Cardiovascular risk in relation to functionality of sequence variants in the gene coding for the low-density lipoprotein receptor: a study among 29 365 individuals tested for 64 specific low-density lipoprotein-receptor sequence variants

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Aims

A plethora of mutations in the LDL-receptor gene (LDLR) underlie the clinical phenotype of familial hypercholesterolaemia (FH). For the diagnosis of FH, it is important, however, to discriminate between pathogenic and non-pathogenic mutations. The aim of the current study was to assess whether true pathogenic mutations were indeed associated with the occurrence of coronary artery disease (CAD) when compared with non-functional variants. The latter variants should not exhibit such an association with CAD.

Methods and results

We assessed 29 365 individuals tested the 64 most prevalent LDLR variants. First, we determined pathogenicity for each of these sequence variants. Subsequently, a Cox-proportional hazard model was used to compare event-free survival, defined as the period from birth until the first CAD event, between carriers and non-carriers of LDLR mutations. Fifty-four sequence variants in the LDLR gene were labelled as pathogenic and 10 as non-pathogenic. The 9 912 carriers of a pathogenic LDLR mutation had a shorter event-free survival than the 18 393 relatives who did not carry that mutation; hazard ratio 3.64 [95% confidence interval (CI): 3.24–4.08; P < 0.001]. In contrast, the 355 carriers of a non-pathogenic LDLR variant had similar event-free survival as the 705 non-carrying relatives; hazard ratio 1.00 (95% CI: 0.52–1.94; P = 0.999).

Conclusion

These findings with respect to clinical outcomes substantiate our criteria for functionality of LDLR sequence variants. They also confirm the CAD risk associated with FH and underline that these criteria can be used to decide whether a specific sequence variant should be used in cascade screening.

Keywords

Familial hypercholesterolemia • Screening • Coronary artery disease • Arteriosclerosis • Functionality

Introduction

Familial hypercholesterolaemia (FH) is a frequent autosomal co-dominant disorder of lipoprotein metabolism, with 1 in 500 persons affected with heterozygous FH in most western countries.1 Patients with FH have elevated plasma low-density lipoprotein cholesterol (LDL-C) levels and an increased risk of premature coronary artery disease (CAD).1,2 Defects in genes that code for proteins involved in hepatic clearance of LDL cholesterol (LDL-C) underlie this hereditary disorder.1 In fact, more than a 100 different mutations in the genes coding for the LDL-receptor (LDLR), apolipoprotein B (APOB), and proprotein...
convertase subtilisin/kexin type 9 (PCSK9) are now known to cause FH.3

The identification of a mutation that underlies FH in a particular family enables genetic testing of family members for the presence of the same mutation and makes it possible to initiate effective medical management before the cardiovascular consequences of FH become clinically manifest. This notion has led to the implementation of a nationwide genetic cascade screening program for FH in the Netherlands and ∼20,000 subjects with FH have been found and treated since 1994.4

However, as widely appreciated, not every sequence variant in LDLR, APOB, or PCSK9 results in an FH phenotype. If a novel sequence variant in one of those genes is identified in a patient with a clear clinical FH diagnosis, one has to rely on in silico or in vitro studies to ascertain pathogenicity. However, in vitro studies are cumbersome and therefore rarely performed. Also, the in silico results do not always correspond with the clinical observations.5 An alternative strategy is to perform cascade screening for such a novel variant and to identify new carriers of that specific variant to determine whether these carriers express an FH phenotype. Accordingly, we recently validated specific criteria that can discriminate pathogenic from non-pathogenic mutations, using lipoprotein and lipid levels as well as the use of lipid-lowering medication.5 Since then, collected a much larger cohort of carriers of non-pathogenic variants in order to assess possible consequences of these variants on the occurrence of cardiovascular disease.

In the present study, we compared cardiovascular risk between individuals with established pathogenic FH mutations and those with sequence variants in LDLR that we consider non-pathogenic. Here, we present our results.

Methods
In this observational study, we used a stepwise approach. First, we applied our criteria to assess functionality of all variants we have identified in LDLR in order to expand the number of individuals with pathogenic and non-pathogenic variants. Secondly, we compared CAD risk between carriers and non-carriers of all non-pathogenic variants and, similarly, of all pathogenic mutations.

Study population
The functionality criteria and event-free survival were assessed in individuals who were screened by genetic testing in the period between January 1994 and December 2010. Of these subjects, lipoprotein profiles, an extensive history of cardiovascular disease, medication use, and specific carrier status were collected at the time of molecular diagnosis. We excluded index patients from the current analysis to avoid clinical sampling bias. The cascade screening programme was launched by the Ministry of Health of the Dutch Government and approved by the National Ethics Committee. All participants gave written informed consent for genetic analysis.

Criteria to test functionality
The three criteria to establish functionality of a specific DNA variant have been described before5 and were: (i) a mean LDL-C level above the 75th percentile for age and gender in untreated individuals carrying such a mutation, (ii) statistically significantly higher mean LDL-C levels in untreated carriers compared with untreated non-carriers, and (iii) a statistically significant higher percentage of medication users among carriers compared with non-carriers at the time of molecular screening. We considered a mutation to be non-pathogenic when none of the three criteria was met.

All genetic variants had to be tested in at least 50 untreated subjects per variant. Additionally, the in silico analyses were applied to all prevalent variants by using the Alamut software (version 2, Interactive Bio-software, Rouen, France: SIFT and PolyPhen prediction). Mutations were described according to the nomenclature as proposed by den Dunnen and Antonarakis,6 and we used the description based on the effect of the mutation on the protein.

Coronary artery disease
Coronary artery disease was defined as a history of one of the following non-fatal cardiac outcomes: (i) sudden cardiac arrest; (ii) myocardial infarction; (iii) coronary artery bypass graft, or (iv) percutaneous transluminal coronary angioplasty. Coronary artery disease event-free survival was defined as the period from year of birth until the year of the first CAD event or censoring at the moment of genetic screening.

Statistical analysis
Differences in LDL-C levels between subgroups were compared by means of the unpaired Student t-test. Proportions of subjects using lipid-lowering medication were compared with the Chi-square test. We compared survival of mutation carriers with that of family members without mutations using the Kaplan–Meier survival analysis. A Cox-proportional hazard model was used to compare CAD risk between the carriers and non-carriers. The non-carriers served as the reference group. We adjusted for gender, hypertension, diabetes, smoking, and body mass index. The Cox-proportional hazard analyses were repeated in R, taking also family ties into account. Sensitivity and specificity were calculated for the variants for which in silico analysis could be performed, using the described criteria as a gold standard for pathogenicity.5 A two-sided P-value < 0.05 was considered statistically significant. Data were analysed with SPSS for Windows 16.0.2 (Chicago, IL, USA).

Results
Test of functionality of new sequence variants
In our study cohort, we extended previous findings with 12 sequence variants that had been tested in more than 50 untreated subjects just by 2010.3 All these variants reside in the LDLR and are presented in Table 1.3,7–11 Three variants involve exonic deletions, whereas the other mutations result in amino acid substitutions.

Table 2 summarizes the in silico analysis and the assessment according to the criteria for all 12 variants. As observed, 11 mutations are pathogenic, whereas G701S (exon 14) is not pathogenic.

Risk of coronary artery disease based on functionality
All tested mutations combined amounted to a total of 64 sequence variants in the LDLR gene that could be labelled as either pathogenic or non-pathogenic.5 The characteristics of these variants including the in silico findings are shown in Supplementary material online, Table S1. The total cohort consisted of 29,365 individuals, who
Cardiovascular risk in relation to functionality of sequence variants

Table 1  Nomenclature of mutations in LDLR

<table>
<thead>
<tr>
<th>Location</th>
<th>Nucleotide change</th>
<th>New nomenclature*</th>
<th>Old nomenclatureb</th>
<th>Referencec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>c.1A&gt;G</td>
<td>p.Met1Val</td>
<td>M-21V</td>
<td>1,3</td>
</tr>
<tr>
<td>Exon 3</td>
<td>c.268G&gt;A</td>
<td>p.Asp90Asn</td>
<td>D69N</td>
<td>2</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.622G&gt;A</td>
<td>p.Glu208Lys</td>
<td>E187K</td>
<td>1</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.662A&gt;C</td>
<td>p.Asp221Gly</td>
<td>D200G</td>
<td>1</td>
</tr>
<tr>
<td>Exon 6</td>
<td>c.858C&gt;A</td>
<td>p.Ser286Arg</td>
<td>S265R</td>
<td>1</td>
</tr>
<tr>
<td>Exon 9</td>
<td>c.1243G&gt;C</td>
<td>p.Asp415His</td>
<td>D394H</td>
<td>1</td>
</tr>
<tr>
<td>Exon 9</td>
<td>c.1297G&gt;T</td>
<td>p.Asp433Tyr</td>
<td>D412Y</td>
<td>1</td>
</tr>
<tr>
<td>Exons 12–18</td>
<td>c.1705+?_2580+1del</td>
<td>Deletion exons 12–18</td>
<td>16 kb deletion of exons 12–18 (Catania-1)</td>
<td>2,4</td>
</tr>
<tr>
<td>Exon 16</td>
<td>c.2311+?_2390+1del</td>
<td>Deletion exon 16</td>
<td>2.0 kb deletion exon 16 (Padova-2)</td>
<td>2,5</td>
</tr>
<tr>
<td>Exons 16–18</td>
<td>c.2311+?_2580+1del</td>
<td>Deletion exons 16–18</td>
<td>8.0 kb deletion exon 16–18</td>
<td>2</td>
</tr>
</tbody>
</table>

Numbering of the nucleotides of the LDLR gene was based on the cDNA, with +1 being the A of the ATG translation initiation codon.

*aNew name represents numbering of the codons with the initiation codon is 1.
*bOld name represents numbering of the codons with initiation codon is –21 for LDLR. Reference sequence LDLR: GenBank Accession NM_000527.3.
*cReferences of the mutations identified in The Netherlands: (1) Fouchier et al.7 (2) Fouchier et al.7 (3) Lombardi et al.12 (4) Bertolini et al.3 (5) Bertolini et al.11

Table 2  Mutations with findings of in silico results and of the application of criteria to assess pathogenicity

<table>
<thead>
<tr>
<th>In silico analysis</th>
<th>In vivo observation</th>
<th>Without cholesterol lowering medication</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All</td>
<td>FH+</td>
</tr>
<tr>
<td>Sequence variant</td>
<td>SIFT</td>
<td>PolyPhen</td>
<td>N</td>
</tr>
<tr>
<td>M1IV</td>
<td>Not tol.</td>
<td>Probably</td>
<td>50</td>
</tr>
<tr>
<td>D90N</td>
<td>Not tol.</td>
<td>Probably</td>
<td>51</td>
</tr>
<tr>
<td>E208K</td>
<td>Tolerated</td>
<td>Benign</td>
<td>50</td>
</tr>
<tr>
<td>L221G</td>
<td>Not tol.</td>
<td>Probably</td>
<td>72</td>
</tr>
<tr>
<td>S286R</td>
<td>Not tol.</td>
<td>Probably</td>
<td>84</td>
</tr>
<tr>
<td>D415H</td>
<td>Not tol.</td>
<td>Benign</td>
<td>50</td>
</tr>
<tr>
<td>D433Y</td>
<td>Not tol.</td>
<td>Probably</td>
<td>50</td>
</tr>
<tr>
<td>W443C</td>
<td>Not tol.</td>
<td>Probably</td>
<td>90</td>
</tr>
<tr>
<td>Deletion exons 12–18</td>
<td>—</td>
<td>—</td>
<td>60</td>
</tr>
<tr>
<td>G701S</td>
<td>Not tol.</td>
<td>Possibly</td>
<td>50</td>
</tr>
<tr>
<td>Deletion exon 16</td>
<td>—</td>
<td>—</td>
<td>80</td>
</tr>
<tr>
<td>Deletion exons 16–18</td>
<td>—</td>
<td>—</td>
<td>87</td>
</tr>
</tbody>
</table>

The in silico analyses could only be performed for mutations that result in an amino acid substitution and not for deletions. Abbreviations for SIFT: not tol., not tolerated. For PolyPhen: possibly, possibly damaging; probably, probably damaging. Other abbreviations: FH+, LDLR mutation carriers; FH–, non-carriers of an LDLR variant; LDL-C, low-density lipoprotein cholesterol; pLDL, mean percentile for age and gender; %Med, percentage cholesterol-lowering medication users.

*aLDL-C levels (mmol/L) are expressed as mean ± standard deviation.
*bP-values based on comparison of LDL-C levels between untreated carriers and non-carriers.

were tested for the presence of one particular LDLR mutation (Table 3). This constitutes 83.3% of the overall population tested in the cascade screening programme. Of these 29 365, 10 60 (3.6%) individuals were tested for 1 of the 10 non-pathogenic sequence variants, of whom 355 subjects carried that variant.

As can be expected, carriers of pathogenic mutations were more often treated with cholesterol lowering medication compared with carriers of non-pathogenic variants in LDLR [4193/ 9912 (42%) vs. 38/355 (11%); P < 0.001]. Also, the mean levels (± SD) of the highest total cholesterol levels measured over lifetime were significantly higher in the carriers of a pathogenic mutation than in those identified with a non-pathogenic variants (9.5 ± 3.1 vs. 6.2 ± 4.3 mmol/L; P < 0.001).

Figures 1 and 2 provide Kaplan–Meier curves for CAD event-free survival for individuals tested for pathogenic and non-pathogenic LDLR variants. The 9912 carriers of a pathogenic
LDLR mutation had on average a shorter event-free survival than the 18,393 relatives who did not carry that mutation. The hazard ratio associated with carriership was 3.64 [95% confidence interval (CI): 3.24–4.08; \( P \), 0.001] (Figure 1). In contrast, the Kaplan–Meier curves overlapped between carriers of the non-pathogenic sequence variants and the relatives that did not carry the variants (Figure 2). In particular, the risk of CAD was similar for both carriers and their unaffected relatives: the hazard ratio was exactly 1.00 (95% CI: 0.52–1.94; \( P = 0.999 \)). The analyses described above were also performed with R statistics—taking family ties into account—and these analyses yielded results that were in essence similar (data not shown).

The in silico analyses could be performed for 34 pathogenic mutations and 9 non-pathogenic variants according to the criteria, as shown in Supplementary material online, Table S1. Of these 43 variants, 33 were amino acid substitutions that were not tolerated according to SIFT, indicating a non-pathogenic nature. The associated specificity was 0.22 (2 out of 9) and the sensitivity was 0.97 (33 out of 34). Polyphen identified 13 variants as benign, i.e. non-pathogenic. Consequently, the specificity was 0.44 (4 out of 9) and the sensitivity 0.74 (25 out of 34).

**Discussion**

In this study, we first applied a set of specific criteria to determine functionality of the 64 most prevalent LDLR sequence variants used in the screening program in our country. Almost 30,000
individuals were tested for one of these variants and, as expected, carriers of pathogenic FH mutations had consistently elevated LDL-C levels. Moreover, FH patients with a pathogenic mutation had a more than three-fold higher hazard ratio for CAD compared with unaffected relatives.

In contrast, lipid levels and the risk of CAD in individuals carrying a non-pathogenic LDLR variant were similar to those of their healthy relatives. As such, this provides further evidence that the proposed criteria to determine non-pathogenicity did label sequence variants correctly.

Only patients with a strong clinical suspicion of genetic FH are referred for analysis of LDLR mutations in the Netherlands. Once a novel sequence variant is identified in such an index patient, the clinical characteristics of the index patient will evidently not reveal whether that variant is functional or not. In fact, we showed previously that the clinical FH diagnostic scores were in essence not different for the index patients in whom an established pathogenic mutation was discovered from the index patients with a novel non-pathogenic variant in the LDLR. Thus, when in vitro tests are lacking and no segregation data are available yet, one often has to rely on the in silico analyses to decide whether further cascade screening should be performed for the novel LDLR variant. Table 1 and Supplementary material online, Table S1, show that the findings in silico do not correspond well with the co-segregation data, with a specificity below 50%. For example, the p.Gly701Ser mutation (c.2101 G > A) was identified as non-pathogenic rather convincingly by co-segregation, while the SIFT and Polyphen indicated the amino acid substitution to be not tolerated and possibly damaging, respectively. Conversely, clinical data on the p.Glu208Lys mutation (c.662 G > A) showed that this mutation is clearly pathogenic, while the in silico analyses suggested that this was a non-pathogenic sequence variant. Thus, the clinical data from the index patients referred for genetic analysis combined with the in silico results are often not conclusive for determining functionality for novel LDLR sequence variants.

Therefore, in case the newly discovered sequence variant in LDLR was identified in a patient with a strong clinical suspicion of FH and the in silico analyses also hinted at pathogenic nature, the further approach was to include it in the genetic cascade screening. After a robust number of individuals was been tested for a variant that was assumed to cause FH, that variant was re-evaluated based on the criteria we proposed. We advocate not to discard a mutation from inclusion in a screening programme based on the pedigrees of less than 50 persons. In addition, three recent publications show that certainly not all carriers of a confirmed pathogenic LDLR mutation show an FH phenotype.

Previously, most other reports on non-pathogenic variants in LDLR focussed on single mutations. Based on in silico, in vitro, or co-segregation analyses or a combination of these, authors then concluded on the functionality of that specific variant. The sample size of the pedigrees that harboured such variants was often modest. In contrast, in our pooled analyses, we were able to assess a large data set of LDLR variants that all were initially thought to be pathogenic and were therefore used for genetic screening in relatives. In fact, at the current time, more than 300 different variants in LDLR have been identified in the Netherlands. Of the 29365 individuals tested for the 64 most prevalent LDLR sequence variants, 355 (1.2%) were identified to carry a variant that was initially thought to cause FH, but was later shown not to be pathogenic. Thus, re-evaluation of functionality of sequence variants seems a reasonable approach to select only those FH mutations for genetic cascade screening that are associated with increased LDL-C levels, and consequently increased CAD risk.

Not surprisingly, we found that sequence variants in LDLR that did not lead to hypercholesterolaemia did not affect CAD risk. In fact, we recently measured carotid intima-media thickness (IMT) in subjects with pathogenic LDLR mutations who had untreated LDL-C levels below the 75th percentile. Their IMT values were similar to those in unaffected relatives and less than those in genetic FH patients with hypercholesterolaemia. The strength of the present study is that we could directly compare CAD risk between carriers of an LDLR variant and unaffected relatives. Several factors other than non-pathogenicity could potentially explain the lack of phenotypic expression of a LDLR mutation. These include healthy lifestyle or co-incidence of neutralizing mutations. However, non-pathogenicity of the sequence variants is by far the most likely explanation, because the risk of CAD was not increased in individuals that carried these variants.

A potential limitation of our event-free survival analysis is that statin use at the time of genetic diagnosis differs between the groups. Particularly, statin use was more prevalent in the group of FH patients with a pathogenic mutation, compared with unaffected relatives and carriers of a non-pathogenic variant. As a consequence, the natural course of FH has likely been altered in this group and the estimate of CAD risk will be an underestimation of the true risk. However, medication use was similar between individuals carrying a non-pathogenic variant and their unaffected relatives. As a consequence, the assessment of the CAD risk in the group of interest, i.e. carriers of a non-pathogenic LDLR sequence variant, was not biased by medication use.

In conclusion, 10 out of 64 sequence variants in the LDLR gene are proven here to be non-pathogenic both in terms of lipids as well as with regard to event-free CAD survival. We therefore have demonstrated that our clinical outcome findings have substantiated the criteria for functionality of LDLR sequence variants. They also confirm the CAD risk associated with FH and underline that these criteria can be used to decide whether a specific sequence variant should be used in cascade screening.

Supplementary material
Supplementary material is available at European Heart Journal online.

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Conflict of interest: none declared.

References


