Atherosclerosis 289 (2019) 101–108

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Impact of *LDLR* and *PCSK9* pathogenic variants in Japanese heterozygous familial hypercholesterolemia patients

Mika Hori[a,*], Naotaka Ohta[b], Atsushi Takahashi[c], Hiroaki Masuda[b], Rieko Isoda[b], Suguru Yamamoto[b], Cheol Son[b], Masatsune Ogura[a], Kiminori Hosoda[b], Yoshihiro Miyamoto[b], Mariko Harada-Shiba[a,**]

[a] Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, 6-1 Kishibe Shinmachi, Suita, Osaka, 564-8565, Japan
[b] Laboratory of Clinical Genetics, National Cerebral and Cardiovascular Center, 6-1 Kishibe Shinmachi, Suita, Osaka, 564-8565, Japan
[c] Department of Genomic Medicine, National Cerebral and Cardiovascular Center Research Institute, 6-1 Kishibe Shinmachi, Suita, Osaka, 564-8565, Japan

HIGHLIGHTS

- *LDLR* and *PCSK9* variants detected in Japanese heterozygous FH were updated.
- Clinical significance of *LDLR* and *PCSK9* variants were annotated in heterozygous FH.
- *LDLR* and *PCSK9* pathogenic variants were found in 46% and 7.8% of FH patients, respectively.
- The proportion of *LDLR* pathogenic variants decreased with increased age of CAD onset.

ARTICLE INFO

Keywords:
Familial hypercholesterolemia
Pathogenic variant
LDLR
PCSK9
Annotation

ABSTRACT

Background and aims: More than 4970 variants in the low-density lipoprotein receptor (*LDLR*) gene and 350 variants in the proprotein convertase subtilisin/kexin 9 (*PCSK9*) gene have been reported in familial hypercholesterolemia (FH) patients. However, the effects of these variants on FH pathophysiology have not been fully clarified. We aimed to update the *LDLR* and *PCSK9* variants in Japanese heterozygous FH (HeFH) patients and annotate their clinical significance for the genetic diagnosis of HeFH.

Methods: A genetic analysis of the *LDLR* and *PCSK9* genes was performed in 801 clinically diagnosed HeFH patients. The association of the pathogenic variants with the clinical FH phenotype was examined.

Results: Pathogenic variants in the *LDLR* and *PCSK9* genes were found in 46% (n = 296) and 7.8% (n = 51) of unrelated FH patients (n = 650), respectively. The prevalence of Achilles tendon thickness was low (44%) in patients harbouring *PCSK9* pathogenic variants. Furthermore, 17% of unrelated FH patients harboured one of five frequent *LDLR* pathogenic variants: c.1845+2T > C, c.1012T > A: p.(Cys338Ser), c.1297G > C: p.(Asp433His), c.1702C > G: p.(Leu568Val), and c.2431A > T: p.(Lys811*). Patients harbouring the c.1845+2T > C and c.1702C > G: p.(Leu568Val) variants had significantly lower serum LDL-cholesterol levels and higher serum HDL-cholesterol levels, respectively, compared with those harbouring the other *LDLR* pathogenic variants. The proportion of *LDLR* pathogenic variants was higher in patients with a younger age of coronary artery disease (CAD) onset and significantly decreased as the age of CAD onset increased.

Conclusions: This study annotated the clinical significance and characteristics of *LDLR* and *PCSK9* pathogenic variants in Japanese HeFH patients.

1. Introduction

Familial hypercholesterolemia (FH) is characterized by high low-density lipoprotein-cholesterol (LDL-C) levels, skin and tendon xanthomas and premature coronary artery disease (CAD) [1], and its prevalence is 1 heterozygous FH (HeFH) patient per 200–500 individuals

* Corresponding author.
** Corresponding author.
E-mail addresses: mihori@ncvc.go.jp (M. Hori), mshiba@ncvc.go.jp (M. Harada-Shiba).

https://doi.org/10.1016/j.atherosclerosis.2019.08.004
Received 27 January 2019; Received in revised form 12 July 2019; Accepted 16 August 2019
Available online 19 August 2019
0021-9150/

in the general population [2,3]. FH patients have an extremely high risk of developing CAD; therefore, an accurate diagnosis of FH should be made as early as possible, a treatment strategy suitable to the patient should then be started as early as possible. FH is caused by mutations in the low-density lipoprotein receptor (*LDLR*) [4], apolipoprotein B (*APOB*) [5] and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes [6], but mutations in the *APOB* gene have not been reported in Japanese FH patients [7]. Gain-of-function variants in the *PCSK9* gene induce further degradation of LDLR and a subsequent increase in the serum LDL-C levels [8], whereas loss-of-function variants cause low LDL-C levels by increasing LDL clearance and reducing the risk of CAD [9]. Over 4970 variants in the *LDLR* gene and 350 variants in the *PCSK9* gene related to FH are described in ClinVar [10]; however, the clinical significance of the variants in the *LDLR* and *PCSK9* genes has not been fully annotated. The characteristics of the detected variants show differences based on the country or race. In addition, *LDLR* and *PCSK9* variants are incidentally detected via genome sequencing [11]. Thus, it is very important to assess the pathogenicity of the variants detected in clinical settings or as incidental findings. In the present study, we updated the *LDLR* and *PCSK9* variants in Japanese HeFH patients and examined the association of the FH phenotype with these variants.

2. Materials and methods

2.1. Patients

A genetic analysis of 801 clinically diagnosed HeFH patients, 650 of whom were unrelated, was performed in the National Cerebral and Cardiovascular Center (NCVC) between May 2005 and January 2018. Five hundred forty-one patients (67.5%) visited the NCVC. The rest of the patients were referred from other hospitals throughout Japan for genetic analysis, and their blood samples were sent to the NCVC. The diagnosis of HeFH was made according to the Japanese FH guidelines, which entail 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 CAD within the second degree of kinship [12]. After excluding 33 patients due to insufficient clinical information and 44 patients who were younger than 20 years, 724 patients were analysed to assess the association of clinical characteristics with gene variants. Of the 724 HeFH patients, 607 were unrelated. The protocol used in this study was approved by the Ethics Review Committee of the NCVC (M17-56). Each patient provided written informed consent to participate in the study.

2.2. DNA analysis

The reference sequences used for the *LDLR* and *PCSK9* genes were NM_000527.4 and NM_174936.3, respectively. Genomic DNA was extracted from the patients' whole blood using an automated DNA extraction machine (QIAsymphony; QIAGEN, Valencia, CA). All coding regions and the exon-intron boundary sequence of the *LDLR* and *PCSK9* genes were examined as described previously [13]. Some patients gave the appearance of homozygosity in exon 1 of the *PCSK9* gene due to the presence of single nucleotide polymorphisms (SNPs) of the primer site. All primers in the *LDLR* and *PCSK9* genes were checked for SNPs. In exons 4 and 11 of the *PCSK9* gene, the presence of SNPs of primer site was confirmed. In the *LDLR* gene, there were no SNPs in the primer sites, but the PCR efficiency was suboptimal for exons 3 and 17. Thus, we designed and used new primers for exons 3 and 17 of the *LDLR* gene and exons 1, 4, and 11 of the *PCSK9* gene from February 2017 (Supplemental Table 1). Patients who underwent genetic analysis before February 2017 and had the appearance of homozygosity in exon 1 or were clinically diagnosed as FH but did not have any pathogenic variants in the *LDLR* and *PCSK9* genes underwent genetic analysis again using the re-designed primers for the *PCSK9* gene. Multiplex ligation-dependent probe amplification was performed to detect large rearrangements of the *LDLR* gene using a P062B LDLR MLPA kit (MRC Holland, Amsterdam, the Netherlands). We did not examine any *APOB* variants in the present study, because variants in the *APOB* gene have not been reported in Japanese FH patients [7].

2.3. Classification of pathogenic variants

In the present study, only nonsynonymous variants were defined as "variants". The variants detected in the *LDLR* or *PCSK9* gene were classified as pathogenic or likely pathogenic using ClinVar [10]; the LOVD database [14]; population data from the Exome Aggregation Consortium (ExAC) [15], the Japanese Human Genetic Variation Database [16], and the Tohoku Medical Megabank Organization [17]; *in silico* tools/software; and functional data [18] based on guidelines edited by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) [19]. If a variant was confirmed to co-segregate in at least two or three families with at least two affected family members, the variant was scored as having moderate to strong evidence for pathogenicity.

2.4. Clinical and laboratory data

Clinical data were retrospectively collected based on the patient's medical records maintained at the NCVC or another hospital. The serum untreated levels of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were measured using standard methods. Unless the untreated TG levels were greater than 400 mg/dL, the LDL-C levels were calculated using the Friedewald formula. The Achilles tendon thickness (ATT) was measured by X-ray analysis [12]. CAD was evaluated based on the presence of myocardial infarction, angina pectoris, or coronary arteries with ≥75% stenosis by coronary angiography or electrocardiogram.

2.5. Statistical analyses

Continuous variables are presented as the mean ± SD or median (interquartile range), and categorical data are presented as percentages. The statistical significance of the differences between two groups was determined using unpaired Student's t-test or Mann–Whitney *U* test for continuous variables and Fisher's exact test, logistic regression test, or Cochran-Armitage test for categorical variables. The statistical significance of the differences among multiple groups was determined by one-way analysis of variance (ANOVA) or Kruskal-Wallis test followed by the Tukey-Kramer or Steel-Dwass test for continuous variables and by the Pearson Chi-square test followed by Fisher's exact test for categorical variables. The differences in the allele frequency were assessed by Fisher's exact test. p-values < 0.05 were considered statistically significant. Bonferroni correction was applied for multiple comparisons of categorical variables. The Cochran-Armitage trend test was performed using R software, and the other statistical analyses were performed using JMP 13.0.0 software (SAS International, Cary, NC, USA).

3. Results

3.1. Annotation of the clinical significance of variants in the LDLR and PCSK9 genes

For the *LDLR* gene, 137 variants, including 18 large deletions/duplications, were detected in 57% (n = 368) of unrelated FH patients (n = 650). According to the ACMG/AMP guidelines, 92 variants were pathogenic or likely pathogenic, 44 variants were variants of uncertain significance (VUS), and one variant was benign [20] (Fig. 1 and Supplemental Table 2). We defined pathogenic and likely pathogenic variants as pathogenic in the present study and thus detected *LDLR* pathogenic variants in 46% (n = 296) of unrelated FH patients. The five most frequently detected variants in the *LDLR* gene were the following:

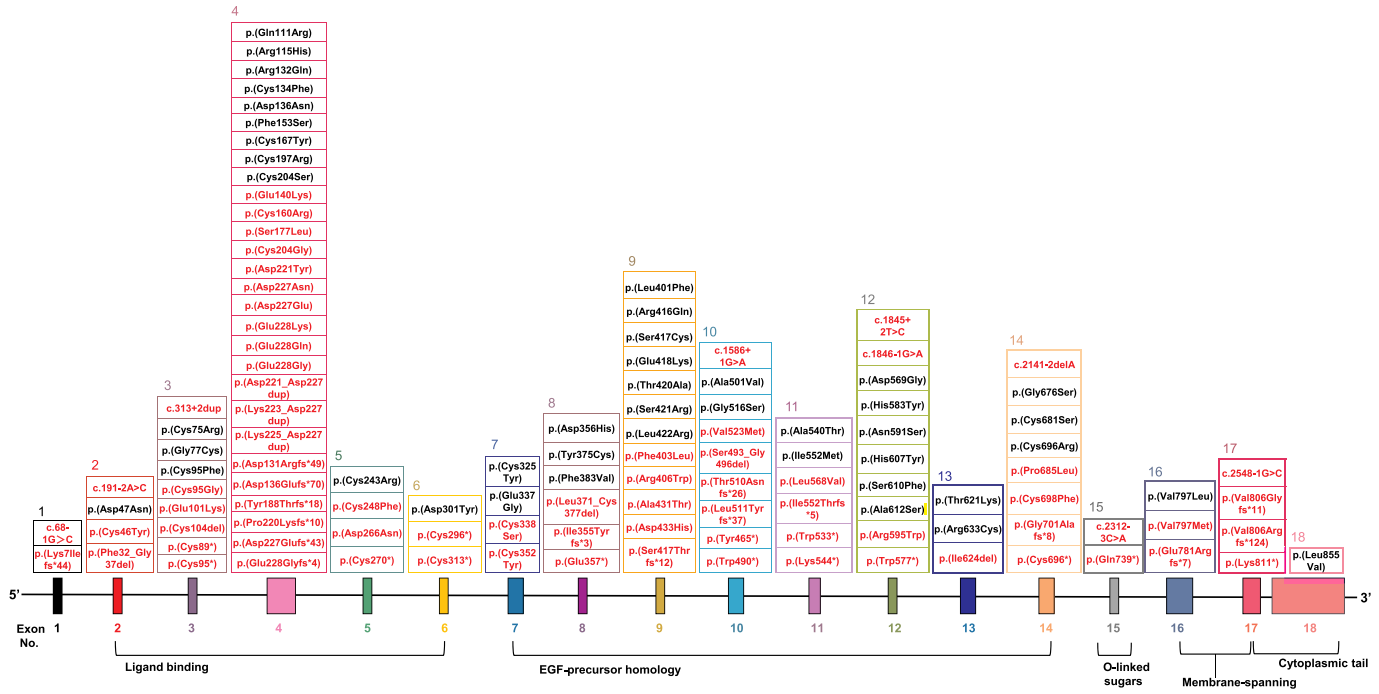

Fig. 1. Location of nonsynonymous variants in the *LDLR* gene detected in Japanese HeFH patients.
The exons in the *LDLR* gene are shown in boxes with the exon number. Point mutations and small deletions/insertions are indicated by red (pathogenic variants) or black text (VUS). The variants shown in this figure correspond to symbols shown in Supplemental Fig. 1. A benign variant p.(Ala860Val) is not included in this figure.

one splicing variant, c.1845+2T > C (n = 27; 7.5%); three missense variants, c.1012T > A: p.(Cys338Ser) (n = 23; 6.4%), c.1297G > C: p.(Asp433His) (n = 20; 5.5%), and c.1702C > G: p.(Leu568Val) (n = 19; 5.2%); and one nonsense variant, c.2431A > T: p.(Lys811*) (n = 21; 5.8%) (Fig. 2). The main cluster of pathogenic variants in the *LDLR* gene was located in exon 4, and a second cluster was located in exon 10 (Supplemental Fig. 1). In Japan and East Asia, 21 *LDLR* variants have been reported in the general population (Supplemental Table 3) [16,17]. The allele frequency of c.1845+2T > C, c.1297G > C: p.(Asp433His), c.1702C > G: p.(Leu568Val), and c.2431A > T: p.(Lys811*) variants in HeFH patients obtained in this study was significantly higher than that found in the Japanese general population. The c.1012T > A: p.(Cys338Ser) variant in the *LDLR* gene was not detected in the Japanese general population.

Table 1 and Supplemental Fig. 2 show the 21 detected *PCSK9* variants. According to the ACMG/AMP guidelines, four variants were pathogenic or likely pathogenic, 10 variants were VUS, and seven variants were benign. *PCSK9* pathogenic variants were found in 7.8% (n = 51) of unrelated FH patients: c.94G > A: p.(Glu32Lys) (n = 45; 6.9%), c.385G > A: p.(Asp129Asn) (n = 1; 0.15%), c.644G > A: p.(Arg215His) (n = 1; 0.15%), and c.1486C > T: p.(Arg496Trp) (n = 4; 0.61%). The c.94G > A: p.(Glu32Lys) variant was the most frequent of the four *PCSK9* pathogenic variants. The allele frequency of the c.94G > A: p.(Glu32Lys) variant in the unrelated HeFH patients was significantly higher than that in the Japanese general population. An *in vitro* functional analysis of PCSK9 p.(Glu32Lys), p.(Asp129Asn), p.(Arg215His) and p.(Arg496Trp) mutants was previously performed [21–23], and we thus defined these variants as pathogenic in the present study.

3.2. Association of pathogenic variants in the LDLR and PCSK9 genes with clinical characteristics of FH

We compared the clinical characteristics of patients aged ≥20 years harbouring *LDLR* pathogenic variants or VUS, *PCSK9* pathogenic variants, and no *LDLR/PCSK9* rare variants (Table 2). The LDL-C levels in the patients harbouring *LDLR* pathogenic variants were significantly higher than those in the patients harbouring either no *LDLR/PCSK9* rare variants (Tukey-Kramer test, $p < 0.0001$) or *PCSK9* pathogenic variants (Tukey-Kramer test, $p < 0.0001$). The prevalence of ATT in the patients harbouring *LDLR* pathogenic variants was significantly higher than that in the patients harbouring either no *LDLR/PCSK9* rare variants (Fisher's exact test and Bonferroni correction, $p < 0.001$) or *PCSK9* pathogenic variants (Fisher's exact test and Bonferroni correction, $p < 0.001$). The prevalence of ATT in patients harbouring *PCSK9* pathogenic variants was 44%. No difference in the prevalence of CAD was found between patients harbouring *LDLR* pathogenic variants and patients harbouring either no *LDLR/PCSK9* rare variants or *PCSK9* pathogenic variants. The prevalence of diabetes *mellitus* in the patients harbouring *LDLR* pathogenic variants was significantly lower than that in the patients harbouring no *LDLR/PCSK9* rare variants (Fisher's exact test and Bonferroni correction, $p < 0.001$). The LDL-C levels and the prevalence of ATT in patients harbouring *LDLR* pathogenic variants were significantly higher than those in patients harbouring the c.94G > A (p.Glu32Lys) variant in the *PCSK9* gene (Supplemental Table 4; Tukey-Kramer test, $p < 0.0001$).

3.3. Effects of five frequent LDLR pathogenic variants on clinical characteristics of FH

The serum lipid levels and prevalence of ATT and CAD in patients aged ≥20 years harbouring the five frequent *LDLR* pathogenic variants were compared with those harbouring the other *LDLR* pathogenic variants (Supplemental Table 5). The serum TC and LDL-C levels in patients harbouring the c.1845+2T > C variant were significantly lower than those in patients harbouring the other *LDLR* pathogenic variants. The prevalence of ATT was significantly higher in patients harbouring the c.1012T > A: p.(Cys338Ser) variant than in those harbouring the other *LDLR* pathogenic variants but was not associated with the variant after adjusting for the prevalence of hypertension (logistic regression test, $p = 0.0718$). The serum TC level and the prevalence of CAD in patients harbouring the c.1297G > C: p.

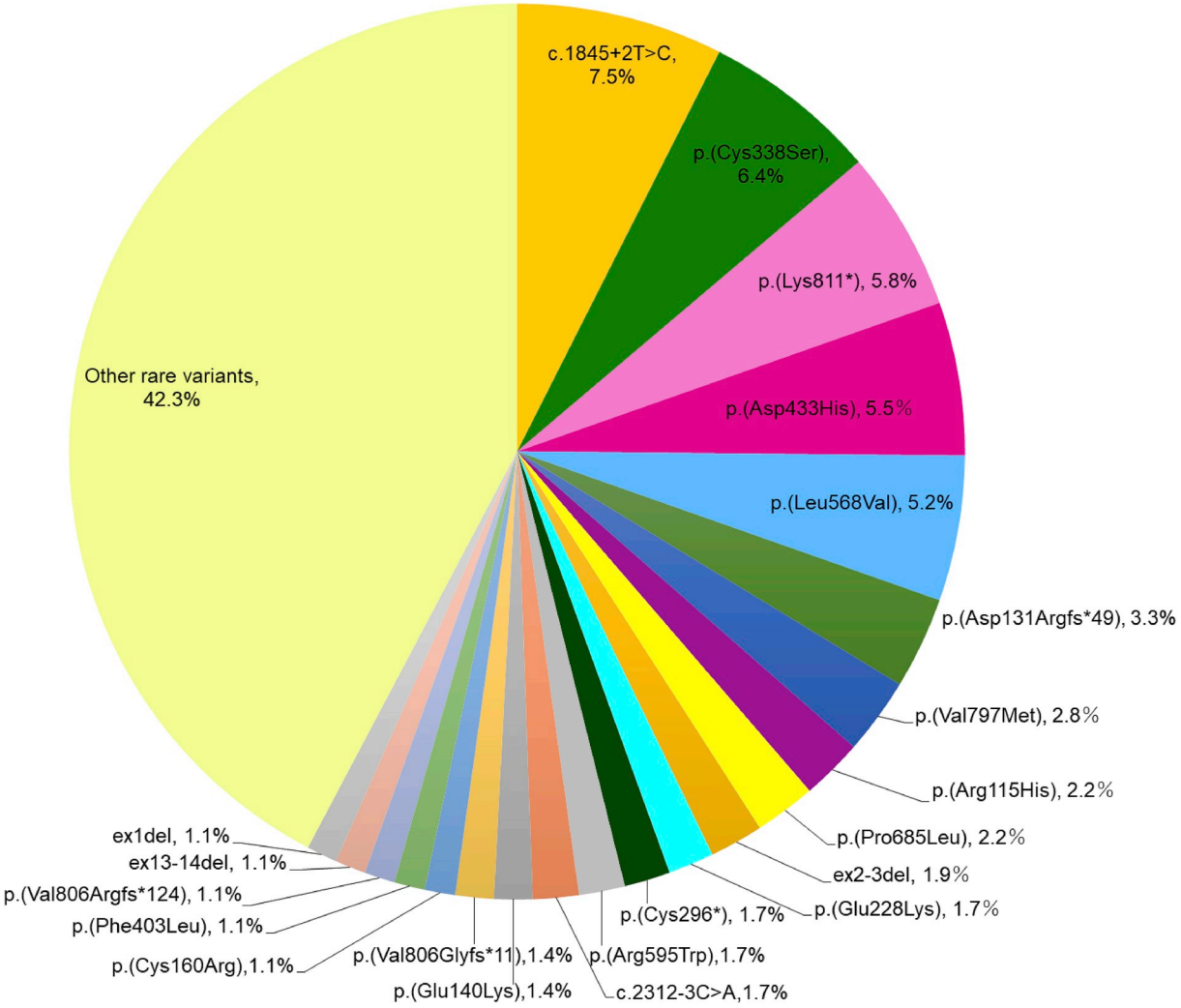

Fig. 2. Distribution of variants in the *LDLR* gene in unrelated Japanese heterozygous FH patients.
The frequency of each variant was calculated as the ratio of the number of each variant to the total number of *LDLR* variants (n = 362) in unrelated patients. A benign variant p.(Ala860Val) is not included in this graph.

(Asp433His) variant were significantly higher than those in patients harbouring the other *LDLR* pathogenic variants. However, the prevalence of CAD was not associated with the c.1297G > C: p.(Asp433His) variant after adjusting for the serum TC level (logistic regression test, $p = 0.0943$). The serum HDL-C level in patients harbouring the c.1702C > G: p.(Leu568Val) variant was significantly higher than that in patients harbouring the other *LDLR* pathogenic variants, and the prevalence of ATT was not associated with the variant after adjusting for the serum HDL-C level (logistic regression test, $p = 0.0997$).

3.4. Impact of LDLR pathogenic variant on age of onset of CAD

We stratified the patients who developed CAD by their age of onset to examine the association between *LDLR* or *PCSK9* pathogenic variants and the age of onset of CAD (Fig. 3, Supplementary Fig. 3). The *LDLR* pathogenic variants were found in 87.5% of the patients who developed CAD in their 20s and in 60.7% of patients who developed CAD in their 30s. The *LDLR* pathogenic variants detected in patients with CAD in their 20s were c.530C > T: p.(Ser177Leu), c.888C > A: p.(Cys296*), c.1297G > C: p.(Asp433His), c.2054C > T: p.(Pro685Leu), c.2389G > A: p.(Val797Met), c.2431A > T: p.(Lys811*), and exon 8–10 duplication. The proportion of patients harbouring *LDLR* pathogenic variants significantly decreased with increases in the age of onset of CAD (Cochran-Armitage trend test, $p = 0.0043$). The proportion of male patients harbouring *LDLR* pathogenic variants significantly decreased with increases in the age of CAD onset (Cochran-Armitage trend test, $p = 0.0084$), but that of female patients did not change with increases in the age of onset of CAD onset (Cochran-Armitage trend test, $p = 0.222$).

4. Discussion

In 801 Japanese FH patients, 137 *LDLR* variants were identified, and 92, 44 and 1 of these variants were classified as pathogenic, VUS, and benign, respectively. Pathogenic variants in the *LDLR* and *PCSK9* genes were found in 46% (n = 296) and 7.8% (n = 51) of unrelated FH patients (n = 650), respectively. Sixty of the 92 *LDLR* pathogenic variants were identified in ClinVar, and 32 were newly identified in this study. The proportion of *LDLR* pathogenic variants was high in patients with a younger age of CAD onset and significantly decreased with increases in the age of onset of CAD. The proportion of male patients harbouring *LDLR* pathogenic variants significantly decreased with increases in the age of CAD onset, but that of female patients did not change with increases in the age of onset of CAD. One possible explanation might be that a lower number of female patients developed CAD compared with the number of male patients.

The *LDLR* pathogenic variants were clustered in exons 4 and 10. The variants in the *LDLR* gene were previously shown to be clustered in

Table 1
Variants detected in the *PCSK9* gene.

Exon No.	Genomic location GRCh38 (Chr1)	Nucleotide change	Effect of protein	No. of patients	No. of families	Risk allele counts in FH	Total allele counts in FH	Allele frequency in FH	Allele frequency in Japan and East Asia (HGVD/ToMMo/ExAC-EAS)[a]	ClinVar	rs number	Variant rating according to ACMG
1	55039847	c.10G > A	p.(Val4Ile)	9	9	9	1300	0.007	0.013/0.014[b]/0.004	Uncertain significance	rs186669805	Benign
1	55039900	c.63_65dup[d]	p.(Leu23dup)	94	86	96	1300	0.074	0.098[b]/-/0.090	Benign/Likely benign	rs35574083	Benign
1	55039931	c.94G > A	p.(Glu32Lys)	53	45	45	1300	0.035	0.006[c]/0.011[c]/0.002[c]	Conflicting interpretations of pathogenicity	rs564427867	Pathogenic
1	55039995	c.158C > T[d]	p.(Ala53Val)	94	86	96	1300	0.074	0.141[c]/0.134[c]/0.230[c]	Benign/Likely benign	rs11583680	Benign
2	55043888	c.253G > A	p.(Glu85Lys)	1	1	1	1300	0.001	−/−/0.0002	N/A	rs774506287	Uncertain significance
2	55043912	c.277C > T	p.(Arg93Cys)	5	5	5	1300	0.004	0.007/0.009/0.008	Benign/Likely benign	rs151193009	Benign
2	55044020	c.385G > A	p.(Asp129Asn)	1	1	1	1300	0.001	−/−/-	Conflicting interpretations of pathogenicity	rs778738291	Likely pathogenic
2	55044031	c.396G > C	p.(Glu132Asp)	1	1	1	1300	0.001	-/0.0001/-	N/A	N/A	Uncertain significance
3	55046626	c.503C > T	p.(Ala168Val)	2	2	2	1300	0.002	-/0.0006/-	N/A	rs770592607	Uncertain significance
4	55052398	c.644G > A	p.(Arg215His)	1	1	1	1300	0.001	-/0.0001/-	Conflicting interpretations of pathogenicity	rs794728683	Likely pathogenic
5	55052779	c.787G > A	p.(Gly263Ser)	6	6	6	1300	0.005	0.008/0.007/0.0002[c]	N/A	rs200146448	Benign
5	55052783	c.791C > T	p.(Thr264Ile)	6	6	6	1300	0.005	0.005/0.006/0.002[b]	Likely benign	rs201789841	Benign
8	55058125	c.1270A > G	p.(Ile424Val)	6	5	5	1300	0.004	0.009/0.008/0.001	N/A	rs759250273	Benign
9	55058576	c.1432G > A	p.(Ala478Thr)	1	1	1	1300	0.001	−/−/0.0007	Uncertain significance	rs375582388	Uncertain significance
9	55058630	c.1486C > T	p.(Arg496Trp)	4	4	4	1300	0.003	−/−/-	Uncertain significance	rs374603772	Likely pathogenic
10	55059492	c.1510G > T	p.(Gly504Trp)	1	1	1	1300	0.001	-/0.0001/-	N/A	rs374455190	Uncertain significance
12	55063391	c.1886G > A	p.(Gly629Asp)	1	1	1	1300	0.001	-/0.0004/-	N/A	N/A	Uncertain significance
12	55063435	c.1930G > A	p.(Val644Ile)	7	6	6	1300	0.005	0.003/0.004/0.0002	N/A	rs143291739	Uncertain significance
12	55063450	c.1945G > A	p.(Ala649Thr)	1	1	1	1300	0.001	−/−/-	N/A	rs371744393	Uncertain significance
12	55063509	c.2004C > A	p.(Ser668Arg)	1	1	1	1300	0.001	0.0004/0.0006/0.0003	Uncertain significance	rs762298323	Uncertain significance
12	55063550	c.2045G > A	p.(Arg682Gln)	1	1	1	1300	0.001	−/−/-	N/A	rs758946245	Uncertain significance

[b] $p < 0.05$, [c] $p < 0.001$ vs allele frequency in FH based on Fisher's exact test.
N/A: not applicable.
[a] Allele frequency in the general population in Japan and East Asia is shown based on the Japanese Human Genetic Variation Database[16], Japanese genome variation database (ToMMo_3.5KJPN)[17], and ExAC-East Asian (ExAC-EAS)[15].
[d] The c.63_65dup: p.(Leu23dup) and c.158C > T: p.(Ala53Val) variants were in linkage disequilibrium with each other, resulting in 10 homozygous and 76 heterozygous variants in these two genes.

Table 2
Clinical characteristics in HeFH patients ≥20 years according to variants in the *LDLR* or *PCSK9* genes.

	Non-carriers of *LDLR/PCSK9* rare variants	Carriers of *LDLR* pathogenic variants	Carriers of *LDLR* VUS	Carriers of *PCSK9* pathogenic variants	*p*-value[a]
N	256	349	64	55	
Age at last follow-up (years)	59 ± 15	52 ± 18	53 ± 18	56 ± 15	< 0.0001
Male (%)	52	50	41	53	0.4460
Total cholesterol (mg/dL)	297 ± 65	351 ± 80	334 ± 72	284 ± 41	< 0.0001
Triglycerides (mg/dL)	134 (95–198)	101 (71–151)	123 (76–201)	155 (100–190)	< 0.0001
HDL-cholesterol (mg/dL)	57 ± 17	53 ± 15	54 ± 14	55 ± 15	0.0669
LDL-cholesterol (mg/dL)	207 ± 50	275 ± 73	251 ± 63	201 ± 42	< 0.0001
Prevalence of diabetes mellitus (%)	18	8	9	4	0.0003
Prevalence of hypertension (%)	14	9	6	11	0.1592
Prevalence of ATT (%)	65	82	55	44	< 0.0001
Smoking (past or current) (%)	44	36	42	36	0.3032
Prevalence of CAD (%)	38	31	34	18	0.0363

Values are the mean ± SD or median (interquartile range). Untreated serum lipid levels are shown.
[a]*p*-values were calculated based on Pearson's Chi-square test for categorical data and based on ANOVA or Kruskal-Wallis test for continuous variables.
ATT: Achilles tendon thickness; CAD: coronary artery disease; VUS: variants of uncertain significance.

exon 4, [18], which is the largest exon in the *LDLR* gene. In the present study, 17% (n = 110) of unrelated FH patients contained at least one of five frequent *LDLR* variants, including c.1845+2T > C, c.1012T > A: p.(Cys338Ser), c.1297G > C: p.(Asp433His), c.1702C > G: p.(Leu568Val) and c.2431A > T: p.(Lys811*). In the Hokuriku district, the following three frequent *LDLR* variants were found in 44.5% of the FH patients (n = 1054) [24]: c.2431A > T: p.(Lys811*) (n = 292; 28%), c.2312-3C > A (n = 119; 11%), and deletion of exon 2–3 (n = 58; 5.5%). Yu and Mabuchi et al. reported that the c.2431A > T: p.(Lys811*) variant is an example of a "founder effect" or a variant that prevailed both locally and widely over a long period of time [2,7]. In the present study, 67.5% of FH patients lived in the Kansai district, which is one of the largest metropolitan areas in Japan and has a greater influx of people, and the rest lived throughout Japan. Therefore, this study could reflect the distribution of *LDLR* variants across Japan.

The clinical phenotypes of FH in carriers of each of the five frequent *LDLR* pathogenic variants were compared with those of the noncarriers in patients harbouring *LDLR* pathogenic variants. Carriers of the c.1845+2T > C and c.1702C > G: p.(Leu568Val) variants had significantly lower serum LDL-C levels and higher serum HDL-C levels than the noncarriers. The serum TC levels in carriers of the c.1297G > C: p.(Asp433His) variant were significantly higher than those in the noncarriers. The prevalence of ATT was significantly higher in carriers of the c.1012T > A: p.(Cys338Ser) variant and significantly lower in carriers of the c.1702C > G: p.(Leu568Val) variant than in the noncarriers. We recently reported that an increased ATT is associated with decreased HDL-C levels, a decreased cholesterol efflux capacity or an increased prevalence of hypertension in FH patients [25]. Thus, after adjusting for the covariates, the prevalence of ATT was not associated with the c.1702C > G: p.(Leu568Val) variant or the c.1012T > A: p.(Cys338Ser) variant. The c.1702C > G: p.(Leu568Val) variant has previously shown to yield a mild phenotype based on a high level of LDLR activity [26], which is consistent with the results of the present study. The c.1845+2T > C variant has been reported to be a receptor-negative variant [27]. The c.1297G > C: p.(Asp433His) variant is associated with impaired processing and rapid degradation of the synthesized receptor [28]. In the future, it is necessary to assess the effect of each variant on phenotype using a large number of samples.

We defined c.94G > A: p.(Glu32Lys), c.385G > A: p.(Asp129Asn), c.644G > A: p.(Arg215His), and c.1486C > T: p.(Arg496Trp) as *PCSK9* pathogenic variants. The prevalence of ATT, the main characteristic of FH, in the patients harbouring *PCSK9* pathogenic variants was 44%, and this value was significantly lower than that observed for the patients harbouring *LDLR* pathogenic variants. In the present study, patients harbouring the c.94G > A: p.(Glu32Lys) variant comprised 88% (n = 45) of unrelated patients harbouring *PCSK9* pathogenic variants (n = 51). The LDL-C levels and the prevalence of ATT in patients harbouring *LDLR* pathogenic variants were significantly higher than those in patients harbouring the c.94G > A: p.(Glu32Lys) variant in the *PCSK9* gene. The c.94G > A: p.(Glu32Lys) variant in the *PCSK9* gene is frequently detected in Japanese FH patients and is a specific variant in East Asian population. Mabuchi et al. has also reported that FH patients harbouring the c.94G > A: p.(Glu32Lys) variant have mild phenotypes compared with FH patients harbouring *LDLR* mutations [29]. In an *in vitro* study, Noguchi et al. have reported that HepG2 cells transfected with PCSK9-p.(Glu32Lys) secreted significantly larger amounts of PCSK9 into the media than those with PCSK9-WT after 24 h

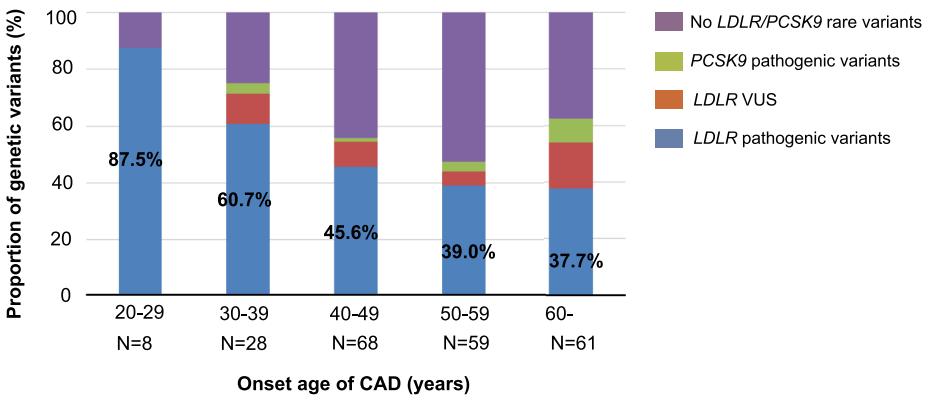

Fig. 3. Proportion of variants in the *LDLR* and *PCSK9* genes stratified by the age of CAD onset.

of incubation (139% ± 13% vs. 100% ± 3%, $p < 0.01$)[23]. Thus, the phenotype of FH patients harbouring the c.94G > A: p.(Glu32Lys) variant differs from that of patients harbouring a gain-of-function mutation, including the c.1120G > T: p.(Asp374Tyr) variant in the *PCSK9* gene detected in the Western countries. The c.94G > A: p.(Glu32Lys) variant in the *PCSK9* gene is defined as pathogenic but shows a mild FH phenotype compared with gain-of-function mutations in the *PCSK9* gene detected in other countries. It was recently reported that the c.94G > A: p.(Glu32Lys) variant in the *PCSK9* gene affects the age of onset of myocardial infarction by increasing the LDL-C levels in the Japanese general population [30]. It is thus likely that FH might be underdiagnosed in patients with the c.94G > A: p.(Glu32Lys) variant in the *PCSK9* gene, despite their genetic risk for CAD.

We previously reported that the c.10G > A: p.(Val4Ile) variant in the *PCSK9* gene increases the prevalence of CAD in accordance with an elevation of the LDL-C level in carriers of an *LDLR* variant [13]. However, in the present study, the c.10G > A: p.(Val4Ile) variant in the *PCSK9* gene was classified as benign according to the ACMG/AMP guidelines, and the allele frequency in unrelated HeFH patients was lower than that in the Japanese general population. In the Korean population, the p.(Val4Ile) variant in the *PCSK9* gene was detected in an individual whose untreated LDL-C levels were < 48 mg/dL [31]. The c.10G > A: p.(Val4Ile) variant in the *PCSK9* gene has been proposed to not affect the phenotype of HeFH by itself.

We predicted the risk of CAD from a genetic analysis of HeFH [13]. In the present study, *LDLR* pathogenic variants were found in 66.7% (n = 24) of the patients who developed CAD at less than 40 years of age and were still observed in 37.7% of patients who developed CAD at 60 years of age or older. Some standard risk factors for CAD, including diabetes, hypertension, and smoking, have been shown to be associated with an increased risk for CAD in HeFH patients [32,33]. In the present study, these risk factors in HeFH patients are expected to increase the risk for developing CAD at a relatively advanced age. Thus, it has been hypothesized that *LDLR* pathogenic variants are related to the development of CAD at a younger age, and the other risk factors for CAD are related to the development of CAD at an advanced age. The genetic analysis of FH is very important for the identification of FH patients at high risk for premature CAD and for improving the prognosis of FH.

This study has some limitations. First, this was a retrospective observational study, and thus, the subjects with missing clinical information were excluded from the analysis of the association of clinical phenotype with pathogenic variants. The exclusion of these patients might have caused a selection bias for the patient phenotype in contrast to genetic data. However, this study was based on one of the largest cohorts of Japanese FH patients and showed that the distribution of *LDLR* pathogenic variants differed from that found in the Hokuriku district, which has a large cohort of Japanese FH patients. Therefore, our results might reflect the distribution of *LDLR* pathogenic variants throughout Japan compared with the Hokuriku district. A second limitation is that only CAD events were considered; this cohort included both patients under treatment and those not receiving treatment, and the treatment period was also not considered. A detailed analysis of CAD events with proper consideration of the treatments and their durations is currently being conducted. A third limitation is that we did not analyse introns in the *LDLR* gene and the other causative genes. Unexplained FH might be partly explained by the accumulation of common SNPs [34] and other causative genes. The fourth limitation is that our center specializes in CAD; thus, the prevalence of CAD in FH patients at our center might be higher than that in the general Japanese population of FH patients.

In conclusion, this study annotated the clinical significance of variants in the *LDLR* and *PCSK9* genes and assessed the impact of their pathogenic variants on the clinical phenotype of Japanese HeFH patients. This study could provide important data for the highly accurate genetic diagnosis of Japanese FH patients.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Financial support

This work was partly supported by grants from the Ministry of Health, Labour and Welfare of Japan for Clinical Research on Intractable Diseases (H26-nanji-ippan-056 and H30-nanji-ippan-003), JSPS KAKENHI (JP17K08681), the Japan Agency for Medical Research and Development (16ek0210075h0001), and the Intramural Research Fund for Cardiovascular Diseases of the National Cerebral and Cardiovascular Center (28-2-2; 29-6-11), by a Japan Heart Foundation & Astellas Grant for Research on Atherosclerosis Update, and by funds from the Takeda Science Foundation, the Kanae Foundation for the Promotion of Medical Science, the Novartis Research Grants, and the Japanese Circulation Society.

Author contributions

M.H. collected the clinical data, drafted the manuscript and contributed to the conception and design of the study. N.O., H.M., R.I., S.Y., and C.S. performed the genetic analyses. A.T. performed the statistical analyses and contributed to the study design. M.O., K.H., and Y.M. interpreted the clinical data. M. H–S. contributed to the study's supervision. All authors gave final approval of the submitted version.

Acknowledgements

We thank Ms. Rie Oishi and Ms. Akiko Yoshida for their clerical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.08.004.

References

[1] J.L. Goldstein, H.H. Hobbs, M.S. Brown, Familial hypercholesterolemia, in: C.R. Scriver, B.A.A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th, McGraw-Hill, Inc., New York, 2001, pp. 2863–2913.
[2] H. Mabuchi, A. Nohara, T. Noguchi, et al., Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan, Atherosclerosis 214 (2011) 404–407.
[3] B.G. Nordestgaard, M.J. Chapman, S.E. Humphries, 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. 34 (2013) 3478–3490a.
[4] M.S. Brown, J.L. Goldstein, Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man, Science 185 (1974) 61–63.
[5] T.L. Innerarity, K.H. Weisgraber, K.S. Arnold, et al., Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 6919–6923.
[6] M. Abifadel, M. Varret, J.P. Rabes, et al., Mutations in PCSK9 cause autosomal dominant hypercholesterolemia, Nat. Genet. 34 (2003) 154–156.
[7] W. Yu, A. Nohara, T. Higashikata, et al., Molecular genetic analysis of familial hypercholesterolemia: spectrum and regional difference of LDL receptor gene mutations in Japanese population, Atherosclerosis 165 (2002) 335–342.
[8] J.D. Horton, J.C. Cohen, H.H. Hobbs, Molecular biology of PCSK9: its role in LDL metabolism, Trends Biochem. Sci. 32 (2007) 71–77.
[9] J.C. Cohen, E. Boerwinkle, T.H. Mosley Jr.et al., Sequence variations in PCSK9, low LDL, and protection against coronary heart disease, N. Engl. J. Med. 354 (2006) 1264–1272.
[10] M.A. Iacocca, J.R. Chora, A. Carrie, et al., ClinVar database of global familial hypercholesterolemia-associated DNA variants, Hum. Mutat. 39 (2018) 1631–1640.
[11] R.C. Green, J.S. Berg, W.W. Grody, et al., ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing, Genet. Med. 15 (2013) 565–574.
[12] M. Harada-Shiba, H. Arai, Y. Ishigaki, et al., Guidelines for diagnosis and treatment

of familial hypercholesterolemia 2017, J. Atheroscler. Thromb. 25 (2018) 751–770.
[13] N. Ohta, M. Hori, A. Takahashi, et al., Proprotein convertase subtilisin/kexin 9 V4I variant with LDLR mutations modifies the phenotype of familial hypercholesterolemia, J. Clin. Lipidol. 10 (2016) 547–555 e545.
[14] S. Leigh, M. Futema, R. Whittall, et al., The UCL low-density lipoprotein receptor gene variant database: pathogenicity update, J. Med. Genet. 54 (2017) 217–223.
[15] M. Lek, K.J. Karczewski, E.V. Minikel, et al., Analysis of protein-coding genetic variation in 60,706 humans, Nature 536 (2016) 285–291.
[16] K. Higasa, N. Miyake, J. Yoshimura, et al., Human genetic variation database, a reference database of genetic variations in the Japanese population, J. Hum. Genet. 61 (2016) 547–553.
[17] M. Nagasaki, J. Yasuda, F. Katsuoka, et al., Rare variant discovery by deep whole-genome sequencing of 1,070 Japanese individuals, Nat. Commun. 6 (2015) 8018.
[18] H.H. Hobbs, M.S. Brown, J.L. Goldstein, Molecular genetics of the LDL receptor gene in familial hypercholesterolemia, Hum. Mutat. 1 (1992) 445–466.
[19] S. Richards, N. Aziz, S. Bale, et al., 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 (2015) 405–424.
[20] M. Hori, E. Miyauchi, C. Son, et al., Detection of the benign c.2579C > T (p.A860V) variant of the LDLR gene in a pedigree-based genetic analysis of familial hypercholesterolemia, J. Clin. Lipidol. 13 (2019) 335–339.
[21] J. Cameron, O.L. Holla, J.K. Laerdahl, et al., Characterization of novel mutations in the catalytic domain of the PCSK9 gene, J. Intern. Med. 263 (2008) 420–431.
[22] T. Fasano, X.M. Sun, D.D. Patel, et al., Degradation of LDLR protein mediated by 'gain of function' PCSK9 mutants in normal and ARH cells, Atherosclerosis 203 (2009) 166–171.
[23] T. Noguchi, S. Katsuda, M.A. Kawashiri, et al., The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolaemia by increasing PCSK9 function and concentration in the circulation, Atherosclerosis 210 (2010) 166–172.
[24] H. Mabuchi, Half a century tales of familial hypercholesterolemia (FH) in Japan, J. Atheroscler. Thromb. 24 (2017) 189–207.
[25] M. Ogura, M. Hori, M. Harada-Shiba, Association between cholesterol efflux capacity and atherosclerotic cardiovascular disease in patients with familial hypercholesterolemia, Arterioscler. Thromb. Vasc. Biol. 36 (2016) 181–188.
[26] Y. Miyake, T. Yamamura, N. Sakai, et al., Update of Japanese common LDLR gene mutations and their phenotypes: mild type mutation L547V might predominate in the Japanese population, Atherosclerosis 203 (2009) 153–160.
[27] T. Funahashi, Y. Miyake, A. Yamamoto, et al., Mutations of the low density lipoprotein receptor in Japanese kindreds with familial hypercholesterolemia, Hum. Genet. 79 (1988) 103–108.
[28] Y. Miyake, S. Tajima, T. Funahashi, et al., A point mutation of low-density-lipoprotein receptor causing rapid degradation of the receptor, Eur. J. Biochem. 210 (1992) 1–7.
[29] H. Mabuchi, A. Nohara, T. Noguchi, et al., Genotypic and phenotypic features in homozygous familial hypercholesterolemia caused by proprotein convertase subtilisin/kexin type 9 (PCSK9) gain-of-function mutation, Atherosclerosis 236 (2014) 54–61.
[30] T. Tajima, H. Morita, K. Ito, et al., Blood lipid-related low-frequency variants in LDLR and PCSK9 are associated with onset age and risk of myocardial infarction in Japanese, Sci. Rep. 8 (2018) 8107.
[31] C.J. Lee, Y. Lee, S. Park, et al., Rare and common variants of APOB and PCSK9 in Korean patients with extremely low low-density lipoprotein-cholesterol levels, PLoS One 12 (2017) e0186446.
[32] M. Paquette, R. Dufour, A. Baass, The Montreal-FH-SCORE: a new score to predict cardiovascular events in familial hypercholesterolemia, J. Clin. Lipidol. 11 (2017) 80–86.
[33] M. Paquette, S. Bernard, I. Ruel, et al., Diabetes is associated with an increased risk of cardiovascular disease in patients with familial hypercholesterolemia, J. Clin. Lipidol. 13 (2019) 123–128.
[34] P.J. Talmud, S. Shah, R. Whittall, et al., Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study, Lancet 381 (2013) 1293–1301.