attributed to the relatively small sample sizes of patients with FH. At present, the peculiarity of the *LDLR* mutation spectrum in Russia cannot be precisely evaluated.

Rapid improvements in next generation sequencing and the increasing number of sequenced genomes are likely to show that genetic variations previously regarded as specific for certain countries are also present in different populations around the world. Data presented in **Supplementary Material** allow calculation of a rough estimate of the peculiarity of the *LDLR* mutation spectrum in Russia. Some calculations were made after excluding silent mutations and neutral polymorphisms, which are more widespread than mutations causing FH.

As mentioned above, approximately 40% of the mixed populations of Moscow and Saint-Petersburg present with mutations specific to Russia. Despite the lower number of families subjected to DNA-testing in Karelia, only 4 of 14 *LDLR* mutations capable of causing FH detected in this region have been described elsewhere in the world, while the other 10 (71%) were reported as novel (Korneva et al., 2017a). Karelian data seem more typical of the Russian population as a whole, relative to those obtained in Moscow, Saint-Petersburg, and Novosibirsk. In the latter megapolis, only 1 of 10 mutations causing FH had not been described in other parts of the world (Shakhtshneider E. et al., 2019). Therefore, to identify "genuine" Russian mutations associated with the development of FH, future research should be conducted in rural settlements, or small towns with majority ethnic Russian populations. To date, collected samples of genetically characterized patients in Russia have been small, and it is difficult to specify major mutations that could reflect national features of FH, particularly as the majority of studies cited in this review did not explore possible associations of specific mutations with the ethnic origin of patients (Zakharova et al., 2005; Voevoda et al., 2008; Meshkov et al., 2009; Komarova et al., 2013c; Korneva et al., 2017a; Shakhtshneider E. et al., 2019; Shakhtshneider E. V. et al., 2019). It seems likely that major achievements in the genetic investigation of the molecular features of FH in Russia are yet to come.

AUTHOR CONTRIBUTIONS

FZ and TB elaborated data, composed tables, and edited the text. MM and VV wrote and composed the text. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.550591/full#supplementary-material

REFERENCES

Aalto-Setälä, K. (1988). The finnish type of the LDL receptor gene mutation: molecular characterization of the deleted gene and the corresponding mRNA. *FEBS Lett.* 234, 411–416. doi: 10.1016/0014-5793(88)80127-3

Aalto-Setälä, K., Gylling, H., Miettinen, T., and Kontula, K. (1988). Identification of a deletion in the LDL receptor gene. A Finnish type of mutation. *FEBS Lett.* 230, 31–34. doi: 10.1016/0014-5793(88)80635-5

Abifadel, M., Rabès, J. P., Devillers, M., Munnich, A., Erlich, D., Junien, C., et al. (2009). Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. *Hum. Mutat.* 30, 520–529. doi: 10.1002/humu.20882

Amsellem, S., Briffaut, D., Carrie, A., Rabes, J. P., Girardet, J. P., Fredenrich, A., et al. (2002). Intronic mutations outside of Alu-repeat-rich domains of the LDL receptor gene are a cause of familial hypercholesterolemia. *Hum. Genet.* 111, 501–510. doi: 10.1007/s00439-002-0813-4

Andersen, L. H., Miserez, A. R., Ahmad, Z., and Andersen, R. L. (2016). Familial defective apolipoprotein B-100: a review. *J. Clin. Lipidol.* 10, 1297–1302. doi: 10.1016/j.jacl.2016.09.009

Astrakhova, K. S., Shakhtshneider, E. V., Ivanoshchuk, D. E., Ragino, Y. I., and Voevoda, M. I. (2016). "Association of rs505151 in PCSK9 gene with lipid profile in Russian population," in *Proceedings of the the International Symposium Systems Biology And Biomedicine (SBIOMED-2016)*, Novosibirsk.

Averkova, A. O., Brazhnik, V. A., Speshilov, G. I., Rogozhina, A. A., Koroleva, O. S., Zubova, E. A., et al. (2018). Targeted sequencing in patients with clinically diagnosed hereditary lipid metabolism disorder and acute coronary syndrome. *Bull. RSMU* 5, 80–86. doi: 10.24075/brsmu.2018.061

Bassam, B. J., Caetano-Anolles, G., and Gresshoff, P. M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196, 80–83. doi: 10.1016/0003-2697(91)90120-i

Bertolini, S., Pisciotta, L., Rabacchi, C., Cefalù, A. B., Noto, D., Fasano, T., et al. (2013). Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy. *Atherosclerosis* 227, 342–348. doi: 10.1016/j.atherosclerosis.2013.01.007

Bourbon, M., Alves, A. C., and Sijbrands, E. J. (2017). Low-density lipoprotein receptor mutational analysis in diagnosis of familial hypercholesterolemia. *Curr. Opin. Lipidol.* 28, 120–129. doi: 10.1097/MOL.0000000000000404

Chakir, K. H., Skobeleva, N. A., Shevtsov, S. P., Konstantinov, V. O., Denisenko, A. D., and Schwartz, E. I. (1998). Two novel Slavic point mutations in the low-density lipoprotein receptor gene in patients with familial hypercholesterolemia from St. Petersburg, Russia. *Mol. Genet. Metab.* 63, 31–34. doi: 10.1006/mgme.1997.2614

Chakir, K., Mandelshtam, M. J., Shevtsov, S. P., Golubkov, V. I., Skobeleva, N. A., Shur, Y. A., et al. (1998). Two novel low-density lipoprotein receptor gene mutations (E397X and 347delGCC) in St. Petersburg familial hypercholesterolemia. *Mol. Genet. Metab.* 65, 311–314. doi: 10.1006/mgme.1998.2762

Chmara, M., Wasag, B., Zuk, M., Kubalska, J., Wegrzyn, A., Bednarska-Makaruk, M., et al. (2010). Molecular characterization of Polish patients with familial hypercholesterolemia: novel and recurrent LDLR mutations. *J. Appl. Genet.* 51, 95–106. doi: 10.1007/BF03195716

Defesche, J. C., Gidding, S. S., Harada-Shiba, M., Hegele, R. A., Santos, R. D., and Wierzbicki, A. S. (2017). Familial hypercholesterolaemia. *Nat. Rev. Dis. Prim.* 3, 1–20. doi: 10.1038/nrdp.2017.93

Durst, R., Colombo, R., Shpitzen, S., Avi, L. B., Friedlander, Y., Wexler, R., et al. (2001). Recent origin and spread of a common Lithuanian mutation, G197del LDLR, causing familial hypercholesterolemia: positive selection is not always necessary to account for disease incidence among Ashkenazi Jews. *Am. J. Hum. Genet.* 68, 1172–1188. doi: 10.1086/320123

Ershova, A. I., Meshkov, A. N., Bazhan, S. S., Storozhok, M. A., Efanov, A. Y., Medvedeva, I. V., et al. (2017). The prevalence of familial hypercholesterolemia in the West Siberian region of the Russian federation: a substudy of the ESSE-RF. *PLoS One* 12:e0181148. doi: 10.1371/journal.pone.0181148

Fouchier, S. W., Kastelein, J. J., and Defesche, J. C. (2005). Update of the molecular basis of familial hypercholesterolemia in the Netherlands. *Hum. Mutat.* 26, 550–556. doi: 10.1002/humu.20256

Goldstein, J. L., Hobbs, H. H., and Brown, M. S. (2001). "Familial hypercholesterolaemia," in *The Metabolic and Molecular Basis of Inherited Disease*, Vol. 3, eds C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (New York, NY: McGraw Hill), 2863–2914.

Hansen, P. S. (1998). Familial defective apolipoprotein B-100. *Dan. Med. Bull.* 45, 370–382.

Hansen, P. S., Rüdiger, N., Tybjaerg-Hansen, A., Faergeman, O., and Gregersen, N. (1991). Detection of the apoB-3500 mutation (glutamine for arginine) by gene amplification and cleavage with Msp I. *J. Lipid Res.* 32, 1229–1233.

Hobbs, H. H., Brown, M. S., and Goldstein, J. L. (1992). Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum. Mutat.* 1, 445–466. doi: 10.1002/humu.1380010602

Jensen, H. K., Jensen, L. G., Hansen, P. S., Faergeman, O., and Gregersen, N. (1996). High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. *Clin. Chem.* 42, 1140–1146. doi: 10.1093/clinchem/42.8.1140

Karpov, Y. A., Kukharchuk, V. V., Boytsov, S. A., Voevoda, M. I., Gafarov, V. V., Gurevich, V. S., et al. (2015). Consensus statement of the Russian National Atherosclerosis Society (RNAS). Familial hypercholesterolemia in Russia: outstanding issues in diagnosis and management. *Atheroscler. Dyslipidem.* 2, 5–15.

Koivisto, U. M., Turtola, H., Aalto-Setälä, K., Top, B., Frants, R. R., Kovanen, P. T., et al. (1992). The familial hypercholesterolemia (FH)-North Karelia mutation of the low density lipoprotein receptor gene deletes seven nucleotides of exon 6 and is a common cause of FH in Finland. *J. Clin. Invest.* 90, 219–228. doi: 10.1172/JCI115839

Komarova, T. Y., Golovina, A. S., Grudinina, N. A., Zakharova, F. M., Korneva, V. A., Lipovetskii, B. M., et al. (2013a). "Finnish" mutations in LDL receptor gene: a rare cause of familial hypercholesterolemia in St. Petersburg and Petrozavodsk. *Bull. Exper. Biol. Med.* 155, 380–383. doi: 10.1007/s10517-013-2159-4

Komarova, T. Y., Golovina, A. S., Grudinina, N. A., Zakharova, F. M., Korneva, V. A., Lipovetsky, B. M., et al. (2013b). New mutations in low-density lipoprotein receptor gene in familial hypercholesterolemia patients from Petrozavodsk. *Russian J. Genet.* 49, 673–676. doi: 10.1134/S1022795413040066

Komarova, T. Y., Korneva, V. A., Kuznetsova, T. Y., Golovina, A. S., Vasilyev, V. B., and Mandelshtam, M. Y. (2013c). Familial hypercholesterolemia mutations in Petrozavodsk: no similarity to St. Petersburg mutation spectrum. *BMC Med. Genet.* 14:128. doi: 10.1186/1471-2350-14-128

Kontula, K., Koivisto, U.-M., Koivisto, P., and Turtola, H. (1992). Molecular genetics of familial hypercholesterolaemia: common and rare mutations of the low density lipoprotein receptor gene. *Ann. Med.* 24, 363–367. doi: 10.3109/07853899209147838

Korneva, V. A., Bogoslovskaya, T. Y., Kuznetsova, T. Y., Mandelshtam, M. Y., and Vasilyev, V. B. (2014). Familial hypercholesterolemia due to a new mutation in the low density lipoprotein receptor gene. *Clin. Med.* 7, 49–53.

Korneva, V. A., Kuznetsova, T. Y., Bogoslovskaya, T. Y., Murtazina, R. Z., Didio, A. V., Serebrenitskaya, M. P., et al. (2016). Modern diagnostics abilities of familial hypercholesterolemia before clinical features of atherosclerosis appearance. *Atheroscler. Dyslipidem.* 1, 22–32.

Korneva, V. A., Kuznetsova, T. Y., Bogoslovskaya, T. Y., Polyakov, D. S., Vasilyev, V. B., Orlov, A. V., et al. (2017a). Cholesterol levels in genetically determined familial hypercholesterolaemia in Russian Karelia. *Cholesterol* 2017:9375818. doi: 10.1155/2017/9375818

Korneva, V. A., Kuznetsova, T. Y., Murtazina, R. Z., Didio, A. V., Bogoslovskaya, T. Y., Mandelshtam, M. Y., et al. (2017b). The familial hypercholesterolemia caused by a novel human low density lipoprotein receptor gene mutation c.1327 T>C (p.W433R). *Kardiologiia* 57, 12–16. doi: 10.18565/cardio.2017.2.12-16

Korneva, V. A., Kuznetsova, T. Y., Komarova, T. Y., Golovina, A. S., Mandelshtam, M. Y., Konstantinov, V. O., et al. (2013). A case of familial hypercholesterolemia caused by a novel mutation p. FsS65: D129X of human low density lipoprotein receptor gene. *Kardiologiia* 53, 50–54.

Krapivner, S. R., Malyshev, P. P., Poltaraus, A. B., Kukharchuk, V. V., and Bochkov, V. N. (2001). A case of familial hypercholesterolemia caused by a novel mutation D461Y in the low density lipoprotein receptor gene. *Kardiologiia* 41, 92–94.

Langlois, S., Kastelein, J. J. P., and Hayden, M. R. (1988). Characterization of six partial deletions in the low-density-lipoprotein (LDL) receptor gene causing familial hypercholesterolemia (FH). *Am. J. Hum. Genet.* 43, 60–68.

Lipovetsky, B. M., Mandelshtam, M. Y., Gaitskhoki, V. S., and Vasilyeva, L. E. (1996). Clinicogenetic aspects of family hypercholesterolemia and their practical implications. *Ter. Arkh.* 68, 24–29.

Lipovetsky, B. M., Mandeslhtam, M. Y., Vasilyeva, L. E., Shakir, K. H., Golubkov, V. I., Shevtsov, S. P., et al. (1998). Prevalence and symptoms of "Lithuanian" mutation in Jews with hyperlipidemia type II and their response to fluvastatin treatment. *Kardiologiia* 38, 39–41.

Malyshev, P. P., Meshkov, A. N., Kotova, L. A., and Kukharchuk, V. V. (2007). Familial defect of apolipoprotein Â-100: molecular disease basis and clinico-biochemical characteristics of the patients. *Cardiovasc. Therapy Prevent.* 6, 40–45. doi: 10.15829/1728-8800-2007-6

Mandelshtam, M. (2011). "Semeynaya giperkholesterinemiya i semeynyy rak molochnoy zhelezy," in *Molekularno-Geneticheskoe Issledovanie Zhiteley Sankt-Peterburga* (Saarbrücken: LAP LAMBERT Academic Publishing GmbH & Co. KG) (in Russian).

Mandelshtam, M., Chakir, K., Shevtsov, S., Golubkov, V., Skobeleva, N., Lipovetsky, B., et al. (1998). Prevalence of Lithuanian mutation among St. Petersburg Jews with familial hypercholesterolemia. *Hum. Mutat.* 12, 255–258. doi: 10.1002/(sici)1098-1004(1998)12:4<255::aid-humu6>3.0.co;2-e

Mandelshtam, M. J., Lipovetskyi, B. M., Schwartzman, A. L., and Gaitskhoki, V. S. (1993). A novel deletion in the low-density lipoprotein receptor gene in a patient with familial hypercholesterolemia from Petersburg. *Hum. Mutat.* 2, 256–260. doi: 10.1002/humu.1380020404

Mandel'shtam, M. Y., Lipovetskii, B. M., Schvartsman, A. L., and Gaitskhoki, V. S. (1995). Molecular heterogeneity of familial hypercholesterolemia in the St.Petersburg population. *Russian J. Genet.* 31, 447–452.

Mandelshtam, M. Y., and Maslennikov, A. B. (2001). Study of familial hypercholesterolemia molecular genetics in Russia. *Mol. Biol. Technol. Med. Pract.* 1, 58–64 (in Russian).

Mandelshtam, M. Y., and Vasilyev, V. B. (2008). Monogenic hypercholesterolemias: new genes, new drug targets. *Russian J. Genet.* 44, 1134–1140. doi: 10.1134/S1022795408100025

Mandelshtam, M. Y., Zakharova, F. M., Golubkov, V. I., Maslennikov, A. B., Tatishcheva, Y. A., Lipovetsky, B. M., et al. (2004). Diagnostics of familial hypercholesterolemia in Russia: achievements and problems. *Mol. Biol. Technol. Med. Pract.* 6, 9–23 (in Russian).

Marduel, M., Carrié, A., Sassolas, A., Devillers, M., Carreau, V., Di Filippo, M., et al. (2010). Molecular spectrum of autosomal dominant hypercholesterolemia in France. *Hum. Mutat.* 31, E1811–E1824. doi: 10.1002/humu.21348

Masana, L., Ibarretxe, D., Rodríguez-Borjabad, C., Plana, N., Valdivielso, P., Pedro-Botet, J., et al. (2019). Toward a new clinical classification of patients with familial hypercholesterolemia: one perspective from Spain. *Atherosclerosis* 287, 89–92. doi: 10.1016/j.atherosclerosis.2019.06.905

Meiner, V., Landsberger, D., Berkman, N., Reshef, A., Segal, P., Seftel, H. C., et al. (1991). A common Lithuanian mutation causing familial hypercholesterolemia in Ashkenazi Jews. *Am. J. Hum. Genet.* 49, 443–449.

Meshkov, A. N., Malyshev, P. P., and Kukharchuk, V. V. (2009). Familial hypercholesterolemia in Russia: genetic and phenotypic characteristics. *Terapevtichesk. Arkhiv.* 81, 23–28.

Meshkov, A. N., Stambol'skii, D. V., Krapivner, S. R., Bochkov, V. N., Kukharchuk, V. V., and Malyshev, P. P. (2004). Low density lipoprotein receptor gene mutations in patients with clinical diagnosis of familial hypercholesterolemia. *Kardiologiia* 44, 58–61.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989a). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. U.S.A.* 86, 2766–2770. doi: 10.1073/pnas.86.8.2766

Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989b). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5, 874–879. doi: 10.1016/0888-7543(89)90129-8

Risch, N., Tang, H., Katzenstein, H., and Ekstein, J. (2003). Geographic distribution of disease mutations in the Ashkenazi Jewish population supports genetic drift over selection. *Am. J. Hum. Genet.* 72, 812–822. doi: 10.1086/373882

Sarraju, A., and Knowles, J. W. (2019). Genetic testing and risk scores: impact on familial hypercholesterolemia. *Front. Cardiovasc. Med.* 6:5. doi: 10.3389/fcvm.2019.00005

Semenova, A. E., Sergienko, I. V., García-Giustiniani, D., Monserrat, L., Popova, A. B., Nozadze, D. N., et al. (2020). Verification of underlying genetic cause in a cohort of Russian patients with familial hypercholesterolemia using targeted next generation sequencing. *J. Cardiovasc. Dev. Dis.* 7:16. doi: 10.3390/jcdd7020016

Shakhtshneider, E., Ivanoshchuk, D., Orlov, P., Timoshchenko, O., and Voevoda, M. (2019). Analysis of the Ldlr, Apob, Pcsk9 and Ldlrap1 genes variability in patients with familial hypercholesterolemia in West Siberia using targeted high throughput resequencing. *Atherosclerosis* 287:e285. doi: 10.1016/j.atherosclerosis.2019.06.883

Shakhtshneider, E. V., Ivanoshchuk, D. E., Fishman, V. S., Belokopytova, P. S., Orlov, P. S., Timoshchenko, O. V., et al. (2019). Target sequencing of genes LDLR, APOB, PCSK9, LDLRAP1 and gene cluster APOA5-A4-C3-A1 in patients with familial hypercholesterolemia. *Complex Prob. Cardiovasc. Dis.* 8:71.

Shakhtshneider, E. V., Ivanoshchuk, D. E., Makarenkova, K. V., Orlov, P. S., Timoshchenko, O. V., Bazhan, S. S., et al. (2017a). Cascade genetic screening in diagnostics of heterozygous familial hypercholesterolemia: clinical case. *Russian J. Cardiol.* 6, 178–179. doi: 10.15829/1560-4071-2017-6-178-179

Shakhtshneider, E. V., Makarenkova, K. V., Astrakova, K. S., Ivanoshchuk, D. E., Orlov, P. S., Ragino, Y. I., et al. (2017b). Targeted next-generation sequencing of PCSK9 gene in patients with familial hypercholesterolemia in Russia. *Kardiologiia* 57, 46–51. doi: 10.18565/cardio.2017.6.46-51

Sözen, M. M., Whittall, R., Oner, C., Tokatli, A., Kalkanoğlu, H. S., Dursun, A., et al. (2005). The molecular basis of familial hypercholesterolaemia in Turkish patients. *Atherosclerosis* 180, 63–71. doi: 10.1016/j.atherosclerosis.2004.12.042

Südhof, T. C., Goldstein, J. L., Brown, M. S., and Russell, D. W. (1985). The LDL receptor: a mosaic of exons shared with different proteins. *Science* 228, 815–822. doi: 10.1126/science.2988123

Tatishcheva, Y. A., Mandelshtam, M. Y., Golubkov, V. I, Lipovetsky, B. M., and Gaitskhoki, V. S. (2001). Four new mutations and two polymorphic variants of the low density lipoprotein receptor gene in familial hypercholesterolemia patients from St. Petersburg. *Russian J. Genet.* 37, 1082–1087. doi: 10.1023/A:1011973817437

Voevoda, M. I., Kulikov, I. V., Shakhtshneider, E. V., Maksimov, V. N., Pilipenko, I. V., Tereschenko, I. P., et al. (2008). The spectrum of mutations in the low-density lipoprotein receptor gene in the Russian population. *Russian J. Genet.* 44, 1191–1194. doi: 10.1134/S1022795408100074

Whittall, R. A., Scartezini, M., Li, K., Hubbart, C., Reiner, Z., Abraha, A., et al. (2010). Development of a high-resolution melting method for mutation detection in familial hypercholesterolaemia patients. *Ann. Clin. Biochem.* 47(Pt 1), 44–55. doi: 10.1258/acb.2009.009076

Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., et al. (1984). The human LDL receptor: cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 39, 27–38. doi: 10.1016/0092-8674(84)90188-0

Zakharova, F. M., Damgaard, D., Mandelshtam, M. Y., Golubkov, V. I., Nissen, P. H., Nilsen, G. G., et al. (2005). Familial hypercholesterolemia in St-Petersburg: the known and novel mutations found in the low density lipoprotein receptor gene in Russia. *BMC Med. Genet.* 6:6. doi: 10.1186/1471-2350-6-6

Zakharova, F. M., Golubkov, V. I., Mandelshtam, M. J., Lipovetskii, B. M., and Gaitskhoki, V. S. (2001). Identification of novel missense mutation G571E, novel silent mutation H229H, nonsense mutation C74X, and four single nucleotide polymorphisms in the low-density lipoprotein receptor gene in patients with familial hypercholesterolemia from St. Petersburg. *Russian J. Bioorgan. Chem.* 27, 349–351. doi: 10.1023/a:1012300632671

Zakharova, F. M., Tatishcheva, Y. A., Golubkov, V. I., Lipovetsky, B. M., Konstantinov, V. O., Denisenko, A. D., et al. (2007). Familial hypercholesterolemia in St. Petersburg: diversity of mutations argues against a strong founder effect. *Russian J. Genet.* 43, 1046–1052. doi: 10.1134/S1022795407090116

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frontiers in Genetics

REVIEW
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The Digenic Causality in Familial Hypercholesterolemia: Revising the Genotype–Phenotype Correlations of the Disease

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Genetically inherited defects in lipoprotein metabolism affect more than 10 million individuals around the globe with preponderance in some parts where consanguinity played a major role in establishing founder mutations. Mutations in four genes have been so far linked to the dominant and recessive form of the disease. Those players encode major proteins implicated in cholesterol regulation, namely, the low-density lipoprotein receptor (LDLR) and its associate protein 1 (LDLRAP1), the proprotein convertase substilin/kexin type 9 (PCSK9), and the apolipoprotein B (APOB). Single mutations or compound mutations in one of these genes are enough to account for a spectrum of mild to severe phenotypes. However, recently several reports have identified digenic mutations in familial cases that do not necessarily reflect a much severe phenotype. Yet, data in the literature supporting this notion are still lacking. Herein, we review all the reported cases of digenic mutations focusing on the biological impact of gene dosage and the potential protective effects of single-nucleotide polymorphisms linked to hypolipidemia. We also highlight the difficulty of establishing phenotype–genotype correlations in digenic familial hypercholesterolemia cases due to the complexity and heterogeneity of the phenotypes and the still faulty *in silico* pathogenicity scoring system. We finally emphasize the importance of having a whole exome/genome sequencing approach for all familial cases of familial hyperlipidemia to better understand the genetic and clinical course of the disease.

Keywords: digenic, familial hypercholesterolemia, *LDLR*, *PCSK9*, *APOB*, *LDLRAP1*

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INTRODUCTION

Familial hypercholesterolemia (FH) (MIM#143890) is a common inherited autosomal codominant disease with complete penetrance that is associated with high serum levels of low-density lipoprotein cholesterol (LDL-C) (Soutar and Naoumova, 2007; Castro-Orós et al., 2010; Singh and Bittner, 2015; Tada et al., 2019). The clinical diagnosis of FH is based on a family history of hypercholesterolemia (mainly in children), plasma LDL-C (>250 mg/dL or 7000 nmol/mL), and the presence of tendon xanthomas (Castro-Orós et al., 2010). Other external manifestations of the disease include xanthelasmas and corneal arcus, all of which are due to high LDL-C level

(Khachadurian, 1988; Goldstein et al., 2001). The clinical distinction of FH was based on phenotypic severity of heterozygous and homozygous forms, where the LDL-C levels being two to four times, respectively, when compared to normal conditions (Khachadurian, 1964).

The prevalence of a heterozygous form of FH (HeFH) has a range of 1 in 250–500 people in most countries and is the most prominent genetic disorder leading to an increased risk of early onset of atherosclerotic cardiovascular disease (CVD) (Akioyamen et al., 2017; Pang et al., 2020). Atherosclerotic CVD is clinically manifested as coronary artery disease (CAD) and ischemic stroke, both of which are considered the leading causes of morbidity and death around the world (Ference et al., 2017; Moldovan and Banescu, 2020). However, the homozygous form of FH (HoFH) is rare and has a prevalence range of 1 in 160,000–1,000,000 worldwide (Brautbar et al., 2015; Singh and Bittner, 2015). The prevalence of HeFH has been reported to be higher in some populations with a pronounced founder effect due to high incidence of consanguineous marriages as is the case with the French Canadians, the South Africans, the Lebanese, and the Finns (Khachadurian, 1988; Koivisto et al., 1992; Goldstein et al., 2001). HeFH prevalence in South Africans and populations of some parts of Europe is 1:71 and 1:200, respectively (Steyn et al., 1989; Ito and Watts, 2015). In Lebanon, in contrast, a high reported frequency of HoFH was estimated to be approximately 1:85 in Christian Lebanese (Fahed et al., 2011; Brautbar et al., 2015). A less common autosomal recessive mode of inheritance has been detected in some of the initial families in Lebanon (Khachadurian, 1964).

For decades, familial autosomal dominant hypercholesterolemia (ADH) has been considered as a monogenic disorder in which more than 85% of the cases are mainly caused by mutations in *LDLR* (*low-density lipoprotein receptor*) (MIM#s 606945, 143890) (Pirillo et al., 2017; Tada et al., 2019; Alnouri et al., 2020). The *LDLR* gene is located on the short arm of chromosome 19 (19p13.1–p13.3) and spans around 45,000 base pairs (Brown and Goldstein, 1986). *LDLR* is made up of 18 exons, which encode a protein of 843 amino acids (Brown and Goldstein, 1986; Marais, 2004). More than 1,700 variants have been detected in the gene so far.[1] Distinct mutations in other genes associated with LDL metabolism have also been shown to cause FH, including *APOB* (*apolipoprotein B*) (MIM #107730), *PCSK9* (*proprotein convertase substilin/kexin type 9*) (MIM # 607786), and *LDLRAP1* (*low-density lipoprotein receptor associate protein 1*) (MIM#605747) (Soutar and Naoumova, 2007; Singh and Bittner, 2015). *APOB* encodes two isoforms (apoB-48 and apoB-100) that constitute the protein component of the LDL-C particle and is considered the natural ligand of LDLR (Rader et al., 2003). PCSK9 encodes a convertase that is implicated in the internalization of the LDLR, whereas the *LDLRAP1* gene encodes a phosphotyrosine protein that directly interacts with the cytoplasmic tail of LDLR (Fahed and Nemer, 2011). Mutations in *LDLR* include deletions, insertions, point, and splicing mutations that disrupt the protein function (Wang et al., 2018). Those mutations lead to protein dysfunction through impairing LDL-C clearance from blood or depleting the number of LDLR within cells. Mutations in the *APOB* hinder the binding of apoB protein on the LDL particle with the LDLR and lead to familial defective APOB (FDB) or ADH2 (Damgaard et al., 2004; Vogt, 2015). Besides, a gain-of-function (GOF) mutation in *PCSK9* generates a hyperfunctioning PCSK9 protein, which in turn increases LDLR degradation and causes ADH3 (Vogt, 2015). Conversely, loss-of-function (LOF) mutations lead to an increase in LDLR expression on the cells and diminished LDL-C levels (Nordestgaard et al., 2013; Cohen et al., 2006). Therefore, the incidence of CVD is significantly reduced in individuals with LOF mutations and lower LDL-C levels, which supports the suggestion that low levels of LDL-C from the time of birth are associated with lower cardiovascular risk (Cohen et al., 2006).

Concomitant with the discovery of the additional genes, high-throughput sequencing analysis has revealed a more complicated "polygenic" form of FH with varying degrees of severity, which was further enhanced by the discovery of modifier genes (Fahed et al., 2016; Tada et al., 2019). The latter are defined as genes involved in altering the course of disease where their protein products become immediate targets for therapeutic intervention (Cutting, 2010). While monogenic FH is predominantly caused by a mutation in a single gene, polygenic FH is usually caused by alterations in two genes or more (Narayanaswamy and Sharma, 2020). Oligenic FH has also been introduced and indicates the presence of damaging mutations in the standard FH genes and LDL-altering accessory genes (Tada et al., 2018). The latter comprises multiple low-frequent variants of genes associated with lipid-related autosomal diseases, including LDLRAP1, adenosine triphosphate–binding cassette subfamily G, member 5/8 (ABCG5/8), and apolipoprotein E attributed to a more severe FH phenotype (Tada et al., 2019). Another severe autosomal recessive FH (ARH, MIM ID #603813) is caused by an LOF mutation in *LDLRAP1* (Soutar and Naoumova, 2007). Comparable to the *LDLR* case, some *LDLRAP1* mutations are geographically associated with a noticeable founder effect such as in Europe and Lebanon (Garcia et al., 2001; Wilund et al., 2002; Cohen et al., 2003; Pisciotta et al., 2006a; Quagliarini et al., 2007; Fahed et al., 2016).

Mutations identified in the primary FH-causing genes (including *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1*) show a similar gene dosage effect and share a comparable clinical phenotype, describing a semidominant inheritance pattern (Nordestgaard et al., 2013). Although mutations in these genes have comparable phenotypes, these still vary in severity and LDL-C blood levels (Brautbar et al., 2015). However, the number of mutant alleles does not always reflect the severity of the disease phenotype, and this was revealed by the variable expressivity of mutations in FH (Fahed and Nemer, 2011; Fahed et al., 2016). Yet, the considerable proportion of adults with FH (∼17–33%) without any detectable variation in the four mentioned genes points out to the likelihood of other unknown genes involved in the pathogenesis of FH (Castro-Orós et al., 2010; Paththinige et al., 2017). For this reason, recent studies are suggesting a combinatorial effect of single mutations that can affect various genes in the LDLR pathway and can yield a phenotype intermediate between heterozygous and homozygous. This, in

[1] https://databases.lovd.nl/shared/genes/LDLR

return, reveals that the genetic heterogeneity of FH among various populations can aid in unraveling other unknown genes that might be involved in FH pathogenesis (Wang et al., 2011). However, the variability in expression creates a gap and makes it challenging to establish phenotype–genotype correlations in FH cases. Genotype–phenotype correlations in FH are so far essential as they permit a better clinical evaluation of the severity of phenotype generated by the mutation and its treatment response (Fahed et al., 2011). Usually, physicians use "homozygous" and "heterozygous" terms to designate the phenotype of FH patients. In this case, HoFH and HeFH should refer only to LDL levels and not to the genotype (Fahed and Nemer, 2011). This could be explained by the fact that some heterozygous mutations in FH patients could be very severe, with LDL-C levels being four times more than normal conditions, thus falling under HoFH. At the same time, the opposite could also occur (Fahed et al., 2016). Therefore, the clinical nomenclature of FH was classified into three categories, including (1) severe, (2) mild, and (3) paradoxical. The latter reveals cases with a confusing presentation of FH. Most FH patients receive high-dose statin therapy. However, many of these patients are still unable to achieve the desired lipid levels and therefore require additional treatments, including LDL apheresis (Moyer and Baudhuin, 2015).

Although the clinical features of both heterozygous and homozygous mutations in monogenic FH have significantly been described, very few is known about "double-heterozygous" or digenic FH phenotypes, where we have a combination of mutations between any two of the known FH-causing genes including *APOB, LDLR, PCSK9*, and *LDLRAP1* (Tada et al., 2011; Cuchel et al., 2014). The Consensus Panel on Familial Hypercholesterolemia of the European Atherosclerosis Society has previously stated that the general levels of LDL-C in double-heterozygous carriers of mutations in *APOB*, *PCSK9*, or *LDLR* to be less severely elevated as compared to homozygous carriers of variations in the same genes (Cuchel et al., 2014). However, data in literature supporting this statement are still lacking (Sjouke et al., 2016b). Therefore, we will review all the reported digenic (double-heterozygotes) cases of FH with *LDLR*, *PCSK9*, *APOB*, and *LDLRAP1* mutations. We will also dissect the pathogenicity of the phenotypes and the molecular effect of the digenic interactions if present, in addition to the efficacy of the proposed therapies on improving the phenotypes. Finally, we will highlight the importance of next-generation sequencing (NGS) testing for all FH cases in unraveling new genes involved in this heterogeneous disease.

DIGENIC FAMILIAL HYPERCHOLESTEROLEMIA MUTATIONS

Digenic inheritance (DI), previously termed epistasis, is the simplest form of oligogenic inheritance for genetically complex diseases (Schäffer, 2013; Gazzo et al., 2016). In oligenic FH, multiple rare mutations contribute to a more severe phenotype (Tada et al., 2019). More recently, Schäffer (2013) has preferably introduced DI as when variant genotypes at two genes explain the phenotypes of some patients and their mildly affected or unaffected relatives more clearly than genotypes in one gene, reflecting a reduced penetrance of the disorder. Rarely, in this case, the patients coinherit two separate genetic mutations, also described as "double" or transheterozygosity (Deltas, 2018). The combinatorial interaction of mutations on two genes is needed in this case to cause the disease (Vockley, 2011). There are very few diseases with a digenic mode of inheritance, and FH has shown to be one of them (Deltas, 2018).

Since the discovery of the *LDLR* gene by Brown and Goldstein (1986), numerous pathogenic mutations, including those in the *APOB* and *PCSK9* genes, have been identified along with the advancement and standardization of genetic analysis (Mabuchi, 2017). In monogenic FH, pathogenic mutations in one gene may be responsible for the expression of the disease phenotype, which also explains a defined diagnosis (Deltas, 2018). The known causative mutations in the *LDLR* gene account for the majority of FH cases with a range of 90–95%, along with 5–10% for *APOB*, and a maximum of 3% for *PCSK9* (Benn et al., 2016). Moreover, pathogenic mutations in the *LDLRAP1* gene are infrequent (up to two variants) in patients with FH (Spina et al., 2018). However, FH patients, in extreme cases, might have *LDLR* mutations with LDL-C levels falling within the normal range, which leads to reduced penetrance. The variability in expression of monogenic FH patients carrying the same gene mutation suggests other genetic or environmental interactions (Benlian et al., 1996).

In some cases reported, FH patients displayed digenic mutations with two different heterozygous mutations in two loci at separate genes, including the involvement of *LDLRAP*, *APOB*, or *PCKS9* (Moyer and Baudhuin, 2015; **Figure 1**). Below, we describe all the FH cases reported in the literature with digenic mutations with an emphasis on the phenotype on one hand and the pathogenicity of mutations on the other hand. The outreaching goal is to highlight the minimal significance of the identified genetic variants in explaining the heterogeneity of the disease even when combining the variants together.

According to our findings, *LDLR/APOB* mutations are the most common among these digenic mutations in FH. While *APOB/PCSK9* mutations are still available, they are less common. Despite being rare, the cases mentioned below provide insight into the molecular mechanisms that underlie the clinical and phenotypic variability in cases with digenic mutations in the primary FH-causing genes (**Table 1**).

LDLR/APOB Mutations

Several genetic defects in the *LDLR* gene, which encodes the LDL receptor (LDLR) is the main cause of FH (ADH1). Generally, FH patients with mutations in *LDLR* reveal the most severe phenotypes, including high LDL-C level and a low response to lipid-lowering therapies (LLT) (Fahed and Nemer, 2011). However, *APOB* mutations have incomplete penetrance, and patients with FDB have less severe phenotypes when compared to FH patients having *LDLR* variants. As the LDL-C uptake is lowered upon *LDLR* mutations, a similar pattern is sometimes obtained if there is a mutation in *APOB* (Soufi et al., 2004). For FDB, the p.(R3500Q) variant is the most prevalent, and the first to be identified. The p.(R3500Q) is common in Europe and

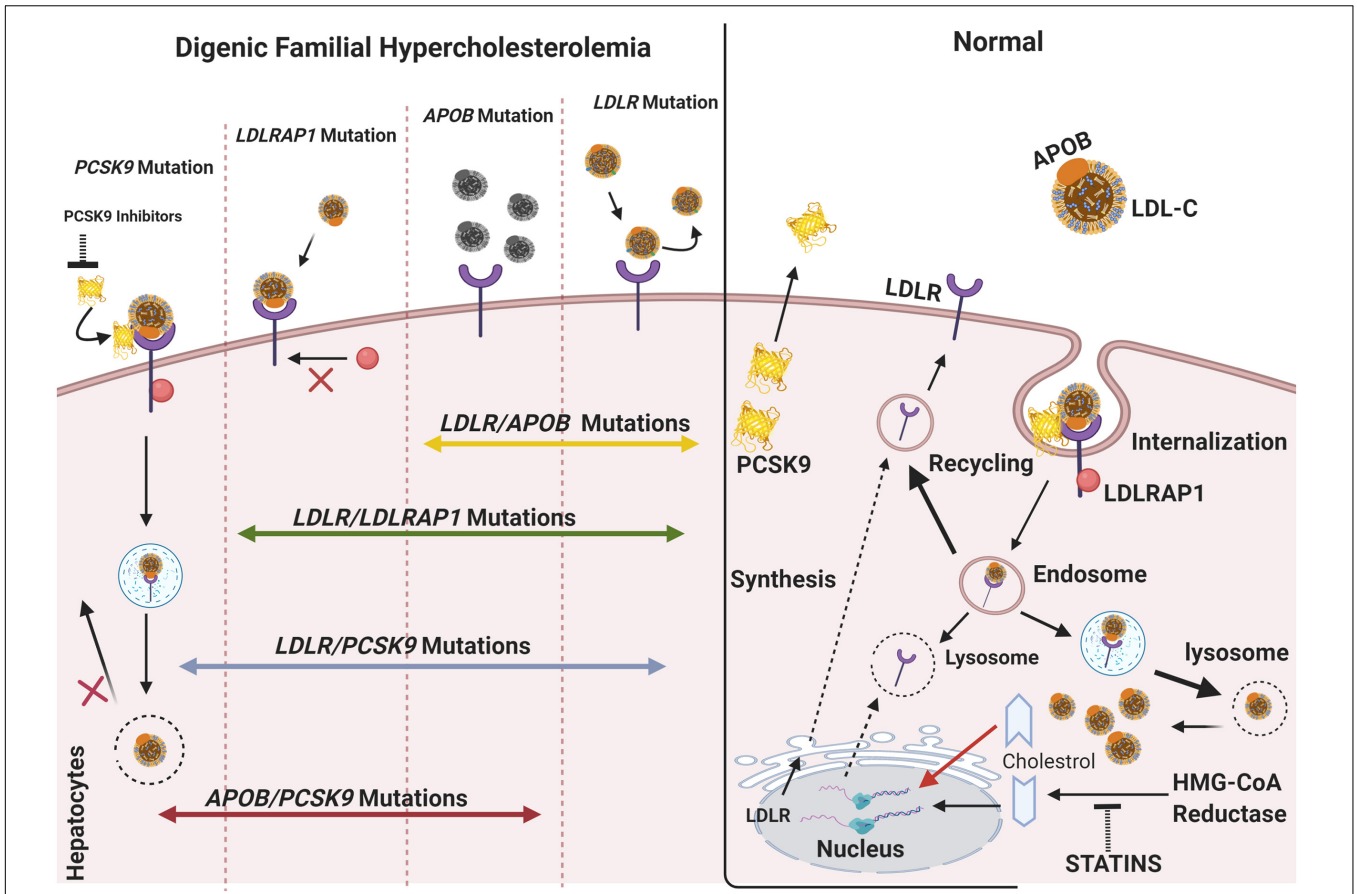

FIGURE 1 | The molecular basis of digenic familial hypercholesterolemia. In normal cases, the LDLR located on the surface of hepatocytes binds APOB/LDL-C forming a complex, which is endocytosed into the cell via the interaction of LDLR with LDLRAP1. As the complex dissociates inside the cell, LDLR usually recycles to the cytoplasmic membrane, and the free cholesterol is consumed inside the cell. Posttranscriptional inhibition of LDLR is mediated by PCSK9 through cell-surface interactions. The transcription of *LDLR* is activated in response to the decrease in cholesterol levels in the cell, such as during treatment with statins. However, an increase in the level of intracellular cholesterol induces ubiquitin-mediated LDLR degradation. In monogenic FH, single mutations in *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1* account for a broad spectrum of phenotypes ranging from mild to severe. *LDLR* mutations affect the binding of LDL-C to LDLR and cause the accumulation of LDL-C in circulation. *APOB* mutations cause protein dysfunction and lead to a limited binding of LDL-C to LDLR. Mutations in *PCSK9*, usually GOF, can account for (1) an increased PCSK9 activity, (2) no LDLR recycling, and (3) accumulation in plasma LDL-C levels. In such a case, anti-PCSK9 therapies, including the emerging PCSK9 inhibitors (e.g., alirocumab, evolocumab), are used to lower LDL-C and prevent CVD. In digenic familial hypercholesterolemia, double-heterozygotes can include an *LDLR* mutation inherited from one parent and a second mutation in *APOB*, *LDLRAP1*, or *PCSK9* inherited from either the same or the other parent. A mutation in *PCSK9* can also occur with another *APOB* mutation. The combination of two mutations in the main FH-causing gene does not account for a more severe phenotype all the time. The phenotype can thus be similar to that of monogenic FH and can cause an impaired LDL-C clearance. The figure was created using https://biorender.com/.

accounts for 2–5% of FH phenotypes (Myant, 1993). Other point mutations in the *APOB*, including p.(R3500W) and p.(R3531C), are rarely occurring, all of which are responsible for impairing the binding of LDL-C to LDLR (Soria et al., 1989; Myant, 1993; Gaffney et al., 1995; Pullinger et al., 1995). FDB patients are assumed to exhibit the same clinical features as FH caused by a defective LDLR (Rauh et al., 1991).

The $Ldlr^{-/-}$ FH mice model revealed a marked elevation in LDL-C and apoB levels and developed acute atherosclerosis upon a chow diet (Powell-Braxton et al., 1998). Patients with compound *LDRL* (ADH) and *APOB* (FDB) mutations are commonly rare, with a prevalence of 1 in 564 (∼0.185), where very few studies have described their occurrence (Myant, 1993; Pirillo et al., 2017). Initially, a study done in 1991 reported an FH double-heterozygous (termed "heterozygous compound") individual with a p.(Arg3500Gln) paternal *APOB* mutation along with a non-reported defective maternal *LDLR*. Unexpectedly, the clinical features of the individual with double mutations were similar to those of family members with either defect, including tendon xanthoma and premature atherosclerosis (Rauh et al., 1991). One explanation for this was that the degree of expression of the mutated *LDLR* and *APOB* in the double-heterozygous individual is different than that of the family members. The prognosis and treatment of this individual were recommended to be different, suggesting that other genes or environmental factors might be affecting this phenotype (Rauh et al., 1991). Similarly, Rubinsztein et al. (1993) reported a South African family who had five double-heterozygous individuals carrying the p.(Asp227Glu)

TABLE 1 | A summary of double-heterozygote FH cases and their characteristics as reported in literature.

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
APOB/LDLR (N=1)	p.Arg3500Gln+LDLR defect (NA)	M	35	7010	Severe (similar to FH and FDB)	NR	NR	Rauh et al., 1991
APOB /LDLR (N=7)	p.Arg3500Gln+p.Asp206Glu	F	48	13600	Intermediate severity with no CAD	Simvastatin 20	25.4%	Rubinsztein et al., 1993; Raal et al., 1997
	p.Arg3500Gln+p.Asp206Glu	F	26	13800	Intermediate severity with no CAD	Simvastatin 20	25.4%	
	p.Arg3500Gln+p.Asp206Glu	F	21	100000	Intermediate severity with no CAD	Simvastatin 20	25.4%	
	p.Arg3500Gln+p.Asp206Glu	F	43	14680	Intermediate severity with no CAD	Simvastatin 20	25.4%	
	p.Arg3500Gln+p.Asp206Glu	M	23	9200	Intermediate severity with no CAD	Simvastatin 20	25.4%	
	p.Arg3500Gln+p.Asp206Glu	F	2	7900	Intermediate severity with no CAD	NR	NR	Rubinsztein et al., 1993
	p.Arg3500Gln+p.Asp206Glu+Val408Met)	M	12	20790	Very Severe with premature atherosclerosis	Partial Ileal bypass surgery	35% reduction in Total cholesterol, Asymptomatic Reduction in LDL-C NR	
APOB/LDLR (N=2)	p.Arg3500Gln +p. Trp66Gly	M	10	15070	Severe	simvastatin 20, cholestyramine 16g	46%	Benlian et al., 1996
	p.Arg3500Gln+p.Glu207Lys	M	55	10630	Severe	simvastatin 40 + cholestyramine 24g	48%	
APOB/LDLR (N=1)	p. Arg3527Gln+p. Gly20Arg	M	48	6260	Mildly severe	10 mg/dl of atorvastatin	43%	DeCampo et al., 2001
APOB/LDLR (N=1)	p.Arg3500Trp+p.Asp407Lys	F	43	9470	Very severe (Exaggerated FH)	Simvastatin 80	49%	Tai et al., 2001
APOB/LDLR (N=1)	p.Arg3527Gln+p.Leu479Pro	F	15	8800	Severe with premature CAD	Atorvastatin 20	34%	Taylor et al., 2010
APOB/LDLR (N=2)	p.Arg3558Cys+p.Tyr19*	M	43	6900	Xanthomas (LDL-c level Similar to LDLR heterozygotes)	NR	NR	Bertolini et al., 2013
	p.Arg3558Cys+p.Tyr19*	M	12	7340	LDL-C level similar to LDLR heterozygotes			

(Continued)

TABLE 1 | Continued

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
APOB/LDLR (N=23)	p.Arg3527Gln+c.31311G>A	M	73	NR	Intermediate Severe	Rosuvastatin 20, Ezetimibe 10	25.4% reduction reported in 5 patients	Sjouke et al., 2016a
	p.Arg3527Gln+c.31311G>A	F	46	NR	Intermediate Severe	Atorvastatin 80, Ezetimibe 10		
	p.Ser4430Thr+p.Trp87Arg	M	37	6000	Intermediate Severe	NR		
	p.Arg3558Cys+p.Trp44*	F	46	NR	Intermediate Severe	Fluvastatin 40, Ezetimibe 10		
	p.Arg3527Gln+ p.Glu228Lys	M	16	8800	Intermediate Severe	Pravastatin 30, Ezetimibe 10		
	p.Arg3527Gln+p.Gly98Ser	M	51	6200	Intermediate Severe	Rosuvastatin 40, Ezetimibe		
	p.Arg3527Gln+p.Gly98Ser	M	24	6800	Intermediate Severe	Rosuvastatin 20		
	p.Arg3527Gln+ p.Asp535Asn	F	61	8200	Intermediate Severe	Rosuvastatin 10		
	p.Arg3527Gln +p.Asp535Asn	F	58	NA	Intermediate Severe	Rosuvastatin 10		
	p.Arg3527Gln+p.Asp535Asn	F	31	7400	Intermediate Severe	Atorvastatin 20		
	p.Arg3500Trp +p.Cys184Arg	F	69	7100	Intermediate Severe	None		
	p.Arg3500Trp+p.Cys184Arg	F	42	13100	Intermediate Severe	Rosuvastatin 40, Ezetimibe 10		
	p.Arg3527Gln+ p.Asn564His/ p.Val800_ Leu802del	M	64	NA	Intermediate Severe	Rosuvastatin 40, Ezetimibe 10		
	p.Arg3527Gln +p.Asn564His/ p.Val800_ Leu802del	F	62	9100	Intermediate Severe	Rosuvastatin 40, Ezetimibe 10		
	p.Arg3527Gln +p.Asn564His/ p.Val800_ Leu802del	M	55	NR	Intermediate Severe	Simvastatin 20, Ezetimibe 10		
	p.Arg3527Gln+p.Asn564His/ p.Val800_ Leu802del	F	36	8700	Intermediate Severe	Rosuvastatin 40		
	p.Arg3527Gln+p.Asn564His/ p.Val800_ Leu802del	M	33	NR	Intermediate Severe	Atorvastatin 40, Ezetimibe 10		
	p.Arg3527Gln +c.31311G > A	F	25	6200	Intermediate Severe	Atorvastatin 40		
	p.Phe807Leufs*10+p.Arg3527Gln	M	6	7400	Intermediate Severe	Pravastatin 40		

(Continued)

TABLE 1 | Continued

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
	p.Arg3527Gln +p.Ala705Pro	F	32	9600	Intermediate Severe	Atorvastatin 80, Ezetimibe 10		
	p.Arg3527Gln+p.Ser306Leu	M	70	NR	Intermediate Severe	Rosuvastatin 10, Ezetimibe 10, Cholestagel 2500, Gemfibrozil 900		
	p.Gln4376*+c.19014A >T	F	53	NR	Intermediate Severe	Atorvastatin 80, Ezetimibe 10		
	p.Arg3558Cys+p.Cys255Arg	F	57	NR	Intermediate Severe	Atorvastatin 40		
APOB/LDLR (N=5)	p.Arg3527Trp+p.Trp490Cys	NR	NR	NR	NR	NR	NR	Pirillo et al., 2017
	p.Arg3527Trp+p.Asp579Asn	NR	NR	NR	NR	NR	NR	
	p.Arg3527Trp+ p.Asp221Gly	NR	NR	NR	NR	NR	NR	
	p.Arg3527Trp+p.Asp221Gly	NR	NR	NR	NR	NR	NR	
	p.Arg3527Trp+p.Asp221Gly	NR	NR	NR	NR	NR	NR	
APOB/LDLR (N=6)	p.Arg3527Gln+c.1846-?_21401?del	F	59	4315	High CVD risk	Rosuvastatin 20, Ezetimibe, Alirocumab 75/150 mg Q2W	39.3% to 55.7%	Hartgers et al., 2018
	p.Arg3527Gln+ .2390-?_25831?del	F	50	4008	High CVD risk	Rosuvastatin 40, Alirocumab 150 mg Q2W	39.3% to 55.7%	
	Arg3527Gln+p.Asp227Glu	F	69	7500	High CVD risk	Atorvastatin 80, Ezetimibe, Alirocumab 75/150 mg Q2W	55.1% to 62.0%	
	p.Arg3527Gln+p.Cys209Tyr	F	47	3879	High CVD risk	Rosuvastatin 40, Ezetimibe, Alirocumab 75/150 mg Q2W	55.1% to 62.0%	
	p.Arg3527Gln+p.Tyr375Trpfs*7	F	58	6956	High CVD risk	Atorvastatin 80 mg Ezetimibe, fish oil, Placebo	NR	
	p.Arg3527Gln+p.Gly478Arg	M	54	4215	High CVD risk	Atorvastatin 80, Ezetimibe, nicotinic acid, Placebo	NR	

(Continued)

TABLE 1 | Continued

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
APOB/PCSK9 (N=1)	p. Ala3396Thr +p. Arg96Cys	M	NR	11378	Severe with a history of MI	Statin treatment	74%	Elbitar et al., 2018
APOB/LDLR* or *LDLR/PCSK9 (N=13)	NR	NR	46 ± 12	6890 ± 1120	Mild, 4 patients with CAD family history	8 treated with Statins	NR	Sun et al., 2018
LDLR/PSKC9 (N=3)	p.Glu228Lys+p.Arg496Trp	F	35	1152	Severe (similar to homozygous FH), Xanthomas and CHD	Atorvastatin 40 alone or more recently in combination with ezetimibe 10, and selective LDL-apheresis (twice a month)	∼ 65–70%	Pisciotta et al., 2006a; Bertolini et al., 2013
	p.Tyr419*+p.Asn425Ser	F	48	13310	Severe (similar to homozygous FH), xanthomas	Simvastatin 80, cholestyramine (24 g/day)	40%	
	p.Tyr419*+p.Asn425Ser	F	62	10470	Severe (similar to homozygous FH)		40%	
LDLR/PCSK9 (N=5)	c.191-2A.G+ p.Arg476Cys	M	51	10300	Intermediate severe (as compared to heterozygous, homozygous and compound heterozygotes)	Simvastatin 80, Ezetimibe 10	40.7%	Sjouke et al., 2016a
	c.1912A.G+p.Arg476Cys	M	79	NR	Intermediate severe (as compared to heterozygous, homozygous and compound heterozygotes)	NR	NR	
	c.191-2A.G+p.Arg476Cys	M	83	NR	Intermediate severe (as compared to heterozygous, homozygous and compound heterozygotes)	Atorvastatin 40, Ezetimibe 10	NR (LDL-C treated= 3200 nmol/mL)	
	p.Val429Met+p.Ala53Gly	F	64	NR	Intermediate severe (as compared to heterozygous, homozygous and compound heterozygotes)	Atorvastatin 80, Ezetimibe 10, cholestagel 2500	NR (LDL-C=3600nmol/mL)	

(Continued)

TABLE 1 | Continued

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
	p.Val429Met+ p.Ala53Gly	M	60	14800	Intermediate severe (as compared to heterozygous, homozygous and compound heterozygotes)	Rosuvastatin 40, Ezetimibe 10	76.35%	
LDLR/PCSK9 (N=17)	c.1187-?_2140þ?dup p.(Gly396_Thr713dup)+c.1327G>A p.(Ala443Thr)	NR	NR	NR	NR	NR	NR	Pirillo et al., 2017
	c.1246C>T (2 subjects) p.(Arg416Trp) +C.137G>T p.(Arg46Leu) LOF variant	NR	NR	NR	NR	NR	NR	
	c.1257C>G p.(Try419*) c.60_65dupGCTGCT (c.61_63triCTG) p.(Leu21tri)	NR	NR	NR	NR	NR	NR	
	c.1257C>G p.(Try419*) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.126C>A p.(Try42*) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.1646G>A p.(Gly549Asp) c.60_65dupGCTGCT (c.61_63triCTG) p.(Leu21tri)	NR	NR	NR	NR	NR	NR	
	c.1783C>T p.(Arg595Trp) c.60_65dupGCTGCT (c.61_63triCTG) p.(Leu21tri)	NR	NR	NR	NR	NR	NR	
	c.1846-?_2583þ?del (2 subjects) p.0 Pathogenic C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.2215C>T p.(Gln739*) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.2312-3C>A p.(Ala771_Ile796del) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.352G>T p.(Asp118Tyr) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.373C>T (HO) p.(Gln125*) c.60_65dupGCTGCT (c.61_63triCTG) p.(Leu21tri)	NR	NR	NR	NR	NR	NR	
	c.418G>T p.(Glu140*) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.418G>T p.(Glu140*) c.60_65dupGCTGCT (c.61_63triCTG) p.(Leu21tri)	NR	NR	NR	NR	NR	NR	
	c.662A>G p.(Asp221Gly) C.137G>T p.(Arg46Leu)	NR	NR	NR	NR	NR	NR	
	c.788A>G p.(Asp263Gly) NEW c.-331C>A	NR	NR	NR	NR	NR	NR	
	c.1547G>A p.(Gly516Asp) c.60_65dupGCTGCT (c.61_63triCTG) p.(Leu21tri)	NR	NR	NR	NR	NR	NR	
LDLR/PCSK9 (N=1)	p.Cys143+p.Leu22_Leu23dup	M	54	3157	NR	Rosuvastatin 40, ezetimibe, alirocumab 150, Q4w	NR	Hartgers et al., 2018

(Continued)

TABLE 1 | Continued

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
LDLR/PCSK9 (N=1)	p.I531TfsX15+ V4I	M	38	7030	Severe with ST MI, Achilles tendon Xanthoma, prone to CAD	Rosuvastatin 10, Ezetimibe 10, Evolocumab 140 mg administred every two weeks	∼ 85.2 %	Shirahama et al., 2018
LDLR/LDLRAP1 (N=4)	p.Q233P+p.Q136X	M	28	14680	Severe	Atorvastatin 80, 12 g cholestyramine 10, Ezetimibe, and LDL Aspheresis	Target was not reached by drugs, Therefore, they did aspheresis	Soufi et al., 2013
	p.Q233P+p.Q136X	M	2	12920	Severe			
	p.Q233P+p.Q136X	M	1	9150	Severe			
	p.Q233P+p.Q136X	F	24	5570	Severe			
LDLR/LDLRAP1 (N=3)	(c.2431A>T)+(c.606dup)	F	79	8223	Severe, xanthomas, Coronary and aortic vulvar Disease	Statin Therapy, suspended during this study	NR	Tada et al., 2011
	(c.2431A>T)+(c.606dup)	M	45	60700		Statin Therapy, suspended during this study	NR	
	(c.2431A>T)/(c.606dup)	F	32	7732		Statin Therapy, suspended during this study	NR	
LDLR/LDLRAP1 (N=4)	c.1255 T > G, p.(Y419D)+ [c.604_605delTCinsA, p.(S202Tfs*2)].	F	45	12490	Severe, STEMI, xanthomas, CAD family history	Rosuvastatin, Ezetimibe	Lowered, but not as recommended by ESC	Alnouri et al., 2018
	c.1255 T > G, p.(Y419D)+ [c.604_605delTCinsA, p.(S202Tfs*2)].	M	17	5500	Severe, CAD family history	None	None	
	c.1255 T > G, p.(Y419D)+ [c.604_605delTCinsA, p.(S202Tfs*2)].	M	14	NA	Severe, CAD family history	None	None	
	c.1255 T > G, p.(Y419D)+ [c.604_605delTCinsA, p.(S202Tfs*2)].	F	8	5.3	Severe, CAD family history	None	None	

(Continued)

TABLE 1 | Continued

Digenic Mutation/ Number of Individuals Identified (N)	Nomenclature of Variants identified	Gender	Age, years	Untreated LDL-C (nmol/mL)	Phenotype severity	Treatment	Response to treatment (%LDL-C reduction)	References
LDLR/LDLRAP1 (N=9)	*LDLR* hetero C681+*LDLRAP* hetero Q136* (N=2)	NR	NR	~6150	No universal pattern identified, Xanthomas (N=1)	NR	NR	Fahed et al., 2016
	LDLR homo C681*+*LDLRAP* hetero Q136* (N=1)	NR	NR	12852	No universal pattern identified Xanthomas, Xanthelasmas	NR	NR	
	LDLR hetero A391T + *LDLRAP* hetero Q136* (N=4)	NR	NR	4500	No universal pattern identified	NR	NR	
	LDLR hetero A391T + *LDLRAP* homo Q136* (N=1)	NR	NR	15050	No universal pattern identified	LDL Aspheresis	NR	
	LDLR hetero C681* + *LDLR* hetero A391T + LDLRAP hetero Q136 (N=1)	NR	NR	7861	No universal pattern identified	NR	NR	
LDLR/LDLRAP1 (N=1)	p.(Glu228Lys)+ p.(Glu250Glyfs*4)	NR	NR	NR	NR	NR	NR	Pirillo et al., 2017

NR: not reported; Drug doses are in mg/day; CVD: Cardiovascular disease; Q2W, every 2 weeks; Q4W, every 4 weeks, MI: Myocardial Infarction, HoFH: homozygous FH; ESC: European Society of Cardiology; STEMI: ST-elevation myocardial infarction.

LDLR and p.(Arg3527Gln) *APOB* mutations, and one "complex" heterozygote who also accounted for a p.(Val429Met) *LDLR* mutation. The double-heterozygous carriers had lipid levels and clinical features that were intermediate in severity between heterozygous and homozygous FH. The daily treatment of these individuals with Simvastatin 20 mg for 6 weeks reduced the LDL-C level by 25.4% (Raal et al., 1997). Also, Benlian et al. (1996) reported a double-heterozygote mutation in *LDLR* and *APOB* in two unrelated French patients with no family history for atherosclerosis. These patients were described as a "new class" of patients with digenic lipid disorders, defined by specific clinical features that result from the combined effects of two independent loci. The two patients were double-heterozygous for the p.(Arg3500Gln) *APOB* mutation and either the p.(Trp66Gly) or p.(Glu207Lys) *LDLR* mutations reflecting an unusual phenotype of "aggravated hypercholesterolemia." The two missense mutations in LDLR were previously detected in a French Canadian cohort, and the p.(Arg3500Gln) *APOB* is a founder mutation in Northern Europe (Leitersdorf et al., 1990; Benlian et al., 1996). Interestingly, their phenotypes were different than those of their heterozygous FH relatives and the homozygous FH and FDB patients. The reported phenotype was also complicated with premature CAD, although remaining responsive to lipid-lowering drugs. The latter included daily uptake of simvastatin 20 mg along with 16 g of cholestyramine. This treatment managed a long-term decrease in the level of plasma lipids (Benlian et al., 1996). The observed phenotype of aggravated hypercholesterolemia gives further evidence that the *LDLR* and *APOB* play distinct roles in regulating the metabolism of LDL. Although both phenotypes were severe, one case expressed a milder phenotype compared with the other, which represented a disease closer to that reported in FH homozygotes carrying the p.(Trp66Gly) mutation (Moorjani et al., 1993). One explanation for this is that the clinical variability of FH depends on the type of mutation (Hobbs et al., 1992; Kotze et al., 1993). It was reported that p.(Glu207Lys) *LDLR* mutation reduces the rate of transport of native LDLR receptors to the surface of the cell, while the p.(Trp66Gly) *LDLR* mutation causes severe functional defects *in vitro* (Leitersdorf et al., 1990; Hobbs et al., 1992). Another explanation for this phenotype is that the abnormal presence of defective LDLR *in vivo* affected the expression of the wild-type allele in FH heterozygotes carrying the p.(Trp66Gly) *LDLR* mutation (Moorjani et al., 1993). At the molecular level, these patients suggested to produce a functional *LDLR* and an apoB ligand. However, they should have a particular therapeutic intervention (Benlian et al., 1996).

However, DeCampo et al. (2001) reported a double-heterozygous case carrying the p.(Arg3500Gln) *APOB* and p.(Gly2Arg) *LDLR* mutations. Yet, his plasma LDL-C level was intermediate when compared to other family members with biallelic mutations. The daily treatment with 10 mg/dL of atorvastatin returned his plasma LDL-C to normal (DeCampo et al., 2001). Another study done by Tai et al. (2001) reported a double-heterozygous case from an Asian family carrying the p.(Asn428Lys) *LDLR* and p.(Arg3527Trp) *APOB* mutations. The detected proband had a pretreatment serum LDL-C level approximately twice as high as her siblings. The reduction

in LDL-C level after the treatment was similar to the two cases reported by Benlian et al. (1996) and Tai et al. (2001) before. The p.(Asp407Lys) mutation in the *LDLR* gene of this patient falls within exon 9. It thus affects the epidermal growth factor (EGF) precursor homology domain of the LDLR protein, encoded by exons 7–14. On the other hand, the positively charged lysine residue found in the detected *LDLR* variant might be affecting the recycling of the receptor (Tai et al., 2001). The p.(Arg3500Trp) *APOB* variant detected in this patient was previously identified in an Asian population and was causing an LOF of *APOB*, a phenotype similar to that produced by p.(Arg3500GLn) *APOB* variant (Choong et al., 1997; Tai et al., 2001). The double *LDLR/APOB* heterozygotes with exaggerated hypercholesterolemia phenotype were still responsive to lipid-lowering treatments. In the same year, another double-heterozygous *LDLR/APOB* carrier was detected and diagnosed with a mild FH phenotype. Even though the double-heterozygous patient carried two variants, his LDL-C levels were lower than that of patients with double mutations in the same *APOB*. Of great interest, the LDL-C level in the patient carrying the p.(G-2) *LDLR* variant, which is located in exon 1, and even in combination with in the (B3500) *APOB* locus, was normalized after being treated with a low dose of statin. This reduction in LDL-C was similar to that of two cases reported previously (Benlian et al., 1996; Tai et al., 2001).

Moreover, Taylor et al. (2010) reported a 15-year-old female carrying p.(Leu479Pro) *LDLR* and p.(Arg3527Gln) *APOB* mutations. The paternal and maternal *LDLR/APOB* variants, respectively, were confirmed by cascade testing (Umans-Eckenhausen et al., 2001; Hadfield et al., 2009). Consequently, the combined effect of both *LDLR/APOB* mutations resulted in a phenotype that is more severe than that of each mutation alone and of homozygous FDB. However, the severity of p.(Leu479Pro) *LDLR* and that of p.(Arg3527Gln) *APOB* variants were less than that of HoFH. The presence of two mutations was only identified with genetic screening and was not clinically assumed given the notable response of the case to statins (Taylor et al., 2010). Bertolini et al. (2013) also detected two related double-heterozygous FH cases carrying the p.(Y419*) *LDLR* and p.(R3558C) *APOB* mutants. Unexpectedly, the LDL-C level of these cases was similar to that observed in the p.(Y419*) *LDLR* heterozygotes (Bertolini et al., 2013). More recently, a wide genetic screening study carried out on affected family members of patients with ADH identified 23 double-heterozygous carriers of the *LDLR/APOB* variants in a cohort of 17 unrelated families from the Netherlands. The levels of LDL-C before treatment were higher in these patients than those of the heterozygotes or the unaffected relatives (Sjouke et al., 2016b). The LDL-C level, however, was significantly lower than that of those patients with homozygous and compound heterozygous *LDLR* variants. Significantly, CVD was present in only 36% of the double-heterozygous *LDLR/APOB* carriers, a value similar to that of homozygous and compound heterozygous ADH (Sjouke et al., 2016b). Although *APOB* mutations are an infrequent cause of FH in Italy, five double-heterozygous carrying the pathogenic *LDLR/APOB* variants were detected in an Italian cohort of 1,076 individuals (Pirillo et al., 2017). The phenotypes of double-heterozygote patients and their response to LLT were not reported by the study. Recently, the sequencing of a cohort of 1,191 patients with FH identified six double-heterozygous patients carrying *APOB/LDLR* mutations. Three of six patients were found to be *APOB*-defective/*LDLR*-negative, and the other three patients were *APOB*-defective/*LDLR*-defective (Hartgers et al., 2018). All patients had a high CVD risk at baseline. The main objectives of the work were to study the efficacy of alirocumab, a PCSK9 antibody, on patients with double-heterozygous FH. For this reason, four patients received alirocumab treatment in addition to a concomitant statin, and two were placebo-treated. The inhibition of PCSK9 with the monoclonal antibody alirocumab reduces LDL-C levels by increasing the level of LDLRs on the liver cell surface, resulting in an increased uptake of the LDL particles. Upon alirocumab treatment, the LDL-C levels dropped down in patients with *APOB*-defective/*LDLR*-negative and *APOB*-defective/*LDLR*-defective at weeks 12 and 24, respectively. These results reveal that double-heterozygous *APOB/LDLR* mutations appeared not to influence the efficacy of alirocumab (Hartgers et al., 2018). Although statins are the first choice for treating FH, PCSK9 inhibitors (including alirocumab) are recently showing more significant LDL-C and atherosclerosis, reducing actions in FH patients.

Usually, the presence of both *LDLR* and *APOB* mutations should be considered in two cases. First, when there is an *LDLR* mutation in some but not all members of a family who exhibit FH, and second, in individuals who have the same *LDLR* mutation but show an exaggerated phenotype compared with other members of the family (Taylor et al., 2010). The severity of the FH phenotype, in addition to CVD risk, may be associated with the combined effect of two pathogenic variations in the two primary candidate genes, *LDLR* and *APOB*. The few studies on the double-heterozygous FH cases carrying both *LDLR* and *APOB* variants reveal that the clinical phenotype is more severe when compared to the simple heterozygous FDB and ADH forms. However, it appears that the phenotype is intermediate between that observed in heterozygous and homozygous FH (Rubinsztein et al., 1993; Benlian et al., 1996; Tai et al., 2001).

APOB/PSCK9 Mutations

The *PCSK9* gene encodes an enzyme that binds to the epidermal growth factor-like repeat A (EGF-A) to promote the degradation of the LDLR in the lysosome of the cell (Sharifi et al., 2017). *PCSK9* has been heavily investigated in many FH populations where a list of more than 161 variants has been identified along with the 12 exons of the gene. The severity of the phenotype is associated with the type of mutation. *PCSK9* LOF mutations are more likely to be associated with a decrease in the cholesterol levels, whereas GOF mutations can lead to a more severe FH associated with a decrease of LDLR on the surface of the cells. Both *in vitro* and *in vivo* studies have significantly aided in unraveling the pathophysiological function of the *PCSK9* gene in human biology in recent years (Lambert et al., 2012). Since the subsequent discovery of the GOF *PCSK9* mutations that result in FH, many research studies have been published about the clinical features and genotypes of patients with ADH. Many

PCSK9 single-nucleotide polymorphisms (SNPs) have also been discovered that have a differential effect on cholesterol regulation in different populations (Fahed and Nemer, 2011). As *PCSK9* promotes LDLR degradation and prevents its recycling to the membrane, studies are now focusing on PCSK9 inhibitors as an emerging safe therapy for dyslipidemia supported by the fact that LOF mutations in *PCSK9* are associated with reduced LDL-C levels and lower CHD risk (Cohen et al., 2006; Benn et al., 2010; Lambert et al., 2012). So far, very few studies have reported digenic mutations in *PCSK9* and other FH genes such *as LDLR* and *APOB*.

The first and only reported *APOB/PCSK9* case was identified in a patient with FH patient from a French cohort with a novel digenic p.(Ala3396Thr) *APOB*/p.(Arg96Cys) *PCSK9* mutations. The patient presented with severe ADH, including an arcus cornea despite treatment with 10 mg of rosuvastatin and ezetimibe. Despite treatment, the detected *APOB/PCSK9* variants resulted in an elevation of total cholesterol (TC) (201 mg/dL; 5,200 nmol/mL) and LDL-C (130 mg/dL; 3,400 nmol/mL) levels (Elbitar et al., 2018). p.(Ala3396Thr). *APOB* had no phenocopy and was shown to have complete penetrance. *In silico* analysis showed that alanine (Ala) at position 3396 in apoB is highly conserved among different species and could be disease-causing. Exome sequencing showed that no one of the other family members carried the *PCSK9* variant. In this study, *in silico* and functional analysis studies were performed to detect the pathogenicity of the detected variants. The *APOB* variant was confirmed to be pathogenic as it affected the apoB binding site to LDLR, suggesting the disruption of apoB–LDLR interaction and LDL internalization (Elbitar et al., 2018).

Also, the identified *PCSK9* variant revealed a 60% increase in the PCSK9 cellular level *in vitro* when compared with the wild-type and to the well-characterized p.(Ser127Arg) GOF variant. This indicates that the novel identified p.(Arg96Cys) is an active GOF mutant as its overexpression results in higher LDLR degradation than the wild-type, causing LDL-C accumulation. Yet, the p.(Arg96Cys) can alone cause ADH as previously reported while aggravating the phenotype when being expressed with another mutant causing ADH (Hopkins et al., 2015; Elbitar et al., 2018).

As studying the effect of the mutations on protein–protein interactions (PPIs) is still lacking in most of the reported studies, we believe that investigating PPIs can be fundamental in understanding the phenotypic changes as the functional interactions are created or disrupted. As other FH cases carrying both *APOB/PCSK9* variants might exist, the molecular identification of FH double-heterozygotes is still essential through genetic cascade screening to provide proper diagnosis and treatment for FH.

LDLR/PCSK9 Mutations

PCSK9 has been identified as an FH modifier gene as it generates significant variable phenotypes even in patients having the same mutation in LDLR. Nowadays, many studies have put looking for *PCSK9* mutations on the top of *LDLR* variants (Abifadel et al., 2009). Mutations in the LDLR protein are distributed through the domains and can lead to various types of dysfunctions. For instance, null mutations generate no LDLRs. In other cases, defects in LDLR can affect its localization to the nuclear membrane, internalization into the cell after binding to LDL-C, or binding to LDL-C particle (Brown and Goldstein, 1986).

It is well known now that homozygous or compound heterozygous FH patients with *LDLR* mutations or double-heterozygotes carrying *LDLR* and p.(R3500Q) *APOB* mutations have more severe phenotypes when compared to the heterozygote FH cases carrying only one mutation in any of the mentioned genes or for missense mutations in the *PCSK9* gene. It was not very clear whether double-heterozygous FH cases carrying *LDLR*/*PCSK9* mutations can lead to similar severe phenotype until Pisciotta et al. (2006a) reported two unrelated double-heterozygous FH carrying *LDLR/PCSK9* mutations with clinical phenotype and family history similar to that of the HoFH. One of the patients carried the two novel p.(N425S) *PCSK9* and p.(Y419X) *LDLR* mutations. The other patient was a carrier of a previously reported variant p.(E228K) *LDLR* variant and a novel p.(R496W) *PCSK9* variant. The cases presented in this study revealed more severe phenotypes as their LDL-C levels were 56 and 44% higher than those relatives who were just carriers of the LDLR heterozygous mutation. However, the *LDLR/PCSK9* double-heterozygotes displayed premature CAD, xanthomatosis, and carotid atherosclerosis (Pisciotta et al., 2006a). As previously reported, the p.(E228K) *LDLR* variant was speculated to produce a severe defective *LDLR* with <2% residual receptor activity, which was similar to the severity of the phenotype of this patient (Hobbs et al., 1992). The novel p.(R496W) *PCSK9* variant, which was also present in the patient's HeFH mother (Bertolini et al., 2004), suggested the high pathogenicity of the *PCSK9* variant might be involved in decreasing the LDLR activity. As the *PCSK9* missense mutations affect the highly conserved amino acids of the proteins, it is suggested that those mutations might be affecting the functional properties of the PCSK9 protein. This implies that a missense mutation in *PCSK9* along with another *LDLR* mutation might lead to a more severe clinical FH phenotype (Taylor et al., 2010; Pisciotta et al., 2006a). Unpredictably, the *LDLR/PCSK9* double-heterozygotes responded to LLT and obtained up to 70% reduction in LDL-C, a level that is rarely attained with the HoFH patients (Pisciotta et al., 2006a). Moreover, in another study with a total of 28 double-heterozygotes, five cases were identified as carriers of *LDLR/PCSK9* mutations. Three patients carried the c.(191-2A. G) *LDLR*/p.(Arg476Cys) PCSK9 variants, and two carried the p.(Val429Met) *LDLR*/(p. Ala53Gly) *PCSK9* variants. Although *LDLR/PCSK9* individuals had higher LDL-C levels than heterozygotes and unaffected individuals, their TC and LDL-C levels were similar to the carriers of homozygous or compound heterozygous *LDLR* mutations (Sjouke et al., 2016b). However, this came in contrast with a previous study that reported the LDL-C levels of *LDLR/PCSK9* double-heterozygous carriers were less severe than those of HoFH (Cuchel et al., 2014).

Another severe double-heterozygous *LDLR/PCSK9* FH case was also recently reported. The patient was identified with p.(Cys143) *LDLR* and p.(Leu22_Leu23dup) *PCSK9* variants. After 12 weeks of treatment with 150 mg alirocumab, a PCSK9 inhibitor, the patient was highly responsive to the treatment

(Hartgers et al., 2018). The inhibition of PCSK9 by alirocumab (monoclonal antibodies) reduces the LDL-C level through increasing the level of LDLR on hepatocytes. In support of previous studies, the results obtained suggest that *PCSK9* GOF variants do not impair alirocumab efficacy (Hopkins et al., 2015; Hartgers et al., 2018). In another recent study, a double-heterozygous *LDLR*/p.(V4I) *PCSK9* FH patient received a PCSK9 antibody associated with coronary plaque regression due to coronary artery syndrome. In addition to CAD, the clinical manifestations included very high levels of LDL-C and extensive xanthomas. The combination of an *LDLR* mutation with a p.(V4I) *PCSK9* mutation in clinically diagnosed FH yields a severe phenotype and makes the patient to be more prone to CAD (Ohta et al., 2016; Shirahama et al., 2018). Lipid-lowering drugs, including rosuvastatin (20 mg) and ezetimibe (10 mg), were not sufficient to achieve a reduction as per the guidelines. Therefore, evolocumab (monoclonal PCSK9 antibody) was also introduced and reduced the LDL-C level (Shirahama et al., 2018).

As PCSK9 inhibits LDLR recycling, which decreases the number of LDLR on the cell surface, inhibiting PCSK9 protein can increase the number of available LDLR protein on the cell surface and increase in return the uptake of LDL-C into the cell (Handelsman and Lepor, 2018). Numerous studies conducted on cells, humans, and animals showed that LOF *PCSK9* mutations could elevate the level of hepatic *LDLR* and thus decrease the amount of LDL-C in circulation (Seidah, 2017). *PCSK9* knockout (KO) mice revealed a hypocholesterolemia phenotype, yielding an 80% decrease in LDL-C. Besides, the KO mice displayed a better response to statins and had a reduced risk for atherosclerosis (Rashid et al., 2005; Zaid et al., 2008; Denis et al., 2012). However, an opposite phenotype was observed in transgenic mice and pigs overexpressing the wild-type *PCSK9* or its GOF mutant p.(D347Y) (Denis et al., 2012; Al-Mashhadi et al., 2013, 2015). Therefore, the promising outcomes of novel therapeutic approaches using PCSK9 inhibitors can improve the severity of the FH phenotype through lowering LDL-C, thus preventing the progression of CAD.

LDLR/LDLRAP Mutations

The *LDLRAP1* gene, located on the short arm of chromosome 1, encodes the cytoplasmic LDLRAP1 protein that acts as an adaptor for LDLR endocytosis in hepatic cells (Sharifi et al., 2017). Usually, mutations in this gene cause premature truncations of the protein, thus leading to the disruption of LDLR. In these cases, the LDL-C level is commonly intermediate between homozygote and heterozygote ADH patients (Austin et al., 2004; Michaely et al., 2004). Mainly, null mutations in *LDLRAP1* can lead to ARH (Garcia et al., 2001). There are some phenotypic differences between ARH and FH-causing *LDLR* mutations, where ARH is typically less severe and more responsive to LLT (Soutar and Naoumova, 2007). However, there is tremendous phenotypic variability among the ARH patients in general, and ARH patients of the same family (Pisciotta et al., 2006b). As ARH is usually uncommon, there are few published data on heterozygous carriers of *LDLRAP1* variants. Data on the clinical manifestation of having *LDLR*/*LDRAP1* double-heterozygotes are also rare. Until now, only four studies have reported cases with digenic mutations in *LDLR* and *LDLRAP1* genes.

The first case of *LDLR/LDLRAP1* was reported in a cohort of 146 individuals where three Japanese FH patients of the same family were double-heterozygous for a nonsense *LDLR* p.(Lys790*) variant and a frameshift p.(Lys204-Glufs*17) *LDLRAP1* variant. The latter was previously reported by the same group to cause elevated LDL-C levels when compared to individuals not carrying this mutant. Only one patient out of three had severe FH phenotype reflected by extensive xanthomas and coronary and aortic valvular disease. However, the LDL-C levels in this patient and the other two patients were similar to individuals with single *LDLR* mutations. Remarkably, the family members showed different LDL-C levels, which suggests the presence of another unknown player (Tada et al., 2011). Soufi et al. (2013) identified four other *LDLR/LDLRAP1* double-heterozygotes in a Turkish FH family. The patients carried the p.(Q136*) *LDLRAP1* homozygous variant along with a p.(Q254P) *LDLR* heterozygous variant. The double-heterozygotes needed weekly apheresis as they did not reach the target LDL-C level by lipid-lowering drugs. The severity of phenotype in the double-heterozygotes was manifested by the presence of xanthomas and CVD. In previous studies, the detected mutation in *LDLR* has shown to affect the ligand-binding domain, while the *LDLRAP1* mutant was generating a truncated protein with a missing phosphotyrosine-binding domain that is needed for LDLR internalization (Garcia et al., 2001; Guardamagna et al., 2009). The authors thus concluded that the combination of the heterozygous *LDLR* and the homozygous *LDLRAP1* variants leads to a more severe phenotype compared to that of homozygous *LDLR* variant alone (Soufi et al., 2013).

Additionally, Fahed et al., 2016 reported Lebanese family members with FH who were double-heterozygotes. The patients carried a p.(Q136*) *LDLRAP1* variant along with homozygous and heterozygous mutations in *LDLR* including p.(C681*), p.(H327fsX5), p.(A391T), and p.(I451T). The prevalence of *LDLRAP1* was unraveled as another founder mutation in the Lebanese population as all the affected family members carried the p.(Q136*) *LDLRAP1* variant. The patients with these mutations, however, revealed variable phenotypic expressivity ranging from mild to severe along with either standard or high LDL-C levels. Although no universal pattern has been identified in this study, the arrangement of the homozygous variant of a gene with a heterozygous variant of another gene accounted for a severe dramatic increase in the levels of LDL-C. Although the occurrence of three heterozygous variants did not similarly increase the levels of LDL-C (Fahed et al., 2016).

Recently, Alnouri et al. (2018) identified four double-heterozygous *LDLR/LDLRAP1* individuals, a mother and three of her children in a Saudi cohort. The reported digenic mutations included the novel p.(Y419D) *LDLR* and p.(S202Tfs*2) *LDLRAP1* variants. The double-heterozygous mother showed a severe FH phenotype (including high LDL-C, xanthomas, and CAD), suggesting that that the additional *LDLRAP1* mutation leads to a more severe phenotype for xanthoma and atherosclerotic CVD in FH patients. Upon using the lipid-lowering drugs,

rosuvastatin (40 mg) and ezetimibe (10 mg), the patient's LDL-C decrease did not reach the optimal level recommended by the European Society of Cardiology. Surprisingly, her children with the same mutations had no clinical manifestations. Severe clinical events obtained in the mother were associated with the effect of the mutations on the functional activity of the protein. Using *in silico* analysis, p.(Y419D) *LDLR* variant was predicted to be deleterious through disrupting the EGF-A domain of the protein. Additionally, the frameshift mutation in *LDLRAP1* generated a truncated protein lacking an essential functional domain that might be affecting the cellular internalization of LDLR/LDL-C (Alnouri et al., 2018).

Although it is not fully understood why the same allele generates subtly or profoundly different phenotypes in most of the FH cases, the outcomes of the studies reporting digenic mutations *LDLR/LDLRAP1*, although very few, can be fundamental for (1) understanding better the phenotype–genotype correlations and (2) further elucidation of the FH heterogeneity and the mutation spectrum.

DIGENIC FH: A SPECTRUM OF PHENOTYPIC DIVERSITY

Familial hypercholesterolemia is a heterogeneous disease where patients show a significant variability of phenotypes that may be explained by the polygenic nature of the disease, which has been lately enhanced by the characterization of modifier genes such as *PCSK9*. Typically, the severity of phenotype varies with the genotype (Sun et al., 2018). This variation is reflected by differences in the phenotypes and the clinical severity of untreated HeFH, double HeFH, compound HeFH, and HoFH, with some overlap within and between genotypes. Also, treated HeFH and HoFH might have an overlap in LDL-C levels (Foody and Vishwanath, 2016). Mutations in more than one gene are predicted, in most cases, to worsen the phenotype and severely increase untreated LDL-C levels. However, this was not the case all the time in FH cases with digenic mutations.

In some cases, the severity of the phenotypes of double-heterozygotes was similar to HeFH, yet not as severe as in HoFH (Mabuchi et al., 2014). For example, the range of LDL-C values associated with double-heterozygotes seems to be broader compared with homozygotes from 196 mg/dL (5,100 nmol/mL; *LDLR/PCSK9*) to 583 mg/dL (15,100 nmol/mL; *LDLR/APOB*) (Benlian et al., 1996; Mabuchi et al., 2011). In one study, 13 heterozygous carriers of either *LDLR/APOB* or *LDLR/PCSK9* mutations were identified by NGS in 285 unrelated Chinese index cases of clinical FH (279 adults, six children). Eight patients out of 13 were treated with statins, and four had a CAD family history. Yet, the double-heterozygotes showed a much milder phenotype compared to the compound and true homozygotes (Sun et al., 2018). Occasionally, double-heterozygotes (a combination of *LDLR* and *APOB/PCSK9* mutations) reflected an intermediate phenotype mainly due to the milder phenotypes of *APOB/PCSK9* carriers (Sánchez-Hernández et al., 2016; Sjouke et al., 2016b). However, as the lipid levels detected in double-heterozygotes were relatively low, the authors claim that this contradiction in data presentation might be related to the small sample size (Sun et al., 2018). Although the range of LDL-C levels is more comprehensive, some double-heterozygotes may often have higher amounts than individuals with true HoFH or compound HeFH. For example, the LDL-C levels in double-heterozygotes having the *LDLR*/*PCSK9* mutations would be expected to vary to a greater or lesser amount compared with levels in individuals with the *LDLR* mutation alone. However, this usually depends on whether the *PCSK9* mutation provides a (1) GOF, leading to FH due to the increase in LDLR degradation, or (2) an LOF leading to hypercholesterolemia associated with the decrease in LDLR degradation (Noguchi et al., 2010; Mabuchi et al., 2011). In one of the cases, the clinical features of the FH individuals with double-heterozygous mutations were similar to those of family members with only one heterozygous mutation (Rauh et al., 1991). One explanation for the lack of difference between double-heterozygous (digenic) and the monogenic heterozygous FH cases is that one of the variants involved is not pathogenic and indeed is a synonymous mutation. It is obviously of great importance to be capable of assessing whether the identified variants are pathogenic or not especially that predicting the pathogenicity of novel variants is not always straightforward. The gold standard to test the pathogenicity of any variant was to perform cosegregation studies with the high LDL-C level among family members. However, the interpretation of clinical data could be puzzling as other environmental and genetic factors variants might be involved in raising up or lowering LDL-C levels (Sharifi et al., 2017). This counterintuitive finding highlights the need for better *in silico* tools and more *in vitro* functional studies to define the effect of a mutation on the protein function and structure. In addition, the differences in phenotypes could be also explained by the presence of other genetic events taking place in some other unknown genes, which reflects the importance of revealing the cause of phenocopies when studying familial segregations.

The heterogeneity and variability in clinical phenotypes within the spectrum of FH mutation carriers propose that the detection and diagnosis of double-heterozygous FH are very difficult and can sometimes be missed. It is not yet clear whether the genetic diagnosis of FH patients as double-heterozygotes is clinically relevant, because in most of the cases, the level of LDL-C is sufficiently treated with lipid-lowering drugs, including statins and PCSK9 inhibitors. Still, many patients who are clinically diagnosed with FH do not reveal any mutation in the four FH genes even with genetic testing. This suggests the presence of many unknown genes involved in cholesterol metabolism that needs to be detected to fill the diagnostic gaps of FH (Fahed and Nemer, 2011).

With the understanding of the FH genetic basis, studies have illustrated that the pronounced increase in LDL-C level was by far due to more than the common monogenic FH. Other causes of FH can be referred to (1) the presence of rare mutations in the known FH genes, (2) mutations in a novel gene, and (3) polygenic FH secondary to the cumulative effect of LDL-C raising SNPs, and (4) other acquired phenocopies (Kwon et al., 2015; Sjouke et al., 2016a). Consequently, performing NGS for all affected and non-affected familial members might aid in filling the gap

for the establishment of phenotype–genotype correlations in FH (Fahed et al., 2016).

PROMISCUITY OF PHENOTYPE–GENOTYPE CORRELATION: NGS AND FH

The introduction of NGS technologies during the last decade is leading to an exponential elevation in the discovery and differentiation between variants of variable functional significance. Those could be classified based on several measures as either clearly or likely pathogenic, of unknown significance, or unlikely pathogenic, or clearly not pathogenic. When DI comes into play, things become more complicated as two or more variants are involved in defining and justifying symptoms and diagnosis, respectively (Deltas, 2018). In other words, NGS is a highly reliable tool in elucidating the entire spectrum of variants in individuals, including those that cause digenic or polygenic disorders. Because of its massively parallel sequencing abilities, NGS is currently considered the primary high-throughput diagnostic tool for FH genetic testing, including all aspects of targeted exome sequencing, whole-exome sequencing (WES), and whole-genome sequencing (WGS). Even though genetic testing and family-based cascade screening are considered high-cost approaches for the diagnosis of FH, they are still useful in distinguishing the different forms of FH (Nherera et al., 2011; Nordestgaard et al., 2013; Talmud et al., 2013).

Although FH diagnosis can be built on several clinical manifestations, the Dutch Lipid Clinic Network Diagnostic Criteria has included genetic testing as an essential tool for more precise disease diagnosis (Defesche et al., 2004; Sturm et al., 2018). Meanwhile, the gold standard for FH diagnosis is to identify a pathogenic variant for FH other than those affecting the function of *LDLR* (Humphries et al., 2008; Watts et al., 2014). Thus, the detection of one or more pathogenic mutations in *LDLR*, *APOB*, or *PCSK9* by genetic testing offers a more definite molecular diagnosis of FH. Detecting a pathogenic variant(s) in the FH proband facilitates the family cascade genetic testing in the at-risk relatives. It can provide precise results for with and without FH relatives.

At the molecular level, genetic testing makes it easier to distinguish FH individuals with (1) heterozygous, (2) double heterozygous, (3) compound heterozygous, (4) homozygous, (5) autosomal recessive mutations, and (6) patients with no detectable pathogenic variant and yet have FH phenotype. The risks of recurrence to relatives and implications for family planning vary between these states. For example, in scenarios where the results of genetic tests recognize double-heterozygotes probands (for example, pathogenic mutations in both *LDLR* and *APOB*), the recurrence risk to relatives is affected by this finding and the recommended approaches to cascade testing (Sturm et al., 2018). As for probands whose genetic testing specifically identifies them as a compound, double-heterozygotes, or homozygotes, parents of the probands should undergo known familial variant testing to identify (1) the maternally and paternally inherited variants, (2) determine whether one of the variants is *de novo*, and (3) which is rare (Tada et al., 2016).

No doubt, NGS technologies have shown to be essential tools for identifying new mutations in FH genes and in revealing double-heterozygous mutations as it improves familial screening, genetic counseling, and understanding disease severity and transmission in different members of the same family (Elbitar et al., 2018). For example, huge genomic testing using WES done on a cohort of 50,726 individuals in a healthcare center in the United States revealed the that frequencies of mutations in *LDLR*, *APOB*, and *PCSK9* were 42.8, 44.5, and 12.7%, respectively (Abul-Husn et al., 2016). Similarly, using WES, Wang et al. (2016) reported 47.3% definite or likely pathogenic variants out of the 313 Ontario-based hypercholesterolemia cohort. Of 105 detected mutations, 87.5% were located with *LDLR* and only 12.5% in *APOB* and *PCSK9*. Interestingly, the study also reported 16 novel mutations, 12 of which in *LDLR* gene, with five missense, five frameshift, and two splicing mutations (Wang et al., 2016). Khera et al. (2019) performed WGS on a 2,081 United States–based cohort. Irrespective of their lipid status, the cohort was recruited after the early onset of myocardial infarction (MI). Sequencing analysis indicated 1.7% of the cases have FH-causing mutations in *LDLR* and surprisingly with no mutations detected in neither *APOB* nor *PCSK9* (Khera et al., 2019). Additionally, WGS data retrieved from 2,081 patients with early-onset MI showed that 17.1% of individuals with a high polygenic score had no mutation in *LDLR*, *APOB*, or *PCSK9*. In contrast, only 0.2% had both high polygenic score and a variation in the FH primary genes (Khera et al., 2019). Genetic cascade testing is recommended by United Kingdom guidelines to identify affected relatives; however, approximately 60% of patients are mutation-negative (Talmud et al., 2013).

Next-generation sequencing has the upper hand in FH diagnosis, and the drop in the cost of NGS made genetic testing more accessible. However, there is a high need for a significant data interpretation. Although a large number of genetic studies have spotted a broad spectrum of mutations in multiple genes to be associated with high LDL-C and FH, the underlying mechanism on how these mutations are linked to elevated LDL-C level might provide better insight on drug/gene or protein targets.

CONCLUSION

As more genes and loci have been additionally identified for monogenic FH, the characterization of new variations in these known genes and loci would assist in the elucidation of novel double-heterozygous FH cases. The polygenic aspect of FH should also be considered, given the fact that some FH phenotypes are due to undescribed variants located in other unknown genomic regions. Although FH is an inherited disorder, genetic testing for the diagnosis of this disease is still infrequently ordered. The genetic testing aids in improving the diagnosis and prognosis of FH, which are hard to be achieved because of the heterogeneity and the variable expressivity of the available mutations and phenotypes. Therefore, additional research is

needed to evaluate how data generated from genetic testing can improve medication and outcomes for FH patients. More likely, and as it was the case with PCSK9 drug targets, this might help in introducing novel lipid-lowering therapeutics needed to treat high-risk patients and families.

AUTHOR CONTRIBUTIONS

AKa compiled all the published data and wrote the first draft of the manuscript. All authors contributed to the critical review of the data, correction, and final write-up of the manuscript.

REFERENCES

Abifadel, M., Rabès, J.-P., Jambart, S., Halaby, G., Gannagé-Yared, M.-H., Sarkis, A., et al. (2009). The molecular basis of familial hypercholesterolemia in lebanon: spectrum of LDLR mutations and role of PCSK9 as a modifier gene. *Hum. Mutat.* 30, E682–E691. doi: 10.1002/humu.21002

Abul-Husn, N. S., Manickam, K., Jones, L. K., Wright, E. A., Hartzel, D. N., Gonzaga-Jauregui, C., et al. (2016). Genetic identification of familial hypercholesterolemia within a single U.S. health care system. *Science* 354:aaf7000. doi: 10.1126/science.aaf7000

Akioyamen, L. E., Genest, J., Shan, S. D., Reel, R. L., Albaum, J. M., Chu, A., et al. (2017). Estimating the prevalence of heterozygous familial hypercholesterolaemia: a systematic review and meta-analysis. *BMJ Open* 7:e016461. doi: 10.1136/bmjopen-2017-016461

Al-Mashhadi, R. H., Bjørklund, M. M., Mortensen, M. B., Christoffersen, C., Larsen, T., Falk, E., et al. (2015). Diabetes with poor glycaemic control does not promote atherosclerosis in genetically modified hypercholesterolaemic minipigs. *Diabetologia* 58, 1926–1936. doi: 10.1007/s00125-015-3637-1

Al-Mashhadi, R. H., Sørensen, C. B., Kragh, P. M., Christoffersen, C., Mortensen, M. B., Tolbod, L. P., et al. (2013). Atherosclerosis: familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of a human PCSK9 gain-of-function mutant. *Sci. Transl. Med.* 5:166ra1. doi: 10.1126/scitranslmed.3004853

Alnouri, F., Al-Allaf, F. A., Athar, M., Abduljaleel, Z., Alabdullah, M., Alammari, D., et al. (2020). Xanthomas can be misdiagnosed and mistreated in homozygous familial hypercholesterolemia patients: a call for increased awareness among dermatologists and health care practitioners. *Glob. Heart* 15, 1–11.

Alnouri, F., Athar, M., Al-Allaf, F. A., Abduljaleel, Z., Taher, M. M., Bouazzaoui, A., et al. (2018). Novel combined variants of LDLR and LDLRAP1 genes causing severe familial hypercholesterolemia. *Atherosclerosis* 277, 425–433. doi: 10.1016/j.atherosclerosis.2018.06.878

Austin, M. A., Hutter, C. M., Zimmern, R. L., and Humphries, S. E. (2004). Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review. *Am. J. Epidemiol.* 160, 407–420. doi: 10.1093/aje/kwh236

Benlian, P., de Gennes, J. L., Dairou, F., Hermelin, B., Ginon, I., Villain, E., et al. (1996). Phenotypic expression in double heterozygotes for familial hypercholesterolemia and familial defective apolipoprotein B-100. *Hum. Mutat.* 7, 340–345.

Benn, M., Nordestgaard, B. G., Grande, P., Schnohr, P., and Tybjærg-Hansen, A. (2010). PCSK9R46L, low-density lipoprotein cholesterol levels, and risk of ischemic heart disease. *J. Am. Coll. Cardiol.* 55, 2833–2842. doi: 10.1016/j.jacc.2010.02.044

Benn, M., Watts, G. F., Tybjærg-Hansen, A., and Nordestgaard, B. G. (2016). Mutations causative of familial hypercholesterolaemia: screening of 98 098 individuals from the copenhagen general population study estimated a prevalence of 1 in 217. *Eur. Heart J.* 37, 1384–1394. doi: 10.1093/eurheartj/ehw028

Bertolini, S., Pisciotta, L., Di Scala, L., Langheim, S., Bellocchio, A., Masturzo, P., et al. (2004). Genetic polymorphisms affecting the phenotypic expression of familial hypercholesterolemia. *Atherosclerosis* 174, 57–65. doi: 10.1016/j.atherosclerosis.2003.12.037

Bertolini, S., Pisciotta, L., Rabacchi, C., Cefalù, A. B., Noto, D., Fasano, T., et al. (2013). Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy. *Atherosclerosis* 227, 342–348. doi: 10.1016/j.atherosclerosis.2013.01.007

Brautbar, A., Leary, E., Rasmussen, K., Wilson, D. P., Steiner, R. D., and Virani, S. (2015). Genetics of familial hypercholesterolemia. *Curr. Atheroscler. Rep.* 17:491.

Brown, M. S., and Goldstein, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis (nobel lecture). *Angew. Chem. Int. Ed.* 25, 583–602. doi: 10.1002/anie.198605833

Castro-Orós, I. D., Pocoví, M., and Civeira, F. (2010). The genetic basis of familial hypercholesterolemia: inheritance, linkage, and mutations. *Appl. Clin. Genet.* 3, 53–64. doi: 10.2147/tacg.s8285

Choong, M. L., Koay, E. S., Khoo, K. L., Khaw, M. C., and Sethi, S. K. (1997). Denaturing gradient-gel electrophoresis screening of familial defective apolipoprotein B-100 in a mixed asian cohort: two cases of arginine3500–>tryptophan mutation associated with a unique haplotype. *Clin. Chem.* 43 (6 Pt 1), 916–923.

Cohen, J. C., Boerwinkle, E., Mosley, T. H. Jr., and Hobbs, H. H. (2006). Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N. Eng. J. Med.* 354, 1264–1272. doi: 10.1056/NEJMoa054013

Cohen, J. C., Kimmel, M., Polanski, A., and Hobbs, H. H. (2003). Molecular mechanisms of autosomal recessive hypercholesterolemia. *Curr. Opin. Lipidol.* 14, 121–127. doi: 10.1097/00041433-200304000-00002

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014). Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the consensus panel on familial hypercholesterolaemia of the european atherosclerosis society. *Eur. Heart J.* 35, 2146–2157. doi: 10.1093/eurheartj/ehu274

Cutting, G. R. (2010). Modifier genes in mendelian disorders: the example of cystic fibrosis. *Ann. N. Y. Acad. Sci.* 1214, 57–69. doi: 10.1111/j.1749-6632.2010.05879.x

Damgaard, D., Jensen, J. M., Larsen, L., Soerensen, R., Jensen, H. K., Gregersen, N., et al. (2004). No genetic linkage or molecular evidence for involvement of the PCSK9, ARH or CYP7A1 genes in the familial hypercholesterolemia phenotype in a sample of danish families without pathogenic mutations in the LDL receptor and ApoB genes. *Atherosclerosis* 177, 415–422. doi: 10.1016/j.atherosclerosis.2004.07.028

DeCampo, A., Schallmoser, K., Schmidt, H., Toplak, H., and Kostner, G. M. (2001). A novel splice-site mutation in intron 7 causes more severe hypercholesterolemia than a combined FH-FDB defect. *Atherosclerosis* 157, 524–525. doi: 10.1016/S0021-9150(01)00535-4

Defesche, J. C., Lansberg, P. J., Umans-Eckenhausen, M. A., and Kastelein, J. J. (2004). Advanced method for the identification of patients with inherited hypercholesterolemia. *Semin. Vasc. Med.* 4, 59–65. doi: 10.1055/s-2004-822987

Deltas, C. (2018). Digenic inheritance and genetic modifiers. *Clin. Genet.* 93, 429–438. doi: 10.1111/cge.13150

Denis, M., Marcinkiewicz, J., Zaid, A., Gauthier, D., Poirier, S., Lazure, C., et al. (2012). Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. *Circulation* 125, 894–901. doi: 10.1161/CIRCULATIONAHA.111.057406

Elbitar, S., Susan-Resiga, D., Ghaleb, Y., El Khoury, P., Peloso, G., Stitziel, N., et al. (2018). New sequencing technologies help revealing unexpected mutations in autosomal dominant hypercholesterolemia. *Sci. Rep.* 8:1943. doi: 10.1038/s41598-018-20281-9

Fahed, A. C., and Nemer, G. M. (2011). Familial hypercholesterolemia: the lipids or the genes? *Nutr. Metab.* 8:23. doi: 10.1186/1743-7075-8-23

Fahed, A. C., Khalaf, R., Salloum, R., Andary, R. R., Safa, R., El-Rassy, I., et al. (2016). Variable expressivity and co-occurrence of LDLR and LDLRAP1 mutations in familial hypercholesterolemia: failure of the dominant and recessive dichotomy. *Mol. Genet. Genomic Med.* 4, 283–291. doi: 10.1002/mgg3.203

Fahed, A. C., Safa, R. M., Haddad, F. F., Bitar, F. F., Andary, R. R., Arabi, M. T., et al. (2011). Homozygous familial hypercholesterolemia in lebanon: a genotype/phenotype correlation. *Mol. Genet. Metab.* 102, 181–188. doi: 10.1016/j.ymgme.2010.11.006

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., et al. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement fromthe european atherosclerosis society consensus panel. *Eur. Heart J.* 38, 2459–2472. doi: 10.1093/eurheartj/ehx144

Foody, J. M., and Vishwanath, R. (2016). Familial hypercholesterolemia / autosomal dominant hypercholesterolemia: molecular defects, the LDL-C continuum, and gradients of phenotypic severity. *J. Clin. Lipidol.* 10, 970–986. doi: 10.1016/j.jacl.2016.04.009

Gaffney, D., Reid, J. M. I, Cameron, M., Vass, K., Caslake, M. J., Shepherd, J., et al. (1995). Independent mutations at codon 3500 of the apolipoprotein B gene are associated with hyperlipidemia. *Arterioscler. Thromb. Vasc. Biol.* 15, 1025–1029. doi: 10.1161/01.ATV.15.8.1025

Garcia, C. K., Wilund, K., Arca, M., Zuliani, G., Fellin, R., Maioli, M., et al. (2001). Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. *Science* 292, 1394–1398. doi: 10.1126/science.1060458

Gazzo, A. M., Daneels, D., Cilia, E., Bonduelle, M., Abramowicz, M., Van Dooren, S., et al. (2016). NAR breakthrough article: DIDA: a curated and annotated digenic diseases database. *Nucleic Acids Res.* 44, D900–D907.

Goldstein, J., Hobbs, H. H., and Brown, M. S. (2001). *The Metabolic and Molecular Bases of Inherited Disease. Familial Hypercholesterolemia*. New York, NY: McGraw-Hill. doi: 10.1036/ommbid.149

Guardamagna, O., Restagno, G., Rolfo, E., Pederiva, C., Martini, S., Abello, F., et al. (2009). The type of LDLR gene mutation predicts cardiovascular risk in children with familial hypercholesterolemia. *J. Pediatr.* 155, 199–204.e2. doi: 10.1016/j.jpeds.2009.02.022

Hadfield, S. G., Horara, S., Starr, B. J., Yazdgerdi, S., Marks, D., Bhatnagar, D., et al. (2009). Family tracing to identify patients with familial hypercholesterolaemia: the second audit of the Department of Health Familial hypercholesterolaemia cascade testing project. *Ann. Clin. Biochem.* 46, 24–32. doi: 10.1258/acb.2008.008094

Handelsman, Y., and Lepor, N. E. (2018). PCSK9 inhibitors in lipid management of patients with diabetes mellitus and high cardiovascular risk: a review. *J. Am. Heart Assoc.* 7:e008953. doi: 10.1161/JAHA.118.008953

Hartgers, M. L., Defesche, J. C., Langslet, G., Hopkins, P. N., Kastelein, J. J. P., Baccara-Dinet, M. T., et al. (2018). Alirocumab efficacy in patients with double heterozygous, compound heterozygous, or homozygous familial hypercholesterolemia. *J. Clin. Lipidol.* 12, 390–396.e8. doi: 10.1016/j.jacl.2017.12.008

Hobbs, H. H., Brown, M. S., and Goldstein, J. L. (1992). Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum. Mutat.* 1, 445–466. doi: 10.1002/humu.1380010602

Hopkins, P. N., Defesche, J., Fouchier, S. W., Bruckert, E., Luc, G., Cariou, B., et al. (2015). Characterization of autosomal dominant hypercholesterolemia caused by PCSK9 gain of function mutations and its specific treatment with alirocumab, a PCSK9 monoclonal antibody. *Circ. Cardiovasc. Genet.* 8, 823–831. doi: 10.1161/CIRCGENETICS.115.001129

Humphries, S. E., Norbury, G., Leigh, S., Hadfield, S. G., and Nair, D. (2008). What is the clinical utility of DNA testing in patients with familial hypercholesterolaemia? *Curr. Opin. Lipidol.* 19, 362–368. doi: 10.1097/MOL.0b013e32830636e5

Ito, M. K., and Watts, G. F. (2015). Challenges in the diagnosis and treatment of homozygous familial hypercholesterolemia. *Drugs* 75, 1715–1724. doi: 10.1007/s40265-015-0466-y

Khachadurian, A. K. (1964). The inheritance of essential familial hypercholesterolemia. *Am. J. Med.* 37, 402–407. doi: 10.1016/0002-9343(64)90196-2

Khachadurian, A. K. (1988). Clinical features, diagnosis and frequency of familial hypercholesterolemia. *Contrib. Infus. Ther.* 23, 26–32.

Khera, A. V., Chaffin, M., Zekavat, S. M., Collins, R. L., Roselli, C., Natarajan, P., et al. (2019). Whole-genome sequencing to characterize monogenic and polygenic contributions in patients hospitalized with early-onset myocardial infarction. *Circulation* 139, 1593–1602. doi: 10.1161/CIRCULATIONAHA.118.035658

Koivisto, U. M., Turtola, H., Aalto-Setälä, K., Top, B., Frants, R. R., Kovanen, P. T., et al. (1992). The familial hypercholesterolemia (FH)-North Karelia mutation the low density lipoprotein receptor gene deletes seven nucleotides exon 6 and is a common cause of FH in Finland. *J. Clin. Invest.* 90, 219–228. doi: 10.1172/JCI115839

Kotze, M. J., De Villiers, W J, Steyn, K., Kriek, J. A., Marais, A. D., Langenhoven, E., et al. (1993). Phenotypic variation among familial hypercholesterolemics heterozygous for either one of two Afrikaner founder LDL receptor mutations. *Arterioscler. Thromb.* 13, 1460–1468. doi: 10.1161/01.atv.13.10.1460

Kwon, M., Han, S. M., Kim, D. I., Rhee, M. Y., Lee, B. K., Ahn, Y. K., et al. (2015). Evaluation of polygenic cause in Korean patients with familial hypercholesterolemia – a study supported by Korean society of lipidology and atherosclerosis. *Atherosclerosis* 242, 8–12. doi: 10.1016/j.atherosclerosis.2015.06.053

Lambert, G., Sjouke, B., Choque, B., Kastelein, J. J. P., and Hovingh, G. K. (2012). The PCSK9 decade. *J. Lipid Res.* 53, 2515–2524. doi: 10.1194/jlr.R026658

Leitersdorf, E., Tobin, E. J., Davignon, J., and Hobbs, H. H. (1990). Common low-density lipoprotein receptor mutations in the French Canadian population. *J. Clin. Invest.* 85, 1014–1023. doi: 10.1172/JCI114531

Mabuchi, H. (2017). Half a century tales of familial hypercholesterolemia (FH) in Japan. *J. Atheroscler. Thromb.* 24, 189–207. doi: 10.5551/jat.RV16008

Mabuchi, H., Nohara, A., Noguchi, T., Kobayashi, J., Kawashiri, M. A., Inoue, T., et al. (2014). Genotypic and phenotypic features in homozygous familial hypercholesterolemia caused by proprotein convertase subtilisin/kexin type 9 (PCSK9) gain-of-function mutation. *Atherosclerosis* 236, 54–61. doi: 10.1016/j.atherosclerosis.2014.06.005

Mabuchi, H., Nohara, A., Noguchi, T., Kobayashi, J., Kawashiri, M. A., Tada, H., et al. (2011). Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan. *Atherosclerosis* 214, 404–407. doi: 10.1016/j.atherosclerosis.2010.11.005

Marais, A. D. (2004). Familial hypercholesterolaemia. *Clin. Biochem. Rev.* 25, 49–68.

Michaely, P., Li, W. P., Anderson, R. G. W., Cohen, J. C., and Hobbs, H. H. (2004). The modular adaptor protein ARH is required for low density lipoprotein (LDL) binding and internalization but not for LDL receptor clustering in coated pits. *J. Biol. Chem.* 279, 34023–34031. doi: 10.1074/jbc.M405242200

Moldovan, V., and Banescu, C. (2020). Molecular diagnosis methods in familial hypercholesterolemia. *Anatol. J. Cardiol.* 23, 120–127. doi: 10.14744/AnatolJCardiol.2019.95038

Moorjani, S., Roy, M., Torres, A., Betard, C., Gagne, C., Lambert, M., et al. (1993). Mutations of low-density- lipoprotein receptor gene, variation in plasma cholesterol, and expression of coronary heart disease in homozygous familial hypercholesterolemia. *Lancet* 34, 1303–1306.

Moyer, A. M., and Baudhuin, L. M. (2015). Genetic considerations in the treatment of familial hypercholesterolemia. *Clin. Lipidol.* 10, 387–403. doi: 10.2217/clp.15.33

Myant, N. B. (1993). Familial defective apolipoprotein B-100: a review, including some comparisons with familial hypercholesterolaemia. *Atherosclerosis* 104, 1–18. doi: 10.1016/0021-9150(93)90171-P

Narayanaswamy, M., and Sharma, S. (2020). *Polygenic Hypercholesterolemia*. *StatPearls*. Treasure Island, FL: StatPearls Publishing.

Nherera, L., Marks, D., Minhas, R., Thorogood, M., and Humphries, S. E. (2011). probabilistic cost-effectiveness analysis of cascade screening for familial hypercholesterolaemia using alternative diagnostic and identification strategies. *Heart* 97, 1175–1181. doi: 10.1136/hrt.2010.213975

Noguchi, T., Shoji, K., Kawashiri, M. A., Tada, H., Nohara, A., Inazu, A., et al. (2010). The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolaemia by increasing PCSK9 function and concentration in the circulation. *Atherosclerosis* 210, 166–172. doi: 10.1016/j.atherosclerosis.2009.11.018

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease. *Eur. Heart J.* 34, 3478–3490. doi: 10.1093/eurheartj/eht273

Ohta, N., Hori, M., Takahashi, A., Ogura, M., Makino, H., Tamanaha, T., et al. (2016). Proprotein convertase subtilisin/Kexin 9 V4I variant with LDLR

mutations modifies the phenotype of familial hypercholesterolemia. *J. Clin. Lipidol.* 10, 547–555.e5. doi: 10.1016/j.jacl.2015.12.024

Pang, J., Sullivan, D. R., Brett, T., Kostner, K. M., Hare, D. L., and Watts, G. F. (2020). Familial hypercholesterolaemia in 2020: a leading tier 1 genomic application. *Heart Lung Circul.*29, 619–633. doi: 10.1016/j.hlc.2019.12.002

Paththinige, C. S., Sirisena, N. D., and Dissanayake, V. H. W. (2017). Genetic Determinants of inherited susceptibility to hypercholesterolemia – a comprehensive literature review. *Lipids Health Dis.* 16:103. doi: 10.1186/s12944-017-0488-4

Pirillo, A., Garlaschelli, K., Arca, M., Averna, M., Bertolini, S., Calandra, S., et al. (2017). Spectrum of mutations in italian patients with familial hypercholesterolemia: new results from the LIPIGEN study. *Atherosc. Suppl.* 29, 17–24. doi: 10.1016/j.atherosclerosissup.2017.07.002

Pisciotta, L., Oliva, C. P., Cefalù, A. B., Noto, D., Bellocchio, A., Fresa, R., et al. (2006a). Additive effect of mutations in LDLR and PCSK9 genes on the phenotype of familial hypercholesterolemia (P. Q254P). *Atherosclerosis* 186, 433–440. doi: 10.1016/j.atherosclerosis.2005.08.015

Pisciotta, L., Oliva, C. P., Pes, G. M., Di Scala, L., Bellocchio, A., Fresa, R., et al. (2006b). Autosomal recessive hypercholesterolemia (ARH) and homozygous familial hypercholesterolemia (FH): a phenotypic comparison. *Atherosclerosis* 188, 398–405. doi: 10.1016/j.atherosclerosis.2005.11.016

Powell-Braxton, L., Véniant, M., Latvala, R. D., Hirano, K. I., Won, W. B., Ross, J., et al. (1998). A mouse model of human familial hypercholesterolemia: markedly elevated low density lipoprotein cholesterol levels and severe atherosclerosis on a low-fat chow diet. *Nat. Med.* 4, 934–938. doi: 10.1038/nm0898-934

Pullinger, C. R., Hennessy, L. K., Chatterton, J. E., Liu, W., Love, J. A., Mendel, C. M., et al. (1995). Familial ligand-defective apolipoprotein B: identification of a new mutation that decreases LDL receptor binding affinity. *J. Clin. Investigat.* 95, 1225–1234. doi: 10.1172/JCI117772

Quagliarini, F., Vallvé, J. C., Campagna, F., Alvaro, A., Fuentes-Jimenez, F. J., Sirinian, M. I., et al. (2007). Autosomal recessive hypercholesterolemia in spanish kindred due to a large deletion in the ARH gene. *Mol. Genet. Metab.* 92, 243–248. doi: 10.1016/j.ymgme.2007.06.012

Raal, F. J., Pilcher, G., Rubinsztein, D. C., Lingenhel, A., and Utermann, G. (1997). Statin therapy in a kindred with both apoliprotein B and low density lipoprotein receptor gene defects. *Atherosclerosis* 129, 97–102. doi: 10.1016/S0021-9150(96)06007-8

Rader, D. J., Cohen, J., and Hobbs, H. H. (2003). Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *J. Clin. Invest.* 111, 1795–1803. doi: 10.1172/jci18925

Rashid, S., Curtis, D. E., Garuti, R., Anderson, N. H., Bashmakov, Y., Ho, Y. K., et al. (2005). Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5374–5379. doi: 10.1073/pnas.0501652102

Rauh, G., Schuster, H., Fischer, J., Keller, C. h., Wolfram, G., and Zöllner, N. (1991). Klinische wochen-schrift identification of a heterozygous compound individual with familial hypercholesterolemia and familial defective apolipoprotein B-100. *Klin. Wochenschr.* 69, 320–324.

Rubinsztein, D. C., Raal, F. J., Seftel, H. C., Pilcher, G., Coetzee, G. A., and Van der Westhuyzen, D. R. (1993). Characterization of six patients who are double heterozygotes for familial hypercholesterolemia and familial defective apo B-100. *Arterioscler. Thromb.* 13, 1076–1081. doi: 10.1161/01.atv.13.7.1076

Sánchez-Hernández, R. M., Civeira, F., Stef, M., Perez-Calahorra, S., Almagro, F., Plana, N., et al. (2016). Homozygous familial hypercholesterolemia in Spain: prevalence and phenotype-genotype relationship. *Circulation* 9, 504–510. doi: 10.1161/CIRCGENETICS.116.001545

Schäffer, A. A. (2013). Digenic inheritance in medical genetics. *J. Med. Genet.* 50, 641–652. doi: 10.1136/jmedgenet-2013-101713

Seidah, N. G. (2017). The PCSK9 revolution and the potential of PCSK9-based therapies to reduce LDL-cholesterol. *Glob. Cardiol. Sci. Pract.* 2017:e201702. doi: 10.21542/gcsp.2017.2

Sharifi, M., Futema, M., Nair, D., and Humphries, S. E. (2017). Genetic architecture of familial hypercholesterolaemia. *Curr. Cardiol. Rep.* 19:44. doi: 10.1007/s11886-017-0848-8

Shirahama, R., Ono, T., Nagamatsu, S., Sueta, D., Takashio, S., Chitose, T., et al. (2018). Coronary artery plaque regression by a PCSK9 Antibody and rosuvastatin in double-heterozygous familial hypercholesterolemia with an LDL receptor mutation and a PCSK9 V4I mutation. *Int. Med.* 57, 3551–3557. doi: 10.2169/internalmedicine.1060-18

Singh, S., and Bittner, V. (2015). Familial Hypercholesterolemia—epidemiology, diagnosis, and screening. *Curr. Atheroscl. Rep.* 1:482. doi: 10.1007/s11883-014-0482-5

Sjouke, B., Defesche, J. C., de Randamie, J. S. E., Wiegman, A., Fouchier, S. W., and Hovingh, G. K. (2016a). Sequencing for LIPA Mutations in patients with a clinical diagnosis of familial hypercholesterolemia. *Atherosclerosis* 251, 263–265. doi: 10.1016/j.atherosclerosis.2016.07.008

Sjouke, B., Defesche, J. C., Hartgers, M. L., Wiegman, A., van Lennep, J. E. R., Kastelein, J. J., et al. (2016b). Double-heterozygous autosomal dominant hypercholesterolemia: clinical characterization of an underreported disease. *J. Clin. Lipidol.* 10, 1462–1469. doi: 10.1016/j.jacl.2016.09.003

Soria, L. F., Ludwig, E. H., Clarke, H. R. G., Vega, G. L., Grundy, S. M., and McCarthy, B. J. (1989). Association between a specific apolipoprotein b mutation and familial defective apolipoprotein B-100. *Proc. Natl. Acad. Sci. U.S.A.* 86, 587–591. doi: 10.1073/pnas.86.2.587

Soufi, M., Rust, S., Walter, M., and Schaefer, J. R. (2013). A combined LDL receptor/LDL receptor adaptor protein 1 mutation as the cause for severe familial hypercholesterolemia. *Gene* 521, 200–203. doi: 10.1016/j.gene.2013.03.034

Soufi, M., Sattler, A. M., Maerz, W., Starke, A., Herzum, M., Maisch, B., et al. (2004). A new but frequent mutation of ApoB-100 – ApoB His3543Tyr. *Atherosclerosis* 174, 11–16. doi: 10.1016/j.atherosclerosis.2003.12.021

Soutar, A. K., and Naoumova, R. P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat. Clin. Pract. Cardiovas. Med.* 4, 214–225. doi: 10.1038/ncpcardio0836

Spina, R., Noto, D., Barbagallo, C. M., Monastero, R., Ingrassia, V., Valenti, V., et al. (2018). Genetic epidemiology of autosomal recessive hypercholesterolemia in sicily: identification by next-generation sequencing of a new kindred. *J. Clin. Lipidol.* 12, 145–151. doi: 10.1016/j.jacl.2017.10.014

Steyn, K., Weight, M. J., Dando, B. R., Christopher, K. J., and Rossouw, J. E. (1989). The use of low density lipoprotein receptor activity of lymphocytes to determine the prevalence of familial hypercholesterolaemia in a rural south african community. *J. Med. Genet.* 26, 32–36. doi: 10.1136/jmg.26.1.32

Sturm, A. C., Knowles, J. W., Gidding, S. S , Ahmad, Z. S., Ahmed, C. D., Ballantyne, C. M. et al. (2018). Clinical genetic testing for familial hypercholesterolemia: JACC scientific expert panel. Convened by the familial hypercholesterolemia foundation. *J. Am. Coll. Cardiol.* 72, 662–680. doi: 10.1016/j.jacc.2018.05.044

Sun, D., Zhou, B. Y., Li, S., Sun, N. L., Hua, Q., Wu, S. L., et al. (2018). Genetic basis of index patients with familial hypercholesterolemia in chinese population: mutation spectrum and genotype-phenotype correlation. *Lipids Health Dis.* 17, 1–8. doi: 10.1186/s12944-018-0900-8

Tada, H., Hosomichi, K., Okada, H., Kawashiri, M. A., Nohara, A., Inazu, A., et al. (2016). A *de novo* mutation of the LDL receptor gene as the cause of familial hypercholesterolemia identified using whole exome sequencing. *Clin. Chim. Acta.* 453, 194–196. doi: 10.1016/j.cca.2015.12.028

Tada, H., Kawashiri, M. A., Nomura, A., Teramoto, R., Hosomichi, K., Nohara, A., et al. (2018). Oligogenic familial hypercholesterolemia, LDL cholesterol, and coronary artery disease. *J. Clin. Lipidol.* 12, 1436–1444. doi: 10.1016/j.jacl.2018.08.006

Tada, H., Kawashiri, M. A., Ohtani, R., Noguchi, T., Nakanishi, C., Konno, T., et al. (2011). A novel type of familial hypercholesterolemia: double heterozygous mutations in LDL receptor and LDL receptor adaptor protein 1 gene. *Atherosclerosis* 219, 663–666. doi: 10.1016/j.atherosclerosis.2011.08.004

Tada, H., Nohara, A., and Kawashiri, M. A. (2019). Monogenic, polygenic, and oligogenic familial hypercholesterolemia. *Curr. Opin. Lipidol.* 30, 300–306. doi: 10.1097/mol.0000000000000609

Tai, E. S., Koay, E. S. C., Chan, E., Seng, T. J., Loh, L. M., Sethi, S. K., et al. (2001). Compound heterozygous familial hypercholesterolemia and familial defective apolipoprotein B-100 produce exaggerated hypercholesterolemia. *Clin. Chem.* 47, 438–443. doi: 10.1093/clinchem/47.3.438

Talmud, P. J., Shah, S., Whittall, R., Futema, M., Howard, P., Cooper, J. A., et al. (2013). Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a

case-control study. *Lancet* 381, 1293–1301. doi: 10.1016/S0140-6736(12)62127-8

Taylor, A., Bayly, G., Patel, K., Yarram, L., Williams, M., Hamilton-Shield, J., et al. (2010). A double heterozygote for familial hypercholesterolaemia and familial defective apolipoprotein B-100. *Ann. Clin. Biochem.* 47, 487–490. doi: 10.1258/acb.2010.010089

Umans-Eckenhausen, M. A., Defesche, J. C., Sijbrands, E. J., Scheerder, R. L., and Kastelein, J. J. (2001). Review of first 5 years of screening for familial hypercholesterolaemia in the Netherlands. *Lancet* 357, 165–168. doi: 10.1016/S0140-6736(00)03587-X

Vockley, J. (2011). "Digenic inheritance," in *Encyclopedia of Life Sciences*, ed. J. Wiley (Chichester: John Wiley & Sons, Ltd). doi: 10.1002/9780470015902.a0005560.pub2

Vogt, A. (2015). The application of clinical genetics dovepress the genetics of familial hypercholesterolemia and emerging therapies. *Appl. Clin. Genet.* 8, 27–36. doi: 10.2147/TACG.S44315

Wang, F., Fan, Q., Tao, R., Gu, G., Zhang, R., and Xi, R. (2018). Genetic analysis in a compound heterozygote family with familial hypercholesterolemia. *Mol. Med. Rep.* 17, 8439–8449. doi: 10.3892/mmr.2018.8904

Wang, J., Dron, J. S., Ban, M. R., Robinson, J. F., McIntyre, A. D., Alazzam, M., et al. (2016). Polygenic versus monogenic causes of hypercholesterolemia ascertained clinically. *Arterioscl. Thromb. Vasc. Biol.* 36, 2439–2445. doi: 10.1161/ATVBAHA.116.308027

Wang, X., Li, X., Zhang, Y. B., Zhang, F., Sun, L., Lin, J., et al. (2011). Genome-wide linkage scan of a pedigree with familial hypercholesterolemia suggests susceptibility loci on chromosomes 3q25-26 and 21q22. *PLoS One*. 6:e24838. doi: 10.1371/journal.pone.0024838

Watts, G. F., Gidding, S., Wierzbicki, A. S., Toth, P. P., Alonso, R., Brown, W. V., et al. (2014). Ìntegrated guidance on the care of familial hypercholesterolemia from the International FH Foundation. *J. Clin. Lipidol.* 8, 148–172. doi: 10.1016/j.jacl.2014.01.002

Wilund, K. R., Yi, M., Campagna, F., Arca, M., Zuliani, G., Fellin, R., et al. (2002). Molecular mechanisms of autosomal recessive hypercholesterolemia. *Hum. Mol. Genet.* 11, 3019–3030. doi: 10.1093/hmg/11.24.3019

Zaid, A., Roubtsova, A., Essalmani, R., Marcinkiewicz, J., Chamberland, A., Hamelin, J., et al. (2008). Proprotein convertase subtilisin/kexin Type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology* 48, 646–654. doi: 10.1002/hep.22354

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Mutational Spectrum of *LDLR* and *PCSK9* Genes Identified in Iranian Patients With Premature Coronary Artery Disease and Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is a common, yet underdiagnosed, genetic disorder characterized by lifelong elevated low-density lipoprotein cholesterol levels, which can increase the risk of early-onset coronary artery disease (CAD). In the present study, we screened the nucleotide variations of the *LDLR and PCSK9* genes, as well as a part of the *APOB* gene, in Iranian patients with FH and premature CAD to find the genetic cause of the disorder. Fifteen unrelated individuals with a clinical diagnosis of FH and premature CAD were recruited. Direct DNA sequencing was applied to screen the whole coding exons and exon–intron boundaries of the *LDLR* and *PCSK9* genes and the main parts of their introns, together with exon 26 of the *APOB* gene. The pathogenicity of the identified mutations was investigated via either segregation analyses in the family or *in silico* predictive software. Six different point mutations (p.Cys148Tyr, p.Cys216Tyr, p.Cys302Trp, p.Cys338Trp, p.Leu479Gln, and p.G593Afs*72) in *LDLR* and a double mutation (p.Asp172His and p.Ala53Val) in both *LDLR* and *PCSK9* genes were identified in seven families with clinically diagnosed FH (43%), whereas no pathogenic mutations were found in eight families with clinically diagnosed FH. This study is the first to identify 1 pathogenic mutation in the *LDLR* gene (c.1014C > G [p.Cys338Trp]) and to cosegregate it from the affected individual in the family. No mutations were found in the *APOB* gene, whereas several silent mutations/polymorphisms were identified in the *LDLR* and *PCSK9* genes. Genetic testing and reports on nucleotide alterations in the Iranian population are still limited. Our findings not only further confirm the significant role of FH in the incidence of premature CAD but also enlarge the spectrum of *LDLR*

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and *PCSK9* variations and exhibit the heterogeneity of FH in Iranians. In patients with no mutation in the examined genes, the disease could be begotten either by a polygenic cause or by gene defects occurring in other related genes and regions not targeted in this study.

Keywords: pre-mature CAD, Familial Hypercholesterolemia, loss-of-function, LDLR, PCSK9 (proprotein convertase subtilisin kexin type 9)

BACKGROUND

Familial hypercholesterolemia (FH) is a monogenic disorder of the metabolism of low-density lipoprotein cholesterol (LDL-C) and is characterized by lifelong elevated levels of LDL particles and LDL-C arterial deposits (Khachadurian, 1964; Brown and Goldstein, 1974). Untreated patients carry the risk of early onset coronary artery disease (premature CAD) and the augmented risk of cardiovascular events (Nordestgaard et al., 2013; Cuchel et al., 2014b; Gidding et al., 2015; Krogh et al., 2016). The current prevalence of FH is estimated to be 1:311 individuals in the general population (Beheshti et al., 2020; Hu et al., 2020).

This monogenic disorder is inherited in two forms of autosomal dominant and autosomal recessive (De Castro-Orós et al., 2010; Cuchel et al., 2014a). The autosomal dominant form of FH is mostly due to the heterozygous and homozygous states of pathogenic variants in the *low-density lipoprotein receptor* (*LDLR*), *apolipoprotein B* (*APOB*), and *proprotein convertase subtilisin/kexin type 9* (*PCSK9*) genes. Between 85 and 90% of the mutations harbored in *LDLR* that result in defective LDL receptors attenuate LDL-C clearance from the blood and, consequently, raise LDL-C plasma levels (Varret and Rabès, 2012).

Likewise, mutations in the *APOB* gene weaken the binding of LDL to LDL receptors, and gain-of-function mutations in the *PCSK9* gene account for 5–15% of cases with FH (El Khoury et al., 2017; Sharifi et al., 2017). PCSK9, a serine protease, mediates the internalization and degradation of LDLR in the lysosome via binding to LDL receptors, leading to diminished LDLR recycling and impaired clearance of LDL-C particles from the plasma (Seidah, 2017). Recently, next-generation sequencing has demonstrated the FH phenotype due to occasional dominant mutations in *apolipoprotein E* (*APOE*) or *signal-transducing adaptor family member 1* (*STAP1*), as well (Nordestgaard et al., 2013; Cuchel et al., 2014b; Fouchier et al., 2014; Berberich and Hegele, 2019).

The autosomal recessive form of FH is frequently in consequence of pathogenic mutations in the *low-density lipoprotein receptor adaptor protein* (*LDLRAP*) gene (Cuchel et al., 2014a). Nevertheless, whole-exome sequencing has revealed that mutations in the *lysosomal acid lipase A* (*LIPA*), *ATP-binding cassette sub-family G member 5 and 8* (*ABCG5/8*), and *cholesterol 7 alpha-hydroxylase* (*CYP7A1*) genes phenotypically cause hypercholesterolemia similar to FH in the recessive status (Brautbar et al., 2015; Berberich and Hegele, 2019).

At present, the diagnosis of FH is frequently based on clinical phenotypes, with the most extensively utilized FH clinical criteria being the Simon Broome register (SBR) (Simon Broome Register Group., 1991) and the Dutch Lipid Clinic Network System (DLCNS) (Nordestgaard et al., 2013). In this study, we analyzed the possible mutations of the *LDLR*, *APOB*, and *PCSK9* genes in Iranian patients suffering from both FH and premature CAD.

MATERIALS AND METHODS

Subjects

The present study enrolled 15 unrelated patients with clinically diagnosed FH and premature CAD who met the SBR and/or DLCNS criteria. The demographic characteristics, clinical features, and cholesterol levels of the study population are presented in **Table 1**. The SB and DLCNS criteria encompass the levels of total cholesterol (TC) and LDL-C, the presence of tendon xanthomas, and a family history of hypercholesterolemia, premature CAD, or cardiovascular events in a first- and/or second-degree relative.

For the purposes of the segregation analysis, the family members of some patients (if available) were involved in the study. The study protocol was approved by the Ethics Committee of Rajaie Cardiovascular Medical and Research Center (RHC.AC.IR.REC.1396.62), and it was conducted in accordance with the Helsinki Declaration.

DNA Extraction

Peripheral blood samples (3–5 mL) were collected from the patients and the available family members in EDTA tubes for DNA extraction. Genomic DNA was isolated from peripheral blood using the Exgene Blood SV Mini Kit (GeneAll Seoul, South Korea). The quantity of the extracted DNA was measured with the NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States).

Polymerase Chain Reaction and Sanger Sequencing

For the amplification of the whole coding exons, exon–intron junctions, and the main parts of the introns of the *LDLR* (NG_009060) and *PCSK9* (NG_009061) genes, specific oligonucleotides were designed (**Supplementary Table 1**).

The forward primer 5′-AGCCTCACCTCTTACTTTTCC ATT-3′ and the reverse primer 5′-CTTTGCTTGTATGTTCT CCGTTGGT-3′ were used to amplify exon 26 of the *APOB* gene (307 base pairs in length), which creates the common FH-causing mutations (p.Arg3500Gln, p.Arg3500Trp, p.Arg3531Cys, and p.His3543Tyr).

TABLE 1 | Clinical characteristics of the enrolled patients.

Patient ID	LDL-C (mg/dL)	Total cholesterol (mg/dL)	Gender	Age	Coronary artery disease	Intervention	Family history		Xanthomas	Corneal ring	Consanguine marriage	SB	DLCNS
							Hypercholes-terolemia	Coronary artery disease or myocardial infarction					
P1	240	417	F	11	Yes	CABG -AVR	Yes	MI,CAD	Yes	–	Yes	Definite	Definite
P2	276	338	F	37	Yes	–	Yes	MI,CAD	–	–	Yes	Possible	Definite
P3	203	288	F	45	Yes	–	Yes	MI	–	–	–	Possible	Definite
P4	370	458	F	40	Yes	CABG	Yes	CAD,MI	Yes	–	–	Definite	Definite
P5	95	179	F	48	Yes	CABG	Yes	CAD,MI	–	–	–	–	Possible
P6	105.6	192	M	47	Yes	CABG-AVR	Yes	CAD	–	Yes	–	Possible	Possible
P7	222	339	F	55	Yes	CABG	Yes	CAD	–	Yes	–	Definite	Definite
P8	391	461	M	24	Yes, MI	CABG-AVR	Yes	MI,CAD	Yes	Yes	Yes	Definite	Definite
P9	340	415	M	22	Yes	–	Yes	MI	Yes	–	Yes	Definite	Definite
P10	153	229	M	30	Yes	CABG	Yes	CAD	–	Yes	Yes	Definite	Definite
P11	250	358	M	32	Yes	PCI	Yes	CAD	–	–	Yes	Possible	Definite
P12	252	355	F	38	Yes	PCI	Yes	MI	–	–	–	Possible	Probable
P13	135	216	F	44	Yes	CABG	Yes	CAD, MI	–	–	Yes	–	Possible
P14	142	192	M	41	Yes	PCI	Yes	MI	–	–	–	–	Possible
P15	204	277	F	46	Yes	–	Yes	CAD, MI	Yes	–	–	Definite	Probable

CAD, coronary artery disease; CABG, coronary artery bypass grafting; AVR, aortic valve replacement; MI, myocardial infarction; PCI, percutaneous coronary intervention.

The primers were designed using Gene Runner (Gene Runner 6.5.50), PerlPrimer (PerlPrimer 1.1.21), Primer3 (Primer3, 4.1.0), and OligoAnalyzer (OligoAnalyzer 3.1) software tools.

The polymerase chain reaction (PCR) test was performed according to an individual setup for each set of oligos, covering the aforementioned regions of the *LDLR*, *PCSK9*, and *APOB* genes. Sanger sequencing was done for all the PCR products using the ABI 3500 DNA Sequencer (Applied Biosystems, CA, United States).

Results of the *in silico* Analysis

BioEdit software (BioEdit 7.2.1) was applied to analyze the sequencing outcomes. The identified variants of the nucleotides were analyzed through the UCSC Genome Browser[1], ClinVar[2], UCL[3], and LOVD[4] databases.

Additionally, SIFT[5], PROVEAN[6], PolyPhen-2[7], and MutationTaster[8] *in silico* predictive software tools were utilized to scrutinize the pathogenesis of the detected mutations.

RESULTS

Clinic Data

Fifteen unrelated individuals with clinically diagnosed FH and premature CAD who met the SB and/or DLCNS criteria were recruited in the current study. According to the SB criteria, of the 15 patients, seven had definite FH and five possible FH. Based on the DLCNS criteria, of the 15 patients, nine had definite, two probable, and four possible FH (**Table 1**). The study population's demographic characteristics, clinical features, and cholesterol levels are demonstrated in **Table 1**. Almost all the recruited patients had premature CAD unrelated to congenital heart diseases, hypertension, arrhythmias, or any other cardiovascular diseases.

Spectrum of the Nucleotide Variants

Direct DNA sequencing of the whole coding exons, with their flanking intron sequences, of the *LDLR* and *PCSK9* genes was performed. Also examined was the presence of the mutations (p.Arg3500Gln, p.Arg3500Trp, p.Arg3531Cys, and p.His3543Tyr) in the gene coding for *APOB*. The FH-causing variants were identified in seven (46.6%) individuals of the 15 screened patients, whereas no mutation was detected in eight patients clinically diagnosed with FH and premature CAD. Among the identified mutations, six occurred in the *LDLR* gene, whereas both *LDLR* and *PCSK9* genes were mutated in one patient (P1). The seven detected mutations were harbored in five different exons (4, 6, 7, 10, and 12) of the *LDLR* gene (**Table 2** and **Figure 1**), and a p.Ala53Val mutation in the *PCSK9* gene was located in exon 1. The bioinformatics analysis of all the identified mutations in the *LDLR* gene categorized them as deleterious, probably damaging, and disease causing (**Table 2**).

Among these seven probands, four (P1, P4, P8, and P9) carried the homozygous FH-causing variants and three probands (P2, P7, and P10) carried a mutation in the heterozygous status. Our data showed that all the mutations in the *LDLR* gene (p.Asp172His, p.Cys338Trp, p.Cys302Trp, p.Cys148Tyr, p.Leu479Gln, and p.Cys216Tyr) and the mutation in the *PCSK9* gene (p.Ala53Val) were of the missense type, except for 1 mutation in the *LDLR* gene (p.G593Afs*72), which was nonsense. The p.Asp172His, p.Cys302Trp, p.Cys148Tyr, p.Cys216Tyr, and p.G593Afs*72 mutations in the *LDLR* gene and the p.Ala53Val mutation in the *PCSK9* gene have been reported in populations other than Iranians, whereas the p.Leu479Gln change has been reported previously in an Iranian patient. Segregation analyses were done on the available family members of the study population.

The homozygous nucleotide variants (c.514G > C [p.Asp172His]), harbored in exon 4 of the *LDLR* gene, and c.158C > T [p.Ala53Val], located in exon 1 of the *PCSK9* gene) were identified in an 11-year-old girl, who had undergone heart valve replacement and had high levels of TC (417 mg/dL), LDL-C (240 mg/dL), and xanthomas, with the latter seen in her knees and elbows and between her fingers. The segregation analysis confirmed that these mutations were disease-causing variants and presented in the proband's parents and two brothers. Both of her parents and both of her siblings were heterozygous for the *LDLR* mutation, although they had different statuses vis-à-vis the *PCSK9* mutation. The c.158C > T *PCSK9* variant was in a heterozygous form in both parents but was inherited in the homozygous status in the elder brother. The younger brother, 2 years of age, was a wild type for this nucleotide change (**Figure 2**). The parents and the siblings had elevated cholesterol levels as well (father: TC = 306 mg/dL and LDL-C = 228 mg/dL; mother: TC = 245 mg/dL and LDL-C = 187 mg/dL; elder brother: TC = 223 mg/dL and LDL-C = 191 mg/dL; and younger brother: TC = 233 mg/dL and LDL-C = 177.8 mg/dL). The mother and father of the proband were first cousins, and there was a positive family history of myocardial infarction, cardiovascular diseases, and hypercholesterolemia in this pedigree (**Figure 2**). The recognized *PCSK9* mutation in this family was reported as a loss-of-function mutation.

Another homozygous mutation (c.1436T > A) in exon 10 of the *LDLR* gene was found in a 24-year-old man (P8). The patient had undergone coronary artery bypass graft (CABG) surgery and valve replacement and had a high, uncontrollable cholesterol level (TC = 461 mg/dL and LDL-C = 391 mg/dL) with detectable xanthomas. This nucleotide variant changes leucine in position 479 to glutamine, which is deleterious, probably damaging, and disease causing according to different *in silico* programs. The patient's parents had a consanguineous marriage and a positive history of myocardial infarction, CABG, and hypercholesterolemia. The cosegregation analysis confirmed that this variant was an FH-causing mutation in the family. The proband's father, mother, and brother were

[1] https://genome.ucsc.edu
[2] www.ncbi.nlm.nih.gov/clinvar
[3] https://www.ucl.ac.uk/fh-old/muttab.html
[4] https://www.LOVD.nl/LDLR
[5] https://sift.bii.a-star.edu.sg/
[6] provean.jcvi.org
[7] genetics.bwh.harvard.edu/pph2
[8] www.mutationtaster.org

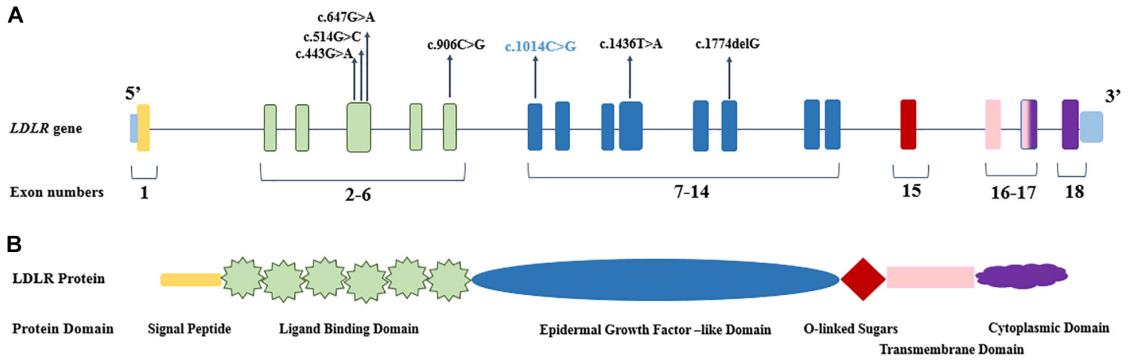

FIGURE 1 | The genomic and protein organization of LDLR. **(A)** The location of identified pathogenic mutations demonstrated in the different exons of *LDLR* gene. The novel mutation presented in blue color. **(B)** The domain organization of LDLR protein, which corresponded to LDLR coding exons (with the common colors).

heterozygous for the mutation, whereas his sister was a wild type (**Supplementary Figure 1**).

A nonsense homozygous nucleotide variant (c.1774delG [p.G593Afs*72]) was identified in a 22-year-old man (**Supplementary Figure 2**). This variant, harbored in exon 12 of the *LDLR* gene, was a frameshift mutation, where the deletion of G led to a shift in the reading frame, created a stop codon 72 amino acids downstream, and produced a truncated protein. The DNA sequencing of the *LDLR* gene demonstrated five G nucleotides in positions 1774–1778, making two glycines at a protein level (592-593 amino acids). Nonetheless, the deletion of G in any of the aforementioned positions altered the second glycine to alanine and generated stop codon 72 amino acids downstream. The software tools of SIFT and MutationTaster predicted this variant to be deleterious and disease causing, respectively. The pedigree analysis of this proband revealed a consanguineous marriage in the family. His mother and father were first cousins with a positive paternal family history of hypercholesterolemia and myocardial infarction. The proband had two younger siblings, who were apparently not affected. The father, who was the only available individual from the family, was heterozygous for this deletion. As the proband's parents were first cousins, and both he and his father were correspondingly homozygous and heterozygous for the variant, the mother of the family, who had hypercholesterolemia, was probably heterozygous for this variant as well.

Additionally, 34 nucleotide variants, dedicated to the exons, introns, and 3-prime untranslated region (3′-UTR) of the *LDLR* gene, were detected in the 15 examined patients (**Supplementary Table 2**). All the nucleotide variants previously described were categorized as benign, likely benign, and with other allele. Moreover, 31 nucleotide variants were identified in the *PCSK9* gene; most of them were categorized as benign, likely benign, with other allele, and with uncertain significance allele (**Supplementary Table 3**). Among them, one exonic (c.1233G > A), one intronic (c.1863+20C > G), and two 3′-UTR nucleotide variants (c.*863 A > G and c.*980 A > G) have not been previously reported. None of the mutations of the *APOB* gene (p.Arg3500Gln, p.Arg3500Trp, p.Arg3531Cys, and p.His3543Tyr) was detected in our 15 screened patients.

Novel Familial Hypercholesterolemia-Causing Variant

One novel nucleotide variant (c.1014C > G) was detected and cosegregated in the available family members as well. The *in silico* mutation prediction tools of SIFT, PROVEAN, PolyPhen-2, and MutationTaster were applied to predict the functionality of the aforementioned variant. The c.1014C > G heterozygous missense mutation was found in a 39-year-old woman. This mutation corresponded to exon 7 of the *LDLR* gene and changed cysteine (C) amino acid in position 338 to tryptophan (W). SIFT, PROVEAN, PolyPhen-2, and MutationTaster predicted this variant to be deleterious, probably damaging, and disease causing, respectively. The family pedigree of the proband featured a positive history of hypercholesterolemia and myocardial infarction on the maternal side (**Figure 3**). The patient had three sisters and a deceased brother. Two younger sisters, aged 36 and 33 years, suffered from hypercholesterolemia as well; the youngest sister, however, was seemingly normal. The only available individual from the family was the proband's 36-year-old sister, who was also heterozygous for this mutation. The mother, who also had hypercholesterolemia, passed away at the age of 60 years due to myocardial infarction. She probably carried the mutation. The proband and her sister, who carried the heterozygous mutation, had consanguineous marriages with their first cousins.

DISCUSSION

Frequent *LDLR*, *APOB*, and *PCSK9* gene mutations have been described in patients suffering from hypercholesterolemia; however, genetic data on the Iranian population are still limited (Jensen et al., 1996; Fardesfahani et al., 2005; Fardesfahani and Khatami, 2010; Farrokhi et al., 2011; Fairoozy et al., 2017; Ekrami et al., 2018; Nikkhooy et al., 2018; Tajamolian et al., 2018).

In this study, we found eight different mutations among 15 patients with clinically diagnosed FH and premature CAD. Among the study population, one patient had double mutations in both *LDLR* and *PCSK9* genes. The present study is the first investigation to report the c.1014C > G mutation. With

TABLE 2 | The pathogenic mutations of *LDLR* and *PCSK9* genes detected in this study.

patient ID	Mutation variants	Protein changes	Genotype	Exon	RS	Pathogenicity	Country distribution	SIFT	Provean	Polyphen2	Mutation-Taster	1000G (allele carriers)	gnomAD	Iranome	UCL (mutation table)	LOVD-LDLR
LDLR																
P1	c.514G > C	p.Asp172-His	CC	4	rs8792-54554	Likely pathogenic	South Africa (Thiart et al., 2000)	Deleterious	Deleterious	Probably Damaging	Disease causing	–	–	–	reported	reported
P7	c.443G > A	p.Cys148-Tyr	GA	4	rs8792-54526	Likely pathogenic	German, United Kingdom (Nauck et al., 2001; Humphries et al., 2006)	Deleterious	Deleterious	Probably Damaging	Disease causing	–	–	–	reported	reported
P10	c.647G > A	p.Cys216-Tyr	GA	4	rs8792-54611	Likely pathogenic	German, Spanish (Dedoussis et al., 2004; Meriño-Ibarra et al., 2007)	Deleterious	Deleterious	Probably Damaging	Disease causing	–	–	–	Not reported	reported
P4	c.906C > G	p.Cys302-Trp	GG	6	rs8792-54716	Likely pathogenic	Iraqi, Turkish patient (Webb et al., 1996)	Deleterious	Deleterious	Probably Damaging	Disease causing	–	–	–	reported	reported
P2	c.1014C > G	p.Cys338-Trp	CG	7	rs879-254755	Pathogenic	Novel	Deleterious	Deleterious	Probably Damaging	Disease causing	–	–	–	Not reported	Not reported
P8	c.1436T > A	p.Leu479-Gln	AA	10	rs879-254900	Likely pathogenic	United Kingdom (Heath et al., 2001) Iranian (Fairoozy et al., 2017)	Deleterious	Deleterious	Probably Damaging	Disease causing	–	–	–	Not reported	Not reported
P9	c.1774 delG	p.G593-Afs*72	delGdelG	12	–	–	Italian N.Irish (Bertolini et al., 2017)	Deleterious	Deleterious	–	Disease causing	–	–	–	reported	Not reported
PCSK9																
P1	c.158C > T	p.Ala53-Val	TT	1	rs115-83680	Pathogenic	South Africa (Thiart et al., 2000)	Deleterious	Neutral	Benign	Polymor-phism	405	6099	5	–	–

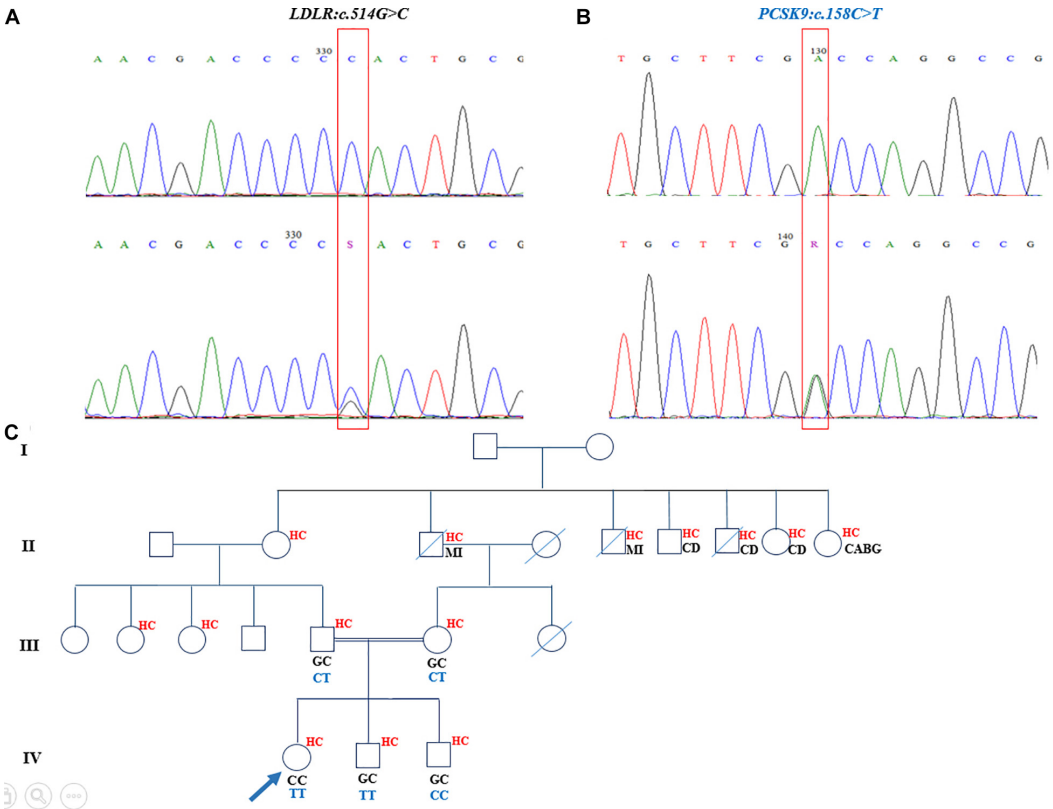

FIGURE 2 | Sequencing analysis and pedigree of proband who had double mutations in *LDLR* and *PCSK9* genes. **(A)** The homozygote and heterozygote nucleotide changes at c.514G > C in *LDLR* demonstrated in upper chromaogram and lower chromatogram, respectively. **(B)** The loss-of-function mutation of *PCSK9* in homozygote and heterozygote states depicted correspondingly in upper panel and lower panel. **(C)** The genotype of proband and her family members for *LDLR* and *PCSK9* nucleotide changes demonstrated, respectively, in black and dark blue in the pedigree. The family history of proband exhibited positive history of hypercholesterolemia and cardiovascular diseases and myocardial infarction. The mother and father of the proband are first cousins. MI, myocardial infarction; HC, hypercholesterolemia; CD, cardiovascular diseases; CABG, coronary artery bypass grafting.

the exception of the c.1436T > A mutation, which has been previously reported in Iran (Fairoozy et al., 2017), all the other mutations have already been reported in populations other than Iranians. Among seven mutations in the *LDLR* gene, only four nucleotide variants are reported in the UCL-FH database (**Table 2**), and none of the pathogenic mutations can be found in 1000Genome, gnomAD, and Iranome databases (**Table 2**).

Previous research on the Iranian population (Jensen et al., 1996; Fardesfahani et al., 2005; Fardesfahani and Khatami, 2010; Khan et al., 2011; Fairoozy et al., 2017; Ekrami et al., 2018; Nikkhooy et al., 2018; Tajamolian et al., 2018) has identified 15 different mutations harbored in exons 3, 4, 9, 10, 11, 12, 14, and 17 of the *LDLR* gene (**Supplementary Table 4**). In 2017, an investigation drawing upon target next-generation sequencing identified seven different mutations (C.389C > G, c.1436T > A, c.1599G > A, c.1729T > C, c.2001_2002delinsGT, and c.2146dupG) that were not previously reported in the Iranian population (Fairoozy et al., 2017). Two different mutations (c.285C > G and c.415G > C) were found in another study conducted on a sample of the Iranian population (Ekrami et al., 2018). The c.445G > T, c. 1246C > T, c.1478-1479delCT, c.660-661InsCC, c.105A > T, and c.1474G > A mutations have also been reported in different studies (Fardesfahani et al., 2005; Fardesfahani and Khatami, 2010; Khan et al., 2011; Nikkhooy et al., 2018; Tajamolian et al., 2018).

The detected mutations in the *LDLR* gene in the present study were scattered over exons 4, 6, 7, 10, and 12, and they encoded the functional domain, the ligand-binding domain, and the epidermal growth factor (EGF)–like of the mature LDLR protein (**Figure 1**). The prevalence of the identified mutations was uppermost in exon 4 (3/7 = 43%) of the *LDLR* gene (Heath et al., 2001; Villéger et al., 2002). Exon 4, along with exons 5 to 7, of the *LDLR* gene encodes the ligand-binding domain of the LDLR protein; nevertheless, exon 4 encodes the critical ligand-binding section of the protein, and any changes in the sequence may affect the folding of the protein and change the functionality of the mature protein. The consequences of missense mutations in this exon may lessen the receptor affinity of LDLR toward both APOE and APOB (Esser et al., 1988). Our data revealed c.514C > G, c.443G > A, and c.647G > A mutations in exon 4; none of these mutations has been previously reported among Iranians. Based on the previously detected mutations in the Iranian population, 5 of the 15 (33.3%) identified mutations occurred in exon 4 of *LDLR* (**Supplementary Table 4**). Accumulating evidence has

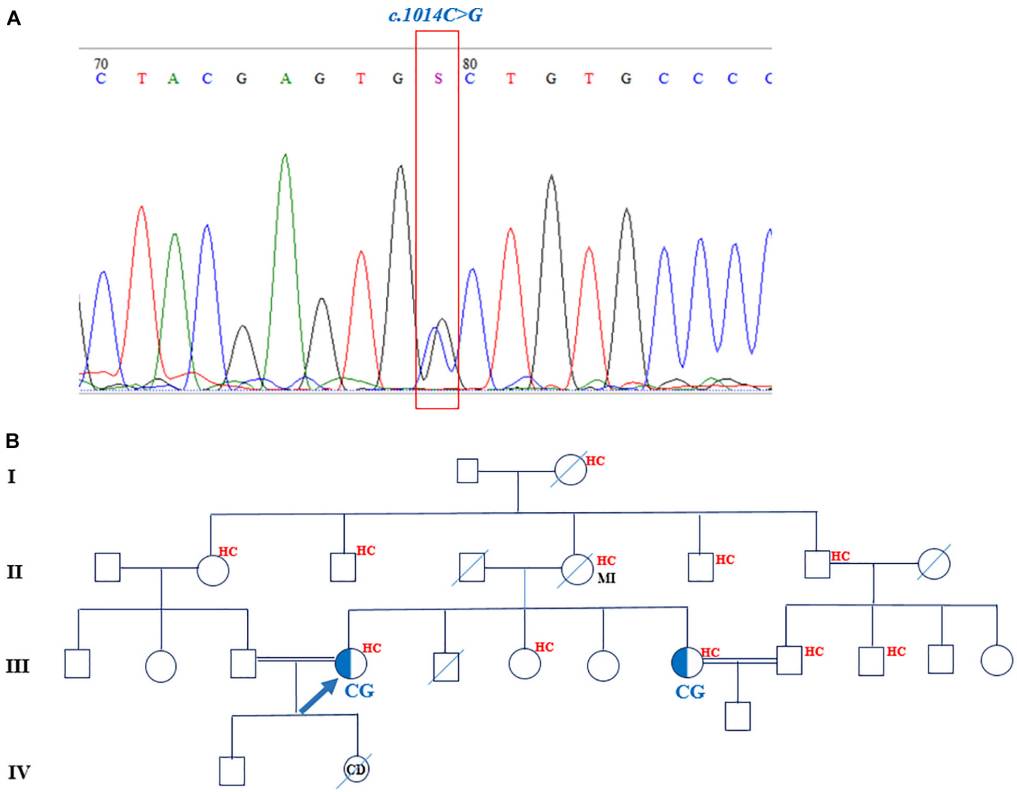

FIGURE 3 | The chromatogram and pedigree of patient carried the novel mutation in *LDLR* gene. **(A)** The identified c.1014C > G nucleotide change shown in a chromatogram. **(B)** The pedigree of proband demonstrated the positive family history of hypercholesterolemia and myocardial infarction. The sister of proband is also heterozygote for the mutation. MI, myocardial infarction; HC, hypercholesterolemia.

demonstrated that individuals carrying mutations in exon 4 have a more severe FH phenotype and congenital heart disease risks; mutations in this region are therefore predominantly deleterious to receptor function (Gudnason et al., 1994; Graham et al., 1999; Humphries et al., 2006). The c.443G > A and c.647G > A mutations have both been described in Germany, the United Kingdom, and Spain (Nauck et al., 2001; Dedoussis et al., 2004; Humphries et al., 2006; Meriño-Ibarra et al., 2007).

The genetic analysis on an 11-year-old girl from the southern Iranian city of Kerman demonstrated a homozygous loss-of-function mutation in the *PCSK9* gene (c.158C > T [p.Ala53Val]) and a pathogenic homozygous mutation in the *LDLR* gene (c.514C > G [p.Asp172His]). The c.158C > T variant in *PCSK9* has also been previously reported in South Africa (Thiart et al., 2000). Several studies have revealed that loss-of-function mutations in *PCSK9* culminate in low LDL-C levels and lead to a diminished risk of CAD (Benjannet et al., 2012; Seidah, 2017; Bayona et al., 2020). Apropos the effect of loss-of-function *PCSK9* variants, some clinical trials have also confirmed that PCSK9 inhibitors lower LDL-C concentrations in the serum (Cohen et al., 2006; Stein et al., 2014; Vuorio et al., 2016; Naeli et al., 2017). Although the index patient carried the homozygous loss-of-function mutation in *PCSK9*, she presented severe phenotypes such as an uncontrollable cholesterol level; several elbows, knees, and hip xanthomas; and aortic valve disease, which necessitated valve replacement surgery. The reason for the patient's presentations may have been completely defective *LDLR* activity owing to homozygous mutations. In addition, she was the offspring of a consanguineous marriage between a couple who presented with moderate hypercholesterolemia but had no cardiovascular diseases. The parents carried both mutations in *LDLR* and *PCSK9* in heterozygous forms. Thus, it seems that the loss-of-function mutation in *PCSK9* has a protective effect against the development of the severe forms of phenotypes (Seidah, 2019) in such individuals, notwithstanding the heterozygous mutation in *LDLR*. The proband had two brothers, who also carried the heterozygous mutation in the *LDLR* gene. In regard to the *PCSK9* gene, the elder brother, similar to the parents, featured a heterozygous mutation, whereas the younger one was a wild type.

To the best of our knowledge, this is the first report on the coincidental inheritance of a pathologic mutation in *LDLR* (p.Asp172His) and a loss-of-function mutation (p.Ala53Val) in *PCSK9* in an Iranian family. The loss-of-function mutation (c.137G > T [p.Arg46Leu]) in *PCSK9* and a pathologic mutation in *LDLR* (c.902A > G [p.Asp301Gly]), both in the heterozygous form, have been previously reported in a Caucasian family (Bayona et al., 2020).

The c.906C > G FH-causing variant (p.Cys302Trp), which was found in this study in exon 6 of *LDLR*, has also been previously identified in an Iraqi–Turkish patient

(Webb et al., 1996). The 40-year-old woman carrying this mutation originated from the north of Iran. Given the close geographical and cultural links between Iraq, Turkey, and Iran, it is probable that the two patients shared some common founder mutations.

The novel mutation found in this study (c.1014C > G), which is located in exon 7 of the *LDLR* gene, changes cysteine to tryptophan. According to the LOVD database, approximately 3% of the total 3731 variants of *LDLR* happen on exon 7 (Fokkema et al., 2005). This variant has not been previously identified; still, the same location features another change that has been previously reported (c.1014C > A). This variation, which is a stop-gained mutation, alters cysteine in position 338 to the termination code and creates a truncated protein (Kotzer and Baudhuin, 2009). Exon 7, along with exons 8–12, of the *LDLR* gene participates in the production of the EGF precursor homology domain of LDLR. This domain of LDLR shares such conserved sequences as cysteine-rich sequences, YWTD repeats, and EGF repeats to EGF (Davis et al., 1987; Springer, 1998). Furthermore, the EGF precursor-like domain comprised two EGF homology domains (EGF-A and EGF-B), dispersed from the third EGF-like domain by a B-propeller domain. Exon 7 of *LDLR* encodes the EGF-A part of the EGF-like domain of LDLR, which encompasses 40-amino-acid residues and six cysteine amino acids, forming three disulfide bonds (Springer, 1998; Jeon et al., 2001). In the molecular mechanisms of LDLR recycling in hepatocytes, PCSK9 binds to the EGF-A domain of LDLR in a PH- and calcium-dependent manner and through lysosomal degradation decreases the total LDLR level in the liver (Zhang et al., 2007; Bottomley et al., 2009). A deficiency in this region interferes with the release of the binding ligand of the internalized LDLR and inhibits its recycling to the cell surface (Davis et al., 1987). On account of the fact that this missense mutation exists in a cysteine residue, it may result in misfolding and defective protein receptors. In addition, all *in silico* predictive programs describe this variant as deleterious, probably damaging, and disease causing. The segregation analysis of this variant in the index patient's sister, who had hypercholesterolemia, confirmed the pathogenicity of this variant as well.

The point mutations (p.leu479Gln and p.G593Afs*72) were respectively harbored in exons 10 and 12, which encode different parts of the LDLR EGF precursor-like domain. Exon 10 encodes part of a 5-repeat region of 40–60 amino acids that has a conserved motif (YWTD). This part is also known to play a role in the intracellular trafficking and recycling of LDLR, which explains why mutations in this section are acknowledged to be the cause of transport-defective or recycling-defective proteins (Brown and Goldstein, 1974; Davis et al., 1987). The p.leu479Gln mutation has been previously described in Iran (Fairoozy et al., 2017) and the United Kingdom (Heath et al., 2001). Our segregation analysis of this mutation in the first degree-relatives of the proband (P8) confirmed the pathogenicity of the mutation too. The translation product of the allele carrying the frameshift mutation (p.G593Afs*72) leads to the truncation of the proteins of 663 amino acids and is deemed a receptor-negative mutation (Bertolini et al., 2017). With respect to the pathogenicity of this mutation, confirmed via the molecular diagnosis of the variation in the family of the proband (P9), the result of the screening of the family demonstrated that the father carried this mutation in a heterozygous state. It is also probable that the proband's mother was also heterozygous for this variation. This mutation was originally found in Italian and Northern Ireland populations (Bertolini et al., 2017).

Furthermore, we succeeded in detecting 31 different nucleotide variants in the *PCSK9* gene. While most of them have been previously reported, there are some unreported variants scattered in exons, introns, and 3′-UTRs (**Supplementary Table 3**). The UTR parts (5′ and 3′) include regulatory elements that interact with such different regulatory factors as transcription factors and microRNAs. Altering the nucleotides in the UTRs of genes may interfere with their interactions and causes the misexpression of their targeted gene (Chatterjee and Pal, 2009; Azad et al., 2020). The existing evidence notwithstanding, this hypothesis still requires more functional analyses to be confirmed.

In the present study, we detected none of the common *APOB* mutations, suggesting that the frequency of *APOB* mutations in Iran may differ from that in other countries. Further research on this point is warranted.

Among our study population, which comprised 15 subjects with clinically diagnosed FH and premature CAD, we detected eight patients who were negative for FH mutations. Accumulating evidence has shown that, in addition to mutations in the *LDLR* and *PCSK9* genes, mutations in other single or multiple genes can contribute to the creation of the FH phenotype (Smilde et al., 2001; Nordestgaard et al., 2013; Cuchel et al., 2014b; Fouchier et al., 2014; Brautbar et al., 2015; Berberich and Hegele, 2019). The accumulation of common small-effect LDL-C–raising alleles can elevate LDL-C levels and, consequently, cause FH (Talmud et al., 2013). Accordingly, the absence of mutations in the FH-mutation–negative probands in our study may be attributable to nucleotide alterations in other genes, nucleotide variations in other parts of the *APOB* gene (not addressed in this study), or a combination of LDL-C-raising alleles. Be that as it may, previous studies on other diseases have demonstrated that the presence and combinations of some polymorphisms can render individuals more susceptible to disease (Khajali and Khajali, 2014; Mesic et al., 2019; Pinheiro et al., 2019).

We succeeded in finding several nucleotide variations/polymorphisms in both *LDLR* and *PCSK9* scattered in exons, introns, and 5′–3′ UTRs. These nucleotide variations/polymorphisms could be the combination of different polymorphisms, predisposing FH-mutation–negative individuals to hypercholesterolemia.

Study Limitations

The results of the present study should be interpreted in light of its limitations. First, our small sample size precluded an accurate estimation of the prevalence of the mutations in the Iranian population. Budget constraints also limited our investigation to the screening of only the *LDLR* and *PCSK9* genes and a part of the *APOB* gene. Indubitably, many other hitherto reported and unreported genes may be the underlying causes of FH-mutation negativity. A large insertion or deletion in the *LDLR* gene causes

FH in 5–10% of patients (Hobbs et al., 1985; Goldmann et al., 2010); we were, unfortunately, unable to detect such mutations. Finally, what has thus far hampered research on polygenic FH in Iran is, aside from financial restraints, the dearth of data on the frequency of polymorphisms in the Iranian population.

CONCLUSION

The existing literature contains a paucity of information regarding genetic testing on nucleotide alterations in the Iranian population. The finding of seven different mutations in this study, along with other studies on samples of the Iranian population, demonstrates the high degree of genetic heterogeneity and the wide spectrum of mutations in Iranians. This domain undoubtedly requires further in-depth research. In daily practice, patients presenting with premature CAD are rarely screened for the genetic analysis of FH; this is a missed opportunity for preventive therapy. Given that the first-degree relatives of patients with FH have a 50% chance of affliction, a targeted molecular genetic screening of individuals with premature CAD and FH is an effective strategy both to prevent cardiovascular diseases in their nascent stages and to confer more desirable management plans.

ACCESSION NUMBER

The accession number of the novel and reported variants are as follows:

LDLR
c.1014C>G variant: [ClinVar]: [SCV001446309]
c.443G>A variant: [ClinVar]: [SCV001467722]
c.647G>A variant: [ClinVar]: [SCV001467723.1]
c.906C>G variant: [ClinVar]: [SCV001467724.1]
c.1436T>A variant: [ClinVar]: [SCV001467725.1]
c.1774delG variant: [ClinVar]: [SUB8843875, processing]
c.514G>C variant: [ClinVar]: [SUB8843863, processing]

PCSK9
c.1233G>A variant: [ClinVar]: [SCV001450531]
c.1863+20C>G variant: [ClinVar]: [SCV001450532]
c.*863A>G variant: [ClinVar]: [SCV001450537]
c.*980A>G variant: [ClinVar]: [SCV001450538]

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Rajaie Cardiovascular, Medical and Research Center Ethics Committees. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AM: experiment design, lab work, data production, data interpretation, and MS edit. MMale, ZG, ZK, and FN: clinical evaluation of patients, data interpretation, and MS edit. MMo: lab work, data production, and MS edit. SK: data interpretation and MS edit. SM and NS: experiment design, data interpretation, and MS edit. MMala: experiment design, data interpretation, writing the first draft of MS, and manuscript final edit. All authors contributed to the article 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.2021.625959/full#supplementary-material

Supplementary Figure 1 | Sequencing analysis and pedigree of proband with c.1436T>A nucleotide change in *LDLR* gene. **(A)** The mutation found in proband in a homozygote form (upper chromatogram) and identified in a heterozygote statue in father of the patient (lower chromatogram). **(B)** The pedigree of the proband demonstrated the consanguineous marriage, positive family history of hypercholesterolemia, myocardial infarction and cardiovascular diseases. The genotypes of his sibling and his parent exhibited in dark blue color as well. MI, HC, and CD stand for myocardial infarction, hypercholesterolemia, and cardiovascular diseases, respectively.

Supplementary Figure 2 | The chromatogram and pedigree of patient who carried the c.1774delG mutation in *LDLR* gene. **(A)** The upper chromatogram demonstrated the sequence in wild type. The homozygote deletion mutation in nucleotide 1774 exhibited in middle chromatogram and the heterozygote form depicted in lower chromatogram. **(B)** The pedigree of the proband showed the consanguineous marriage and positive family history of hypercholesterolemia and MI. MI and HC stand for Myocardial infarction and Hypercholesterolemia, respectively.

Supplementary Table 1 | The sequences of all designed oligonucleotides utilized in this study to perform the PCRs and Sanger sequencing.

Supplementary Table 2 | All nucleotide variations of *LDLR* gene found in this study.

Supplementary Table 3 | All nucleotide variations of *PCSK9* gene found in this study.

Supplementary Table 4 | The previously reported pathogenic mutations in Iranian population.

REFERENCES

Azad, F. M., Arabian, M., Maleki, M., and Malakootian, M. (2020). Small Molecules with Big Impacts on Cardiovascular Diseases. *Biochem. Genet.* 58, 359–383. doi: 10.1007/s10528-020-09948-z

Bayona, A., Arrieta, F., Rodríguez-Jiménez, C., Cerrato, F., Rodríguez-Nóvoa, S., Fernández-Lucas, M., et al. (2020). Loss-of-function mutation of PCSK9 as a protective factor in the clinical expression of familial hypercholesterolemia: A case report. *Medicine* 99:e21754. doi: 10.1097/md.0000000000021754

Beheshti, S. O., Madsen, C. M., Varbo, A., and Nordestgaard, B. G. (2020). Worldwide Prevalence of Familial Hypercholesterolemia. *Meta Analy. Million Sub.* 75, 2553–2566. doi: 10.1016/j.jacc.2020.03.057

Benjannet, S., Hamelin, J., Chrétien, M., and Seidah, N. G. (2012). Loss-and gain-of-function PCSK9 variants cleavage specificity, dominant negative effects, and low density lipoprotein receptor (LDLR) degradation. *J. Biol. Chem.* 287, 33745–33755.

Berberich, A. J., and Hegele, R. A. (2019). The complex molecular genetics of familial hypercholesterolaemia. *Nat. Rev. Cardiol.* 16, 9–20. doi: 10.1038/s41569-018-0052-6

Bertolini, S., Pisciotta, L., Fasano, T., Rabacchi, C., and Calandra, S. (2017). The study of familial hypercholesterolemia in Italy: A narrative review. *Atheroscl. Suppl.* 29, 1–10. doi: 10.1016/j.atherosclerosissup.2017.07.003

Bottomley, M. J., Cirillo, A., Orsatti, L., Ruggeri, L., Fisher, T. S., Santoro, J. C., et al. (2009). Structural and biochemical characterization of the wild type PCSK9-EGF (AB) complex and natural familial hypercholesterolemia mutants. *J. Biol. Chem.* 284, 1313–1323. doi: 10.1074/jbc.m808363200

Brautbar, A., Leary, E., Rasmussen, K., Wilson, D. P., Steiner, R. D., and Virani, S. (2015). Genetics of familial hypercholesterolemia. *Curr. Atheroscl. Rep.* 17:20.

Brown, M. S., and Goldstein, J. L. (1974). Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proc. Natl. Acad. Sci. U. S. A.* 71, 788–792. doi: 10.1073/pnas.71.3.788

Chatterjee, S., and Pal, J. K. (2009). Role of 5′-and 3′-untranslated regions of mRNAs in human diseases. *Biol. Cell* 101, 251–262. doi: 10.1042/bc20080104

Cohen, J. C., Boerwinkle, E., Mosley, T. H. Jr., and Hobbs, H. H. (2006). Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N. Engl. J. Med.* 354, 1264–1272. doi: 10.1056/nejmoa054013

Cuchel, M., Bruckert, E., Ginsberg, H., Raal, F., Santos, R., Hegele, R., et al. (2014a). European Atherosclerosis Society Consensus Panel on Familial Hypercholesterolaemia. Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. *Eur. Heart J.* 35, 2146–2157.

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014b). Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. *Eur. Heart J.* 35, 2146–2157.

Davis, C. G., Goldstein, J. L., Südhof, T. C., Anderson, R. G., Russell, D. W., and Brown, M. S. (1987). Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature* 326, 760–765. doi: 10.1038/326760a0

De Castro-Orós, I., Pocoví, M., and Civeira, F. (2010). The genetic basis of familial hypercholesterolemia: inheritance, linkage, and mutations. *Appl. Clin. Genet.* 3:53. doi: 10.2147/tacg.s8285

Dedoussis, G. V., Genschel, J., Bochow, B., Pitsavos, C., Skoumas, J., Prassa, M., et al. (2004). Molecular characterization of familial hypercholesterolemia in German and Greek patients. *Hum. Mutat.* 23, 285–286. doi: 10.1002/humu.9218

Ekrami, M., Torabi, M., Ghafouri-Fard, S., Mowla, J., Soltani, B. M., Hashemi-Gorji, F., et al. (2018). Genetic analysis of iranian patients with familial hypercholesterolemia. *Iran. Biomed. J.* 22:117.

El Khoury, P., Elbitar, S., Ghaleb, Y., Abou Khalil, Y., Varret, M., Boileau, C., et al. (2017). PCSK9 mutations in familial hypercholesterolemia: from a groundbreaking discovery to anti-PCSK9 therapies. *Curr. Atheroscl. Rep.* 19:49.

Esser, V., Limbird, L., Brown, M. S., Goldstein, J. L., and Russell, D. W. (1988). Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *J. Biol. Chem.* 263, 13282–13290. doi: 10.1016/s0021-9258(18)37702-0

Fairoozy, R., Futema, M., Vakili, R., Abbaszadegan, M., Hosseini, S., Aminzadeh, M., et al. (2017). The genetic spectrum of familial hypercholesterolemia (FH) in the Iranian population. *Scient. Rep.* 7, 1–9.

Fardesfahani, P., and Khatami, S. (2010). Familial hypercholesterolemia in Iran: A novel frameshift mutation in low density lipoprotein receptor (LDLR) gene.

Fardesfahani, P., Rouhi, D. S., Khatami, S., Zeinali, C., and Taghikhani, M. (2005). A Novel Mutation in exon 4 of the low density lipoprotein (LDL) receptor gene in an Iranian Familial Hypercholesterolemia patient. *Iran. Biomed. J.* 3:9.

Farrokhi, E., Shayesteh, F., Mobarakeh, S. A., Dehkordi, F. R., Samani, K. G., and Chaleshtori, M. H. (2011). Molecular characterization of Iranian patients with possible familial hypercholesterolemia. *Ind. J. Clin. Biochem.* 26, 244–248. doi: 10.1007/s12291-011-0113-7

Fokkema, I. F., den Dunnen, J. T., and Taschner, P. E. (2005). LOVD: easy creation of a locus-specific sequence variation database using an "LSDB-in-a-box" approach. *Hum. Mutat.* 26, 63–68. doi: 10.1002/humu.20201

Fouchier, S. W., Dallinga-Thie, G. M., Meijers, J. C., Zelcer, N., Kastelein, J. J., Defesche, J. C., et al. (2014). Mutations in STAP1 are associated with autosomal dominant hypercholesterolemia. *Circul. Res.* 115, 552–555. doi: 10.1161/circresaha.115.304660

Gidding, S. S., Ann Champagne, M., de Ferranti, S. D., Defesche, J., Ito, M. K., Knowles, J. W., et al. (2015). The agenda for familial hypercholesterolemia: a scientific statement from the American Heart Association. *Circulation* 132, 2167–2192. doi: 10.1161/cir.0000000000000297

Goldmann, R., Tichý, L., Freiberger, T., Zapletalová, P., Letocha, O., Soška, V., et al. (2010). Genomic characterization of large rearrangements of the LDLR gene in Czech patients with familial hypercholesterolemia. *BMC Med. Genet.* 11, 1–8. doi: 10.1186/1471-2350-11-115

Graham, C. A., McClean, E., Ward, A. J., Beattie, E. D., Martin, S., O'Kane, M., et al. (1999). Mutation screening and genotype: phenotype correlation in familial hypercholesterolaemia. *Atherosclerosis* 147, 309–316.

Gudnason, V., Day, I., and Humphries, S. (1994). Effect on plasma lipid levels of different classes of mutations in the low-density lipoprotein receptor gene in patients with familial hypercholesterolemia. *Arterioscl. Thromb. J. Vascul. Biol.* 14, 1717–1722. doi: 10.1161/01.atv.14.11.1717

Heath, K. E., Humphries, S. E., Middleton-Price, H., and Boxer, M. (2001). A molecular genetic service for diagnosing individuals with familial hypercholesterolaemia (FH) in the United Kingdom. *Eur. J. Hum. Genet.* 9, 244–252. doi: 10.1038/sj.ejhg.5200633

Hobbs, H. H., Lehrman, M. A., Yamamoto, T., and Russell, D. W. (1985). Polymorphism and evolution of Alu sequences in the human low density lipoprotein receptor gene. *Proc. Natl. Acad. Sci.* 82, 7651–7655. doi: 10.1073/pnas.82.22.7651

Hu, P., Dharmayat, K. I., Stevens, C. A., Sharabiani, M. T., Jones, R. S., Watts, G. F., et al. (2020). Prevalence of familial hypercholesterolemia among the general population and patients with atherosclerotic cardiovascular disease: a systematic review and meta-analysis. *Circulation* 141, 1742–1759. doi: 10.1161/circulationaha.119.044795

Humphries, S. E., Cranston, T., Allen, M., Middleton-Price, H., Fernandez, M. C., Senior, V., et al. (2006). Mutational analysis in UK patients with a clinical diagnosis of familial hypercholesterolaemia: relationship with plasma lipid

traits, heart disease risk and utility in relative tracing. *J. Mole. Med.* 84, 203–214. doi: 10.1007/s00109-005-0019-z

Jensen, H., Jensen, L., Hansen, P., Faergeman, O., and Gregersen, N. (1996). An Iranian-Armenian LDLR frameshift mutation causing familial hypercholesterolemia. *Clin. Genet.* 49, 88–90. doi: 10.1111/j.1399-0004.1996.tb04334.x

Jeon, H., Meng, W., Takagi, J., Eck, M. J., Springer, T. A., and Blacklow, S. C. (2001). Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nat. Struct. Biol.* 8, 499–504.

Khachadurian, A. K. (1964). The inheritance of essential familial hypercholesterolemia. *Am. J. Med.* 37, 402–407. doi: 10.1016/0002-9343(64)90196-2

Khajali, F., and Khajali, Z. (2014). L-carnitine supplementation decreases lipid peroxidation and improves cardiopulmonary function in broiler chickens reared at high altitude. *Acta Veter. Hungar.* 62, 489–499. doi: 10.1556/avet.2014.022

Khan, S. P., Ghani, R., Ahmed, K. Z., and Yaqub, Z. (2011). Genetic basis for diagnosis of novel mutation of LDL receptor gene. *Int. J. Collabor. Res. Int. Med. Publ. Health* 3, 0–0.

Kotzer, M., and Baudhuin, L. (2009). Novel human pathological mutations. Gene symbol: LDLR. Disease: hypercholesterolemia. *Hum. Genet.* 126:352.

Krogh, H. W., Mundal, L., Holven, K. B., and Retterstøl, K. (2016). Patients with familial hypercholesterolaemia are characterized by presence of cardiovascular disease at the time of death. *Eur. Heart J.* 37, 1398–1405. doi: 10.1093/eurheartj/ehv602

Meriño-Ibarra, E., Puzo, J., Jarauta, E., Cenarro, A., Recalde, D., García-Otín, A., et al. (2007). Hyperlipoproteinaemia (a) is a common cause of autosomal dominant hypercholesterolaemia. *J. Inher. Metab. Dis.* 30:970. doi: 10.1007/s10545-007-0585-z

Mesic, A., Rogar, M., Hudler, P., Bilalovic, N., Eminovic, I., and Komel, R. (2019). Characterization and risk association of polymorphisms in Aurora kinases A, B and C with genetic susceptibility to gastric cancer development. *BMC Cancer* 19:919.

Naeli, P., Mirzadeh Azad, F., Malakootian, M., Seidah, N. G., and Mowla, S. J. (2017). Post-transcriptional regulation of PCSK9 by miR-191, miR-222, and miR-224. *Front. Genet.* 8:189. doi: 10.3389/fgene.2017.00189

Nauck, M., Köster, W., Dörfer, K., Eckes, J., Scharnagl, H., Gierens, H., et al. (2001). Identification of recurrent and novel mutations in the LDL receptor gene in German patients with familial hypercholesterolemia. *Hum. Mutat.* 18, 165–166. doi: 10.1002/humu.1171

Nikkhooy, M., Foroughmand, A. M., Galehdari, H., and Yazdankhah, S. (2018). The First Report of GAC< inline-formula id=. *Zahedan J. Res. Med. Sci.* 8:20.

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur. Heart J.* 34, 3478–3490. doi: 10.1093/eurheartj/eht273

Pinheiro, D. S., Santos, R. S., Jardim, P. C. V., Silva, E. G., Reis, A. A., Pedrino, G. R., et al. (2019). The combination of ACE I/D and ACE2 G8790A polymorphisms revels susceptibility to hypertension: A genetic association study in Brazilian patients. *PLoS One* 14:e0221248. doi: 10.1371/journal.pone.0221248

Seidah, N. G. (2017). The PCSK9 revolution and the potential of PCSK9-based therapies to reduce LDL-cholesterol. *Glob. Cardiol. Sci. Pract.* 2017:e201702.

Seidah, N. G. (2019). The elusive inhibitory function of the acidic N-terminal segment of the prodomain of PCSK9: the plot thickens. *J. Mol. Biol.* 431, 904–907. doi: 10.1016/j.jmb.2019.01.015

Sharifi, M., Futema, M., Nair, D., and Humphries, S. E. (2017). Genetic architecture of familial hypercholesterolaemia. *Curr. Cardiol. Rep.* 19:44.

Simon Broome Register Group. (1991). Risk of fatal coronary heart disease in familial hypercholesterolaemia. *BMJ* 303, 893–896. doi: 10.1136/bmj.303.6807.893

Smilde, T. J., van Wissen, S., Awollersheim, H., Trip, M. D., Kastelein, J., and Stalenhoef, A. F. (2001). Effect of aggressive versus conventional lipid lowering on atherosclerosis progression in familial hypercholesterolemia (ASAP): a prospective, randomised, double-blind trial. *Lancet* 357, 577–581. doi: 10.1016/s0140-6736(00)04053-8

Springer, T. A. (1998). An extracellular β-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. *J. Mole. Biol.* 283, 837–862. doi: 10.1006/jmbi.1998.2115

Stein, E. A., Giugliano, R. P., Koren, M. J., Raal, F. J., Roth, E. M., Weiss, R., et al. (2014). Efficacy and safety of evolocumab (AMG 145), a fully human monoclonal antibody to PCSK9, in hyperlipidaemic patients on various background lipid therapies: pooled analysis of 1359 patients in four phase 2 trials. *Eur. Heart J.* 35, 2249–2259. doi: 10.1093/eurheartj/ehu085

Tajamolian, M., Kolahdouz, P., Nikpour, P., Forouzannia, S. K., Sheikhha, M. H., and Yazd, E. F. (2018). A rare missense mutation and a polymorphism with high frequency in LDLR gene among iranian patients with familial hypercholesterolemia. *Adv. Biomed. Res.* 7:37.

Talmud, P. J., Shah, S., Whittall, R., Futema, M., Howard, P., Cooper, J. A., et al. (2013). Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. *Lancet* 381, 1293–1301. doi: 10.1016/s0140-6736(12)62127-8

Thiart, R., Scholtz, C. L., Vergotine, J., Hoogendijk, C. F., de Villiers, J. N. P., Nissen, H., et al. (2000). Predominance of a 6 bp deletion in exon 2 of the LDL receptor gene in Africans with familial hypercholesterolaemia. *J. Med. Genet.* 37, 514–519. doi: 10.1136/jmg.37.7.514

Varret, M., and Rabès, J.-P. (2012). Missense mutation in the LDLR gene: a wide spectrum in the severity of familial hypercholesterolemia. *Mutat. Hum. Genet. Dis.* 73, 55–74.

Villéger, L., Abifadel, M., Allard, D., Rabès, J. P., Thiart, R., Kotze, M. J., et al. (2002). The UMD-LDLR database: additions to the software and 490 new entries to the database. *Hum. Mutat.* 20, 81–87. doi: 10.1002/humu.10102

Vuorio, A., Watts, G. F., and Kovanen, P. T. (2016). Initiation of PCSK9 inhibition in patients with heterozygous familial hypercholesterolaemia entering adulthood: a new design for living with a high-risk condition? *Eur. Heart J.* 37, 1353–1356. doi: 10.1093/eurheartj/ehw010

Webb, J. C., Sun, X.-M., McCarthy, S., Neuwirth, C., Thompson, G., Knight, B., et al. (1996). Characterization of mutations in the low density lipoprotein (LDL)-receptor gene in patients with homozygous familial hypercholesterolemia, and frequency of these mutations in FH patients in the United Kingdom. *J. Lip. Res.* 37, 368–381. doi: 10.1016/s0022-2275(20)37623-9

Zhang, D.-W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., et al. (2007). Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *J. Biol. Chem.* 282, 18602–18612. doi: 10.1074/jbc.m702027200

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frontiers in Pharmacology

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Risk of Recurrent Coronary Events in Patients With Familial Hypercholesterolemia; A 10-Years Prospective Study

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Background and Aim: Real world evidence on long term treatment of patients with familial hypercholesterolemia (FH) is important. We studied the effects of intensive lipid lowering medication (LLM) and optimized lifestyle in the study TTTFH–Treat To Target FH.

Materials and Methods: Adults with a first known total cholesterol of mean (95% CI) 9.8 mmol/L (9.5, 10.1) were included consecutively in their routine consultation during 2006. Of the patients 86.4% had a pathogenic FH-mutation and the remaining were clinically diagnosed. We included 357 patients and 279 met for follow-up after median 10.0 (min 8.1, max 12.8) years.

Results: Mean (95% CI) low density lipoprotein (LDL-C) was reduced from 3.9 (3.8, 4.1) to 3.0 (2.9, 3.2). More men than women used high intensity statin treatment, 85.2 and 60.8%, respectively. Women (n = 129) had higher LDL-C; 3.3 mmol/L (3.0, 3.5), than men; (n = 144) 2.8 mmol/L (2.6, 3.0), p = 0.004. Add-on PCSK9 inhibitors (n = 25) reduced mean LDL-C to 2.0 (1.4, 2.6) mmol/L. At enrollment 57 patients (20.4%) had established atherosclerotic cardiovascular disease (ASCVD), and 46 (80.4%) of them experienced a new event during the study period. Similarly, 222 (79.6%) patients had no detectable ASCVD at enrollment, and 29 of them (13.1%) experienced a first-time event during the study period.

Conclusion: A mean LDL-C of 3.0 mmol/L was achievable in FH, treated intensively at a specialized clinic with few users of PCSK9 inhibitors. LDL-C was higher (0.5 mmol/L) in women than in men. In patients with ASCVD at enrollment, most (80.7%) experienced a new ASCVD event in the study period. The FH patients in primary prevention had more moderate CV risk, 13% in ten years.

Keywords: familial hypercholesterolemia, ASCVD, side effect, TTTFH, statin, PCSK9-inhibitor, ezetrimibe, colesevelam

INTRODUCTION

The introduction of statins in the early 1990s dramatically improved treatment options for patients with heterozygous familial hypercholesterolemia (FH). However, apart from the introduction of ezetimibe, no important new drugs were developed for these patients until the approval of Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors in 2015. Effect and safety of statins is very well documented through large cardiovascular (CV) endpoint studies (Cholesterol Treatment Trialists' (CTT) et al., 2010). No such CV end point studies exist to document effect in patients with FH, since in these patients, it would be unethical to use placebo. The treatment of FH thus rests on the extrapolation of results of trials in hyperlipidemic patients in the general population (Cholesterol Treatment Trialists' (CTT) et al., 2010; Ference et al., 2017). Long term prospective cohort studies reporting cardiovascular outcomes are therefore important. In the Simon Broome Register studies (Scientific-Steering-Committe, 1999; Neil et al., 2008; Humphries et al., 2019) analyses were performed before and after statins became available, and excess CHD mortality decreased from 3.4-fold before to a 2.1-fold excess CHD mortality after statins became available (Scientific-Steering-Committe, 1999). Effect of statins in FH was also investigated in a Dutch study reporting that statins decreased risk of CHD by 76% vs. those not treated (Versmissen et al., 2008). Further, several studies on real world data have shown that few FH subjects achieve their LDL cholesterol (LDL-C) treatment goals (Bogsrud et al., 2019; Duell et al., 2019; Iyen et al., 2019; Perez de Isla et al., 2019). In the most recent of these studies mean LDL-C was decreased to 2.90 mmol/L in specialized lipid clinics (Duell et al., 2019). This included, however, treatment with PCSK9 inhibitors in 30% of the patients.

The aim of the present study was to reduce LDL-C to treatment goals as recommended in guidelines in the period 2006–2018, using statins, ezetimibe, resins and diet and lifestyle treatment at study start, and at the end of the study PCSK9 inhibitors in 7.5% of the patients.

MATERIALS AND METHODS

During January to July 2006, all FH patients between the age of 18–75 (n = 426) meeting for a routine consultation at the Lipid Clinic (LC) were consecutively invited to participate in the study TTTFH–Treat To Target Familial Hypercholesterolemia. Inclusion and exclusion criteria are given in **Table 1**. Of 426 patients invited, 357 signed an informed consent at visit 1 (V1). One year later, 332 patients met for the second visit (V2). From 2015 to 2019, median 10 (min 8.1, max 12.8) years later 279 patients met for the third and final visit (V3). Data from the 279 patients who completed the full study period are presented in this report. The most common reasons for exclusion were 1) patient could not be reached (n = 41); 2) did not wish to/could not participate (n = 28); 3) death (n = 12). (**Figure 1**, flow chart). The diagnosis was verified by genetic testing at 86.4% of the patients or clinically by the Dutch Lipid Clinic Network (DLCN) criteria; definite FH at 6%, probable 5% and possible FH at 2.5% (Nordestgaard et al., 2013). Patients underwent a physical examination and a review of diet and lifestyle. Blood samples were analyzed for the routine biological analyses at the Department of Medical Biochemistry, Oslo University Hospital. In few cases blood samples provided by their RGP were used. Medical history was retrieved from the study case report form and the patients' medical records. Follow-up data was collected after 1 year and at end of study after median 10 years (**Figure 1**). The diagnosis of metabolic syndrome (MetS) was based on the criteria from NCEP/ATP III (Grundy et al., 2004). In 2006 and 2007 the study was approved by the Regional Ethics Committee as a quality assurance study. The informed consent that was used in 2006 needed to be replaced with a new in 2015, because then the study was approved as a research study. We follow the principles of Good Research for Comparative Effectiveness when possible (Benchimol et al., 2015; Dreyer et al., 2016) using a study protocol with a broad and pre-specified aim.

Routine Biochemistry Measurement

Total cholesterol (TC), HDL cholesterol (HDL-C), LDL-C, triglycerides (TG), apolipoprotein A1 (ApoA1), apolipoprotein B (apoB), glucose, Glycated hemoglobin ($HbA1_c$), C-reaction protein (CRP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), Creatine kinase, Gamma GT and lipoprotein (a) (Lp (a)) was measured. In case of missing Lp (a) values the highest value was retrieved from medical records. Values for non-HDL-C and ApoB/ApoA1 ratio were calculated. Measurements at enrollment and end of study are given in **Table 2**. The patients' first measured TC and LDL-C values were retrieved from their medical records.

Diet and Body Mass Index

All patients received dietary counseling at each visit, and at least once they consulted a clinical nutritionist. Visit 3 (V3) was an ordinary medical consultation by the MDs in the study, followed by consultations of students in clinical nutrition. We used a validated questionnaire (SmartDiet®) to evaluate the patients' diet (Svilaas et al., 2002). Body mass index was calculated at enrollment and at end of study.

Adverse Effects

Adverse effects of the LLM were classified according to the likelihood of whether the effects were caused by the LLM. They were classified as definite, probable, or possible. Adverse effects were classified as definite if it disappeared after termination of the medication and in addition reoccurred at re-challenge with same medication in similar dose, and in addition to that a re-challenge procedure was repeated at least twice. Less certain adverse side effects were classified as probable or possible by clinical assessment.

Cardiovascular Event and Death

Patients who died (n = 12) were not included in the analysis since we did not have access to the death certificates. Of those who died, eight were male and four females. Median age (95% CI) at time of death was 64.8 (47.3, 67.5) years, the youngest being 38.3, and the

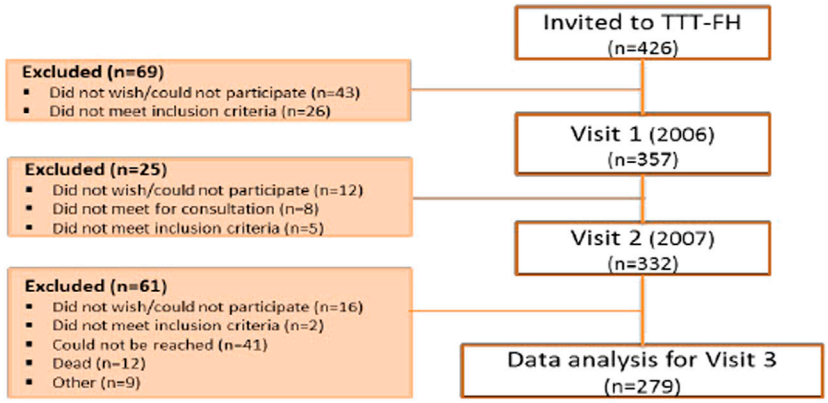

FIGURE 1 | Flow chart.

oldest 76.1 years. From the hospital medical records, we found that cause of death was myocardial infarction in three patients. One patient died in a traffic accident, one by suicide and in the remaining 7 deaths the cause was unknown. Two of these patients had established atherosclerotic cardiovascular disease (ASCVD) and three patients had cancer.

The number of ASCVD events presented in this study were retrieved from the study case report forms and from medical records. The most common events were percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG) and acute myocardial infarction (AMI).

Statistics

We used IBM SPSS Statistics version 25.0 (SPSS Inc, Chicago) for statistical analysis. Continuous variables with Normal distribution were presented as mean and 95% confidence interval (95% CI), while skewed variables were presented median and 25^{th}–75^{th} percentiles (25–75p) or minimum and maximum values (min-max). Categorical variables were portrayed as number of cases and percentages. Student's t-test, either independent or paired, were used when comparing normally distributed continuous variables, and Mann-Whitney U test or Wilcoxon signed rank test in case of non-normally distributed variables. Differences between categorical variables were tested using Chi square test for independence, or Fisher's exact test if the assumptions for using Chi-scare test were violated. A p-value <0.05 was considered statistically significant. Analysis of variance (ANOVA) was used to test for statistical significance for three or more groups. Stratification was used as the main method to adjust for factors like sex or types of treatment. Tests were performed both with and without outliers. In case that removal of outliers affected the significance of the results, this was noted in the tables or the text.

TABLE 1 | Inclusion and exclusion criteria.

Inclusion criteria
i. Age 18 to 75
ii. Signed informed consent
iii. Confirmed genetic FH or by Dutch Lipid Clinic Network (DLCN) criteria
Exclusion criteria
i. Participating in other on-going study
ii. Receiving LDL apheresis
iii. Dropping out of scheduled consultations during the half year
iv. Not able to fill out questionnaires or join telephone interviews
v. Not willing to participate
vi. Serious concomitant disease e.g. malignant disease

RESULTS

Measurements in the study population at enrolment and end of study are given in **Table 2**. Measurements at end of study according to use of statins, ezetimibe, resins or additional treatment with PCSK9-inhibitors are given in **Table 3**. In 86.4% of the patients a FH mutation was identified. The remaining patients were diagnosed according to the DLCN diagnostic criteria for FH (Haase and Goldberg, 2012). Criteria of definite clinical FH (score >8 points) was fulfilled in 6.1%, probable FH (6–8 points) in 5.0% and possible FH (3–5 points) in 2.5%. In 66 patients (24.4%), the diagnosis of FH was made after the age of 40 years (**Table 4**). Of the 66 patients with late diagnose there were 40 women and 26 men. In the total cohort, mean age of first known high TC measurement was 27.9 years, and only 30.0% of the patients were diagnosed with FH before the age of 20 years.

Recurrent and First Time ASCVD Events

At enrollment 57 patients (20.4%) had established ASCVD and 46 (80.4%) of them experienced a recurrent event during the follow-up period of 8–12 years (**Table 5**). Of those 222 (79.4%) without ASCVD a new event occurred only in 29 (13.1%) during the study period. Among patients diagnosed after 40 years of age, 76.5% had ASCVD at follow-up, as compared to 27.0% among those diagnosed before age of 40 years. As shown in **Table 5**, patients with recurrent events were older and had significantly higher LDL-C at first time measurement than patients with only

TABLE 2 | Measurements at enrollment and at end of study.

		Study start	Study end	P*
	n	Mean (95% CI)	Mean (95% CI)	
Total-C, mmoL/L	272	5.7 (5.5, 5.9)	4.9 (4.7, 5.1)	**<0.001**
HDL-C, mmoL/L	271	1.4 (1.3, 1.4)	1.4 (1.3, 1.4)	0.530
LDL-C, mmoL/L	272	3.9 (3.8, 4.1)	3.0 (2.9, 3.2)	**<0.001**
TG, mmoL/L	268	1 (1, 1.1)	1.2 (1.1, 1.2)	**<0.001**
Non-HDL-C, mmoL/L	270	4.3 (4.1, 4.5)	3.5 (3.3, 3.6)	**<0.001**
ApoA1 g/L	251	1.4 (1.3, 1.4)	1.5 (1.4, 1.5)	**<0.001**
ApoB g/L	252	1 (1, 1.1)	1.1 (1.0, 1.1)	0.122
ApoB/ApoA1 ratio	251	0.78 (0.74, 0.82)	0.74 (0.71, 0.78)	0.101
Glucose, mmoL/L	217	5.1 (5, 5.2)	5.5 (5.3, 5.7)	**<0.001**
HbA1c, %	195	5.4 (5.3, 5.5)	5.7 (5.6, 5.8)	**<0.001**
Systolic BP, mmHG	163	129 (126.8, 131.1)	128.6 (127, 130.4)	0.413
Diastolic BP, mmHG	163	78.2 (76.8, 79.6)	77.2 (76, 78.5)	0.824

$p < 0.05$ is considered statistically significant differences between enrolment and end of study. Tested with paired samples T-test. Significant values in bold. Corrected for multiple testing using False Discovery Rate.

one event, and those with one event were older than those without ASCVD. HDL-C and TG did not differ significantly between the groups. Patients with recurrent ASCVD had higher levels of Lp (a) and HbA1c, higher age, and higher prevalence of diabetes, hypertension, smoking and FH diagnosed after 40 years of age (**Table 5**). The average age at first ASCVD event in the total cohort, was 47.6 years which is in the same range as observed in several other studies (Mata et al., 2011; degoma et al., 2016; Mundal et al., 2016).

Metabolic Syndrome

In those who suffered from METS during the whole observation time 68.4% had CVD, and among those who had developed METS at last visit 53.3% had CVD. In those with no METS over the ten-year period 23.3% suffered from CVD, and those who normalized their METS during the observation period 27.3% had CVD (**Table 5**).

Lipids and Lipid Lowering Treatment

Mean TC, LDL-C, and non-HDL-C were significantly reduced from start to end of study as shown in **Table 2**. BMI increased from mean (95% CI) 26.2 (25.5, 26.9) to 27.3 (26.7, 28) kg/m^2, but blood pressure remained unchanged during the period (**Table 2**). At the end of the study period, 73.8% of all patients used high intensity statins and 47.8% used add-on ezetimibe (**Table 5**). PCSK9 inhibitors were used by 9%, as it became available during the last years of the study period. The 25 patients using PCSK9 inhibitors had even lower mean LDL-C (95% CI) 2.0 (1.4, 2.6) (**Table 3**). Overall, LDL-C ≤1.8 mmol/L was achieved only in 22 patients, representing 8% of the patients in the cohort.

Adverse Effects

Adverse effects of the LLM were mostly associated with muscle pain for statins, and GI problems for colesevelam. In total, adverse effects were reported by 123 patients (44.1%). The study physicians classified them as definite in 44 patients (16.5%), and 23 patients (8.3%) could not use statins at all due to adverse effects. Characteristics of those not using statins is given in **Table 6**. Five of the statin intolerant patients used ezetimibe, two used colesevelam and one used PCSK9-inhibitor. In total, 18 patients (6.5%) did not use any kind of LLM, 13 females and five males. Reason for not using LLM was adverse effects in 10 patients, six patients did not report any specific reasons, four women tried to become pregnant and three patients were skeptic to statins.

Diet and Lifestyle

The patients improved their diet from enrolment to the last visit as measured by SmartDiet score ($p < 0.05$ for all). At end of study they used less butter and/or margarine as spread on bread, less meat as cold cuts, less low-fiber bread, more fish for dinner and more vegetables, although mean score of fruits and vegetables corresponds to a maximum intake of 4 units per day, which is lower than the national recommendations. On the other hand, patients reported eating more high fat cheese ($p = 0.006$) than recommended. Over half of the population was physically active for at least 1.5 h per week at the end of study, and the majority had an alcohol intake between 0–7 units per week, which did not change significantly during the study period. The number of persons who smoked was reduced by 32.0% from enrolment to end of study, but the number of cigarettes smoked per smoker was not reduced.

DISCUSSION

In the present study, more than 80% of the FH patients with established ASCVD at enrollment experienced a new event over a 10-year period, despite being treated to mean (95%CI) LDL-C 3.0 (2.9, 3.2) mmol/L, which is a 55% reduction compared to the untreated LDL-C of 6.6 (5.3, 7.9) mmol/L. As observed in other trials on treatment LDL-C is much too high in FH, Duell et al. reported 2.9 mmol/L using PCSK9 inhibitors in 30% of the patients (Duell et al., 2019), Langslet et al. reported 3.2 mmol/L in 909 FH patients of whom 47% had ASCVD (Langslet et al., 2020) and in another recent trial on FH patients LDL-C was 4.0 mmol/L on conventional treatment (Raal et al., 2020). Taken together, this suggests that a mean LDL-C around 3.0 mmol/L is about as low as it is possible to achieve in real-world practice in patients with FH. In those with additional treatment with PCSK9 inhibitors, a mean LDL-C around 2.0 mmol/L is achievable. A treatment goal of ≤1.8 mmol/L was achieved in no more than 8% of FH patients, which is in line with other reports (Bogsrud et al., 2019; Duell et al., 2019; Perez de Isla et al., 2019).

Age at diagnosis and treatment start is an important risk factor in FH; high age at diagnosis resulting in high lifetime LDL-C load and high risk of ASCVD (Nordestgaard et al., 2013). Looking at new and recurrent ASCVD events, we observed that patients with recurrent events were younger when they had their first event (mean 44.0 years) as compared to those who experienced ASCVD only once, who had a mean age of 51.8 years. Thus, the atherosclerosis in patients with recurrent events seems to be more aggressive than in those with only one event. Further, those with recurrent events were slightly older at diagnosis (35.5 years) compared with those

TABLE 3 | Measurement at end of study in patients using traditional treatment or PCSK9-inhibitor add-on.

	Total			Traditional treatment (n=255)			PCSK9 add-on (n=25)			P*
	n	Mean	(95% CI)	n	Mean	(95% CI)	n	Mean	(95% CI)	
Total-C, mmol/L	273	4.9	(4.7, 5.1)	252	5.0	(4.8, 5.2)	21	3.9	(3.3, 4.5)	**0.002**
HDL-C, mmol/L	272	1.4	(1.3, 1.4)	252	1.4	(1.3, 1.4)	20	1.5	(1.3, 1.7)	0.262
LDL-C, mmol/L	273	3.0	(2.9, 3.2)	252	3.1	(2.9, 3.3)	21	2.0	(1.4, 2.6)	**<0.001**
TG, mmol/L	271	1.2	(1.1, 1.2)	251	1.2	(1.1, 1.2)	20	1.1	(0.9, 1.3)	0.595
Non-HDL-C, mmol/L	272	3.5	(3.3, 3.6)	251	3.6	(3.4, 3.7)	21	2.2	(1.6, 2.8)	**<0.001**
Lp(a) mg/L	242	562.2	(478.1, 646.3)	218	527.6	(442.5, 612.7)	21	988.8	(595.1, 1382.5)	**0.004**
ApoAI g/L	260	1.5	(1.4, 1.5)	245	1.5	(1.4, 1.5)	15	1.5	(1.4, 1.7)	0.578
ApoB, g/L	262	1.1	(1.0, 1.1)	246	1.1	(1, 1.1)	16	0.8	(0.7, 1)	**0.004**
ApoB/ApoAI ratio	260	0.74	(0.71, 0.78)	245	0.76	(0.72, 0.79)	15	0.54	(0.42, 0.67)	**0.003**
Glucose, mmol/L	235	5.5	(5.3, 5.7)	221	5.5	(5.3, 5.7)	14	5.5	(5.1, 5.8)	0.786
HbAlc, %	238	5.7	(5.6, 5.8)	226	5.7	(5.6, 5.8)	12	5.4	(5.2, 5.6)	0.203
Systolic BP, mmHG	214	129	(127, 130)	197	129	(127, 130)	17	129	(123, 134)	0.952
Diastolic BP, mmHG	214	77	(76, 79)	197	77	(76, 78)	17	81	(76, 85)	0.101

*Traditional treatment is statins and/or ezetimibe and/or colesevelam. *P<0.05 is considered statistically significant differences between the group with traditional treatment and those with PCSK9 inhibitor add-on, tested with Independent samples T-test. Significant values in bold. Corrected for multiple testing using False Discovery Rate.*

TABLE 4 | Measurementas at end of study in female male and in those who initiated treatment >age 20 years.

	Untreated before 40 years (N=68)			Male (N=149)			Female (N=30)			*p**
Total-C, mmol/L	66	4.9	(4.5, 5.2)	144	4.5	(4.3, 4.8)	129	5.3	(5.1, 5.6)	**<0.001**
HDL-C, mmol/L	66	1.5	(1.4, 1.6)	144	1.3	(1.2, 1.3)	128	1.5	(1.5, 1.6)	**<0.001**
LDL-C, mmol/L	66	2.9	(2.6, 3.2)	144	2.8	(2.6, 3.0)	129	3.3	(3.0, 3.5)	**0.004**
TG, mmol/L	66	1.3	(1.2, 1.4)	144	1.2	(1.1, 1.3)	127	1.2	(1.1, 1.3)	0.742
Non-HDL-C, mmol/L	66	3.3	(3.0, 3.6)	144	3.3	(3.0, 3.5)	128	3.7	(3.5, 4)	**0.006**
Lp (a) mg/L	57	730.7	(472.6, 988.8)	126	591.3	(478.0, 704.7)	113	534.1	(405.1, 663.1)	0.598
ApoA1 g/L	63	1.6	(1.5, 1,6)	133	1.4	(1.3, 1.4)	127	1.6	(1.5, 1.6)	**<0.001**
ApoB g/L	64	1.0	(0.95, 1.1)	135	1.0	(0.97, 1.1)	127	1.1	(1.0, 1.2)	0.071
ApoB/ApoA1 ratio	63	0.70	(0.6, 0.7)	133	0.76	(0.71, 0.8)	127	0.73	(0.68, 0.78)	0.298
Glucose, mmol/L	55	5.8	(5.4, 6.3)	119	5.7	(5.4, 5.9)	116	5.4	(5.1, 5.7)	0.243
HbA1, %	59	5.7	(5.6, 5.8)	123	5.8	(5.6, 6.0)	115	5.6	(5.5, 5.7)	**0.031**
Systolic BP, mmHg	43	131	(126, 136)	116	131	(128, 133)	98	126	(124, 129)	**0.016**
Diastolic BP, mmHg	43	78	(75, 81)	116	79	(78.2, 81.1)	98	75	(73.6, 76.9)	**<0.001**

**p < 0.05 is considered statistically significant differences between male and female, tested with Independent samples T-test. Significant values in bold. Corrected for multiple testing using False Discovery Rate.*

with ASCVD once (32.2 years), and patients free of ASCVD were even younger at diagnosis (mean age 25.2 years). Recent studies have underlined the importance of additional risk factors to predict ASCVD risk in FH (Martín-Campos et al., 2018; Perez-Calahorra et al., 2019). We observed (Martín-Campos et al., 2018; Perez-Calahorra et al., 2019) a striking relationship between METS and the prevalence of CVD, in those who developed METS during the observation period 53.3% had ASCVD compared to 23.3% in those with no METS. This highlights the utmost importance of diet and physical activity among FH-patients.

The risk of recurrent ASCVD in FH has not been much investigated previously. A Dutch study of 345 FH patients found that in patients with a history of ASCVD the event risk of was almost 30% per year under age 40 years and 15% in patients older than 60 years (Mohrschladt et al., 2004). The high risk of recurrent ASCVD in the present study might be explained by high age at diagnosis of FH (Krogh et al., 2015), and the fact that so few reaches LDL-C treatment target as compared to people without FH. In the general population, a Danish study from 2016 reported that only 11% of patients experiencing a first time myocardial infarction used statins prior to the event (Kulenovic et al., 2016). After the first-time myocardial infarction, these patients receive statins leading to a major reduction in LDL-C. In contrast, many FH patients are already on intensive per-oral treatment prior to the first event, and further lowering of LDL-C may be difficult to achieve without PCSK9-inhibitors. Persistent high LDL-C after a CVD event in patients with FH could explain the risk of recurrent events in FH demonstrated in the present study. However, no more than 29 (13.1%) of 222 the patients who were free of ASCVD at study start, had a first time ASCVD event during the follow-up, underlining the effect of early primary prevention. Taken together, these data suggest that early treatment start is of major importance to reduce ASCVD events.

We observed that women had 0.5 mmol/L higher LDL-C than men (**Table 5**), and the higher LDL may be related to the previous

TABLE 5 | Comparison between patients with only one ASCVD event, multiple ASCVD events and those free of ASCVD at end of study.

	NO ASCVD			ASCVD once			Recurrent ASCVD		
	N			N			N		
MI	193	27	(26.3, 27.7)	40	27.3	(25.5, 29.1)	46	27.4	(25.9, 28.9)
Current smokers	186	25	(13.4%)	38	4	(10.5%)	45	9	(20.0%)
Former smokers	166	65	(39.2%)	35	20	(57.1%)	44	24	(54.5%)
	n	mean	(95% CI)	n	mean	(95% CI)	n	mean	(95% CI)
Age at last follow-up[abc]	193	51.3	(49.5, 53.1)	40	60.5	(57.1, 63.9)	40	66.7	(64.3, 69.2)
Age first known total-C[ac]	192	25.2	(23.2, 27.2)	39	32.2	(27.5, 36.9)	39	35.5	(32.6, 38.4)
Age at first ASCVD[c]				38	51.8	(48.3, 55.3)	45	44.0	(40.9, 47.2)
Blood parameters									
Total-C, mmol/L	190	5	(4.8, 5.2)	39	4.5	(4.1, 5)	44	4.7	(4.3, 5.1)
HDL-C, mmol/L	190	1.4	(1.4, 1.5)	39	1.3	(1.2, 1.5)	44	1.3	(1.2, 1.5)
LDL-C, mmol/L[a]	190	3.2	(3.0, 3.3)	39	2.5	(2.2, 2.9)	44	2.9	(2.5, 3.2)
TG, mmol/L[b]	189	1.1	(1.1, 1.2)	39	1.2	(1.1, 1.4)	43	1.3	(1.2, 1.5)
Lp(a), mg/L[b]	161	454	(382, 526)	37	673	(385, 962)	41	898	(593, 1202)
Glocuse, mmol/L[ab]	163	5.3	(5.1, 5.5)	33	6.1	(5.4, 6.8)	38	6.1	(5.5, 6.7)
HbA1c, %[ab]	166	5.6	(5.5, 5.7)	33	5.8	(5.6, 6.1)	39	6.1	(5.8, 6.5)
Co-morbidities	N	n	%	N	N	%	N	n	%
Mets[ab]	193	24	(12.4%)	40	16	(40%)	46	18	(39.1%)
Diabetes[b]	193	14	(7.3%)	40	5	(12.5%)	46	9	(19.6%)
Hypertension[abc]	193	22	(11.4%)	40	21	(52.5%)	46	35	(76.1%)
Lipid lowering Medication									
High intensity statin therapy	193	132	(68.4%)	40	33	(82.5%)	46	41	(83.1%)
Moderate intensity statin therapy	193	43	(22.3%)	40	5	(12.5%)	46	2	(4.3%)

[a]*Significant difference between «No ASCVD» and «ASCVD once».*

[b]*Significant differences between «No ASCVD» and «Recurrent ASCVD once».*

[c]*Significant differences between «ASCVD once» and «Recurrent ASCVD». Tested with ANOVA with Bonferroni correction, or fisher's exact test (2 sided Sign level $p < 0.05$).*

TABLE 6 | Measurements according to use of lipdi lowering medication at end of study.

	Patients not using lipid lowering medication (n = 18)			Patients not using statins (n=23)			patients using lipid lowering medication (n = 256)			*P**
	n (%)			n (%)			n (%)			
Women		13	(72 2%)		16	(69.6%)		114	(44.5%)	**0.028**
Secondary prevention		3	(16.7%)		5	(21.7%)		104	(40.6%)	0.260
	n	Mean	(95% CI)	n	Mean	(95% CI)	n	Mean	(95% CI)	
Age at V3, years	18	48.3	(40.8, 55.9)	23	49.0	(42.1, 55.8)	256	55.7	(54.2, 57.3)	**0.017**
BMI, kg/m^2	18	25.4	(23.5, 27.3)	23	25.9	(23.6, 28.1)	255	27.2	(26.6, 27.9)	0.229
First measured total-C, mmol/L	18	9.4	(8.4, 10.5)	23	9.6	(8.7, 10.6)	249	9.8	(9.5, 10.1)	0.681
First measured LDL-C, mmol/L	12	6.6	(5.3, 7.9)	14	6.9	(5.6, 8.3)	121	7.5	(7.1, 7.9)	0.361
Lipdi at V3										
Total-C, mmol/L*	17	7.6	(6.4, 8.7)	22	7.5	(6.5, 8.5)	251	4.7	(4.5, 4.8)	**<0.001**
HDL-C, mmol/L	17	1.4	(1.2, 1.6)	22	1.5	(1.3, 1.7)	250	1.4	(1.3, 1.4)	0.022
LDL-C, mmol/L*	17	5.6	(4.6, 6.6)	22	5.6	(4.7, 6.4)	251	2.8	(2.7, 2.9)	**<0.001**
TG, mmol/L*	17	1.0	(0.8, 1.2)	22	0.9	(0.8, 1.1)	249	1.2	(1.1, 1.3)	**0.022**
Non-HDL-C, mmol/L*	17	6.2	(5.1, 7.2)	22	6.0	(5.1, 6.9)	250	3.3	(3.1, 3.4)	**<0.001**
Lp (a), mg/L	15	360.1	(141.5, 578.6)	20	376.7	(203.3, 550.1)	219	581.4	(490.2, 675.5)	0.189
ApoA1 g/L	16	1.4	(1.3, 1.6)	20	1.5	(1.3, 1.6)	240	1.4	(1.4, 1.5)	0.706
ApoB, g/L*	16	1.6	(1.4, 1.8)	21	1.6	(1.4, 1.8)	241	1.0	(0.98, 1.0)	**<0.001**
ApoB/ApoA1 ratio*	16	1.2	(1, 1.3)	20	1.12	(0.98, 1.27)	240	0.71	(0.68, 0.74)	**<0.001**
Glucose, mmol/L	14	5.1	(4.7, 5.5)	17	5.2	(5.8, 5.6)	217	5.6	(5.4, 5.7)	0.254
HbA1c, %*	15	5.3	(5.1, 5.4)	18	5.2	(5.1, 5.4)	220	5.7	(5.6, 5.8)	**0.008**
Systolic BP, mmHG	10	121	(115, 127)	13	121	(116, 126)	200	129	(127, 131)	**0.029**
Diastolic BP, mmHG	10	74	(68, 79)	13	76	(71, 82)	200	78	(77, 79)	0.063

**$p < 0.05$ is considered statistically significant differences between those on statins (n=256) vs those off stains (n=23) tested with independent samples T-test, Mann-Whitney U-test and Chi square test or Fisher's exact test. Significant values in bold. Corrected for multiple testing False Discovery Rate.*

finding that women with FH had their first ASCVD event as young as men (Mundal et al., 2016). Usually men are younger at first ASCVD but not in this FH cohort (Mundal et al., 2016).

Adverse effects were reported in 44.1% of the patients, and 16.5% had definite side effects as classified by the study physicians. This implies that many patients accept to use statins despite side effects. However, 8.6% of the patients had stopped using statins and 6.5% did not use any kind of LLM despite having a mean (95CI%) LDL-C of 5.6 (4.6, 6.6) mmol/L.

Routinely collected health data, obtained for administrative and clinical purposes without specific a priori research goals, are increasingly used for research (Benchimol et al., 2015; Dreyer et al., 2016; Sherman et al., 2016). The present study is a prospective cohort study with a pre-specified research protocol. However, using real-world data is challenging. Importantly, missing data on specific measurements was common in the present study, as shown in the tables.

In conclusion, LDL-C ≤1.8 mmol/L was achieved in no more than 8% of the patients despite the intense mostly per oral lipid treatment, with only 9% using PCSK9 inhibitors. As much as 80.7% of the patients with ASCVD at study start had recurrent ASCVD event over a period of median 10 years. The FH patients in primary prevention had a more moderate CV risk (13% in ten years). With maximally tolerated LLM without use of PCSK9 inhibitors mean (95% CI) LDL-C was reduced to 3.1 (2.9, 3.3), which is much higher than the recommended levels in patients with established ASCVD or with FH, and especially in the combination of both. This illustrates the need for more extensive use of PCSK9-inhibitors among the FH-patients.

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 The Regional Ethics Committee Oslo/Norway. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors have participated in recruiting and examining patients. They have informed the patients about the the study intention, and collected the patients consents. They have all contributed in the writing process.

REFERENCES

Benchimol, E. I., Smeeth, L., Guttmann, A., Harron, K., Moher, D., Petersen, I., et al. (2015). The REporting of studies Conducted using Observational Routinely-collected health Data (RECORD) statement. *PLoS Med.* 12, e1001885. doi:10.1371/journal.pmed.1001885

Bogsrud, M. P., Græsdal, A., Johansen, D., Langslet, G., Hovland, A., Arnesen, K. E., et al. (2019). LDL-cholesterol goal achievement, cardiovascular disease, and attributed risk of Lp(a) in a large cohort of predominantly genetically verified familial hypercholesterolemia. *J. Clin. Lipidol.* 13, 279–286. doi:10.1016/j.jacl.2019.01.010

Cholesterol Treatment Trialists' (CTT)Baigent, C., Blackwell, L., Emberson, J., Holland, L. E., Reith, C., et al. 2010). Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet* 376, 1670–1681. doi:10.1016/S0140-6736(10)61350-5

Degoma, E. M., Ahmad, Z. S., O'brien, E. C., Kindt, I., Shrader, P., Newman, C. B., et al. (2016). Treatment gaps in Adults with heterozygous familial hypercholesterolemia in the United States: data from the CASCADE-FH registry. *Circ. Cardiovasc. Genet.* 9, 240–249. doi:10.1161/CIRCGENETICS.116.001381

Dreyer, N. A., Bryant, A., and Velentgas, P. (2016). The grace checklist: a validated assessment tool for high quality observational studies of comparative effectiveness. *J. Manag. Care Spec. Pharm.* 22, 1107–1113. doi:10.18553/jmcp.2016.22.10.1107

Duell, P. B., Gidding, S. S., Andersen, R. L., Knickelbine, T., Anderson, L., Gianos, E., et al. (2019). Longitudinal low density lipoprotein cholesterol goal achievement and cardiovascular outcomes among adult patients with familial hypercholesterolemia: the CASCADE FH registry. *Atherosclerosis* 289, 85–93. doi:10.1016/j.atherosclerosis.2019.08.007

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., et al. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel, *Eur. Heart J.* 38, 2459–2472. doi:10.1093/eurheartj/ehx144

Grundy, S. M., Brewer, H. B., Jr., Cleeman, J. I., Smith, S. C., Jr., and Lenfant, C. (2004). Definition of metabolic syndrome: report of the national heart, lung, and blood institute/American heart association conference on scientific issues related to definition. *Circulation* 109, 433–438. doi:10.1161/01.CIR.0000111245.75752.C6

Haase, A., and Goldberg, A. C. (2012). Identification of people with heterozygous familial hypercholesterolemia. *Curr. Opin. Lipidol.* 23, 282–289. doi:10.1097/MOL.0b013e3283556c33

Humphries, S. E., Cooper, J. A., Capps, N., Durrington, P. N., Jones, B., Mcdowell, I. F. W., et al. (2019). Coronary heart disease mortality in severe vs. non-severe familial hypercholesterolaemia in the Simon Broome Register. *Atherosclerosis* 281, 207–212. doi:10.1016/j.atherosclerosis.2018.11.014

Iyen, B., Qureshi, N., Kai, J., Akyea, R. K., Leonardi-Bee, J., Roderick, P., et al. (2019). Risk of cardiovascular disease outcomes in primary care subjects with familial hypercholesterolaemia: a cohort study. *Atherosclerosis* 287, 8–15. doi:10.1016/j.atherosclerosis.2019.05.017

Krogh, H. W., Mundal, L., Holven, K. B., and Retterstol, K. (2015). Patients with familial hypercholesterolaemia are characterized by presence of cardiovascular disease at the time of death *Eur. Heart J.* 37, 1398–1405. doi:10.1093/eurheartj/ehv602

Kulenovic, I., Mortensen, M. B., Bertelsen, J., May, O., Dodt, K. K., Kanstrup, H., et al. (2016). Statin use prior to first myocardial infarction in contemporary patients: inefficient and not gender equitable. *Prev. Med.* 83, 63–69. doi:10.1016/j.ypmed.2015.12.001

Langslet, G., Hovingh, G. K., Guyton, J. R., Baccara-Dinet, M. T., Letierce, A., Manvelian, G., et al. (2020). Regional variations in alirocumab dosing patterns in patients with heterozygous familial hypercholesterolemia during an open-label extension study. *Cardiovasc. Drugs Ther.* 34, 515–523. doi:10.1007/s10557-020-06984-0

Martín-Campos, J. M., Plana, N., Figueras, R., Ibarretxe, D., Caixàs, A., Esteve, E., et al. (2018). Autosomal dominant hypercholesterolemia in Catalonia: correspondence between clinical-biochemical and genetic diagnostics in 967

patients studied in a multicenter clinical setting. *J. Clin. Lipidol.* 12, 1452–1462. doi:10.1016/j.jacl.2018.09.002

Mata, N., Alonso, R., Badimón, L., Padró, T., Fuentes, F., Muñiz, O., et al. (2011). Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish familial hypercholesterolemia longitudinal cohort study (SAFEHEART). *Lipids Health Dis.* 10, 94. doi:10.1186/1476-511X-10-94

Mohrschladt, M. F., Westendorp, R. G., Gevers Leuven, J. A., and Smelt, A. H. (2004). Cardiovascular disease and mortality in statin-treated patients with familial hypercholesterolemia. *Atherosclerosis* 172, 329–335. doi:10.1016/j.atherosclerosis.2003.11.007

Mundal, L., Veierød, M. B., Halvorsen, T., Holven, K. B., Ose, L., Iversen, P. O., et al. (2016). Cardiovascular disease in patients with genotyped familial hypercholesterolemia in Norway during 1994-2009, a registry study. *Eur. J. Prev. Cardiol.* 23, 1962–1969. doi:10.1177/2047487316666371

Neil, A., Cooper, J., Betteridge, J., Capps, N., Mcdowell, I., Durrington, P., et al. (2008). Reductions in all-cause, cancer, and coronary mortality in statin-treated patients with heterozygous familial hypercholesterolaemia: a prospective registry study. *Eur. Heart J.* 29, 2625–2633. doi:10.1093/eurheartj/ehn422

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., et al. (2013). Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur. Heart J.* 34, 3478–3490a. doi:10.1093/eurheartj/eht273

Pérez de Isla, L., Arroyo-Olivares, R., Muñiz-Grijalvo, O., Diaz-Díaz, J. L., Zambón, D., Fuentes, F., et al. (2019). Long-term effect of 2 intensive statin regimens on treatment and incidence of cardiovascular events in familial hypercholesterolemia: the SAFEHEART study. *J. Clin. Lipidol.* 13, 989–996. doi:10.1016/j.jacl.2019.10.005

Perez-Calahorra, S., Laclaustra, M., Marco-Benedí, V., Lamiquiz-Moneo, I., Pedro-Botet, J., Plana, N., et al. (2019). Effect of lipid-lowering treatment in cardiovascular disease prevalence in familial hypercholesterolemia. *Atherosclerosis* 284, 245–252. doi:10.1016/j.atherosclerosis.2019.02.003

Raal, F. J., Kallend, D., Ray, K. K., Turner, T., Koenig, W., Wright, R. S., et al. (2020). Inclisiran for the treatment of heterozygous familial hypercholesterolemia. *N. Engl. J. Med.* 382, 1520–1530. doi:10.1056/NEJMoa1913805

Scientific-Steering-Committe (1999). Mortality in treated heterozygous familial hypercholesterolaemia: implications for clinical management. Scientific Steering Committee on behalf of the Simon Broome Register Group. Scientific Steering Committee on behalf of the Simon Broome Register Group. *Atherosclerosis* 142, 105–112.

Sherman, R. E., Anderson, S. A., Dal Pan, G. J., Gray, G. W., Gross, T., Hunter, N. L., et al. (2016). Real-world evidence - what is it and what can it tell us?. *N. Engl. J. Med.* 375, 2293–2297. doi:10.1056/NEJMsb1609216

Svilaas, A., Ström, E. C., Svilaas, T., Borgejordet, A., Thoresen, M., and Ose, L. (2002). Reproducibility and validity of a short food questionnaire for the assessment of dietary habits. *Nutr. Metabol. Cardiovasc. Dis.* 12, 60–70.

Versmissen, J., Oosterveer, D. M., Yazdanpanah, M., Defesche, J. C., Basart, D. C., Liem, A. H., et al. (2008). Efficacy of statins in familial hypercholesterolaemia: a long term cohort study. *BMJ* 337, a2423. doi:10.1136/bmj.a2423

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