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► **To cite this version:**

Roeland Huijgen, Iris Kindt, Sigrid W Fouchier, Joep C Defesche, Barbara A Hutten, et al.. Functionality of Sequence Variants in the Genes Coding for the Low-Density Lipoprotein Receptor and Apolipoprotein B in Individuals with Inherited Hypercholesterolemia. *Human Mutation*, 2010, 31 (6), pp.752. 10.1002/humu.21258 . hal-00552383

HAL Id: hal-00552383

<https://hal.science/hal-00552383v1>

Submitted on 6 Jan 2011

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Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2009-0421.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	18-Feb-2010
Complete List of Authors:	Huijgen, Roeland; Academic Medical Center Amsterdam, Vascular Medicine Kindt, Iris; Foundation for the Identification of Persons with Inherited Hypercholesterolemia Fouchier, Sigrid; Academic Medical Center Amsterdam, Vascular Medicine Defesche, Joep; Academic Medical Center Amsterdam, Vascular Medicine Hutten, Barbara; Academic Medical Center Amsterdam, Clinical Epidemiology, Biostatistics and Bioinformatics Kastelein, John; Academic Medical Center Amsterdam, Vascular Medicine Vissers, Maud; Academic Medical Center Amsterdam, Vascular Medicine
Key Words:	Familial Hypercholesterolemia, Genetic screening, LDLR, APOB, Functionality



Humu-2009-0421

Research Article

Functionality of Sequence Variants in the Genes Coding for the Low-Density Lipoprotein Receptor and Apolipoprotein B in Individuals with Inherited Hypercholesterolemia

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Abstract

Objectives: Patients with familial hypercholesterolemia (FH) have elevated LDL-C levels, usually above the 90th percentile (P90) for age and gender. However, large scale genetic cascade screening for FH showed that 15% of the LDL-receptor (LDLR) or Apolipoprotein B (APOB) mutation carriers have LDL-C levels below P75. Non-pathogenicity of sequence changes may explain this phenomenon.

Methods: To assess pathogenicity of a mutation we proposed three criteria: 1) mean LDL-C >P75 in untreated mutation carriers; 2) higher mean LDL-C level in untreated carriers than in untreated non-carriers; and 3) higher percentage of medication users in carriers than in non-carriers at screening. We considered a mutation non-pathogenic when none of the three criteria were met. We applied these criteria to mutations that had been determined in more than 50 untreated adults. Segregation analysis was performed to confirm non-pathogenicity.

Results: Forty-six mutations had been tested in more than 50 untreated subjects and three were non-pathogenic according to our criteria: one in *LDLR* (c.108C>A, exon 2) and two in *APOB* (c.13154T>C and c.13181T>C, both in exon 29). Segregation analysis supported their non-pathogenic nature.

Conclusions: According to our criteria, three sequence variants were non-pathogenic. The criteria may help to identify non-pathogenic sequence changes in genetic cascade screening programmes.

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Key words: familial hypercholesterolemia, genetic screening

For Peer Review

Introduction

Familial hypercholesterolemia (FH; MIM# 143890) is an inherited disorder of lipoprotein metabolism with a prevalence of 1:400 in the Netherlands. It is characterized by an autosomal dominant inheritance pattern of high levels of low-density lipoprotein cholesterol (LDL-C).[Goldstein et al., 2001; Lansberg et al., 2000] Patients with FH have a severely increased risk for premature cardiovascular disease which can be substantially reduced by LDL-C lowering treatment.[Huijgen et al., 2008; Versmissen et al., 2008]

The clinical phenotype of FH is predominantly caused by mutations in the LDL- receptor (*LDLR*; MIM# 606945) or the apolipoprotein B (*APOB*; MIM# 107730).[Goldstein et al., 2001; Pullinger et al., 1995] The identification of these mutations in probands is essential for effective screening of family members. Systematic tracing of relatives of the probands is actively being carried out in several European countries.[Thorsson et al., 2003; Pocovi et al., 2004; Leren et al., 2004; Bhatnagar et al., 2000] In the Netherlands, such a programme is carried out by the Foundation for the Identification of Persons with Inherited Hypercholesterolemia (In Dutch: StOEH).[Umans-Eckenhuis et al., 2001] Currently more than 500 *LDLR* or *APOB* mutations are used for screening of probands and their relatives.[Fouchier et al., 2001; Fouchier et al., 2005a] Since 1994, over 16,000 relatives with an FH mutation have been identified.

In order to diagnose FH in a patient or family, DNA samples from clinically suspected FH patients are analysed for the presence of an *LDLR* or *APOB* mutation.[Defesche, 2000] A patient is considered a proband for family screening when a pathogenic mutation has been ascertained. Subsequently, first-degree relatives of the identified probands are offered DNA analysis for the presence of the specific FH causing mutation. Cascade screening is extended to

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3 identify distant relatives of the probands by using the inheritance pattern across the pedigree.

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5 [Umans-Eckenhause et al., 2001]

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8 FH patients usually have plasma LDL-C levels above the 90th percentile (P90) for age
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10 and gender. However, in our observation, 15% of the mutation carriers had pre-treatment LDL-C
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12 levels below the P75 for age and gender.[Huijgen et al., 2009] This may be to the consequence of
13
14 a number of reasons but non-pathogenicity of detected sequence changes is the most likely
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16 explanation. *In vitro* expression assays are the preferred method to assess the pathogenic nature
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18 of mutations of unknown functionality. However, these have been performed on only a very
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20 small number of variants as they are costly and time-consuming. Therefore, web-based tools
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22 (PolyPhen and SIFT, also referred to as *in silico* analysis) predicting the effect on LDLR activity
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24 or APOB100 binding properties are most frequently used to determine whether or not a sequence
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26 is pathogenic. [Fouchier et al., 2005a; Leigh et al., 2008] However, also the use of these web-
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28 based methods cannot always accurately predict the pathogenicity of gene variants.[Leigh et al.,
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30 2008]

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36 Conversely, examination of pedigree data can also ascertain mutation pathogenicity as
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38 shown by several reports.[Defesche et al., 2009; Fouchier et al., 2005a; Leigh et al., 2008;
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40 Lombardi et al., 1997] However, in most previous studies, decisions on pathogenicity of
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42 mutations were mainly based on judgement by experts on a relative small number of pedigrees.
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44 In order to discriminate between pathogenic and non-pathogenic mutations in a more systematic
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46 way we sought to formulate a set of criteria for application in larger cohorts. Evidently, these
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48 criteria should be based on untreated LDL-C levels since truly pathogenic mutations give rise to
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50 clinically relevant higher levels compared to those in relatives without such a mutation.
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52 Moreover, the criteria should also take medication use into account because patients with
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3 severely elevated LDL-C levels are more likely to use lipid-lowering medication, even without
4 awareness of genetic predisposition. So, in case of a pathogenic mutation, a higher percentage of
5 medication users at molecular diagnosis in carriers than in non-carriers can be expected. Based
6 on these considerations we propose a number of criteria to assess pathogenicity of *LDLR* and
7 *APOB* mutations. In the current study, we tested the ability of these criteria to discriminate
8 established pathogenic mutations from non-pathogenic variants in a large cohort of individuals
9 that had been molecularly screened for FH. Finally, with the use of the criteria we identified
10 potential non-pathogenic variants among *LDLR* and *APOB* mutations that were currently
11 assumed to be pathogenic.
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27 **Materials and Methods**

28 *Criteria for Functionality*

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30 Three criteria were proposed to test pathogenicity of a specific DNA variant: 1) a mean LDL-C
31 percentile for age and gender above P75 in untreated individuals carrying such a mutation; 2) a
32 statistically significant higher mean LDL-C level in untreated carriers compared to untreated
33 non-carriers; and 3) a statistically significant higher percentage of medication users in carriers
34 than in non-carriers at the time of molecular screening. We considered a mutation to be non-
35 pathogenic when none of the three criteria were met.
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46 Age and gender specific percentiles of LDL-C were calculated using the reference values
47 of the Caucasian population.[Gotto, Jr. et al., 1984] Mutations were described according to the
48 nomenclature as proposed by den Dunnen and Antonakaris.[den Dunnen et al., 2000]
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55 *Study Cohort and Comparisons within this Cohort*

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3 The proposed criteria were tested in a cohort of persons, who were screened for FH by the
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5 Foundation for the Identification of Persons with Inherited Hypercholesterolemia in the period
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7 between January 2003 and September 2008. Of these subjects, lipid profiles, clinical information,
8
9 medication use and specific carrier status were collected at the time of molecular diagnosis. We
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11 excluded probands, subjects younger than 18 and older than 80 years, and subjects of whom a
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13 complete lipid profile was not available.
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17 We first tested the criteria in subjects from families with one of the six most prevalent
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19 and established pathogenic mutations: c.10580G>A (in *APOB*) and c.[1690A>C;2393_2401del],
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21 c.313+1/2 (this term is used to describe a group of three variants: c.313+1G>A; c.313+1G>C or
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23 c.313+2T>C), c.1359-1G>A (*LDLR*), c.1027G>A (*LDLR*) and c.131G>A (all in *LDLR*) as well
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25 as in subjects with one of nine already established non-pathogenic mutations c.2140+5G>A,
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27 c.148G>T, c.757C>T, c.2231G>A, c.1729T>C, c.2177C>T, c.2282C>T and c.2479G>A (all in
28
29 *LDLR*), and c.13130T>C (in *APOB*). [Defesche & Kastelein, 2009; Fouchier et al., 2005a;
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31 Lombardi et al., 1997; Fouchier et al., 2001] Subsequently, all mutations that were used for
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33 genetic screening in at least 50 untreated subjects were evaluated by the proposed criteria, in
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35 order to identify potential non-pathogenic variants. Additionally, the *in silico* analysis results
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37 based on SIFT and PolyPhen prediction were retrieved for the variants that were tested by the
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39 criteria.
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48 ***Segregation analysis for newly identified non-pathogenic sequence variants***

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50 Segregation analysis was performed to evaluate non-pathogenicity of sequence variants that had
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52 been identified as such by our criteria. All probands and subjects that used cholesterol-lowering
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54 medication were excluded from segregation analysis. The statistical power of the family was
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3 assessed by simulations using SLINK v2.51 under the assumptions of autosomal dominant
4 inheritance with complete penetrance and no phenocopies, no heterogeneity and a disease allele
5 frequency of 0.001. Subjects with untreated LDL-C levels above P90 were labelled as affected
6 with clinical FH. The same assumptions were used for the two-point parametric linkage analysis
7 with Fastlink v4.1. All linkage programs were accessed through the EasyLinkage
8 package.[Hoffman et al., 2005] A negative LOD score was considered as highly suggestive for
9 no linkage, and a LOD score below -2 as definite proof of no linkage.
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20 21 22 *Dutch Lipid Network Criteria in Probands*

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24 We retrieved clinical data consisting of both personal and family history on LDL-C levels,
25 medical history of CVD, and stigmata of FH (tendinous xanthomata and arcus cornealis) of all
26 probands with newly identified non-pathogenic mutations. These data were reviewed and scored
27 according to published criteria for the clinical diagnosis of FH.[Defesche, 2000] The mean
28 diagnostic score of probands of a newly identified non-pathogenic sequence variant was
29 compared to that of the probands diagnosed with a frequent and established pathogenic mutation.
30 For each of the five pathogenic LDLR mutations (Table 2), we selected the proband that was
31 diagnosed with FH successively after the proband with the newly identified non-pathogenic
32 variant. Similarly, for each proband with an identified non-pathogenic *APOB* variant, we
33 selected the two successive probands with the c.10580G>A mutation, a proven pathogenic
34 mutation in *APOB*. [Innerarity et al., 1987]
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51 52 53 *Statistical Analysis*

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3 The proposed criteria were tested for each mutation that was assessed in at least 50 untreated
4 persons. We estimated that 30% of these screened persons would have the mutation. With a
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6 sample size of 15 untreated carriers of a mutation and 35 non-carriers and an assumed common
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8 standard deviation (SD) of 1.1 mmol/L, we were able to detect a difference of 0.97 mmol/L in
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10 mean LDL-C between these groups (two-sided, with $\alpha=0.05$ and $\beta=0.2$).
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15 Differences in LDL-C levels between subgroups were compared by means of the
16
17 unpaired Student t-test. By means of linear regression models we adjusted for age and gender.
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19 Proportions of subjects using lipid-lowering medication were compared with the Chi-square test.
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22 A p-value < 0.05 was considered statistically significant. Data were analyzed with SPSS
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24 for Windows 16.0.2, Chicago, IL, USA.
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29 **Results**

30 *Study Cohort*

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32 Clinical data and mutation results were obtained from 23,874 individuals. All subjects consented
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34 to participate in the cascade screening programme for FH. The screening programme was
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36 prospectively reviewed and approved by the consulted Medical Ethical Committee. Complete
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38 lipid profiles were retrieved of 16,935 persons aged between 18 and 80 years (non-probands), in
39
40 which a total of 214 different mutations in *LDLR* or *APOB* were detected. In total 5,467 (32%)
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42 subjects were shown to carry one of these mutation, of whom 2703 (49%) used lipid-lowering
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44 medication at time of genetic testing. Of the 11,468 non-mutation carriers, 1,345 (13%) used
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46 medication. Among the untreated subjects, mean LDL-C levels (\pm SD) were 4.6 ± 1.4 mmol/L
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48 (P86) in mutation carriers and 3.2 ± 0.9 (P43) mmol/L in the non-carriers ($p<0.001$).
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3 The nomenclature of all variants we referred to in this manuscript is described in Table 1. This
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5 comprises the description of the gene, location of the mutation, the effect on the protein, current
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7 name, old name and references.
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10 11 12 ***Established Pathogenic and Non-Pathogenic Mutations***

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15 Untreated patients with one of the six most prevalent and established pathogenic mutations had
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17 statistically significant elevated LDL-C levels as compared to their unaffected relatives and more
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19 often used lipid-lowering medication (Table 2). Of these mutations, c.313+1/2, c.1359-1G>A
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21 and c.131G>A in *LDLR* resulted in the most severe LDL-C elevations and the highest proportion
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23 of medication users at molecular screening.
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27 Table 3 presents LDL-C levels and medication use in subjects with nine established non-
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29 pathogenic mutations.[Defesche & Kastelein, 2009; Fouchier et al., 2005a; Lombardi et al.,
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31 1997] The 171 untreated carriers of these mutations had a mean LDL-C level of 3.4 ± 1.0
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33 mmol/L (P53). This level did not significantly differ from the mean LDL-C level of 3.3 ± 0.9
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35 mmol/L (P43) in 370 untreated unaffected relatives ($p=0.12$). The same pattern was observed for
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37 each sequence variant separately. When we adjusted for age and gender, results were not
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39 essentially different, except for the c.2479G>A variant, in which the difference in LDL-C levels
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41 became significant ($p=0.02$). The proportion of persons using cholesterol-lowering medication at
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43 the time of screening did not differ significantly between carriers and non-carriers for each of the
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45 mutations, except for the c.2140+5G>A variant, in which the proportion of treated persons was
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47 higher compared to the non-carriers (40% vs 11%, respectively, $p=0.02$).
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55 56 ***Newly Identified Putative Non-Pathogenic Mutations***

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3 Of the 214 mutations in our study cohort, 52 had been analysed in more than 50 untreated adults
4 and were therefore evaluated by the three criteria of pathogenicity. Among these, 46 mutations
5 were currently assumed to be pathogenic, whereas six were already assumed to be non-
6 pathogenic and no longer used for genetic cascade screening (see Table 3). Of these 46, three
7 mutations did not meet any of the criteria: the *LDLR* variant c.108C>A (exon 2) and the *APOB*
8 mutations c.13154T>C and c.13181T>C (both in exon 29) and were therefore labelled as non-
9 pathogenic by our criteria (Table 4). Taken together, the 72 untreated carriers of these three
10 mutations had a mean LDL-C level of 3.3 ± 1.0 mmol/L (P52) compared to 3.3 ± 1.0 mmol/L
11 (P41) in untreated non-carriers ($p=0.88$). Adjustments for age and gender by means of
12 multivariate models did not essentially change the results. The average medication use was 14%
13 in the carriers and 10% in non-carriers ($p=0.29$).
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29 Table 5 summarises the outcomes of the proposed criteria in the groups with the
30 established pathogenic and non-pathogenic variants as well as the three variants which were
31 identified as non-pathogenic according to the criteria. The proposed criteria accurately identified
32 14 (93%) of the 15 established pathogenic and non-pathogenic mutations as such.
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41 ***Application of the criteria for all prevalent mutations***

42 Table 6 summarises the outcomes of all variants tested according to the proposed criteria and the
43 *in silico* results.
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51 ***Segregation analysis for newly identified putative non-pathogenic sequence variants***

52 Anonymous pedigree information could be retrieved for the two c.108C>A (*LDLR*) pedigrees,
53 six out of eight c.13154T>C (*APOB*) pedigrees and four out of six pedigrees with the
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3 c.13181T>C (*APOB*) variants. The c.108C>A and c.13154T>C families lacked power to obtain
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5 significant LOD scores, with ranges of LOD scores that could be detected of -0.55 to 1.21 and -
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7 0.68 to 1.62, respectively. Two point linkage analyses showed negative LOD scores of -0.041
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9 and -0.22 for the c.108C>A and c.13154T>C variants, respectively, indicating a trend towards no
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11 linkage. Only the c.13181T>C families showed sufficient power to detect significant LOD
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13 scores; range -3.59 to 7.17. Two-point parametric linkage analysis with Fastlink showed a LOD
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15 score of -3.25 for all c.13181T>C families combined. This negative LOD score was driven by
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17 the data of the largest of those four families, which in itself also yielded that LOD score of -3.25.
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19 This indicated that the c.13181T>C variant is definitely not linked to an autosomal dominant
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21 hypercholesterolemia phenotype.
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29 ***Prevalence of Newly Identified Non-Pathogenic Mutations***

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31 The cohort of those tested for one of the 46 relative prevalent mutations that were currently
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33 assumed to be pathogenic consisted of 14,030 subjects. Of these, 241 subjects had been tested for
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35 sequence variants that were newly identified to be non-pathogenic according to our criteria and
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37 the segregation analysis (1.7%).
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43 ***FH Diagnostic Scores of Probands***

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45 We were able to retrieve clinical data of 9 of the in total 16 probands with the identified non-
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47 pathogenic variants: c.108C>A (n=2), c.13154T>C (n=5) and 2 with c.13181T>C (n=2) and 30
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49 of the 36 pre-selected probands with one of the six most prevalent and established pathogenic
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51 mutations. The mean of clinical FH diagnostic scores of the probands with one of the identified
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53 non-pathogenic variant was 4.8 ± 1.2 points compared to a mean of 5.3 ± 2.6 points in the
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3 probands with a pathogenic mutation ($p=0.63$). Four of the nine probands with a newly identified
4 non-pathogenic mutation (44%) had a probable or definite clinical diagnosis (≥ 6 points) based on
5 the Dutch Lipid Network Criteria, whereas 47% of the probands with a prevalent pathogenic
6 mutation had a probable or definite clinical diagnosis ($p=0.91$). The mean pre-treatment LDL-C
7 levels were 6.0 ± 0.9 and 6.4 ± 1.3 mmol/L in probands with a newly identified non-pathogenic
8 and in those with an established pathogenic mutation, respectively ($p=0.37$).
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20 Discussion

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22 In this study we evaluated three criteria to assess pathogenicity of sequence variants in the LDLR
23 and APOB genes that were assumed to cause FH. Our criteria discriminated prevalent and
24 established pathogenic mutations from established non-pathogenic sequence variants. Of the
25 subsequent DNA variants that were evaluated, three emerged as non-pathogenic according to our
26 proposed criteria. Segregation analysis supported the conclusion to label these three variants as
27 non-pathogenic.
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37 We hypothesized that non-pathogenicity of mutations might explain in part the fact that
38 15 percent of mutation carriers have a pre-treatment LDL-C level below P75.[Huijgen et al.,
39 2009] However, the discovery of three non-pathogenic variants in our cohort clarifies only a
40 small proportion ($<2\%$) of the low LDL-C levels in mutation carriers. Other reasons for non-
41 penetrance may be related to healthy lifestyle or diet or to other mutations in the APOB and
42 PCSK9 genes that neutralize the effect of the FH mutation.[Fouchier et al., 2005b; Leren et al.,
43 2008; van der Graaf et al., 2008; Abifadel et al., 2009]
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53 The determination of the non-pathogenic nature of three sequence variants raises the
54 question whether selection of the probands was appropriately performed. The c.108C>A variant
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3 is an exonic mutation in *LDLR* that is predicted by SIFT to be a pathogenic mutation. The *APOB*
4 mutations are in exon 29 positions 4385 and 4394, which is the carboxyl terminus of APOB100.
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6 Mutations in this region, for example the c.[13187G>A;13188G>C] (also described as
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8 p.Trp4396Tyr), have been elegantly illustrated to affect normal LDLR binding by interaction
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10 between the arginine 3527 and the carboxyl tail and, thus, were – theoretically – proven to be
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12 pathogenic.[Boren et al., 2001] Furthermore, the clinical characteristics and mean LDL-C levels
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14 in the index patients with these three non-pathogenic sequence variants did not differ from
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16 probands with established mutations, as illustrated by the similar FH diagnostic scores. Thus
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18 after complete analysis of *LDLR* and *APOB* in these probands, only these three variants were
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20 identified and therefore initially thought to be pathogenic. Hence, the initiation of cascade
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22 screening seemed reasonable. However, now that it is evident that the c.108C>A, c.13154T>C
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24 and c.13181T>C variants are likely non-pathogenic, the presence of other mutations or
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26 secondary causes likely explain the FH phenotype in these individuals. All index patients with
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28 these sequence variants require further examination and reanalysis for the presence of other
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30 mutations.
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39 The first two criteria we proposed to test pathogenicity of a specific DNA variant were
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41 related to LDL-C levels of untreated individuals. The use of only those two criteria would
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43 presumably underestimate the severity of FH-causing gene variants since persons with high
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45 LDL-C levels are more likely to use medication, also without awareness of their genetic
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47 predisposition before screening. To take this effect into account, the criterion of medication use
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49 was introduced to avoid misinterpretation of the LDL-C data and the pathogenicity of a sequence
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51 variant. The consequence of using criteria based on LDL-C levels only and denying medication
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53 use is illustrated by data on deletion of exon 16 (*LDLR*) (c.2311+?_2390_?del) (Table 6). Out of
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3 the 27 carriers with this mutation only one was not treated (3.7%) by the time of genetic
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5 diagnosis: a 28 -year old male with a LDL-C level of 3.9 mmol/L (P84). The difference in LDL-
6
7 C levels between this one carrier and his 50 non-carrying untreated relatives, with a mean level
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9 of $(3.2 \pm 1.0 \text{ mmol/L})$, did not reach statistical significantly difference ($p=0.53$). This may have
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11 cast doubt about the pathogenicity of this mutation. However, if medication use was taken into
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13 account (96% in carriers vs. 9% in non-carriers), there is no longer any doubt about
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15 pathogenicity of the mutation. Therefore, medication use should be considered to assess
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17 pathogenicity. We realise, however, that the medication use is likely to depend on the
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19 organisation of the health care system in a country or region and should be interpreted with
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21 caution when used to evaluate large datasets in other countries.
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27 In general, when family data is used to assess the consequences of gene variants on a trait,
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29 several problems can be expected. This also may hold true for our approach. For example,
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31 several mutations were only identified in one family or a few small families. Co-segregation of a
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33 mutation with hypercholesterolemia may be considered as proof that the mutation causes FH.
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35 But in small groups of individuals this pattern may be present merely due to chance. An incorrect
36
37 classification of a sequence variant as non-pathogenic would have serious consequences for the
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39 quality of the screening programme. To avoid this, the minimum number of untreated subjects
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41 that was assessed for each mutation was set at 50.
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46 As a result of the requirement of a minimum number of untreated subjects, certain
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48 mutations suspected to be non-pathogenic were not evaluated. For these variants, assessment of
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50 pathogenicity will rely on the combination of in silico or in-vitro analysis and segregation
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52 analysis in small pedigrees. Alternatively, those variants could also be re-tested by means of our
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54 criteria once a sufficient number of patients or families with these variants have been identified.
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3 The evaluation of pathogenicity by our criteria was not always straightforward (Table 6)
4 and revealed some uncertain variants that met part of the criteria. For instance, the deletion of
5 promoter and exon 1 (c.1-?_c.67+? del), c.1057G>A and c.1898G>T (all in *LDLR*) did not meet
6 criterion 1 since subjects with these variants had LDL-C levels on average below P75. However,
7 these variants did meet criterion 2 since carriers had a significantly higher mean LDL-C level
8 than non-carriers. Carriers of the deletion of promoter and exon 1 also did meet criterion 3. The
9 deletion of exon 16 (*LDLR*) did only not meet criterion 2, and was discussed in an earlier
10 paragraph. Lastly, the c.1247G>A (*LDLR*) mutation only failed to meet criterion 3, i.e. carriers
11 were not more often treated than non-carriers at the time of molecular diagnosis. Based on the
12 data of all three criteria combined, we would argue to consider some of them (the deletion of
13 promoter and exon 1, c.1247G>A (*LDLR*) and the deletion of exon 16) likely to be pathogenic,
14 whereas other variants (c.1057G>A and c.1898G>T) seem to result in only modest LDL-C
15 elevations. In uncertain cases, segregation analysis might give additional clues on pathogenicity
16 of the mutation. Furthermore, mutations of which the issue of pathogenicity remains doubtful
17 should be followed critically when more carriers are identified with screening in order to
18 definitely characterize their functionality.
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41 A potential drawback of our approach could be the use of p-values (see criteria 2 and 3)
42 for the decision on pathogenicity of a mutation. For example, the p-value for criteria 2 is highly
43 dependent on the number of subjects in whom genetic screening has been performed but also on
44 the variation of the LDL-C levels in the groups. With large numbers and small variations, p-
45 values will become easily significant. For those mutations, the chance to be designated as a non-
46 pathogenic variant will be reduced. Conversely, in case of small numbers and large variation, a
47 mutation will become more suspicious to be non-pathogenic. We therefore believe that the first
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3 criterion, i.e. a mean age- and gender-specific percentile for LDL-C higher than P75, is
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5 particularly important for differentiation of pathogenicity for variants that were tested either in
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7 few or many subjects. Thus, although our criteria can readily be used to classify variants with
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9 respect to non-pathogenicity, common sense and clinical judgement remain crucial for the
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11 interpretation of the data.
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15 In conclusion, examination of pedigree data and medication use at the time of genetic
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17 testing of LDLR or APOB mutations can help to ascertain mutation pathogenicity. A need for
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19 such an additional method to characterize functionality exists, at least in the Netherlands, because
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21 segregation analysis lacked power for showing non-pathogenicity and also *in silico* analysis
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23 often yielded inconclusive results. The proposed criteria differentiated well between established
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25 pathogenic and non-pathogenic mutations and enabled us to identify three non-pathogenic
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27 variants that were previously assumed to cause FH. The prevalence of these newly discovered
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29 non-pathogenic sequence variants was low. Nevertheless, the fact these non-pathogenic variants
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31 were identified underline that novel sequence changes in *LDLR* and *APOB* should be interpreted
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33 with caution before being incorporated within the cascade screening program. We believe such
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35 uniform criteria could have future wider relevance, especially with regard to recently initiated
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37 genetic cascade screening programmes in other countries.
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Table 1. Nomenclature of mutations in *LDLR* and *APOB*

Gene	Location	Nucleotide change (cDNA)	Effect on protein	New name*	Old name**	References***
<i>LDLR</i>	P-exon 1	c.1-?_67-?del	-	-	13kb del	Novel
<i>LDLR</i>	exon 2	c.108C>A	p.Asp36Glu	D36E	D15E	Hum Genet
<i>LDLR</i>	exon 2	c.148G>T	p.Ala50Ser	A50S	A29S	Hum Genet
<i>LDLR</i>	exon 2	c.131G>A	p.Trp44X	W44X	W23X	Hum Genet
<i>LDLR</i>	intron 2	c.191-2A>G	-	-	191-2	Hum Genet
<i>LDLR</i>	exon 3	c.241C>A	p.Arg81Cys	R81C	R60C	Hum Genet
<i>LDLR</i>	intron 3	c.313+1G>A	-	-	313+1 or 313+1/2	Hum Genet
<i>LDLR</i>	intron 3	c.313+1G>C	-	-	313+1 or 313+1/2	Hum Genet
<i>LDLR</i>	intron 3	c.313+2T>C	-	-	313+2 or 313+1/2	Hum Genet
<i>LDLR</i>	intron 3	c.314-1G>A	-	-	314-1	Hum Genet
<i>LDLR</i>	exon 4	c.429C>A	p.Cys143X	C143X	C122X	Hum Genet
<i>LDLR</i>	exon 4	c.518delG	p.Cys173fsX205	-	518delG	Hum Genet
<i>LDLR</i>	exon 4	c.550T>C	p.Cys184Arg	C184R	C163R	Hum Genet
<i>LDLR</i>	exon 4	c.621C>T	p.Gly207Gly	G207G	G186G	Clin Genet
<i>LDLR</i>	exon 4	c.682G>A	p.Glu228Lys	E228K	E207K	Hum Genet
<i>LDLR</i>	exon 5	c.742T>G	p.Cys248Gly	C248G	C227G	Hum Genet
<i>LDLR</i>	exon 5	c.757C>T	p.Arg253Trp	R253W	R232W	Hum Genet
<i>LDLR</i>	exon 5	c.763T>C	p.Cys255Arg	C255R	C234R	Hum Genet
<i>LDLR</i>	exon 6	c.877delG	p.Asp293fsX348	-	877delG	Hum Genet
<i>LDLR</i>	exon 6	c.917C>T	p.Ser306Leu	S306L	S285L	Hum Genet
<i>LDLR</i>	exon 6	c.[932A>G;938G>A]	p.[Lys311Arg;Cys313Trp]	K311R/C313W	K290R/C292W	Hum Genet
<i>LDLR</i>	exon 7-8	c.940+?_1187-?del	-	-	2.5kb del (Cape Town-2)	Hum Genet
<i>LDLR</i>	exon 7	c.1004G>T	p.Gly335Val	G335V	G314V	Hum Genet
<i>LDLR</i>	exon 7	c.1027G>A	p.Gly343Ser	G343S	G332S	Hum Genet
<i>LDLR</i>	exon 7	c.1048C>T	p.Arg350X	R350X	R329X	Hum Genet
<i>LDLR</i>	exon 7	c.1057G>A	p.Glu353Lys	E353K	E332K	Hum Genet
<i>LDLR</i>	exon 8	c.1069G>A	p.Glu357Lys	E357K	E336K	Hum Genet
<i>LDLR</i>	exon 9-12	c.1186+?_1846-?dup	-	-	4.4kb dub (Leiden-3)	Hum Genet
<i>LDLR</i>	exon 9	c.1247G>A	p.Arg416Gln	R416Q	R395Q	Hum Genet
<i>LDLR</i>	exon 9	c.1265T>C	p.Leu422Pro	L422P	L401P	Hum Genet
<i>LDLR</i>	exon 9	c.1285G>A	p.Val429Met	V429M	V408M	Hum Genet
<i>LDLR</i>	exon 9	c.1291G>A	p.Ala431Thr	A431T	A410T	Hum Genet
<i>LDLR</i>	intron 9	c.1358+1G>A	-	-	1358+1	Hum Genet

1	<i>LDLR</i>	intron 9	c.1359-1G>A	-	-	1359-1	Hum Genet
2	<i>LDLR</i>	exon 10	c.1549T>C	p.Ser517Pro	S517P	S496P	Hum Genet
3	<i>LDLR</i>	exon 11+17	c.[1690A>C;2393_2401del]	p.[Asn564His;799_801del]	N564H/2393del19bp	N543H/2393del19bp	Hum Genet
4	<i>LDLR</i>	exon 12	c.1775G>A	p.Gly592Glu	G592E	G571E	Hum Genet
5	<i>LDLR</i>	exon 12	c.1729T>C	p.Ser587Arg	S587R	S566R	Hum Genet
6	<i>LDLR</i>	exon 12	c.1820A>G	p.His607Arg	H607R	H586R	novel
7	<i>LDLR</i>	exon 12	c.1835G>C	p.Leu611Phe	L611F	L590L	Hum Genet
8	<i>LDLR</i>	exon 12	c.1843G>A	p.Glu615Lys	E615K	E594K	Hum Genet
9	<i>LDLR</i>	exon 13	c.1898G>A	p.Arg633His	R633H	R612H	Hum Mutat
10	<i>LDLR</i>	exon 13	c.1898G>T	p.Arg633Leu	R633L	R612L	Hum Mutat
11	<i>LDLR</i>	exon 14	c.2054C>T	p.Pro685Leu	P685L	P664L	Hum Genet
12	<i>LDLR</i>	exon 14	c.2096C>T	p.Pro699Leu	P699L	P678L	Hum Genet
13	<i>LDLR</i>	exon 14	c.2113G>C	p.Ala705Pro	A705P	A684P	Hum Genet
14	<i>LDLR</i>	intron 14	c.2140+5G>A	-	-	2140+5	Hum Genet
15	<i>LDLR</i>	exon 15	c.2177C>T	p.Thr726Ile	T726I	T705I	Hum Genet
16	<i>LDLR</i>	exon 15	c.2231G>A	p.Arg744Gln	R744Q	R723Q	Hum Mutat
17	<i>LDLR</i>	exon 15	c.2282C>T	p.Thr761Met	T761M	T740M	Hum Mutat
18	<i>LDLR</i>	exon 16	c.2311+?_2390-?del	-	-	0.4kb del (Leiden-2)	Hum Genet
19	<i>LDLR</i>	intron 16	c.2390-2A>G	-	-	2390-2	Hum Genet
20	<i>LDLR</i>	exon 17	c.2479G>A	p.Val827Ile	V827I	V806I	Hum Genet
21	<i>APOB</i>	exon 26	c.10580G>A	p.Arg3527Gln	R3527Q	R3500Q	Hum Genet
22	<i>APOB</i>	exon 29	c.13130T>C	p.Ile4377Thr	I4377T	I4350T	novel
23	<i>APOB</i>	exon 29	c.13154T>C	p.Arg4385His	R4385H	R4358H	novel
24	<i>APOB</i>	exon 29	c.13181T>C	p.Val4394Ala	V4394A	V4367A	novel

Numbering of the nucleotides of the *LDLR* and *APOB* genes was based on the cDNA, with +1 being the A of the ATG translation initiation codon. *New name represent numbering of the codons with the initiation codon is 1. **Old name represent numbering of the codons with initiation codon is -21 for *LDLR* and -27 for *APOB*. Reference sequence *LDLR*: GenBank Accession NM_000527.3. Reference sequence *APOB*: GenBank Accession NM_000384.2. *** References of the mutations identified in the Netherlands: Human Genetics 2001;109:602-615, Clinical Genetics 2008;73:573-578, Human Mutation 2005;26:550-556.

Table 2. The six most prevalent known pathogenic mutations in the Netherlands: low-density lipoprotein cholesterol levels and percentage medication use in mutation carriers and non-carriers

Mutations	Gene	Subjects without cholesterol-lowering medication							All subjects				N of families
		FH+			FH-			FH+ vs FH-		FH+	FH-	FH+ vs FH-	
		N	LDL-C*	pLDL	N	LDL-C*	pLDL	$p^{\#}$	p^{\ddagger}	%Med	%Med	p	
c.131G>A	<i>LDLR</i>	60	5.8 ± 1.7	91	289	3.2 ± 0.9	42	<0.001	<0.001	62	7	<0.001	89
c.313+1/2**	<i>LDLR</i>	110	5.7 ± 1.4	93	661	3.2 ± 0.9	41	<0.001	<0.001	61	9	<0.001	127
c.1027G>A	<i>LDLR</i>	84	4.0 ± 0.9	76	306	3.3 ± 0.9	44	<0.001	<0.001	37	10	<0.001	34
c.1359-1G>A	<i>LDLR</i>	67	5.6 ± 1.8	90	646	3.1 ± 0.9	40	<0.001	<0.001	75	11	<0.001	129
c.[1690A>C;2393_2401del]	<i>LDLR</i>	397	4.6 ± 1.2	85	1327	3.2 ± 0.9	43	<0.001	<0.001	43	13	<0.001	259
c.10580G>A	<i>APOB</i>	487	4.6 ± 1.1	85	1266	3.3 ± 1.0	45	<0.001	<0.001	38	11	<0.001	251
Total	-	1211	4.8 ± 1.4	85	4506	3.2 ± 0.9	43	<0.001	<0.001	48	11	<0.001	889

*LDL-C levels (mmol/L) are expressed as mean ± standard deviation. [#]p-values based on univariate comparison of LDL-C levels between untreated carriers and non-carriers. [‡]p-values based on comparison of LDL-C levels between untreated carriers and non-carriers, adjusted for age and gender.

** Because a single PCR detects three variants (c.313+1G>A; c.313+1G>C; c.313+2T>C) without discriminating between the different variants, these are depicted here as one group. *APOB*, apolipoprotein B gene; FH, familial hypercholesterolemia; FH+, FH carriers; FH-, non-carriers who in principle were 1st degree relatives of carriers; LDL-C, low-density lipoprotein cholesterol; *LDLR*, LDL-receptor gene; N, number; pLDL, mean percentile for age and gender; p , p-value; %Med, percentage cholesterol-lowering medication users.

Table 3. The known non-pathogenic mutations in the Netherlands: low-density lipoprotein cholesterol levels and percentage medication use in mutation carriers and non-carriers

Mutations	Gene	Subjects without cholesterol-lowering medication								All subjects			N of families
		FH+			FH-			FH+ vs FH-		FH+	FH-	FH+ vs FH-	
		N	LDL-C*	pLDL	N	LDL-C*	pLDL	$p^{\#}$	p^{\ddagger}	%Med	%Med	p	
c.148G>T ^a	<i>LDLR</i>	2	2.6 ± 0.9	22	3	3.1 ± 1.2	41	0.62	0.79	0	0	n.p.	8
c.757C>T ^a	<i>LDLR</i>	20	3.5 ± 0.9	53	43	3.3 ± 0.9	45	0.45	0.43	17	15	0.86	17
c.1729T>C ^a	<i>LDLR</i>	26	3.3 ± 1.0	52	30	3.1 ± 1.0	37	0.53	0.06	21	27	0.58	3
c.2140+5G>A ^a	<i>LDLR</i>	6	3.1 ± 0.6	48	52	3.0 ± 0.8	34	0.87	0.42	40	12	0.042	8
c.2177C>T ^{a+b}	<i>LDLR</i>	35	3.2 ± 1.1	47	61	3.1 ± 0.9	42	0.67	0.54	5	15	0.13	11
c.2231G>A ^a	<i>LDLR</i>	39	3.5 ± 0.9	53	111	3.5 ± 1.0	50	0.95	0.89	9	13	0.44	12
c.2282C>T ^a	<i>LDLR</i>	9	4.0 ± 0.6	69	8	3.2 ± 0.9	45	0.06	0.81	10	0	0.38	2
c.2479G>A ^a	<i>LDLR</i>	27	3.6 ± 1.1	61	56	3.3 ± 0.8	44	0.21	0.02	7	7	0.97	11
c.13130T>C ^a	<i>APOB</i>	7	3.1 ± 0.5	50	6	2.7 ± 0.8	38	0.26	0.40	0	14	0.30	1
Total	-	171	3.4 ± 1.0	53	370	3.3 ± 0.9	43	0.12	0.01	12	14	0.61	81

*LDL-C levels (mmol/L) are expressed as mean ± standard deviation. [#]p-values based on univariate comparison of LDL-C levels between untreated carriers and non-carriers. [‡]p-values based on comparison of LDL-C levels between untreated carriers and non-carriers, adjusted for age and gender. ^aNon-pathogenicity of sequence variant made public online on Dutch FH-website: www.jojogenetics.nl. ^bEvidence for the non-pathogenic nature of c.2177C>T was published separately as well: Human Genetics 1997;99:106-107. *APOB*, apolipoprotein B gene; FH, familial hypercholesterolemia; FH+, FH carriers; FH-, non-carriers who in principle were 1st degree relatives of carriers; LDL-C, low-density lipoprotein cholesterol; *LDLR*, LDL-receptor gene; N, number; n.p., not performed (in both groups none of the individuals used medication); pLDL, mean percentile for age and gender; p , p-value; %Med, percentage cholesterol-lowering medication users.

Table 4. Characteristics of the new discovered non-pathogenic mutations in the Netherlands, and lipid levels and medication use in mutation carriers versus non-carriers

Mutations	Gene	Subjects without cholesterol-lowering medication								All subjects			N of families
		FH+			FH-			FH+ vs FH-		FH+	FH-	FH+ vs FH-	
		N	LDL-C*	pLDL	N	LDL-C*	pLDL	$p^{\#}$	p^{\ddagger}	%Med	%Med	p	
c.108C>A	<i>LDLR</i>	23	3.1 ± 0.9	45	43	3.2 ± 1.0	41	0.77	0.64	4	10	0.37	2
c.13154T>C	<i>APOB</i>	32	3.2 ± 0.8	52	58	3.3 ± 1.0	37	0.90	0.50	18	7	0.08	8
c.13181T>C	<i>APOB</i>	17	3.8 ± 1.1	63	40	3.5 ± 0.8	47	0.27	0.20	19	13	0.52	6
Total	-	72	3.3 ± 1.0	52	141	3.3 ± 1.0	41	0.88	0.31	14	10	0.29	16

*LDL-C levels (mmol/L) are expressed as mean ± standard deviation. $^{\#}$ p-values based on univariate comparison of LDL-C levels between untreated carriers and non-carriers. ‡ p-values based on comparison of LDL-C levels between untreated carriers and non-carriers, adjusted for age and gender. *APOB*, apolipoprotein B gene; FH, familial hypercholesterolemia; FH+, FH carriers; FH-, non-carriers who in principle were 1st degree relatives of carriers; LDL-C, low-density lipoprotein-cholesterol; *LDLR*, LDL-receptor gene; N, number; pLDL, mean percentile for age and gender; p , p-value; %Med, percentage cholesterol-lowering medication users.

Table 5. Application of the proposed criteria for three mutation groups with more than 50 untreated adults (i.e. most prevalent, known non-pathogenic, new non-pathogenic)

	<i>Criterion 1</i>	<i>Criterion 2</i>	<i>Criterion 3</i>
	Untreated adults FH+ LDL-C>P75?	Untreated adults FH+ vs. FH- LDL-C higher?	All adults tested FH+ vs. FH- Medication use higher?
Most prevalent pathogenic			
c.131G>A	yes	yes	yes
c.313+1/2*	yes	yes	yes
c.1027G>A	yes	yes	yes
c.1359-1G>A	yes	yes	yes
c.[1690A>C;2393_2401del]	yes	yes	yes
c.10580G>A	yes	yes	yes
Known non-pathogenic			
c.757C>T	no	no	no
c.2140+5G>A	no	no	yes
c.2177C>T	no	no	no
c.2231G>A	no	no	no
c.2479G>A	no	no	no
New non-pathogenic			
c.108C>A	no	no	no
c.13154T>C	no	no	no
c.13181T>C	no	no	no

*Group of three variants: c.313+1G>A; c.313+1G>C; c.313+2T>C. FH, familial hypercholesterolemia; FH+, FH carriers; FH-, non-carriers who in principle were 1st degree relatives of carriers; LDL-C, low-density lipoprotein-cholesterol; P75, 75th percentile for age and gender.

Table 6. The 52 mutations with data on untreated low-density lipoprotein cholesterol levels of > 50 untreated subjects

Mutation	Gene	<i>In silico</i> analysis		<i>In vivo</i> observation								
		SIFT	PolyPhen	Subjects without cholesterol lowering medication						All subjects		
				N	LDL-C	pLDL	LDL-C	pLDL	+ vs. - <i>p</i> [#]	FH+ %Med	FH- %Med	+ vs. - <i>p</i>
c.10580G>A	<i>APOB</i>	not tol.	possibly	1753	4.6 ± 1.1	85	3.3 ± 1.0	45	<0.001	38	11	<0.001
c.[1690A>C;2393_2401del]	<i>LDLR</i>	not tol./-	probably/-	1724	4.6 ± 1.2	85	3.2 ± 0.9	43	<0.001	43	13	<0.001
c.313+1/2 ^{**}	<i>LDLR</i>	-	-	771	5.7 ± 1.4	93	3.2 ± 0.9	41	<0.001	61	9	<0.001
c.1359-1G>A	<i>LDLR</i>	-	-	713	5.6 ± 1.8	90	3.1 ± 0.9	40	<0.001	75	11	<0.001
c.1027G>A	<i>LDLR</i>	not tol.	probably	390	4.0 ± 0.9	76	3.3 ± 0.9	44	<0.001	37	10	<0.001
c.131G>A	<i>LDLR</i>	-	-	349	5.8 ± 1.7	91	3.2 ± 0.9	42	<0.001	62	7	<0.001
c.682G>A	<i>LDLR</i>	not tol.	probably	318	5.6 ± 1.6	95	3.1 ± 0.9	40	<0.001	75	10	<0.001
c.917C>T	<i>LDLR</i>	not tol.	probably	312	5.0 ± 1.4	89	3.1 ± 1.0	42	<0.001	42	11	<0.001
c.191-2A>G	<i>LDLR</i>	-	-	306	5.4 ± 1.6	92	3.3 ± 0.9	42	<0.001	68	10	<0.001
c.241C>A	<i>LDLR</i>	not tol.	benign	250	4.4 ± 1.2	78	3.0 ± 0.8	34	<0.001	31	9	<0.001
c.621C>T	<i>LDLR</i>	-	-	219	4.9 ± 1.2	89	3.0 ± 0.8	36	<0.001	51	14	<0.001
c.2113G>C	<i>LDLR</i>	not tol.	benign	204	5.0 ± 1.4	91	3.3 ± 1.0	45	<0.001	48	5	<0.001
c.1291G>A	<i>LDLR</i>	not tol.	benign	185	5.0 ± 1.2	89	3.2 ± 0.9	40	<0.001	46	14	<0.001
c.1835G>C	<i>LDLR</i>	not tol.	possibly	173	5.6 ± 1.8	92	3.3 ± 1.0	47	<0.001	82	7	<0.001
c.940-?_c.1187+? del	<i>LDLR</i>	-	-	162	5.3 ± 1.4	89	3.1 ± 0.8	40	<0.001	77	6	<0.001
c.2231G>A ^{tab3}	<i>LDLR</i>	tolerated	benign	150	3.5 ± 0.9	53	3.5 ± 1.0	50	0.95	9	13	0.44
c.763T>C	<i>LDLR</i>	not tol.	probably	141	4.4 ± 1.4	83	3.4 ± 1.1	46	<0.001	50	12	<0.001
c.1775G>A	<i>LDLR</i>	not tol.	probably	115	4.6 ± 1.0	88	3.2 ± 0.9	42	<0.001	44	5	<0.001
c.2096C>T	<i>LDLR</i>	not tol.	probably	111	4.7 ± 0.9	91	3.4 ± 1.1	48	<0.001	43	14	0.001
c.2177C>T ^{tab3}	<i>LDLR</i>	not tol.	probably	96	3.2 ± 1.1	47	3.1 ± 0.9	42	0.67	5	15	0.13
c.1898G>A	<i>LDLR</i>	not tol.	probably	95	4.3 ± 1.5	75	3.2 ± 1.0	38	<0.001	35	8	<0.001
c.1549T>C	<i>LDLR</i>	not tol.	possibly	95	4.2 ± 1.2	75	3.0 ± 0.9	34	<0.001	34	9	0.002
c.1285G>A	<i>LDLR</i>	not tol.	possibly	93	5.9 ± 2.0	88	3.3 ± 0.9	43	<0.001	61	17	<0.001
c.13154T>C ^{tab4}	<i>APOB</i>	tolerated	possibly	90	3.2 ± 0.8	52	3.3 ± 1.0	37	0.90	18	7	0.08
c.1048C>T	<i>LDLR</i>	-	-	86	5.4 ± 0.9	97	3.0 ± 1.0	37	<0.001	70	9	<0.001
c.2054C>T	<i>LDLR</i>	not tol.	probably	81	5.2 ± 0.8	95	3.3 ± 0.8	45	<0.001	68	17	<0.001
c.2390-2A>G	<i>LDLR</i>	-	-	80	5.3 ± 1.4	88	3.4 ± 1.1	47	<0.001	54	12	<0.001
c.1069G>A	<i>LDLR</i>	not tol.	probably	80	6.1 ± 2.1	94	3.4 ± 0.8	52	<0.001	74	11	<0.001
c.1247G>A	<i>LDLR</i>	not tol.	benign	80	4.2 ± 0.9	84	3.0 ± 0.9	40	<0.001	21	18	0.80
c.1265T>C	<i>LDLR</i>	not tol.	benign	77	4.3 ± 1.0	79	3.1 ± 0.8	43	<0.001	54	18	<0.001

1	c.429C>A	<i>LDLR</i>	-	-	75	5.8 ± 1.5	97	3.2 ± 1.0	42	<0.001	74	17	<0.001
2	c.1004G>T	<i>LDLR</i>	not tol.	probably	73	5.5 ± 1.4	92	3.1 ± 1.0	41	<0.001	56	8	<0.001
3	c.2479G>A ^{tab3}	<i>LDLR</i>	not tol.	probably	73	3.6 ± 1.1	61	3.3 ± 0.8	44	0.21	7	7	0.97
4	c.[932A>G;938G>A]	<i>LDLR</i>	not tol.;	benign;	70	6.3 ± 0.3	97	3.1 ± 0.7	37	<0.001	67	7	0.001
5			not tol.	probably									
6													
7	c.314-1G>A	<i>LDLR</i>	-	-	69	5.0 ± 1.9	75	2.9 ± 0.9	31	<0.001	61	8	<0.001
8	c.108C>A ^{tab4}	<i>LDLR</i>	not tol.	benign	66	3.1 ± 0.9	45	3.2 ± 1.0	41	0.77	4	10	0.37
9	c.757C>T ^{tab3}	<i>LDLR</i>	not tol.	benign	63	3.5 ± 0.9	53	3.3 ± 0.9	45	0.45	17	15	0.86
10	c.742T>G	<i>LDLR</i>	not tol.	probably	60	5.2 ± 1.3	91	3.3 ± 0.9	51	<0.001	52	0	<0.001
11	c.108C>A	<i>LDLR</i>	-	-	58	6.2 ± 1.3	95	3.1 ± 1.0	35	<0.001	69	18	<0.001
12													
13	c.2140+5G>A ^{tab3}	<i>LDLR</i>	-	-	58	3.1 ± 0.6	48	3.0 ± 0.8	33	0.87	40	12	0.042
14	c.13181T>C ^{tab4}	<i>APOB</i>	not tol.	benign	57	3.8 ± 1.1	63	3.5 ± 0.8	47	0.27	19	13	0.52
15	c.1-?_c.67+?del	<i>LDLR</i>	-	-	57	4.6 ± 1.7	74	3.3 ± 0.9	44	0.023	73	16	<0.001
16	c.1729T>C ^{tab3}	<i>LDLR</i>	not tol.	benign	56	3.3 ± 1.0	52	3.1 ± 1.0	36	0.53	21	27	0.58
17	c.1820A>G	<i>LDLR</i>	not tol.	probably	56	4.4 ± 0.7	83	3.2 ± 0.8	42	0.002	62	18	0.001
18	c.1843G>A	<i>LDLR</i>	not tol.	probably	55	4.8 ± 1.0	95	3.2 ± 1.1	46	<0.001	59	14	0.001
19	c.1057G>A	<i>LDLR</i>	not tol.	benign	54	3.8 ± 1.1	62	3.1 ± 0.9	38	0.029	25	15	0.49
20	c.1898G>T	<i>LDLR</i>	not tol.	probably	54	3.9 ± 1.3	68	2.9 ± 0.6	40	<0.001	38	22	0.19
21	c.550T>C	<i>LDLR</i>	not tol.	probably	53	6.6 ± 1.3	97	2.9 ± 0.4	34	<0.001	33	7	<0.001
22	c.1187-?_c.1845+? dup	<i>LDLR</i>	-	-	53	6.7 ± 1.9	97	3.1 ± 0.8	42	<0.001	72	16	<0.001
23	c.1358+1G>A	<i>LDLR</i>	-	-	51	5.1 ± 1.2	88	3.3 ± 1.0	45	<0.001	82	16	<0.001
24	c.2311-?_c.2390+?del	<i>LDLR</i>	-	-	51	3.9 ⁺	86	3.2 ± 1.0	47	0.53	96	9	<0.001
25	c.877delG	<i>LDLR</i>	-	-	51	6.5 ± 1.9	95	3.2 ± 0.6	34	<0.001	65	6	<0.001

The mutations are listed descending according to prevalence in The Netherlands. *LDL-C levels (mmol/L) are expressed as mean ± standard deviation. #*p*-values based on univariate comparison of LDL-C levels between untreated carriers and non-carriers. +Based on one carrier therefore no standard deviation. ** Group of three variants: c.313+1G>A; c.313+1G>C; c.313+2T>C. ^{tab3/4} (Putative) Non-pathogenic variant also described in Table 3 or 4. The *in-silico* analyses could only be performed for mutations that result in an amino-acid substitution and not for stop codon mutations (e.g. c.131G>A) and splicing mutations (e.g. c.1359-1G>A and c.621C>T). Abbreviations for SIFT: not tol., not tolerated. For PolyPhen: possibly, possibly damaging; probably, probably damaging. Other abbreviations: *APOB*, apolipoprotein B gene; FH, familial hypercholesterolemia; FH+, FH carriers; FH-, non-carriers who in principle were 1st degree relatives of carriers; LDL-C, low-density lipoprotein cholesterol; *LDLR*, LDL-receptor gene; N, number; *p*, *p*-value; pLDL, mean percentile for age and gender; %Med, percentage cholesterol-lowering medication users.