

# CLINICAL VERSUS MOLECULAR DIAGNOSIS OF HETEROZYGOUS FAMILIAL HYPERCHOLESTEROLAEMIA IN THE DIVERSE SOUTH AFRICAN POPULATION

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*Objective.* Familial hypercholesterolaemia (FH) is a common genetic disease characterised by strikingly elevated plasma cholesterol concentration, which can lead to premature coronary death if left untreated. In this study DNA diagnosis of FH, which allows detection before onset of clinical symptoms, was evaluated against biochemical parameters routinely used to identify subjects with FH.

*Design.* A population-based strategy was used to identify low-density lipoprotein receptor (LDLR) gene defects in South Africans with clinical signs of FH, followed by a family-based DNA screening approach for presymptomatic diagnosis of FH.

*Results.* DNA screening of 790 at-risk relatives for the FH-related mutations identified in 379 index cases, allowed accurate disease diagnosis in an additional 338 relatives and exclusion of the relevant mutation in 452 individuals. The sensitivity and specificity of the diagnosis, based on total cholesterol values measured in family members of FH heterozygous index cases with one of the three founder-related mutations, D154N, D206E and V408M, were 89.3% and 81.9%, respectively.

*Conclusion.* The predominance of 10 LDLR gene mutations in the local population justifies population-directed DNA diagnosis of FH in South Africa on a routine basis, particularly since expression of the defective gene measured in biochemical tests does not allow accurate diagnosis of FH in all cases. DNA testing provides a definitive tool for family tracing aimed at pre-clinical diagnosis and preventive treatment of FH.

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1053

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The diagnosis of familial hypercholesterolaemia (FH) is based on clinical findings and elevated low-density lipoprotein (LDL) cholesterol levels. The rare homozygous form of the disease is characterised by severe clinical features, including tendon xanthomas and atherosclerosis, usually associated with early coronary death during childhood. Most FH heterozygotes carrying one defective low-density lipoprotein receptor (LDLR) gene do not present with cholesterol deposits in the skin and tendons, which complicates disease diagnosis and consequently preventive treatment.<sup>1</sup> DNA analysis may therefore be more appropriate for the diagnosis of heterozygous FH, particularly in homogeneous populations where a limited number of LDLR gene mutations account for the disease in the majority of cases.

The diverse South African population provides a valuable source of material for genetic studies. Initial studies of FH patients focused on the Afrikaner population, considered to be a genetic isolate because of its geographical and historical situation. The predominance of specific haplotypes in this population group<sup>2,3</sup> confirmed the occurrence of a founder effect,<sup>4</sup> and led to the identification of three mutations that are responsible for the disease in approximately 90% of Afrikaner FH patients.<sup>5,7</sup> The value of a routinely used DNA-based test was demonstrated,<sup>8,9</sup> necessitating the development of a cost-effective method to screen for multiple mutations in a single reaction.<sup>10</sup> Population screening by direct detection of the founder mutations D154N, D206E and V408M, confirmed the high prevalence of heterozygous FH (1/70) in Afrikaners.<sup>11</sup> Loubser *et al.*<sup>12</sup> demonstrated that these mutations are also responsible for the disease in 15 - 20% of South African FH patients of mixed ancestry (coloured population). This finding, as well as frequent detection of two LDLR gene mutations causing FH in the majority of South African Jews (del 3-bp)<sup>13</sup> and Indians (P664L),<sup>14,15</sup> provided direct genetic evidence that Caucasoid admixture contributes significantly to the FH phenotype in South Africans of mixed ancestry. A 6-bp deletion identified in a Xhosa with homozygous FH<sup>16</sup> appeared to be absent in the coloured population.<sup>12</sup> Frequent detection of this deletion-mutation in FH patients from the South African black population,<sup>17</sup> where this disease appears to be extremely rare, suggests that FH may be underdiagnosed in this group. This finding supports the notion that clinical criteria for the diagnosis of FH need to differ by country/population.<sup>18</sup>

DNA screening for FH should be a priority in South Africa where specific founder-type mutations contribute significantly to the high death rate from coronary heart disease (CHD) in several population groups. However, FH is largely underdiagnosed locally, such that most affected individuals are not treated to prevent unnecessary early deaths due to heart attacks. Routine screening is ongoing to identify the disease-causing mutation in patients referred for molecular diagnosis, but family follow-up is rarely performed. This is an

unfortunate situation, since tracing of defective LDLR genes in families would facilitate accurate diagnosis of FH or exclusion of the disease in at-risk relatives. Most importantly, it would identify those family members at high risk of developing CHD, so that cholesterol levels can be lowered or normalised by dietary and/or drug treatment.

In an attempt to identify and assist families with FH, an international project aimed at Making Early Diagnosis to Prevent Early Deaths in MEDical PEDigrees (MED-PED) was initiated, which currently involves more than 30 countries worldwide. In a report by Williams *et al.*<sup>19</sup> on this family-based case-finding approach, it was suggested that more rigid cholesterol screening should be used for persons in the general population whose chance before cholesterol testing may be only 1 in 500 (1 in 70 among Afrikaners), in contrast to first-degree relatives of a confirmed FH case whose chance before cholesterol testing is 50%. In this study the MED-PED approach was followed to screen family members of molecularly characterised FH index patients for known mutations in the LDLR gene. In order to extend the spectrum of LDLR gene mutations, clinically diagnosed FH index cases without known mutations were subjected to extensive screening of the promoter and coding region of the LDLR gene. The overall objective of the study was to determine what percentage of adult FH patients heterozygous for mutations D154N, D206E or V408M could be diagnosed accurately on the basis of raised total cholesterol levels.

## MATERIALS AND METHODS

### Subjects

The initial study population consisted of hypercholesterolaemics referred for a molecular diagnosis of FH, based on previously described criteria.<sup>2,9</sup> For this study, follow-up mutation screening was performed/extended in families where the FH-related mutation had been identified in the index case. Index cases without known mutations were subjected to extensive mutation screening followed by mutation screening in relevant at-risk family members. In these index patients pretreatment total serum cholesterol (TC) levels had to be at least equal to the 90th percentile for age and gender,<sup>20</sup> with normal serum triglyceride (TG) levels (< 2.3 mmol/l). In addition, an FH study participant had to have either clinical features of FH (tendon xanthoma of the Achilles tendon or tendons on the dorsum of the hand with or without xanthelasma) or a family history of early CHD. We report on all the mutation-positive cases identified in this study, together with mutation data described previously in South African families (Table I). Known mutations causing familial defective apolipoprotein B-100 (FDB) were excluded in all the study participants using previously described methods.<sup>21,22</sup>



Table I. Spectrum of mutations identified in the LDLR gene in different South African population groups

Exon/ intron	Molecular event	Designation	No.	Relatives tested
Afrikaner population				
Exon 3	T→G at 259	W66G*	1	None
Exon 4	G→A at 523	D154N	31	55 +, 105 -
Exon 4	A→G at 662	D200G	5	3 +, 1 -
Exon 4	Ins 18-bp after 681	Ins AA 201-206	1	1 +, 5 -
Exon 4	C→G at 681	D206E	144	144 +, 194 -
Exon 6	C→T at 917	S285L	3	2 +, 2 -
Exon 7	C→T at 1048	R329X	1	None
Exon 8	G→A at 1130	C356Y†	4	5 +, 1 -
Exon 8	G→T at 1145	G361V†	2	3 +, 4 -
Exon 9	G→A at 1285	V408M	57	51 +, 61 -
Exon 16	G→A at 2389	V776M	1	None
Mixed ancestry (coloured population)				
Promoter	C→T at -59	-59c→t	1	3 +, 14 -
Exon 1	T→C at 28	W-12R	1	None
Exon 2	G→A at 148	A29T	1	None
Exon 3	C→T at 232	R57C	1	None
Exon 3	C→T at 241	R60C	1	None
Exon 4	del TC after 368	del 2-bp	1	None
Exon 4	G→A at 523	D154N	2	None
Exon 4	Del GGT after 651	del 3-bp	4	None
Exon 4	A→G at 662	D200G	1	None
Exon 4	A→G at 680	D203A	1	None
Exon 4	C→G at 681	D206E	19	4 +, 4 -
Exon 4	G→A at 682	E207K	1	None
Exon 4	T→G at 691	C210G	1	None
Exon 5	G→A at 772	E237K*	1	4 +, 7 -
Intron 6	G→A at 941-4	941-4 G→A	1	None
Intron 6-8	del 2.5-kb	del 2.5-kb	10	2 +
Exon 8	T→G at 1154	L364R	1	None
Exon 9	G→A at 1285	V408M	13	
Exon 14	C→T at 2054	P664L	3	None
Black population				
Promoter	del CTC after -92	del 3-bp	1	None
Exon 2	del 6-bp after 138	del 6-bp	4	1 +, 2 -
Exon 2	del G at 172	del 1-bp	1	None
Intron 3	G→A at 313+1	313+1G→A	1	4 -
Exon 4	G→C at 514	D151H	1	None
Exon 5	C→T at 756	R232W	1	1 +, 3 -
Exon 9	G→A at 1217	R385Q	1	None
Exon 9	G→A at 1222	E387K	1	5 +, 5 -
Exon 14	C→T at 2096	P678L	1	None
Exon 17	G→A at 2441	R793Q	1	None
Indian population				
Exon 1	A→T at 1	M-21L	1	2 +, 1 -
Exon 3	C→T at 232	R57C	1	None
Exon 3	G→T at 268	D69Y	1	None
Exon 4	G→A at 418	E119K	1	None
Exon 4	G→T at 661	D200Y	1	2 +, 3 -
Exon 4	G→A at 682	E207K	2	8 +, 7 -
Exon 8	C→A at 1175	C371X	1	11 +, 9 -
Exon 9	C→G at 1215	N384K	1	3 +, 2 -
Exon 14	C→T at 2054	P664L	10	None
Exon 16	A→T at 2356	S765C	1	4 +, 2 -



Table I. Continued

		Jewish population		
Exon 3	C→T at 253	Q64X	2	1 +
Exon 4	C→T at 373	Q104X*	1	None
Exon 4	del GGT after 651	del 3-bp	5	1 +
Exon 4	C→G at 681	D206E	1	2 -
Intron 9	G→A at 1358+1	1358+1 G→A*	1	None
Exon 9	C→G at 1284	N407K*	1	2+
Exon 9	G→A at 1285	V408M	1	None
Intron 14	G→A at 2140+5	2140+5G→A	1	None
		European ancestry		
Exon 3	C→T at 232	R57C	1	None
Exon 4	Ins G at 558	558 ins G	1	1 +, 3 -
Exon 4	del G at 617	617 del G	1	None
Exon 4	C→G at 681 <sup>§</sup>	D206E	1	None
Exon 6	G→A at 910	D283N	1	None
Exon 7	C→T at 1048	R329X	1	None
Exon 8	A→C at 1133	Q357P	1	None
Exon 8	C→T at 1150	Q363X	1	3 +, 4 -
Exon 8	C→G at 1156	D365E	1	3 +, 4 -
Exon 9	G→C at 1329	W422C	1	None
Exon 10	Complex del/ins	16-bp del/5-bp inst <sup>†</sup>	1	None
Exon 10	T→C at 1447	W462R	1	None
Exon 11	G→A at 1646	G528D	1	2+
Exon 11	A→C at 1690	N543H	1	None
Exon 14	C→A at 2043	C660X	2	7 +, 2 -
Exon 14	del T at 2092	2092 del T	1	4+, 1 -
Intron 14	G→A at 2140+5	2140+5G→A	1	None
Exon 17	del 9-bp after 2393	2393 del 9-bp	1	None

\* M Callis and R Thiart — unpublished data.

† Novel mutation identified in this study.

The majority of mutations summarised in this table are included in FH databases <http://www.umd.necker.fr> and <http://www.ucl.ac.uk/fh>. Relatively common mutations that may represent founder mutations are highlighted. The number of family members who tested positive (+) or negative (-) for the mutation identified in the index case is indicated.

The study protocol was approved by the Ethics Review Committee of the University of Stellenbosch and all blood samples were obtained with informed consent. In this study 'White or Afrikaner' refers to an individual of European descent, mainly Dutch, French, German and British origin; 'coloured' refers to an individual of mixed ancestry, including San, Khoi, African Negro, Madagascar, Javanese and European origin; and 'black' refers to South Africans of central African descent.

### DNA analysis

Genomic DNA was extracted from whole blood collected in EDTA-containing tubes, according to a standard technique.<sup>23</sup> Polymerase chain reaction (PCR) amplification of the LDLR gene was performed using the exon-specific primers described by Jensen *et al.*,<sup>24</sup> or allele-specific primers specially designed for detection of mutations known to be common in South Africa.<sup>10</sup> Heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis<sup>10</sup> was performed using three different gel systems to improve mutation detection efficiency: 10% polyacrylamide gel supplemented with 7.5%

urea; 10% polyacrylamide gel supplemented with 5% glycerol and 20% polyacrylamide gel. Electrophoresis was carried out overnight at room temperature and at 4°C on a 20 cm Hoeffer gel apparatus. When no aberrant patterns could be detected using the HEX-SSCP method, denaturing gradient gel electrophoresis (DGGE) was performed as described by Nissen *et al.*<sup>22</sup> PCR products showing aberrant patterns were sequenced with the automated ABI 373 system. Where appropriate, restriction enzyme analysis was performed for confirmation, to screen for known mutations, and to trace specific mutations in families.

### Clinical evaluation

Evaluation of biochemical versus DNA diagnosis was performed in families with the Afrikaner founder mutations D206E (FH1), V408M (FH2) or D154N (FH3), the deleterious effects of which have previously been confirmed at the cellular level.<sup>25,26</sup> Since index patients had been selected on the basis of elevated TC levels, genotype/phenotype correlation studies were only performed on family members recruited through tracing of defective genes in the pedigrees.



## RESULTS

The spectrum of mutations in the South African population, including 65 different mutations identified in 379 index cases, is summarised in Table I. Of the 790 at-risk relatives analysed, 338 inherited the disease-related LDLR gene mutation. In addition to the earlier reports on Afrikaners<sup>8,9</sup> and Jews,<sup>13</sup> detailed data have recently been published on families from the coloured,<sup>12</sup> black<sup>17</sup> and Indian<sup>14,15</sup> populations.

Segregation analysis of mutation S285L identified in an Afrikaner family (Fig. 1) demonstrated that one of the mutation-negative children (II-3) had a TC concentration above the 80th percentile (4.9 mmol/l), which might have been falsely classified as heterozygous FH in the absence of the DNA test. This finding was in accordance with previous data of Kotze *et al.*<sup>27</sup> who demonstrated that a value of 6 mmol/l might best discriminate between mutation-positive and -negative children within Afrikaner FH families.

In Table II, 443 at-risk family members (above the age of 18 years) screened for one of the three Afrikaner founder mutations (D154N, D206E or V408M) previously identified in the index case, were grouped according to the presence of TC levels above the 80th and 95th percentile for age and gender. Evaluation of biochemical versus DNA diagnosis revealed that

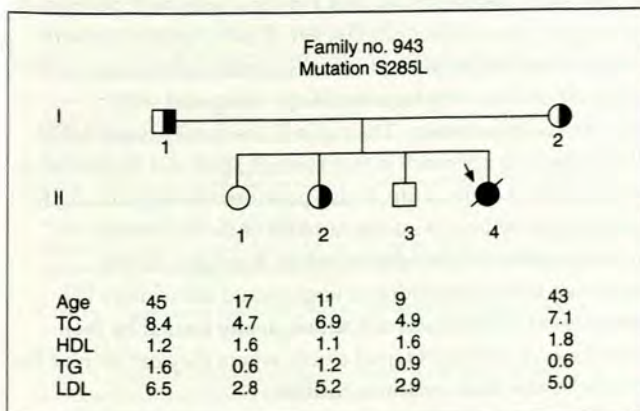


Fig. 1. Segregation analysis of mutation S285L in an Afrikaner family where one of the children (indicated by an arrow) died at the age of 6 years of a heart attack. Mutation-positive cases are indicated by dark-shaded symbols. (TC = total cholesterol; HDL = high-density lipoprotein cholesterol; TG = triglycerides; LDL = low-density lipoprotein cholesterol.)

15.6% of cases may be misdiagnosed when the 80th percentile is used as a biochemical cut-off point for a diagnosis of FH, compared with 12.4% using the 95th percentile<sup>20</sup> for age and gender. In total, 16/150 relatives (10.7%) with an FH mutation were falsely classified as normal (negative predictive value of 89.3%), while 53/293 (18.1%) without the mutation were falsely classified as FH heterozygotes (positive predictive value of 81.9%). The sensitivity and specificity of FH diagnosis according to TC values (80th percentile) were therefore 89.3% and 81.9%, respectively.

## DISCUSSION

The mutational spectrum underlying lipid abnormalities differs among population groups. Knowledge of the spectrum of gene defects causing primary hypercholesterolaemia in a specific population and in affected families allows accurate disease diagnosis and preventive treatment. The presence of at least 10 founder-type LDLR gene mutations in the South African population (highlighted in Table I) enhances the prospects of DNA-based diagnosis of FH in this country. After exclusion of the three previously-described Afrikaner founder mutations, D206E, V408M and D154N, a further eight mutations were identified in this population group. Four of these mutations, D200G, S285L, C356Y and G361V, detected in 14 Afrikaner families, probably represent minor founder mutations of which the associated haplotypes have been defined previously using 10 LDLR gene polymorphisms.<sup>3</sup>

The novel insertion/deletion mutation identified in exon 10 was absent in more than 100 normal chromosomes screened. This complex mutation deletes 16 bases from nucleotide 1379 to 1394 (ACGGCGTCTCTCCTA) and is replaced by five bases (CAGCT); and six amino acids, His439-Gly440-Val441-SER442-Ser443-Tyr444, were replaced by two amino acids, Pro439-Ala440. The insertion results in a stop codon at amino acid position 441 and is therefore highly likely to affect the LDLR function and cause FH.

In the family with mutation S285L where both parents were heterozygous for this mutation, one of the children died at the age of 6 years of a heart attack. This event was most likely caused by the inheritance of two copies of mutation S285L. Since termination of a pregnancy is justified in the case of an

Table II. Evaluation of biochemical versus DNA diagnosis in family members of Afrikaner index patients (> 18 years) with disease-causing LDLR gene mutations

Mutation	Number of relatives	> 95th percentile (%)	> 80th percentile (%)
D206E	93	61 (66)	82 (88)
V408M	27	20 (74)	23 (85)
D154N	30	25 (83)	29 (97)
No mutation	293	12 (4)	53 (18)
Total	443		



FH homozygous fetus, a prenatal diagnosis of FH was recently performed in an Afrikaner family with the founder mutation D206E.<sup>28</sup> The parents requested this procedure because they already had one severely affected child shown to be homozygous for this LDLR gene mutation. The DNA test results indicated a normal fetus, which led to continuation of the pregnancy. Since the risk of homozygous FH offspring is extremely high (25%) in cases where both parents have heterozygous FH, prenatal diagnosis of this condition is of particular importance in the South African population with an increased incidence of certain disease-related LDLR gene mutations.

In this study the evaluation of biochemical versus DNA diagnosis, previously performed in 220 children between the ages of 2 and 18 years,<sup>27</sup> was extended in an Afrikaner adult group including 443 close relatives of 232 index patients with one of the common founder mutations D154N, D206E or V408M. Although the majority of mutation-positive cases were identified for the first time during mutation screening, the relatively low sensitivity value of 89.3%, compared with 93% in the sample of children, could be attributable to altered dietary habits in FH families or influence of other external factors that are more prevalent in adulthood (e.g. smoking, diabetes). The specificity value also differed between the adult (81.9%) and child (89%) samples, which demonstrated that TC levels as a diagnostic means are more accurate in children than in adults. DNA tests are therefore the preferred method for the diagnosis of FH.

Separate evaluation of the Afrikaner founder mutations versus TC levels (> 80th percentile) suggests that the penetrance of mutation D154N is the highest of the three Afrikaner founder mutations. The estimated sensitivity of detecting this receptor-defective mutation<sup>26</sup> using biochemical parameters was 97%, compared with 88% for mutation D206E and 85% for mutation V408M. The finding that mutation V408M, previously shown to result in less than 2% of receptor activity (receptor-negative) is biochemically expressed to a lesser or equal degree than mutations D154N and D206E (receptor-defective, 20% activity), may be due to the small number of V408M-positive relatives analysed. Another explanation for this phenomenon may, however, be related to the finding that mutation D154N occurs *in cis* with mutation A370T, representing a *StuI* polymorphism<sup>29</sup> that may have a subtle effect on plasma lipid levels.<sup>30</sup>

Interestingly, one of the male patients with mutation V408M exhibited a normal TC value. This finding provides us with another example of the extent of clinical variability in FH.<sup>31,32</sup> The normal TC value in this V408M heterozygote could be due to interaction with a cholesterol-lowering gene.<sup>33,34</sup> Sass *et al.*<sup>35</sup> hypothesised that the apo E2 allele of the apolipoprotein E polymorphism may be a potent cholesterol-lowering factor. The likelihood of apo E allelic status as a contributing factor

has been excluded in the South African normocholesterolaemic individual with mutation V408M, since this subject was homozygous for the neutral E3 allele (J N P de Villiers — unpublished data). The genetically homogeneous Afrikaner population provides a valuable source of material for gene-gene interaction studies and is increasingly used for this purpose.<sup>36</sup>

Approximately half of the offspring of an affected parent can be expected to have a severely elevated plasma cholesterol level from birth onwards, as was demonstrated in the child<sup>27</sup> and adult samples. Cardiovascular disease usually manifests in patients with FH before the age of 55 years,<sup>1</sup> which appears to correspond with data for the Afrikaner population.<sup>11,31</sup> Notably, none of the black FH patients shown to be heterozygous for a 6-bp deletion in exon 2 presented with CHD.<sup>17</sup> Although none of these subjects displayed variation in the LDLR promoter region shown to be associated with altered phenotypic expression of FH in African populations where these mutations prevail,<sup>17,37</sup> other modifier genes and/or environmental factors may be involved. It is tempting to speculate that the prevalence of (clinical) FH is likely to increase with westernisation of the black population, once the apparently aggravating effect of the relatively common -175g →t polymorphism<sup>17</sup> is manifested in the presence of other causative FH mutations.

This study demonstrated that FH has a very high penetrance in populations of European descent, which justifies a genetic diagnosis of this treatable disease. The main advantage of DNA diagnostics is its very high specificity compared with biochemical parameters. The value of the family-based MED-PED screening approach is that identification and treatment of FH is assured early in life. It also provides the opportunity for genetic counselling to inform families of the importance of mutation screening in other relatives. Extension of this approach to the population at large would allow more FH patients to be diagnosed and subsequently treated by their clinicians, or referred to lipid clinics where they can receive the intensive care their condition justifies.

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