Mutations in the lipoprotein lipase Gene Associated With Ischemic Heart Disease in Men
The Copenhagen City Heart Study

Hans H. Wittrup, Anne Tybjærg-Hansen, Rolf Steffensen, Samir S. Deeb, John D. Brunzell, Gorm Jensen, Børge G. Nordestgaard

Abstract—The aim of this study was to test the hypothesis that the Asp9Asn substitution and the $T(-93)\rightarrow G$ mutation in the promoter of the lipoprotein lipase gene affect plasma lipid levels and thereby the risk of ischemic heart disease (IHD). We genotyped 9033 men and women from a general population sample and 940 patients with IHD. The frequency of both the $G$ allele and the Asn9 allele in the general population sample was $\approx 0.015$ for both men and women. These 2 mutations appeared together in 95% of carriers. The average triglyceride-raising effect associated with double heterozygosity for the $T(-93)\rightarrow G$ mutation and the Asp9Asn substitution was 0.28 mmol/L ($P=0.004$) and 0.16 mmol/L ($P=0.10$) in men and women, respectively. On logistic regression analysis allowing for age, the risk of IHD for double heterozygous men and women was increased 90% (95% confidence interval [CI], 20% to 200%) and 30% (95% CI, −40% to 170%), respectively, compared with noncarriers. When, in addition, other conventional cardiovascular risk factors were allowed for, the risk of IHD for double heterozygous men and women was increased 70% (95% CI, 0% to 190%) and 20% (95% CI, −50% to 180%), respectively. Of the overall risk of IHD in men in the general population, the fraction attributable to double heterozygosity was 3%, similar to the 5% attributable to diabetes mellitus. These results demonstrate that the Asp9Asn substitution is in linkage disequilibrium with the $T(-93)\rightarrow G$ mutation and that the double-heterozygous carrier status is associated with elevated plasma triglycerides and an increased risk of IHD in men. (Arterioscler Thromb Vasc Biol. 1999;19:1535-1540.)

Key Words: atherosclerosis ■ coronary disease ■ genes ■ enzymes ■ lipids

Lipoprotein lipase (LPL) hydrolyzes triglycerides contained in the core of both chylomicrons and VLDLs, thus causing these particles to be transformed into chylomicron remnants and IDLs/LDLs, respectively; excess surface molecules are transferred to the HDL fraction.1,2 More than 60 different rare, structural mutations in the lipoprotein lipase gene have been described in either the homozygote or compound heterozygote form in patients with severe hypertriglyceridemia and reduced HDL levels.1,3 As a result of such mutations, the enzyme is either not produced or becomes catalytically ineffective. In a previous study including 10 207 individuals, we demonstrated that 1 of these rare mutations, causing the Gly188Glu substitution, in the heterozygous state is associated with increased plasma triglyceride levels, reduced HDL cholesterol levels, and a 5-fold increase in risk of ischemic heart disease (IHD).4 In another recent study of the same individuals, we observed that the more common Asn291Ser substitution in LPL in the heterozygous state was associated with an increase in plasma triglycerides mainly in women, a decrease in plasma HDL cholesterol in both sexes, and a 2-fold increase in risk of IHD in women.5

A different class of genetic variation is represented by regulatory mutations.6 Such mutations may either increase or reduce the level of mRNA gene transcripts and consequently the level of protein, rather than alter the structure of the protein itself. Recently, a compound mutation $T(-39)\rightarrow C/ T(-93)\rightarrow G$ in the promoter of the lipoprotein lipase gene was found in a patient with familial combined hyperlipidemia and decreased postheparin plasma LPL activity and mass;7 this individual was also heterozygous for the common Asp9Asn substitution in LPL, which has previously been described.8 Familial combined hyperlipidemia is associated with an increased risk of IHD.9–11

The main objective of the present study was to test the hypothesis that the $T(-93)\rightarrow G$ mutation and/or the Asp9Asn substitution in the heterozygous form leads to dyslipidemia and to increased susceptibility to IHD in carriers. For this purpose, we analyzed a sample of 9033 men and women from the Danish general population (the Copenhagen City Heart...
Study) and 940 patients with verified IHD for the presence of these 2 mutations in the lipoprotein lipase gene.

Methods

Subjects
A general population sample (the Copenhagen City Heart Study) with an almost-equal number of men and women (55%) stratified into 10-year age groups from 20 to 80 years and above was drawn randomly from the Copenhagen Central Population Register with the aim to obtain a representative sample of the adult Danish general population.15 Less than 1% were nonwhites, and ~98.8% were of Danish descent. The Copenhagen City Heart Study is a prospective cardiovascular population study; a detailed description of the first (1976 to 1978) and second (1981 to 1983) examinations has previously been published.12 We studied individuals participating in the third examination of this study from 1991 through 1994.4,5,13–15 In brief, 10,049 individuals participated, 9258 gave blood, and 9033 were genotyped for both the T(-93)→G mutation and the Asp9Asn substitution. Among those genotyped, 548 individuals had IHD (World Health Organization International Statistical Classification of Diseases and Related Health Problems, 8th ed, 1986; No. 410 to 414), and 327 had suffered a nonfatal myocardial infarction (MI; World Health Organization No. 410). These diagnoses were verified by reviewing all hospital admissions (via the Danish National Patient Register) and, if necessary, medical records from hospitals or general practitioners; data derived from the Danish National Patient Register have previously been shown to have high validity.16 Plasma lipids and lipoproteins were measured in the nonfasting state. Plasma triglycerides were measured at the first and third examinations; HDL cholesterol at the second and third; plasma cholesterol at all 3; and apolipoprotein AI, apolipoprotein B, and lipoprotein(a) at the 136-bp PCR product, which was digested with the restriction enzyme TaqI, this yielded a 136-bp PCR product, which was digested to yield a 117- and a 19-bp product for normal homozygous Asp9 individuals; 136-, 117-, and 19-bp products for heterozygous probands; and a 136-bp product only for homozygous Asn9 probands. Both primers were used in concentrations of 1.0 μmol/L.

Other Analyses
Colorimetric and turbidimetric assays were used to measure nonfasting (general population sample) or fasting (patients with IHD) plasma levels of total cholesterol, HDL cholesterol, triglycerides, glucose, apolipoprotein B, apolipoprotein AI, and fibrinogen (CHOD-PAP, precipitation of apolipoprotein B–containing lipoproteins followed by CHOD-PAP, GPO-PAP, hexokinase method, sheep anti-human apolipoprotein B, sheep anti-human apolipoprotein AI, and fibrinogen kinetic kits, respectively, all from Boehringer Mannheim) and lipoprotein(a) [rabbit anti-human lipoprotein(a) from DAKO A/S, Glostrup, Denmark]. Blood pressure was determined as described previously.17 Body mass index was weight divided by height squared. Waist-hip ratio was the body circumference measured midway between the lower rib margin and the iliac crest, divided by the maximum circumference over the buttocks.

Analysis of Results
Data on women and men were analyzed separately using the SPSS software package.18 A value of P<0.05 on 2-sided tests was considered significant. Only data for the double heterozygous carriers were included; there was not enough power to evaluate the effect of single heterozygosity for the T(-93)→G mutation (n=13), single homozygosity for the T(-93)→G mutation (n=1), or double homozygosity (n=2), although both single T(-93)→G heterozygous individuals and double homozygous individuals had a trend toward higher plasma triglyceride levels like those seen for double heterozygous individuals.

The differences between genotypes in plasma levels of lipids and lipoproteins were analyzed in the general population sample of 9033 individuals by Student’s t test; the distributions of plasma triglyceride and lipoprotein(a) values were skewed, requiring logarithmic transformation of these parameters before t tests were performed.

Homogeneity of the association of genotype with plasma triglyceride, HDL cholesterol, and apolipoprotein AI levels between tertiles of continuous covariates [age, cholesterol, apolipoprotein B, lipoprotein(a), body mass index, waist-hip ratio, glucose, systolic blood pressure, and diastolic blood pressure] or between the presence or absence of categorical covariates (smoking, diabetes mellitus, and menopausal status and postmenopausal hormonal replacement therapy in women) was tested using interaction terms in an ANOVA including genotype and the covariate in question. No statistically significant interactions were revealed in these analyses.

Logistic regression analysis with forced entry was performed to investigate the role of genotype and other covariates in predicting IHD and MI, either in a bivariate analysis allowing for age only, or in a multivariate analysis wherein other cardiovascular risk factors were also allowed for.19,20 Results are given as ORs (e^β) with 95% CIs (e^β±1.96 SE); for continuous covariates, ORs correspond to an increase of 1 SD. Homogeneity of the ability of conventional cardiovascular risk factors to predict IHD among genotypes was tested by the introduction, 1 at a time, of all possible 2-factor interaction terms between genotype and conventional cardiovascular risk factors in the logistic regression analysis. There was no statistically significant evidence for interaction in any of these analyses. Overall model fit for covariates or interaction terms was tested using the likelihood ratio test between complete and reduced models.19,20

To examine the relative importance of genotype compared with conventional cardiovascular risk factors on the total risk of IHD in the population at large, an attributable fraction was calculated as f(R−1)/[1+f(R−1)], where f is the frequency of the covariate (eg, diabetes mellitus or genotype) in the population at large, and R is the OR for IHD associated with that covariate.21

Results

Frequencies of Mutations in the General Population Sample
The frequency of the −93G allele for men and women in the general population sample was 0.015 (95% CI, 0.013 to
TABLE 1. Lipids and Lipoproteins in Individuals From the General Population Sample by Carrier Status of the Asp9Asn Substitution and T(−93)G Mutation in lipoprotein lipase

<table>
<thead>
<tr>
<th></th>
<th>No Mutation</th>
<th>Double Heterozygotes</th>
<th>Student’s t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Asn9 and −93G</td>
<td></td>
</tr>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of individuals*†</td>
<td>3904</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>Age, y†</td>
<td>57 (56–57)</td>
<td>55 (52–58)</td>
<td>0.26</td>
</tr>
<tr>
<td>Plasma cholesterol, mmol/L†</td>
<td>6.0 (5.9–6.0)</td>
<td>5.9 (5.7–6.1)</td>
<td>0.61</td>
</tr>
<tr>
<td>Plasma apolipoprotein B, mg/dL†</td>
<td>86 (86–87)</td>
<td>86 (81–90)</td>
<td>0.70</td>
</tr>
<tr>
<td>Plasma lipoprotein(a), mg/L†‡</td>
<td>292 (281–304)</td>
<td>272 (204–341)</td>
<td>0.24</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mmol/L†</td>
<td>1.38 (1.37–1.40)</td>
<td>1.33 (1.25–1.42)</td>
<td>0.24</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mmol/L§</td>
<td>1.04 (1.03–1.05)</td>
<td>1.01 (0.93–1.09)</td>
<td>0.34</td>
</tr>
<tr>
<td>Plasma apolipoprotein A1, mg/dL†</td>
<td>130 (129–131)</td>
<td>127 (122–132)</td>
<td>0.20</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L†‡</td>
<td>2.13 (2.06–2.20)</td>
<td>2.41 (2.12–2.69)</td>
<td>0.004</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L†‡</td>
<td>2.02 (1.96–2.07)</td>
<td>2.78 (1.88–3.68)</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of individuals*†</td>
<td>4790</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>Age, y†</td>
<td>58 (58–59)</td>
<td>58 (56–60)</td>
<td>0.79</td>
</tr>
<tr>
<td>Plasma cholesterol, mmol/L†</td>
<td>6.3 (6.3–6.3)</td>
<td>6.3 (6.1–6.5)</td>
<td>0.77</td>
</tr>
<tr>
<td>Plasma apolipoprotein B, mg/dL†</td>
<td>86 (86–87)</td>
<td>88 (84–92)</td>
<td>0.34</td>
</tr>
<tr>
<td>Plasma lipoprotein(a), mg/L†‡</td>
<td>324 (313–336)</td>
<td>307 (244–369)</td>
<td>0.86</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mmol/L†</td>
<td>1.73 (1.71–1.74)</td>
<td>1.65 (1.58–1.72)</td>
<td>0.06</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mmol/L§</td>
<td>1.26 (1.25–1.27)</td>
<td>1.22 (1.16–1.28)</td>
<td>0.24</td>
</tr>
<tr>
<td>Plasma apolipoprotein A1, mg/dL†</td>
<td>151 (150–152)</td>
<td>147 (143–152)</td>
<td>0.12</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L†‡</td>
<td>1.68 (1.65–1.71)</td>
<td>1.84 (1.63–2.05)</td>
<td>0.10</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L†‡</td>
<td>1.37 (1.34–1.39)</td>
<td>1.55 (1.34–1.76)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values are shown as means and (95% CIs).
*Because some of the characteristics in this Table were not determined for all individuals, the number of individuals varies between characteristics.
†Values from the third examination of the Copenhagen City Heart Study (1991 to 1994).
‡To approach a normal distribution, the values were transformed logarithmically before statistical tests, but mean values and 95% CIs are shown for untransformed values.
§Values from the second examination of the Copenhagen City Heart Study (1981 to 1983); numbers of men and women were 2711 and 3489 for no mutation and 71 and 106 for double heterozygotes, respectively.
∥Values from the first examination of the Copenhagen City Heart Study (1976 to 1978); numbers of men and women were 2392 and 3420 for no mutation and 71 and 101 for double heterozygotes, respectively.

0.018) and 0.015 (0.013 to 0.018), respectively (χ² P=0.85). Genotype frequencies predicted by Hardy-Weinberg equilibrium were not significantly different from those observed (χ² P<0.99).

The frequency in the general population sample of the allele causing the Asp9Asn substitution was 0.014 (95% CI, 0.012 to 0.017) and 0.015 (0.013 to 0.018) for men and women, respectively. The genotype frequencies predicted by Hardy-Weinberg equilibrium did not differ significantly from those observed (χ² P<0.70).

Among the 274 heterozygous carriers of the T(−93)G mutation in the general population sample, 261 probands (95%) were also carriers of the Asp9Asn substitution in the heterozygous state, so-called double heterozygotes. Thirteen probands were heterozygous for the T(−93)G mutation alone, but no carriers of the Asp9Asn substitution alone were found.

Phenotype of Double Heterozygous Carriers
The average increase in plasma triglycerides in double heterozygous carriers of the T(−93)G mutation and Asp9Asn substitution in the general population sample was 0.028 mmol/L in men and 0.16 mmol/L in women (Table 1). Plasma HDL cholesterol and apolipoprotein A1 levels were not affected in double heterozygotes compared with noncarriers. When values from the first (1976 to 1978) and second (1981 to 1983) examinations were analyzed, the data confirmed that double heterozygote male carriers had elevated plasma triglycerides but unaffected HDL cholesterol levels (Table 1). The observed effects in women were independent of menopausal status and, in postmenopausal women, independent of hormonal replacement therapy (data not shown).

Plasma cholesterol, apolipoprotein B, lipoprotein(a), glucose, and fibrinogen; body mass index; waist-hip ratio; systolic blood pressure; and diastolic blood pressure did not
Men and in women, menopausal status, plasma triglycerides, and body mass index, diabetes mellitus, hypertension, smoking, and in women, menopausal status, plasma triglycerides were similar (Table 2).

Risk of IHD and Conventional Cardiovascular Risk Factors

On bivariate logistic regression analysis allowing for age only, diabetes mellitus, hypertension, plasma apolipoprotein B, lipoprotein(a), and HDL cholesterol were moderate to strong predictors of IHD in both men and women (ORs of 1.5 to 2.2 and 1.6 to 6.0, respectively; Table 2). An increase of 1 SD in plasma triglycerides was a weak predictor of IHD in both sexes, resulting in a 30% and 20% increase in risk of IHD in men and women, respectively. Plasma cholesterol and apolipoprotein AI, smoking, and body mass index did not predict IHD consistently in both sexes. Results for nonfatal MI were similar (Table 2).

On multivariate logistic regression analysis allowing for age, plasma cholesterol, apolipoprotein B, lipoprotein(a), body mass index, diabetes mellitus, hypertension, smoking, and in women, menopausal status, plasma triglycerides were still a weak predictor of IHD in both men and women (Table 3).

Risk of IHD in Double Heterozygous Carriers

On bivariate logistic regression analysis allowing for age only, double heterozygous men had a 90% and 120% increased risk of IHD and nonfatal MI, respectively, compared with noncarriers (Table 2). In contrast, female double heterozygous carriers did not have an increased risk.

On multivariate logistic regression analysis allowing for age, plasma cholesterol, apolipoprotein B, lipoprotein(a), body mass index, diabetes mellitus, hypertension, smoking, and in women, menopausal status, double heterozygous men had an increase in risk of IHD and nonfatal MI of 70% and 110%, respectively, while double heterozygous women were unaffected (Table 3).

Attributable Fraction for IHD

Of the overall risk of IHD in men in the general population, the fraction attributable to double heterozygosity was 3%, similar to the 5% attributable to diabetes mellitus (Table 4).

Discussion

The present study demonstrates that the common Asp9Asn substitution and the T(−93)→G mutation in lipoprotein lipase were weak predictors of IHD in both sexes. Results for nonfatal MI were similar (Table 2).

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lipase in the double heterozygous state are associated with increased levels of plasma triglycerides in men. Increased triglyceride levels have been found to be associated with increased susceptibility to IHD in this and other studies,\textsuperscript{22–24} and all in all, these findings suggest an association of the double-heterozygous carrier status in men with increased risk of IHD. This hypothesis is supported by the \( \approx 2 \)-fold increased risk of IHD in men observed in this study. Furthermore, it is also supported by our previous observations in the same samples that heterozygous carriers of the Gly188Glu substitution in LPL have increased plasma triglycerides of 0.8 mmol/L as well as a 5-fold increased risk of IHD\textsuperscript{4} and that heterozygous female carriers of the Asn291Ser substitution in LPL have increased plasma triglycerides of 0.23 mmol/L as well as an \( \approx 2 \)-fold increased risk of IHD.\textsuperscript{5} Finally, our findings are supported by a recent study of 762 Dutch men with coronary artery disease (37 double heterozygous carriers for the \( -93G^T\rightarrow G \) and 296 controls (4 double heterozygous carriers), suggesting that double-heterozygous carrier status was associated with a nonsignificant elevation in plasma triglycerides, a decrease in HDL cholesterol, and an increased risk of coronary artery disease\textsuperscript{25}.

The increased OR for IHD and MI among double heterozygous carriers in the present study was statistically significant in men but not in women. Two explanations for this sex difference are possible. The simplest interpretation of the data is that double heterozygosity is not a risk factor for IHD in women. Another possible explanation is that there is an effect in women, but we were unable to detect it for 1 or more of the following 3 reasons: (1) The average increase in plasma triglycerides in women is only half that in men, and at most, of only borderline significance. (2) The statistical power for the analysis in women is weaker. (3) At a mean age of 57 years, potential susceptibility mutations like those studied may not yet have manifested their effects fully in women. Sex-specific effects associated with polymorphisms in apolipoprotein genes have been reported in other studies.\textsuperscript{5,26–27}

Whether it is the Asp9Gln substitution, the \( T(-93)\rightarrow G \) mutation, or the combination of the 2 that causes the increase in plasma triglycerides and in risk of IHD in men is unsettled.\textsuperscript{8,28–35} In favor of the Asp9Gln substitution is the change in charge of LPL induced by the substitution of aspartate with asparagine: located in the N-terminal end of LPL, this amino acid substitution may influence catalytic activity.\textsuperscript{3} Furthermore, in contrast to whites, single heterozygotes for the \( T(-93)\rightarrow G \) mutation were found in 50% to 60% of 161 black South Africans\textsuperscript{34} and of 81 Afro-Caribbeans,\textsuperscript{35} and in these populations the \( -93G \) allele was found to be associated with lower plasma triglyceride levels compared with carriers of the \( -93T \) allele; this suggests that the Asp9Gln substitution may be the determining factor for the elevation of plasma triglycerides in whites.

Mechanistically, it seems plausible that modest elevations in plasma triglycerides may lead to an increased risk of IHD for the following reasons: (1) Impaired hydrolysis of triglycerides in chylomicron and VLDL particles leads to accumulation of chylomicron remnants, small VLDLs, and IDLs in plasma, which appear to be trapped in the vessel wall\textsuperscript{36,37} and thereby may promote atherosclerosis. (2) Elevated plasma triglycerides have been found to be associated with a subclass of small, dense LDL particles, which on their own have been suggested to be atherogenic.\textsuperscript{22} (3) Finally, even though plasma levels of HDL cholesterol in double heterozygous carriers were not significantly reduced, impaired triglyceride catabolism could decrease transfer of excess surface material from chylomicron and VLDL particles to HDL particles, resulting either in fewer such particles or a different distribution among HDL fractions, thereby impairing reverse cholesterol transport.\textsuperscript{38} It is unclear at present which of these 3 potential atherogenic mechanisms is more important; however, all 3 support the hypothesis that a mutation in the lipoprotein lipase gene, which leads to impaired metabolism of triglycerides, may also increase the susceptibility of carriers to IHD.

Plasma lipids and lipoproteins in individuals from the general population sample were measured in the nonfasting state, whereas patients with IHD were fasting. Because the present results on the effect of double heterozygosity on lipids and lipoproteins relate only to individuals in the general population sample, this difference in fasting versus nonfasting measurements has no direct implications for the results. With respect to plasma triglycerides, it is possible, however, that the present results may differ from results obtained in the fasting state; however, the 28 double heterozygous men among patients with IHD showed a similar effect on plasma triglycerides in the fasting state as observed among carriers in the general population sample (data not shown). Because nonfasting plasma triglyceride levels are higher than fasting levels, the present study probably underestimated the role of triglycerides in predicting IHD (Tables 2 and 3). Furthermore, variability due to only single determinations of plasma triglycerides cannot account for the observed association; rather, the opposite is true, since any misclassification due to laboratory errors would bias the results toward the null hypothesis, which would also tend to underestimate the effect of plasma triglycerides on the risk of IHD as well as the effect of genotype on triglyceride levels.

In conclusion, double heterozygous men have increased plasma triglyceride levels and an increased risk of IHD. Double heterozygous women have a smaller and, at most, a borderline-significant increase in triglyceride levels and no increase in risk of IHD.

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