Influence of LDL-receptor mutation type on age at first cardiovascular event in patients with familial hypercholesterolaemia

Olga W. Souverein1*, Joep C. Defesche2, Aeilko H. Zwinderman1, John J.P. Kastelein2, and Michael W.T. Tanck1

1 Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, PO Box 22700, 1100 DE Amsterdam, the Netherlands and 2 Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands

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Aims To investigate the influence of different LDL-receptor (LDLR) gene mutations on age at first cardiovascular event in familial hypercholesterolaemia (FH) patients.

Methods and results Dutch FH patients (n = 862) with known LDLR mutations from a retrospective cohort study were included. A gamma frailty Cox model was used. An event was defined as the first cardiovascular event. Gender, hypertension, smoking, diabetes, HDL cholesterol, LDL cholesterol (LDL-C), or triglycerides were included as covariates. Furthermore, the effect of LDLR mutation type on LDL and HDL cholesterol levels was investigated using mixed effects models, including gender, smoking, body mass index, and age at time of lipid measurement as covariates. A total of 86 different LDLR mutations from a retrospective cohort study were included. A gamma frailty Cox model was used. An event was defined as the first cardiovascular event. Gender, hypertension, smoking, diabetes, HDL cholesterol, LDL cholesterol (LDL-C), or triglycerides were included as covariates. Furthermore, the effect of LDLR mutation type on LDL and HDL cholesterol levels was investigated using mixed effects models, including gender, smoking, body mass index, and age at time of lipid measurement as covariates. A total of 86 different LDLR mutations were present in this cohort. Twenty-two percent of patients experienced an event (median age: 47.1 year; range: 25.6–85.8 years). The effect of LDLR mutation type on event-free survival is only significant in the models without LDL-C levels. Also, LDLR mutation type was significantly associated with LDL-C levels (P = 0.007), but not with HDL cholesterol levels (P = 0.12).

Conclusion In the present study, LDL-C levels are a more important risk factor of event-free survival than the type of LDLR mutation.

Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant trait, primarily caused by mutations in the LDL-receptor (LDLR) gene.1 It is characterized by elevated levels of LDL cholesterol (LDL-C), the presence of tendon xanthomas, and early onset of coronary atherosclerosis.1,2 The prevalence of heterozygous FH is estimated to be about 1 in 500 persons.1

The LDLR gene is located on chromosome 19.1 In recent years, over 700 different mutations in the LDLR gene have been described,3,4 of which over 350 were found in the Dutch population.5 Phenotypic expression of FH varies between patients. Differences between mutation types have been found for total cholesterol, LDL-C, HDL cholesterol, and response to treatment with simvastatin.6–19 Although these results have not been unequivocal. Furthermore, results have also been ambiguous concerning the expression of cardiovascular disease (CVD). Bertolini et al.12 found that CVD occurred at a younger age in receptor-negative patients than in receptor-defective patients, and Vohl et al.14 found that carriers of null alleles were younger at first revascularization than carriers of defective alleles at the LDLR locus. Also, homozygous carriers of a >10 kb deletion showed onset of CVD at a younger age than homozygous carriers of the W66G mutation in exon 3.16 On the other hand, Chaves et al.19 found no difference in the presence of CVD between carriers of null mutations and carriers of defective mutations, and Sijbrands et al.18 found no difference in mortality risk between first-degree relatives of carriers of either null alleles or other types of mutations. Given these previous results it might be expected that different mutations are associated with different ages at which the first cardiovascular event is reported.

However, a major drawback of the previous studies is that they investigated only a small number of mutations or grouped different mutations to ease analysis. The latter limitation as well as the current ambiguous results, prompted us to investigate the influence of individual LDLR mutations on age at first cardiovascular event in a cohort of patients with FH. Since many mutations occur only very rarely, it is impossible to obtain stable estimates of the relative risks (RRs) for these different mutations. Instead, we estimate the distribution of the RRs over all
mutation classes. Therefore, we use a frailty model, which is able to deal with the many different categories associated with the different mutations in our cohort. Furthermore, the model allows the sizes of the individual groups to differ from group to group, and for group size to be one.

Methods

The shared frailty model

Frailty models are random-effects models for survival data, and may be used to model association between individual survival times within subgroups. The shared frailty model can be defined as an extension of the proportional hazards regression model. It is assumed that the hazard rate for the $j$th individual ($j = 1, \ldots, n$) in the $i$th subgroup, defined by the $i$th mutation type in the LDLR gene, is a non-parametric baseline hazard rate, $h_0(t)$, the vector of covariates, $\beta$ is the vector of regression coefficients, $w_1, \ldots, w_m$ are the log RRs (also called frailties) associated with the $i$th mutation, and $\alpha = \exp(\sigma w_i)$. We assume that the $\alpha$’s are gamma distributed with shape and scale parameters both equal to $\delta$, so that the mean of the $\alpha$’s is one and the variance is $1/\delta$. The choice for the gamma distribution is based on convenience because the posterior distribution of the $\alpha$’s given the data is a gamma distribution with parameters $[\delta + \sum_{j=1}^{n_i} d_{ij}]$ and $[\delta + \sum_{j=1}^{n_i} H_0(t_{ij}) \exp(\beta Z_{ij})]$, where $\sum_{j=1}^{n_i} d_{ij}$ is the number of events in the $i$th subgroup, and $H_0(t_{ij})$ is the cumulative baseline hazard function. It follows that

$$E[\alpha_i|\text{Data}] = \frac{[\delta + \sum_{j=1}^{n_i} d_{ij}]}{[\delta + \sum_{j=1}^{n_i} H_0(t_{ij}) \exp(\beta Z_{ij})]}$$

The variance of $\alpha_i$ is calculated as follows

$$\text{Var}[\alpha_i|\text{Data}] = \frac{[\delta + \sum_{j=1}^{n_i} d_{ij}]}{[\delta + \sum_{j=1}^{n_i} H_0(t_{ij}) \exp(\beta Z_{ij})]^2}$$

and these statistics can be used as a quantification of the RR of each mutation type present in this cohort.

Study population

We studied FH heterozygotes from a Dutch, retrospective, multicentre, cohort study, who were 18 years or older and had a documented LDLR mutation. The study design and population have been described elsewhere. In short, 4000 patients were randomly selected from a DNA bank database consisting of DNA samples from clinically suspected FH patients (n = 9300) referred from lipid clinics throughout the Netherlands. A total of 2400 subjects met the diagnostic criteria for FH, whereas 1600 were excluded because they did not meet these criteria. A team of 13 especially trained data-collectors reviewed all patients’ hospital records. Subsequently, extensive information on demographics, classical risk factors, laboratory parameters, and CVD endpoints was acquired. To ensure data completeness, additional information was sought from general practitioners, patients, and hospitals that patients had visited formerly. To obtain consistent data sets, quality guidelines were implemented. The present study excluded 1145 subjects because they were included in the cohort based on other diagnostic criteria than the presence of a LDLR mutation. Of the remaining 1255 subjects, 393 had missing values for at least one of the variables used in the frailty analysis (see Statistical analysis).

The study complies with the Declaration of Helsinki. Written informed consent was obtained from all living patients. The Ethics Institutional Review Board of each participating hospital approved the protocol.

Cardiovascular events

The combination of cardiovascular mortality and CVD was the primary measure of outcome. CVD was defined by the presence of at least one of the following: (i) myocardial infarction, proved by at least two of the following: (a) classical symptoms (>15 min), (b) specific ECG abnormalities, (c) elevated cardiac enzymes (>2x upper limit of normal); (ii) percutaneous coronary intervention or other invasive procedures; (iii) coronary artery bypass grafting; (iv) angina pectoris, diagnosed as classical symptoms in combination with at least one unequivocal result of one of the following: (a) exercise test, (b) nuclear scintigram, (c) dobutamine stress ultrasound, (d) a more than 70% stenosis on a coronary angiogram; (v) ischaemic stroke, demonstrated by CT or MRI scan; (vi) documented transient ischaemic attack; (vii) peripheral arterial bypass graft; (viii) peripheral percutaneous transluminal angioplasty or other percutaneous invasive intervention; (ix) intermittent claudication defined as classical symptoms in combination with at least one unequivocal result of one of the following: (a) ankle/arm index <0.9, (b) a stenosis (>50%) on an angiogram or duplex scan.

If information on CVD did not strictly fulfil the above-mentioned criteria, or if any suspect history, symptoms, or diagnostic evaluations were found in the record, the case was presented to an independent adjudication committee.

Laboratory analysis

All laboratory parameters were measured in fasting blood samples. Lipid levels, as stated in the medical record, were determined after at least 6 weeks of withdrawal of any lipid-lowering medication. LDL-C levels were calculated using the Friedewald formula. Mutations in the LDLR gene were assessed by methods described previously. In short, all patients were analysed by PCR for the 14 most common mutations found in the Netherlands, if no mutation was found, the promoter and all coding regions of the LDLR gene were analysed by denaturing gradient gel electrophoresis and aberrant bands were sequenced. In addition, long-range PCR and Southern blotting techniques were used to identify large deletions and insertions. However, since these techniques were time-consuming and not very discriminating, a different approach was used in a later stage: the promoter region and all exons (including exon–intron boundaries) of the LDLR gene were sequenced directly and major LDLR gene rearrangements were identified by the multiplex ligation-dependent probe amplification (MLPA) technique with the Salsa P062 LDLR Exon Deletion Test Kit (MRC-Holland, Amsterdam, The Netherlands). This MLPA kit is designed to detect deletions/duplications of one or more exons of the LDLR gene. To denote mutations, we use the nomenclature system described by Antonarakis and den Dunnen and Antonarakis.

Statistical analysis

The gamma frailty model as described earlier was used in the analysis. Five different models were analysed. The covariates gender, hypertension (yes/no; time-dependent), smoking (ever/never; time-dependent with a linearly decreasing risk effect for the 3 years after cessation), and diabetes (yes/no; time-dependent) were included in all these five models, while LDLR mutation type was included as the frailty term as described earlier. The five models differed in the number of lipid variables that were included as covariates. We investigated the effect of including either LDL-C levels, HDL cholesterol levels, or triglyceride levels. Therefore, the first model did not include any lipid variables, the second model included only HDL cholesterol levels, the third model included only LDL-C levels, the fourth model included only triglyceride levels, and the fifth model included all these three lipid variables. These lipid variables were entered as continuous variables.
Also, random effects models were used to estimate the effect of LDLR mutation type on LDL and HDL cholesterol, respectively. In these models, gender, smoking (ever/never), body mass index, and age at time of lipid measurement were included as fixed effects, and type of LDLR mutation was included as a random effect. For these models, the intraclass correlation coefficient was calculated, which is defined as the variance attributed to LDLR mutation type divided by the total variance (i.e., sum of variance of the random effects of LDLR mutation type and the residual variance).

For each individual, follow-up started at birth and ended at the date of first cardiovascular event defined as described earlier. Subjects without CVD were censored at the date of the last Lipid Clinic visit or at the date of death attributable to other causes. Analyses were performed using the SAS System (version 9.1 for Windows) and S-plus 6.0. The variance test was one-sided, all other tests were two-sided. A \( P \)-value smaller than 0.05 was regarded as statistically significant.

## Results

In the present study, 862 FH patients with documented LDLR mutations were included. A total of 86 different LDLR mutation types were present in these patients. Table 1 shows the number of patients and the number of events for each of the 15 most frequent LDLR mutation types. These 15 most common mutations represent 736 subjects, averaging 49.1 patients per group. The remaining 126 patients have one of the other 71 mutations, which is an average of 1.8 patient per group.

Some characteristics of the FH patients in this study are shown in Table 2. Of the 862 patients, 190 (22%) experienced a cardiovascular event. The median follow-up duration was 43.8 years, ranging from 18.4 to 85.8 years. For patients who were censored, the median follow-up was 43.0 years (range: 18.4–80.8 years), and the median age at which patients experienced their first event was 47.1 (range: 25.6–85.8).

The hazard ratios (HRs) and 95% confidence intervals (CI) of the fixed effects of the different frailty models are shown in Table 3. It can be seen that male gender, presence of hypertension, presence of diabetes, smoking, and higher levels of LDL-C were all associated with increased risk. On the other hand, higher levels of HDL cholesterol were associated with a lower risk. Triglyceride levels were not statistically significantly associated with age at first cardiovascular event.

The last row in Table 3 shows the overall significance of the different LDLR mutation types. It can be seen that LDLR mutation type was significantly associated with event-free survival only in those models that did not include LDL-C as a fixed effect. In other words, when LDL-C was included in the model, there was no significant effect of LDLR mutation type on event-free survival.

The HRs for each individual LDLR mutation type, calculated as described in equation (2), are plotted in Figure 1 for the model in which all lipid variables are included as covariates (model 5). The different groups are ordered according to their value of HR. The error bars indicate the limits according to two times the standard deviation [i.e., square root of equation (3)]. Furthermore, Table 4 shows the HRs for the five mutation types with the lowest HRs and for the five mutation types with the highest HRs for the model in which no lipid variables were included as covariates (model 1) and for the model in which all lipid variables were included as covariates (model 5).

The individual HRs of the mutation types for the models where only HDL cholesterol or only triglycerides were entered as covariates (besides gender, hypertension, smoking, and diabetes) were very similar to the HRs in the model with no correction for lipid variables (data not shown), while the HRs for mutation type in the model where only LDL-C was entered as covariate (besides gender, hypertension, smoking, and diabetes), were very similar to the HRs in the model where all lipid variables were entered as covariates (data not shown). In Table 4, it can be seen that including LDL-C as a covariate shrank all HRs towards 1. Furthermore, including LDL-C reduced the variance of the estimated HRs.

Because both LDL-C and HDL cholesterol were associated with event-free survival in the frailty model, we investigated the effect of LDLR mutation type on both these variables, using a mixed effects model. Body mass index, gender, smoking, and age at time of lipid measurement were included as fixed effects in both these models, while...
**Table 3** HRs and 95% CIs from the different frailty models

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Model 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>2.82 (2.08–3.83)</td>
<td>2.44 (1.77–3.35)</td>
<td>3.01 (2.21–4.10)</td>
<td>2.78 (2.04–3.78)</td>
<td>2.61 (1.89–3.61)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.69 (1.12–2.56)</td>
<td>1.71 (1.14–2.58)</td>
<td>1.70 (1.13–2.55)</td>
<td>1.69 (1.12–2.55)</td>
<td>1.74 (1.15–2.61)</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.50 (1.11–2.05)</td>
<td>1.50 (1.11–2.05)</td>
<td>1.52 (1.12–2.06)</td>
<td>1.50 (1.10–2.04)</td>
<td>1.52 (1.12–2.07)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2.91 (1.21–7.00)</td>
<td>2.65 (1.10–6.37)</td>
<td>2.65 (1.11–6.32)</td>
<td>2.70 (1.12–6.52)</td>
<td>2.40 (0.99–5.81)</td>
</tr>
<tr>
<td>HDL</td>
<td>—</td>
<td>0.45 (0.27–0.75)</td>
<td>—</td>
<td>—</td>
<td>0.46 (0.26–0.79)</td>
</tr>
<tr>
<td>LDL</td>
<td>—</td>
<td>—</td>
<td>1.10 (1.03–1.18)</td>
<td>—</td>
<td>1.10 (1.03–1.19)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.14 (0.97–1.36)</td>
<td>1.03 (0.85–1.25)</td>
</tr>
<tr>
<td><em>P</em>-value of frailty</td>
<td>0.04</td>
<td>0.04</td>
<td>0.10</td>
<td>0.04</td>
<td>0.11</td>
</tr>
</tbody>
</table>

**Discussion**

The present study investigated the effect of LDLR mutation type on age at first cardiovascular event in a cohort of Dutch FH patients using a frailty model. The results indicate that LDL-C levels, as well as gender, smoking, hypertension, diabetes and HDL cholesterol levels, are important predictors for age at first event. Furthermore, when LDL-C levels are known, the type of LDLR mutation does not seem to add information regarding the age at first cardiovascular event. Moreover, LDL-C levels are influenced by LDLR mutation type.

Our analysis identified mutations that seem to be associated with a better prognosis (e.g. [N543H; 2393–2401delTCCTCGTCT], S285L), as well as mutations that seem to be associated with a worse prognosis (e.g. V408M, E207K) than this cohort of heterozygous FH patients had on average. In agreement with our results, Castillo et al. classified the [N543H; 2393–2401delTCCTCGTCT] mutation as a mild mutation, and it was also found to be associated with a better CVD-free survival in a similar cohort of FH patients.

On the other hand, three other mutations associated with lower RRs compared with the average in this cohort are S285L, R329X, and C146X. The S285L mutation results in an LDLR protein with 2–5% activity compared to normal, while the R329X and C146X mutations are class 1 mutations, meaning the LDLR protein is not synthesized. Of the mutations associated with increased RRs, the V408M and E207K mutations have <2% LDLR activity, and the EX7_8del (Cape Town-2) and W23X have between 2 and 5% activity. Therefore, these preliminary results are the first to indicate that clustering based on functionality might not yield accurate results concerning the effect of LDLR mutation type on age of onset of CVD.
Furthermore, the effects of the \textit{LDLR} mutation types on event-free survival and the effects of mutation type on LDL-C levels are weakly correlated, meaning that, for instance, the \textit{LDLR} mutation types that are associated with a short event-free survival are not necessarily the mutation types associated with high LDL-C levels. This was not an unexpected finding, since it has been found that the LDL-C levels of FH heterozygotes vary widely even in those that carry the same mutation, and that other genetic and environmental risk factors are important determinants of FH phenotype.\cite{10,15,32–34} Therefore, this result indicates once more that other factors are also important in determining LDL-C levels and event-free survival of FH heterozygotes.

This is the first study which uses a frailty model to investigate the effect of \textit{LDLR} mutation type on age at first cardiovascular event in a cohort of patients with FH. This method enables the study of the effect of each \textit{LDLR} mutation type separately. This is a major advantage compared with previous studies that have analysed the effect of \textit{LDLR} mutation type by clustering different types to obtain groups that were large enough for analysis, since this may obscure important effects of individual mutation types. However, many mutation types were present in only one or a few FH patients in this cohort. For these mutations, the present analysis is not conclusive about their influence on event-free survival due to lack of power. However, since these mutation types are rare in the Dutch population, they will not be important on a population level.

The 190 events in our study can be divided into 158 cardiac events, 16 cerebral events, 15 peripheral events, and 1 subject had a cardiac and peripheral event reported on the same day. This heterogeneity may have diluted the effect of mutation type on age at first event, but since the number of events is limited, we do not think that, given power, more specific analysis are warranted.

One of the inclusion criteria of this study was that subjects had to be at least 18 years old. Since the probability of dying before the age of 18 years is small, it is not likely that this has led to ‘survivor bias’.

One limitation of the shared frailty model is that it forces the unobserved covariates to be identical within clusters. To cope with this, we included the most important (known) covariates as fixed effects in our model. Nevertheless, it cannot be excluded that other unobserved covariates still play a role, and these might not be the same for all patients who share the same \textit{LDLR} mutation.

In conclusion, the present study shows LDL-C levels are more important than \textit{LDLR} mutation type for event-free survival in FH patients. Furthermore, the present analysis enabled estimation of RRs for each individual \textit{LDLR} mutation type, resulting in an indication that [N543H; 2393–2401delTCCTCGTCT] is associated with a longer event-free survival, while V408M might be associated with an earlier age at first cardiovascular event.

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\section*{Conflict of interest}
none declared.

\section*{References}


