The use of low density lipoprotein receptor activity of lymphocytes to determine the prevalence of familial hypercholesterolaemia in a rural South African community

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SUMMARY  The diagnosis of heterozygous familial hypercholesterolaemia in three rural South African communities in which hypercholesterolaemia is very prevalent could be confirmed by the measurement of low density lipoprotein (LDL) receptor activity in circulating lymphocytes. A nominal cut off point could be proposed which separated the LDL receptor activity of 24 clinically diagnosed heterozygous FH patients and 31 healthy people. LDL receptor activity was measured as total degradation of $^{125}$I-LDL and expressed as ng LDL/mg cell protein/6 hours. The cut off point was set at 970 ng/mg protein/6 hours. This proposed cut off point was tested by assaying the LDL receptor of three homozygous FH patients and seven of their obligate heterozygous FH first degree relatives. The three homozygous FH patients showed no receptor activity and the activity of the seven obligate heterozygous first degree relatives fell below the proposed cut off point. To determine the prevalence of FH in the study population, all persons aged 15 to 24 years whose total cholesterol levels fell above the 80th centile for their age and sex, as well as their families, were approached (n=114). The LDL receptor activity in lymphocytes of 77 of these persons aged 15 to 24 years was determined after applying the exclusion criteria. Ten of the 77 participants had LDL receptor activity below 970 ng LDL/mg protein/6 hours and were therefore diagnosed as being heterozygous FH patients. The calculation of the prevalence (corrected for exclusions) revealed that one in 71 of the 15 to 24 year old permanent residents in the predominantly Afrikaans speaking community suffered from heterozygous FH. This is higher than any FH prevalence previously reported for any group.

The high coronary heart disease (CHD) mortality rate in South African whites, and particularly the Afrikaans speaking members of that ethnic group, has in part been ascribed to the high prevalence of familial hypercholesterolaemia (FH). The Witwatersrand area, where the disease has been reported to have a particularly high prevalence rate in the Afrikaans community, the disease appears to cluster among members of a particular Afrikaans religious denomination. It has been postulated that this finding points towards a genetic isolate. Seftel et al showed that in this group the homozygote prevalence was approximately 1:30 000. This could have been an underestimate of the true prevalence as these researchers could not be sure that they had traced every homozygote in the study area. The finding of a high prevalence of FH in the southwestern Cape area in a predominantly Afrikaans speaking community, the majority of whom belong to religious groups other than to the one described in the Witwatersrand, identified the need to determine accurately the true prevalence of FH in the Afrikaans speaking white community of South Africa.

The FH prevalence rates described above were based on clinical observations and total serum cholesterol level determinations. It remains a problem to diagnose heterozygous FH patients positively in populations in which polygenic hypercholester-
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To determine the prevalence of FH in a young, white, predominantly Afrikaans speaking, rural population of three south-western Cape towns, all subjects between the ages of 15 and 24 years resident in this region were approached. The response rate was 72%. Non-fasting total cholesterol (TC) levels were determined on the 976 permanently resident responders. Those whose TC levels were above the 80th centile for their age and sex were identified as the target population. The 114 such subjects and their first degree relatives (at least one parent and one sib per family) were invited to participate in a further investigation which included fasting plasma TC determinations, lipoprotein fractionation, apoprotein A1 and B determinations, clinical examinations for stigmata of FH, and the taking of a family history of CHD. These results will be reported separately.

Of these 114 index patients, the LDL receptor activity was determined in 77 participants. The remainder were excluded on one or more of the following grounds: pregnancy, refusal, another sib in the study, insufficient family members available, or index patients who could not be studied (for example, because of military training).

LABORATORY METHODS
The serum total cholesterol was measured on a Gilford autoanalyser using the Boehringer CHOD-PAP enzymatic method and calibrated against the Boehringer Mannheim Precilip EL control sera. Two control samples were included in each batch analysed. Human LDL (density=1.019 to 1.063 g/ml, 1019 to 1063 kg/m³) was isolated by differential ultracentrifugation at 10°C from plasma donated by normolipidaemic subjects. The LDL was labelled by a modification of the iodine monochloride method of MacFarlane.

Specific radioactivity levels were in the range of 150 to 350 cpm/ng of protein and less than 1% of the

Subjects and methods

LDL RECEPTOR CUTOFF POINT DETERMINATION
A study was undertaken to establish a cut off point for the LDL receptor activity which would best distinguish between the LDL receptor activity in circulating lymphocytes of known heterozygous FH patients and that of normal persons. Twenty-four clinically diagnosed heterozygous FH patients (11 women and 13 men aged seven to 62 years) were compared with 31 normal persons (11 women and 20 men aged 15 to 53 years). To be included as a heterozygous FH patient, the subjects had to have a total serum cholesterol value ≥7.0 mmol/l and also to have at least two of the following: tendon xanthomata, a family history of CHD in at least one first degree relative under the age of 50 years, or a first degree relative with similarly raised plasma cholesterol and tendon xanthomata. Participants were classified as normal when their total cholesterol values were ≤5.7 mmol/l and no family history of CHD or clinical stigmata of FH were present.

The LDL receptor activity cut off point suggested from the above study was tested by determining the LDL receptor activity of seven obligate heterozygote FH patients (five women and two men aged seven to 52 years) and three of their homozygote FH first degree relatives (three women aged 15 to 33 years). The homozygote FH were classified as such on the basis of a total cholesterol value of ≥15 mmol/l before treatment and the presence of tendon or cutaneous xanthomata at an early age, as well as an autosomal dominant pattern of inheritance of FH in their relatives from both parental lines.

The reproducibility of the assay was estimated by studying the lymphocytes from the same subjects on at least three occasions. Two heterozygote participants and two normal participants were studied.

THE FH PREVALENCE STUDY

Prevalence of familial hypercholesterolaemia in South Africa. It was therefore clear that a more accurate estimate of the prevalence of heterozygous FH would have to be based on a more immediate expression of the pathophysiology of this disease than serum cholesterol.

Studies of cultured skin fibroblasts and freshly isolated human lymphocytes have shown that cells from FH homozygotes express little or no low density lipoprotein (LDL) receptor activity even when maximally 'upregulated' and therefore show no high affinity degradation of LDL. Fibroblasts and lymphocytes from FH heterozygotes usually express half the normal number of LDL receptors when fully 'upregulated' and hence degrade LDL at about half the normal rate. The deficiency of LDL receptors in liver and perhaps also in peripheral tissues accounts for the greatly increased plasma concentrations of LDL in FH. Bilheimer et al have successfully used the direct LDL receptor assay in freshly isolated mononuclear cells to discriminate between small numbers of normal healthy subjects, non-FH hyperlipidaemic subjects, and true FH heterozygotes.

In this study we attempted to determine the FH prevalence in three rural South African communities directly by means of the LDL receptor activity measured in freshly isolated lymphocytes by methodology adapted from that described by Bilheimer et al.
total radioactivity in the final preparations came from free iodide (quantified as trichloroacetic acid soluble radioactivity). The LDL was used within 10 days of being labelled. Lipoprotein deficient serum (LPDS) was prepared from the plasma of healthy subjects by differential ultracentrifugation at a density of 1.215 g/ml (1215 kg/m^3).17

Lymphocytes were isolated from 50 ml blood drawn from the subjects and defibrinated in sterile glass tubes. After centrifugation at 800 g for 30 minutes, mononuclear cells were isolated by first diluting the buffy coat in one volume of RPMI 1640 tissue culture medium (Flow Laboratories, Irving, Scotland) and then the resultant cell suspension 1:1 with more RPMI 1640. Of the diluted cells, 6 ml were then layered onto 3 ml Ficoll-Hypaque of density 1.077 g/ml (1077 kg/m^3)18 and centrifuged for 40 minutes at 400 g. The interface of mononuclear cells was harvested and washed three times by recentrifugation through Eagle's medium. Of the mononuclear cells prepared in this manner, more than 95% were lymphocytes, confirmed by the trypan blue exclusion tests.

The maximum LDL receptor activity was quantified by the proteolytic degradation of 125I-LDL by blood mononuclear cells after prior incubation in the absence of lipoproteins to 'upregulate' the LDL receptor number, using a method similar to that of Bilheimer et al.13 LDL catabolism was quantified from the accumulation of trichloroacetic acid soluble non-iodide radioactivity in the medium. Cellular degradation of 125I-LDL was calculated as the difference between results obtained from identical incubations with and without cells, and the results expressed as ng 125I-LDL/mg cell protein/6 hours. Mistry et al.19 have shown that, at the concentration of 125I-LDL used (10 μg protein/ml), approximately 90% of the cellular degradation occurs via the receptors with high affinity for LDL. Measurements were performed in triplicate and normal and heterozygote subjects were assayed on the same day.

**Calculation of Prevalence**

Subjects with an LDL receptor activity below the selected cut off point (970 ng LDL/mg cell protein/6 hours) were labelled as FH heterozygotes. The prevalence of FH in this community was estimated and was corrected for the exclusions mentioned above.

**Results**

The LDL receptor activity in freshly isolated mononuclear cells of clinically diagnosed heterozygous FH patients and normal participants are shown in the figure (a). These results showed that the receptor activity expressed as total degradation of 125I-LDL in lymphocytes can discriminate between FH heterozygotes and healthy subjects. A cut off point of 970 ng LDL catabolised per mg cell protein in six hours allowed optimal separation of FH heterozygote and normal subjects.

Repeat measurements on the two known heterozygote patients and the two normal participants had a mean coefficient of variance (CV) of 12% for heterozygote participants and 14.5% for the normal participants. This is of the same magnitude as the CV reported by Bilheimer et al.13

The testing of the cut off point against the LDL receptor assay results of obligate heterozygotes and homozygote FH patients is shown in the figure (b). The homozygote FH patients showed no LDL receptor activity and none of the heterozygous FH patients had LDL receptor activities above the suggested cut off point. The heterozygous patients were found to have an LDL receptor activity between 429 and 960 ng LDL catabolised per mg cell protein in six hours. The range of the receptor activity of normal participants was between 999 and 10,000 ng LDL/mg protein/6 hours.

**Figure** Low density lipoprotein receptor activity expressed as ng LDL/mg cell protein/6 hours was determined in lymphocytes of (a) 24 clinically identified heterozygous familial hypercholesterolaemic (FH) patients and compared to those of 31 normal subjects, with the range of values of the two groups suggesting a nominal cut off point of 970 ng LDL/mg cell protein/6 hours; (b) three homozygous FH patients and seven of their obligate heterozygous FH first degree relatives, lending support to the proposed cut off point; (c) a group of 77 hypercholesterolaemic participants in which 10 heterozygous FH patients could be identified on the basis of the proposed cut off point.
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3530 ng LDL catabolised per mg cell protein in six hours.

The cut off point of 970 ng LDL degraded per mg cell protein per six hours identified 10 of the 77 index patients aged 15 to 24 years with LDL receptor activity corresponding to that of heterozygous FH patients. These results are shown in the figure (c).

The prevalence of heterozygous FH in the 15 to 24 year old permanent residents in the predominantly Afrikaans speaking rural population of three southwestern Cape towns was calculated, and this calculation included a correction for the exclusions. Table 1 shows the prevalence of FH to be one in 71 in the study population.

The usual diagnostic features of heterozygous FH in the group identified as such is compared with those participants with normal LDL receptor activity in table 2. The heterozygous FH group had significantly higher total and LDL cholesterol values and significantly more first degree relatives with a history of CHD and tendon xanthomata. Of importance is the fact that many of the first degree relatives of the non-FH index patients also suffered from CHD and were found to have tendon xanthomata.

**Discussion**

Although an arbitrary discrete cut off point of 970 ng LDL catabolised per mg cell protein in six hours was chosen to distinguish between FH and non-FH hypercholesterolaemic index cases, it is clear from the coefficients of variation that misclassification can occur which will affect the calculated prevalence of heterozygous FH in the study population. The clinical finding of tendon xanthomata in the first degree relatives of non-FH index patients may point to such a misclassification. While fully acknowledging this limitation of the choice of a discrete cut off point, we felt justified in using it after verifying it against LDL receptor activities of obligate heterozygous patients, and in view of the suspected magnitude of this undiagnosed disease in the study population the need for a defined diagnostic criterion, even with limitations, became necessary.

The estimated prevalence of FH of 1:71 in the Afrikaans speaking South African study community is ev. in higher than the 1:100 reported by Seftel et al\(^2\) using the Hardy-Weinberg equation\(^20\) in the Afrikaans group of a specific religious denomination\(^5\) in the Witwatersrand. Jooste et al\(^6\) estimated the prevalence to be at least 1:87, based on TC values, in the same communities as the present study. The FH prevalence rate based on the LDL receptor assay is the highest ever reported and supports the suggestion made by Seftel et al\(^2\) and Pretorius\(^1\) that FH has made a significant contribution to the extremely high CHD mortality among South African whites. To our knowledge this is the first study which has used the LDL receptor assay to estimate directly the FH prevalence in a population.

A plausible explanation for the high FH prevalence in South African whites compared to other populations\(^21\) could be the founder gene effect suggested by Seftel et al\(^2\) and Torrington et al\(^3\). A

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**TABLE 1.** The figures used in the calculation of the prevalence of familial hypercholesterolaemia in the rural predominantly Afrikaans speaking white population of three western Cape towns.

| Permanent residents aged 15 to 24 years | 976 |
| Residents with total cholesterol levels ≥80th centile | 114 |
| Residents excluded on various criteria | 37 |
| Participants with LDL receptor assays | 77 |
| Participants with LDL receptor levels below the cut off point of 970 ng/ml protein/6 hours heterozygous FH patients | 10 |
| % heterozygous participants | 12.99 |
| FH prevalence not corrected (%) | 1.02 |
| FH prevalence corrected for exclusions (%) | 1.41 |
| Prevalence of FH in study population | 1.71 |

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**TABLE 2.** Comparison of diagnostic features of familial hypercholesterolaemia in the two groups of index patients aged 15 to 24 years.

<table>
<thead>
<tr>
<th>Participants with LDL receptor activity suggestive of FH</th>
<th>Participants with normal LDL receptor activity</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>Total serum cholesterol in mmol/l (SD)</td>
<td>7.13 (1.20)</td>
<td>6.10 (0.83)</td>
</tr>
<tr>
<td>Low density lipoprotein cholesterol in mmol/l (SD)</td>
<td>5.30 (1.21)</td>
<td>4.21 (0.85)</td>
</tr>
<tr>
<td>History of coronary heart disease in first degree relatives of index patients</td>
<td>65%</td>
<td>29%</td>
</tr>
<tr>
<td>Tendon xanthomata in index patients</td>
<td>10%</td>
<td>0%</td>
</tr>
<tr>
<td>Tendon xanthomata in first degree relatives of index patients</td>
<td>50%</td>
<td>11.9%</td>
</tr>
</tbody>
</table>
study by Van der Westhuysen et al\textsuperscript{22} indicated that the LDL receptor abnormality found in South African homozygous FH patients was predominantly of the receptor defective type. This finding supports the founder gene effect hypothesis, since Tolleshaug et al\textsuperscript{23} have described at least seven mutations that could affect the LDL receptor phenotype where the receptor defective type did not predominate, as found in the reported\textsuperscript{22} South African study.

The results of the present study found the high prevalence of FH in a white Afrikaans speaking community which does not belong to the specific church affiliation described by Torrington et al.\textsuperscript{5} Therefore, if the founder gene effect is to be validated, it would probably have to be illustrated in forebears who were not exclusively members of the South African Gereformeerde Church.

A recent study by Kotze et al\textsuperscript{24} identified three DNA polymorphisms at the human LDL receptor gene locus of South African families clinically diagnosed as suffering from FH. Although one of the DNA polymorphisms was found more often than the other two, the presence of the latter two DNA polymorphisms provides evidence against a single mutation causing the founder gene effect. These findings, as well as the variation in receptor activity in the receptor defective FH homozygotes described by Van der Westhuysen et al\textsuperscript{22} and the variation in clinical manifestation of the disease in affected families, weighs against a single founder gene effect as the only explanation for the high prevalence of FH in white Afrikaans speaking South Africans.

It is likely that our estimated 1:71 prevalence of FH is representative of Afrikaans speaking whites, since it agrees with previous estimates in other parts of South Africa.\textsuperscript{2} The methodology used is based on direct measurement of the cellular abnormality found in FH. The possibility for misclassification is present, but unlikely to be of a magnitude that will negate the finding of a high prevalence of FH in the study population. Until gene markers suitable for population screening programmes are found, the present method is a useful alternative.

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