The spectrum of premature atherosclerosis: from single gene to complex genetic disorder
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Molecular genetic testing in Familial Hypercholesterolemia: spectrum of LDL receptor gene mutations in the Netherlands

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Abstract

Mutations in the LDL receptor are responsible for familial hypercholesterolemia (FH). At present, more than 600 mutations of the LDL receptor gene are known to underlie FH. However, the array of mutations varies considerably in different populations. Therefore, the delineation of essentially all LDL receptor gene mutations in a population represents a prerequisite for the implementation of nation-wide genetic testing for FH. In this study, the frequency and geographical distribution of 13 known mutations were evaluated in a cohort of 1223 FH patients. We identified 358 mutation carriers, representing 29% of the FH cohort. Four mutations (N543H-2393del9, 1359 - 1 G ->, 313 + 1 G -> A and W23X) occurred with a relatively high frequency, accounting for 22.4% of the entire study cohort. Two of these common FH mutations (N543H-2393del9 and 1359 - 1 G -> A) showed a preferential geographic distribution. Second, to further expand the array of LDL receptor gene mutations, we conducted mutation analysis by denaturing gradient gel electrophoresis (DGGE) in 141 children with definite FH. A mutation was identified in 111 patients, involving 16 new single base substitutions and four small deletions and insertions, which brings the number of different FH-causing mutations in our country up to 61. Our data indicate that an estimate of the prevalence of specific mutations, as well as the compilation of a database of all FH-causing mutations in a given country, can facilitate selection of the most appropriate molecular diagnostic approach.

Introduction

Familial hypercholesterolemia (FH) is a common autosomal, dominantly inherited disorder of lipoprotein metabolism\(^1\), caused, in the vast majority of cases, by mutations in the low density lipoprotein (LDL) receptor gene. Also, mutations in the apolipoprotein B100 gene are known to cause a phenotype undistinguishable from FH.\(^2\)

Recently, evidence for the existence of a third gene that might be involved in an ‘FH-like’ phenotype became available.\(^3\)

Historically, FH is diagnosed on typical clinical traits: elevation of LDL cholesterol up to twice the normal level, presence of xanthomata and a family history of premature coronary artery disease (CAD). However, a clinical diagnosis of FH is not always unequivocal, especially in young patients in which physical stigmata are
often not present. In these cases, a molecular assay would be desirable for certain diagnosis in families and for genetic counselling. Therefore, the elucidation of essentially all LDL receptor gene mutations in our FH patients is a prerequisite for the development of nation-wide genetic testing for FH. At present, more than 600 mutations of the LDL receptor gene are known to underlie FH worldwide. However, the spectrum of mutations in different populations varies to a large extent. 

Previously, a number of LDL receptor mutations in the Dutch FH population were reported, including ten large rearrangements and 26 point mutations. While some mutations were also identified in other European countries, the majority were so far unique to the Dutch FH population. In the present study, we first sought to identify prevalent LDL receptor gene mutations and we therefore evaluated the frequency and geographical distribution of 13 known mutations in a cohort of 1223 FH patients, dispersed throughout the low countries. Second, in order to extend the spectrum of LDL receptor gene mutations, we conducted DNA analysis by denaturing gradient gel electrophoresis (DGGE) in 141 heterozygous FH patients with a definite clinical diagnosis based on internationally accepted criteria.

Material and methods

Patients
All patients were of Dutch descent and met the criteria for a diagnosis of heterozygous FH: LDL cholesterol above the 95th percentile for sex and age, presence of tendon xanthomata and history of premature atherosclerosis in the patient or in a first-degree relative.

For mutation analysis, 141 patients, referred to the Amsterdam Lipid Research Clinic of the Academic Medical Centre and the Slotervaart University Teaching Hospital, were recruited. In all patients, the presence of large rearrangements in the LDL receptor gene and of the R3500Q mutation of the ApoB gene was excluded. For the assessment of the frequency of specific mutations, 1223 samples were collected from 46 lipid clinics evenly distributed over the different regions of the country. All patients were referred to the regional lipid clinic by cardiologists, internists and general practitioners (GPs), based on suspected lipid disorders. A diagnosis of FH was given according to uniform diagnostic criteria established within the lipid clinic network. All patients were informed of the outcome of the test
by their GP. To the best of our knowledge, all patients, both in the small group for mutation analysis as well as in the large cohort for frequency of known mutations, were not related.

**DNA analysis**

Genomic DNA was isolated from whole blood samples, as previously described\(^ {18} \). For DGGE analysis, all 18 exons and the promoter region of the LDL receptor gene were individually amplified from genomic DNA by PCR using Super Taq DNA polymerase (HT Biotechnology, Cambridge, United Kingdom) as described previously\(^ {11} \), with the following modifications. Exon 4 was amplified in two overlapping fragments with pairs of primers as described by Hobbs et al.\(^ {4} \). PCR amplification of the promoter region was performed as described by Nissen et al.\(^ {19} \). All fragments, except for the 3' half of exon 4, were amplified with the same cycling protocol, which consisted of 94°C 5 min and 32 cycles of 94°C 1 min, 55°C 30 s, 72°C 1.5 min in an OmniGene Thermal cycler (Hybald, Ashford, United Kingdom). The 3' half of exon 4 required an annealing temperature of 62°C for PCR amplification. DGGE analysis was conducted as previously described.\(^ {11} \) Exons displaying aberrant migration patterns on DGGE gels were amplified and direct-solid phase DNA sequencing was performed as described by Hultman et al.\(^ {20} \). Sequencing data were compared to the normal LDL receptor cDNA sequence.\(^ {21} \)

**Mutation analysis**

Small deletions (up to five nucleotides) are detected by PCR amplification of the corresponding fragment followed by agarose gel electrophoresis in a 5% gel. Smaller rearrangements or single base substitutions are detected by restriction digest analysis of the PCR amplification product, in case the nucleotide change creates or abolishes a restriction site. In all other cases, mutation analysis is conducted by PCR-primer-introduced restriction analysis (PCR-PIRA). Primers required for PCR-PIRA are listed in Table 1. Restriction enzyme digest analysis was then conducted according to the manufacturer’s recommendation. Discrepancies were resolved by repeated PCR and analysis of independent DNA samples.
Table 1: List of oligonucleotides for PCR-primer-introduced restriction analysis

<table>
<thead>
<tr>
<th>Name of mutation</th>
<th>Location</th>
<th>5' oligonucleotide</th>
<th>3' oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>191-2 A→G</td>
<td>Intron 2</td>
<td>5'-TGACAGTTCAATCCTGTCTCTTCGAG-3'</td>
<td>5'-ACTCCCGACGACTGATAGCC-3'</td>
</tr>
<tr>
<td>C163R</td>
<td>Exon 4</td>
<td>5'-GTTCCGAGACTTACGCGGTAGGG-3'</td>
<td>5'-CTACTCTCTCTCTGGGAAAGACTAAACCCGGC-3'</td>
</tr>
<tr>
<td>C201X</td>
<td>Exon 4</td>
<td>5'-CAAGACAGGCGCTCTGGGCGG-3'</td>
<td>5'-CCATACCCAGATTTCTCTCGTACAGATTGTCCCT-3'</td>
</tr>
<tr>
<td>C234R</td>
<td>Exon 5</td>
<td>5'-GCCATCTCTCTGCGGCTCTCCGGC-3'</td>
<td>5'-CGCTGATCTGTGACAGATACG-3'</td>
</tr>
<tr>
<td>E273K</td>
<td>Exon 6</td>
<td>5'-GCCGCCCTCCGAGCCGAGAGATGAGCTGGTCTCAGACACAG-3'</td>
<td>5'-CGGCCGCCGAGCCGAGAGATGAGCTGGTCTCAGACACAG-3'</td>
</tr>
<tr>
<td>2140+5 G→A</td>
<td>Intron 14</td>
<td>5'-CGCCCCGGCGCGCGCGCGCGCGAGAACGACCGCGAGCTGGTCTCAGACACAG-3'</td>
<td>5'-GGTACCAGATAGCTGACAGATAGCAG-3'</td>
</tr>
<tr>
<td>V776L</td>
<td>Exon 16</td>
<td>5'-TCTGTGCCATTGTGCTTCTGCCAGGC-3'</td>
<td>5'-GTGATAACGAGACCGAGCTGCGGCC-3'</td>
</tr>
</tbody>
</table>

a The underline represents the mismatched nucleotide.

Results

Analysis of frequency and geographical distribution of known FH-causing mutations in the Dutch population

Restriction enzyme digest analysis of 13 different point mutations that are known to be responsible for FH in the Dutch population was conducted in a cohort of 1223 heterozygous patients. The type, location and frequency of the mutations analysed are summarised in Table 2.

Four mutations were found with a relatively high frequency. The most common mutations, with a frequency of 8%, are N543H and 2393del9, which are linked on the same allele.12 This double mutation has also been identified in Denmark22, although with a low frequency.

A splicing mutation in intron 9, 1359 - 1 G → A, is the second most common mutation with a frequency of 6.9%. This mutation appears to be frequent also in the neighbouring country of Belgium.23

The cluster of splicing mutations at intron 3, 313 + 1 G → A and G → C, 313 + 2 T → C, represents the third most frequent mutation (4.9%). At first, having no prior indication of their relative frequency11, we applied a protocol that allows their simultaneous detection. However, subsequent analysis tailored to the detection of each single defect revealed that all positives for this test were carriers of the 313 + 1 G → A variant. This finding is in agreement with the data on the frequency of this mutation in other countries.24,25

Finally, the W23X mutation, occurring with a frequency of 3.1%, is a recurrent
Table 2: Frequency of known mutations

<table>
<thead>
<tr>
<th>Name</th>
<th>Location Ref.</th>
<th>Detection method</th>
<th>Number of carriers (%)</th>
<th>Cohort size</th>
</tr>
</thead>
<tbody>
<tr>
<td>W23X</td>
<td>Exon 2</td>
<td>Stul+ (PIRA)</td>
<td>38 (3.1)</td>
<td>1 223</td>
</tr>
<tr>
<td>313+1 G → A</td>
<td>Intron 3</td>
<td>NdeI+ (PIRA)</td>
<td>61 (4.9)</td>
<td>1 223</td>
</tr>
<tr>
<td>C146X</td>
<td>Exon 4</td>
<td>Ddel +</td>
<td>5 (0.4)</td>
<td>869</td>
</tr>
<tr>
<td>E207X</td>
<td>Exon 4</td>
<td>MnII -</td>
<td>21 (1.7)</td>
<td>1 223</td>
</tr>
<tr>
<td>S285L</td>
<td>Exon 6</td>
<td>Msel +</td>
<td>11 (0.9)</td>
<td>1 223</td>
</tr>
<tr>
<td>R329X</td>
<td>Exon 7</td>
<td>HhaI- (PIRA)</td>
<td>0</td>
<td>563</td>
</tr>
<tr>
<td>V408M</td>
<td>Exon 9</td>
<td>NlaIII +</td>
<td>11 (0.9)</td>
<td>1 223</td>
</tr>
<tr>
<td>1358+1 G → A</td>
<td>Intron 9</td>
<td>BstEII- (PIRA)</td>
<td>1</td>
<td>563</td>
</tr>
<tr>
<td>1359-1 G → A</td>
<td>Intron 9</td>
<td>Ddel -</td>
<td>78 (6.9)</td>
<td>1 223</td>
</tr>
<tr>
<td>N543H</td>
<td>Exon 11</td>
<td>NcoI+</td>
<td>98* (8)</td>
<td>1 223</td>
</tr>
<tr>
<td>P664L</td>
<td>Exon 14</td>
<td>PstI +</td>
<td>4 (0.32)</td>
<td>1 223</td>
</tr>
<tr>
<td>2393del19</td>
<td>Exon 17</td>
<td>S% agarose</td>
<td>98* (8)</td>
<td>1 223</td>
</tr>
<tr>
<td>R35OM(Apo13)</td>
<td>Exon 26</td>
<td>Mspl- (PIRA)</td>
<td>30 (2.4)</td>
<td>1 223</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>358 (29.2)</td>
<td></td>
</tr>
</tbody>
</table>

*N543H and 2393del9 are linked on the same allele.

mutation in several European countries and in the United States. During the course of screening, it became apparent that some mutations are rare and, hence, a smaller number of patients were screened. To evaluate whether a correlation between the frequency and the geographical distribution of these mutations can be established, each mutation was classified according to the region of origin of the carriers (data not shown).

Interestingly, the two most common mutations are most frequently found in specific regions of the country (Figure 1). Fifty-four carriers (69%) of the 1359-1 G → A mutation originate from the province of North-Brabant, in the mid-southern part of the country, while 65 carriers (67%) of the N543H-2393del9 mutation originate from the province of North-Holland in the north-west part of The Netherlands and, more specifically, from the district West-Friesland. The intron 3 mutation, 313 + 1 G → A is more frequently found in provinces along the eastern border of the country (Groningen and Gelderland). The W23X mutation in exon 2 is particularly frequent in the northern provinces (Friesland, Groningen and Drenthe). All other mutations were too rare to establish a local preference.
Identification and characterisation of new mutations

One hundred and forty-one, apparently unrelated, heterozygous FH patients were screened for sequence alterations in all 18 exons of the LDL receptor gene and in the promoter region by DGGE. Prior to DGGE analysis, the presence of large rearrangements(26) and of the R3500Q variant of the ApoB gene(2) was excluded in this group. Nucleotide alterations were revealed by the presence of two or more DNA bands on DGGE gels compared with a single band in normal samples and the underlying molecular defect was then characterised by direct sequencing. A mutation was identified in 111 patients, yielding a detection rate of 80%. In addition to a number of mutations that have been previously reported in the Dutch FH population11-17, we found a number of mutations that were previously not known in the Dutch population, but that have been found in other countries (Table 3). These include the A29S(27), the C134G FH-Germany(4), the 1061-8
(T → C)(28), the E336K FH-Paris 7(4) and the V8061 FH-New York 5(4).

However, the vast majority (20 out of 25) of the variants do not appear to have been reported previously in the literature (Table 3). One new polymorphism in exon 4 (C → T at 621, G186G) is also presented in Table 3.

All mutations and polymorphisms have been identified in only 1 or 2 individuals, indicating that these variants are rare.

**Missense mutations**

Twelve missense mutations, which occur most frequently[^4], were identified. Five of them, C113R and C163R in exon 4, C234R in exon 5, C331W and C317G in exon 7, involve a cysteine residue, indicating that these mutations are most probably pathogenic.

The V776L mutation is caused by a G → T transversion at the last nucleotide of exon 16, which changes the codon for valine at position 776 into a leucine. Therefore, we classified it as a missense mutation. However, this nucleotide is also part of the signal sequence required for correct splicing of the intron[^29] and it is most likely that his mutation is responsible for FH also through impairment of this mechanism.

**Splicing mutations**

Four new splicing mutations were identified. Two of them, 191 - 2 A → G in intron 2 and 2389 + 1 G → T in intron 16, affect the invariant AG and T dinucleotide of acceptor and donor sites, respectively[^29]. The third, 67-5del4, is a four nucleotide deletion which severely disrupts the splicing consensus sequence at the acceptor site of intron 1, including the AG invariant dinucleotide. The fourth splicing mutation, 2140 + 5 G → A in intron14, does not involve nucleotides that are essential or splicing. However, a G nucleotide at position + 5 of the 5' donor splice site consensus sequences is known to be associated with a consensus value of 0.84[^29]. Therefore, it is plausible to assume that the 2140 + 5 G → A mutation is also an FH causing mutation.

**Insertions and deletions**

We have reported two new deletions in Table 3. One is a five nucleotide deletion in exon 16, which results in a frameshift and premature termination at codon 765, just a few bases downstream from the deletion. The second one is an in-frame 12 bp deletion in exon 14, which deletes four amino acids (Gln657 - Tyr658 - Leu659 - Cys660) of the protein sequence, including a cysteine residue.

The only insertion detected (Table 3) consists of a duplication of 18 nucleotides in
<table>
<thead>
<tr>
<th>Location Method</th>
<th>Nucleotide change</th>
<th>Effect on coding sequence</th>
<th>Name</th>
<th>Type</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 1*</td>
<td>DelCTCA at 67-5</td>
<td>3' splice acceptor</td>
<td>67-5del4</td>
<td>Splicing</td>
<td>Ddel-</td>
</tr>
<tr>
<td>Exon 2</td>
<td>G → T at 147</td>
<td>Ala → Ser at 29</td>
<td>A295</td>
<td>Missense</td>
<td>TspRI+ (PIRA)</td>
</tr>
<tr>
<td>Intron 2</td>
<td>A → G at 191-2</td>
<td>3' splice acceptor</td>
<td>190-2 A → G</td>
<td>Splicing</td>
<td>Avall+</td>
</tr>
<tr>
<td>Exon 4</td>
<td>T → C at 400</td>
<td>Cys → Arg at 113</td>
<td>C113R</td>
<td>Missense</td>
<td>Hvpl+</td>
</tr>
<tr>
<td>Exon 4</td>
<td>T → G at 463</td>
<td>Cys → Gly at 134</td>
<td>C134G</td>
<td>Missense</td>
<td>MspI+ (PIRA)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>C → A at 666</td>
<td>Cys → Stop at 201</td>
<td>C201X</td>
<td>Nonsense</td>
<td>PstI+ (PIRA)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>C → T at 621</td>
<td>Gly → Gly at 186</td>
<td>G186G</td>
<td>Silent</td>
<td>Pvull</td>
</tr>
<tr>
<td>Exon 4</td>
<td>T → C at 551</td>
<td>Cys → Arg at 163</td>
<td>C163R</td>
<td>Missense</td>
<td>Hhal+ (PIRA)</td>
</tr>
<tr>
<td>Exon 5</td>
<td>G → A at 759</td>
<td>Arg → Tryp at 232</td>
<td>R232W</td>
<td>Missense</td>
<td>MnlI+ (PIRA)</td>
</tr>
<tr>
<td>Exon 5</td>
<td>T → C at 765</td>
<td>Cys → Arg at 234</td>
<td>C234R</td>
<td>Missense</td>
<td>Acc+</td>
</tr>
<tr>
<td>Exon 6</td>
<td>A → G at 880</td>
<td>Lys → Glu at 273</td>
<td>E273K</td>
<td>Missense</td>
<td>SfoNl-</td>
</tr>
<tr>
<td>Exon 7</td>
<td>G → T at 1004</td>
<td>Gly → Val at 314</td>
<td>C314V</td>
<td>Missense</td>
<td>Clal-</td>
</tr>
<tr>
<td>Exon 7</td>
<td>C → G at 1056</td>
<td>Cys → Trp at 331</td>
<td>C331W</td>
<td>Missense</td>
<td></td>
</tr>
<tr>
<td>Exon 7</td>
<td>T → G at 1012</td>
<td>Cys → Gly at 317</td>
<td>C317G</td>
<td>Missense</td>
<td></td>
</tr>
<tr>
<td>Intron 7</td>
<td>T → C at 1061-8</td>
<td>3' splice acceptor</td>
<td>I334V</td>
<td>Splicing</td>
<td></td>
</tr>
<tr>
<td>Exon 8</td>
<td>A → G at 1063</td>
<td>Ile → Val at 334</td>
<td>E336K</td>
<td>Missense</td>
<td></td>
</tr>
<tr>
<td>Exon 8</td>
<td>G → A at 1067</td>
<td>Glu → Lys at 336</td>
<td>D412Y</td>
<td>Missense</td>
<td>Rsal+</td>
</tr>
<tr>
<td>Exon 9</td>
<td>G → T at 1298</td>
<td>Asp → Tyr at 412</td>
<td>P542H</td>
<td>Missense</td>
<td>HaeIII-</td>
</tr>
<tr>
<td>Exon 11</td>
<td>C → A at 1688</td>
<td>Pro → His at 542</td>
<td>2032 (12del)</td>
<td>Frameshift</td>
<td>5% agarose gel</td>
</tr>
<tr>
<td>Exon 14</td>
<td>A → G at 2140+5</td>
<td>Del Gin657 Tyr588 Leu658, Cys660</td>
<td>2140+5 G → A</td>
<td>Frameshift</td>
<td>BfaI+ (PIRA)</td>
</tr>
<tr>
<td>Exon 16</td>
<td>DelGAAGA at 2343</td>
<td>5' splice donor</td>
<td>2343 del5 Stop 765</td>
<td>Splicing</td>
<td>5% agarose gel</td>
</tr>
<tr>
<td>Exon 16</td>
<td>G → T at 2389</td>
<td>Val → Leu at 776</td>
<td>V776L</td>
<td>Missense/Splicing</td>
<td>Pvull+ (PIRA)</td>
</tr>
<tr>
<td>Intron 16</td>
<td>G → T at 2389+1</td>
<td>5' splice donor</td>
<td>2389+1 G → T</td>
<td>Splicing</td>
<td>Msel+</td>
</tr>
<tr>
<td>Exon 17</td>
<td>18 bp duplication</td>
<td>Duplication aa 782-787</td>
<td>ins 782-787</td>
<td>Insertion (in-frame)</td>
<td>5% agarose gel</td>
</tr>
<tr>
<td>Exon 17</td>
<td>G → A at 2479</td>
<td>Val → Ile at 806</td>
<td>Y8061</td>
<td>Missense</td>
<td></td>
</tr>
</tbody>
</table>

* Bold characters indicate novel mutations.
exon 17, resulting in the in-frame insertion of six amino acids (Cys782 - Leu783 - Gly784 - Val785 - Phe786 - Leu787) at the end of the transmembrane domain of the receptor protein.

**Discussion**

The main objective of our analysis was to delineate the full spectrum of mutations that underlie FH in the Dutch population, which represents a first step towards the implementation of nation-wide DNA testing for this disease. Two different approaches were used: first, we estimated the prevalence of a number of mutations that are already known to be responsible for FH in the Dutch population and, second, we searched for additional defects by DGGE screening of a group of patients with an unequivocal clinical diagnosis of FH.

We believe that a molecular diagnosis for FH offers several advantages. It is the only approach that provides a definitive diagnosis, which might become essential before considering gene therapy. Molecular diagnosis is also crucial in family studies, allowing identification of affected family members, who require cholesterol lowering, while non-affected sibs can be reassured. Identification of a significant number of carriers of a similar mutation will also aid in studying genotype-phenotype relationships as well as establish the relationship between a given mutation and the response to lipid-lowering therapy.

In order to evaluate the prevalence of mutations already known to be responsible for FH in the Dutch population, we selected 13 mutations that in previous studies appeared in more than 1 individual. Their frequency was tested in a large cohort of FH patients, collected through 46 lipid clinics, evenly distributed among our provinces. Therefore, we consider these patients as representative of the overall FH population in The Netherlands. Out of 1223 FH patients, 358 carriers of the 13 different mutations were identified, representing 29% of the patients analysed. In this group, we identified 30 carriers of the R3500Q mutation in the ApoB gene, which represents 2.4% of the cohort examined. These data are consistent with the frequency reported in other countries. Contrary to the small group of patients analyzed for new variants, we did not analyze this large cohort of patients for the presence of large rearrangements. Considering that these have been reported with a frequency of about 6% in the Dutch population, inclusion of the large rearrangements in the screening strategy of this group would have increased the number of carriers identified up to 35%.
Four LDL receptor gene mutations were detected with a relatively high frequency: the double mutation N543H and 2393del9, 1359 - 1 G → A, 313 + 1 G → A and W23X. Screening for these four mutations alone would have resulted in the identification of 275 carriers (22.4% of all patients analyzed). This indicates that an estimate of the prevalence of specific mutations in a given population can facilitate the selection of a strategy for the implementation of mutation analysis.

With the exception of a few populations, including the French-Canadians, Christian Lebanese, Druze, Finns and Afrikaner, in which a small number of mutations explain the majority of FH cases, in most European countries and in North America founder effects do not exist. The preferential geographical distribution of the two most common Dutch FH mutations in specific areas of the country, Brabant for the 1359 - 1 G → A and West-Friesland for the double N543H and 2393del9 mutation, represents an unexpected finding. While the origin of a possible founder effect in the mid-southern part of the country is unclear, the high prevalence of the double mutation in West-Friesland may be attributed to geographical isolation. From the 13th century onward, this region was separated from the mainland by water and marshes and this situation remained unaltered until the 17th century, when large areas in North-Holland were reclaimed due to rapid expansion of the population. Interestingly, the same double mutation has also been recently identified in two small FH families in Denmark.

We also report the identification of 16 new single base substitutions and four small deletions and insertions, which, added to the point mutations and large rearrangements previously identified in the LDL receptor gene of Dutch FH patients, brings the number of FH-causing mutations in this country up to 61. Although the actual frequency of the newly identified mutations needs to be evaluated in a larger group of patients, none of them were detected in more than 1 or 2 patients, suggesting that these mutations are rare.

In general, several arguments suggest that the newly identified mutations are pathogenic. First, with the only exception of the 2140 + 5 mutation in intron 14, these were the only nucleotide changes found after scanning of the entire coding and splice site consensus sequences of the LDL receptor gene. Second, these changes do not occur in the normal Dutch population, as assessed by DGGE screening of 100 control DNA samples. In addition, with the exception of only one family in which the 2140 + 5 mutation is found in combination with the E207K variant, in all other cases family analysis shows a clear pattern of co-segregation of the mutation with clinical signs of FH, such as an elevated LDL cholesterol.

Out of the 12 missense mutations, five involve cysteine residues and are most likely
to result in defective protein receptors due to misfolding. For the remaining seven, expression of the mutant genes in mammalian cells followed by functional studies of the mutant receptors is required to definitively demonstrate their pathogenicity. In 30 out of the 141 FH patients initially included in the screening strategy for new LDL receptor defects, we were not able to detect any variant by DGGE, in spite of the definite clinical diagnosis, of the exhaustive screening approach, with the inclusion of all exons, promoter region and splice site consensus sequences and of the exclusion of large rearrangements, that cannot be detected by DGGE and of the R3500Q variant in the ApoB gene.

A similar detection rate (~80%) was also obtained in a previous study, in which a smaller group of patients was examined. One possible explanation lies in the detection limit of the DGGE technique itself. Although DGGE has been proven to be a powerful detection method, it is known that it is not able to detect all mutations, possibly because of their peculiar position in the fragment or because of the surrounding sequence composition in the fragment to be analysed. In these cases a second round of screening with a different detection method (e.g. single strand conformation polymorphism (e.g. [SSCP]), increases the chances of detecting nearly all variants.

A second plausible explanation implies that the mutation is located in a gene other than the LDL receptor. New evidence supporting this hypothesis has recently been given by Varret et al. and implicates a new locus, named FH 3, that might be responsible for a phenotype indistinguishable from the classical FH according to the clinical traits.

Considering the large variability of the spectrum of Dutch FH mutations and the fact that most mutations are rare, the application of a molecular diagnostic test for routine screening of all mutations requires a strenuous effort.

In this respect, the recent development of high-throughput methodologies like the oligonucleotide ligation assay (OLA) or multiplex allele-specific diagnostic assay (MASDA) that enable simultaneous analysis of a large number of known mutations (> 100) in a single assay might offer a valid alternative to the laborious and time consuming assays for specific mutations.

Although the number of Dutch FH mutations identified is high, the database of all FH-causing mutations in the Dutch population is far from complete. It has been estimated that there are about 40,000 FH heterozygotes in the Dutch population. Considering that so far in this as well as in previous studies only a limited number of patients have been scanned for unknown defects and that most mutations are present in single families, the actual number of Dutch FH mutations may be much
higher. Therefore, predictions on the total number of LDL receptor defects accounting for FH in our population cannot be made and, as a consequence, estimates of the predictive value of a multiplex testing protocol remain inaccurate. Continuation of this approach may increase the number of LDL receptor mutations to several hundreds. For this reason, screening for new mutations by DGGE remains a major objective of our laboratory.

In addition, strict selection criteria were applied to the patients included in this study. Therefore, mutations leading to a mild phenotype of FH might have been missed. A multiplex testing protocol would therefore result in a relatively low predictive value, inherent to the selection criteria applied.

In summary, with the work described in this paper we have obtained more insight into the molecular basis of FH in The Netherlands. Sixty-one different mutations have been found to be responsible for the disease: these include ten large rearrangements, reported elsewhere\textsuperscript{8,9} and 51 point mutations. Out of these, 20 are novel mutations. The vast majority of mutations have been found in 1 or 2 individuals. Only four mutations present with a relatively high frequency and, altogether, account for 22% of all patients examined.

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