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Haplotype associations of three DNA polymorphisms at the human low density lipoprotein receptor gene locus in familial hypercholesterolaemia

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SUMMARY The frequency and inheritance of three restriction fragment length polymorphisms (RFLPs) of the low density lipoprotein (LDL) receptor gene were investigated in 27 South African families with familial hypercholesterolaemia. Four haplotypes, defined by the enzymes *Pvu*II, *Stu*I, and *Nco*I, were found to segregate in this population. The frequency of the rare allele detected by *Nco*I was found to be 0·53 in 45 unrelated familial hypercholesterolaemic (FH) patients compared to 0·33 in 60 normal controls ($p<0·005$). In 71% of the families studied, a haplotype with common alleles for *Pvu*II and *Stu*I and the rare allele for *Nco*I cosegregated with the defective gene. In 20% of the families, a second haplotype with rare alleles for *Pvu*II and *Stu*I and common allele for *Nco*I segregated with FH. In these families the haplotypes unambiguously cosegregate with the disease and can therefore be used for early diagnosis of FH.

Mutations in the human gene for the low density lipoprotein (LDL) receptors produce the clinical syndrome of familial hypercholesterolaemia (FH).[1] These receptors are located in coated pits on the cell surface, bind plasma LDL, and carry it into cells by receptor mediated endocytosis. In patients with FH, the genetically defective receptors cause LDL to accumulate to high levels in plasma, which eventually leads to atherosclerosis.

At least 10 different mutant alleles at the LDL receptor locus can be distinguished through biochemical studies of cultured fibroblasts from FH patients.[2] A cDNA clone for the human LDL receptor gene has recently been isolated,[3] which enables one to study these receptor defects at DNA level. DNA restriction site polymorphisms (RFLPs) around or within structural genes allow identification of alleles, which can be used to study the association with a disease within a population, or segregation of marker alleles and disease within a family. Previous studies have shown that a *Pvu*II restriction enzyme polymorphism of the LDL receptor gene can be used to show that alleles of this genetic marker coinherit with familial hypercholesterolaemia.[4]

We have previously reported two new RFLPs at the LDL receptor gene locus.[5,6] Both the restriction enzymes *Stu*I and *Nco*I identify two allele polymorphisms which are informative for family studies and early diagnosis of FH. In this study we report the frequency of the three LDL receptor gene restriction site polymorphisms in the normocholesterolaemic and FH populations. A study of haplotype associations with FH in 27 informative families show a predominant association of two haplotypes with the disease.

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Subjects and methods

Blood samples were collected from 45 selected FH patients attending the lipid clinic at Tygerberg Hospital, Tygerberg, South Africa. Pedigree analyses were performed on 27 of these patients and

blood samples were collected from 292 of the family members. The patients were selected as having FH when the following criteria were fulfilled. Total serum cholesterol levels of all patients had to be at least equal to the 90th centile for their age and sex categories of a typical South African white population.[7] If patients received cholesterol lowering drugs (cholestyramine, probucol, bezafibrate, or nicotinic acid) in full dose, singly or in combination, they were included in the FH study group only if their total cholesterol level was at least equal to the 70th centile for the age and sex group. All FH study participants had normal serum triglyceride levels equal to or lower than 2·0 mmol/l. In addition, a 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 coronary heart disease (CHD). An early CHD family history was defined as being present when the FH participant reported that at least two first or second degree relatives suffered CHD before the age of 50 years.

Blood samples from 60 normocholesterolaemic, Afrikaans speaking, white subjects were obtained from the laboratory population and from unaffected spouses of subjects with FH. Total serum cholesterol levels of the normocholesterolaemic study group were not higher than the 50th centile for their age and sex categories of a typical white South African population.[7] Their serum triglyceride levels were also normal (≤ 2 mmol/l). All the other criteria for FH were absent in this study group.

LIPID DETERMINATION

The total cholesterol and high density lipoprotein (HDL) cholesterol levels were measured on a Gilford auto-analyser using the Boehringer CHOD-PAP enzymatic method. HDL cholesterol was measured after precipitation of the apoprotein B containing lipoproteins with dextran sulphate-magnesium chloride. The triglyceride levels were determined by the Boehringer enzymatic Peridochrom method. In each case the Gilford auto-analyser was calibrated against Precilip or Precilip EL control sera, which were corrected by Boehringer Mannheim for the specific test kit in question. Two control samples were included in each batch analysed.

DNA PREPARATION AND RFLP ANALYSIS

DNA was prepared from the blood samples by a Triton-X100 lysis method.[8] Aliquots of the DNA (5 µg) were digested with the restriction enzymes *Pvu*II, *Stu*I, and *Nco*I, using conditions recommended by the suppliers (Boehringer Mannheim). DNA fragments were separated by size on a 0·6% agarose gel and transferred to nitrocellulose filters (Schleicher and Schnell, BA85, 0·45 nm) by the Southern blotting technique.[9]

The human DNA probe for the LDL receptor gene, pLDLR-2HH1, was obtained from Dr D W Russell of Dallas. It consists of a 1·9 kb fragment of the 3′ end of the LDL receptor cDNA clone and subcloned into a *Bam*HI site of the vector pSP64.[3] The insert was excised with *Bam*HI, separated from the vector on a 1% agarose gel, and recovered. The probe DNA was labelled in vitro by nick translation to a specific activity of 10^8 cpm/µg (BRL Kit, Amersham International).

Filters were prehybridised for three hours at 65°C in 3×SSC (1×SSC: 0·15 mol/l NaCl, 0·015 mol/l sodium citrate), 0·1% sodium dodecyl sulphate (SDS), 10×Denhardt's solution, 50 µg/ml denatured salmon sperm DNA (Sigma), and 10 µg/ml poly A (Sigma). The probe was then added at a concentration of 50 ng/ml and hybridisations were carried out for 22 hours at 65°C. Filters were finally washed in 1×SSC, 0·1% SDS and exposed to Kodak XAR film for one to three days.

STATISTICAL ANALYSIS

χ^2 tests were performed to document that the populations investigated were or were not in Hardy-Weinberg equilibrium and to compare the chromosomal frequencies.

Results

Table 1 shows the results of lipid analysis, the family history of early CHD, and presence of tendon xanthomas in 45 FH patients and 60 controls. Family history denotes the presence of a myocardial infarct or sudden death before the age of 50 years in first or second degree relatives.

Fig 1 shows representative autoradiographs of Southern blots of genomic DNA digested with *Pvu*II, *Stu*I, and *Nco*I, hybridised to the radiolabelled LDL receptor gene probe. *Hind*III digested

TABLE 1 *Clinical data of unrelated normocholesterolaemic and FH subjects.*

	FH	*Normal*
Number of subjects	45	60
Mean age (y)	44 ±12·9	45 ±12·5
Mean serum cholesterol (mmol/l)	9·2±1·57	5·2±0·68
Mean plasma HDL (mmol/l)	1·2±0·31	1·4±0·58
Mean serum triglyceride (mmol/l)	1·3±0·36	1·2±0·42
Male/female ratio	22:23	20:40
% with tendon xanthomas	62	0
% with family history of CHD ≤50 years	78	0

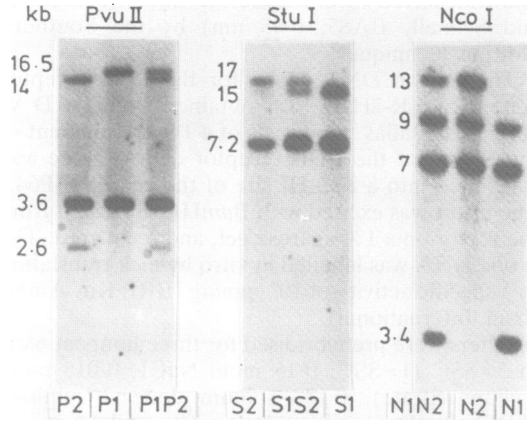

FIG 1 *Southern blot analysis of the LDL receptor gene DNA polymorphisms. The hybridisation pattern obtained from digests of 5 μg DNA with PvuII, StuI, and NcoI from three normal subjects are shown. Fragment sizes are indicated in kb. The common alleles are designated P1, S1, and N1 and the rare alleles designated P2, S2, and N2, respectively.*

lambda phage fragments were used as DNA size markers with each batch of digests. The genomic DNA of heterozygotes showing recognition sites of *Pvu*II, *Stu*I, and *Nco*I are illustrated diagrammatically in fig 2a, b, and c respectively. The alleles defined using the restriction enzyme *Pvu*II are characterised by DNA fragments of 16·5 and 3·6 kbp (P1), and 14, 3·6, and 2·6 kbp (P2).[4] The alleles defined by *Stu*I are characterised by two fragments of 15 and 7·2 kbp (S1), and 17 and 7·2 kbp (S2),[5] and the alleles defined by *Nco*I by fragments of 9, 7, and 3·4 kbp (N1), and 9, 7, and 13 kbp (N2) respectively.[6] The approximate map of fragment sizes in fig 2 is derived from Südhof *et al*[10] and from DNA double digests using sets of different restriction enzymes. The variable *Stu*I and *Nco*I sites are respectively located in exons 8 and 18 of the LDL receptor gene.

RFLP GENOTYPES AND ALLELE FREQUENCIES IN FH AND NORMOCHOLESTEROLAEMIC POPULATIONS

The distribution of genotypes and frequencies of the three RFLP alleles were determined in 60 normocholesterolaemic and 45 unrelated FH subjects (table 2). The distribution of genotypes is close to the expected value if the population is in Hardy-Weinburg equilibrium, but a statistically significant increase in the rare allele of the *Nco*I polymorphism above the values in the normocholesterolaemic group was found in the FH population ($\chi^2 = 17{\cdot}2$, $p<0{\cdot}005$). This indicates population association of this allele with a gene predisposing to hypercholesterolaemia. For the *Stu*I and *Pvu*II RFLPs there is no statistically significant difference in allele frequency between the normocholesterolaemic and FH populations.

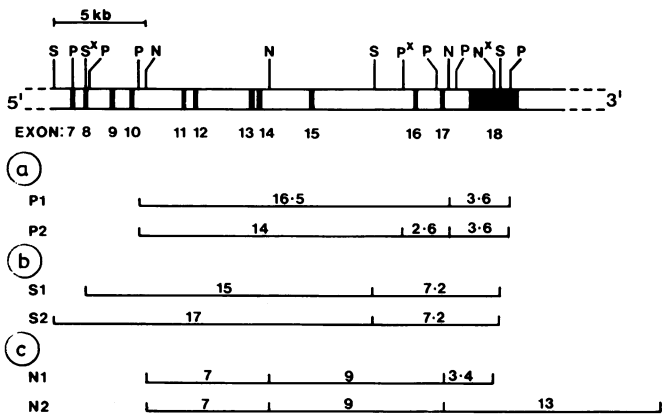

FIG 2 *Diagrammatic representation of sites and approximate fragment sizes of the human LDL receptor gene RFLPs. P^x, S^x, and N^x represent the polymorphic sites for the enzymes PvuII, StuI, and NcoI respectively. The diagrams show genomic DNA fragments detected with the LDL receptor probe pLDLR-2HH1 in P1P2 (a), S1S2 (b), and N1N2 (c) heterozygotes.*

A study of the genotype combinations of the three RFLPs show that only 10 of the possible 27 genotype combinations occur in both populations (table 3), with four of the possible eight haplotypes (table 4). Genotype combinations were obtained directly from Southern blot analysis, while haplotypes were deduced if the subjects were homozygous for at least two of the polymorphisms. Where possible, the haplotypes of double heterozygotes were inferred from the pedigree. (There were 14 double heterozygotes in the normal population and two in the FH population for whom pedigree information was unavailable, so they are therefore not included.) Haplotypes for 43 unrelated FH (86 haplotypes) and 46 normocholesterolaemic subjects (92 haplotypes) are shown in table 4.

TABLE 2 *Comparison of genotype distribution and allele frequencies of the PvuII, StuI, and NcoI RFLPs in the normocholesterolaemic and FH populations.*

Enzyme	*No of subjects*			*Allele frequency*	
*Pvu*II	P1-1	P1-2	P2-2	P1	P2
Normal n=60	28	26	6	0·68	0·32
FH n=45	27	14	4	0·76	0·24
*Stu*I	S1-1	S1-2	S2-2	S1	S2
Normal n=60	51	9	0	0·93	0·07
FH n=45	37	7	1	0·90	0·10
*Nco*I	N1-1	N1-2	N2-2	N1	N2
Normal n=60	26	28	6	0·67	0·33
FH n=45	7	28	10	0·47	0·53

Normal vs FH frequency χ^2 = 17·2 (1 df), p<0·005.

TABLE 3 *Genotype frequencies of the three polymorphisms of the LDL receptor gene in the normal and FH populations.*

Genotype	*FH subjects (n=45)*		*Normal subjects (n=60)*	
	No	*%*	*No*	*%*
P1P1S1S1N1N1	0	0	9	15
P1P1S1S1N1N2	17	38*	14	23*
P1P1S1S1N2N2	10	22*	5	8*
P1P2S1S1N1N1	1	2	8	13
P1P2S1S1N1N2	8	18	11	18
P1P2S1S2N1N1	2	4	3	5
P1P2S1S2N1N2	3	7	4	7
P2P2S1S1N1N1	1	2	4	7
P2P2S1S2N1N1	2	4	2	3
P2P2S2S2N1N1	1	2	0	0

*See text.

TABLE 4 *Haplotype frequencies of the three polymorphisms of the LDL receptor gene in the normal and FH populations.*

Haplotype	*FH population (n=86)*		*Normal population (n=92)*	
	No of chromosomes	*%*	*No of chromosomes*	*%*
P1S1N1	20	23	39	42
P1S1N2	46	54*	29	32*
P1S2N1	0	0	0	0
P1S2N2	0	0	0	0
P2S1N1	11	13	21	23
P2S1N2	0	0	0	0
P2S2N1	9	10	3	3
P2S2N2	0	0	0	0

*See text.

The demonstration of only a few genotype and haplotype combinations in the populations can be interpreted as non-random association of the three DNA markers of the LDL receptor gene. One of the haplotypes, P1S1N2, shows a significantly higher incidence in the FH group (32% in normocholesterolaemics compared with 54% in hypercholesterolaemics). The predominance of the N2 allele of the *Nco*I polymorphism in FH subjects is clear in tables 2 and 3.

SEGREGATION OF THE RFLP HAPLOTYPES IN FH FAMILIES

We have used the *Pvu*II, *Stu*I, and *Nco*I polymorphisms to follow the inheritance of the LDL receptor gene in 27 informative families in which FH occurs. In 17 families (46 normal and 75 affected offspring) the P1S1N2 haplotype and the FH phenotype cosegregated. An example of one extended family is shown in fig 3a. The FH phenotype was associated with the P2S2N1 haplotype in seven informative families, where 38 normal and 46 affected offspring were analysed (example of one family in fig 3b). The P1S1N1 haplotype has shown association with the FH phenotype in two families (four normal and eight affected offspring) and the P2S1N1 haplotype in only one family (seven normal and eight affected offspring).

Table 5 shows the frequencies of the different haplotypes associated with FH in 34 unrelated FH heterozygotes. The FH haplotypes were deduced from the pedigree analyses in the 27 families and from seven patients homozygous for all three RFLPs. In 71% of the FH families studied, the P1S1N2 haplotype cosegregates with the defective gene, while the P2S2N1 haplotype segregates in 20% of families. The P1S1N1 and P2S1N1 haplotypes are associated with the FH phenotype in only 6% and 3% of families studied, respectively.

TABLE 5 *Frequencies of haplotypes associated with the FH phenotype.*

Haplotype	*No of subjects (n=34)*	*%*
P1S1N1	2	6
P1S1N2	24	71
P2S1N1	1	3
P2S2N1	7	20

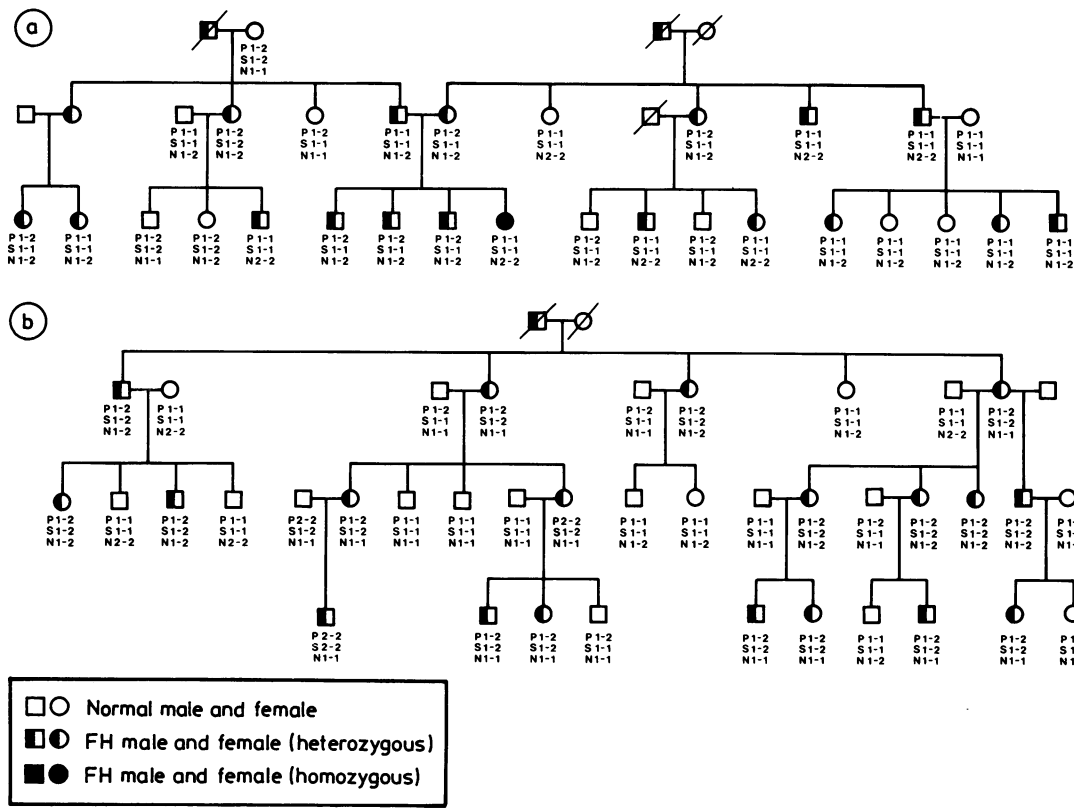

FIG 3 *Segregation of FH and the LDL receptor gene RFLPs in two families. (a) FH phenotype associated with the P1S1N2 haplotype. (b) FH phenotype associated with the P2S2N1 haplotype.*

The association of the P1S1N2 and P2S2N1 haplotypes with FH in the white South African population were further confirmed in five clinical FH homozygotes. Four have shown P1P1S1S1N2N2 genotypes, while one is a heteroallelic genetic compound with a P1P2S1S2N1N2 genotype, containing both the P1S1N2 and P2P2N1 haplotypes. The haplotypes were deduced from family studies. The haplotypes and lipid results of five FH homozygotes are shown in table 6.

Discussion

The prevalence of FH in South Africa is unusually high and a 'founder gene' effect in the Afrikaner population has been proposed.[11] LDL receptor studies of South African FH homozygotes have shown a predominance of a receptor defective type of abnormality.[12]

In our study using three DNA polymorphisms at the human LDL receptor gene in 45 FH and 60

TABLE 6 *Haplotypes and lipid results of five homozygous FH patients.*

Case	*Age (y)*	*Cholesterol (mmol/l)*		*Total triglyceride (mmol/l)*	*Haplotype*
		Total	*HDL*		
FH 8	32	16·5	0·9	1·1	P1S1N2, P2S2N1
FH 35	28	19·2	1·3	1·3	P1S1N2
FH 109	14	15·7	0·1	1·3	P1S1N2
FH 225	13	17·6	1·0	0·7	P1S1N2
FH 226	7	20·4	0·9	1·1	P1S1N2

normocholesterolaemic subjects, we have found a statistically significant excess of the rare N2 allele of the *Nco*I RFLP in the FH population. The *Pvu*II, *Stu*I, and *Nco*I RFLPs have been used to follow the inheritance of the LDL receptor gene in 27 informative FH families. In 71% of these the P1S1N2 haplotype cosegregated with the defective gene. This association was further confirmed in five FH homozygotes. Four were homozygous for the P1S1N2 haplotype and one a genetic compound with both the P1S1N2 and P2S2N1 haplotypes; in 20% of FH families studied the FH phenotype cosegregated with the P2S2N1 haplotype. The P1S1N1 and P2S1N1 haplotypes have been associated with the FH phenotype in only 6% and 3% of families studied respectively. Studies are under way to confirm these rare FH haplotypes in clinical FH homozygotes.

If, as expected for polymorphisms around a single gene, there is no recombination between the three RFLPs, it will be possible to combine the information on all three RFLPs to form an unequivocal haplotype for any subject. In the 27 families studied so far, we have observed no recombination within the DNA region of the LDL receptor gene covered by the three RFLPs. Analyses of associations of RFLPs have revealed only four of the possible eight haplotypes. This may be an indication that the *Pvu*II, *Stu*I, and *Nco*I RFLPs are non-randomly associated. Since the S2 allele is found only in association with the P2 allele, whereas the S1 allele is found with both the P1 and P2 allele, it is likely that the *Pvu*II polymorphism evolved before the *Stu*I polymorphism. The base pair changes creating the loss of a *Stu*I site must have arisen on a chromosome 19 with a P2 genotype. Similarly we can postulate that the *Pvu*II polymorphism has arisen in the presence of the N1 allele, since the N2 allele is never found in association with P2S1 or P2S2 genotypes.

Haplotype analysis alone is not sufficient to distinguish normal from FH chromosomes, since the same haplotypes were found in both the normal and FH populations. This finding is interpreted as an indication that the LDL receptor gene haplotypes were established at a point in evolution before the mutations causing FH. It further implies that the FH mutations evolved independently from one another, suggesting that a particular mutation is likely to be associated with a specific haplotype. Variations at these three polymorphic sites may serve as valuable genetic markers, for the identification of different FH mutations, for population studies, and as a basis for prenatal diagnosis of many FH mutations.

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References

1 Goldstein JL, Brown MS. Familial hypercholesterolemia. In: Stanbury JB, Wyngaarden JB, Frederickson DS, Goldstein JL, Brown MS, eds. *The metabolic basis of inherited disease*. 5th ed. New York: McGraw-Hill, 1983: 672–712.

2 Goldstein JL, Brown MS. Progress in understanding the LDL receptor and HGM-CoA reductase, two membrane proteins that regulate the plasma cholesterol. *J Lipid Res* 1984;**25**: 1450–61.

3 Yamamoto T, Davis CG, Brown MS, *et al.* The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 1984;**39**:27–38.

4 Humphries SE, Horsthemke B, Seed M, *et al.* A common DNA polymorphism of the low-density lipoprotein (LDL) receptor gene and its use in diagnosis. *Lancet* 1985;**i**:1003–5.

5 Kotze MJ, Retief AE, Brink PA, Weich HFH. A DNA polymorphism in the human low-density lipoprotein receptor gene. *S Afr Med J* 1986;**70**:77–9.

6 Kotze MJ, Langenhoven, E, Dietzsch E, Retief AE. A RFLP associated with the low-density lipoprotein receptor gene. *Nucleic Acids Res* 1987;**15**:376.

7 Rossouw JE, Jooste PL, Steyn K, Benadé AJS. Serum total and high-density lipoprotein cholesterol—reference values obtained in the Coronary Risk Factor Study baseline survey. *S Afr Med J* 1985;**67**:533–8.

8 Kunkel LM, Smith KD, Boyer SH, *et al.* Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. *Proc Natl Acad Sci USA* 1977;**74**:1245–9.

9 Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975;**98**: 503–17.

10 Südhof TC, Goldstein JL, Brown MS, Russel DW. The LDL receptor gene: a mosaic of exons shared with different proteins. *Science* 1985;**228**:815–22.

11 Seftel HC, Baker SG, Sandler MP, *et al.* A host of hypercholesterolemic homozygotes in South Africa. *Br Med J* 1980;**281**:633–6.

12 Coetzee GA, Van der Westhuizen DR. Familial hypercholesterolemia – a receptor defect. *S Afr J Cont Med Educ* 1984;**2**: 49–56.

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