Common paraoxonase gene variants, mortality risk and fatal cardiovascular events in elderly subjects

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Abstract

Recent studies indicate that the enzyme paraoxonase may be an important modulator of cardiovascular disease risk because of its ability to protect LDL from oxidation. We tested for association between two functional variants of the paraoxonase gene (Met-55:Leu and Gln-192:Arg) and both all-cause mortality and fatal cardiovascular disease. This was done within a population-based study among subjects aged 85 years and over in a cross-sectional and a prospective design. In the cross-sectional analysis, the distribution of both paraoxonase genotypes was found to be similar in the subset of 364 elderly subjects who were born in Leiden, The Netherlands, as compared with 250 young subjects whose families originated from the same geographical region. The polymorphisms were in strong linkage disequilibrium ($P < 0.00001$) and the frequency of the haplotype carrying both risk alleles was not lower in the elderly than in the young (0.313 vs. 0.284). The complete cohort of 666 elderly subjects was followed over 10 years. The risk of all-cause and cardiovascular mortality was not increased in elderly subjects with the paraoxonase Leu:Leu (RR, 1.1 [95% CI, 0.9–1.5] and 1.3 [95% CI, 0.8–2.0], respectively) or the Arg:Arg genotype (RR, 0.9 [95% CI, 0.7–1.2] and 0.7 [95% CI, 0.4–1.3], respectively). In a subset of patients with diabetes, the all-cause mortality risk was elevated in Arg:Arg carriers (RR, 2.1 [95% CI, 0.8–5.8]) but this did not reach statistical significance. Analysis of genotype combinations did not yield significant associations with mortality. The paraoxonase gene variants, previously associated with coronary artery disease, are thus not likely to have a major effect on the risk of fatal cardiovascular disease in the population at large. Adverse effects of the gene variants might be observed in subjects exposed to factors that enhance oxidative stress such as diabetes. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Paraoxonase; Polymorphism; oxLDL; Cardiovascular disease; Mortality

1. Introduction

Oxidised low-density lipoprotein (oxLDL) is thought to play a central role in atherogenesis [1,2]. Evidence is accumulating that the enzyme paraoxonase protects LDL from oxidation. Paraoxonase, which is physically associated with apolipoprotein A-I in HDL, inhibits Cu$^{2+}$-induced oxidation of LDL in vitro [3] by destroying proinflammatory lipid peroxides [4]. Subsequent studies using a cell co-culture model showed that HDL from wild-type mice but not from paraoxonase deficient mice inhibits the monocyte chemotactic activity of LDL, which becomes oxidised in the subendothelial matrix if HDL is absent [5]. Moreover, paraoxonase deficient mice are more susceptible to atherosclerosis than wild-type mice when fed a high-fat/high-cholesterol diet [5].

The contribution of oxLDL to cardiovascular disease in humans may thus be evaluated by studying functional variants of the paraoxonase gene (PON1). Two variants in the coding region have been identified leading to a methionine-55 to leucine and a glutamine-192 to arginine amino acid substitution [6]. The inter-individual variation in the ability of paraoxonase to hy-
Hydrolyse organophosphorous compounds is determined by the Gln-192/Arg polymorphism. The effect, however, is substrate-dependent [7,8]. In vitro, the Arg-isof orm was found to be less effective in preventing LDL from oxidation by Cu$^{2+}$ than the Gln-isof orm [9]. Hence, the Arg-allele may be a risk factor for cardiovascular disease. Evidence for this hypothesis was obtained in three studies in Caucasian subjects, which found the Arg-allele to be more common in type 2 diabetic patients with coronary heart disease [10,11] and in patients with more than 75% stenosis in a coronary artery [12] than in controls. Two small studies suggested that the Arg-allele was associated with coronary heart disease in the Japanese [13,14]. In contrast, no increased frequency of the Arg-allele was observed in myocardial infarct patients in two studies [11,15], in Finnish patients who underwent coronary bypass surgery [16] and in Italian patients with more than 50% stenosis [17].

The Met-55/Leu polymorphism has been associated with the level of paraoxonase in serum [8] and mRNA in liver biopsies [18]. Surprisingly, it was the high-level associated Leu-allele that was found to represent an increased risk of coronary heart disease in Caucasian patients with type 2 diabetes [8]. This might suggest that the Leu-allele is not only associated with paraoxonase serum level, but also has a detrimental effect on enzyme function. In a study among Asian Indians and Chinese, the Met-55/Leu polymorphism was not associated with coronary heart disease [19].

Until now, none of the studies that assessed the possible disease association of the paraoxonase polymorphisms were prospective, nor did they include fatal cases. Therefore, we explored whether both polymorphisms, either separately or in combination, are associated with all-cause and cardiovascular mortality in the general population. This was done within a population-based study among subjects aged 85 years and over (Leiden 85-plus Study [20]) using two designs [21]. The impact of the gene variants on mortality before the age of 85 years was studied cross-sectionally, by comparing the paraoxonase genotype distribution between subjects aged 85 years and over and young subjects with families from the same geographical region. In a prospective study with a 10-year follow-up period, the relation of the gene variants to all-cause and cardiovascular mortality above the age of 85 years was investigated. During follow-up, the all-cause mortality was 89% and the cardiovascular mortality 38%.

2. Methods

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part [20]. Out of a total of 1258 eligible subjects, 221 died before enrolment. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. Diabetes was diagnosed on the basis of the medical interview, use of medication for the treatment of diabetes and/or a serum glucose level over 11.0 mmol/l in a non-fasting blood sample. After exclusion of subjects with a non-Dutch ($n = 29$) or unknown ($n = 69$) place of birth, sufficient cell material was available from 666 (188 men/478 women) subjects for the present genetic study. Subjects from whom a DNA sample was available did not significantly differ from subjects from whom a DNA sample was not available, with respect to age ($P = 0.2$), gender ($P = 0.2$), smoking ($P = 0.8$), the prevalence of diabetes ($P = 0.8$) or the prevalence of hypertension ($P = 0.8$). DNA was extracted by protein precipitation using potassium acetate and chloroform extraction [22]. The paraoxonase Met-55/Leu and Gln-192/Arg genotypes were determined by PCR-amplification followed by digestion with NlaIII and AlvI, respectively [6]. For genotyping of the Gln-192/Arg polymorphism an alternative downstream primer was used (5′-GAGAATCTGAGTAAATCCAC-TACATTTCAG) which results in a 64 bp and 172 bp DNA fragment after digestion if the Arg-allele is present. Digestion products were separated on 7.5% polyacrylamide MADGE-gels (microtitre array diagonal gel electrophoresis) [23]. Paraoxonase genotypes were assessed independently by two observers. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified. In all cases the previously assigned genotype was confirmed. The study was approved by the Medical Ethics Committee of Leiden University and informed consent was obtained from all participants.

2.1. Cross-sectional analysis

Paraoxonase genotype distributions were compared among elderly subjects aged 85 years and over and young controls. To avoid false associations with the paraoxonase polymorphisms due to differences in geographical origin rather than age, only those subjects aged 85 years and over who were born in Leiden ($n = 364$; 55%) were compared with a control population which consisted of 250 (139 men/111 women) blood donors aged 18–40 years of Dutch descent with either two Leiden-born parents or one Leiden-born parent and the other born within a 12-km distance of Leiden. Information regarding the birthplace of their grandparents was obtained from a written questionnaire.

The elderly subjects were survivors of a cohort born in Leiden between 1887 and 1901. An investigation was made of whether the young control population was likely to represent the Leiden genotype distribution of
two generations before. The frequencies of the Leu/Leu genotype were 41.4, 41.0, 42.5 and 42.1% among young subjects with either one or more \((n = 203)\), two or more \((n = 178)\), three or more \((n = 120)\) and four \((n = 76)\) Leiden-born grandparents, respectively. For the Arg/Arg genotype these frequencies were 9.4, 9.6, 11.7 and 9.2%, respectively. Thus, the paraoxonase genotype frequencies in control subjects were independent of the number of grandparents born in Leiden. This indicates that no specific Leiden paraoxonase genotype distribution existed around 1900.

2.2. Prospective follow-up study

All participants in the Leiden 85-plus Study were followed for mortality until October 1, 1996. Among the 666 subjects of the cohort studied, two were lost to follow-up. The primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorised for cardiovascular disease (ICD-9 codes [24] 390–459), ischaemic heart disease (410–414), cerebrovascular disease (430–438).

2.3. Statistical analysis

When analysing the paraoxonase polymorphisms separately, the genotypes were not grouped because previous studies reported a co-dominant association with the risk of coronary heart disease [10–12]. In the cross-sectional analysis, distributions of genotypes and haplotypes were compared by the \(\chi^2\)-test. Mortality risks up to the age of 85 years and 95% confidence intervals (CIs) were estimated using the exposure odds ratio. Linkage disequilibrium between the two paraoxonase polymorphisms was tested using a likelihood-ratio test and maximum-likelihood haplotype frequencies were computed using an expectation–maximisation algorithm. Both procedures were performed using ARLEQUIN software version 1.1 [25]. The latter procedure is an iterative process aiming at obtaining maximum–likelihood estimates of haplotype frequencies from multi-locus genotypic data when the gametic phase is unknown. In this case, simple gene counting is not possible because several haplotypes are possible for individuals heterozygous at both paraoxonase loci. The expectation–maximisation algorithm starts with arbitrary (random) estimates of haplotype frequencies. These estimates are used to compute expected genotype frequencies assuming Hardy–Weinberg equilibrium (expectation step). The relative genotype frequencies obtained are used as weightings for their two constituting haplotypes in a gene counting procedure leading to new estimates of haplotype frequencies (maximisation step). The expectation and maximisation steps are repeated until the haplotype frequencies reach equilibrium. The maximum likelihood estimate of the population linkage-disequilibrium parameter \(D\) and the percent of its maximum possible value were calculated as described by Thompson et al. [26].

In the prospective follow-up study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause or October 1, 1996. Survival was estimated using the Kaplan–Meier product limit method. Age and gender adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models. Causes of death were assumed to be independent. \(P\)-values of less than 0.05 were considered to indicate statistical significance and all \(P\)-values were based on two-sided tests.

3. Results

3.1. Cross-sectional analysis

The genotype distributions of the paraoxonase polymorphisms were determined in a cohort of 666 subjects aged 85 years and over. The frequencies for the Met-55/Leu polymorphism were 10.8% (Met/Met), 48.3% (Met/Leu) and 40.8% (Leu/Leu); for the Gln-192/Arg polymorphism the frequencies were 47.0% (Gln/Gln), 44.9% (Gln/Arg) and 8.1% (Arg/Arg). Excess mortality before the age of 85 years among carriers of the putative risk-genotypes would be reflected in a reduced frequency of these genotypes in the elderly population. Therefore, paraoxonase genotype frequencies in the elderly subjects born in Leiden \((n = 364; 55%\) of the cohort studied) were compared with those in young subjects aged 18–40 years whose families originated from the Leiden area \((n = 250)\) (Table 1). The genotype distributions of both polymorphisms were in Hardy–Weinberg equilibrium. The Met-55/Leu genotype distribution was virtually identical among elderly and young subjects \((P = 0.73)\). The estimated mortality risk up to the age of 85 years associated with the Leu/Leu genotype was not increased compared with the Met/Met genotype \((OR, 0.8 [95\% CI, 0.5–1.4])\). The Gln-192/Arg genotype distribution was also similar in the elderly and the young \((P = 0.26)\). The mortality risk associated with the Arg/Arg genotype was not increased \((OR, 0.9 [95\% CI, 0.5–1.6])\) as compared to that with the Gln/Gln genotype. Separate analysis of men and women yielded similar results. The frequencies of the Leu/Leu and Arg/Arg genotypes observed for elderly men \((n = 115; 33.9\text{ and } 8.7\%, \text{ respectively})\) and for elderly women \((n = 249; 43.4\text{ and } 8.4\%, \text{ respectively})\) were not significantly different from those in young subjects \((39.6\text{ and } 8.4\%, \text{ respectively})\).

The Met-55/Leu and Gln-192/Arg polymorphisms were in strong negative linkage disequilibrium [26] \((P < 0.00001; \ D = 100\%\text{ in young and } D = 96\%\text{ in elderly})\).
Table 1
Paraoxonase genotype distributions in subjects aged 85 years and over and young subjects whose families originated from the same geographical region

<table>
<thead>
<tr>
<th>Paraoxonase genotype</th>
<th>Subjects</th>
<th>Elderly a (n = 364)</th>
<th>Young b (n = 250)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Met-55/Leu</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met/Met</td>
<td>39 (10.7%)</td>
<td>32 (12.8%)</td>
<td></td>
</tr>
<tr>
<td>Met/Leu</td>
<td>178 (48.9%)</td>
<td>119 (47.6%)</td>
<td></td>
</tr>
<tr>
<td>Leu/Leu</td>
<td>147 (40.4%)</td>
<td>99 (39.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Gln-192/Arg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln/Gln</td>
<td>164 (45.1%)</td>
<td>129 (51.0%)</td>
<td></td>
</tr>
<tr>
<td>Gln/Arg</td>
<td>169 (46.4%)</td>
<td>100 (40.0%)</td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>31 (8.5%)</td>
<td>21 (8.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Met-55/Leu–Gln-192/Arg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met/Met–Gln/Gln</td>
<td>39 (10.7%)</td>
<td>32 (12.8%)</td>
<td></td>
</tr>
<tr>
<td>Met/Leu–Arg/Arg</td>
<td>83 (22.8%)</td>
<td>56 (22.4%)</td>
<td></td>
</tr>
<tr>
<td>+ Leu/Leu–Gln/Arg</td>
<td>29 (8.0%)</td>
<td>21 (8.4%)</td>
<td></td>
</tr>
</tbody>
</table>

a Median age: 89 years (range 85–100).
b Median age: 31 years (range 18–40).

The analysis of combinations of genotypes also did not reveal any differences between the two groups (Table 1). The estimated mortality risk up to the age of 85 years associated with homozygosity for both putative risk alleles was 1.1 (95% CI, 0.5–2.4) as compared with subjects homozygous for the Met and Gln-allele. It should be noted that because of the negative linkage disequilibrium, in the young all of the 21 Arg/Arg carriers and in the elderly 29 of the 31 Arg/Arg carriers were also homozygous for the Leu-allele.

3.2. Prospective follow-up study

During the 10-year follow-up period 89% of the 666 subjects died of any cause, 38% of the 666 of cardiovascular disease, 9% of ischaemic heart disease and 13% of cerebrovascular disease. The 10-year survival of 666 elderly subjects according to paraoxonase Met-55/Leu and Gln-192/Arg genotypes is shown in Fig. 1. The all-cause mortality risk was not increased among carriers of the Leu/Leu genotype, nor among carriers of the Arg/Arg genotype, nor in carriers homozygous for both putative risk alleles (RR, 1.1 [95% CI, 0.9–1.5], 0.9 [95% CI, 0.7–1.2] and 0.9 [95% CI, 0.6–1.4], respectively; Table 3). No significantly increased risks of cardiovascular death causes were observed for (combinations of) paroxonase genotypes, except for the heterozygous Gln/Arg genotype that was associated with an increased risk of ischaemic heart disease. This finding is, however, not compatible with a recessive or subjects). Thus, the rare Arg-allele almost always occurred in combination with the frequent Leu-allele, which gives rise to a common haplotype carrying both putative risk-alleles. The estimated haplotype frequencies were similar in elderly and young subjects (P = 0.34; Table 2). The analysis of combinations of genotypes also did not reveal any differences between the two groups (Table 1). The estimated mortality risk up to the age of 85 years associated with homozygosity for both putative risk alleles was 1.1 (95% CI, 0.5–2.4) as compared with subjects homozygous for the Met and Gln-allele. It should be noted that because of the negative linkage disequilibrium, in the young all of the 21 Arg/Arg carriers and in the elderly 29 of the 31 Arg/Arg carriers were also homozygous for the Leu-allele.

Table 2
Estimated paraoxonase haplotypes frequencies in subjects aged 85 years and over and young subjects whose families originated from the same geographical region

<table>
<thead>
<tr>
<th>Paraoxonase haplotype</th>
<th>Elderly (728 chromosomes)</th>
<th>Young (500 chromosomes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met–Gln</td>
<td>0.348</td>
<td>0.366</td>
</tr>
<tr>
<td>Leu–Gln</td>
<td>0.335</td>
<td>0.350</td>
</tr>
<tr>
<td>Met–Arg</td>
<td>0.004</td>
<td>0.000</td>
</tr>
<tr>
<td>Leu–Arg</td>
<td>0.313</td>
<td>0.284</td>
</tr>
</tbody>
</table>

Fig. 1. Kaplan–Meier estimate of 10-year cumulative survival according to paroxonase Met-55/Leu and Gln-192/Arg genotype for subjects aged 85 years and over. Survival was not significantly different for the various paroxonase genotypes.
Table 3
Ten-year all-cause and cardiovascular disease mortality risks according to paraoxonase genotype in subjects aged 85 years and over

<table>
<thead>
<tr>
<th>Paraoxonase genotype</th>
<th>No (n = 593)</th>
<th>Cardiovascular disease (n = 250)</th>
<th>Ischaemic heart disease (n = 62)</th>
<th>Cerebrovascular disease (n = 84)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR a</td>
<td>95% CI</td>
<td>RR 95% CI</td>
<td>RR 95% CI</td>
</tr>
<tr>
<td>Met-55/Leu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met/Met</td>
<td>72</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Met/Leu</td>
<td>320</td>
<td>1.0</td>
<td>0.8–1.4</td>
<td>0.8–1.9</td>
</tr>
<tr>
<td>Leu/Leu</td>
<td>272</td>
<td>1.1</td>
<td>0.9–1.5</td>
<td>0.8–2.0</td>
</tr>
<tr>
<td>Gln-192/Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln/Gln</td>
<td>312</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gln/Arg</td>
<td>298</td>
<td>1.2</td>
<td>1.0–1.4</td>
<td>1.0–1.7</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>54</td>
<td>0.9</td>
<td>0.7–1.2</td>
<td>0.4–1.3</td>
</tr>
<tr>
<td>Met-55/Leu+ Gln-192/Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met/Met–Gln/Gln</td>
<td>71</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Met/Leu-Arg/Arg</td>
<td>147</td>
<td>1.3</td>
<td>0.9–1.7</td>
<td>0.9–2.4</td>
</tr>
<tr>
<td>+ Leu/Leu–Gln/Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu/Leu–Arg/Arg</td>
<td>51</td>
<td>0.9</td>
<td>0.6–1.4</td>
<td>0.4–1.6</td>
</tr>
</tbody>
</table>

a RR indicates the mortality risk as estimated with a Cox proportional hazard model adjusted for gender and age at baseline.

(co-)dominant effect. Analysis of men and women separately yielded similar results. The Leu/Leu genotype was associated with an all-cause mortality of 1.1 (95% CI, 0.7–1.8) in men (n = 189) and 1.1 (95% CI, 0.8–1.6) in women (n = 475) compared with the Met/Met genotype. For cardiovascular mortality these risks were 1.5 (95% CI, 0.7–3.6) and 1.1 (95% CI, 0.7–1.9), respectively. The Arg/Arg genotype was associated with an all-cause mortality of 0.7 (95% CI, 0.4–1.3) in men and 1.0 (95% CI, 0.7–1.5) in women compared with the Gln/Gln genotype. For cardiovascular mortality these risks were 0.7 (95% CI, 0.3–2.1) and 0.7 (95% CI, 0.4–1.4), respectively.

It has been hypothesised that the effects of the putative paraoxonase risk-alleles may be enhanced by factors that increase oxidative stress such as diabetes and smoking [4]. Therefore, we repeated our analyses for elderly subjects with diabetes (n = 72) and for those who smoked (n = 109). In the subset of patients with diabetes, the all-cause mortality risk was elevated in Arg/Arg carriers (RR, 2.1 [95% CI, 0.8–5.8]) but this did not reach statistical significance. The relative risk of cardiovascular disease mortality was 1.8 (95% CI, 0.4–8.5) in carriers of the Arg/Arg genotype and 1.9 (95% CI, 0.5–6.6) in carriers of the Leu/Leu genotype. Among the subset of smoking elderly no elevated all-cause or cardiovascular mortality risks were observed (data not shown).

4. Discussion

Polymorphisms in the paraoxonase gene are associated with paraoxonase levels in serum (Met-55/Leu) [8] and differential susceptibility of LDL to oxidation in vitro (Gln-192/Arg) [9]. In this study we investigated the contribution of these paraoxonase polymorphisms to mortality in a cohort born between 1887 and 1901. We found that paraoxonase genotypes previously associated with an increased risk of cardiovascular disease (i.e. containing a Leu [8] or an Arg-allele [10,12]) were not associated with mortality in middle or old age. The prevalence of the putative risk genotypes was not less among elderly subjects (> 85 years) than among young subjects (18–40 years) nor were the genotypes associated with all-cause mortality or fatal cardiovascular events in elderly subjects followed over a 10-year period.

It has been hypothesised that the effects of the paraoxonase polymorphisms are enhanced in subjects with type 2 diabetes, because these patients are exposed to higher levels of oxidative stress [4]. Additional findings that suggest such enhanced effects may be the elevated levels of acute-phase proteins observed in these patients [27,28] since the acute-phase response has been related to a decline in HDL-associated paraoxonase activity in humans, rabbits and experiments in vitro [29]. Indications for increased deleterious effects of the paraoxonase gene variants were found in the two-fold higher cardiovascular mortality among diabetes patients with the Arg/Arg or the Leu/Leu genotype. These associations did, however, not reach statistical significance and should be further explored in more extensive studies.

The two paraoxonase polymorphisms were found to be in a strong negative linkage disequilibrium in the Dutch population: almost all of the rare Arg-alleles occur on a haplotype carrying the common Leu-allele and, vice versa, about half of the Leu-alleles are found
on a haplotype carrying the Arg-allele. This should be taken into account when studying the effects of the polymorphisms on enzyme function or disease risk. In fact, it is impossible to study the influence of the Arg-allele independently of the effect of the Leu-allele. If the linkage disequilibrium between the two polymorphisms is weaker in non-Caucasian populations, these may be used to study the effects independently.

The absence of an association with all-cause mortality in middle and old age does not exclude the possibility that the polymorphisms are associated with an increased risk of fatal cardiovascular disease. It indicates, however, that this potential increase in risk is limited. The finding that the paraoxonase gene variants were not a risk factor for fatal cardiovascular disease in old age substantiates this interpretation. It may be hypothesised that paraoxonase gene variants do contribute to coronary artery disease as suggested by several other studies [8,10–14], but not to the acute complications of atherosclerosis leading to fatal cardiovascular events. The absence of an association with myocardial infarction would be in agreement with this view [11,15]. Assuming that the paraoxonase gene variants are a causal factor in atherogenesis because they affect the degree of LDL oxidation and oxidised LDL is central to the pathogenesis of atherosclerosis, our results may imply that not atherosclerosis as such, but other factors (for example those controlling plaque stability) are critical in determining cardiovascular mortality.

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