The V73M mutation in the hepatic lipase gene is associated with elevated cholesterol levels in four Dutch pedigrees with familial combined hyperlipidemia

Mariëtte J.V. Hoffer a,1, Harold Snieder b, Sebastian J.H. Bredie c, Pierre N.M. Demacker c, Johannes J.P. Kastelein d, Rune R. Frants a,*, Anton F.H. Stalenhoef c

a MGC-Department of Human Genetics, Leiden University Medical Centre, PO Box 9503, 2300 RA Leiden, The Netherlands
b Twin Research Unit, St Thomas' Hospital, London, UK
c Department of Medicine, Division of General Internal Medicine, University Hospital Nijmegen, Nijmegen, The Netherlands
d Department of Vascular Medicine, Academic Medical Centre, Amsterdam, The Netherlands

Received 21 December 1998; received in revised form 20 September 1999; accepted 4 October 1999

Abstract

Familial combined hyperlipidemia (FCHL) is a heritable lipid disorder characterized by multiple lipoprotein phenotypes within a single family. Previously, we have shown an increased incidence of mutations in the LPL gene which was associated with elevated levels of very low density lipoprotein (VLDL) and decreased levels of high density lipoprotein among the families studied. Now, we report the results of our study on the hepatic lipase gene. We found the HL V73M variant to be present in four FCHL families. By means of a pedigree-based maximum log-likelihood method we analyzed the effect of this variant on the lipid levels in these families. Carriers of the HL V73M variant revealed significantly higher levels of total cholesterol (P < 0.01) and apoB (P < 0.01). These findings show that the HL V73M mutant explains another part of the variability in the phenotype observed among FCHL family members, compared with mutations in the LPL gene. Family analysis shows that in these FCHL families, carriers of mutations in the LPL or HL genes have an increased risk for FCHL compared with their non-carrier relatives. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hepatic lipase; Familial combined hyperlipidaemia; HLV73M mutation; apoB100; Cholesterol

1. Introduction

Familial combined hyperlipidemia (FCHL) is the most common genetic cause of lipid disorders and is associated with coronary artery disease (CAD) [1,2]. In FCHL families, affected relatives exhibit different combinations of elevated plasma cholesterol and elevated triglyceride levels. In addition, characteristics such as elevated levels of plasma apolipoprotein (apo) B100, increased very low density lipoprotein (VLDL) production, predominance of small dense low density lipoprotein (LDL), and insulin resistance have been reported [3–6].

So far, no unique metabolic defect has been identified in FCHL. Several kinetic studies have shown hepatic overproduction of VLDL apoB 100 [7–9]. In addition, impaired lipolysis of VLDL [8] and increased conversion of VLDL to LDL has been observed [10]. Therefore, in general, defective regulation of the apoB metabolism may be responsible for the development of FCHL.

Considering elevated levels of either VLDL, LDL or both as affected phenotype in family studies, FCHL was initially suggested to be an autosomal dominant disorder [1,2]. Using complex segregation analysis, Cullen et al. [11] found evidence for a major gene acting on triglyceride levels in families with FCHL. Additional
studies, using apoB levels and LDL subclass phenotype as quantitative traits in complex segregation analysis, showed that these traits appear to be influenced by genetic effects which are of use in the prediction of FCHL [12–15]. Taken together, FCHL can be considered a complex disorder with a heterogeneous background.

Suggestive linkage results have been demonstrated between FCHL and several loci [16–18], but so far these results have not been confirmed [19,20]. It was also shown that specific mutations in the FABP2, APOA4, LPL and HL genes have higher frequencies among FCHL patients when compared with healthy control subjects [21–25]. We speculate that the lipoprotein lipase (LPL) and hepatic lipase (HL) genes could be good candidate genes causing FCHL because of their central roles in apoB and triglyceride metabolism. Previously, we have shown that the D9N and N291S mutations in the LPL gene are associated with elevated lipid levels of VLDL and decreased levels of HDL among carriers compared with their non-carrier relatives in FCHL families [26].

In the present study, we investigated whether the HL gene is an additional genetic determinant of the variability in phenotypic expression of FCHL. Therefore, 39 probands were screened for a HL protein isoform, V73M. Subsequently, using a pedigree-based maximum log-likelihood method, we investigated the influence of this HL variant on lipid and lipoprotein levels in four extended FCHL families.

2. Methods

2.1. Subjects

The FCHL pedigrees considered here have previously been used to investigate the inheritance of LDL subclass profiles and plasma apoB levels [14,15]. The families were ascertained through probands selected from patients attending the University Lipid Research Clinics at the Universities of Nijmegen and Amsterdam. Probands fulfilled the following criteria:

1. elevated levels of both total cholesterol and triglycerides at first measurement;
2. cholesterol and triglyceride levels above the 90th percentile using the age- and sex-related percentile levels of the Prospective Cardiovascular Munster (PROCAM) study [27].

These values were consistent over several measurements in which probands had not been given any lipid lowering drugs. Families were included when a multiple-type hyperlipidemia with levels of total plasma cholesterol and/or triglycerides above the 90th percentile (single measurement) was present in at least one first-degree relative of the proband. Children were 15 years or older.

None of the FCHL probands had specific clinical signs, like tendon xanthomata, and none were homozygous for the APOE*2 allele. For all probands, a secondary cause of hyperlipidaemia (i.e. diabetes mellitus, hypothyroidism, and hepatic or renal impairment) was excluded by standard laboratory tests. The study protocol was approved by the ethical committee of the participating universities and each subject gave informed consent. Normal lipidemic individuals, randomly selected from three different geographic areas in the Netherlands were used as a control population [28].

2.2. Measurement of lipids, lipoproteins and apolipoproteins

EDTA blood samples were obtained from the probands and family members after overnight fasting. No lipid lowering drugs were administered to the subjects for at least 4 weeks prior to the onset of the study. Plasma was separated from cells by centrifugation at 500 × g for 10 min at room temperature, and used immediately for lipid and lipoprotein analysis. VLDL (d < 1.006 g/ml) was isolated by ultracentrifugation for 16 h at 36 450 rpm in a fixed-angle TFT 45.6 rotor (Kontron, Zurich), in a Beckman L7-55 ultracentrifuge [29]. Plasma and lipoprotein cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (No. 237574; Boehringer–Mannheim, FRG; Sera-pak, No. 6639; Tournai, Belgium). HDL-cholesterol was determined in whole plasma using the polyethylene glycol 6000 method [30]. The apolipoproteins apoA1 and apoB were determined by immunonephelometry as previously described for apoB [31]. LDL subtraction pattern was determined as described by Bredie et al. [14].

2.3. DNA analysis

Genomic DNA was isolated from leukocytes according to Miller et al. [32]. Identification of carriers of the HL V73M variant was performed by PCR using the primers: 5'-AGC TGG AGA AGG AAG GG-3' (sense) and 5'-ATT AAC TCT CAG AAG AAG GG-3' (anti-sense) [33]. The reaction mixture included 15 pmol of each primer, 0.5 μg genomic DNA, 0.2 mmol/l of each dNTP, 10 mmol/l Tris–HCl; pH 9.0, 1.5 mmol/l MgCl2, 50 mmol/l KCl, 0.01% (w/v) gelatin, 0.1% Triton X-100, 0.5 unit Taq polymerase (Amplitaq, Perkin Elmer), in a total volume of 30 μl. Amplification was performed for 32 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, with an initial denaturation period of 3 min. Some 30 μl of PCR products were digested with the restriction enzyme SpH1 according to recommendations of the supplier (Pharmacia). Thereafter, fragments were separated on a 4% MP agarose gel (Boehringer Mannheim, FRG) and stained with
ethidium bromide. In the case of the mutant allele, a restriction site is formed due to the G → A change, and therefore digestion of the PCR product will reveal one fragment of 189 bp for the wildtype allele, and two fragments of 137 and 52 bp for the variant allele. Identification of the LPL N291S allele carriers in the two families originates from an earlier study [26].

2.4. Statistical analysis

To test for the statistical significance of the effect of the LPL N291S and HL V73M mutations on lipoprotein traits using all carriers in multiple families, a pedigree-based maximum likelihood method developed by Lange et al. [34,35], was used, as previously described [26,36].

For a given pedigree of \( n \) individuals a vector of observations \( (x) \) is defined and a vector of expected values \( E(x) \), that can depend on measured variables such as gender or genotype. The covariance between the residual part of the observations, i.e. the part that is not accounted for by the measured genotype or other variables, depends on the relationship between the pedigree members and on the genetic model assumed for the observations. Throughout, we have modelled the variances not accounted for by the measured genotype as consisting of additive genetic and random environmental variance, recognizing that the genetic part may also reflect environmental influences shared by family members. However, our main interest is to test for the influence of the measured genotype. For a given \( E(x) \) and expected covariance matrix \( \Sigma \), the log likelihood of obtaining the observation vector \( x \) is: \( L = -\frac{1}{2} \text{Ln}[\Sigma] - \frac{1}{2}[x - E(x)]\Sigma^{-1}[x - E(x)] + \text{constant}; \) where ’ denotes matrix transposition. The joint log-likelihood of obtaining all pedigrees is the sum of the log-likelihood of the separate pedigrees. Estimation involves selection of parameter values under a specific model that maximizes the joint likelihood of all pedigrees. The likelihood obtained for different models can be compared with chi-squared difference tests where \( \chi^2 = 2(L_1 - L_0) \) and \( L_1 \) and \( L_0 \) denote the log likelihood for the general (\( H_1 \)) and the constrained (\( H_0 \)) hypothesis. The degrees of freedom (\( df \)) for this test are equal to the number of independent parameters between \( H_1 \) and \( H_0 \) [35]. The Fisher package [34] was used for genetic modelling. Preliminary analysis did not give any evidence for gene × gender interactions unlike our previous paper on LPL-93 G → T/D9N [37]. The most general (full) model allowed for: age regression; gender-difference; differences between carriers and non-carriers of LPL N291S and/or HL V73M which were defined by four categories. For each of those four categories a different mean was estimated in the full model. By setting the regression for age or gender to zero or by constraining estimated means to be equal across carrier status of LPL N291S or HL V73M in the specification of the submodels, it was possible to test for each of those effects separately. A significant decrease in log-likelihood of the submodel compared to the full model, as apparent from the chi-square difference test, indicates that a model which allows for the effect shows a better fit, i.e. the specific effect is significant.

In the model fitting to the pedigree data with the Fisher package, ascertainment correction was carried out by conditioning on the probands. The data from the probands were omitted from statistical calculations (e.g. the estimation of the means) to avoid possible ascertainment bias. Because the distribution of total triglyceride, VLDL-cholesterol and VLDL-triglyceride was skewed, these data were transformed by natural logarithm in order to obtain a normal distribution.

3. Results

As a part of an ongoing study aiming at the identification of genetic risk factors underlying FCHL we screened 39 patients for the presence of a HL isoform, V73M. This revealed a substantially higher frequency of this variant in unrelated FCHL patients (allele frequency 6.4%, 5 out of 39 probands) when compared with healthy controls (2.8%, 6/109), (OR 2.3, 95% CI 0.67–8.09). Subsequently, all relatives of four patients were analysed (Fig. 1). For one patient no relatives were available. Surprisingly, two of these families were previously identified for presence of the LPL N291S variant [26]. Table 1 shows the descriptive biochemical parameters of all the relatives used in this study.

The effect of the HL V73M variant on plasma lipid levels in family members of FCHL patients was analyzed by a pedigree-based maximum likelihood method, as described in materials and methods. Because the LPL N291S variant was also present within these families, the effect of both mutations was taken into account, resulting in four different groups defined by carrier status of the two variants. The most general (full) model allowed for the effects of age, gender, and carrier status for the HL V73M and the LPL N291S variant by estimating a regression for age, gender and separate means for the four different categories, which were defined by carrier status of the HL V73M and LPL N291S variant. Table 2 shows log-likelihoods of the full model and the four submodels which provide a test for each of the above mentioned effects (age, gender, LPL N291S and HL V73M) on BMI and lipid variables. No significant effect of either of the mutations on BMI is found within the families studied. Therefore, BMI was excluded as a parameter in all further models. As shown in Table 2, age has a significant effect on
Fig. 1. Pedigrees of FCHL families. Open symbols indicate the non-carrier relatives (○, female; □, male). Filled symbols indicate individuals with cholesterol (CHOL) and/or triglyceride (TG) levels above the age-sex specific 90th percentile. Heterozygous carriers for the HL V73M and/or LPL N291S variant are indicated below the symbols. Arrows indicate the proband.
Table 1
Characteristics of relatives of patients with familial combined hyperlipidemia

<table>
<thead>
<tr>
<th></th>
<th>Non-carrier relatives</th>
<th>HL V73M</th>
<th>LPL N291S</th>
<th>HL V73M + LPL N291S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 19</td>
<td>n = 24</td>
<td>n = 8</td>
<td>n = 2</td>
</tr>
<tr>
<td>Age</td>
<td>41 ± 15</td>
<td>45 ± 19</td>
<td>42 ± 16</td>
<td>42 ± 25</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>7/12</td>
<td>11/13</td>
<td>4/4</td>
<td>1/1</td>
</tr>
<tr>
<td>BMI</td>
<td>24.41 ± 1.03</td>
<td>24.22 ± 1.14</td>
<td>26.34 ± 1.48</td>
<td>25.90 ± 2.45</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.69 ± 0.42</td>
<td>6.55 ± 0.48</td>
<td>5.64 ± 0.62</td>
<td>5.87 ± 1.03</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.91 ± 0.08</td>
<td>1.34 ± 0.09</td>
<td>1.81 ± 0.12</td>
<td>1.62 ± 0.19</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.36 ± 0.11</td>
<td>1.27 ± 0.13</td>
<td>0.97 ± 0.16</td>
<td>1.45 ± 0.25</td>
</tr>
<tr>
<td>VLDL-cholesterol</td>
<td>0.24 ± 0.14</td>
<td>0.34 ± 0.17</td>
<td>0.54 ± 0.21</td>
<td>0.34 ± 0.33</td>
</tr>
<tr>
<td>VLDL-triglycerides</td>
<td>0.44 ± 0.11</td>
<td>0.61 ± 0.14</td>
<td>1.16 ± 0.17</td>
<td>0.73 ± 0.28</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>4.04 ± 0.42</td>
<td>4.63 ± 0.42</td>
<td>4.27 ± 0.62</td>
<td>4.01 ± 1.02</td>
</tr>
<tr>
<td>apoB100</td>
<td>1.13 ± 0.12</td>
<td>1.38 ± 0.17</td>
<td>1.39 ± 0.21</td>
<td>-</td>
</tr>
<tr>
<td>apoA1</td>
<td>1.51 ± 0.07</td>
<td>1.48 ± 0.09</td>
<td>1.34 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td>LDL subtraction</td>
<td>0.01 ± 0.09</td>
<td>-0.17 ± 0.11</td>
<td>-0.47 ± 0.15</td>
<td>-</td>
</tr>
</tbody>
</table>

* All levels are given as mean ± SE in mmol/l except age; year, BMI; kg/m² and apolipoprotein levels: g/l.

Table 2
Log-likelihood for five different models testing the effects of age, gender, and carrier status for LPL N291S or HL V73M for lipid traits in FCHL pedigrees

<table>
<thead>
<tr>
<th>Model</th>
<th>BMI</th>
<th>Plasma CHOL</th>
<th>Plasma TG</th>
<th>VLDL-CHOL</th>
<th>VLDL-TG</th>
<th>LDL-CHOL</th>
<th>HDL-CHOL</th>
<th>apoB</th>
<th>apoA1</th>
<th>LDL subtraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Full model</td>
<td>-73.18</td>
<td>-24.86</td>
<td>52.72</td>
<td>31.4</td>
<td>37.76</td>
<td>-28.46</td>
<td>44.62</td>
<td>45.62</td>
<td>65.96</td>
<td>47.62</td>
</tr>
<tr>
<td>2 Age</td>
<td>-80.49b</td>
<td>-35.18b</td>
<td>50.79d</td>
<td>30.26</td>
<td>36.87</td>
<td>-35.51b</td>
<td>44.42</td>
<td>35.85b</td>
<td>64.75</td>
<td>46.85</td>
</tr>
<tr>
<td>3 Gender</td>
<td>-73.37</td>
<td>-25.17</td>
<td>48.96c</td>
<td>26.36c</td>
<td>31.63b</td>
<td>-28.79</td>
<td>32.71b</td>
<td>44.37</td>
<td>55.64b</td>
<td>40.03b</td>
</tr>
<tr>
<td>4 LPLN291S</td>
<td>-74.04</td>
<td>-24.86</td>
<td>49.14c</td>
<td>29.58</td>
<td>34.18c</td>
<td>-28.49</td>
<td>40.78c</td>
<td>43.59d</td>
<td>64.46</td>
<td>41.05b</td>
</tr>
<tr>
<td>5 HLV73M</td>
<td>-73.24</td>
<td>-28.56c</td>
<td>50.84</td>
<td>31.16</td>
<td>37.33</td>
<td>-30.21</td>
<td>44.59</td>
<td>40.59c</td>
<td>65.92</td>
<td>46.35</td>
</tr>
</tbody>
</table>

* Log-likelihoods for five models. Model definition; full: most general model allowing for: (i) age regression (ii) gender-difference (iii) difference between carriers and non-carriers of LPL N291S and (iv) HL V73M. The other (sub)models provide a test for each of the effects separately. Testing procedure: each model is tested against the full model. A significant decrease in log-likelihood of the submodel compared to the full model indicates a significant effect of the tested parameter. When twice the difference in log-likelihood of these models is higher than the \( \chi^2 \) corresponding to \( df = 1 \), this indicates a significant difference as indicated. Model 2–5: \( df = 1 \).

To find out whether the mutations in the HL and LPL genes are modifying or causal factors for FCHL we went back to the pedigrees of these families and counted the presence and absence of these mutations among affected and non-affected family members. If no association was present, one would expect similar numbers of carriers among affected and non-affected individuals. However, counting affected individuals as being carrier or non-carrier of the HL V73M or LPL N291S variant revealed that all affected individuals in these four families were carrier of either one or both of these two variants (Fig. 1, Table 3).

Table 3
Distribution of affected and non-affected individuals in four Dutch FCHL families

<table>
<thead>
<tr>
<th></th>
<th>Affected</th>
<th>Non-affected</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL V73M and/or LPL N291S</td>
<td>19</td>
<td>19</td>
<td>38</td>
</tr>
<tr>
<td>Non-carrier</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Totals</td>
<td>19</td>
<td>31</td>
<td>50</td>
</tr>
</tbody>
</table>
4. Discussion

In this paper, using four FCHL families, we have shown that the HL V73M variant is associated with elevated levels of total cholesterol and apoB. Neither VLDL nor LDL cholesterol levels were elevated, suggesting that an increased number of VLDL remnant particles is responsible for the increase in cholesterol levels. Although it has previously been shown that allelic variation in the HL gene accounts for 25% of the variability in HDL cholesterol [38], no effect of HL V73M was found on HDL levels which may be masked by additional modifying genes present within these FCHL families.

Hepatic lipase is involved in the hydrolysis of triglycerides in IDL and HDL, as well as phospholipids in HDL. In addition, HL can facilitate chylomicron remnant uptake independently of lipolysis by acting as a bridging protein [39]. Individuals with HL deficiency may present with features like accumulation of triglyceride-rich lipoproteins as well as β-VLDL. In addition, patients have increased plasma apoB concentrations [40]. Recently, an association was found between a promoter variation and hepatic lipase activity [41]. Although no specific association with hyperlipidemia and the V73M variant was found in a previous study using patients with classical hyperlipoproteinemias, i.e. type I, III, IV and V hyperlipoproteinemia [33], our study revealed an increased frequency of HL V73M among patients with FCHL (type IIB hyperlipoproteinemia) compared with control subjects. Similar results were found by Gehrisch et al. [25]. The amino acid at position 73 is not conserved in other lipases in human or other species but a mild effect on enzyme activity can not be excluded [33]. Functional studies supporting this hypothesis have not been performed so far.

The present study revealed that the LPL N291S mutation is significantly associated with a negative K value, indicating predominance of heavy, dense LDL particles. A previous segregation analysis in a large sample of Dutch FCHL families (including the families used in our study) [14] had already shown that the LDL subfraction profile can be used as an additional diagnostic marker [4,14]. However, this association seems to be restricted to FCHL because recently, a study focusing on the identification of genetic loci underlying the small dense LDL particle phenotype in CAD families excluded the LPL gene as a candidate gene locus [42].

Recently, the HL gene has been excluded as a major candidate gene for FCHL using linkage analysis in Finnish families [43]. Due to non-affected carriers, the HL V73M variant was also not stringently linked with the expression of FCHL in our families. This suggests that the HL gene could be excluded as the principal genetic factor for FCHL. However, counting affected individuals for being carrier or non-carrier of the HL V73M or LPL N291S variant revealed that almost all affected individuals in these four families were carrier of either one or both of these two variants. We found a similar result (OR 3.2, CI 1.6–6.6) in all FCHL families when we combined the LPL-93 G→T/D9N, LPL N291S and HL V73M variants [26,37]. Together with the finding that carriers of these mutations have significantly higher lipid levels then their noncarrier relatives this strongly suggest that the phenotypic expression of FCHL within our families cannot be explained by one major gene but is due to a more complex genetic mode of inheritance. Because not all carriers of mutations in the LPL and HL genes have a FCHL phenotype our results suggest that these genes are more likely modifying than the major genes. This fits into the ‘double hit’ hypothesis first suggested by Kwiterovitz [44], i.e. a combination of mutations in two different genes produces a full expression of the FCHL phenotype. However, further investigation will be needed to explain these findings for the HL gene within our FCHL families.

All together, our study showed an association between the V73M mutation in the HL gene and elevated cholesterol and apoB levels. The inheritance patterns of FCHL within our families suggest the involvement of multiple loci. Our results also indicate that both the LPL and HL gene are among the modifier genes of the FCHL phenotype but additional genes have to be identified to fully explain the observed inheritance pattern.

Acknowledgements

We are grateful to Janine Vogelaar (Nijmegen) for help in collecting blood samples from family members and for technical assistance in lipoprotein analysis. The authors acknowledge Dr P. de Knijff for his helpful discussions. This research was supported by the Præventiefonds (project 28-2230) and by the Netherlands Heart Foundation (NHS 92.056).

References


