THE MED-PED PROJECT: PRESYMPTOMATIC DIAGNOSIS IN FAMILIES WITH DISEASE-RELATED LDL RECEPTOR GENE MUTATIONS

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Medical Sciences at the University of Stellenbosch.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

SUMMARY

Familial hypercholesterolaemia (FH) contributes significantly to the high death rate from cardiovascular disease worldwide. FH is a common autosomal co-dominant disease characterised by raised cholesterol levels and premature coronary heart disease (CHD). Whilst these features usually are very prominent in homozygotes the clinical diagnosis of heterozygotes is complicated by variable phenotypic expression. Specific founder genes in the low-density lipoprotein receptor (LDLR) gene have increased the prevalence of FH in South African Afrikaners, Indians, Jews and Coloureds, and screening for these known mutations allows unequivocal diagnosis of FH-affected individuals. The systematic molecular analysis of FH resulted in the identification of at least ten founder-type LDLR gene mutations among the 56 different gene defects described to date in the diverse South African population. DNA screening of 792 at-risk family members for the FH-related mutations identified in 379 index cases, allowed accurate disease diagnosis in an additional 340 relatives and exclusion of the relevant mutation in 452 individuals. This effort forms part of the MED PED FH initiative, a collaborative project to "Make Early Diagnosis and Prevent Early Deaths in MEDical PEDigrees with FH".

Evaluation of clinical criteria versus DNA diagnosis of three founder-related mutations (D154N, D206E and V408M) in the South African population demonstrated that the sensitivity and specificity of diagnoses, based on total cholesterol values measured in family members of index cases recruited for this study, were 88% and 77%, respectively. A population-directed DNA diagnosis of FH

is therefore justified in South Africa on a routine basis, since expression of the defective gene measured in biochemical tests does not allow accurate diagnosis of FH in all cases.

The application of mutation detection was illustrated by prenatal diagnosis of FH performed for a couple who are both heterozygous for the most common Afrikaner mutation, D206E. The mutation was absent in the foetus and a normocholesterolaemic infant was born. Prenatal diagnosis of FH, aimed at the detection of homozygous cases, is particularly applicable in populations and families with molecularly defined LDLR gene mutations.

The MED-PED approach resulted in accurate diagnosis and subsequent treatment of FH in more patients, and referral to lipid clinics where they could receive the intensive care their condition justifies. Molecularly diagnosed FH patients will be the first to benefit from future treatment approaches based on mutation type.

OPSOMMING

Familiële hiprcholesterolemie dra grootliks by tot die wêreldwye hoë sterftesyfer van kardiovaskulêre siekte. FH is 'n algemene outosomale ko-dominante siekte wat gekenmerk word deur verhoogde cholesterolvlakke en vroeë koronêre hartsiekte. Terwyl hierdie kenmerke prominent is in homosigote, word die kliniese diagnose van heterosigote bemoeilik deur variasie in fenotipiese uitdrukking. Spesifieke stigtergene in die lae-digtheids lipoproteien reseptor (LDLR) geen het die voorkomssyfer van FH verhoog in Suid Afrikaanse Afrikaners, Indiërs, Jode en Kleurlinge. Sifting vir hierdie bekende mutasies maak akkurate diagnose van FH-geaffekteerde individue moontlik. Die sistematiese molekulêre analise van FH het aangetoon dat ten minste tien van die 56 verskillende geen defekte wat tot dusver beskryf is in die Suid-Afrikaanse populasie stigtertipe LDLR geen mutasies is. DNA sifting van 792 familielede vir die FH-verwante mutasie in 379 indeksgevalle geïdentifiseer is, het akkurate diagnose moontlik gemaak in 340 addisionele familielede, en uitsluiting daarvan in 452 individue. Hierdie poging vorm deel van die MED-PED FH ("Make Early Diagnosis and Prevent Early Deaths in MEDical PEDigrees with FH) inisiatief.

Evaluering van kliniese kriteria teenoor DNA diagnose van drie stigter verwante mutasies (D154N, D206E en V408M) in die Suid Afrikaanse populasie het getoon dat die sensitiwiteit en spesifisiteit van die diagnose, wat gebasseer is op totale cholesterol waardes in familielede van indeksgevalle, onderskeidelik 88% en 77%

was. 'n Populasie gerigte DNA diagnose van FH is dus geregverdig in Suid-Afrika op 'n roetine basis, omdat die defektiewe geen nie altyd in biochemiese toetse uitgedruk word nie.

Die waarde van mutasie opsporing is geillustreer deur 'n voorgeboortelike diagnose van FH wat aangevra is vir ouers wat beide heterosigoties is vir die mees algemene Afrikaner mutasie, D206E. Die mutasie was afwesig in die fetus en 'n normocholesterolemiese baba is gebore. Voorgeboortelike diagnose van FH, wat gemik is op die opsporing van homosigotiese gevalle, is veral van toepassing in populasies en families met bekende LDLR geen mutasies.

Die MED-PED benadering het gelei tot akkurate diagnose en daaropvolgende behandeling van FH in meer pasiënte, en verwysings na lipiedklinieke waar hulle intensiewe aandag kan geniet. Molekulêre gediagnoseerde FH pasiënte sal die eerste wees om baat te vind by toekomstige behandeling wat moontlik gebasseer sal word op mutasie status.

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LIST OF ABBREVIATIONS

A and/or a	adenine
AA	acrylamide
AMP	adenosine mono phosphate
apo	apolipoprotein
APS	ammonium persulphate
ARMS	amplification refractory mutation system
bp	base-pair
C	cross-linking
C and/or c	cytosine
CAD	coronary artery disease
CHD	coronary heart disease
ddH_2O	double distilled water
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	2'-deoxy-nucleoside-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
F	forward
FH	familial hypercholesterolaemia
g	gram

G and/or g	guanosine
HDL	high density lipoprotein
HDLC	high density lipoprotein cholesterol
HEX-SSCP	heteroduplex-single strand conformation polymorphism
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
kb	kilobase
kD	kilo Dalton
LDL	low density lipoprotein
LDLC	low density lipoprotein cholesterol
LDLR	low density lipoprotein receptor
Lp(a)	lipoprotein (a)
M	moles per litre
m/v	mass per volume
mg/dl	milligram per decilitre
mg/ml	milligram per millilitre
ml	millilitre
mm	millimetre
Mr	molecular weight
mRNA	messenger ribonucleic acid
OD	optical density
PAA	polyacrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PE	Perkin-Elmer
pH	hydrogen ion concentration
pmol	picomole
RBC	red blood cells
rpm	revolutions per minute
SSCP	single strand conformation polymorphism
T and/or t	thymine
Taq	*Thermus aquaticus*
TBE	tris-borate/EDTA
TEMED	N, N, N' N',-tetramethylethylenediamine
U	units
UV	ultra violet
V	volt
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

1.1 The MED-PED Project

MED-PED (**M**ake **E**arly **D**iagnosis to **P**revent **E**arly **D**eaths in **MED**ical **PED**igrees) is an international project aimed at the identification of family members at risk of developing a treatable dominantly inherited disorder. Following index case identification, many close relatives are identified with the same genetic disease by this efficient and cost effective family-based approach. In addition, education, treatment, long term support and compliance are thought to be more effective when involving families instead of individuals (Williams et al., 1993).

The common lipid disorder, familial hypercholesterolaemia (FH), represents an excellent first model for the MED-PED approach. The goal is to identify persons with FH who are undiagnosed and therefore not treated to protect or at least delay onset of coronary heart disease (CHD). Only a small proportion of the estimated 10 million FH heterozygotes and 5000 FH homozygotes worldwide are receiving the benefits of recent advances in the diagnosis and treatment of FH. Heterozygous FH patients do not require specialised experimental genetic treatment to live longer. Changes in diet and administration of cholesterol-lowering drugs are relatively easy to implement. MED-PED currently involves over 180 collaborators from at least 30 countries worldwide. FH cases in the MED-PED registry has risen from 800 in 1994 to approximately 30 000 in 1998 (FH: report of a WHO consultation., 1997).

MED-PED provides a method to avoid costly general population screening, yet is practical and easy to implement. The first step in a genetic approach is to obtain a blood sample for DNA isolation and mutation screening of the low-density lipoprotein receptor

(LDLR) gene from an individual with a clinical diagnosis of FH. Once the disease-causing mutation has been identified in the index patient, all relatives can be screened for the gene defect. This allows presymptomatic diagnosis or exclusion of FH in at-risk family members. Figure 1.1 illustrates the outline of the MED-PED approach. MED-PED guidelines have been described and documented to be cost effective. Since treatment is available for FH, patients should be identified and informed of the ability to significantly reduce their risk of heart disease.

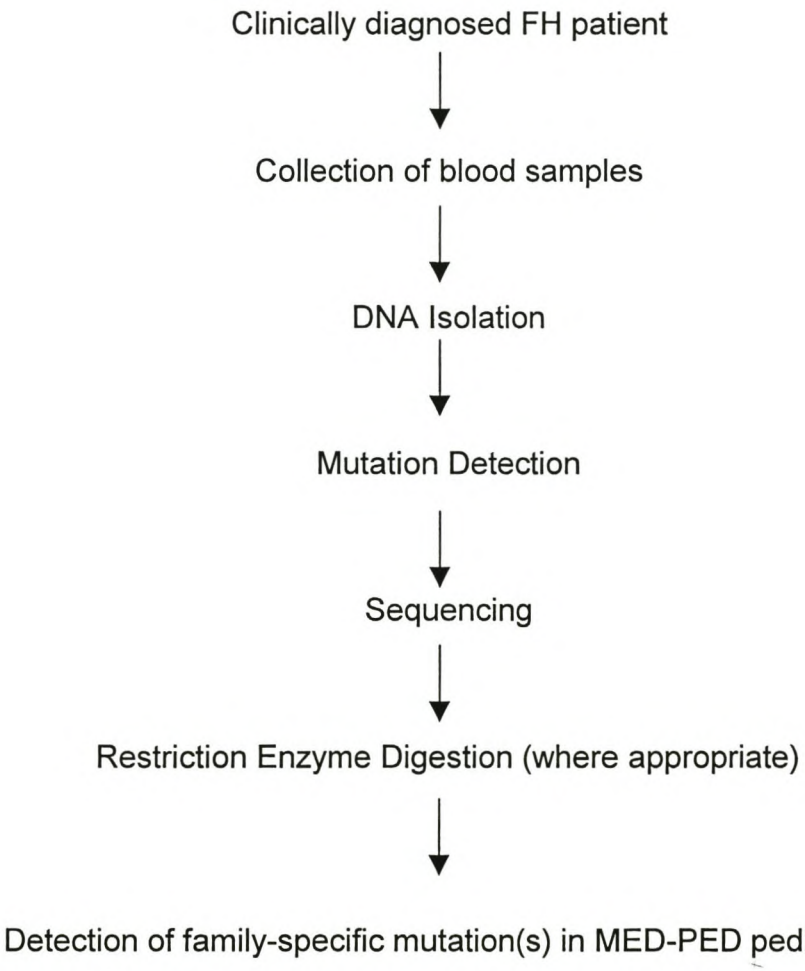

Figure 1.1 MED-PED approach for identification of FH mutations in families

The MED-PED approach for case finding involves five different steps.

Step 1: Index case finding. This is the first person identified as having FH and represents a newly discovered FH pedigree.

Step 2: Relative case finding. The index case is asked to give consent for MED-PED staff to contact relatives who may have high plasma cholesterol levels and/or early CHD. This family information is collected by various methods but usually involves the index case providing information such as full names, addresses and telephone numbers of their relatives.

Step 3: Diagnosis. Medical staff review patient and relative data and make a diagnosis based on biochemical and/or mutation data.

Step 4: Enter in MED-PED registry. After an individual is diagnosed with FH, they are notified and entered into the MED-PED registry.

Step 5: Continuation of relative case finding. After identifying family members with FH, several new FH cases can be found among more distant relatives. The probability of a family member carrying an LDLR mutation is well defined; the likelihood of having FH is 50% for each parent, sibling and child; 25% for each aunt, uncle, grandparent, niece and nephew; 12.5% for first cousins and siblings of grandparents (FH: report of a WHO consultation., 1997).

Other dominantly inherited diseases where the MED-PED approach can be applied include familial defective apolipoprotein B (FDB) (clinically indistinguishable from FH), sudden arrhythmic death syndrome (long QT), glucocorticoid remediable aldosteronism (GRA) with hypertension and early strokes and possibly several cancers of the breast and colon (Williams et al., 1993). In South Africa, this family-based approach has been extended to identify affected relatives of patients suffering from several genetic diseases that occur at increased frequencies due to founder gene mutations (Kotze and Callis, 1999).

1.2 Familial hypercholesterolaemia (FH)

The features of hypercholesterolaemia, xanthomatosis and angina pectoris, have been recognised as a dominantly inherited disease since the work of Müller in 1939. Further studies outlined in Table 1.1 led to cloning of the LDLR gene underlying this common lipid disorder. Heterozygous FH is characterised by a two-fold increase of cholesterol in LDL. VLDL cholesterol and triglyceride levels may also occasionally be increased. The increase of LDL cholesterol is seen even in childhood, usually being present already at birth (Kwiterovich et al., 1974). Clinical symptoms of the disease include tendon xanthomas, xanthelasma, arcus corneae and atherosclerosis. These symptoms usually develop gradually from the second decade until the fourth decade of life. Half of all FH heterozygotes develop xanthomas and arcus corneae by the third decade of life. Eventually 80% of FH patients will be affected with xanthomas. The complications resulting from atherosclerosis are myocardial infarction and/or stroke. The mean age of

onset of CHD is about 45 years in affected males and 53 years in affected females (Goldstein et al., 1995).

In the homozygous state this condition is much more severe with plasma LDL levels elevated at least four times the normal value. Severe hypercholesterolaemia is present at birth and persists throughout life. Xanthomas are sometimes present at birth but by age 4 it is evident in nearly every patient. Arcus corneae develop in early childhood. Atherosclerosis results in death from myocardial infarction usually before thirty years of age (Goldstein et al., 1995).

The world prevalence of heterozygous FH is 1:500 and that of homozygotes is 1:1000000 (Goldstein et al., 1995). In certain homogeneous populations, such as the South African Afrikaner population, the frequency is as high as 1/70 due to a founder effect (Seftel et al., 1980; Steyn et al., 1996).

Table 1.1 History of Familial Hypercholesterolemia (FH)

1784	Discovery of cholesterol
Pre 1900	Association of xanthomas in tendons and atheromas in arteries
1930s	Müller & Thannhauser recognised familial clustering of patients exhibiting xanthomas, premature CHD and hypercholesterolaemia
1940s/50s	Wilkinson and Aldersberg substantiated genetic basis for hypercholesterolaemia by family studies
mid 1950s	Gofman showed hypercholesterolaemia in FH due to a selective increase in the plasma concentration of low density lipoprotein
early 1960s	Khachadurian delineated differences between heterozygotes and homozygotes – first evidence of a single gene inheritance
1960s	Fredrickson, Levy and Lees conclude that FH is a disorder involving the metabolism of both the apolipoprotein and cholesterol components of LDL
1970s	Brown and Goldstein discovered that FH is caused by mutations in the cell surface LDLR gene
1982	Purification of the LDLR gene
1983	Cloning of the cDNA
1984	Isolation and characterisation of the gene at a locus on human chromosome 19
1985	Brown and Goldstein were awarded the Nobel Prize in Medicine

1.3 The low density lipoprotein receptor (LDLR)

The LDLR gene is located on the short arm of chromosome 19. The gene encodes a single-chain cell-surface glycoprotein comprising 839 amino acids in its mature form. It contains approximately two asparagine-linked oligosaccharide chains and nearly eighteen serine/threonine-linked oligosaccharide chains. The LDLR binds two protein ligands, apolipoprotein B-100 and apolipoprotein E (Schneider et al., 1982).

The mRNA of the LDLR is 5.3 kb long and encodes a protein of 860 amino acids. Nearly half of the mRNA constitutes a long 3' untranslated region with two and a half copies of the Alu family of middle repetitive DNAs. The gene is divided into 18 exons and 17 introns (Yamamoto et al., 1984).

The LDLR pathway starts in the endoplasmic reticulum (ER) where the protein is synthesised. It contains high mannose *N*-linked carbohydrate chains and *O*-linked chains. After 30 minutes the high mannose N-linked oligosaccharide chains are converted to the complex endoglycosidase H-resistant form. At the same time, the addition of one galactose and one or two sialic acid residues elongate each O-linked chain. Next, the LDLRs appear on the cell surface where they gather in coated pits. These coated pits then form coated vesicles. Many of these coated vesicles will fuse to form endosomes. Due to a low pH, the receptor returns to the surface where it binds with another lipoprotein particle and begins another cycle of endocytosis. The LDL that dissociates from the receptor moves to the lysosome, where it is degraded by acid hydrolytic enzymes. The apoprotein of LDL is hydrolysed by a lysosomal lipase. The

unesterified cholesterol is used for membrane synthesis and as a regulator of intracellular homeostasis (Soutar et al., 1986).

1.4 Classification of LDLR gene mutations

LDLR mutations can be divided into five classes based on their phenotypic effects on the protein. Class 1 mutations fail to produce immunoprecipitable protein (null alleles). Class 2 mutations encode proteins that are blocked, either completely or partially, in transport between the ER and the Golgi apparatus (transport-defective alleles). Class 3 mutations encode proteins that are synthesised and transported to the cell surface, but fail to bind LDL normally (binding-defective alleles). Class 4 mutations encode proteins that move to the cell surface and bind LDL normally, but are unable to cluster in clathrin-coated pits and thus do not internalise LDL (internalisation-defective alleles). Class 5 mutations encode receptors that bind and internalise in coated pits, but fail to discharge the ligand in the endosome and fail to recycle to the cell surface (recycling-defective alleles) (Hobbs et al., 1990; Goldstein et al., 1995).

1.5 Aim of study

FH is a largely underdiagnosed disease and therefore most affected individuals are not treated to prevent unnecessary early deaths due to heart attacks. Studies are ongoing to identify the disease-causing mutation in index patients being referred for molecular diagnosis, and tracing of the defective LDLR genes in families would enable accurate diagnosis of FH or exclusion of the disease in at-risk family members. 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.

The objective of this study was to screen relatives of molecularly-characterised FH index patients for a known mutation in the family, to incorporate this data in the MED-PED database. The specific aims were:

(1) to identify FH-related mutation(s) in clinically diagnosed FH patients and their at- risk family members

(2) determine the best age-sex specific lipid/clinical criteria for diagnosing FH in the South African population

(3) use DNA data to determine what percentage of FH patients could be diagnosed by clinical criteria

(4) determine the rate at which specific genes for FH are unexpressed in lipid tests

(5) determine whether clinical criteria need to be different by country/population.

REFERENCES

Familial hypercholesterolaemia: report of a WHO consultation. World Health Organisation, Human Genetics programme, Division of Noncommunicable Diseases. Paris, October 1997.

Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolaemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*, 7th edn. McGraw-Hill, New York, 1995: 1981-2030.

Hobbs HH, Russel DW, Brown MS, Golstein JL. The LDL receptor locus and familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu Rev Genet* 1990; 24: 133-137.

Kotze MJ, Callis M. MedPed FH: a paradigm for other common monogenic diseases in South Africa. *Atherosclerosis* 1999; 144: 467-468.

Kwiterovich PO Jr, Fredrickson DS, Levy RI: Familial hypercholesterolemia (one form of familial type II hyperlipoproteinemia). A study of its biochemical, genetic and clinical presentation in childhood. *J Clin Invest* 1974; 53: 1237-1241.

Schneider WJ, Beisiegel U, Goldstein JL, Brown MS. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164 000 molecular weight. *J Biol Chem* 1982; 257: 2664-2668.

Seftel HC, Baker SG, Sandler MP, Forman MB, Joffe BI, Mendelsohn D, Jenkins T, Mieny CJ. A host of hypercholesterolaemic homozygotes in South Africa. *Br Med J* 1980; 281: 633-636.

Soutar AK, Harders-Spengel K, Wade DP, Knight BL. Detection and quantitation of low-density lipoprotein (LDL) receptors in human liver by ligand blotting, immunoblotting and radioimmunoassay. *J Biol Chem* 1986; 261: 17127-17133.

Steyn K, Goldberg YP, Kotze MJ, Steyn M, Swanepoel ASP, Fourie JM, Coetzee GA, Van der Westhuyzen DR. Estimation of the prevalence of familial hypercholesterolaemia in a rural Afrikaner community by direct screening for three Afrikaner founder low density lipoprotein receptor gene mutations. *Hum Genet* 1996; 98: 95-97.

Williams RR, Schumacher MC, Barlow GK, Hunt SC, Ware JL, Pratt M, Latham BD. Documented need for more effective diagnosis and treatment of familial hypercholesterolemia according to data from 502 heterozygotes in Utah. *Am J Cardiology* 1993; 72: 18D-24D.

Yamamoto T, Davis CG, Brown MS, Scneider WJ, Casey ML, Goldstein JL, Russel DW. The human LDL receptor: A cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 1984; 39: 27-36.

CHAPTER 2

MATERIALS AND METHODS

DETAILED EXPERIMENTAL PROCEDURES

2.1 Isolation of DNA

Four different DNA isolation methods were used during this study.

Method 1: This method was used for the isolation of DNA from whole blood. EDTA-preserved blood was mixed with 40 ml cold lysis buffer (155 mM NH_4Cl; 10 mM KHCO3; 0.1 mM EDTA with pH 7.4) in a 50 ml polypropylene Falcon tube. The lysis mixture was placed on ice until lysis of RBS was apparent. Then the mixture was centrifuged at 1500 rpm for 10 minutes. The supernatant was removed with care as not to disturb the pellet. The cell pellet was washed carefully by gently mixing with 10 ml PBS (dissolve 10 tablets in 1 litre of ddH_2O, OXOID) buffer. The supernatant was removed and the pellet resuspended in 3 ml nuclei lysis buffer (10 mM Tris-Cl; 400 mM NaCl; 2 mM EDTA at pH 8.2), 50 μl proteinase K (10 mg/ml, Boehringer Mannheim) and 300 μl 10% SDS (w/v). This solution was then mixed on a vortex for 5 seconds and incubated overnight at 55°C.

The next morning 1ml of saturated 6 M NaCl solution was added to each tube and then shaken vigorously for approximately 1 minute followed by centrifugation at 2500 rpm for 15 minutes. The precipitated pellet was left at the base of the tube and the supernatant that contains the DNA transferred to a fresh 50 ml Falcon tube. Two volumes of cold absolute ethanol was added to the tube and left at room temperature for the DNA to precipitate. The precipitated DNA strands were subsequently removed with a glass pipette and transferred to a labelled 1.5 ml microcentrifuge tube. Then 70% ethanol was added to rid the DNA of excess salt followed by centrifugation. Excess ethanol was

removed and the DNA left to air-dry at room temperature. The DNA pellet was then dissolved in 300-500 µl of sterile ddH_2O (Miller et al., 1988).

Method 2: This method involved the use of DNAzol™ (GibcoBRL). Approximately 200 µl whole blood was mixed with 1 ml lysis buffer (155 mM NH_4Cl; 10 mM KHCO3; 0.1 mM EDTA with pH 7.4) and placed on ice until RBC underwent lysis – usually after 10 minutes. The solution was centrifuged in a benchtop centrifuge at 13 000 rpm for 1 minute and the supernatant carefully removed. The pellet was then washed with 1 ml PBS (Dissolve 10 tablets in 1 litre of ddH_2O, OXOID) and centrifuged for 30 seconds. The supernatant was removed and 100-200 µl DNAzol™ (GibcoBRL) added depending on pellet size, vortexed and left on the bench for 5 minutes. The preparation was then centrifuged at 13 000 rpm to remove cell debris. Two volumes of cold 100% absolute ethanol were added and the solution left at room temperature for DNA to precipitate. Precipitated DNA strands was removed with a glass pipette and placed in a labelled 1.5 ml microcentrifuge tube containing 70% ethanol. The sample was then centrifuged and excess ethanol removed. DNA was washed with 1 ml of SABAX water before centrifugation. The resultant DNA pellet was dissolved in 500 µl 8 mM NaOH and buffered with 25 µl Tris-Cl (pH 8.0).

Method 3: This method was employed for the isolation of DNA from buccal swabs and cultured amniotic cells. Perkin Elmer (CheekSwab) mouth brushes were used to collect buccal cells in cases where blood samples could not be obtained. The cells were pelleted by centrifuging for 10 minutes at 2500 rpm. The supernatant was discarded and

the pellet resuspended in 200 µl 50 mM NaOH. The mixture was placed in a fresh microcentrifuge tube. A hole was made in the top of the tube with a hypodermic needle and boiled for 10 minutes on a floating tray in boiling water. The solution was buffered with 25 µl 1 M Tris-Cl (pH 8.0). The DNA was then pipette into a fresh tube (Talmud et al., 1991).

Method 4: This method is a shortened version of the first method. Approximately 400 µl freshly prepared 170 mM NH_4Cl was mixed with 100 µl whole blood and incubated at room temperature for 20 minutes. By centrifugation for 30 s at 13 000 rpm the white blood cells were pelleted and the supernatant discarded. The pellet was washed several times with cold 0.9% NaCl (commercial available saline). After resuspending the pellet in 200 µl 50 mM NaOH the mixture was boiled for 10 minutes on a floating tray in boiling water. To buffer the solution 25 µl 1M Tris-Cl (pH 8.0) was added. The DNA was then placed in labelled tubes.

2.2 Determination of DNA concentration

DNA concentration was determined by spectrophotometry. The readings were taken at UV wavelengths of 260 nm and 280 nm. An OD of 1 at 260 nm corresponds to approximately 50 µg/ml of double stranded DNA. The purity of the sample was calculated by the ratio of readings at 260 nm and 280 nm (OD_{260}/OD_{280}). Pure DNA showed a value of 1.8 while a value above 1.8 indicated RNA contamination and a value below 1.8 protein or phenol contamination. Contaminated DNA samples were subjected to DNA purification techniques.

2.3 DNA Purification

Those DNA samples which did not yield a product following PCR amplification, were subjected to ethanol precipitation and phenol-chloroform purifications.

2.4 Polymerase Chain Reaction (PCR)

Genomic DNA was amplified by the polymerase chain reaction in a Hybaid Omnigene Thermal Cycler (Hybaid, Teddington, Middlesex, UK) or a Perkin Elmer Thermal Cycler, PE 9700 (Perkin Elmer, South Africa Pty Ltd., Johannseburg, SA). The PCR primers used in this study are listed in Tables 2.1 (Jensen et al., 1996) and 2.2 (Nissen et al., 1996). Each PCR reaction consisted of 20-50 ng genomic DNA, 20 pmol each of forward and reverse primers, 100 μM of each dNTP, 10X Buffer with/without 1.5 mM $MgCl_2$, 25 mM $MgCl_2$ (optional), 0.5 U *Taq* DNA polymerase, 200 μM Cresol red loading buffer and nuclease-free water to a final volume of either 25 or 50 μl.

2.5 Multiplex PCR

Three founder-related mutations (D154N, D206E and V408M) causing FH in 90% of affected Afrikaners (Kotze et al., 1991) were screened with the use of multiplex amplification refractory mutation system (ARMS-PCR). Three common (COMM 1-3) and three ARMS primers specific for the mutant alleles of the LDLR gene were used (Kotze et al., 1995). Reactions included genomic 0.5 μg DNA, 2 U of *Taq* DNA polymerase (Boehringer Mannheim), 100 mmol/l Tris-HCl, 15 mmol/l $MgCl_2$, 500 mmol/l KCl with pH 8.3 at 20°C, 200 μM of each dNTP, 25 pmol of primer COMM 1, 50 pmol of primer

ARMS 1, 10 pmol of primers COMM 2 and ARMS 2, 100 pmol of primers COMM 3 and ARMS 3, 2 mM tetramethylammonium chloride and 15% glycerol overlaid with light mineral oil (Sigma).

Thermal cycling was performed in a Perkin-Elmer Thermal Cycler, PE 9700. The PCR program included an initial denaturation step of 5 minutes at 94°C, followed by 15 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and an extension at 72°C for 2 minutes. This was followed by 20 cycles of the same conditions but with an annealing temperature of 55°C, followed by a final step at 35°C for 10 seconds.

Table 2.1 Primers used to amplify the promoter and coding region of the LDLR gene.

Region/ Exon	Oligonucleotide sequence (5' to 3')	Fragment size (bp)
Promoter	F GAGGCAGAGAGGACAATGGC R CACGACCTGCTGTGTCCAAGCTTGAAACCC	277
1	F CACATTGAAATGCTGTAAATGACG R CTATTCTGGCGCCTGGAGCAAGCC	215
2	F TTGAGAGACCCTTTCTCCTTTTCC R GCATATCATGCCCAAAGGGG	183
3	F TTCCTTTGAGTGACAGTTCAATCC R GATAGGCTCAATAGCAAAGGCAGG	196
4A	F GTGGTCTCGGCCATCCATCC R AGCCATCTTCGCAGTCGGGG	242
4B	F CCCCCAGCTGTGGGCCTGCG R CGCCCCCACCCTGCCCCGCC	237
5	F AGAAAATCAACACACTCTGTCCTG R GGAAAACCAGATGGCCAGCG	180
6	F TCCTCCTTCCTCTCTCTGGC R TCTGCAAGCCGCCTGCACCG	179
7	F GGCGAAGGGATGGGTAGGGG R GTTGCCATGTCAGGAAGCGC	236
8	F CATTGGGGAAGAGCCTCCCC R GCCTGCAAGGGGTGAGGCCG	220
9	F CCCCTGACCTCGCTCCCCGG R GCTGCAGGCAGGGGCGACGC	224
10	F ATGCCCTTCTCTCCTCCTGC R AGCCCTCAGCGTCGTGGATA	278
11	F TCCTCCCCCGCCCTCCAGCC R GCTGGGACGGCTGTCCTGCG	194
12	F ACTGGCATCAGCACGTGACC R CGTGTGTCTATCCGGCCACC	236
13	F GTCATCTTCCTTGCTGCCTG R TTCCACAAGGAGGTTTCAAGGTTGGGGGGG	329
14	F AAATTTCTGGAATCTTCTGG R GCAGAGAGAGGCTCAGGAGG	268
15	F AGAAGACGTTTATTTATTCTTTC R GTGTGGTGGCGGGCCCAGTCTTT	217
16	F CCTTCCTTTAGACCTGGGCC R CATAGCGGGAGGCTGTGACC	173
17	F GGGTCTCTGGTCTCGGGGGC R GGCTCTGGCTTTCTAGAGAGGG	242
18	F GCCTGTTTCCTGAGTGCTGG R TCTCAGGAAGGGTTCTGGGC	135

Table 2.2 Primers used to amplify the promoter and coding region of the LDLR gene for DGGE.

Region/ Exon	Oligonucleotide sequence (5' to 3')	Fragment size (bp)
30% to 70% denaturant gradient		
Promoter	F 40-bp GC-clamp-AGGACTGGAGTGGGAATCAGAGC R TGCTGTCCTAGCTGGAAACCC	252
2	F 40-bp GC-clamp-CGTGGTCAGTTTCTGATTCTGGCG R ATAAATGCATATCATGCCCAAAGG	253
3	F 40-bp GC-clamp-TCGGCCTCAGTGGGTCTTTC R ACTCCCCAGGACTCAGATAGGC	268
5	F 40-bp GC-clamp-GGCCCTGCTTGTTTTTCTCTGG R AGCAGCAAGGCACAGAGAATGG	282
6	F 40-bp GC-clamp-ACGAAACTGAGGCTCAGACACACC R GCTCCCCACAAACTCTGCAAGC	262
10	F GCAGTGAGATGAGGGCTCCTGG R 40-bp GC-clamp-CCTGCAGCCCTCAGCGTCG	349
11	F 40-bp GC-clamp-GGATCCTCCCCCGCCCTC R TGGCTGGGACGGCTGTCC	239
12	F GGCCCTCAGGCCCTCTGG R 40-bp GC-clamp-CCGAGTTTTCTGCGTTCATCTT	336
13	F 50-bp GC-clamp-GTCATCTTCCTTGCTGCCTG R CACAAGGAGGTTTCAAGGTTGG	264
17	F 50-bp GC-clamp-GGGCAGCTGTGTGACAGAGCG R CATGGCTCTGGCTTTCTAGAGAGG	279
ApoB	F 40-bp GC-clamp-GGAGCAGTTGACCACAAGCTTAGC R GGTGGCTTTGCTTGTATGTTCTCC	382
40% to 80% denaturant gradient		
1	F 50-bp GC-clamp-TTGAAATGCTGTAAATGACGTGG R CTGGCGCCTGGAGCAAGC	256
4A	F 40-bp GC-clamp-ACTGCGGCAGCGTCCCCGGC R GGATGCAGGTGGAGCTGTTGC	297
4B	F ACCTGTGGTCCCGCCAGC R 40-bp GC-clamp-CCAGGGACAGGTGATAGGACG	345
7	F 40-bp GC-clamp-AGAGTGACCAGTCTGCATCCCTGG R TTGGTTGCCATGTCAGGAAGC	253
8	F 40-bp GC-clamp-TCCCCACCAAGCCTCTTTCTCTC R CCACCCGCCGCCTTCC	222
9	F 46-bp GC-/10-bp AT-clamp-CTGACCTCGCTCCCCGGACC R GGCTGCAGGCAGGGGCGACG	278
14	F 50-bp GC-clamp-TCTCGTTCCTGCCCTGACTCC R GACACAGGACGCAGAAACAAGG	274
15	F 3-bp GC-clamp-GGCACGTGGCACTCAGAAGACG R 50-bp GC-clamp-GTGTGGTGGCGGGCCCAGTCTTT	288
16	F 50-bp GC-clamp-CTCCATTTCTTGGTGGCCTTCC R CATAGCGGGAGGCTGTGACCTGG	239
18	F 50-bp GC-clamp-CCTGAGTGCTGGACTGATAGTTTCC R AAGGCCGGCGAGGTCTCAGG	190

3-bp GC-clamp: CGG

40-bp GC-clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

46-bp GC-/10-bp AT-clamp: CGCCCGCGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCGAAATAATAAA

50-bp GC-clamp: CGCCCGCCGCCGCCCGCCGCGCCCCGCGCCCGTCCCGCC GCCCCCGCCCG

2.6 Agarose gel electrophoresis

The PCR products were loaded on a 2% agarose gel, electrophoresed for 30 minutes at 100 V, stained for 5 minutes in 1x TBE containing ethidium bromide (10 mg/ml) and examined under ultraviolet light.

2.7 Heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis

HEX-SSCP analysis was performed on a 20 cm vertical gel apparatus. The gel preparation was as follows: firstly the two glass plates were clamped together with binder clips and an agarose plug was poured at the bottom. The polyacrylamide (PAA) gel was poured between the two glass plates, the gel allowed to polymerise (6-10 minutes) and a 1mm comb was inserted between the glass plates. The PCR products (10-15 μl) were denatured for 5 minutes at 95°C (Kotze et al., 1995) in the presence of cresol red loading buffer, loaded on the PAA gel and electrophoresed at room temperature overnight at 100 V in 1X TBE buffer. The following morning the apparatus was disassembled and the gel stained for 10 minutes in 1X TBE containing ethidium bromide (10 mg/ml) and then destained in distilled water for 5 minutes. Finally the DNA was visualised on the UVP Image Store 500 Ultra-Violet transilluminator system, and photographed with the Sony Video Graphic Printer (UP 860 LE) system.

The buffers used in this study were prepared as described in "Molecular Cloning: A Laboratory Manual" (Sambrook et al., 1989). The stock solutions for HEX-SSCP analysis were:

1. 40% stock (1% C)

    39.6 g acrylamide

    0.4 g bisacrylamide

    Made up to a total volume of 100 ml with ddH_20

2. 10% Ammoniumpersulphate (APS)

    2 g Ammoniumpersulphate

    20 ml ddH_20

3. Loading buffer

Formamide Blue (25 ml): 95% formamide (de-ionised) 22.7 ml, 20mM EDTA (disodiumsalt) 0.168 g, 0.05% Xylene Cyanol 0.0125 g, 0.05% Bromophenol blue 0.0125 g.

Ficoll Orange G (100 ml): 0.1% Orange G 0.1 g, 20% Ficoll 20 g, 10 mM EDTA 0.29 g.

Cresolred: 1 mmol/l Cresolred 3.82 g, 60% Sucrose 60 g.

Three different PAA gel systems were used:

System 1. 10% Urea gel (combined heteroduplex and SSCP)

1.5X TBE final buffer concentration

4.5 g Urea

18 ml 5X TBE

26 ml ddH_2O

15 ml of 40% stock solution (1% C)

800 µl APS (10% stock)

80 µl TEMED

System 2. 5% Glycerol gel

0.5X TBE final buffer concentration

3 ml glycerol

6 ml 5X TBE

36 ml ddH_2O

15 ml of 40% stock (1% C)

800 µl APS (10% stock)

80 µl TEMED

System 3. 20% Mighty Small

0.5X TBE final buffer concentration

1 ml 5X TBE

4 ml ddH_2O

5 ml of 40% stock (1% C)

80 µl APS (10% stock)

40 µl TEMED

2.8 Denaturing gradient gel electrophorese (DGGE)

Two different denaturant concentrations (30-70% and a 40-80%) were used to prepare the gels. Two glass plates with 1mm spacers were clamped together with binder clips. A gradient mixer was put above the DGGE frame. Exactly 14 ml of the lower denaturant concentration was poured in the left side and 14 ml of the higher concentration in the

right chamber of the gradient mixer. The connection between the two chambers was opened to let the solution passes through the plastic tube between the two glass plates. The gel was then allowed to polymerise for an hour. After one hour, the gel apparatus was placed into the bath containing 1X TAE buffer heated to 60°C and then loaded 7.5-10 µl PCR product on the gel. Electrophoresis was performed for 5 hours at 150 V or overnight at 50 V at a constant temperature of 60°C. Staining of the gel was done in 1X TAE buffer containing ethidium bromide (10 mg/ml) for 10 minutes, destained in distilled water for 5 minutes. Then visualised on the UVP Image Store 5000 Ultra-Violet transilluminator system and photographed with the Sony Video Graphic Printer (UP 860 LE) system.

2.9 Restriction enzyme analysis

A reaction for restriction enzyme digestion was prepared by adding the following reagents to a microcentrifuge tube: PCR product (1-10 µl, >40 ng), 10X enzyme specific buffer (1/10th volume), restriction enzyme (1-10 units per 10 µl PCR product) and filled up as appropriate with sterile distilled water. The solution was gently mixed by pipetting and the reaction incubated at the specified temperature (usually 37°C) for at least 1 hour, but preferably overnight. The enzyme was inactivated by heating at 70-100°C for 10 minutes (see instructions for specific enzymes). Then 5 µl of loading buffer was added and the product loaded on a PAA gel.

The stock solutions for restriction enzyme analysis were:

30% stock (3.4% C)

58.8g acrylamide

1.2g bisacrylamide

Make up to a total volume of 200 ml with dH_2O.

2.10 Sequencing

Automated sequencing was performed when aberrations were detected. For automated sequencing, the Perkin Elmer 310 sequencer was used.

REFERENCES

Jensen HK, Jensen LG, Hansen PS, Faergeman O, Gregerson N. High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. *Clin Chem* 1996; 42: 1140-1146.

Kotze MJ, Langenhoven E, Warnich L, Du Plessis L, Retief AE. The moleceular basis and diagnosis of familial hypercholesterolaemia in South African Afrikaners. *Ann Hum Genet* 1991; 55: 115-121.

Kotze MJ, Theart L, Callis M, Peeters AV, Thiart R, Langenhoven E. Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low-density lipoprotein receptor gene mutations. *PCR Methods Appl.* 1995; 4: 352-356.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 1988; 16: 1215.

Nissen H, Guldberg P, Hansen AB, Petersen NE, Horder M. Clinically applicable mutation screening in familial hypercholesterolemia. *Hum Mutat* 1996; 8: 168-177.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual (2nd edn) 1989 Cold Spring Harbour Laboratory Press.

Talmud P, Tybjaerg-Hansen A, Bhatnagar D, Mbewu A, Miller JP, Durrington P, Humphries S. Rapid screening for specific mutations in patients with a clinical diagnosis of familial hypercholesterolaemia. *Atherosclerosis* 1991; 89: 137-141.

CHAPTER 3

RESULTS AND DISCUSSION

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

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Abstract

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. The systematic molecular analysis of FH resulted in the identification of at least ten founder-type LDLR gene mutations among the 65 different gene defects described to date in the diverse South African population. DNA screening of 790 at-risk family members 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 index cases recruited for this study, were 89.3% and 81.9%, respectively.

Conclusions. The predominance of at least ten 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 provide a definitive tool for family tracing aimed at pre-clinical diagnosis and preventive treatment of FH.

INTRODUCTION

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 (Goldstein et al., 1995). 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 focussed on the Afrikaner population, considered to be a genetic isolate due to its unique geographic and historical situation. The predominance of specific disease-associated chromosomes in this population group (Kotze et al., 1987; 1989a) confirmed the occurrence of a founder effect (Seftel et al., 1980), and led to the identification of three mutations that are responsible for the disease in approximately 90% of Afrikaner FH patients (Leitersdorf et al., 1989; Kotze et al., 1989b; 1990). The value of a routinely used DNA-based test was demonstrated (Kotze et al., 1992; 1994), necessitating the development of a cost-effective method to screen for multiple mutations in a single reaction (Kotze et al., 1995b). Population screening by direct detection of the founder mutations D154N, D206E and V408M, confirmed the high

prevalence of heterozygous FH (1/70) in Afrikaners (Steyn et al., 1996). Loubser et al. (1999) demonstrated that these mutations are also responsible for the disease in up to 15% 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) (Meiner et al., 1991) and Indians (P664L) (Rubinsztein et al., 1992; Kotze et al., 1997), 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 appeared to be absent in the Coloured population (Leitersdorf et al., 1988). Frequent detection of this deletion-mutation in FH patients from the South African Black population (Thiart et al., in press), 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 be different by country/population (Primstone et al., 1998).

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 of the affected individuals are not treated to prevent unnecessary early deaths due to heart attacks. Studies are ongoing to identify the disease-causing mutation in patients being referred for molecular diagnosis, but family follow-up is rarely performed, partly due to costs involved. This is an unfortunate situation, since tracing of defective LDLR genes in families would enable accurate diagnosis of FH or exclusion of the disease in at-risk family members. 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. (1993) 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 in 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 objectives of the study were to (i) determine what percentage of FH patients could be diagnosed by clinical criteria and (ii) determine the rate at which specific genes for FH are unexpressed in lipid tests.

MATERIALS AND METHODS

Subjects

Patients were selected for mutation screening using previously-described clinical criteria for a diagnosis of FH (Kotze et al., 1987). Pretreatment total serum cholesterol (TC) levels had to be at least equal to the 90th percentile for age and gender (Rossouw et al., 1985), with normal serum triglyceride (TG) levels (<2.3 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 CHD. Family follow-up was performed/extended in families where the FH-related mutation has been identified in the index case, while index cases without known mutations were subjected to extensive mutation screening and/or family follow-up. Known mutations causing familial defective apolipoprotein B-100 (FDB) (Soria et al., 1989) were excluded in all the study participants using previously-described methods (Kotze et al., 1994; Jensen et al., 1996).

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 stock; "Coloured" refers to an individual of mixed ancestry, including San, Khoi, African Negro, Madagascar, Javanese and Western European origin; "Black" refers to South Africans of central African descent.

Methods

Genomic DNA was extracted from whole blood collected in EDTA-containing tubes, according to a standard technique (Miller et al., 1987). PCR amplification of the coding region of the LDLR gene was performed using the exon-specific primers described by Jensen et al. (1996), or allele-specific primers specially designed for detection of mutations known to be common in South Africa (Kotze et al., 1995b). The promoter region of the LDLR gene was amplified using primers 5'-GAGGCAGAGAGGACAATGGC-3' and 5'-CCACGTCATTTACAGCATTTCAATG-3'. Heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis was performed using three different gel systems to improve mutation detection efficiency: 10% (1%C) polyacrylamide gel supplemented with 7.5% urea; 10% (1%C) polyacrylamide gel supplemented with 5% glycerol and 20% (1%C) 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 with the HEX-SSCP method, denaturing gradient gel electrophoresis (DGGE) was performed as described by Nissen et al. (1996). 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.

Evaluation of biochemical versus DNA diagnosis was performed in families with the Afrikaner founder mutations D206E (FH1), V408M (FH2) or D154N (FH3), of which the deleterious effects have previously been confirmed at the cellular level (Fourie et al.,

1988; Graadt van Roggen et al., 1995). Since index patients had been selected on the basis of elevated TC levels, genotype/phenotype correlations were only performed in family members recruited through tracing of defective genes in the pedigrees.

RESULTS

The spectrum of mutations in the diverse South African population, including four novel mutations detected for the first time during the course of this study, are summerised in Table 1. Sixty-five different disease-related mutations were identified in 379 index patients. Of the 790 at-risk relatives analysed, 338 inherited the disease-related LDLR gene mutation.

Table 1. Spectrum of mutations identified in the LDLR gene in different South African population groups

Exon/ Intron	**Molecular Event**	**Designation**	**No.**	**Relatives Tested**	**References**
AFRIKANER POPULATION					
Exon 3	T→G at 259	W66G	1	None	Leitersdorf et al., 1990
Exon 4	G→A at 523	**D154N**	31	55 +, 105 -	Kotze et al., 1989b
Exon 4	A→G at 662	**D200G**	5	3 +, 1 -	Hobbs et al., 1992
Exon 4	Ins 18-bp after 681	Ins AA 201-206	1	1 +, 5 -	Kotze et al., 1995a
Exon 4	C→G at 681	**D206E**	144	144 +, 194 -	Kotze et al., 1990
Exon 6	C→T at 917	**S285L**	3	2 +, 2 -	Hobbs et al., 1992
Exon 7	C→T at 1048	R329X	1	None	Solberg et al., 1994
Exon 8	G→A at 1130	**C356Y**	4	5 +, 1 -	This study
Exon 8	G→T at 1145	**G361V**	2	3 +, 4 -	This study
Exon 9	G→A at 1285	**V408M**	57	51 +, 61 -	Kotze et al., 1989b
Exon 16	G→A at 2389	V776M	1	None	Pereira et al., 1995
MIXED ANCESTRY (COLOURED POPULATION)					
Promoter	C→T at –59	-59c→t	1	3 +, 14 -	Scholtz et al., 1999
Exon 1	T→C at 28	W-12R	1	None	Marx et al., 1997
Exon 2	G→A at 148	A29T	1	None	Loubser et al., 1999
Exon 3	C→T at 232	R57C	1	None	Day et al., 1997
Exon 3	C→T at 241	R60C	1	None	Nissen et al., 1998
Exon 4	del TC after 368	del 2-bp	1	None	Loubser et al., 1999
Exon 4	G→A at 523	D154N	2	None	Kotze et al., 1989b

Exon 4	Del GGT after 651	del 3-bp	4	None	Meiner et al., 1991
Exon 4	A→G at 662	D200G	1	None	Hobbs et al., 1992
Exon 4	A→G at 680	D203A	1	None	Loubser et al., 1999
Exon 4	C→ G at 681	D206E	19	4 +, 4 -	Kotze et al., 1990
Exon 4	G→A at 682	E207K	1	None	Leitersdorf et al., 1990
Exon 4	T→G at 691	C210G	1	None	Sundvold et al., 1996
Exon 5	G→A at 772	E237K	1	4 +, 7 -	Loubser et al., 1999
Intron 6	G→A at 941-4	941-4 G→A	1	None	Loubser et al., 1999
Intron 6-8	del 2.5-kb	del 2.5-kb	10	2 +	Henderson et al., 1988
Exon 8	T→G at 1154	L364R	1	None	Loubser et al., 1999
Exon 9	G→A at 1285	V408M	13		Kotze et al., 1989b
Exon 14	C→T at 2054	P664L	3	None	Hobbs et al., 1992
BLACK POPULATION					
Promoter	del CTC after –92	del 3-bp	1	None	Peeters et al., 1998
Exon 2	del 6-bp after 138	**del 6-bp**	4	1 +, 2 -	Leitersdorf et al., 1988
Exon 2	del G at 172	del 1-bp	1	None	Hobbs et al., 1992
Intron 3	G→A at 313+1	313+1G→A	1	4 -	Thiart et al., in press
Exon 4	G→C at 514	D151H	1	None	Thiart et al., in press
Exon 5	C→T at 756	R232W	1	1 +, 3 -	Thiart et al., in press
Exon 9	G→A at 1217	R385Q	1	None	Thiart et al., in press
Exon 9	G→A at 1222	E387K	1	5 +, 5 -	Hobbs et al., 1992
Exon 14	C→T at 2096	P678L	1	None	Schuster et al., 1995
Exon 17	G→A at 2441	R793Q	1	None	Thiart et al., in press
INDIAN POPULATION					
Exon 1	A→T at 1	M-21L	1	2 +, 1 -	Langenhoven et al. 1996
Exon 3	C→T at 232	R57C	1	None	Kotze et al. 1997
Exon 3	G→T at 268	D69Y	1	None	Rubinsztein et al. 1993
Exon 4	G→A at 418	E119K	1	None	Rubinsztein et al. 1993
Exon 4	G→T at 661	D200Y	1	2 +, 3 -	Koivisto et al., 1995
Exon 4	G→A at 682	E207K	2	8 +, 7 -	Leitersdorf et al., 1990
Exon 8	C→A at 1175	C371X	1	11 +, 9 -	Langenhoven et al., 1996
Exon 9	C→G at 1215	N384K	1	3 +, 2 -	Kotze et al., 1997
Exon 14	C→T at 2054	**P664L**	10	None	Soutar et al., 1989
Exon 16	A→T at 2356	S765C	1	4 +, 2 -	Kotze et al., 1997
JEWISH POPULATION					
Exon 3	C→T at 253	Q64X	2	1 +	Schuster et al., 1995
Exon 4	C→T at 373	Q104X	1	None	This study
Exon 4	del GGT after 651	**del 3-bp**	5	1 +	Meiner et al., 1991
Exon 4	C→ G at 681	D206E	1	2 -	Kotze et al., 1990
Intron 9	G→A at 1358+1	1358+1 G→A	1	None	Callis (unpublished results)
Exon 9	C→G at 1284	N407K	1	2 +	Callis (unpublished results)
Exon 9	G→A at 1285	V408M	1	None	Kotze et al., 1989b
Intron 14	G→A at 2140+5	2140+5G→A	1	None	Heath et al., 1999
EUROPEAN ANCESTRY					
Exon 3	C→T at 232	R57C	1	None	Callis et al., 1998
Exon 4	Ins G at 558	558 ins G	1	1 +, 3 -	Thiart et al., 1998

Exon 4	del G at 617	617 del G	1	None	Callis et al., 1998
Exon 4	C→ G at 681 [5]	D206E	1	None	Kotze et al., 1990
Exon 6	G→A at 910	D283N	1	None	Bilheimer et al., 1985
Exon 7	C→T at 1048	R329X	1	None	Solberg et al., 1994
Exon 8	A→C at 1133	Q357P	1	None	Callis et al., 1998
Exon 8	C→T at 1150	Q363X	1	3 +, 4 -	Kotze et al., 1997
Exon 8	C→G at 1156	D365E	1	3 +, 4 -	Kotze et al., 1997
Exon 9	G→C at 1329	W422C	1	None	Hobbs et al., 1992
Exon 10	Complex del/ins	16-bp del/5-bp ins	1	None	This study
Exon 10	T→C at 1447	W462R	1	None	Ward et al., 1995
Exon 11	G→A at 1646	G528D	1	2 +	Hobbs et al., 1992
Exon 11	A→C at 1690	N543H	1	None	Tricot-Guerber et al., 1995
Exon 14	C→A at 2043	C660X	2	7 +, 2 -	Lehrman et al., 1987
Exon 14	del T at 2092	2092 del T	1	4 +, 1 -	Hobbs et al., 1992
Intron 14	G→A at 2140+5	2140+5G→A	1	None	Heath et al., 1999
Exon 17	del 9-bp after 2393	2393 del 9-bp	1	None	Lombardi et al., 1996

The majority of mutations summerised in this table were included in a recent mutation update (Varret et al., 1998).

Table 2 shows the characteristics of 11 hypercholesterolaemics diagnosed with FH on the basis of clinical features, but in whom no mutations could be identified despite extensive analysis of the LDLR and apo B genes.

Table 2. Characteristics of 11 molecularly-uncharacterised patients diagnosed with FH on the basis of clinical and biochemical features

Index	Sex	Age	LDLC	Clinical	Origin
RM	f	54	9.3	Arc, Xan	Black
AM	f	58	5.7	Arc, Xan	European
CN	f	58	5.3	Arc	Black
CK	m	26	12.1	Arc, Xan	Black
LM	f	56	3.5	Arc, CHD	Afrikaner
TM	m	53	5.0	Arc, MI	Black
PL	m	36	7.5	Arc, Xan, CHD	Indian
ST	f	62	6.1	Arc	European
IG	f	54	5.1	Xan	Afrikaner
AM	f	70	6.3	Arc, Xan	Black
JD	f	64	4.7	Arc, Xan	European

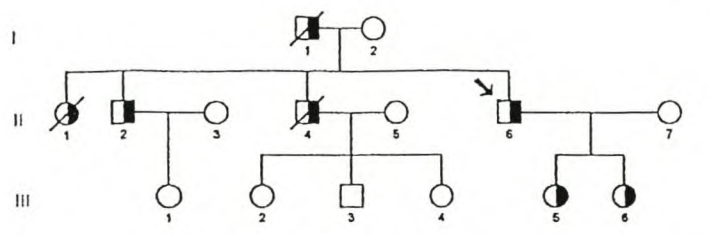
LDLC, low-density lipoprotein cholesterol; CHD, coronary heart disease; Arc, arcus cornealis; Xan, xanthomata; MI, myocardial infarction.

Mutations D200G (exon 4), S285L (exon 6), C356Y (exon 8) and G361V (exon 8) were identified in 14 unrelated Afrikaner families without the previously described founder mutations. The pedigrees of four of these families are illustrated in Figure 1, together with the results obtained after HEX-SSCP and restriction enzyme analysis. Mutation D200G in exon 4 creates a *Msp*I restriction enzyme recognition site, and after digestion three bands of 237-bp (normal allele), 176-bp and 61-bp (mutant allele) are observed in heterozygous individuals. Mutation C356Y in exon 8 creates a *Rsa*I restriction enzyme recognition site and after digestion three bands of 220-bp (normal allele), 115-bp and 105-bp (mutant allele) were observed in FH heterozygotes.

Figure 1. Identification of mutations D200G, S285L, C356Y and G361V in 4 Afrikaner families. Mutation-positive family members are indicated in dark-shaded symbols.
(A)Pedigree structures with ages and lipid profiles indicated (TC, Total cholesterol; HDL, High density lipoprotein cholesterol; TG, Triglyceride; LDL, Low density lipoprotein cholesterol). (B) Detection of mutations in the LDLR gene using HEX-SSCP and restriction enzyme analysis. Lane 1 and 2 represent mutation positive and control cases, respectively [see next page].

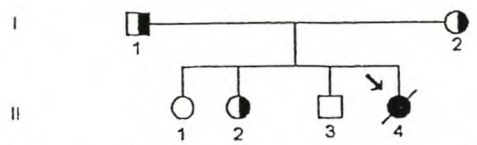
A

Family no. 129
Mutation D200G



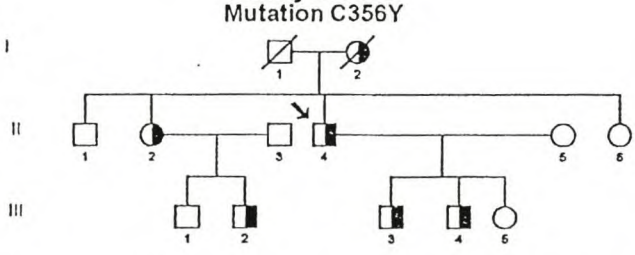
Age	52	13	25	23	50	21	47	13	9	41
TC	8.9	4.1	4.8	5.1	4.1	5.0	9.8	6.5	7.5	5.2
HDL	1.1	1.1	0.9	1.2	1.9	1.7	1.1	1.4	1.6	1.3
TG	1.9	1.3	1.3	2.0	2.2	1.6	1.7	1.1	1.0	1.9
LDL	6.9	2.4	3.3	3.0	1.2	2.6	7.9	4.6	5.4	3.0

Family no. 943
Mutation S285L



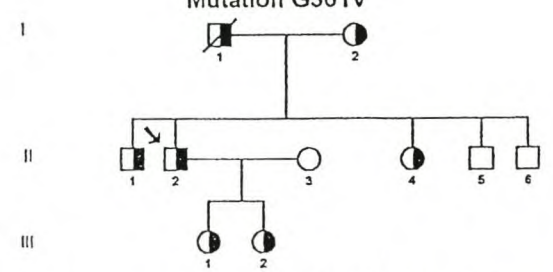
Age	45	17	11	9	43
TC	8.4	4.7	6.9	4.9	7.1
HDL	1.2	1.6	1.1	1.6	1.8
TG	1.6	0.6	1.2	0.9	0.6
LDL	6.5	2.8	5.2	2.9	5.0

Family no. 186
Mutation C356Y



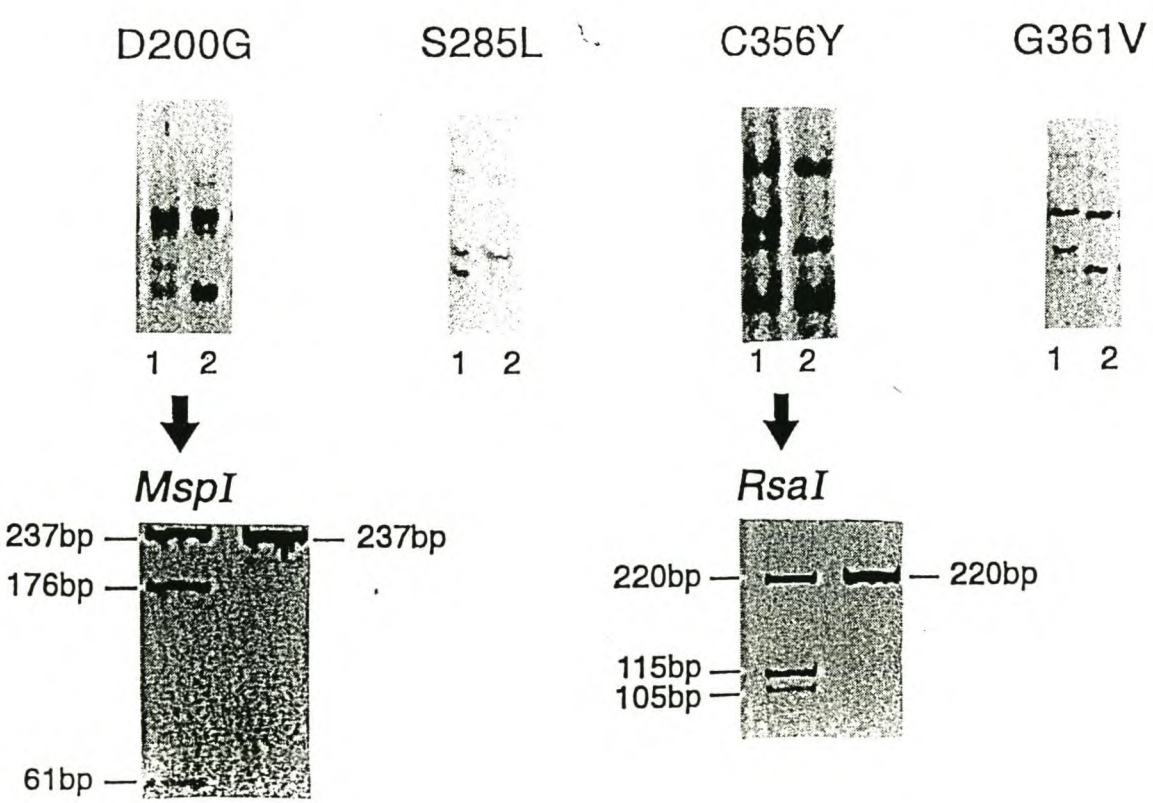
Age	58	31	43	22	12	40	--
TC	9.6	9.5	9.6	10.0	9.9	6.1	5.5
HDL	1.0	1.6	2.2	1.2	1.5	1.2	1.5
TG	1.5	1.6	2.7	0.9	1.6	1.4	1.1
LDL	7.9	7.2	6.2	8.4	7.7	4.3	3.5

Family no. 262
Mutation G361V



Age	44	41	9	5	37	68	38	34	33
TC	9.5	7.5	7.7	7.2	7.4	9.6	9.2	5.0	6.3
HDL	0.9	0.8	0.9	1.1	0.9	0.6	0.8	1.0	0.8
TG	5.9	1.3	1.2	0.8	2.1	3.6	3.2	1.9	5.3
LDL	5.9	6.1	6.3	5.7	5.5	7.3	6.9	3.1	3.1

B



In Table 3, 448 at-risk family members (>18 years old) screened for one of the three Afrikaner founder mutations 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 11.8% may be misdiagnosed when the 80th percentile is used as a biochemical cut-off point for a diagnosis of FH, compared to 29.4% using the 95th percentile for age and gender (Rossouw et al., 1985). In total, 18/153 relatives with a FH mutation were falsely classified as normal (negative predictive value of 88%), whilst 68/296 without the mutation were falsely classified as FH heterozygotes (positive predictive value of 77%). The sensitivity and specificity of FH diagnosis according to TC values (80th percentile) were therefore 88% and 77%, respectively.

Table 3. 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	94	62 (65%)	83 (88%)
V408M	28	21 (75%)	24 (86%)
D154N	30	25 (83%)	29 (97%)
No mutation	296	13 (4%)	55 (19%)
Total	448		

In Figure 2 total cholesterol (TC) concentrations are shown as a function of age in 349 women (A) and 316 men (B) older than 18 years. There is a small overlap in TC levels between normal and affected individuals. For FH women and men, average TC concentrations were 7.98 (SD=2.49) and 7.61 (SD=2.30) mmol/l, respectively.

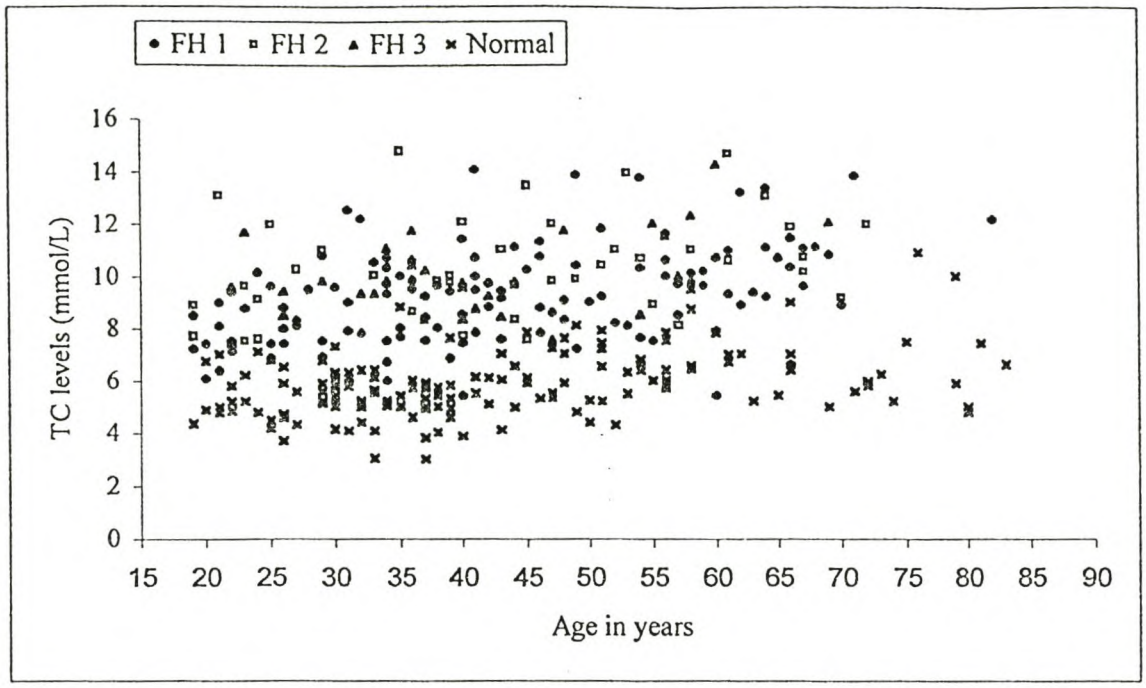
In Table 3, 443 at-risk family members (>18 years old) screened for one of the three Afrikaner founder mutations previously identified in the index case were grouped according to the presence of TC levels above the 80th and 95th percentile for age and gender. Evaluation of biochemical parameters revealed that 15.6% may be misdiagnosed when the 80th percentile is used as a biochemical cut-off point for a diagnosis of FH, compared to 12.4% using the 95th percentile for age and gender (Rossouw et al., 1985). In total, 16/150 relatives with a FH mutation were falsely classified as normal (negative predictive value of 89.3%), whilst 53/293 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.

Table 3. 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		

In Figure 2 total cholesterol (TC) concentrations are shown as a function of age in 349 women (A) and 316 men (B) older than 18 years. There is a small overlap in TC levels between normal and affected individuals. For FH women and men, average TC concentrations were 7.98 (SD=2.49) and 7.61 (SD=2.30) mmol/l, respectively.

A



B

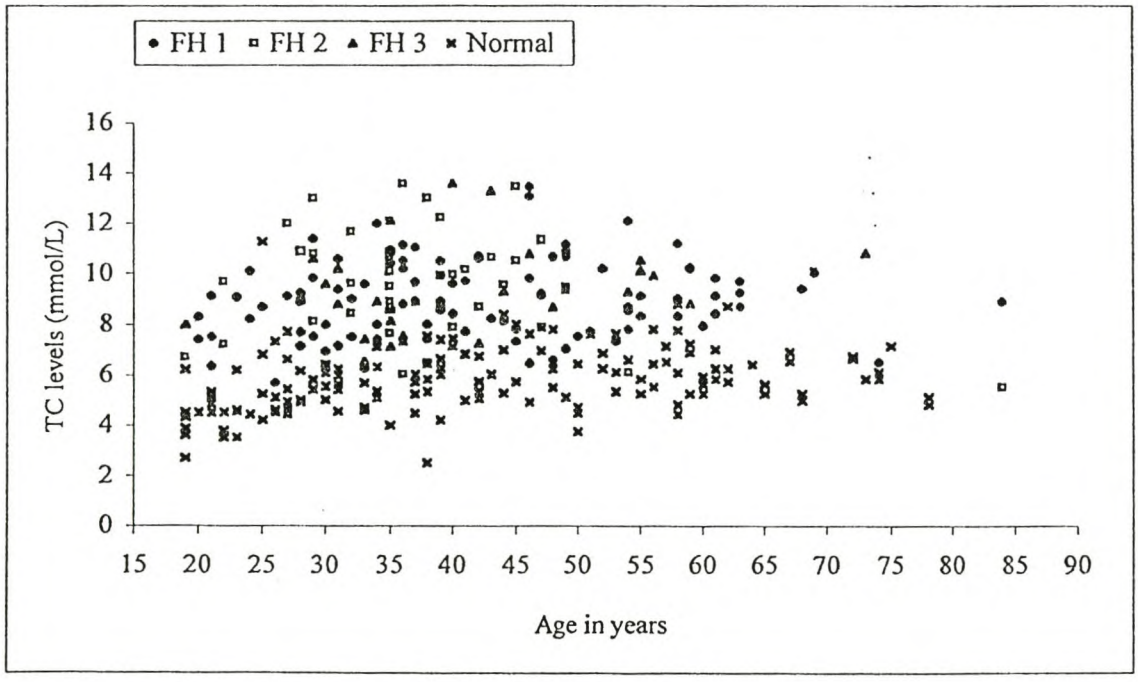


Figure 2. Total cholesterol (TC) concentrations as a function of age in 349 women (A) and 316 men (B).

DISCUSSION

The mutational spectrum underlying lipid abnormalities differs among population groups. Knowledge of the exact gene defects causing primary hypercholesterolaemia in a specific population or family allows accurate disease diagnosis and preventive treatment. The presence of at least ten founder-type LDLR gene mutations in the South African population (highlighted in Table 1) 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 (Table 1). Haplotype studies indicated that four of these mutations (D200G, S285L, C356Y and G361V) detected in 12 Afrikaner families may represent minor founder mutations (Thiart, 2000).

Mutations C356Y and G361V have not previously been described in other population groups. Both involve evolutionary conserved residues (Mehta et al., 1991) and occur in the epidermal growth factor (EGF) precursor homology domain of the LDLR gene that is responsible for recycling. Evidence in favour of the causitive nature of the two exon 8 mutations furthermore includes the finding that both co-segregate with elevated cholesterol levels in the families with these mutations, and failure to identify additional potential gene defects in the remaining part of the LDLR gene. The exon 8 mutations were also absent in more than 100 normal chromosomes screened. Mutations D200G and S285L, previously described in European populations from which Afrikaners originated, resides in the important ligand-binding region of the LDLR gene (Hobbs et al., 1992; Schuster et al., 1995). In pedigree number 943 (Figure 1), both parents were

heterozygous for mutation S285L. One of their children died at the age of 6 years of a heart attack, which most likely was caused by the inheritance of two copies of mutation S285L. Subject II-2 shown to be heterozygous for mutation S285L, presented with TC above the 70th percentile for age and gender. One of the mutation-negative children (II-3) had a TC concentration above the 80th percentile, which might have been falsely classified as heterozygous FH in the absence of the DNA test. This was in accordance with the data previously reported by Kotze et al. (1998) who found that a value of 6 mmol/l may best discriminate between mutation-positive and -negative children within Afrikaner FH families.

In this study the evaluation of biochemical versus DNA diagnosis, previously performed in 220 children between the ages of 2-18 years (Kotze et al., 1998), were 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 (>90%) were identified for the first time during mutation screening, the relative low sensitivity value of 89.3%, compared to that of 93% in the children sample, could be attributed to some of the patients being on medication. The specificity value also differed between the adult (81.9%) and children (89%) studies, which demonstrated that TC levels as a diagnostic means are even less accurate in the adult population. DNA tests are therefore the preferred method, particularly in populations where specific mutations predominate.

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 (Graadt van Roggen et al., 1995) using biochemical parameters was 97%, compared to 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 relatives analysed for mutation V408M. Notably, one of the male patients with mutation V408M revealed a normal TC value. This finding provides us with another example of the extent of clinical variability in FH (Kotze et al.,1993a; b). The normal TC value in this V408M heterozygote could be due to interaction with a cholesterol lowering gene(s) as previously suggested by Hobbs et al. (1989). Ekstrom et al. (1999) have also described a pathogenic mutation (C240F) in a clinically normal 17-year old subject. Sass et al. (1995) hypothesised that the apo E2 allele of the apolipoprotein E polymorphism may be a potent cholesterol-lowering gene. The likelihood of apo E allelic status as a contributing factor has been excluded in the South African normocholesterolaemic subject with mutation V408M, since this patient was homozygous for the neutral E3 allele (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 (Lingenhel et al., 1998).

Failure to identify FH- or FDB-related mutations in 11 clinically diagnosed FH patients included in this study may be considered a reflection of locus heterogeneity in autosomal dominant hypercholesterolaemia (ADH). Recently, a novel locus for ADH was mapped on human chromosome 1 (Varret et al., 1999). Studies are underway to identify

and characterise the causative gene, since this may lead to the development of novel therapeutic strategies targeted at the cause of the disease in a subset of hypercholesterolaemic families without mutations in the LDLR or apolipoprotein B genes. Mutation detection could also be compromised by the limitations imposed by the mutation detection methods used, since the HEX-SSCP and DGGE screening methods used cannot identify large gene rearrangements.

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 children (Kotze et al., 1998) and adult samples. Cardiovascular disease usually becomes manifested in male and female patients with FH before 55 years of age (Goldstein et al., 1995), which appear to correspond with data in the Afrikaner population (Kotze et al., 1993c; Steyn et al., 1996). However in the Black population none of the FH heterozygotes with the relatively severe 6-bp deletion in exon 2 presented with CHD (Thiart et al., in press).

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 to clinical criteria. 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 counseling 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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REFERENCES

Bilheimer DW, East C, Grundy SM, Nora JJ. Clinical studies in a kindred with a kinetic LDL receptor mutation causing familial hypercholesterolemia. *American Journal of Medical Genetics* 1985; 22: 593-598.

Callis M, Jansen S, Thiart R, de Villiers JNP, Raal FJ, Kotze MJ. Mutation analysis in familial hypercholesterolemia patients of different ancestries: identification of three novel LDLR gene mutations. *Molecular and Cellular Probes* 1998; 12: 149-152.

Day INM, Whittall RA, O'Dell SD, Haddad L, Bolla MK, Gudnason V, Humphries SE. Spectrum of LDL receptor gene mutations in heterozygous familial hypercholesterolemia. *Hum Mut* 1997; 10: 116-127.

Ekstrom U, Abrahamson M, Flor—n C-H, Tollig H, Wettrell G, Nilsson G, Sun X-M, Soutar AK, Nilsson-Ehle P. An individual with a healthy phenotype in spite of a pathogenic LDL receptor mutation (C240F). *Clin Genet* 1999; 55: 332-339.

Fourie AM, Coetzee GA, Gevers W, van der Westhuyzen DR. Two mutant low-density-lipoprotein receptors in Afrikaners slowly processed to forms surface forms exhibiting rapid degradation or functional heterogeneity. *Biochem J* 1988; 255: 411-415.

Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolaemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*, 7th edn. McGraw-Hill, New York, 1995: 1981-2030.

Graadt van Roggen JF, van der Westhuyzen DR, Coetzee GA, Marais AD, Steyn K, Langenhoven E, Kotze MJ. FH Afrikaner-3 LDL receptor mutation results in defective LDL receptors and causes a mild form of familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 1995; 15: 765-772.

Heath KE, Gudnason V, Humphries SE, Seed M. The type of mutation in the low density lipoprotein receptor gene influences the cholesterol-lowering response of the HMG-CoA reductase inhibitor simvastatin in patients with heterozygous familial hypercholesterolaemia. *Atherosclerosis* 1999; 143: 41-54.

Henderson HE, Berger GM, Marais AD. A new LDL receptor gene deletion mutation in the South African population. *Hum Genet* 1988; 80: 371-374.

Hobbs HH, Leitersdorf E, Leffert CC, Cryer DR, Brown MS, Goldstein JL. Evidence for a dominant gene that suppresses hypercholesterolemia in a family with defective low density lipoprotein receptors. *J Clin Invest* 1989; 84: 656-644.

Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* 1992; 1: 445-466.

Jensen HK, Jensen LG, Hansen PS, Faergeman O, Gregerson N. High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. *Clin Chem* 1996; 42: 1140-1146.

Koivisto U-M, Viikari JS, Kontula K. Molecular characterization of minor gene rearrangements in Finnish patients with heterozygous familial hypercholesterolemia: identification of two common missense mutations (Gly823→Asp and Leu380→His) and eight rare mutations of the LDL receptor gene. *Am J Hum Genet* 1995; 57: 789-797.

Kotze MJ, Langenhoven E, Retief AE, Steyn K, Marais MP, Grobbelaar JJ, Oosthuizen CJJ, Weich HFH, Benade AJS. Haplotype associations of three DNA polymorphisms at the human low density lipoprotein receptor gene locus in familial hypercholesterolaemia. *J Med Genet* 1987; 24: 750-755.

Kotze MJ, Langenhoven E, Retief AE, Seftel HC, Henderson HE, Weich HFH. Haplotypes identified by 10 DNA restriction fragment length polymorphisms at the low density lipoprotein gene locus. *J Med Genet* 1989a; 26: 255-259.

Kotze MJ, Langenhoven E, Warnich L, du Plessis L, Marx MP, Oosthuizen CJJ, Retief AE. The identification of two low-density lipoprotein receptor gene mutations in South African familial hypercholesterolaemia. *S Afr Med J* 1989b; 76: 399-401.

Kotze MJ, Warnich L, Langenhoven E, du Plessis L, Retief AE. An exon 4 mutation identified in the majority of South African familial hypercholesterolaemics. *J Med Genet* 1990; 27: 298-302.

Kotze MJ, Langenhoven E, Kriek JA, Oosthuizen CJJ, Retief AE. DNA screening of hyperlipidemic Afrikaners for familial hypercholesterolemia. *Clin Genet* 1992; 42: 43-46.

Kotze MJ, de Villiers WJS, Steyn K, Kriek JA, Marais AD, Langenhoven E, Herbert JS, Graadt van Roggen JF, Van der Westhuyzen DR, Coetzee GA. Phenotypic variation among familial hypercholesterolemics heterozygous for either one of two Afrikaner founder LDL receptor mutations. *Arterioscler Thromb* 1993a; 13: 1460-1468.

Kotze MJ, Davis HJ, Bissbort S, Langenhoven E, Brusnicky J, Oosthuizen CJJ. Intrafamilial variability in the clinical expression of familial hypercholesterolemia: importance of risk factor determination for genetic counselling. *Clin Genet* 1993b; 43: 295-299.

Kotze MJ, De Villiers WJ, Steyn K, Kriek JA, Marais AD, Langenhoven E, Herbert JS, Graadt van Roggen JF, Van der Westhuyzen DR, Coetzee GA. Phenotypic variation among familial hypercholesterolemics heterozygous for either one of two Afrikaner founder LDL receptor mutations. *Arterioscler Thromb* 1993c; 13: 1460-1468.

Kotze MJ, Langenhoven E, Theart L, Marx MP, Oosthuizen CJJ. Report on a molecular diagnostic service for familial hypercholesterolemia in Afrikaners. *Genet Counsel* 1994; 5: 15-22.

Kotze MJ, Langenhoven E, Theart L, Loubser O, Micklem A, Oosthuyzen CJJ. Recurrent LDL-receptor mutation causes familial hypercholesteraemia in South African coloureds and Afrikaners. *SAMJ* May 1995a; 85: 357-361.

Kotze MJ, Theart L, Callis M, Peeters AV, Thiart R, Langenhoven E. Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low density lipoprotein receptor gene mutations. *PCR Methods Applic* 1995b; 4: 352-356.

Kotze MJ, Loubser O, Thiart R, de Villiers JNP, Langenhoven E, Theart L, Steyn K, Marais AD, Raal FJ. CpG hotspot mutations at the LDL receptor locus are a frequent cause of familial hypercholesterolemia among South African Indians. *Clin Genet* 1997; 51: 394-398.

Kotze MJ, Peeters AV, Loubser O, Theart L, du Plessis L, Hayes VM, de Jong G, de Villiers JN, Lombard CJ, Hansen PS, Raal FJ. Familial hypercholesterolemia: potential diagnostic value of mutation screening in a pediatric population of South Africa. *Clin Genet* 1998; 54: 74-78.

Langenhoven E, Warnich L, Thiart R, Rubinsztein DC, van der Westhuyzen DR, Marais AD, Kotze MJ. Two novel point mutations causing receptor-negative familial

hypercholesterolemia in a South African Indian homozygote. *Atherosclerosis* 1996; 125: 111-119.

Lehrman MA, Schneider WJ, Brown MS, Davis GC, Elhamer A, Russell DW, Goldstein JL. The Lebanese allele at the low-density lipoprotein receptor locus. *J Biol Chem* 1987; 262: 401-410.

Leitersdorf E, Hobbs HH, Fourie AM, Jacobs M, van der Westhuyzen DR, Coetzee GA. Deletion in the first cysteine-rich repeat of low density lipoprotein receptor impairs its transport but not lipoprotein binding in fibroblasts from a subject with familial hypercholesterolemia. *Proc Natl Acad Sci USA* 1988; 85: 7912-7916.

Leitersdorf E, Van der Westhuyzen DR, Coetzee GA, Hobbs HH. Two common low density lipoprotein receptor gene mutations cause familial hypercholesterolemia in Afrikaners. *J Clin Invest* 1989; 84: 954-961.

Leitersdorf E, Tobin EJ, Davignon J, Hobbs HH. Common low-density lipoprotein receptor mutations in the French Canadian population. *J Clin Invest* 1990; 85: 1014-1023.

Lingenhel A, Kraft HG, Kotze MJ, Peeters AV, Kronenberg F, Kruse R, Utermann G. Concentrations of the atherogenic Lp(a) are elevated in FH. *Eur J Hum Genet* 1998; 6: 50-60.

Lombardi P, Kamerling SWA, Defesche JC, Kastelein JJP, Havekes LM. Identification of a double mutation in the low-density lipoprotein receptor gene causing familial hypercholesterolemia. *Clin Genet* 1996; 50: 525-526.

Loubser O, Marais AD, Kotze MJ, Godenir N, Thiart R, Scholtz CL, de Villiers JN, Hillermann R, Firth JC, Weich HF, Maritz F, Jones S, van der Westhuyzen DR. Founder mutations in the LDL receptor gene contribute significantly to the familial hypercholesterolemia phenotype in the indigenous South African population of mixed ancestry. *Clin Genet* 1999; 55: 340-345.

Marx MP, Thiart R, de Villiers JNP, Kotze MJ. Spectrum of LDLR gene mutations in South Africa. *Med Genetika* 1997; 9 (Suppl):369 (Abstract)

Mehta KD, Chen WJ, Goldstein JL, Brown MS. The low density lipoprotein receptor in Xenopus laevis. 1. Five domains that resemble the human receptor. *Journal of Biological Chemistry* 1991; 266: 10406-10414.

Meiner V, Landsberger D, Berkman N, Reshef A, Segal P, Seftel HC, van der Westuyzen DR, Jeenah MS, Coetzee GA, Leitersdorf E. A common Lithuanian mutation causing familial hypercholesterolemia in Askenazi Jews. *Am J Hum Genet* 1991; 49: 443-449.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from nucleated cells. *Nucleic Acids Res* 1988; 16: 1215.

Nissen H, Guldberg P, Hansen AB, Petersen NE, Horder M. Clinically applicable mutation screening in familial hypercholesterolemia. *Hum Mut;* 1996 8: 168-177.

Nissen H, Hansen AB, Guldberg P, Hansen TS, Petersen NE, Horder M. Evaluation of a clinically applicable mutation screening technique for genetic diagnosis of familial hypercholesterolemia and familial defective apolipoprotein B. *Clin Genet* 1998; 53: 433-439.

Peeters AV, Kotze MJ, Scholtz CL, de Waal LF, Rubinsztein DC, Coetzee GA, Zuliani G, Streiff R, Liu J, van der Westhuyzen DR. A 3-basepair deletion in repeat 1 of the LDL receptor promoter reduces transcriptional activity in a South African Pedi. *J Lipid Res* 1998; 39:1021-1024.

Pereira E, Ferreira R, Hermelin B, Thomas G, Bernard C, Bertrand V, Nassiff H, Mendez del Castillo D, Bereziat G, Benlian P. Recurrent and novel LDL receptor gene mutations causing heterozygous familial hypercholesterolemia in La Habana. *Hum Genet* 1995; 96: 319-322.

Pimstone SN, Sun X-M, du Souich C, Frohlich JJ, Hayden MR, Soutar AK. Phenotypic variation in heterozygous familial hypercholesterolaemia. A comparison of Chinese patients with the same or similar mutations in the LDL receptor gene in China or Canada. *Arterioscler Thromb Vasc Biol* 1998; 18: 309-315.

Rossouw JE, Jooste PL, Steyn K, Benade 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: 533-538.

Rubinsztein DC, Coetzee GA, Marais AD, Leitersdorf E, Seftel HC, Van der Westhuyzen DR. Identification and properties of the proline 664-leucine mutant LDL receptor in South Africans of Indian origin. *J lipid Res* 1992; 33: 1647-1655.

Rubinsztein DC, Jialal I, Leitersdorf E, Coetzee GA, van der Westhuyzen DR. Identification of two new LDL-receptor mutations causing homozygous familial hypercholesterolemia in a South African of Indian origin. *Biochim Biophys Acta* 1993; 1182: 75-82.

Sass C, Giroux LM, Ma Y, Roy M, Lavigne J, Lussier-Cacan S, Davignon J, Minnich A. Evidence for a cholesterol-lowering gene in a French-Canadian kindred with familial hypercholesterolemia. *Hum Genet* 1995; 96: 21-26.

Scholtz CL, Peeters AV, Hoogendijk CF, Thiart R, Hillermann R, Liu J, Marais AD, Kotze MJ. A novel –59 C/T mutation in repeat 2 of the LDLR gene promoter: Reduction in transcriptional activity and possible allelic interaction in a South African family with familial hypercholesterolaemia. *Hum Mol Genet* 1999 (in press).

Schuster H, Keller C, Wofram G, Zollner N. Ten LDL receptor mutants explain one third of familial hypercholesterolemia in a German sample. *Arterioscler Thromb Vasc Biol* 1995; 15: 2176-2180.

Seftel HC, Baker SG, Sandler MP, Forman MB, Joffe BI, Mendelsohn D, Jenkins T, Mieny CJ. A host of hypercholesterolaemic homozygotes in South Africa. *Br Med J* 1980; 281: 633-636.

Solberg K, Rodningen OK, Tonstad S, Ose L, Leren TP. Familial hypercholesterolaemia caused by a non-sense mutation in codon 329 of the LDL receptor gene. *Scand J Clin Lab Invest* 1994; 54: 605-609.

Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA* 1989; 86: 587-591.

Soutar AK, Knight BL, Patel DD. Identification of a point mutation in growth factor repeat C of the low density lipoprotein-receptor gene in a patient with homozygous familial hypercholesterolemia that affects ligand binding and intracellular movement of receptors. *Proc Natl Acad Sci USA* 1989; 86: 4166-4170.

Steyn K, Goldberg YP, Kotze MJ, Steyn M, Swanepoel ASP, Fourie JM, Coetzee GA, van der Westhuyzen DR. Estimation of the prevalence of familial hypercholesterolemia

in a rural Afrikaner community by direct screening for three Afrikaner founder low density lipoprotein receptor gene mutations. *Hum Genet* 1996; 98: 479-484.

Sundvold H, Solberg K, Tonstad S, Rodningen OK, Ose L, Berg K, Leren TP. A common missense mutation (C210G) in the LDL receptor gene among Norwegian familial hypercholesterolemia subjects. *Hum Mutat* 1996; 7: 70-71.

Thiart R, Loubser O, De Villiers JNP, Marx MP, Zaire R, Raal FJ, Kotze MJ (1998) Two novel and two known low-density lipoprotein receptor gene mutations in German patients with familial hypercholesterolemia. *Hum Mut* 1(Suppl):S232-S233.

Thiart R, Scholtz C, Vergotine J, Hoogendijk CF, de Villiers JNP, Nissen H, Brusgaard K, Gaffney D, Hoffs MS, Vermaak WJH, Kotze MJ. Predominance of a 6-bp deletion in exon 2 of the LDL receptor gene in Africans with familial hypercholesterolaemia. *J Med Genet* (in press) (see Appendix 3).

Thiart R. Comparative analysis of familial hypercholesterolaemia in different population groups. PhD. University of Stellenbosch. 2000.

Tricot-Guerber F, Saint-Jore B, Valenti K, Foulon T, Hadjian AJ. Identification of a mutation, N543H, in exon 11 of the low-density lipoprotein receptor gene in a French family with familial hypercholedsterolemia. *Hum Mut* 1995; 6: 87-88.

Varret M, Rabés JP, Thiart R, Kotze MJ, Baron H, Cenarro A, Descamps O, Ebhardt M, Hondelijn JC, Kostner GM, Miyake Y, Pocovi M, Schmidt H, Schuster H, Stuhrmann M, Yamamura T, Junien C, Beroud C, Boileau C. LDLR database (second edition): new additions to the database and the software, and results of the first molecular analysis. *Nucl Acid Res* 1998; 26: 250-255.

Varret M, Rabés JP, Saint-Jore B, Cenarro A, Marinoni JC, Civeira F, Devillers M, Krempf M, Coulon M, Thiart R, Kotze MJ, Schmidt H, Buzzi JC, Kostner GM, Bertolini S, Pocovi M, Rosa A, Farnier M, Martinez M, Junien C, Boileau C. A third major locus for autosomal dominant hypercholesterolemia maps to 1p34.1-p32. *Am J Hum Genet* 1999; 64: 1378-1387.

Ward AJ, O'Kane M, Young I, Nicholls DP, Nevin NC, Graham CA. Three novel mutations in the EGF precursor homology domain of the low-density lipoprotein receptor gene in Northern Irish patients with familial hypercholesterolemia. *Hum Mut* 1995; 6: 254-256.

Williams RR, Schumacher MC, Barlow GK, Hunt SC, Ware JL, Pratt M, Latham BD. Documented need for more effective diagnosis and treatment of familial hypercholesterolemia (FH): Data from 502 heterozygotes in Utah. *Am J Cardiol* 1993; 72: 18D-24D.

PRENATAL DIAGNOSIS IN A SOUTH AFRICAN FAMILY WITH FAMILIAL HYPERCHOLESTEROLAEMIA CAUSED BY MUTATION D206E IN THE LOW DENSITY LIPOPROTEIN RECEPTOR GENE

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Objective. In this report on the outcome of the first prenatal diagnosis performed for familial hypercholesterolaemia (FH) in a South African family, we aim to demonstrate the value of a population-directed screening strategy to identify FH patients in the South African population, where enrichment of particular gene mutations has significantly increased the disease prevalence.

Design. Prenatal diagnosis was offered to an Afrikaner heterozygous FH couple who has two affected daughters, of whom the youngest is severely affected due to the inheritance of two defective genes. Genomic DNA isolated from amniotic fluid and peripheral blood of the parents were amplified by the polymerase chain reaction (PCR) and subjected to mutation analysis.

Results. The carrier status of mutation D206E was confirmed in the parents, whilst this mutation was not detected in DNA directly amplified from amniotic fluid. To exclude the possibility of a false-negative result due to the limited number of cells in the uncultured amniotic fluid sample, an aliquot of liquor was also cultured *in vitro*, and the DNA extracted and subjected to a second round of analysis. This confirmed the absence of mutation D206E in the foetus.

Conclusions. This case illustrates the application of a DNA-based mutation detection technique as a simple and rapid diagnostic aid that can be carried out at a relatively early gestational stage. Prenatal diagnosis of FH, aimed at the detection of homozygous cases, is particularly feasible in populations and families with molecularly defined LDLR gene mutations.

INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal co-dominant disease caused by mutations in the low density lipoprotein receptor (LDLR) gene. Characteristic features include elevated levels of low density lipoprotein (LDL), the major cholesterol-transport lipoprotein in human plasma, and deposition of LDL-derived cholesterol in tendons, skin (xanthomas) and arteries (atheromas). The world prevalence of heterozygous FH is approximately 1:500 whilst that of homozygotes is estimated at approximately 1:1000000. The heterozygous form is associated with a high risk of myocardial infarction usually during the fourth or fifth decade of life, while the rare homozygous condition is characterised by early atherosclerosis, often resulting in myocardial infarction before the age of 20 years (Goldstein et al., 1995).

FH heterozygous parents have a 1:4 risk of having a homozygous child. In a relatively homogeneous population group such as the South African Afrikaner, where the prevalence of FH has been increased to approximately 1/70 due to a founder effect (Steyn et al., 1996), the likelihood of unions which could result in FH homozygous offspring is significantly increased (Seftel et al., 1980). Consequently, prenatal diagnosis of FH is particularly feasible in this population, where three LDLR gene mutations (D154N, D206E and V408M) are responsible for the disease in approximately 90% of affected individuals (Leitersdorf et al., 1989; Kotze et al., 1989, 1991). The significance of DNA screening in populations with a high prevalence of FH due to a founder effect and/or a high rate of consanguinity has previously been demonstrated by prenatal

diagnosis performed in a Christian-Arab family with a disease-causing point mutation in exon 14 of the LDLR gene (Reshef et al., 1992).

The first documented prenatal diagnosis of FH was reported in 1978 and involved the use of functional assays for quantitative assessment of LDLR activity in cultured amniotic fluid cells (Brown et al., 1978). This laborious procedure was only applicable to FH homozygotes with so-called receptor-negative mutations (< 2% of activity), since the technique could not reliably discriminate between homozygotes with detectable receptor activity and heterozygotes. Measurement of the cholesterol concentration in a foetal blood sample obtained at the twenty-fourth week of gestation, demonstrating a total cholesterol concentration of 543 mg/dl compared with the mean value of 66 mg/dl in 48 control foetuses, was subsequently reported by deGennes et al. (1985). The diagnosis of homozygous FH was confirmed by analysis of foetal skin fibroblasts obtained at the time of termination. Indirect molecular-genetic approaches using intragenic restriction fragment length polymorphisms (RFLPs) (Coviello et al., 1993) and microsatellite markers flanking the LDLR gene (De Oliveira et al., 1998), may be applicable in families where the disease-causing mutation is uncharacterised. In this study we report the exclusion of FH in a foetus, using direct mutation detection in an Afrikaner family with the founder-related LDLR gene mutation D206E. The value of the population-directed mutation screening strategy used in South Africa, under the auspices of the international MED-PED (Make Early Diagnosis to Prevent Early Deaths) initiative (Willliams et al., 1993), is demonstrated.

SUBJECTS AND METHODS

Subjects

Prenatal diagnosis was requested by an Afrikaner FH heterozygous couple whose first child is a FH heterozygote and the second, clinically homozygous (Figure 1). Cutaneous xanthomas were evident at the age of one year and eight months in the homozygous child, whose total cholesterol (TC) concentration was 18 mmol/l at the time. The first TC value measured in this child at the age of two months was reported to be approximately 9 mmol/l. The disease-causing LDLR gene mutation D206E has previously been identified in both parents and the clinical FH homozygote. Parental bloods and an amniotic fluid sample were obtained with informed consent.

DNA analysis

Genomic DNA was extracted from whole blood obtained from both parents and directly from amniotic fluid using a standard technique (Miller et al., 1988). An aliquot of the foetal sample was also grown to confluency in DMEM medium at 37°C (5% CO_2) and the DNA extracted. DNA was amplified using the polymerase chain reaction (PCR) technique (Saiki et al., 1988). Multiplex PCR using three sets of allele-specific primers was performed to simultaneously screen for three founder-related LDLR gene mutations (Kotze et al., 1995) in order to confirm the presence of mutation D206E in both parents and determine the disease status in the foetus. Each reaction was performed in a total volume of 50 µl containing the PCR primers, 0.5 µg genomic DNA, 2 U *Taq* DNA polymerase (Boehringer Mannheim), PCR buffer with $MgCl_2$ (Boehringer Mannheim), 200 µM each dATP, dCTP, dGTP and dTTP, 2 mM tetramethylammonium chloride

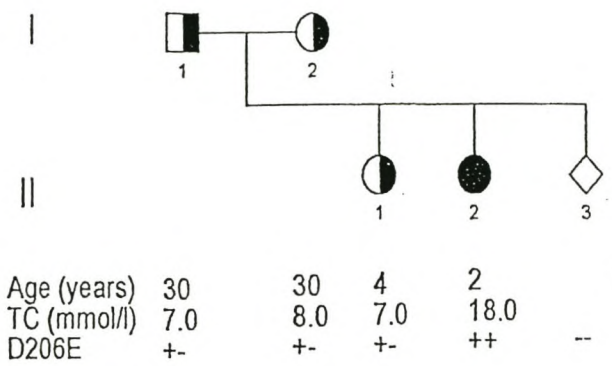
(Me_4NCl), and 15% glycerol. The PCR program included an initial denaturation at 94°C for 5 min and then two consecutive amplification steps (1) 15 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 2 min; and (2) 20 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min. Aliquots of the amplified DNA were resolved by electrophoresis on a 10% polyacrylamide gel. In order to verify the results obtained by the allele-specific PCR, *Dde*I digestion of amplified DNA (5-10µl aliquots) was also performed (Kotze et al., 1991). Primers CCCCCAGCTGTGGGCC and CCGCCCCGTCCCACCCCCGC were used to amplify exon 4 according to Jensen at al. (1996). Approximately 10 µl of loading dye (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) was added to the reaction and the resulting products resolved on a 10% polyacrylamide gel (in 0.5x TBE buffer) for 1½ hours at 250V. The gel was stained in ethidium bromide (10 mg/ml) for 10 minutes, destained in distilled water for another 10 minutes, and photographed on an UV transilluminator.

RESULTS

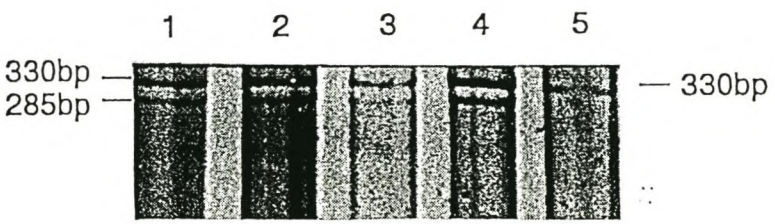
The pedigree of the family is illustrated in Figure 1A. TC levels determined approximately three years ago in family members were compatible with heterozygous (I-1, I-2, II-1) or homozygous (II-2) FH, as previously reported following the identification of mutation D206E in this family. The multiplex PCR confirmed heterozygosity for mutation D206E in both parental samples, while the mutation-specific 285-bp band was absent in DNA of the foetus (Figure 1B). Since mutation D206E creates a *Dde*I restriction enzyme recognition site, absence of mutation D206E was also confirmed by restriction enzyme analysis (Figure 1C) of foetal DNA samples extracted from both uncultured amniotic fluid sample and the cultured amniocytes. This finding also excluded the possibility that the absence of the 285-bp PCR fragment in the foetal sample could have been due to inadequate DNA.

Figure 1. DNA screening for mutation D206E in a family who requested prenatal diagnosis of FH. (A) Pedigree structure with ages and total cholesterol levels indicated. "+" and "-" indicate the presence and absence of the mutation, respectively. (B) Multiplex PCR. The five lanes in the polyacrylamide gel contain amplified DNA from the heterozygous father (1), heterozygous mother (2), heterozygous first child (3), homozygous second child (4) and normal fetus (5). (C) Gel electrophoresis of *Dde*I digested PCR-amplified exon 4 DNA. Lanes: (1) DNA from homozygous daughter (2) DNA from heterozygous daughter (3) DNA from normal fetus (4) DNA from heterozygous mother (5) DNA from heterozygous father. [see next page]

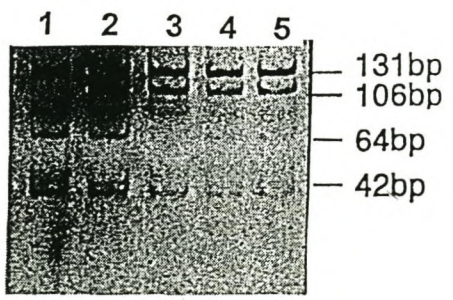
A



B



C



DISCUSSION

This study describes the first prenatal DNA diagnosis that has been performed for FH in South Africa, approximately ten years after molecular analysis demonstrated that three LDLR gene mutations are responsible for the disease in 90% of Afrikaners (Leitersdorf et al., 1989; Kotze et al., 1989). The development of a rapid, nonradioactive screening method to simultaneously screen for these founder-related mutations D154N, D206E and V408M, facilitates an improved diagnostic service for FH in South Africa (Kotze et al., 1995). This method is currently used routinely at four academic institutions in South Africa, to screen new index cases of European descent for LDLR gene mutations and to trace the defective gene in families. In the family presented in this study, the value of such a population-based screening strategy is demonstrated, since the couple who requested prenatal diagnosis of FH had previously been diagnosed with heterozygous FH after identification of mutation D206E. Their first-born child is a FH heterozygote and the second, a homoallelic homozygote.

Analysis of DNA isolated directly from amniotic fluid at the 16th week of gestation, and subsequently from cultured amniocytes, excluded the presence of mutation D206E in the foetus. Both allele-specific PCR and restriction enzyme analysis methods were utilised for the diagnosis, since the multiplex assay cannot distinguish between heterozygote and homoallelic FH homozygote status (Kotze et al., 1995). Sufficient high-quality DNA is furthermore required for optimal results with the multiplex assay, which can cause a problem when DNA is extracted from amniotic fluid. *Dde*I digestion,

however, provides clear distinction between a FH homozygote and heterozygote or an individual without the D206E mutation. The reason for not using the latter method routinely in the identification of patients with mutation D206E is firstly, because it is more cost-effective to simultaneously screen for all three founder mutations in a single multiplex PCR and secondly, because the small fragment sizes (64- and 42-bp) generated by *Dde*I digestion in the mutant allele frequently complicates clear distinction between FH heterozygotes and unaffected individuals. The non-specific band below the 42-bp band in Figure 1C is due to primer dimer.

Due to the sensitivity of the PCR technique, extreme caution should be taken to ensure that the cells obtained for DNA extraction are foetal in origin and not contaminated by maternal cells. Since chromosome analyis indicated a male foetus (data not shown) and mutation D206E previously identified in the mother was absent, further studies to verify the origin of the DNA analysed were not performed. The finding of a normal lipid profile (TC 1.4 mmol/l) in cord blood obtained at birth supported the results of the prenatal diagnosis. Previous studies have indicated that there is a clear distinction in plasma cholesterol levels between normal and heterozygous FH children at birth (Kwiterovich et al., 1990).

We believe that prenatal diagnosis of FH and termination of the foetus is justified in homozygous cases, because the condition is associated with a very high morbidity. This has application in populations where FH is prevalent due to a founder effect, multiple entries of disease genes into an isolated population or recurrent mutational events. The Afrikaner population descending from ~2000 original settlers who emigrated to the Cape

of South Africa in the 17th and 18th centuries provide an excellent example of the founding phenomena (Botha et al., 1983). The fertility rate was high and the population expanded to the current ~4 million of today, with a concurrent increase in specific defective genes introduced by European settlers. The three LDLR gene mutations responsible for the high prevalence of FH in Afrikaners have also been shown to contribute significantly to the FH phenotype in the South African population of mixed ancestry (Loubser et al., 1999). Molecular analysis of FH in other population groups has furthermore revealed the presence of founder-type mutations in South African Jewish (Meiner et al., 1991; Kotze et al., 1997) and Indian communities (Rubinsztein et al., 1992), as well as in Lebanese (Callis et al., 1998) and Black FH patients (Leitersdorf et al., 1988; Thiart et al., in press).

Knowledge of the spectrum of causative LDLR gene mutations in the diverse South African population has led to a population-directed strategy to identify affected families with FH. This approach provides the opportunity for accurate genetic counseling to inform families of the importance of mutation screening in other at-risk relatives. Genetic testing has made pre-clinical diagnosis of FH, including prenatal diagnosis, a reality.

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REFERENCES

Botha MC, Beighton P. Inherited disorders in the Afrikaner population of southern Africa. Part 1. Historical and demographic background, cardiovascular, neurological, metabolic and intestinal conditions. *S Afr Med J* 1983; 64: 609-612.

Brown MS, Kovanen PT, Goldstein JL, Eeckles R, Vandenberghe K, van den Berghe H, Fryns JP, Cassiman JJ. Prenatal diagnosis of homozygous familial hypercholesterolaemia: Expression of a genetic receptor disease *in utero*. *Lancet* 1978; 1: 526-529.

Callis M, Jansen S, Thiart R, de Villiers JNP, Raal FJ, Kotze MJ. Mutation analysis in familial hypercholesterolemia patients of different ancestries: identification of three novel LDLR gene mutations. *Mol Cell Probes* 1998; 12: 149-152.

Coviello D, Bertolini S, Masturzo P, Ghisellini M, Tiozzo R, Zambelli F, Stefanutti C, Torcia F, Pachi A, Ricci G, Calandra S. Chronic DNA analysis for the prenatal diagnosis of familial hypercholesterolaemia. *Hum Genet* 1993; 92: 424-426.

deGennes JL, Daffos F, Dairou F, Forestier F, Capella-Pavlosky M, Truffert J, Gaschard JC, Darbois Y. Direct fetal blood examination for prenatal diagnosis of homozygous familial hypercholesterolemia. *Arteriosclerosis* 1985; 5: 440-442.

De Oliveira e Silva ER, Haddad L, Kwiterovich Jr PO, Humphries SE, Day INM. Applicability of LDLR flanking microsatellite polymorphisms for prenatal diagnosis of homozygous state for familial hypercholesterolaemia. *Clin Genet* 1998; 53: 375-378.

Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolaemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*, 7th edn. McGraw-Hill, New York, 1995: 1981-2030.

Jensen HK, Jensen LG, Hansen PS, Faergeman O, Gregersen N. High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. *Clin Chem* 1996; 42: 1140-1146.

Kotze MJ, Langenhoven , Warnich L, Du Plessis L, Marx MP, Oosthuizen CJJ, Retief AE. The identification of two low-density lipoprotein receptor gene mutations in South African familial hypercholesterolaemia. *S Afr Med J* 1989; 76: 399-401.

Kotze MJ, Langenhoven E, Warnich L, Du Plessis L, Retief AE. The molecular basis and diagnosis of familial hypercholesterolaemia in South African Afrikaners. *Ann Hum Genet* 1991; 55: 115-121.

Kotze MJ, Theart L, Callis M, Peeters AV, Thiart R, Langenhoven E. Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low-density lipoprotein receptor gene mutations. *PCR Methods Appl.* 1995; 4: 352-356.

Kotze MJ, Loubser O, Thiart R, de Villiers JNP, Langenhoven E, Theart L, Steyn K, Marais AD, Raal FJ. CpG hotspot mutations at the LDL receptor locus are a frequent cause of familial hypercholesterolaemia among South African Indians. *Clin Genet.* 1997; 51: 394-398.

Kwiterovich PO Jr. Diagnosis and management of familial dyslipoproteinemia in children and adolescents. *Pediatr Clin North Am* 1990; 37: 1489-1523.

Leitersdorf E, Hobbs HH, Fourie AM, Jacobs M, Van der Westhuyzen DR, Coetzee GA. Deletion in the first cysteine-rich repeat of low density lipoprotein receptor impairs its transport but not lipoprotein binding in fibroblasts from a subject with familial hypercholesterolemia. *Proc Natl Acad Sci U S A*. 1988; 85: 7912-7916.

Leitersdorf E, Van Der Westhuyzen DR, Coetzee GA, Hobbs HH. Two common low-density lipoprotein receptor gene mutations cause familial hypercholesterolemia in Afrikaners. *J Clin Invest* 1989; 84: 954-961.

Loubser O, Marais AD, Kotze MJ, Godenir N, Thiart R, Scholtz CL, de Villiers JNP, Hillermann R, Firth JC, Weich HFH, Maritz F, Jones S, van der Westhuyzen DR. Founder mutations in the LDL receptor gene provide evidence of Caucasoid admixture and contribute significantly to the familial hypercholesterolemia phenotype in an indigenous South African population of mixed ancestry. *Clin Genet* 1999; 55: 340-345.

Meiner V, Landsberger D, Berkman N, Reshef A, Segal P, Seftel HC, van der Westhuyzen DR, Jeenah MS, Coetzee GA, Leitersdorf E. A common Lithuanian mutation causing familial hypercholesterolemia in Ashkenazi Jews. *Am J Hum Genet* 1991; 49: 443-449.

Miller SA, Dykes DD, Polesky HE. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Research* 1988; 16: 1215.

Reshef A, Meiner V, Dann EJ, Granat M, Leitersdorf E. Prenatal diagnosis of familial hypercholesterolemia caused by the "Lebanese" mutation at the low density lipoprotein receptor gene. *Hum Genet* 1992; 89: 237-239.

Rubinsztein DC, Coetzee GA, Marais AD, Leitersdorf E, Seftel HC, van der Westhuyzen DR. Identification and properties of the proline664-leucine mutant LDL receptor in South Africans of Indian origin. *J Lipid Res.* 1992; 33: 1647-1655.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988; 239: 487-491.

Seftel HC, Baker SG, Sandler MP, Forman MB, Joffe BI, Mendelsohn D, Jenkins T, Mieny CJ. A host of hypercholesterolaemic homozygotes in South Africa. *Br Med J* 1980; 281: 633-636.

Steyn K, Goldberg YP, Kotze MJ, Steyn M, Swanepoel ASP, Fourie JM, Coetzee GA, Van der Westhuyzen DR. Estimation of the prevalence of familial hypercholesterolaemia in a rural Afrikaner community by direct screening for three Afrikaner founder low density lipoprotein receptor gene mutations. *Hum Genet* 1996; 98: 95-97.

Thiart R, Scholtz C, Vergotine J, Hoogendijk CF, de Villiers JNP, Nissen H, Brusgaard K, Gaffney D, Hoffs MS, Vermaak WJH, Kotze MJ. Predominance of a 6-bp deletion in exon 2 of the LDL receptor gene in Africans with familial hypercholesterolaemia. *J Med Genet* (in press) (see Appendix 3).

Williams RR, Schumacher MC, Barlow GK, Hunt SC, Ware JL, Pratt, Latham BD. Documented need for more effective diagnosis and treatment of familial hypercholesterolemia according to data from 502 heterozygotes in Utah. *Am J Cardiol* 1993; 72: 18D-24D.

CHAPTER 4

CONCLUSIONS

Familial hypercholesterolaemia (FH) has been a focus of research in South Africa since its recognition as a common disease among Afrikaners nearly two decades ago (Seftel et al., 1980). Subsequent studies have shown that FH is also prevalent among Indians, Jews and Coloureds, but rare among Blacks in South Africa. The majority of people with FH is not diagnosed and therefore is not treated with cholesterol-lowering medication and modified diets, which may prevent or delay early death from heart attack. The solution to this problem is directly dependent on the success of finding individuals with FH. This led to the establishment of the MED-PED project, which is based on the concept that tracing of the defective gene in close relatives of an index patient would allow accurate disease diagnosis in approximately 50% of family members (Williams et al., 1993).

Systematic molecular analysis was used in this study to identify low-density lipoprotein receptor (LDLR) gene defects in South Africans with a clinical diagnosis of FH, followed by a family-based DNA screening approach for presymptomatic diagnosis of FH. At least ten of the 56 different gene defects described to date in the diverse South African population represents founder type mutations. DNA screening of 792 at-risk family members for the FH-related mutation identified in 379 index cases, allowed accurate disease diagnosis in 340 relatives and exclusion of the relevant mutation in 452 individuals. The characteristics of 232 index patients with the FH Afrikaner 1, -2 and –3 mutations are shown in Appendix 1, together with that of 658 relatives screened for the relevant family-specific mutation. The pedigrees of 106 families where several affected members were traced via the MED-PED approach are illustrated in Appendix 2. The

sensitivity and specificity of DNA diagnosis, based on total cholesterol values measured in family members of index cases recruited for this study, were 88% and 77%, respectively in Afrikaner families with founder-related LDLR gene mutations. These values were obtained when applying the 80th percentile for age and gender, the TC cut-off point found to discriminate between mutation-positive and –negative cases.

The first prenatal DNA diagnosis of FH has been undertaken during the course of this study, some ten years after it was shown that mutation D206E predominates in the Afrikaner FH population (Leitersdorf et al., 1989; Kotze et al., 1989, 1991). In the family analysed both the mother and father were found to be heterozygous for mutation D206E, while their youngest daughter was shown to be homozygous for the mutation. The mutation was not detected in DNA amplified directly from amniotic fluid or in cultured cells of the foetus which led to continuation of the pregnancy. This case illustrates the application of a DNA-based mutation detection technique as a simple and rapid diagnostic aid that can be carried out at a relatively early gestational stage. Prenatal DNA diagnosis of FH aimed at the detection of homozygous cases is an important consideration in populations and families with molecularly LDLR gene mutations.

The predominance of at least ten 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. DNA testing provides definitive tool for family tracing aimed at pre-clinical diagnosis and preventive treatment of FH. DNA data was used to determine

the percentage of FH patients that could be diagnosed by clinical criteria. It revealed a sensitivity value of 88%. The rate at which specific genes for FH are unexpressed in lipid tests was also determined. A specificity value of 77% was calculated. In the Black population of South Africa hypercholesterolaemics with lipid profiles compatible with the diagnosis of heterozygous FH frequently lack xanthomata characteristic of this condition (Marais and Berger, 1986). These findings provide evidence that FH is probably underdiagnosed in the South African Black population, most likely as a result of altered expression of FH-related mutations. It is therefore suggested that clinical/biochemical criteria for the diagnosis of FH need to be different by country/population and DNA methods may assist in making a definitive disease diagnosis.

Future prospects of the MED-PED initiative would be to extend this family-based effort to other treatable genetic diseases, as suggested by Kotze and Callis (1999). Molecularly uncharacterised patients with a clinical diagnosis of FH will be subjected to further genomic analysis to identify possible new gene(s) causing autosomal dominant hypercholesterolaemia (ADH). Recently, a novel locus for ADH was mapped on human chromosome 1p (Varret et al., 1999; Hunt et al., 1999). Identification and characterisation of the causative gene may lead to the development of novel therapeutic strategies targeted at the cause of the disease in a subset of hypercholesterolaemic families without mutations in the LDLR or apolipoprotein B genes.

REFERENCES

Hunt SC, Hopkins P, Thorne T, Bulka K, Wardell B, McDermott M, Bandley A, Fraser J, Skolnick M, Williams R, Samuels M. A genetic locus near 1p32 causes autosomal dominant hypercholesterolemia in an extended Utah pedigree. *Am J Hum Genet* 1999; 65: A77.

Kotze MJ, Langenhoven E, Warnich L, du Plessis L, Marx MP, Oosthuizen CJJ, Retief AE. The identification of two low-density lipoprotein receptor gene mutations in South African familial hypercholesterolaemia. *S Afr Med J* 1989; 76: 399-401.

Kotze MJ, Langenhoven E, Warnich L, du Plessis L, Retief AE. The molecular basis and diagnosis of familial hypercholesterolemia in South African Afrikaners. *Ann Hum Genet* 1991; 55: 115-121.

Kotze MJ, Callis M. MEDPED FH: a paradigm for other common monogenic diseases in South Africa. *Artheriosclerosis* 1999; 144: 467-468.

Leitersdorf E, van der Westhuyzen DR, Coetzee GA, Hobbs HH. Two common low density lipoprotein receptor gene mutations cause familial hypercholesterolemia in Afrikaners. *J Clin Invest* 1989; 84: 954-961.

Marais AD, Berger GM. A diversity of genetic hyperlipoproteinaemias in Black patients. Experience at the lipid clinics at the Grootte Schuur Hospital and the Red Cross War Memorial Children's Hospital, Cape Town. *S Afr Med J* 1986; 70: 583-587.

Seftel HC, Baker SG, Sandler MP, Forman MB, Joffe BI, Mendelsohn D, Jenkins T, Mieny CJ. A host of hypercholesterolaemic homozygotes in South Africa. *Br Med J* 1980; 281: 633-636.

Varret M, Rabés JP, Saint-Jore B, Cenarro A, Marinoni JC, Civeira F, Devillers M, Krempf M, Coulon M, Thiart R, Kotze MJ, Schmidt H, Buzzi JC, Kostner GM, Bertolini S, Pocovi M, Rosa A, Farnier M, Martinez M, Junien C, Boileau C. A third major locus for autosomal dominant hypercholesterolemia maps to 1p34.1-p32. *Am J Hum Genet* 1999; 64: 1378-1387.

Williams RR, Schumacher MC, Barlow GK, Hunt SC, Ware JL, Pratt M, Latham BD. Documented need for more effective diagnosis and treatment of familial hypercholesterolemia (FH): Data from 502 heterozygotes in Utah. *Am J Cardiol* 1993; 72: 18D-24D.

APPENDIX 1

Table 1. Characteristics of Afrikaner index patients with mutations D206E (FH1), V408M (FH2) and D154N (FH3) and their relatives.

ID	L-No.	SEX	AGE	F.H.	arc	xant	CHD	TC	HDL	TG	LDL	FH1	FH2	FH3	PERCENTILE
0001	L146	m	46	e		y	n	10,79	0,8	1,6	9,26	n	n	y	>95th
0002	L145	f	48	u		n	n	7,0	1,3	1,7	4,92	n	n	n	>60th
0003	L144	m	13	e		n	n	6,5	1,1	0,5	5,17	n	n	y	>95th
0004	L142	m	16	e		n	n	9,13	0,9	0,59	7,95	n	n	y	>95th
0005	L143	m	15	e		n	n	4,9	1,2	1,0	3,24	n	n	n	80th
0445		m	28	e			n	5,0	1,5	1,4	2,86	n	n	n	>30th
0456		f	56	n			n	5,9	2,6	1,2	2,75	n	n	n	>10th
0457		f	66	e			u	9,0	1,4	3,7	5,9	n	n	n	>90th
0458		m	22	e			n	3,8	1,6	1,2	1,65	n	n	n	>10th
0459		m	23	e			n	3,5	1,5	0,7	1,68	n	n	n	>10th
0460		m	24	e			n	4,4	1,9	0,7	2,18	n	n	n	>30th
0547		m	67	e			u	6,5	1,4	1,4	4,46	n	n	n	60th
0567		m	33	e			n	7,4	1,9	0,7	5,18	n	n	y	>90th
0574		m	30	e			n	6,1	1,4	1,3	4,1	n	n	n	70th
0575		m	72	n			n	6,7	1,5	1,5	4,51	n	n	n	>60th
0591		f	30	e			n	7,3	2,2	1,6	4,37	n	n	n	>90th
0614		m	59	e			n	7,2	0,9	2,5	5,15	n	n	n	80th
0615		f	51	n			n	7,2	1,5	1,7	4,92	n	n	n	70th
0616		f	36	e			n	5,7	0,9	1,3	4,2	n	n	n	50th
0617		m	27	e			n	7,7	0,9	3,5	5,19	n	n	n	95th
0618		m	62	e			n	5,7	1,2	1,3	3,9	n	n	n	30th
0648		f	51	e			n	6,5	1,7	1,7	4,02	n	n	n	>50th
0649		m	16	e			n	5,8	1,1	3,3	3,19	n	n	n	>90th
0650		f	81	e			n	7,4	1,0	3,0	5,02	n	n	n	>60th
0651		m	39	n			n	4,2	0,9	1,4	2,66	n	n	n	5th
0652		f	16	e			n	4,4	1,3	0,9	2,69	n	n	n	>30th
0653		f	10	e			n	5,0	1,4	1,3	3,0	n	n	n	>60th
0654		f	39	e			n	7,6	1,7	3,4	4,34	n	n	n	>90th
0677		m	56	e			n	7,8	1,0	2,7	5,56	n	n	n	>90th
0800		f	22	e			n	5,2	1,6	0,8	3,23	n	n	n	60th
0801		m	53	e			n	7,6	1,2	1,4	5,76	n	n	n	>80th
0802		m	59	n			n	5,2	1,3	0,8	3,53	n	n	n	>10th
0860		m	25	e			n	5,2	1,6	1,5	2,91	n	n	n	40th
0010		f	8	e		n	n	6,92	1,6	0,55	5,07	n	n	y	>95th
12	L023	m	33	e	y	y	n	6,53	1,3	0,62	4,95	n	n	y	>70th
0251	L022	f	33	n		n	n	5,6	2,2	1,0	2,94	n	n	n	60th
0252	L021	f	13	e		n	n	5,7	2,1	1,0	3,14	n	n	n	>80th
0253	L020	m	9	e		n	n	9,2	2,0	0,8	6,83	n	n	y	>95th
0254		m	46	n			n	4,9	1,3	1,9	2,73	n	n	n	10th
0255		f	44	e		n	n	6,5	1,4	1,4	4,46	n	n	n	>70th
0256		f	17	e			n	4,2	1,0	1,0	2,74	n	n	n	20th
0257		f	15	e			n	4,6	1,3	0,6	3,02	n	n	n	40th
0258		m	34	e		n	n	6,3	0,6	1,3	5,1	n	n	n	>70th
0259		f	32	n			n	6,4	1,3	1,6	4,37	n	n	n	>80th
0260		f	8	e			n	5,9	1,7	0,9	3,79	n	n	n	>80th
0261		f	11	e			n	4,7	1,5	0,8	2,83	n	n	n	50th
0274		f	33	e			n	6,1	2,1	0,8	3,63	n	n	n	>70th
0310		m	52	n			n	6,2	1,4	1,6	4,07	n	n	n	50th
0311		f	47	e			n	5,5	1,8	1,4	3,06	n	n	n	20th

0312		m	29	e		n	n	10,6	1,3	2,0	8,38	n	n	y	>95th
0313		f	23	e			n	5,2	1,3	1,0	3,44	n	n	n	60th
0314		f	34	e			n	5,2	1,4	2,0	2,88	n	n	n	>40th
0315		f	26	e			n	4,6	1,4	1,5	2,51	n	n	n	20th
0317		f	58	n			n	9,5	2,0	1,2	6,95	n	n	n	95th
0318		m	65	e			n	5,2	1,3	1,0	3,44	n	n	n	>10th
0323		f	48	n			n	5,9	1,2	1,6	3,97	n	n	n	>30th
0324		m	19	e			n	3,6	1,1	0,9	2,09	n	n	n	>10th
0342		m	26	e			n	7,3	1,2	2,0	5,18	n	n	n	90th
0348		m	31	n			n	5,4	1,3	0,56	3,84	n	n	n	>40th
0361		f	61	e			n	6,7	1,2	1,8	4,67	n	n	n	40th
0362		m	65	n			n	5,6	1,4	1,1	3,7	n	n	n	30th
0495		f	69	e			n	5,0	1,8	1,6	2,47	n	n	n	<5th
0496		m	75	n			n	7,1	0,9	5,7	3,59	n	n	n	80th
0722		f	32	e			n	9,3	1,3	0,9	7,59	n	n	y	>95th
0723		f	55	n			n	6,0	1,0	2,7	3,76	n	n	n	20th
0823	L110	f	37	n			n	3,0	0,8	1,6	1,47	n	n	n	<5th
0824	L111	m	14	e			n	6,0	1,1	0,5	4,67	n	n	y	>95th
0825	L109	f	12	e			n	6,7	0,9	0,6	5,52	n	n	y	>95th
0013		m	36	e		y	n	13,6	1,7	1,9	11,03	n	y	n	>95th
1081		f	38	n		n	n	5,0	1,47	1,86	2,68	n	n	n	60th
1082		f	9	e		n	n	5,12	1,3	1,24	3,25	n	n	n	>60th
1099		m	13	e		n	n	9,9	1,09	0,59	8,54	n	y	n	>95th
1100		m	8	e		n	n	8,0	1,12	0,83	6,5	n	y	n	>95th
0014	L302	f	34	l		y	n	10,7	1,3	0,54	9,15	y	n	n	>95th
0015	L301	m	34	n			u	5,1	1,5	1,3	3,0	n	n	n	>30th
1347	L303	f	19	l		n	n	7,22	1,91	0,63	5,02	y	n	n	>95th
1348	L304	f	15	l		n	n	8,23	1,62	0,67	6,3	y	n	n	>95th
1349	L299	m	17	l		n	n	7,68	1,53	0,53	5,91	y	n	n	>95th
1350	L300	m	10	l		n	n	4,36	2,11	0,39	2,07	n	n	n	>50th
0017		m	45	e		y	y	13,5	1,2	1,5	11,61	n	y	n	>95th
1083		f	30	e		n	n	6,1	1,1	1,4	4,36	n	n	n	>70th
1084		f	31	e		n	n	5,76	1,38	0,99	3,93	n	n	n	>60th
1085		f	47	e		n	n	7,21	1,41	5,52	3,27	n	n	n	70th
1086		m	18	e		n	n	5,47	1,32	1,33	3,54	n	n	n	>90th
1087		m	23	e		n	n	6,19	1,31	2,92	3,54	n	n	n	90th
1088		f	18	e		n	n	11,33	1,12	1,34	9,6	n	y	n	>95th
0019		m	54	e		y	y	9,27	0,74	1,59	7,8	n	n	y	>95th
0390		m	27	e			n	4,5	1,0	1,8	2,67	n	n	n	>10th
0430		m	61	e			n	7,0	1,2	2,1	4,84	n	n	n	>70th
0493		f	23	e			n	6,2	2,6	1,0	3,14	n	n	n	>80th
0494		f	58	u			n	8,7	1,6	3,1	5,68	n	n	n	>80th
0027		m	63	e		n	y	9,23	1,3	0,5	7,7	y	n	n	>95th
0852		m	38	e			n	6,4	0,7	2,0	4,78	n	n	n	>50th
0029		f	24	e		n	n	10,15	1,07	1,56	8,36	y	n	n	>95th
0232		f	48	e			u	8,3	1,5	0,8	6,43	y	n	n	>80th
0330		f	44	e		y	y	11,1	1,2	2,6	8,71	y	n	n	>95th
0331		m	14	e		n	n	8,33	1,16	0,75	6,83	y	n	n	>95th
0031	L151	m	61	e	y	y	y	9,1	0,94	2,9	6,83	y	n	n	>95th
1243	L152	f	56	n			n	5,7	1,84	1,67	3,09	n	n	n	10th
1306	L247	m	62	e		n	n	6,22	1,49	1,31	4,13	n	n	n	50th
1307	L255	m	64	e	y	n	n	6,36	1,48	2,44	3,76	n	n	n	>50th

1308	L254	f	15			n	n	4,94	0,84	2,44	2,98	n	n	n	60th
1309	L256	f	75	e	y	n	n	7,48	1,78	0,86	5,31	n	n	n	>60th
1310	L259	f	4	e		n	n	4,73	1,39	0,84	2,95	n	n	n	50th
1311	L260	f	31	e		n	n	12,54	2,17	1,96	9,47	y	n	n	>95th
1312	L296	m	31	n			n	4,53	1,26	1,81	2,44	n	n	n	>10th
1313	L297	f	1	e		n	n	2,08	0,89	0,47	0,97	n	n	n	<5th
1314	L273	m	28	e		n	n	9,02	1,38	2,01	6,72	y	n	n	>95th
1361	L347	f	58	e		n	n	6,53	1,81	1,36	4,1	n	n	n	>30th
1362	L358	f	66	e	y	n	n	11,46	1,35	1,34	9,5	y	n	n	>95th
1363	L399	f	37	e		n	n	7,51	1,15	0,78	6,0	y	n	n	>90th
1364	L400	f	8	e		n	n	7,44	1,21	1,0	5,77	y	n	n	>95th
1365	L401	f	12	e		n	n	3,83	1,47	1,26	1,78	n	n	n	10th
1366	L387	f	47	e		u	u	7,55	1,16	1,18	5,85	n	n	y	>70th
1367	L388	f	15	e		n	n	5,93	1,18	0,54	4,5	n	n	y	>80th
1368	L389	f	13	e		n	n	6,88	1,02	0,56	5,6	n	n	y	>95th
1369	L393	m	50	n		n	n	4,46	0,94	1,2	2,97	n	n	n	<5th
1432	L437	m	38	n		n	n	2,52	0,99	2,56	0,36	n	n	n	<5th
0032		f	49	I		n	n	10,4	1,95	0,9	8,04	y	n	n	>95th
0036		f	49	I		y	y	13,9	1,82	2,3	11,02	y	n	n	>95th
0037		f	22	e		n	n	7,5	0,95	0,8	6,18	y	n	n	>95th
0935		f	51	e		y	n	11,81	0,91	2,36	9,82	y	n	n	>95th
0040		m	54	I		y	y	7,8	0,76	1,2	6,49	y	n	n	>80th
0694		f	51	n			n	7,9	1,2	3,2	5,23	n	n	n	>80th
0695	L248	m	20	I			n	7,4	1,0	0,8	6,03	y	n	n	>95th
0696	L272	m	18	I			n	6,5	0,9	2,1	4,64	y	n	n	>95th
0041		f	44	I	y	y	n	9,6	0,9	1,3	8,1	n	y	n	>95th
0042		m	40	I	y	y	n	9,6	1,0	1,6	7,87	y	n	n	>95th
0043		f	42	I	y	n	n	8,8	2,0	0,6	6,52	y	n	n	>95th
0044		m	28	e	y	y	y	10,9	0,53	1,85	9,52	n	y	n	>95th
0045		f	30	n		n	n	5,2	1,7	1,1	3,0	n	n	n	>40th
0055		m	54	I		n	y	12,1	1,1	1,9	10,13	y	n	n	>95th
0792		f	82	I		y	y	12,2	1,3	1,1	10,4	y	n	n	>95th
0793		f	59	I		y	n	9,6	1,3	1,4	7,66	y	n	n	>95th
0061		m	30	n		n	n	8,0	1,4	1,2	6,05	y	n	n	>95th
0062		f	52	n		y	y	8,2	2,1	1,2	5,55	y	n	n	>80th
0069		f	72	I		y	y	12,0	1,2	2,4	9,7	n	y	n	>95th
0068	L233	m	74	n			n	5,8	1,3	2,1	3,54	n	n	n	>30th
1301	L240	m	48	n		n	n	5,48	1,69	1,19	3,24	n	n	n	>20th
1294	L232	f	24	e			n	7,59	1,55	0,65	5,74	n	y	n	>95th
1295	L234	f	50	e			n	5,23	2,36	1,31	2,27	n	n	n	>10th
1296	L235	f	16	e		n	n	3,31	1,87	0,42	1,25	n	n	n	<5th
1297	L236	m	19	e		n	n	6,69	0,94	1,36	5,13	n	y	n	>95th
1298	L237	m	14	e		n	n	4,43	1,34	0,9	2,68	n	n	n	60th
1299	L238	m	22	e		n	n	7,21	1,24	1,55	5,26	n	y	n	>95th
1300	L239	m	47	e		y	n	7,87	1,09	0,97	6,34	n	y	n	>80th
1302	L241	f	39	e		n	n	4,94	2,44	0,87	2,1	n	n	n	>20th
1303	L242	m	32	e		u	n	8,42	1,94	0,87	6,08	n	y	n	>95th
1304	L243	m	35	e		u	n	7,64	1,7	0,83	5,56	n	y	n	>80th
1389	L292	m	54	e		n	y	6,09	0,66	1,84	4,59	n	y	n	>40th
1390	L340	f	18	e		n	n	5,82	2,15	0,59	3,4	n	n	n	>80th
1391	L397	m	23	e		n	n	4,53	1,03	0,83	3,12	n	n	n	>30th

1392	L398	f	52	n		n	n	4,31	1,58	0,75	2,39	n	n	n	<5th
0070	L138	f	51	e		y	n	9,2	2,1	0,9	6,69	y	n	n	>95th
0089		f	38	l			n	5,7	1,7	0,9	3,59	n	n	n	50th
1237	L137	f	79	e		y	y	9,97	1,85	2,45	7,0	n	n	n	>95th
0076	L216	f	62	e		y	n	8,9	1,5	1,3	6,8	y	n	n	>90th
0620		f	21	e			n	8,1	1,3	1,7	6,02	y	n	n	>95th
0621		m	26	e			n	5,1	1,3	1,0	3,34	n	n	n	>40th
0712		m	55	n			n	5,2	1,2	2,2	2,99	n	n	n	>10th
0713		m	21	e			n	4,5	1,0	1,1	3,0	n	n	n	40th
0714		f	56	e			n	6,4	1,7	1,1	4,2	n	n	n	30th
0750		m	63	e			n	9,7	1,0	1,3	8,1	y	n	n	>95th
0751		m	31	e		n	n	9,4	0,8	1,1	8,1	y	n	n	>95th
0097		f	60	e		y	n	10,7	1,1	1,2	9,05	y	n	n	>95th
0096		m	74	n			n	6,1	1,0	4,6	2,99	n	n	n	>40th
0104	L044	m	39	l	y	n	n	8,9	1,7	1,7	6,42	y	n	n	>95th
1192	L041	m	13	l			n	4,21	1,89	0,7	2,0	n	n	n	50th
1193	L042	m	18	l			n	6,07	1,35	0,72	4,39	y	n	n	95th
1194	L043	f	44	n			n	4,95	1,38	0,82	3,19	n	n	n	>20th
1321	L222	m	40	n		n	n	7,1	1,08	2,84	4,72	n	n	n	>80th
1322	L223	f	10	l		n	n	4,8	1,19	1,22	3,05	n	n	n	>50th
1323	L224	m	7	l		n	n	5,36	1,43	1,08	3,43	n	n	n	90th
1324	L225	f	37	l		n	n	5,71	1,51	1,3	3,6	n	n	n	50th
0106	L096	m	49	e		y	n	10,92	0,55	1,94	9,48	y	n	n	>95th
0108		m	21	e			n	9,13	0,6	1,56	7,81	y	n	n	>95th
0110		m	16	e			n	7,01	0,77	1,1	5,74	y	n	n	>95th
0328		m	24	e			n	8,2	0,8	2,3	6,34	y	n	n	>95th
0365		m	22	e			n	3,5	1,1	1,6	1,67	n	n	n	10th
0367		f	37	e			n	3,8	1,5	0,8	1,93	n	n	n	<5th
0375		f	21	e			n	4,8	1,9	1,1	2,4	n	n	n	50th
0376		m	23	e			n	4,6	1,3	1,0	2,84	n	n	n	>40th
0380		f	54	e			n	6,4	1,5	1,9	4,03	n	n	n	50th
0381		m	16	e			n	3,4	1,3	0,6	1,82	n	n	n	>50th
0382		m	14	e			n	4,2	1,6	0,8	2,23	n	n	n	50th
0383		f	20	e			n	4,9	1,4	1,5	2,81	n	n	n	>50th
0391		f	22	e			n	5,8	1,5	1,1	3,8	n	n	n	80th
0411		f	39	e			n	5,8	1,4	1,6	3,67	n	n	n	>50th
0412		m	40	n			n	7,4	1,1	2,4	5,2	n	n	n	>80th
0413		f	15	e			n	5,1	1,4	1,2	3,15	n	n	n	>60th
0414		f	11	e			n	5,1	1,5	1,1	3,1	n	n	n	>60th
0418		f	76	e			u	10,9	0,9	5,9	7,29	n	n	n	>95th
0419		f	45	e		n	n	7,8	1,3	1,7	5,72	n	n	n	>80th
0420		m	48	n			n	7,8	0,7	4,7	4,94	n	n	n	>80th
0421		f	15	e			n	6,2	1,4	1,7	4,02	n	n	n	>90th
0422		m	12	e			n	6,9	1,5	1,6	4,67	n	n	n	>95th
0492		m	31	e			n	5,6	1,7	1,5	3,21	n	n	n	>50th
0512		f	47	e			u	8,6	1,6	2,4	5,9	y	n	n	>90th
0513		m	49	n			n	5,1	1,3	5,0	1,51	n	n	n	>10th
0514		m	15	e			n	6,5	1,6	1,1	4,4	y	n	n	>95th

0539		f	17	e			n	7,2	1,5	0,6	5,42	y	n	n	>95th
0586		f	26	e			n	6,5	1,4	2,0	4,18	n	n	n	>80th
0647		f	28	e			n	9,5	1,4	0,9	7,69	y	n	n	>95th

0807		m	33	e			n	9,6	1,2	0,5	8,17	y	n	n	>95th
0107	L095	f	46	e		y	n	11,31	1,21	0,8	9,73	y	n	n	>95th
0352		f	54	n			n	6,8	1,5	1,4	4,66	n	n	n	>60th
0353		m	16	e			n	8,2	0,9	1,6	6,57	y	n	n	>95th
0354		f	62	e			n	7,0	1,3	2,3	4,64	n	n	n	50th
0355		m	55	e			n	8,3	0,9	0,9	6,99	y	n	n	95th
0366		m	19	e			n	3,9	1,1	0,8	2,43	n	n	n	30th
0374		m	58	e			u	8,8	1,3	3,1	6,08	n	n	n	>95th
0377		f	29	e			n	5,2	1,8	1,5	2,71	n	n	n	>40th
0378		f	25	e			n	4,2	1,8	0,6	2,12	n	n	n	10th
0379		f	21	e				9,0	2,3	0,7	6,38	y	n	n	>95th
0403		f	27	e		n	n	8,3	1,5	1,1	6,3	y	n	n	>95th
0408		f	23	e			n	6,2	1,7	0,8	4,13	n	n	n	>80th
0464		f	36	e			n	6,0	1,5	1,6	3,77	n	n	n	60th
0465		m	39	n			n	6,6	1,2	2,1	4,44	n	n	n	70th
0466		m	7	e			n	5,1	1,3	0,8	3,43	n	n	n	>80th
0523		f	64	e			n	13,4	1,4	2,2	10,99	y	n	n	>95th
0524		f	26	e			n	7,4	1,5	0,7	5,58	y	n	n	>90th
0548		m	23	e			n	4,6	1,1	1,1	3,0	n	n	n	>40th
0560		m	30	e			n	5,0	1,2	1,1	3,3	n	n	n	>30th
0703		f	30	e			n	6,3	1,2	0,8	4,73	n	n	n	>80th
0112	L157	m	49	e		y	y	10,7	1,2	3,0	8,12	y	n	n	>95th
1040		m	84	e		n	n	8,9	1,4	1,4	6,86	y	n	n	>95th
1041		f	83	n		n	n	6,6	0,9	4,7	3,54	n	n	n	>95th
1068		f	22	e		n	n	7,14	1,58	1,42	4,91	y	n	n	95th
1070		m	47	e		n	n	7,86	1,53	1,75	5,53	n	n	n	>80th
1071	L156	f	37	n			n	4,98	2,07	0,51	2,68	n	n	n	>20th
1072	L155	m	9	e		n	n	7,01	1,07	0,96	5,5	y	n	n	>95th
1073	L158	m	8	e		n	n	7,84	1,22	0,76	6,27	y	n	n	>95th
1074		m	51	e		n	n	7,6	1,13	2,85	5,16	n	n	n	>80th
1075		f	45	n			n	5,9	1,5	1,6	3,67	n	n	n	>30th
1076		m	12	e			n	4,53	1,74	0,91	2,37	n	n	n	>60th
0113		m	12	n		n	n	9,39	0,84	1,05	8,07	y	n	n	>95th
0114		m	42	n			n	6,7	1,0	1,0	5,24	n	n	n	>70th
0118	L081	m	37	e	y	y	n	8,9	0,9	1,3	7,4	n	n	y	>95th
0006	L073	f	9	e		n	n	8,22	1,48	0,83	6,36	n	n	y	>95th
0007	L075	f	37	u			n	5,9	1,0	1,3	4,3	n	n	n	>50th
0116		f	56	n			n	5,9	1,7	1,8	3,37	n	n	n	>10th
0117		f	21	e			n	7,0	2,2	2,1	3,84	n	n	n	>90th
0119	L074	m	7	e			n	5,49	1,54	0,34	3,79	n	n	n	>90th
0155		f	32	e			n	4,4	1,4	0,9	2,59	n	n	n	>10th
0156		f	7	n			n	4,3	1,4	1,0	2,44	n	n	n	30th
0157		f	5	n			n	4,1	1,2	0,7	2,58	n	n	n	20th
0699		f	42	e		n	n	9,2	1,2	0,9	7,59	n	n	y	>95th
0700		m	20	e			n	4,5	1,1	1,2	2,85	n	n	n	40th
0701		f	17	e			n	4,4	1,4	0,9	2,59	n	n	n	>30th
0702		m	19	e			n	4,5	1,0	1,6	2,77	n	n	n	>60th
0126		f	56	l		n	n	10,6	1,6	1,4	8,36	y	n	n	>95th
0088		f	27	n			n	8,1	1,3	2,0	5,88	y	n	n	>95th
0127		m	56	e			y	6,4	1,0	3,9	3,61	n	n	n	>50th
0171		m	25	n			n	6,8	1,0	3,9	4,01	n	n	n	>80th
0133		f	61	e		y	y	9,3	1,5	1,3	7,2	y	n	n	>90th

0115		f	37	e		n	n	9,2	1,4	1,3	7,2	y	n	n	>95th
0134		m	67	n			n	6,9	1,1	2,3	4,74	n	n	n	>70th
0169		m	61	e	y		y	8,4	1,7	1,3	6,1	y	n	n	>95th
0697		m	20	e		n	n	8,3	0,8	1,5	6,81	y	n	n	>95th
0698		f	56	n			n	6,0	2,1	0,7	3,58	n	n	n	20th
0709		m	60	e	y		n	5,6	1,3	1,1	3,8	n	n	n	30th
0710		f	74	e			n	5,2	1,3	1,4	3,26	n	n	n	>5th
0711		f	72	e			n	5,8	1,8	1,5	3,31	n	n	n	>10th
0721		f	34	e	y		n	6,0	1,2	0,7	4,48	y	n	n	>70th
0735		m	31	e			n	6,2	1,0	2,2	4,19	n	n	n	>70th
0736		m	30	e			n	6,3	0,8	2,2	4,49	y	n	n	>70th
0752	L263	f	57	e		n	n	9,7	1,2	1,2	7,95	y	n	n	>95th
0882		m	63	e			n	8,7	1,0	1,4	7,06	y	n	n	>95th
1332	L257	m	17	e		n	n	5,6	1,56	0,87	3,64	n	n	n	>90th
1333	L258	m	15	e		n	n	4,25	1,18	0,89	2,66	n	n	n	>40th
1334	L261	m	44	e		n	n	8,36	1,37	1,98	6,08	n	n	n	>90th
1335	L262	f	12	e		n	n	5,72	1,5	1,68	3,45	n	n	n	>80th
0170		m	32	l		n	n	9,03	1,41	1,01	7,16	y	n	n	>95th
0177		m	52	e		y	y	10,22	1,12	1,2	8,55	y	n	n	>95th
0038		f	54	e		y	n	10,3	1,38	1,2	8,37	y	n	n	>95th
0178		f	12	e			n	8,43	1,15	1,3	6,68	y	n	n	>95th
0179		f	15	e			n	8,6	0,94	1,04	7,18	y	n	n	>95th
0830		m	28	e		n	n	8,89	0,98	0,77	7,56	y	n	n	>95th
0845		m	25	e			n	4,2	1,3	0,5	2,67	n	n	n	>5th
0847		f	51	e			n	5,2	1,2	0,8	3,63	n	n	n	>10th
0182		m	36	l	y	y	n	11,15	1,0	1,08	9,65	y	n	n	>95th
0189	L268	f	68	e		n	n	11,14	1,3	1,42	9,19	y	n	n	>95th
0190	L383	m	60	n			n	5,2	1,0	1,9	3,33	n	n	n	>10th
0191	L266	f	20	l		n	n	7,4	0,9	1,0	6,04	y	n	n	>95th
0201	L394	f	32	l			n	5,2	1,2	1,2	3,45	n	n	n	>40th
1336	L264	f	6	e		n	n	8,5	1,31	0,97	6,75	y	n	n	>95th
1337	L265	f	4	e		n	n	7,69	0,88	1,34	6,2	y	n	n	>95th
1338	L267	m	2	e		n	n	4,99	1,44	0,57	3,29	n	n	n	>80th
1339	L269	f	36	e		n	n	10,59	1,04	3,74	7,83	y	n	n	>95th
1340	L270	m	7	e		n	n	5,34	1,21	1,46	3,46	n	n	n	>90th
1393	L382	m	39	n		n	n	7,36	1,2	2,82	4,87	n	n	n	>80th
1394	L396	m	27	n		n	n	4,92	1,04	1,48	3,2	n	n	n	30th
0217	L148	f	50	e		n	n	9,0	0,7	2,0	7,38	y	n	n	95th
0218	L149	m	56	n			n	5,5	1,0	2,1	3,54	n	n	n	>20th
0219	L360	f	15	e			n	5,3	1,5	1,3	3,2	n	n	n	>70th
0220	L361	f	26	e			n	5,9	0,9	5,4	2,52	n	n	n	70th
0221	L359	f	18	e			n	8,1	0,9	2,6	6,01	y	n	n	>95th
0553		f	29	e			n	5,9	1,4	2,2	3,49	n	n	n	70th
0238		m	53	l		y	n	7,3	0,7	1,6	5,87	y	n	n	80th
0233		m	21	l		n	n	5,3	1,1	1,2	3,65	n	n	n	>70th
0234		m	18	l			n	4,5	1,1	1,3	2,8	n	n	n	>60th
0235		m	16	l		n	n	3,7	1,0	1,0	2,24	n	n	n	20th
0236		f	13	l		n	n	4,1	1,2	1,1	2,4	n	n	n	20th
0237		f	49	n		n	n	4,8	1,3	1,1	3,0	n	n	n	>5th
0737		f	56	l		y	n	10,0	2,7	1,9	6,43	y	n	n	>95th
0738		m	34	l		y	n	8,0	0,9	0,9	6,69	y	n	n	>95th
0755		f	29	l			n	6,9	1,3	0,9	5,19	y	n	n	90th

0756		m	26	l			n	4,5	1,2	0,9	2,89	n	n	n	>10th
0239		f	43	e		y	y	11,0	1,0	2,2	8,99	n	y	n	>95th
0240		m	22	e		n	n	4,5	1,3	1,0	2,74	n	n	n	>60th
0241		m	17	e		n	n	5,2	1,2	4,7	1,84	n	n	n	>80th
0319		m	50	n			n	6,4	1,3	2,7	3,86	n	n	n	>50th
0242	L230	f	67	l	y		y	11,06	1,26	1,06	9,31	y	n	n	>95th
0688		f	35	l			n	10,0	1,3	0,9	8,29	y	n	n	>95th
0689		m	37	l			n	6,0	1,4	0,8	4,23	n	n	n	50th
0690		m	55	l			n	9,1	0,6	1,3	7,9	y	n	n	>95th
0691		m	27	l			n	6,6	1,0	2,5	4,45	n	n	n	80th
0692		m	31	l		n	n	9,4	0,7	2,0	7,78	y	n	n	>95th
0243		f	40	e	y	y	y	8,5	1,3	1,1	6,7	y	n	n	>95th
0246	L034	f	34	e		n	y	9,7	1,3	,8	8,03	y	n	n	>95th
0245		m	37	n		n	n	5,7	1,5	2,7	2,96	n	n	n	40th
0247	L033	f	11	e			n	8,2	1,3	1,0	6,44	y	n	n	>95th
0248	L032	f	10	e			n	7,9	1,2	1,2	6,15	y	n	n	>95th
1188	L035	m	73	n			n	5,78	1,46	0,98	3,87	n	n	n	>30th
1326	L218	f	61	n		n	n	6,99	1,24	1,82	4,92	n	n	n	>40th
1325	L217	m	46	e		n	n	6,45	1,03	2,29	4,37	y	n	n	>50th
0266	L193	m	45	e		y	y	7,3	1,3	0,9	5,59	y	n	n	80th
0265	L114	f	6	n			n	5,51	1,47	1,15	3,51	n	n	n	>80th
0267	L115	f	14	e		n	n	6,57	1,4	0,75	4,83	y	n	n	>95th
0268		m	16	e			n	6,5	1,17	1,01	4,87	y	n	n	>95th
0483	L116	f	39	n			n	4,6	1,8	1,3	2,2	n	n	n	>10th
0619	L196	f	64	e			n	9,2	1,4	1,3	7,2	y	n	n	>90th
0622		f	67	e			n	9,6	1,6	1,6	7,27	y	n	n	>95th
0623		f	46	e			n	5,3	1,5	0,6	3,52	n	n	n	>10th
0624		m	21	e		n	n	7,5	1,2	1,0	5,84	y	n	n	>95th
0625		m	68	n			n	5,2	1,3	0,8	3,53	n	n	n	>10th
0626	L198	m	40	e			n	8,4	1,2	3,3	5,69	y	n	n	95th
0627		f	38	l			n	5,5	1,8	0,7	3,38	n	n	n	>40th
0944		m	35	e	y	y	y	10,96	0,98	1,28	9,39	y	n	n	>95th
1266	L197	m	78	e			n	4,79	1,3	1,85	2,64	n	n	n	>5th
1267	L199	f	27	e			n	5,59	1,13	2,1	3,5	n	n	n	>50th
1268	L200	f	21	e			n	6,4	1,18	1,24	4,65	y	n	n	90th
1269	L201	f	18	e			n	4,6	1,28	1,15	2,79	n	n	n	>40th
1270	L202	m	28	e			n	7,7	0,83	1,31	6,27	y	n	n	95th
1317	L221	f	20	n		n	n	6,09	1,07	0,59	4,75	y	n	n	>80th
0269		m	39	e		y	y	8,53	1,36	1,28	6,58	y	n	n	>95th
0298		f	23	e		n	n	11,7	1,28	0,94	9,99	n	n	y	>95th
0461	L364	f	29	e			n	9,8	1,0	2,0	7,88	n	n	y	>95th
0462		f	56	e			n	7,5	0,9	6,0	3,85	n	n	n	>60th
0484		m	31	e		y	y	10,2	0,3	2,2	8,89	n	n	y	>95th
0485	L053	f	31	n			n	4,1	1,6	1,2	1,95	n	n	n	>5th
0486	L055	m	8	e			n	4,2	1,4	2,3	1,74	n	n	n	50th
0487	L057	f	4	e			n	8,3	1,6	1,6	5,97	n	n	y	>95th
0488	L056	m	11	e			n	6,0	1,3	1,2	4,15	n	n	y	>95th
0503		m	34	e			n	7,1	1,4	2,0	4,78	n	n	n	>80th
0561	L366	f	13	e			n	9,1	1,7	1,3	6,8	n	n	y	>95th
0562	L365	f	14	e			n	5,2	1,5	1,3	3,1	n	n	n	70th
1204	L054	f	9	e			n	8,34	1,28	0,99	6,61	n	n	y	>95th
1351	L363	m	44	n		n	n	6,95	1,14	0,76	5,46	n	n	n	>70th

0299		f	56	I		y	y	11,62	1,16	1,31	9,86	y	n	n	>95th
0301		f	41	e		y	y	9,47	1,61	,83	7,48	y	n	n	>95th
0344		m	37	e			n	11,03	1,1	2,5	8,78	y	n	n	>95th
0599		f	18	e			n	5,9	1,6	1,9	3,43	n	n	n	>80th
0600		m	9	e			n	5,0	1,7	2,1	2,34	n	n	n	>80th
0601		m	12	e			n	7,7	1,6	3,0	4,72	y	n	n	>95th
0602		m	17	e			n	8,2	1,3	1,5	6,21	y	n	n	>95th
0720		m	33	e			n	4,7	1,2	1,0	3,04	n	n	n	>20th
0724		f	26	n			n	4,7	1,2	1,1	3,0	n	n	n	>20th
0725		f	8	e			n	4,3	1,1	0,7	2,88	n	n	n	30th
0726		f	3	e			n	5,0	1,5	0,9	3,09	n	n	n	>60th
0727		m	29	e			n	7,5	0,8	0,8	6,33	y	n	n	>90th
0728		f	35	n			n	5,0	1,0	1,2	3,45	n	n	n	>20th
0729		m	16	e			n	4,7	0,9	0,9	3,39	n	n	n	70th
0730		m	15	e			n	3,8	0,7	1,4	2,46	n	n	n	>20th
0731		f	11	e			n	4,5	1,4	0,7	2,78	n	n	n	40th
0734		f	14	e			n	5,8	1,3	1,8	3,67	n	n	n	>80th
0739		m	41	e			n	7,7	0,9	1,9	5,93	y	n	n	90th
0740		f	37	n			n	5,8	1,5	1,1	3,8	n	n	n	>50th
0741		m	12	e			n	7,5	1,2	0,8	5,93	y	n	n	>95th
0742		m	15	e			n	6,8	0,9	0,9	5,49	y	n	n	>95th
0743		m	9	e			n	8,5	1,1	0,7	7,08	y	n	n	>95th
0768		f	31	e		n	n	7,9	1,2	2,0	5,78	y	n	n	>95th
0769		m	11	e		n	n	6,5	1,4	1,1	4,6	y	n	n	>95th
0304		f	38	e		y	n	9,8	1,1	1,7	7,92	n	y	n	>95th
0305		m	42	n			n	5,3	1,1	3,0	2,82	n	n	n	>20th
0611		f	13	e		n	n	6,1	0,64	1,39	4,82	n	y	n	>90th
0612		m	14	I			n	4,3	1,2	1,0	2,64	n	n	n	>50th
0329	L087	f	57	e		y	n	10,0	1,1	1,6	8,17	n	n	y	>95th
0332		f	19	e		y	n	8,5	1,4	1,5	6,41	y	n	n	>95th
0343	L227	f	36	e		y	n	9,83	0,88	0,85	8,56	y	n	n	>95th
0603		f	34	e			n	9,3	1,3	1,4	7,36	y	n	n	>95th
0613		f	66	n		n	n	7,0	1,6	2,3	4,34	n	n	n	50th
0787		m	30	n			n	6,4	1,1	1,4	4,66	n	n	n	>70th
0788		m	5	e			n	7,1	0,8	0,8	5,93	y	n	n	>95th
1293	L228	m	11	e		n	n	7,08	0,91	0,68	5,86	y	n	n	>95th
0345		f	63	e	y	y	y	9,37	1,12	1,73	7,46	y	n	n	>90th
0357		m	14	n		n	n	6,68	1,47	0,34	5,05	y	n	n	>95th
0358		f	40	n	y		n	5,4	1,3	0,5	3,87	y	n	n	40th
0368		f	39	e		n	n	10,0	1,3	1,0	8,24	n	y	n	>95th
0369		m	19	e			n	4,3	1,3	1,0	2,54	n	n	n	>50th
0370		f	12	e			n	10,53	0,73	1,25	9,23	n	y	n	>95th
0385	L211	f	58	e		n	y	10,1	1,3	1,27	8,22	y	n	n	>95th
0009		m	39	n			n	6,0	1,2	1,5	4,11	n	n	n	50th
0011		f	5	e			n	7,25	1,74	0,55	5,26	y	n	n	>95th
0386		f	38	n	y		n	9,62	0,58	1,8	8,21	y	n	n	>95th
1038		f	11	n		n	n	8,7	1,3	0,6	7,12	y	n	n	>95th
0399	L092	f	42	I		y	n	9,7	1,5	1,0	7,74	y	n	n	>95th
0410		m	14	I			n	5,1	1,1	1,4	3,36	n	n	n	>80th
0704		m	50	I			n	4,7	1,0	1,0	3,24	n	n	n	>5th
0705		m	19	I		n	n	6,2	1,1	1,5	4,41	n	n	n	>95th
0706		f	22	I			n	9,4	1,2	0,9	7,79	y	n	n	>95th

0707		f	56	l			n	7,8	1,9	0,9	5,49	n	n	n	>70th
0708		f	43	l		n	n	7,0	1,7	1,2	4,75	n	n	n	>80th
0715		f	49	e		n	n	8,1	1,2	3,0	5,52	n	n	n	>80th
0716		m	47	l	y	y	n	9,2	0,5	2,9	7,37	y	n	n	>95th
0717		f	33	l			n	6,4	1,6	1,4	4,16	n	n	n	>80th
0718		m	30	n			n	6,3	0,9	4,0	3,57	n	n	n	>70th
0719		f	10	l			n	5,7	1,3	1,1	3,9	y	n	n	>80th
0732		m	57	l		n	n	7,1	0,8	4,0	4,47	n	n	n	80th
0733		f	51	n			n	7,2	1,4	2,0	4,88	n	n	n	70th
0450	L295	f	57	e		y	n	8,51	1,15	0,98	6,91	y	n	n	>80th
0272		f	53	e			y	6,29	1,25	0,7	4,72	n	n	n	>40th
0453		m	34	l		y	n	7,4	1,0	1,8	5,57	y	n	n	>90th
0454		m	29	n	y		n	9,85	0,78	1,12	8,56	y	n	n	>95th
0796		f	27	n			n	4,4	1,7	0,6	2,42	n	n	n	>10th
0455		m	39	e		y	y	9,9	1,3	2,6	7,41	n	y	n	>95th
0542		f	33	n			n	4,1	1,1	1,7	2,22	n	n	n	>5th
0543		f	6	e			n	3,6	1,3	1,3	1,7	n	n	n	>5th
0544		m	2	e			n	7,2	0,7	1,5	5,81	n	y	n	>95th
0545		f	9	e			n	3,9	1,1	1,1	2,3	n	n	n	>10th
0516		f	40	e			n	8,3	1,3	1,1	6,5	n	n	y	>95th
0533	L219	f	41	l		y	n	10,7	1,01	2,52	8,53	y	n	n	>95th
0534		m	45	n			n	5,7	0,7	6,6	1,97	n	n	n	>30th
0535		f	17	l			n	8,5	1,0	1,2	6,95	y	n	n	>95th
0536		f	18	l			n	4,8	1,3	0,8	3,13	n	n	n	>50th
0537		f	14	l			n	8,9	1,1	1,0	7,34	y	n	n	>95th
1292	L220	f	12	l		n	n	5,2	1,2	1,0	3,54	n	n	n	70th
0550		m	36	e	y		y	10,2	1,0	1,5	8,51	y	n	n	>95th
0551		f	5	e			n	7,2	1,6	0,8	5,23	y	n	n	>95th
0678		f	36	e		n	n	4,6	1,3	1,1	2,8	n	n	n	>10th
0693		m	3	l			n	5,9	1,6	1,4	3,66	n	n	n	>95th
0813		m	72	n			n	6,6	0,9	4,0	3,87	n	n	n	>60th
0814		f	64	e			n	11,1	1,2	1,6	9,17	y	n	n	>95th
0815		f	40	e		n	n	11,4	1,0	1,1	9,9	y	n	n	>95th
0816		m	42	n			n	5,3	1,5	0,6	3,52	n	n	n	>20th
0817		m	14	e			n	3,2	1,1	1,0	1,64	n	n	n	<5th
0818		f	12	e			n	3,9	1,4	0,7	2,18	n	n	n	>10th
0552		f	23	e		n	n	7,5	1,4	0,9	5,69	n	y	n	>95th
0554		m	32	e	y		y	7,5	1,2	0,78	5,94	y	n	n	>90th
0555	L293	m	46	l		y	y	13,11	1,13	0,98	11,53	y	n	n	>95th
0826	L371	f	42	l			n	5,1	1,7	1,6	2,67	n	n	n	30th
0827	L374	m	11	l			n	4,4	1,4	0,4	2,82	n	n	n	60th
0828	L373	m	9	l			n	8,1	1,5	0,5	6,37	y	n	n	>95th
0829	L372	m	7	l			n	6,1	1,3	0,5	4,57	y	n	n	>95th
0556		f	46	l		n	n	7,8	1,2	1,0	6,14	y	n	n	>80th
0557		m	53	n			n	6,1	1,5	2,3	3,54	n	n	n	>40th
0558		f	15	l			n	5,4	1,1	0,9	3,89	y	n	n	80th
0559		m	12	l			n	5,8	1,5	1,1	3,8	y	n	n	>90th
0580		f	50	l			n	4,4	1,0	2,3	2,34	n	n	n	<5th
0584		f	23	e		y	n	8,76	1,58	1,25	6,61	y	n	n	>95th
0609		m	27	u		u	u	9,1	1,1	1,3	7,4	y	n	n	>95th
0610		f	56	n			n	5,8	0,8	1,6	4,27	n	n	n	>10th
0658	L278	f	60	e		y	y	14,3	0,87	1,07	12,94	n	n	y	>95th

0316		f	55	e			y	12,0	1,1	2,4	9,8	n	n	y	>95th
0326		f	60	e			n	7,8	1,2	10,3	1,88	n	n	n	>70th
0327		m	62	n			n	8,7	2,7	1,2	5,45	n	n	n	>95th
0351		f	35	e			n	8,8	2,0	2,7	5,56	n	n	n	>95th
0401		m	28	e			n	4,9	1,5	2,1	2,44	n	n	n	30th
0402		m	58	n			n	4,8	1,0	2,9	2,47	n	n	n	>5th
0423		f	25	e		n	n	6,8	1,3	0,7	5,18	n	n	y	>80th
0424		m	59	e		n	y	8,8	1,0	1,3	7,2	n	n	y	>95th
0425		m	31	e		n	n	8,8	1,2	1,8	6,77	n	n	y	>95th
0508		f	31	e			n	6,3	1,9	1,7	3,62	n	n	n	>80th
0509		m	11	e			n	6,3	1,3	0,9	4,59	n	n	y	>95th
0510		f	36	e		y	n	10,4	1,8	1,4	7,96	n	n	y	>95th
0511		m	42	n			n	5,7	1,6	1,0	3,64	n	n	n	40th
0518		m	10	e			n	10,3	1,4	0,9	8,49	n	n	y	>95th
0519		f	34	e			n	9,8	1,0	1,5	8,11	n	n	y	>95th
0520		m	39	n			n	6,2	1,2	2,4	3,9	n	n	n	>50th
0521		f	8	e			n	10,3	1,5	1,4	8,16	n	n	y	>95th
0522		f	5	e			n	5,6	1,5	0,7	3,78	n	n	n	>80th
0525		m	61	n			n	6,2	1,6	1,1	4,1	n	n	n	50th
0526		f	58	e		y	y	12,3	1,1	1,9	10,33	n	n	y	>95th
0527		m	17	e			n	5,0	1,0	0,8	3,63	n	n	n	>80th
0540		m	18	e			n	3,4	0,8	0,4	2,42	n	n	n	>5th
0541		f	80	e		n	n	5,0	0,8	2,8	2,92	n	n	n	<5th
0549		m	31	n			n	6,0	0,9	7,4	1,71	n	n	n	>60th
0566		f	48	e		n	n	7,6	1,4	2,9	4,87	n	n	n	>70th
0587		f	53	e			n	5,5	1,2	2,2	3,29	n	n	n	>20th
0588		m	14	e			n	3,7	1,1	2,5	1,45	n	n	n	>5th
0657		m	61	n		n	n	5,8	0,8	1,1	4,5	n	n	n	>30th
0658	L278	f	60	e		y	y	14,3	0,87	1,07	12,94	n	n	y	>95th
0659	L349	m	40	e		y	y	13,6	0,7	1,02	12,43	n	n	y	>95th
0660	L351	f	41	n			n	5,5	1,0	1,0	4,04	n	n	n	>40th
0661	L348	f	11	e			n	7,3	1,0	0,6	6,02	n	n	y	>95th
0662	L350	f	8	e			n	3,2	0,8	0,6	2,12	n	n	n	<5th
0663	L430	f	26	e		y	n	8,5	1,1	0,9	6,99	n	n	y	>95th
0664	L274	f	34	e		y	n	10,4	1,0	0,4	9,22	n	n	y	>95th
0665	L392	m	38	n			n	7,5	0,9	1,3	6,0	n	n	n	>80th
0667		m	12	e			n	4,6	0,9	0,4	3,52	n	n	n	>60th
0668	L370	m	11	e			n	9,9	1,0	0,2	8,81	n	n	y	>95th
0669	L279	f	36	e			y	11,73	1,53	0,94	9,77	n	n	y	>95th
0670	L280	m	36	n			n	7,3	1,0	3,3	4,79	n	n	n	>80th
0671	L277	m	10	e			n	8,95	1,67	,98	6,83	n	n	y	>95th
0672	L276	f	8	e			n	7,93	1,15	0,78	6,42	n	n	y	>95th
0679		f	4	e			n	4,6	1,5	1,1	2,6	n	n	n	>40th
0819		m	38	e			n	5,8	0,9	2,8	3,62	n	n	n	>40th
0820		f	41	e			n	6,1	1,0	2,0	4,18	n	n	n	>60th
0843		f	80	e		n	n	4,8	1,3	0,3	3,36	n	n	n	<5th
1341	L275	f	13	e		n	n	6,64	1,44	1,37	4,57	n	n	n	>95th
1395	L431	m	37	n		n	n	4,46	1,15	1,0	2,85	n	n	n	>5th
1396	L432	f	12	n		n	n	6,55	1,28	0,78	4,91	n	n	y	>95th
1397	L433	m	10	n		n	n	4,84	1,69	0,58	2,88	n	n	n	>70th
1398	L434	m	4	n		n	n	5,64	1,17	0,75	4,13	n	n	y	>90th
0673		f	43	e		y	y	9,4	1,2	1,6	7,47	y	n	n	>95th

0785		f	13	e			n	7,75	1,24	0,6	6,23	y	n	n	>95th
0784		m	34	e		y	n	12,0	0,84	1,6	10,43	y	n	n	>95th
0856		f	32	l			n	5,0	1,4	1,0	3,14	n	n	n	40th
0794		f	30	l		n	n	9,57	0,96	0,89	8,2	y	n	n	>95th
0795		f	37	e		n	n	10,17	1,8	0,6	8,09	n	n	y	>95th
0853		f	35	n			n	5,4	1,0	1,4	3,76	n	n	n	40th
0854		m	34	e			n	8,9	1,6	1,7	6,52	n	n	y	>95th
0855		m	6	e			n	4,1	1,1	3,0	1,62	n	n	n	40th
0832		m	43	e		n	y	8,2	0,63	1,6	6,84	y	n	n	>90th
0833		f	41	e		n	n	7,8	0,75	1,4	6,41	y	n	n	>90th
0839		f	6	e		n	n	7,5	0,99	0,71	6,18	y	n	n	>95th
0838		m	8	e		n	n	6,1	1,4	0,4	4,52	y	n	n	>95th
0841		m	58	e		y	y	8,3	0,41	1,25	7,32	y	n	n	95th
0850	L246	f	33	l		n	n	10,5	0,8	1,8	8,87	y	n	n	>95th
0848		f	63	n			n	5,2	1,2	0,8	3,63	n	n	n	>5th
0849		m	69	l		n	y	10,0	1,1	2,0	7,98	y	n	n	>95th
0851	L244	f	26	l			n	8,0	1,0	0,7	6,68	y	n	n	>95th
1327	L245	m	29	l		n	n	7,52	1,3	1,55	5,51	y	n	n	>90th
0862		f	40	e		n	y	12,05	1,29	1,84	9,92	n	y	n	>95th
0864		m	58	e		y	y	9,0	0,65	1,5	7,66	y	n	n	>95th
0865		f	55	e		n	y	8,9	0,73	1,0	7,71	n	y	n	>90th
0870		m	42	e		y	y	10,7	0,59	2,17	9,11	y	n	n	>95th
0871		f	40	e		n	n	7,4	1,11	0,69	5,97	y	n	n	>90th
0873		f	24	e		n	y	9,13	0,92	1,39	7,57	n	y	n	>95th
0874		m	31	e		n	n	7,15	0,53	1,53	5,92	y	n	n	>80th
0876		f	2	e		n	n	5,2	1,0	1,1	3,7	n	n	n	70th
0880		f	43	e		n	n	7,57	1,01	0,7	6,24	y	n	n	>90th
0883		f	40	e		n	n	9,72	1,03	0,7	8,37	n	n	y	>95th
0886		f	49	e		y	n	9,87	1,71	0,73	7,83	n	y	n	>95th
0888		f	25	e		n	n	7,4	0,99	0,93	5,98	y	n	n	>90th
0889		m	35	e		y	y	9,5	0,8	1,7	7,92	n	y	n	>95th
0892		f	33	u		u	u	9,3	2,1	3,8	5,46	n	n	y	>95th
0893		m	41	n			n	6,8	1,4	1,5	4,71	n	n	n	>70th
0911		f	16	e		y	y	8,16	1,4	0,95	6,32	y	n	n	>95th
0912		f	69	e	y	y	y	10,82	1,14	1,33	9,07	y	n	n	>95th
0913		m	55	e		y	y	10,12	1,05	2,04	8,13	n	n	y	>95th
0915		m	35	e		y	n	8,86	1,09	0,58	7,5	n	y	n	>95th
0916		m	28	e		y	y	9,24	0,97	0,86	7,88	y	n	n	>95th
0917		f	67	e	y	y	n	10,15	1,39	1,63	8,01	n	y	n	>95th
0918		m	61	e	y	y	y	9,82	1,36	0,77	8,11	y	n	n	>95th
0919		m	37	e	y	y	y	9,66	0,99	0,97	8,23	y	n	n	>95th
0920		f	58	e	y	y	n	11,0	0,91	1,86	9,24	n	y	n	>95th
0921		f	36	e	y	y	y	10,6	1,0	1,5	8,91	n	n	y	>95th
0923		f	45	e		y	y	10,23	1,21	2,03	8,09	y	n	n	>95th
0924		f	39	e		y	n	9,7	1,21	1,34	7,88	n	y	n	>95th
0927		m	45	e		y	y	10,54	0,94	1,06	9,11	n	y	n	>95th
0929		m	35	e	y	y	n	10,1	1,02	2,31	8,02	n	y	n	>95th
0930		f	66	e	y	y	y	11,9	0,98	3,66	9,24	n	y	n	>95th
0931		m	38	e	y	y	n	7,41	1,18	0,79	5,87	y	n	n	>80th
0932		m	58	e	y	y	y	8,32	0,69	1,13	7,11	y	n	n	95th
0936		m	48	e		y	y	8,67	1,3	0,68	7,06	n	n	y	>90th

0940		m	55	e		y	y	10,52	1,0	2,02	8,59	n	n	y	>95th
0943		f	29	e		n	n	11,01	1,5	0,89	9,1	n	y	n	>95th
0945		f	48	e	y	y	n	9,05	1,68	1,01	6,91	y	n	n	>95th
0948		m	50	e		n	n	7,5	0,7	4,11	4,91	y	n	n	>80th
0949		m	49	e	y	y	n	10,78	1,0	1,0	9,32	y	n	n	>95th
0952		f	29	e		y	n	10,8	1,2	1,0	9,14	y	n	n	>95th
0954		m	25	e	y	y	n	8,69	0,71	1,32	7,37	y	n	n	>95th
0955		f	38	e		n	n	8,0	1,23	0,4	6,59	y	n	n	>95th
0956		m	32	e		y	n	9,0	1,16	1,03	7,37	y	n	n	>95th
0957		f	25	e		y	n	9,6	1,81	1,25	7,22	y	n	n	>95th
0959		f	35	e		n	n	8,0	1,53	1,34	5,86	y	n	n	>95th
0963		m	27	u		u	u	12,0	0,9	2,3	10,04	n	y	n	>95th
0964		m	45	u		u	u	7,8	1,2	1,0	6,14	y	n	n	>80th
0965		m	36	u		u	u	7,3	1,8	0,7	5,18	n	n	y	>80th
0966		m	58	u		u	u	11,2	1,2	1,7	9,22	y	n	n	>95th
0967		m	48	u		u	u	10,7	1,0	1,1	9,2	y	n	n	>95th
0968		m	35	u		u	u	8,1	1,1	1,2	6,45	n	n	y	>90th
0969		m	29	u		u	u	8,1	1,3	1,5	6,11	n	y	n	>95th
0970		f	26	u		u	u	9,4	1,0	0,8	8,03	n	n	y	>95th
0971		m	49	u		u	u	9,34	1,01	0,6	8,05	n	y	n	>95th
0972		m	37	u		u	u	8,93	0,55	1,7	7,6	y	n	n	>95th
0973		m	35	u		u	u	8,59	0,8	1,55	7,08	y	n	n	>95th
0974		m	35	u		u	u	8,87	0,55	1,49	7,64	n	y	n	>95th
0975		m	49	u		u	u	11,16	0,76	1,8	9,57	y	n	n	>95th
0976		m	46	u		u	u	9,82	0,64	2,77	7,91	y	n	n	>95th
0977		m	40	u		u	u	9,94	0,71	1,18	8,69	n	y	n	>95th
0978		f	53	u		u	u	13,95	1,09	1,72	12,07	n	y	n	>95th
0979		m	32	u		u	u	9,63	0,79	1,42	8,19	n	y	n	>95th
0980		m	47	u		u	u	9,15	1,34	0,98	7,36	y	n	n	>95th
0981		m	49	u		u	u	7,03	1,24	0,54	5,54	y	n	n	>95th
0982		m	41	u		u	u	9,68	0,6	1,29	8,49	y	n	n	>95th
0983		f	46	u		u	u	10,72	1,41	2,33	8,24	y	n	n	>95th
0984		m	35	u		u	u	10,55	0,61	1,67	9,17	n	y	n	>95th
0985		f	43	u		u	u	9,15	0,59	1,24	7,99	y	n	n	>95th
0986		m	44	u		u	u	8,1	1,39	0,72	6,38	y	n	n	>95th
0987		m	54	u		u	u	8,7	1,16	0,44	7,34	y	n	n	>95th
0988		f	61	u		u	u	10,97	1,03	0,88	9,54	y	n	n	>95th
0989		f	43	u		u	u	8,41	0,95	1,22	6,9	n	n	y	>95th
0990		m	54	u		u	u	8,54	0,8	1,19	7,19	y	n	n	>95th
1000	L426	f	26	e		n	y	8,82	1,69	1,06	6,64	y	n	n	>95th
1001	L427	m	58	n			n	6,04	1,29	1,11	4,24	n	n	n	40th
1002	L428	m	28	e		n	n	7,15	1,14	1,1	5,51	y	n	n	>80th
1003	L419	f	55	e		y	y	7,5	1,4	0,96	5,66	y	n	n	>60th
1004		m	29	n			n	5,79	0,97	2,2	3,81	n	n	n	>50th
1427	L429	m	30	e		n	n	5,55	1,37	1,85	3,33	n	n	n	50th
1005	L051	m	36	e	y		n	8,8	0,9	1,1	7,4	y	n	n	>95th
1202	L052	m	35	u			n	4,0	1,39	0,45	2,4	n	n	n	<5th
1203	L106	f	34	e			n	6,7	1,44	0,64	4,97	y	n	n	>80th
1320	L231	m	34	e		n	n	5,34	1,18	0,79	3,8	n	n	n	>40th
1006		f	34	e		n	n	11,04	1,1	0,64	9,65	n	n	y	>95th
1007	L005	m	29	e	y	y	y	13,0	1,2	2,23	10,78	n	y	n	>95th

1150	L019	f	52	e		y	y	11,0	0,14	1,29	10,27	n	y	n	>95th
1163	L001	f	29	n		n	n	5,57	1,84	1,2	3,18	n	n	n	>50th
1164	L002	m	33	e		n	n	4,6	0,77	3,15	2,39	n	n	n	20th
1165	L003	f	29	n		n	n	5,14	1,26	0,62	3,6	n	n	n	>40th
1166	L004	f	7	e		n	n	6,86	1,5	0,65	5,06	n	y	n	>95th
1167	L006	f	5	e		n	n	7,69	1,4	0,77	5,94	n	y	n	>95th
1168	L007	f	30	e		n	n	4,17	1,12	1,6	2,32	n	n	n	>5th
1169	L016	m	57	n		n	n	6,46	0,99	3,26	3,97	n	n	n	>50th
1170	L017	f	79	n		n	n	5,88	1,45	1,16	3,9	n	n	n	>10th
1171	L018	f	27	e		n	n	10,27	1,41	1,81	8,03	n	y	n	>95th
1008	L323	f	41	e		n	n	9,94	1,28	1,19	8,11	y	n	n	>95th
1428	L324	f	12	e		n	n	5,79	1,21	0,78	4,22	y	n	n	>80th
1429	L320	f	9	e		n	n	4,18	1,26	0,76	2,57	n	n	n	20th
1430	L321	f	13	e		n	n	6,77	1,11	1,46	4,99	y	n	n	>95th
1431	L322	m	50	n		n	n	3,73	1,78	1,24	1,38	n	n	n	<5th
1009		m	29	l		n	n	9,85	0,78	1,12	8,56	y	n	n	>95th
1010	L059	m	43	e		n	y	13,31	1,06	2,25	11,22	n	n	y	>95th
1156	L086	f	48	e		n	y	11,73	1,63	0,64	9,81	n	n	y	>95th
1205	L058	f	11	e			n	9,09	1,66	0,57	7,17	n	n	y	>95th
1206	L060	f	34	u			n	5,06	1,33	0,81	3,36	n	n	n	40th
1207	L061	m	14	e			n	5,51	1,21	1,04	3,82	n	n	n	>90th
1208	L133	m	39	e		n	n	6,25	1,65	0,85	4,21	n	n	n	>50th
1209	L134	m	4	e		n	n	8,12	1,58	0,5	6,31	n	n	y	>95th
1318	L326	f	54	e		n	n	8,49	2,07	1,23	5,86	n	n	y	90th
1319	L327	m	58	e	y	y	n	7,73	1,11	0,99	6,17	n	n	n	90th
1011	L168	f	51	e		y	y	10,4	1,2	2,63	7,99	n	y	n	>95th
1250	L167	m	55	e			n	5,79	1,09	1,33	4,09	n	n	n	>30th
1012		f	32	e		y	n	12,2	1,5	0,97	10,26	y	n	n	>95th
1013		f	65	l	y	y	y	10,7	0,9	1,4	9,16	y	n	n	>95th
1014	L187	f	64	l		y	n	13,1	1,8	1,26	10,72	n	y	n	>95th
1263	L186	f	61	l			n	10,6	1,4	1,11	8,69	n	y	n	>95th
1264	L188	m	78	n			n	5,08	0,62	1,5	3,77	n	n	n	>10th
1015	L153	f	56	l		y	y	11,47	0,81	2,48	9,52	n	y	n	>95th
1244	L159	m	39	l		y	n	12,22	1,39	1,09	10,33	n	y	n	>95th
1245	L161	m	41	l		y	n	10,13	1,1	0,81	8,66	n	y	n	>95th
1256	L195	f	65	l			n	5,44	0,96	1,64	3,73	n	n	n	>5th
1370	L395	m	60	n		n	n	5,89	1,02	2,35	3,79	n	n	n	>30th
1371	L407	m	38	l		n	n	5,32	0,95	2,37	3,28	n	n	n	>20th
1016		f	41	e		n	y	14,1	1,2	2,94	11,55	y	n	n	>95th
1017		m	22	e		n	n	9,7	1,1	0,6	8,32	n	y	n	>95th
1018	L027	m	42	e		n	y	7,2	1,3	1,7	5,12	n	n	y	>80th
1033	L024	f	12	e			n	8,6	1,15	1,45	6,78	n	n	y	>95th
1183	L025	f	10	e		n	n	4,51	1,44	1,2	2,52	n	n	n	40th
1184	L026	f	39	u		n	n	5,3	1,36	1,55	3,23	n	n	n	>30th
1019		f	12	e		n	n	8,3	1,1	1,4	6,56	n	y	n	>95th
1020		f	61	e		n	y	14,7	1,8	1,16	12,37	n	y	n	>95th
1021		m	29	e		y	n	11,4	1,07	1,48	9,65	y	n	n	>95th
1022	L093	f	66	l		y	n	10,35	1,17	0,77	8,83	y	n	n	>95th
1224	L118	f	34	l			n	10,29	0,91	1,72	8,59	y	n	n	>95th
1225	L119	m	8	l			n	4,03	1,03	,95	2,56	n	n	n	>30th
1226	L120	f	31	l			n	8,99	0,93	1,22	7,5	y	n	n	>95th
1227	L121	m	36	l			y	10,52	0,87	1,56	8,93	y	n	n	>95th

1023		f	70	e		y	y	8,89	1,46	0,84	7,04	y	n	n	90th
1024	L165	f	35	n		n	n	7,67	1,4	0,8	5,9	y	n	n	>90th
1025	L229	f	62	l		y	n	13,23	1,07	2,28	11,11	y	n	n	>95th
1026	L011	m	38	e	y	y	y	6,43	0,66	1,81	4,94	n	y	n	>60th
1172	L008	m	14	e		n	n	4,08	0,81	1,33	2,66	n	n	n	>30th
1173	L009	m	11	e		n	n	4,81	1,2	0,99	3,16	n	n	n	>70th
1174	L010	f	38	n		n	n	4,04	1,55	0,81	2,12	n	n	n	<5th
1175	L012	m	5	e		n	n	6,95	0,88	1,18	5,53	n	y	n	>95th
1176	L013	m	8	e		n	n	6,34	1,0	0,65	5,04	n	y	n	>95th
1177	L014	m	36	e		u	y	5,98	0,73	1,05	4,77	n	y	n	>40th
1178	L015	f	33	n		n	n	3,03	1,28	0,62	1,47	n	n	n	<5th
1027		m	39	e		y	y	10,49	0,99	1,03	9,03	y	n	n	>95th
1028		f	17	e		n	n	8,05	1,12	1,55	6,22	y	n	n	>95th
1029		m	21	e		n	n	6,35	1,26	0,78	4,73	y	n	n	>90th
1030		m	10	l		n	n	9,37	1,41	0,66	7,66	y	n	n	>95th
1031		m	35	l		n	y	10,85	1,73	2,21	8,11	y	n	n	>95th
1032		m	3	l		n	n	7,77	1,23	0,67	6,23	n	y	n	>95th
1034		m	18	l		n	n	9,84	1,3	3,12	7,11	y	n	n	>95th
1035	L281	f	22	n		n	n	9,57	1,6	0,61	7,69	n	n	y	>95th
1342	L282	m	27	n		n	n	5,41	1,39	1,19	3,47	n	n	n	>40th
1343	L283	f	51	n		n		7,42	1,25	1,47	5,5	n	n	n	>70th
1344	L284	m	30	e		n	n	9,59	1,0	1,14	8,07	n	n	y	>95th
1345	L285	f	24	e		n	n	7,11	1,3	1,4	5,17	n	n	n	95th
1346	L286	m	56	e	y		n	9,92	1,02	3,09	7,48	n	n	y	>95th
1399	L436	m	28	e		n	n	6,16	1,11	1,63	4,3	n	n	n	70th
1037		f	21	e	y		n	13,12	1,06	1,78	11,24	n	y	n	>95th
1039	L045	f	39	e		y	n	9,41	1,15	0,47	8,04	y	n	n	>95th
1195	L046	f	25	e			n	6,88	1,28	1,81	4,77	y	n	n	>80th
1196	L047	m	26	u			n	4,59	0,94	2,09	2,69	n	n	n	>10th
1197	L048	m	2	e			n	3,59	0,97	0,5	2,39	n	n	n	10th
1198	L077	f	21	u			n	4,99	1,11	0,53	3,64	n	n	n	>50th
1199	L078	m	23	e			n	9,08	0,82	0,71	7,93	y	n	n	>95th
1200	L079	m	43	u			n	5,98	0,98	3,13	3,56	n	n	n	>40th
1201	L091	m	26	e			n	5,67	1,16	0,57	4,25	y	n	n	>50th
1101		m	47	e		n	y	11,35	1,26	1,0	9,63	n	y	n	>95th
1102		f	15	e		n	n	9,9	1,56	0,87	7,94	n	y	n	>95th
1103		m	9	e		n	n	8,49	0,9	3,22	6,11	n	y	n	>95th
1104		m	35	e		y	y	10,44	0,54	1,14	9,38	y	n	n	>95th
1105		m	14	e		n	n	9,45	0,49	5,38	6,49	y	n	n	>95th
1106		f	46	e		n	n	8,76	1,32	2,59	6,25	y	n	n	>90th
1257	L179	m	45	n			n	8,0	1,0	4,98	4,72	n	n	n	>80th
1258	L181	m	21	e			n	5,16	0,84	2,17	3,32	u	n	n	>60th
1259	L182	m	19	e			n	2,69	0,95	0,72	1,41	n	n	n	<5th
1260	L183	f	15	e			n	3,95	1,16	0,68	2,48	n	n	n	>10th
1261	L184	f	43	e			n	6,03	0,97	2,72	3,81	y	n	n	60th
1262	L185	f	71	n			n	5,57	2,01	2,71	2,32	n	n	n	>5th
1265	L180	m	68	e	y		n	9,4	0,79	2,5	7,46	y	n	n	>95th
1107		m	38	e		y	n	13,0	0,9	1,8	11,27	n	y	n	>95th
1109		f	1	e			n	7,6	0	0	7,6	n	y	n	>95th
1108		f	31	l		n	n	6,0	1,6	1,2	3,85	y	n	n	>70th
1110		f	36	n		y	n	8,61	1,16	0,65	7,15	n	y	n	>95th
1111		m	39	u		u	u	8,7	2,0	1,07	6,21	y	n	n	>95th

1112		f	54	e		y	y	10,67	1,6	1,61	8,33	n	y	n	>95th
1113		f	8	e		n	n	6,24	1,04	0,54	4,95	n	y	n	>95th
1114		m	11	e		n	n	7,17	1,75	0,72	5,09	n	y	n	>95th
1115		m	32	e		y	y	11,7	0,98	0,71	10,39	n	y	n	>95th
1116		f	32	e		y	n	7,8	0,76	0,7	6,72	y	n	n	>95th
1117		m	59	e		u	u	10,24	0,68	1,89	8,69	y	n	n	>95th
1118		f	5	e		n	n	7,3	0,53	0,6	6,49	y	n	n	>95th
1119		m	9	e		n	n	8,4	1,37	0,9	6,62	y	n	n	>95th
1123		m	38	l		n	n	8,0	1,0	0,9	6,59	y	n	n	>90th
1124		m	2	e		n	n	8,5	2,0	1,0	6,04	n	n	y	>95th
1125			0					8,0	1,0	1,0	6,54	y	n	n	>95th
1130		m	42	e		u		8,66	1,01	2,03	6,72	n	y	n	>95th
1128		f	8	e		n		4,64	1,0	1,02	3,17	n	n	n	>40th
1131		m	12	e		n		6,75	0,98	1,24	5,2	n	y	n	>95th
1132		f	14	e		n		2,73	0,97	0,81	1,39	n	n	n	<5th
1127		f	16	e		n	n	11,53	1,38	1,28	9,56	n	y	n	>95th
1133		m	44	e		u		9,54	1,6	1,01	7,48	n	y	n	>95th
1129		f	7	e		n	n	8,21	1,21	0,81	6,63	n	n	y	>95th
1134	L099	f	69	e		y	y	12,06	1,14	1,49	10,24	n	n	y	>95th
1135	L375	m	36	e		y	n	7,54	0,83	2,13	5,73	n	n	y	>80th
1136		f	34	e		y	n	7,51	0,92	1,65	5,83	y	n	n	95th
1154	L136	f	58	e		y	y	9,83	1,0	2,0	7,91	n	n	y	>95th
1239	L169	m	35	e			n	8,6	0,91	1,29	7,1	n	n	y	>95th
1271	L209	f	72	l		n	y	5,94	1,2	3,05	3,34	n	n	n	>10th
1353	L328	f	42	n		n	n	6,09	1,39	1,26	4,12	n	n	n	60th
1354	L329	m	3	e		n	n	6,46	1,36	0,56	4,84	n	y	n	>95th
1355	L330	f	8	e		n	n	5,21	1,78	0,58	3,16	n	n	n	70th
1356	L331	f	44	e		y	y	9,73	1,34	4,03	6,54	n	n	y	>95th
1357	L378	m	6	e		n	n	4,04	0,96	1,61	2,34	n	n	n	>30th
1358	L390	m	48	n		n	n	6,2	1,15	1,62	4,31	n	n	n	50th
1359	L415	m	27	e		n	n	4,66	0,64	2,87	2,7	n	n	n	20th
1360	L420	f	18	e			n	4,84	1,32	1,41	2,87	n	n	n	>50th
1137	L037	f	54	l	y		y	7,64	1,21	1,03	5,96	y	n	n	>70th
1189	L038	f	27	e			n	4,33	1,1	0,85	2,84	n	n	n	>10th
1190	L039	m	59	n			n	6,85	1,11	1,51	5,05	n	n	n	70th
1191	L040	m	30	e			n	6,91	1,09	0,5	5,59	y	n	n	>80th
1138	L113	m	30	n		y	n	6,36	1,19	1,18	4,63	y	n	n	>70th
1139		m	60	e	y		y	7,91	1,76	0	6,15	y	n	n	>90th
1140		m	29	e		y	n	10,8	0,9	0,7	9,58	n	y	n	>95th
1141	L271	f	54	e		n	n	13,8	1,87	1,43	11,27	y	n	n	>95th
1142	L171	f	71	n		n	y	13,9	1,81	2,75	10,83	y	n	n	>95th
1251	L172	f	39	n			n	6,82	1,21	1,68	4,84	y	n	n	>80th
1252	L173	m	21	n			n	4,86	1,12	1,49	3,06	n	n	n	50th
1372	L346	f	40	l		n	n	9,51	1,0	15,02	1,62	n	n	n	>95th
1373	L344	f	20	l		n	n	6,72	1,5	2,13	4,24	n	n	n	>90th
1143	L062	m	44	e		y	y	9,27	0,79	0,86	8,09	n	n	y	>95th
1210	L063	f	18	e			n	7,31	0,93	0,95	5,94	n	n	y	>95th
1211	L064	m	10	e			n	4,62	1,16	1,2	2,91	n	n	n	>60th
1212	L065	f	43	u			n	4,12	0,89	1,06	2,74	n	n	n	5th
1144		m	46	e		n	y	13,5	0,9	5,17	10,23	y	n	n	>95th
1145	L135	f	25	u		y	n	12,0	1,0	0,47	10,78	n	y	n	>95th
1146	L170	f	66	e		y	y	6,6	1,78	1,53	4,12	y	n	n	>30th

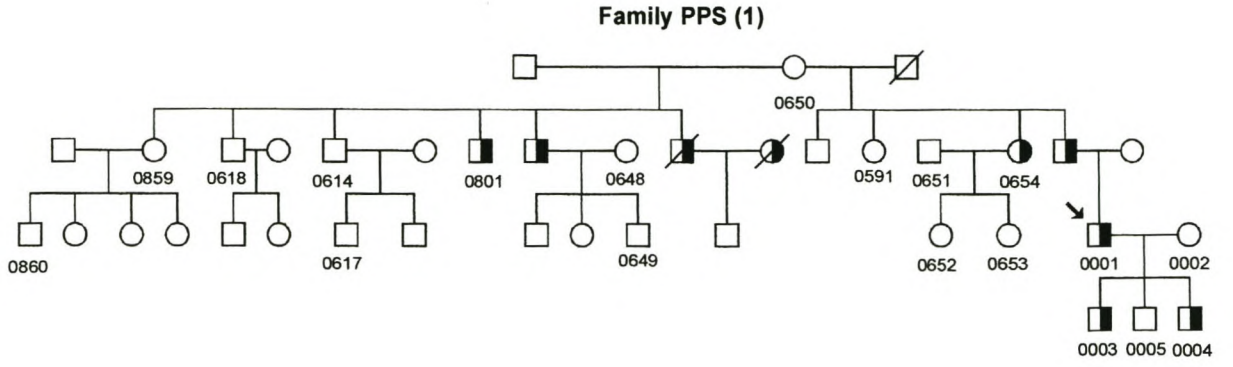
1147	L192	f	59	e		y	n	10,16	1,45	1,45	8,04	y	n	n	>95th
1253	L174	f	56	e	y		y	5,9	1,23	1,27	4,09	y	n	n	>10th
1254	L175	f	60	e		y	n	7,9	1,2	1,04	6,22	y	n	n	>70th
1255	L176	f	53	e		n	n	8,08	1,52	2,0	5,64	y	n	n	>80th
1148	L288	m	31	n		n	n	10,6	1,5	3,0	7,72	y	n	n	>95th
1151		m	19	e		n	n	8,0	1,15	1,27	6,27	n	n	y	>95th
1152	L069	m	39	e		n	n	9,9	1,79	1,97	7,21	y	n	n	>95th
1213	L066	f	37	n			n	4,93	1,55	1,14	2,86	n	n	n	>20th
1214	L067	m	10	n			n	4,4	1,58	0,4	2,64	n	n	n	60th
1215	L068	f	13	n			n	4,7	1,4	0,8	2,93	n	n	n	50th
1153	L125	f	36	e		y	n	9,8	0,61	1,88	8,33	y	n	n	>95th
1233	L122	m	11	e			n	4,44	1,52	0,52	2,68	n	n	n	60th
1234	L123	m	8	e			n	8,15	1,09	0,59	6,79	y	n	n	>95th
1235	L124	m	37	n			n	5,23	1,15	1,08	3,58	n	n	n	>20th
1155	L140	m	35	e		y	n	12,12	1,04	2,19	10,08	n	n	y	>95th
1238	L139	f	66	e			n	6,4	0,95	2,85	4,14	n	n	n	30th
1240	L177	f	30	e			n	5,01	0,77	0,5	4,01	n	n	n	40th
1241	L178	f	30	e			n	5,76	1,04	1,47	4,05	n	n	n	>60th
1316	L298	f	33	e		y	n	10,02	1,29	1,05	8,25	n	y	n	>95th
1157	L097	f	41	l	y	y	n	8,7	0,95	0,44	7,55	n	n	y	>95th
1158	L101	f	45	e		y	y	13,5	1,36	1,36	11,52	n	y	n	>95th
1232	L100	f	54	n			n	6,56	1,07	1,79	4,67	n	n	n	>50th
1159	L031	m	42	e		n	n	10,6	1,1	2,98	8,13	y	n	n	>95th
1185	L028	m	12	e		n	n	4,65	1,09	0,3	3,42	y	n	n	>60th
1186	L029	f	13	e		n	n	6,47	,94	0,66	5,23	y	n	n	>95th
1187	L030	f	40	n		n	n	3,87	1,64	0,55	1,98	n	n	n	<5th
1160	L071	m	24	e		n	n	10,12	1,1	2,98	7,65	y	n	n	>95th
1216	L070	f	49	e			n	7,18	1,56	,53	5,38	y	n	n	>60th
1217	L072	f	18	e			n	3,77	1,36	0,7	2,09	n	n	n	>5th
1352	L345	m	44	n		n	n	5,26	1,02	2,06	3,3	n	n	n	>20th
1161	L164	f	35	e		y	n	14,82	1,67	1,05	12,67	n	y	n	>95th
1162		m	12	e		n	n	7,0	1,37	1,9	4,76	n	y	n	>95th
1247	L163	m	52	e		n	n	6,84	0,92	1,58	5,2	n	n	n	>60th
1248	L162	f	29	e			n	6,75	1,72	0,74	4,69	n	n	n	>80th
1305	L253	m	12	e		n	n	9,95	1,27	1,72	7,89	n	y	n	>95th
1179		f	37	e		y	n	8,28	1,38	1,06	6,41	n	y	n	>95th
1180		m	49	e	y	y	y	9,45	0,77	0,95	8,24	n	y	n	>95th
1181		f	6	e		n	n	5,01	1,0	0,71	3,68	n	n	n	>60th
1182		f	19	e		n	n	4,38	1,0	,63	3,09	n	n	n	30th
1184	L208	f	70	e	y	y	y	9,15	2,16	1,14	6,47	n	y	n	>90th
1272	L189	m	41	e			n	4,95	1,38	1,24	3,0	u	u	u	>10th
1273	L190	m	14	e			n	4,22	1,64	0,65	2,28	n	n	n	50th
1274	L191	m	47	e			n	6,91	1,61	1,3	4,7	u	n	n	70th
1275	L194	f	58	e			n	6,42	1,51	2,33	3,84	u	u	u	30th
1276	L205	f	47	e			n	5,33	1,55	0,83	3,4	n	n	n	>10th
1277	L206	m	54	e			n	6,56	1,0	2,81	4,27	n	n	n	60th
1278	L204	f	22	e			n	4,85	1,71	,87	2,74	n	n	n	50th
1279	L203	f	40	e			n	7,69	1,08	1,28	6,02	n	y	n	>90th
1280	L207	f	73	e			n	6,24	1,8	1,01	3,98	n	n	n	>20th
1281	L210	m	29	e			n	5,42	1,0	1,65	3,66	n	n	n	>40th
1374	L379	m	11	e		n	n	4,17	1,56	1,37	1,98	n	n	n	40th
1375	L380	m	15	e		n	n	4,25	1,31	1,66	2,18	n	n	n	50th

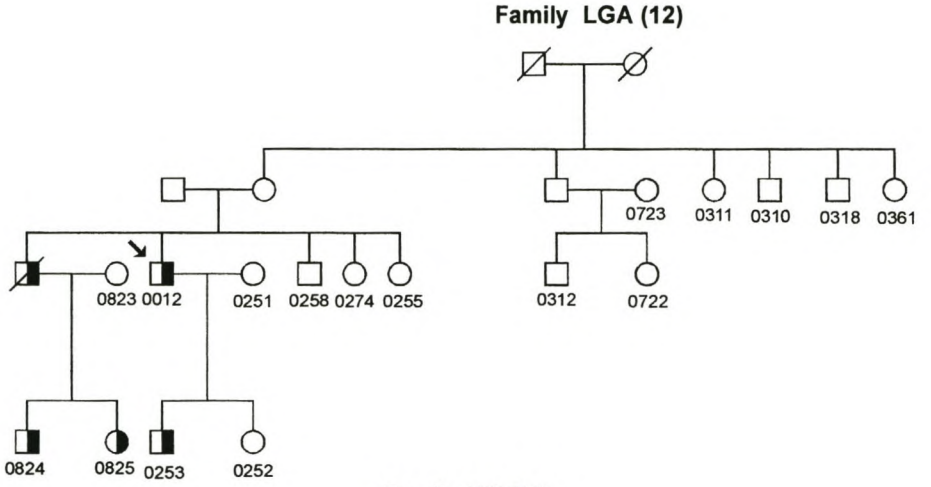
1376	L381	m	42	n		n	n	5,01	1,25	1,49	3,08	n	n	n	>10th
1377	L402	m	43	e	y		n	10,67	1,06	5,51	7,08	n	y	n	>95th
1378	L403	m	48	e	y	y	y	6,57	0,96	1,32	5,0	y	n	n	60th
1379	L404	f	45	e		y	n	7,52	1,57	0,91	5,53	n	y	n	>70th
1380	L405	f	33	e		n	n	5,53	1,85	0,73	3,35	n	n	n	>50th
1381	L406	f	19	e		n	n	8,91	1,36	1,51	6,86	n	y	n	>95th
1382	L408	m	69	e	y	y	y	10,07	1,03	1,61	8,3	n	y	n	>95th
1383	L409	f	67	e		n	n	10,71	1,02	3,95	7,88	n	y	n	>95th
1384	L410	m	25	e		n	n	11,28	0,8	1,9	9,61	n	n	n	>95th
1385	L418	f	19	e	y		n	7,73	1,29	1,39	5,8	n	y	n	>95th
1433	L438	f	37	n		n	n	5,29	1,09	1,32	3,59	n	n	n	>30th
1434	L439	f	12	e		n	n	5,28	0,86	4,03	2,57	n	n	n	70th
1435	L440	f	7	e		n	n	4,19	1,33	0,79	2,5	n	n	n	20th
1236	L112	m	35	u		u	n	7,13	0,93	1,17	5,66	n	n	y	>80th
1246	L160	m	74	l			y	6,47	0,76	2,42	4,6	y	n	n	>50th
1249	L166	m	68	e			n	4,96	0,97	0,76	3,64	u	n	u	10th
1291	L080	m	51	e		n	n	7,69	1,06	1,38	6,0	y	n	n	>80th
1219	L082	f	22	e			n	7,34	1,21	0,61	5,85	y	n	n	>95th
1220	L083	m	12	e			n	4,15	1,4	0,66	2,45	n	n	n	40th
1221	L084	f	30	e			n	5,41	1,51	1,16	3,37	n	n	n	>50th
1222	L089	f	45	n			n	6,07	1,34	0,98	4,28	n	n	n	>30th
1223	L090	f	29	e			n	7,49	0,91	0,8	6,21	y	n	n	>90th
1291	L212	m	73	e		n	y	10,8	1,81	1,16	8,46	n	n	y	>95th
1292	L094	m	59	n		y	y	10,19	1,3	0,67	8,58	y	n	n	>95th
1229	L117	f	18	n			n	4,25	1,68	0,58	2,3	n	n	n	>20th
1230	L131	f	24	n			n	4,8	1,23	0,54	3,32	n	n	n	50th
1231	L132	f	26	n			n	3,73	1,49	0,42	2,05	n	n	n	<5th
1293	L147	f	56	n		y	n	11,6	1,8	1,8	8,97	y	n	n	>95th
1315	L141	f	60	l		y	n	5,43	1,15	1,44	3,62	y	n	n	>5th
1328	L250	m	40	e		n	y	7,84	1,34	1,61	5,76	n	y	n	>90th
1331	L252	f	38	n		n	n	5,38	1,55	0,84	3,44	n	n	n	>30th
1329	L249	f	13	e		n	n	7,81	1,23	1,27	6,0	n	n	y	>95th
1330	L251	m	10	e		n	n	5,95	1,69	1,4	3,62	n	n	n	>90th
1386	L213	f	47	e	y	y	n	12,0	1,22	0,76	10,43	n	y	n	>95th
1387	L214	m	10	e		n	n	6,11	1,0	0,37	4,94	n	y	n	>95th
1388	L215	m	17	e		n	n	8,09	1,31	0,74	6,44	n	y	n	>95th
1400	L305	m	33	e	y	y	y	6,26	1,32	1,35	4,32	y	n	n	>70th
1401	L306	f	12	e		n	n	9,16	1,45	1,56	6,99	y	n	n	>95th
1402	L307	f	32	n		n	n	5,14	1,27	0,8	3,5	n	n	n	>40th
1403	L308	f	7	e		n	n	6,59	1,26	0,71	5,0	y	n	n	>95th
1404	L352	f	3	e		n	n	7,1	1,81	0,68	4,98	n	n	n	>95th
1405	L353	f	25	e		n	n	4,5	1,41	1,13	2,57	n	n	n	>10th
1406	L354	f	36	e		y	n	9,5	1,39	0,82	7,73	y	n	n	>95th
1407	L355	f	11	e		n	n	5,05	1,94	0,72	2,78	n	n	n	>60th
1408	L356	m	9	e		n	n	7,06	2,18	0,58	4,61	y	n	n	>95th
1409	L357	m	17	e		n	n	8,0	1,79	0,8	5,84	y	n	n	>95th
1410	L332	m	35	e		y	n	12,07	1,27	2,73	9,55	n	y	n	>95th
1411	L333	f	35	n		n	n	5,03	1,8	0,72	2,9	n	n	n	>20th
1412	L334	m	8	e		n	n	7,07	1,84	0,57	4,97	n	y	n	>95th
1413	L335	m	5	e		n	n	7,15	1,48	0,89	5,26	n	y	n	>95th
1418	L411	f	57	e		y	y	8,1	1,35	3,38	5,2	n	y	n	80th
1414	L341	m	46	n		n	n	7,59	1,74	1,11	5,34	n	n	n	>80th

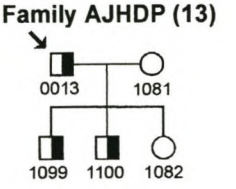
1415	L342	f	44	e		y	n	8,3	0,99	1,49	6,63	n	y	n	>95th
1416	L343	f	7	e		n	n	9,94	1,48	1,11	7,95	n	y	n	>95th
1417	L362	m	53	e		n	y	5,31	1,03	0,97	3,84	n	n	n	>20th
1419	L412	m	58	n		n	n	4,41	0,62	1,5	3,1	n	n	n	<5th
1420	L413	f	33	e		n	n	5,66	0,73	2,93	3,59	n	n	n	60th
1421	L414	f	30	e		n	n	6,03	1,15	2,19	3,88	n	n	n	>70th
1422	L416	f	47	e		n	y	9,79	1,13	1,33	8,05	n	y	n	>95th
1423	L417	f	23	e		n	n	9,62	0,89	2,47	7,6	n	y	n	>95th
1424	L435	m	84	e		n	n	5,51	0,58	2,25	3,9	n	y	n	>20th
1425	L367	f	8	e		n	n	5,6	1,18	0,67	4,11	y	n	n	>80th
1426	L368	f	30	I		n	n	5,87	1,7	0,63	3,88	n	n	n	>60th
1436	L451	f	37	e		y	n	8,41	0,49	1,18	7,38	y	n	n	>95th

ID = Identity; L numbers represent patients followed-up for genotype/phenotype correlation studies performed by Lingenhel et al., 1998; F.H. = family history; CHD = coronary heart disease; act = arcus corneae; xant = xanthomata; TC = total cholesterol; HDLC = High density lipoprotein cholesterol; TG = Triglyceride; LDLC = Low density lipoprotein cholesterol;
Percentiles according to Coris TC levels of 1979; Index cases are highlighted with relatives below.

APPENDIX 2


Family PPS (1)
0650
0859
0618
0614
0801
0648
0591
0651
0654
0860
0617
0649
0652
0653
0001
0002
0003
0005
0004


Family LGA (12)
0723
0311
0310
0318
0361
0823
0012
0251
0258
0274
0255
0312
0722
0824
0825
0253
0252


Family AJHDP (13)
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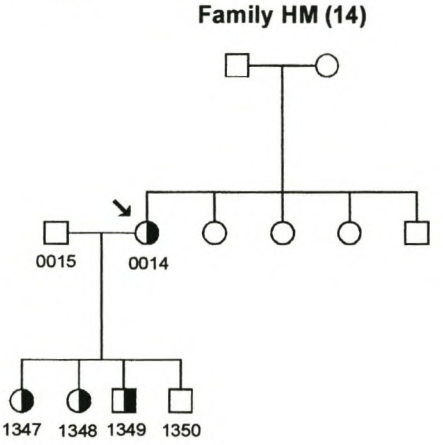
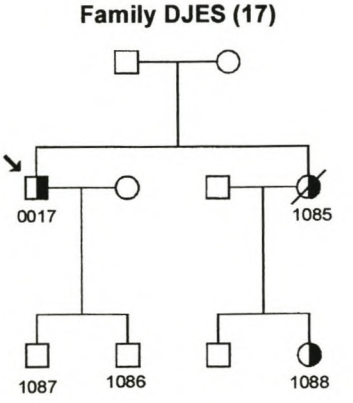
1081
1099
1100
1082


Family HM (14)
0015
0014
1347
1348
1349
1350


Family DJES (17)
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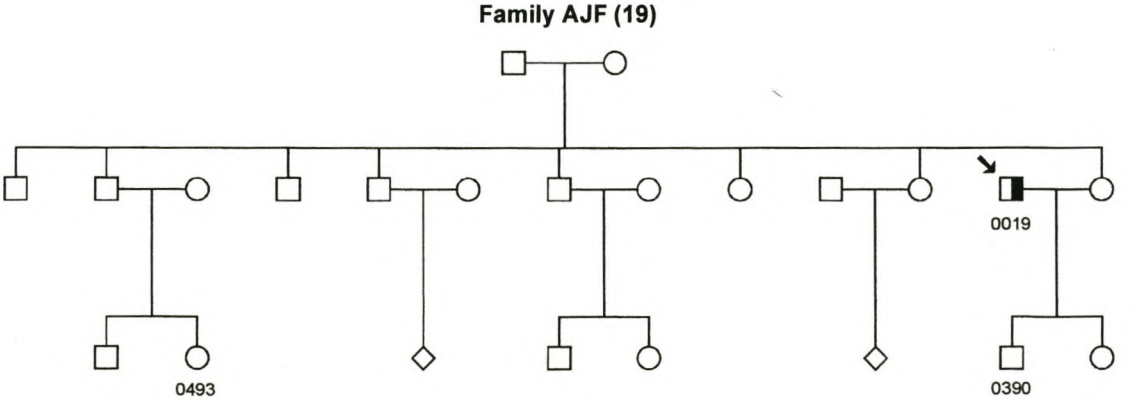
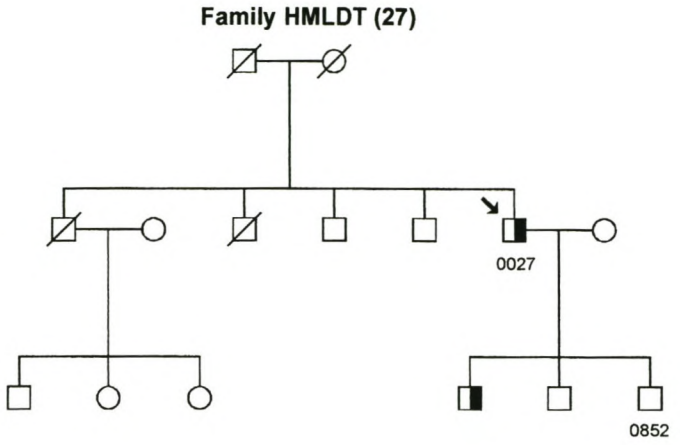
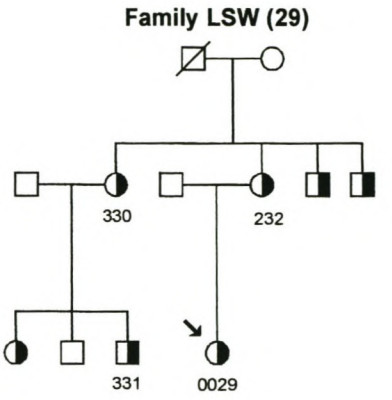
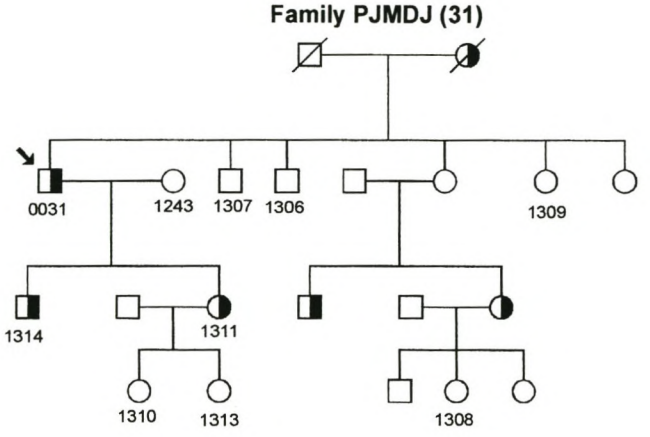
1085
1087
1086
1088


Family AJF (19)
0019
0493
0390


Family HMLDT (27)
0027
0852


Family LSW (29)
330
232
331
0029


Family PJMDJ (31)
0031
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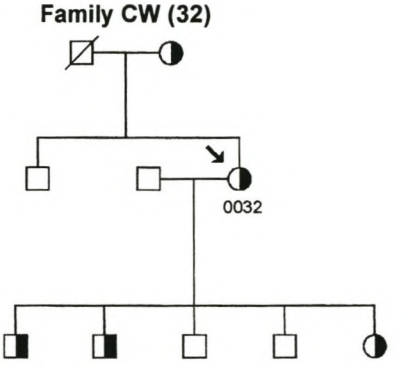
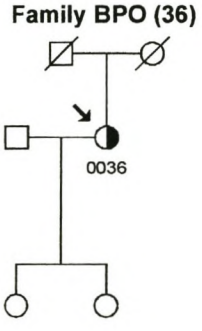
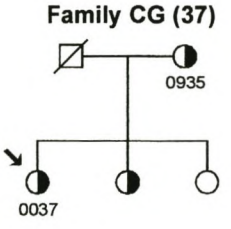
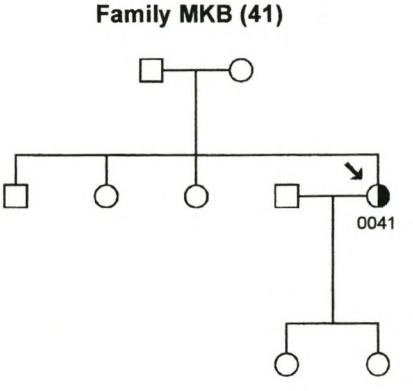
1307
1306
1309
1314
1311
1310
1313
1308


Family CW (32)
0032


Family BPO (36)
0036


Family CG (37)
0935
0037


Family MKB (41)
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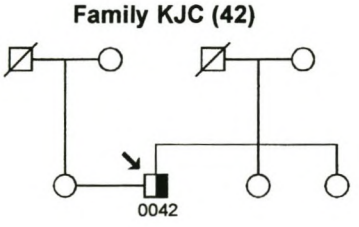
Family KJC (42)
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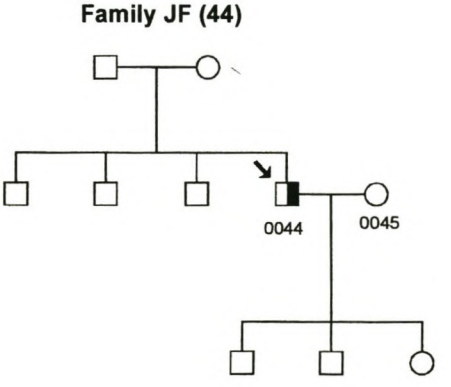
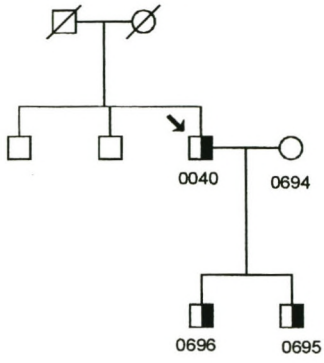
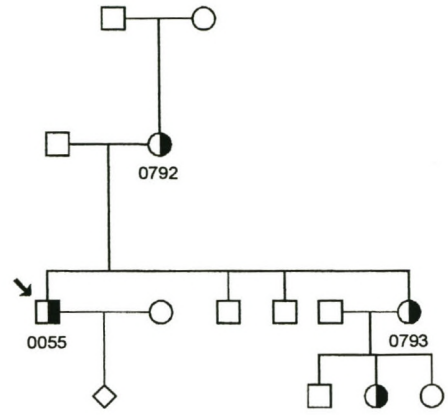

Family JF (44)
0044
0045

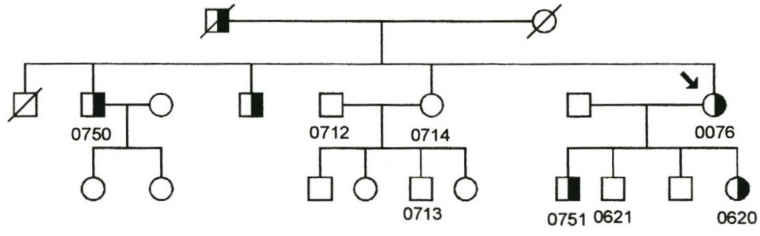
Family 40 (GWB)



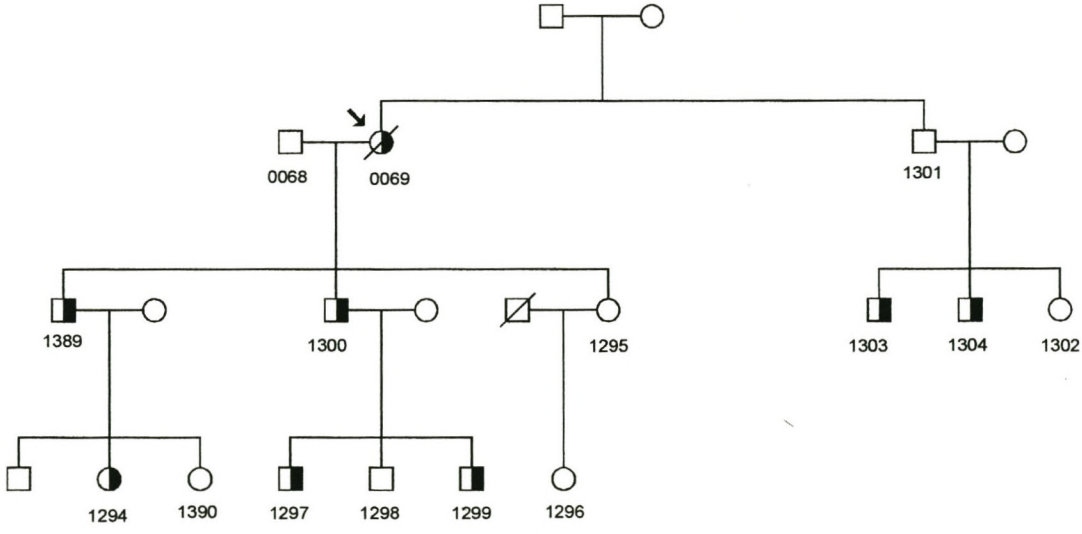
Family 55 (MJVDW)

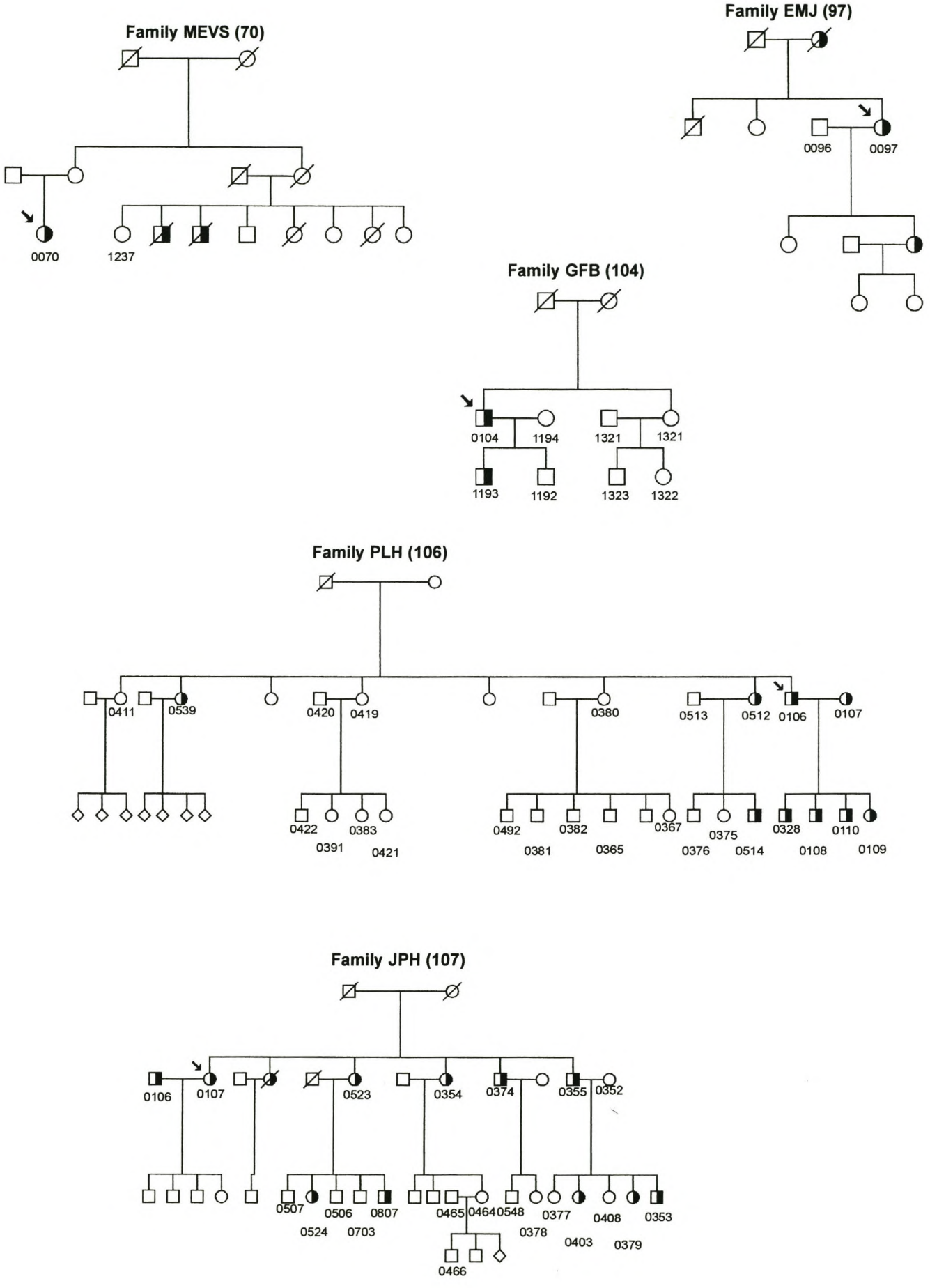


Family LMS (76)

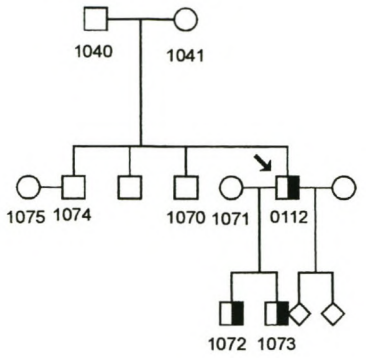


Family SMM (69)

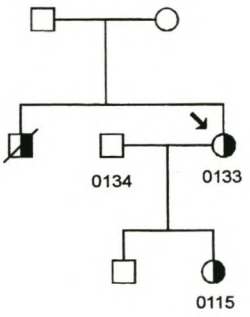



Family MEVS (70)
0070
1237
Family EMJ (97)
0096
0097
Family GFB (104)
0104
1194
1321
1321
1193
1192
1323
1322
Family PLH (106)
0411
0539
0420
0419
0380
0513
0512
0106
0107
0422
0383
0391
0421
0492
0382
0367
0375
0381
0365
0376
0514
0328
0110
0108
0109
Family JPH (107)
0106
0107
0523
0354
0374
0355
0352
0507
0506
0807
0524
0703
0465
0464
0548
0377
0408
0353
0378
0403
0379
0466

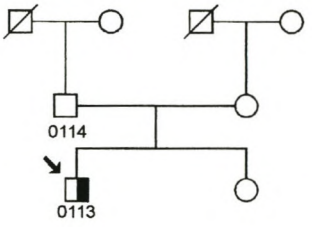
Family DHC (112)



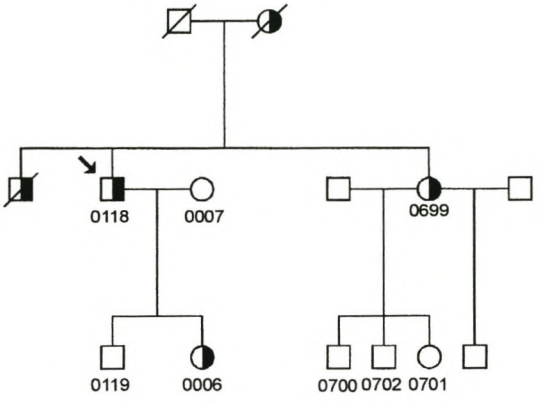
Family HW (133)



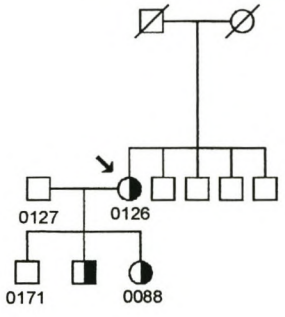
Family CJV (113)



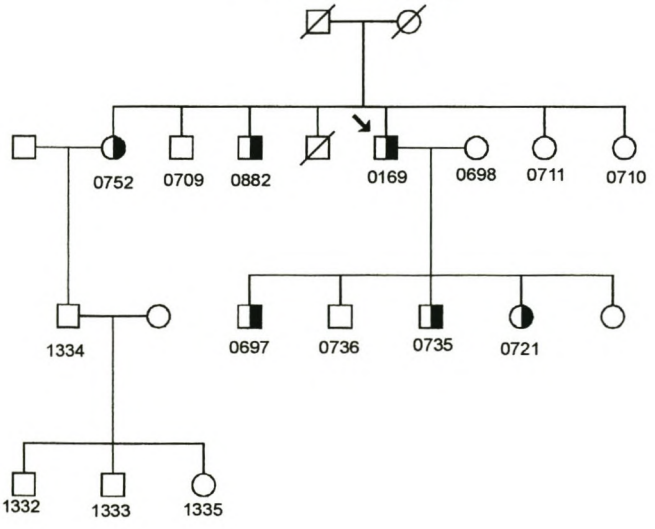
Family CAM (118)



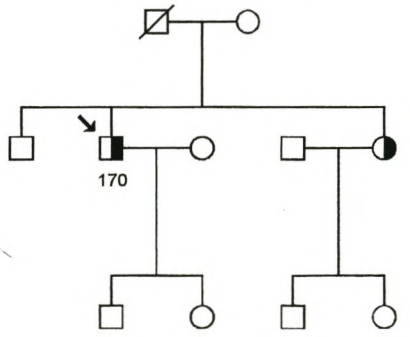
Family HJS (126)

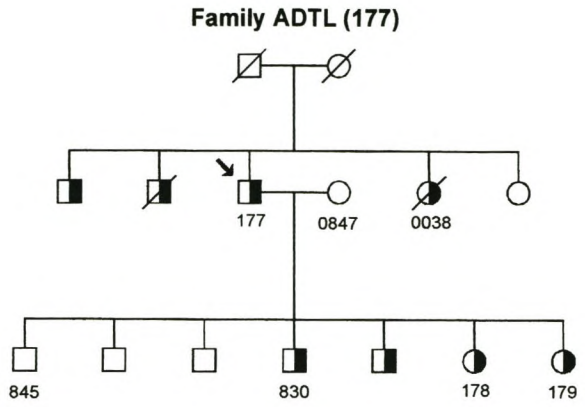


Family FAJ (169)



Family JPV (170)




Family ADTL (177)
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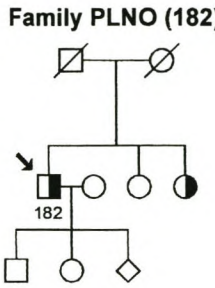
179


Family PLNO (182)
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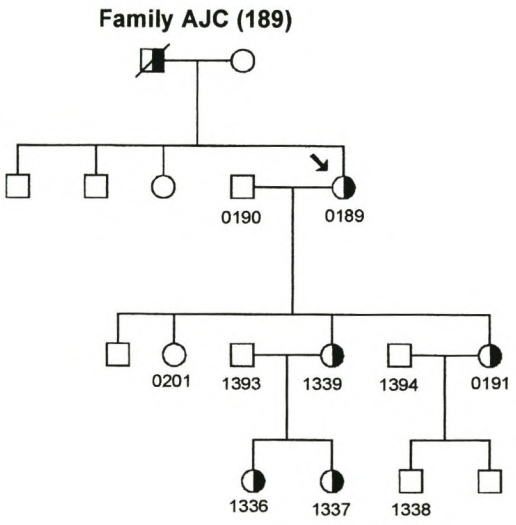
Family AJC (189)
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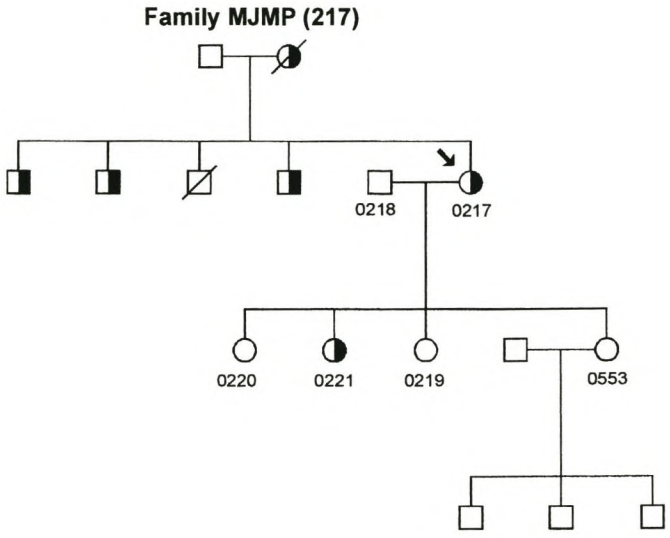
Family MJMP (217)
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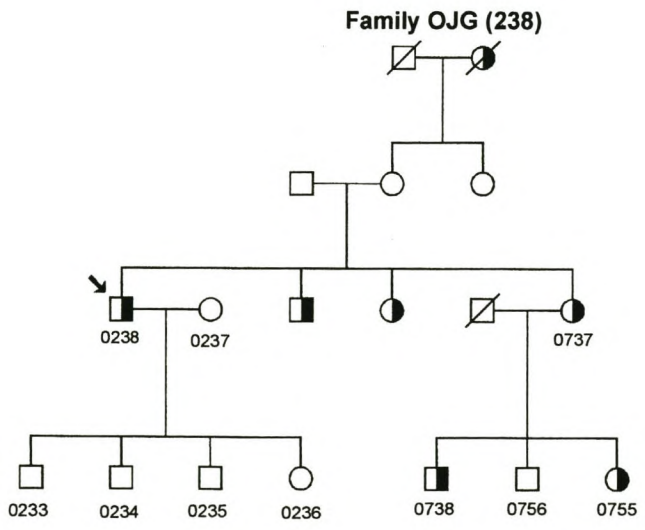
Family OJG (238)
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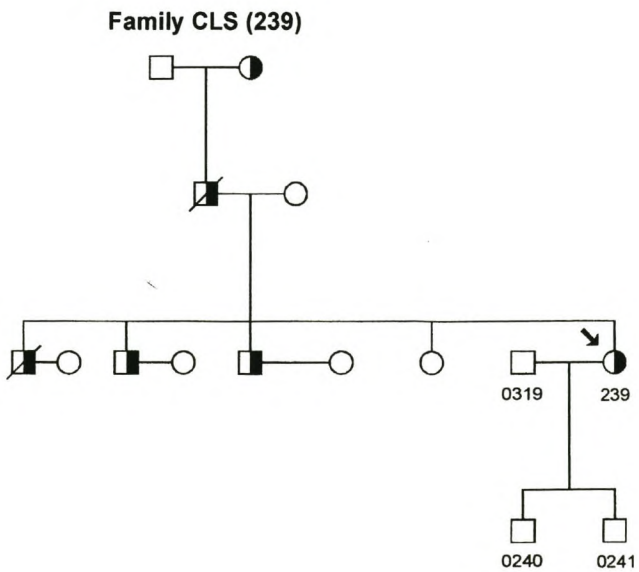
Family CLS (239)
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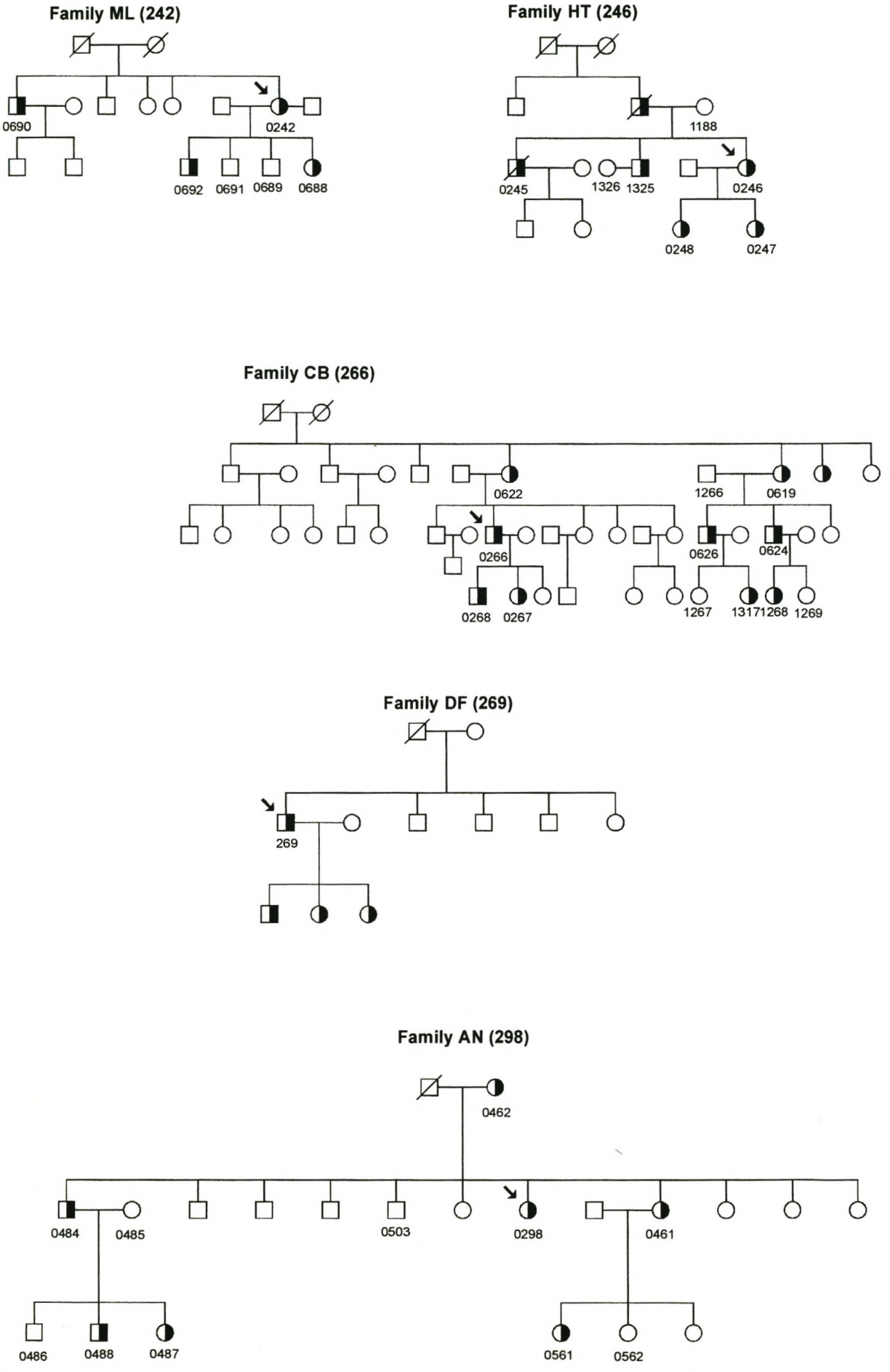
Family ML (242)
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0688
Family HT (246)
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0248
0247
Family CB (266)
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0267
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13171268
1269
Family DF (269)
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Family AN (298)
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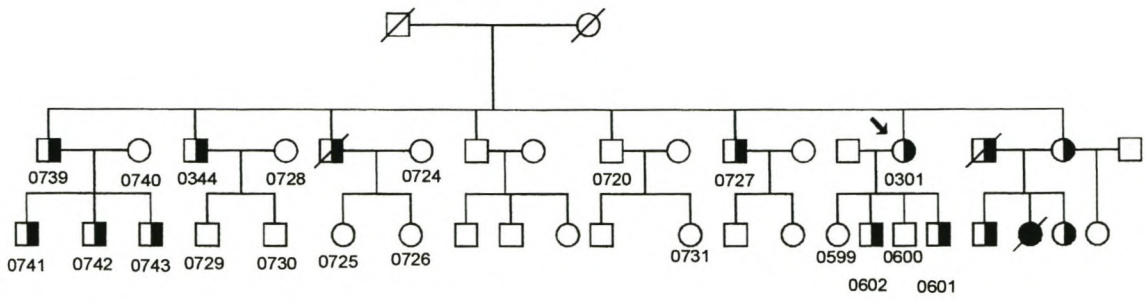
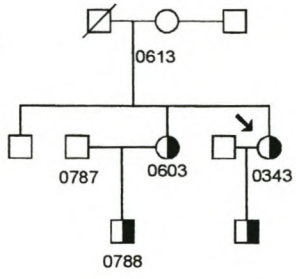
0562

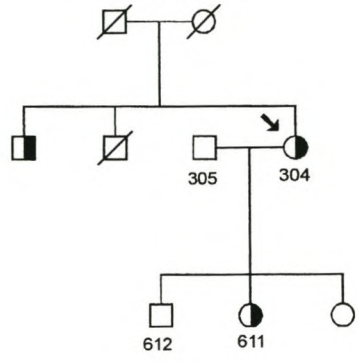
Family SDV (301)



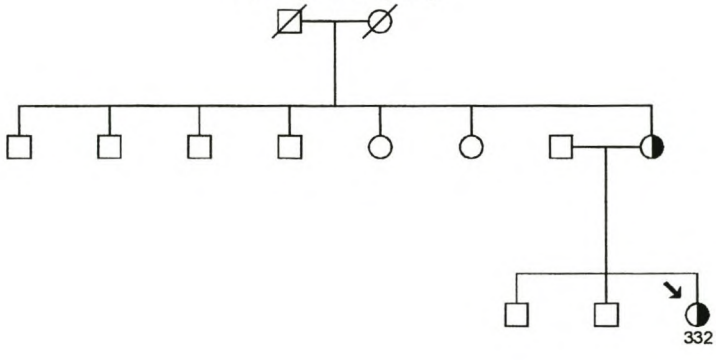
Family LD (343)



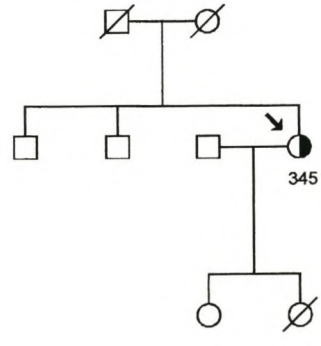
Family WT (304)



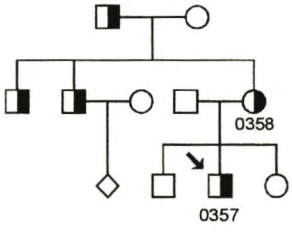
Family AWV (332)



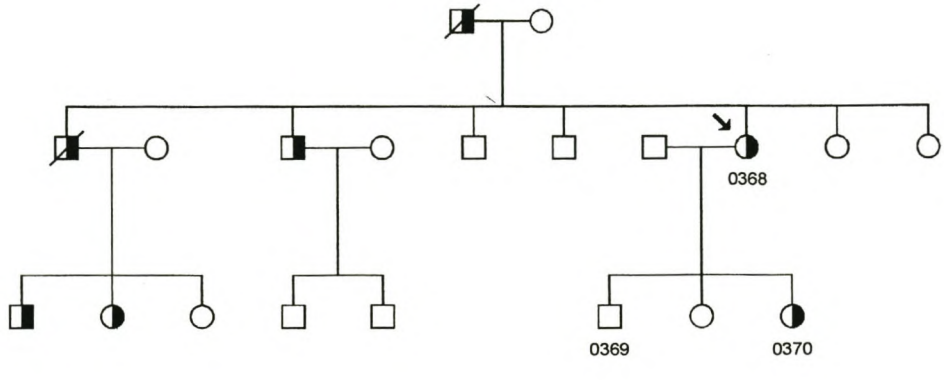
Family CP (345)



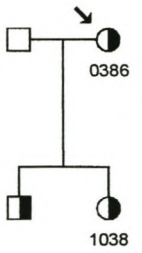
Family JP (357)



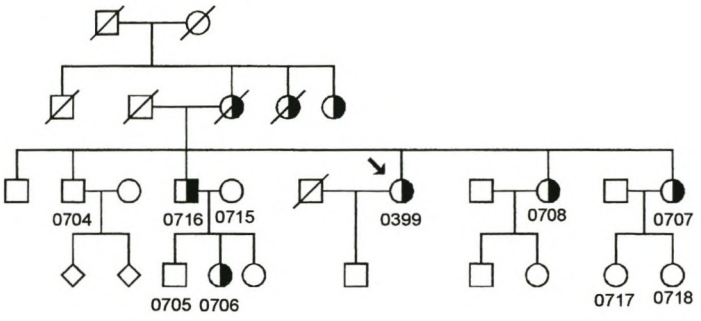
Family AFD (368)



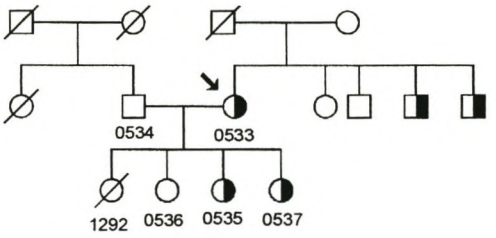
Family HM (386)



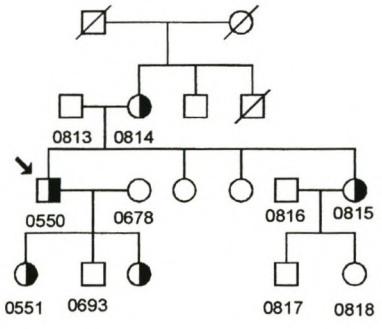
Family BK (399)



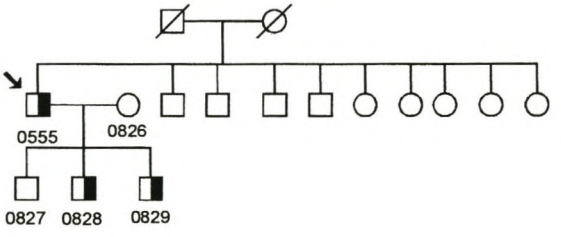
Family CJVC (533)



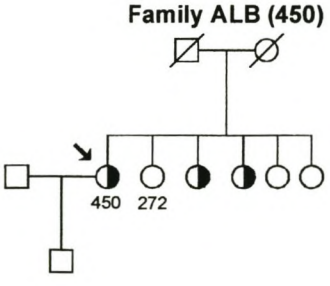
Family SGJ (550)



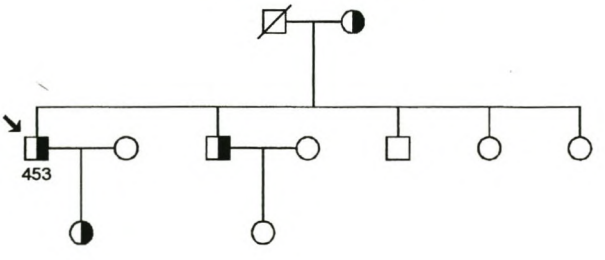
Family MLS (555)

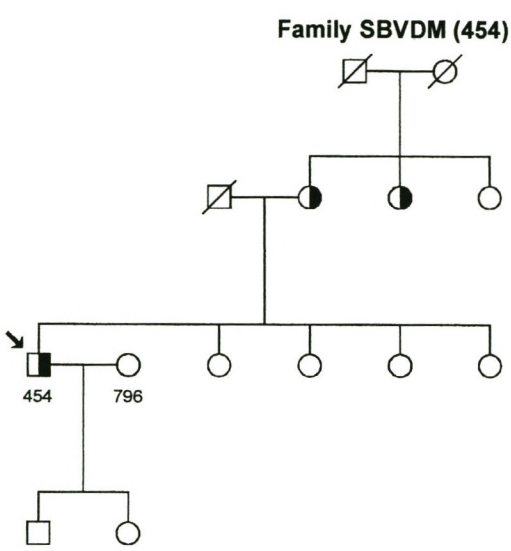


Family ALB (450)



Family AEHDV (453)




Family SBVDM (454)
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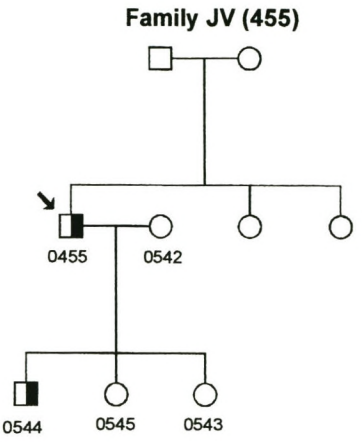
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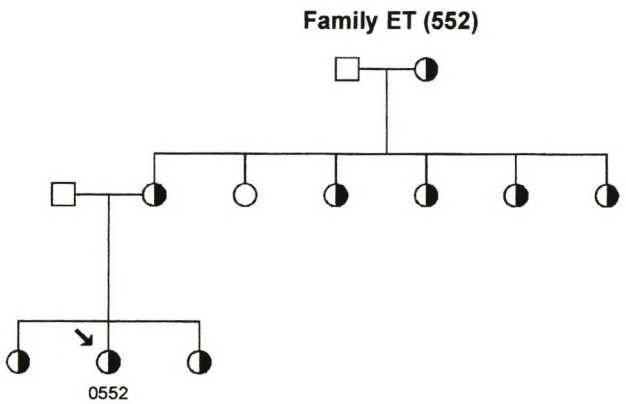
Family ET (552)
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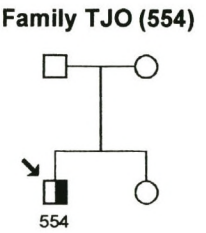
Family TJO (554)
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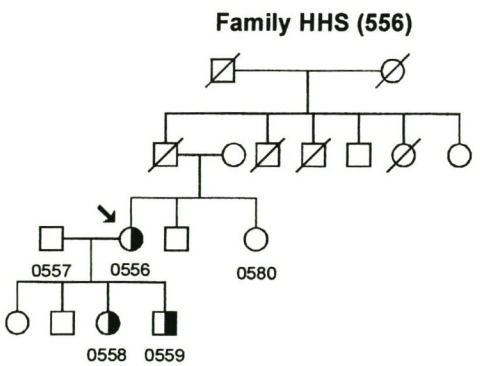
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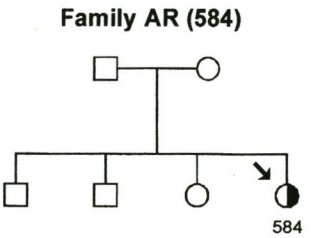
Family AR (584)
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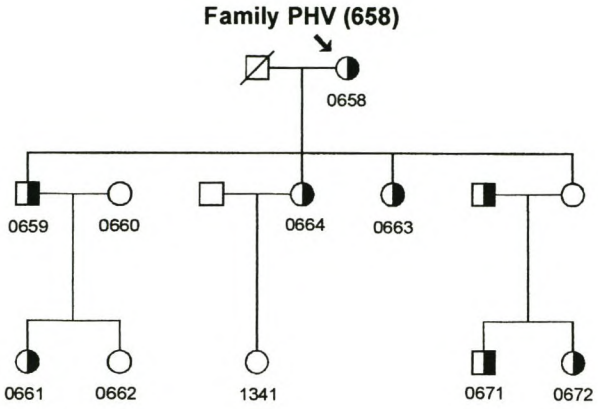
Family PHV (658)
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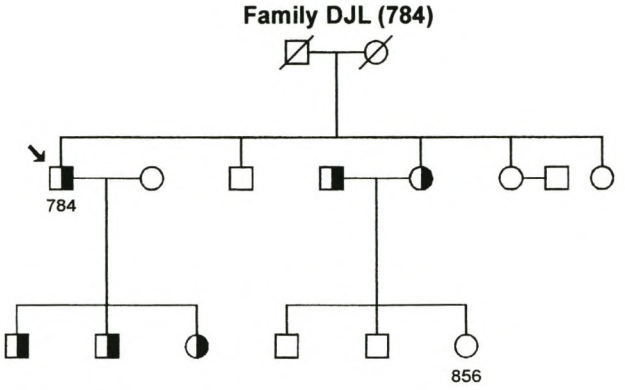
Family DJL (784)
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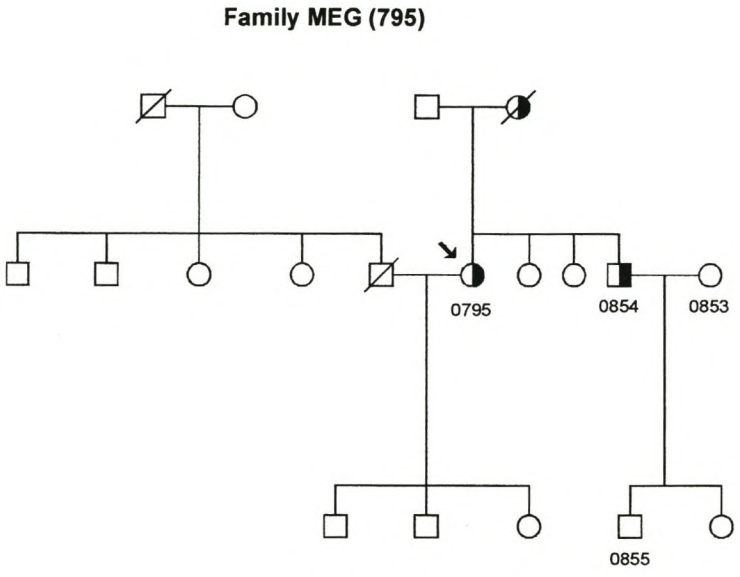
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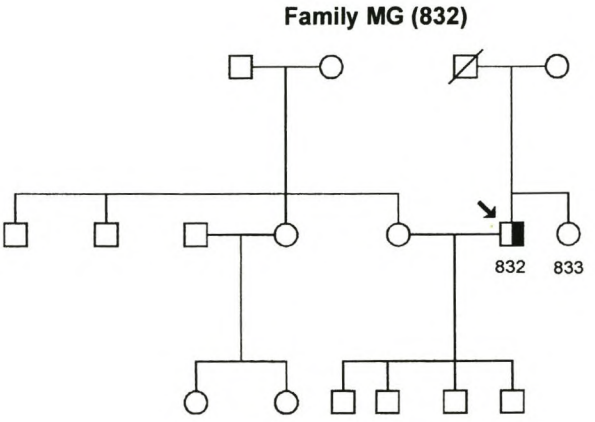
Family MG (832)
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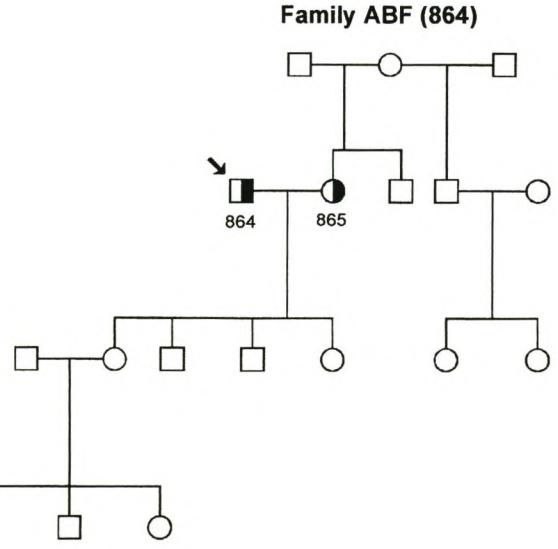
Family ABF (864)
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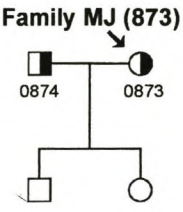
Family MJ (873)
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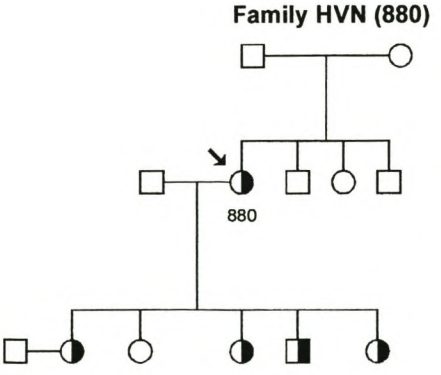
Family HVN (880)
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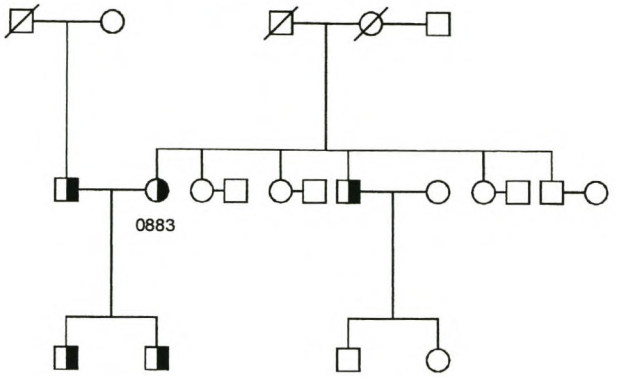
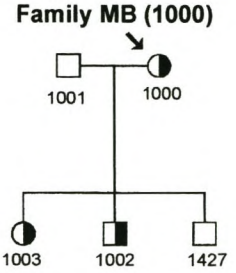
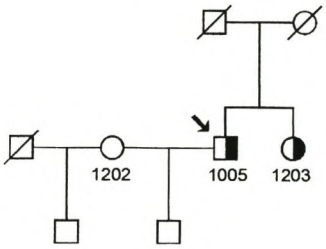
Family MSEW (883)



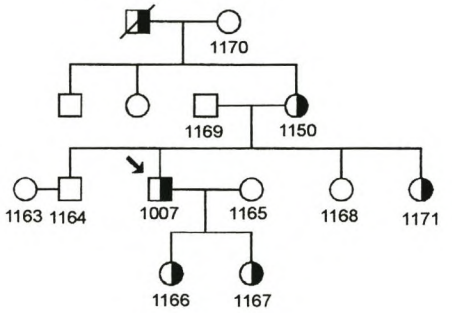
Family MB (1000)



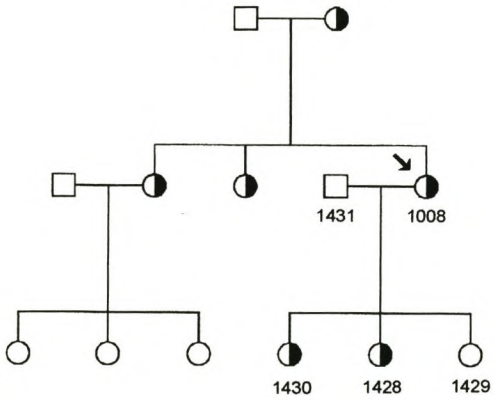
Family GE (1005)



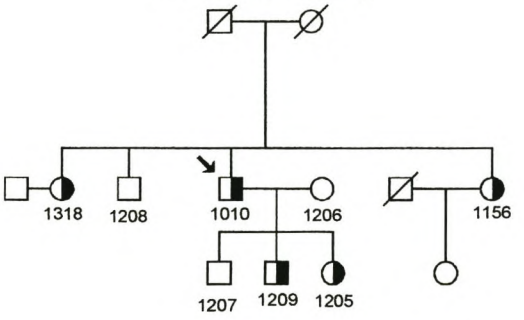
Family PB (1007)



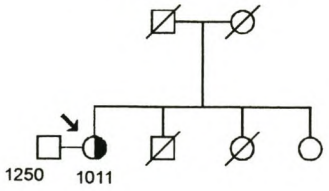
Family LD (1008)



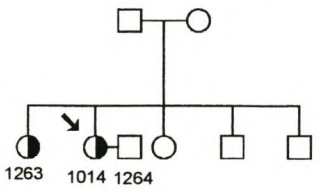
Family JJPV (1010)



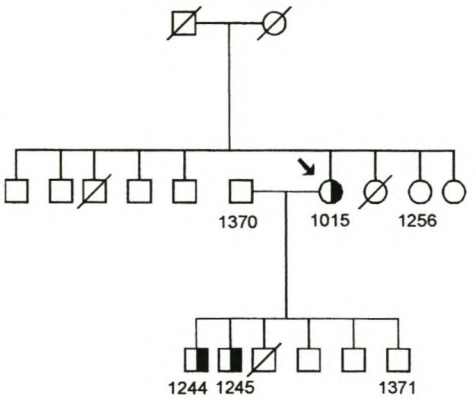
Family JG (1011)



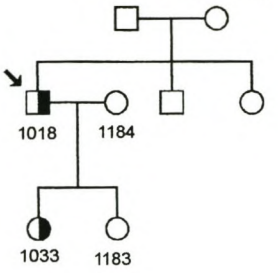
Family JGSC (1014)



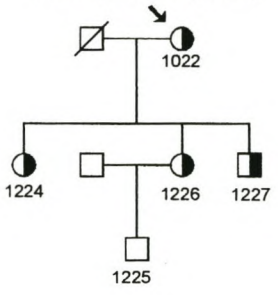
Family HCG (1015)



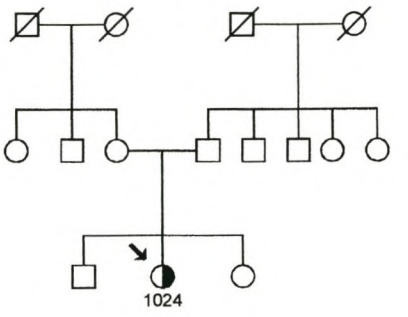
Family LM (1018)



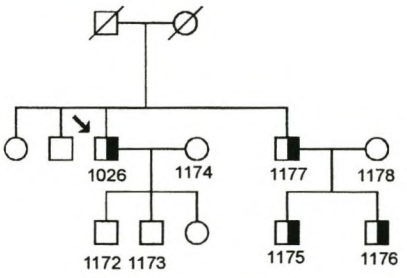
Family SCVN (1022)



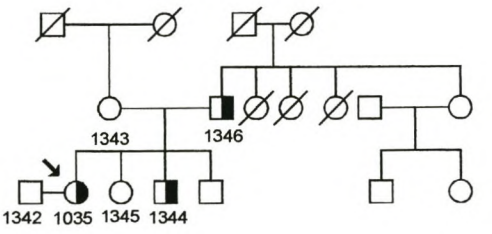
Family AB (1024)



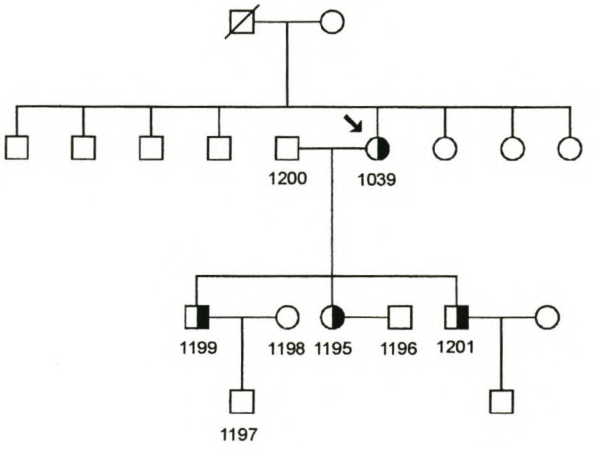
Family FB (1026)



Family CL (1035)



Family ADW (1039)



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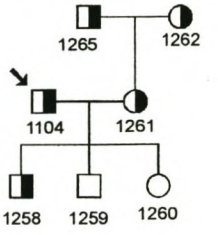

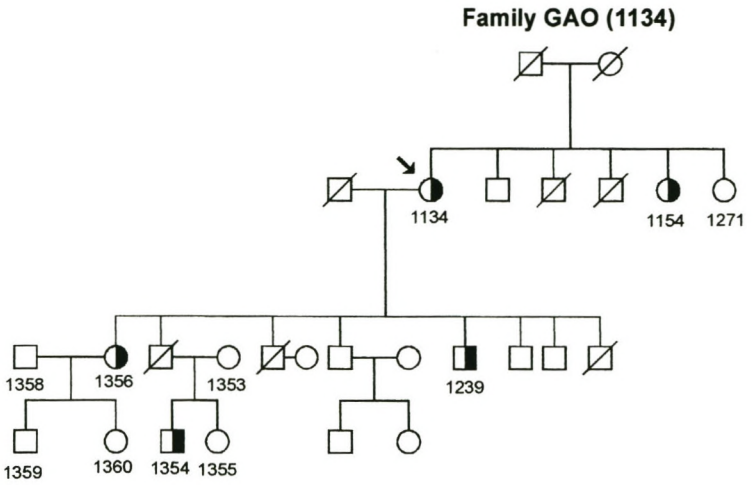
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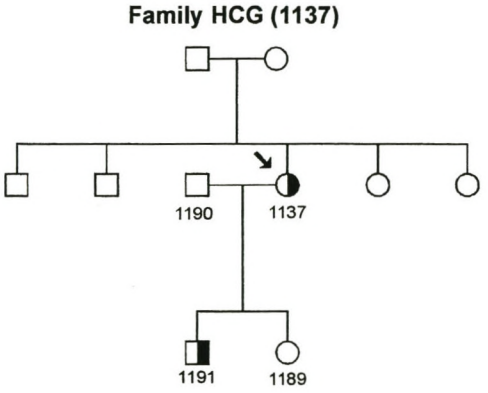
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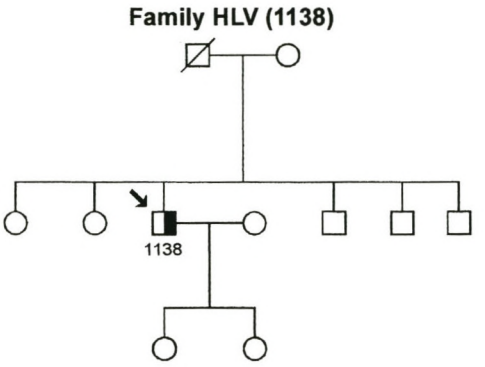
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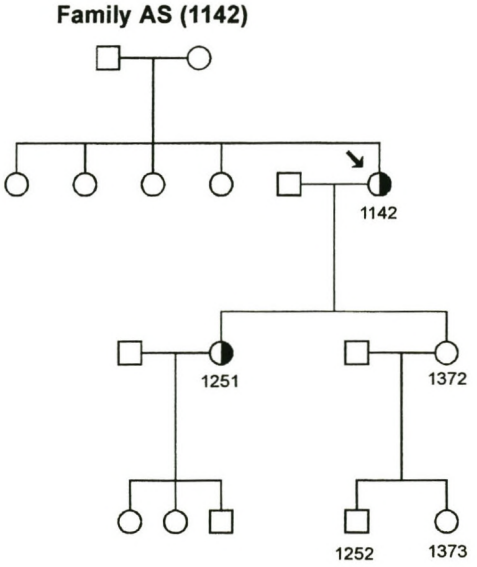
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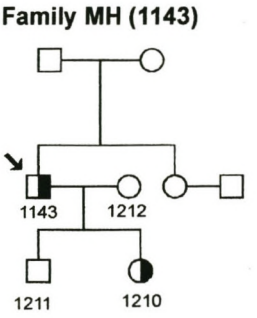
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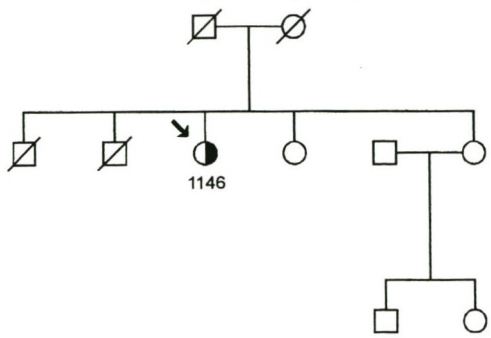

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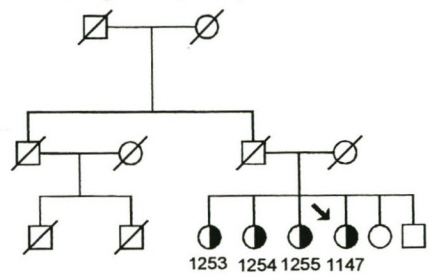

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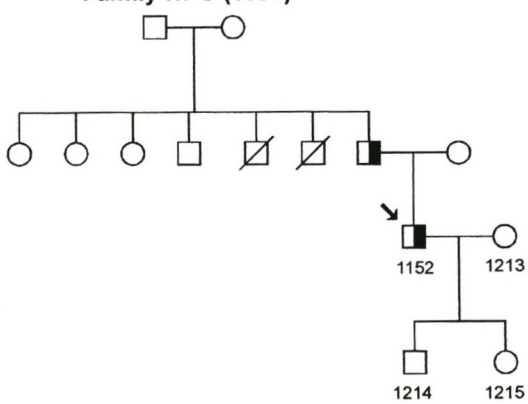

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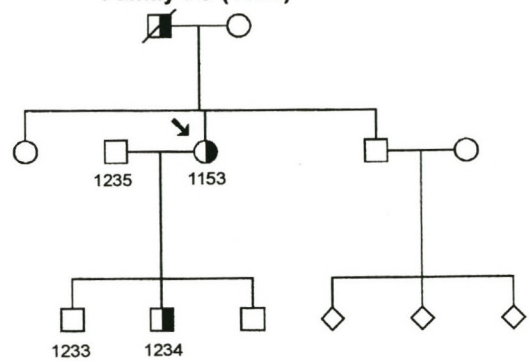

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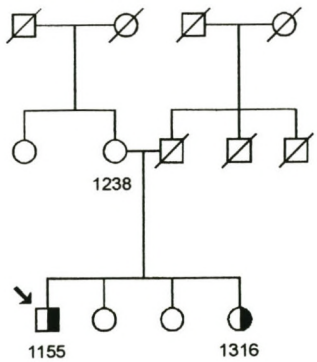

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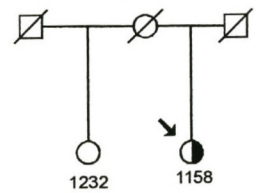

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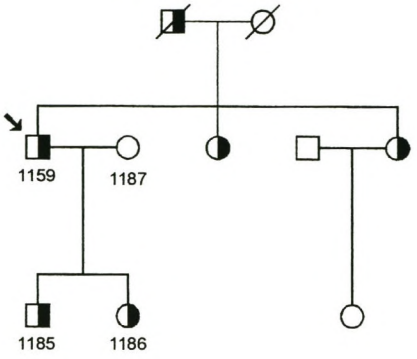

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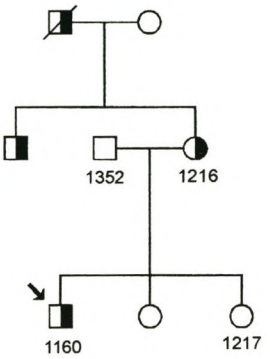

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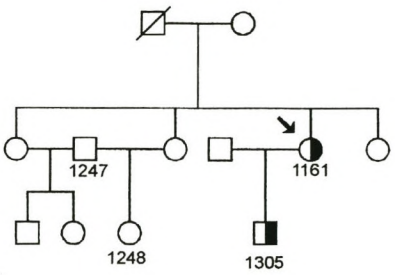

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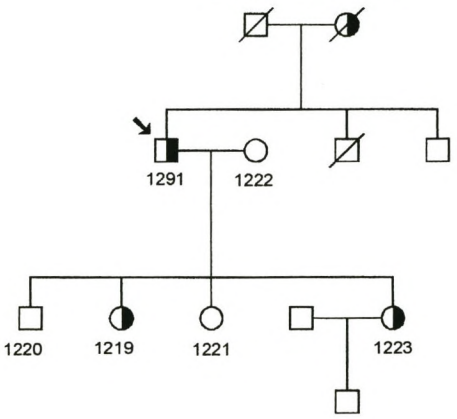

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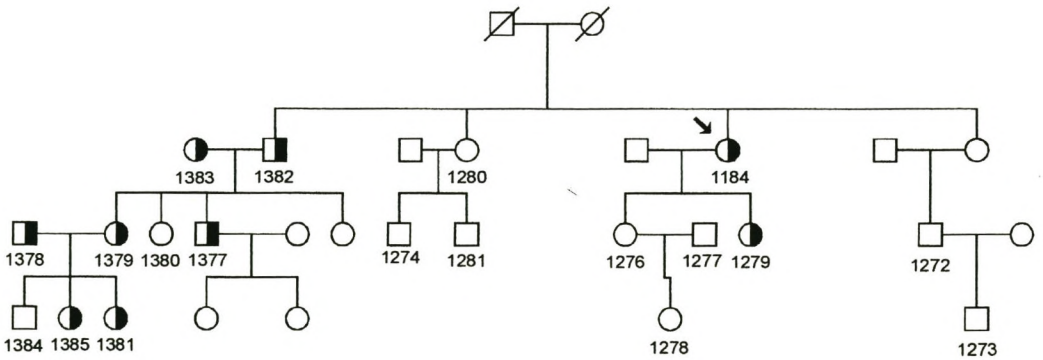

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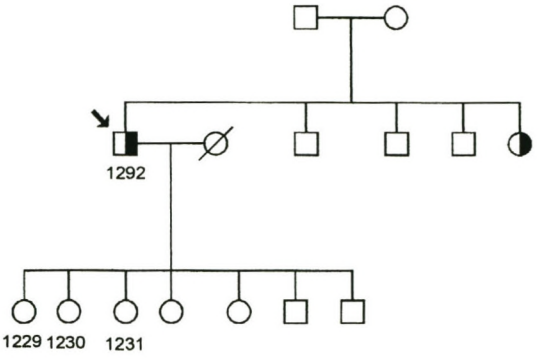

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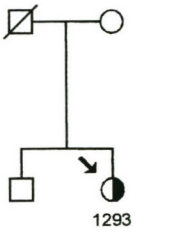

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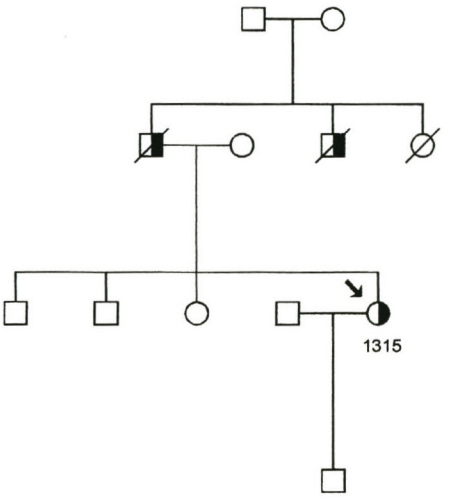

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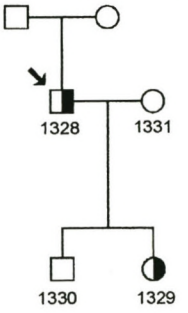

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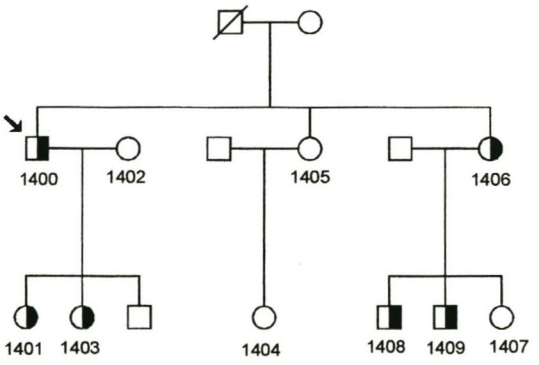

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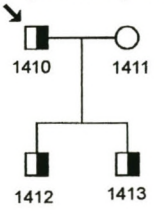

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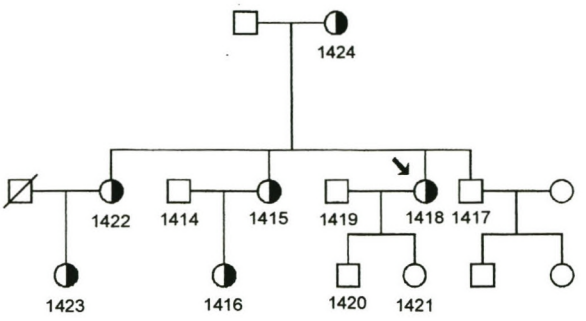

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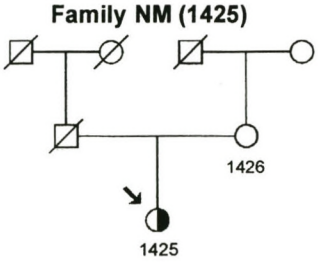

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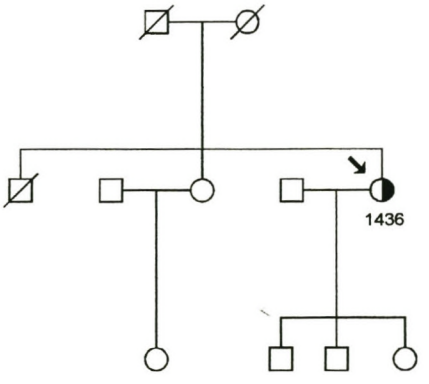

APPENDIX 3

Predominance of a 6-bp deletion in exon 2 of the LDL receptor gene in Africans with familial hypercholesterolaemia

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Journal of Medical Genetics

Abstract

In South Africa the high prevalence of familial hypercholesterolaemia (FH) among Afrikaners, Jews and Indians due to founder genes is in striking contrast to its reported virtual absence in the Black population in general. In this study the molecular basis of primary hypercholesterolaemia was studied in 16 Africans diagnosed with FH. DNA analysis using three screening methods resulted in the identification of seven different mutations in the coding region of the low-density lipoprotein (LDLR) gene in 10 of the patients analysed. These included a 6-bp deletion (GCGATG) accounting for 28% of defective alleles, and six point mutations (D151H, R232W, R385Q, E387K, P678L, R793Q) detected in single families. The Sotho patient with missense mutation R232W was also heterozygous for a *de novo* splicing defect 313+1G→A. Several silent mutations/polymorphisms were detected in the LDLR and apolipoprotein B genes, including a base change (g→t) at nucleotide position -175 in the FP2 LDLR regulatory element. This promoter variant was detected at a significantly higher frequency ($P<0.05$) in FH patients compared to controls, and occurred *in cis* with mutation E387K in one family. Analysis of four intragenic LDLR gene polymorphisms demonstrated that the same chromosomal background was identified at this locus in the four FH patients with the 6-bp deletion. Detection of the 6-bp deletion in Xhosa, Pedi and Tswana FH patients suggests that it is an ancient mutation predating tribal separation approximately 3000 years ago.

Introduction

Autosomal dominant hypercholesterolaemia (ADH) is most commonly caused by mutations in the low-density lipoprotein receptor (LDLR) gene causing familial hypercholesterolaemia (FH), or in the apolipoprotein B (apo B) gene causing familial defective apo B (FDB).[1, 2] These biochemical defects result in the precipitation of excess cholesterol, and clinical characteristics include tendon xanthomata and premature coronary heart disease (CHD). The estimated incidence of both FH and FDB is approximately 1 in 500 in most Caucasian populations.

In the Afrikaner population of South Africa, the prevalence of FH has been increased to approximately 1 in 70, as a consequence of a founder effect following the introduction of at least three defective LDLR gene alleles by European settlers.[3-5] This is in striking contrast to the apparently low prevalence of FH in the Black population, reported to have migrated from Central Africa to the South in three main groups, the Nguni's (Xhosa, Tembu, Swazi and Zulu) along the east coast, the Sotho's (South Sotho, North Sotho/Pedi, West Sotho/Tswana) who settled further west on the Transvaal highveld, and the Venda's living in the Northern Transvaal area.[6, 7] We suspect that FH is not frequently recognised in Africans due to altered clinical expression, and not because of a lower mutation prevalence compared to most other populations. Previous studies have indicated that the mutational mechanisms giving rise to germ-line mutations is largely a function of the local DNA sequence environment.[8-10]

Since the situation in South Africa is ideal for studies of underlying lipid-related genetic differences among population groups,[11] we attempted to identify Black hypercholesterolaemics to determine the spectrum of mutations in the promoter and coding region of the LDLR gene and in exon 26 of the apoB gene. FDB has not previously been studied in the South African Black population, but was found to be rare in other South African populations, most likely due to a "negative" founder effect that diluted the frequency of the common apo B3500 mutation in the immigrants relative to their parent populations.[12]

Subjects and Methods

Subjects

Blood samples were collected from 56 Black patients attending lipid clinics in South Africa, after obtaining informed consent and ethical approval by the regional Review Committees. Details on clinical features and ethnicity were provided by the referring clinicians. Sixteen patients with a diagnosis of "classical" or "probable" FH, including two FH homozygotes, were selected for extensive mutation analysis for the coding and promoter region of the LDLR gene and exon 26 of the apo B gene. Blood samples were also obtained from 38 of their family members (table 1). Classical FH (12 probands) was defined as the occurrence of pretreatment total cholesterol (TC) >7 mmol/l, with the presence of tendon xanthomata and/or premature CHD in the index case or a first-degree relative. Probable FH (4 probands) was defined by the same pretreatment cholesterol level and primary hypercholesterolaemia and/or

premature CHD in the family (table 1). DNA samples of the 40 lipid clinic patients without the FH phenotype, but who had hyperlipidaemia or normal lipid profiles in the presence of vascular disease, were included for analysis of specific regions of the LDLR gene. Ninety-six individuals drawn from the same population (19 Pedi's, 21 Sotho's, 27 Xhosa's, 29 Zulu's) were sampled as controls. TC, high-density lipoprotein cholesterol (HDLC) and triglyceride (TG) determinations and extraction of genomic DNA were performed using standard methods[13] Plasma LDL-cholesterol (LDLC) concentrations were calculated with the Friedewald formula [LDLC=TC-(HDL+TG/2.18)].[14]

Mutation detection

Heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis was performed in South Africa,[15] and denaturing gradient gel electrophoresis (DGGE) in Denmark[16] and Scotland,[17] to screen polymerase chain reaction (PCR)-amplified genomic DNA for mutations in the LDLR and apo B genes. For HEX-SSCP analysis, the exon-specific primers described by Jensen et al.[18] were used, while the promoter region of the LDLR gene was amplified using primers 5'-GAGGCAGAGAGGACAATGGC-3' and 5'-CCACGTCATTTACAGCATTTCAATG -3'. Base changes in the promoter region were numbered according to Hobbs et al.[19] after adding an additional A within the AAAA stretch preceding repeat 1, which is missing from the published sequence.[20] PCR products showing aberrant electrophoresis patterns were sequenced on both strands with a PCR Product Sequencing kit (Amersham) and/or an automated sequencer ABI 373A.

Haplotype analysis

Haplotype analysis using four LDLR gene polymorphisms were performed according to Theart et al.[21] Microsatellite markers VWA31, F1A1 and TH01 (Profiler kit, Applied Biosystems) were used to test for biological consistency in two families.

Statistical analysis

Allele frequencies were determined by allele counting. Testing for significance of heterogeneity in mutation frequencies among patient and control groups was based on the Chi-square and Fisher's exact tests.

Results

Extensive DNA screening of the LDLR gene in 16 Black FH patients, using both the DGGE and HEX-SSCP screening methods, revealed 6 missense mutations in individual families and a 6-bp deletion in four probands (table 1).[22] The deletion (FH Cape Town-1), previously described in a Xhosa FH homozygote,[23] and missense mutations D151H and R385Q have not (yet) been reported in other populations. Haplotype SmaI+/StuI+/AvaII- was associated with the deletion in all three FH heterozygotes and a homoallelic FH homozygote. Screening of the coding region in DNA of the four FH patients heterozygous for a base change (g→t) at nucleotide position -175 of the LDLR gene promoter, resulted in the detection of a recycling-

deficient mutation E387K[19] in the DNA of subject EF. Interestingly, this Pedi proband was found to be extremely heterogeneous at the DNA level, since a silent C to T base change was furthermore detected at nucleotide position 1104 in exon 8, in addition to two silent mutations in the apo B gene. The G to C change in the third base of codon 3540 (T3540T) and the T to C change in the third base of codon 3552 (T3552T) in the apo B gene have previously been reported in Nigerian and African American subjects, respectively[17] One of the daughters of proband EF (II-3 in fig. 1) carried two copies of the silent apo B mutation at codon 3540. RFLP analysis indicated that haplotype SmaI+/StuI+/AvaII-/NcoI+ co-segregated with the -175t allele in the family (fig. 1). This chromosomal background was also identified in two of the other probands with the sequence substitution at -175 in the LDLR promoter region, while haplotype SmaI-/StuI+/AvaII+/NcoI+ was associated with the t allele in the Tswana proband (LM), who also carried the T3552T variant in the apo B gene.

In order to determine whether the two mutations identified in each of probands EF and SH occur *in cis* or *in trans* on their respective chromosomes, blood samples were obtained from additional family members for segregation analysis. Pedigree analysis in the family of EF demonstrated that mutation E387K and the -175g→t variant occur on the same chromosome (fig.1). All the family members who inherited the 387K/-175t haplotype (I-1, I-2, II-2, II-4, III-2 and III-5) had abnormally high TC and LDLC levels. Individual II-2, with a clinical diagnosis of heterozygous FH, was homozygous for the t allele at nucleotide position -175. This implies that her deceased father (husband of the index case) also carried the -175g→t promoter variant, but in the absence of mutation E387K. Her normocholesterolaemic son (III-

1), as well as her brother inherited this paternal chromosome, the latter presenting with a moderately raised TC value. The proband's son (II-1) and one of her daughters (II-3) (confirmed by marker studies using highly informative microsatellites) had moderately raised plasma cholesterol concentrations in the absence of either the promoter variant or the exonic mutation, indicating that another unknown factor contributes to the abnormal lipid profile observed in this family. TC concentrations were found to be very low in the general Black population (approximately 3 mmol/l) compared with other South African groups.[24, 25]

DNA screening of the 53-year old father of proband CK, diagnosed with homozygous FH, revealed homozygosity for the t allele at nucleotide position -175. His TC and LDLC levels were 6.11 mmol/l and 4.29 mmol/l, respectively, which is comparable to that of a FH heterozygote. Plasma TG and HDLC concentrations were 1.49 mmol/l and 1.14 mmol/l, respectively, and the only clinical feature indicative of hyperlipidaemia in this obligate FH heterozygote was corneal arcus.

HEX-SSCP analysis indicated that the splicing defect identified in exon 3 represents a *de novo* event in the family of SH, since it was not present in any of his close relatives analysed. Familial relationship was illustrated by transmission of the exon 5 mutation (R232W) from the father (72 years, TC 4.1 mmol/l), and was further substantiated by marker studies using three highly informative microsatellites (data not shown). Mutation R232W was absent in the normocholesterolaemic brother (30 years, TC 3.5 mmol/l) and sister (42 years, TC 3.3 mmol/l) of the proband. Their mother aged 62 years presented with a TC level of 2.9 mmol/l. It was therefore not possible to determine whether the splice mutation occurred *in cis* in the proband on

the paternal chromosome bearing mutation R232W, or *in trans* on the normal maternal chromosome.

Subsequent DNA screening of 96 control individuals from the general Black population comprising 56 Nguni's (27 Xhosa's, 29 Zulu's) and 40 Sotho's (19 Pedi's, 21 Sotho's) resulted in the identification of 6 individuals [4 Nguni's (1 Xhosa, 3 Zulu's) and 2 Sotho's (1 Pedi, 1 Sotho)] heterozygous (6%) for the -175t allele. Although the number of patients analysed is small, the frequency of this allele appeared to be higher within each tribal group (2/6 Nguni's and 2/10 Sotho's with FH) compared to the controls (4/56 Nguni's and 2/40 Sotho's). An overall statistically significant difference ($P<0.05$) was observed between the presence of the rare t allele in the general Black population (0.03) compared to its frequency of 0.13 in the patients diagnosed with classical or probable FH ($\chi^2=5.916$, 1df, $P=0.0149$). We furthermore detected 5 carriers of the -175g→t polymorphism among 40 lipid clinic patients without the FH phenotype (13%), demonstrating an intermediate allele frequency of 0.06. This was not significantly different from the frequencies observed in the FH ($\chi^2=1.326$, 1df, $P=0.249$) or control ($\chi^2=1.474$, 1df, $P=0.224$) groups. Variant -175g→t was also detected in 1/47 DNA samples of control individuals from the Venda tribe studied by Ehrenborg et al.[25] while absent in more than 300 Caucasians screened.[26]

Discussion

Numerous LDLR gene mutations (>600) have been identified in FH patients world-wide, but genetic data on Black African populations are rare.[19, 22, 23, 27] A striking finding is that a 6-bp deletion predominate in a small number (5/18) of FH patients [19, 23, 28, this study] identified in the South African Black population, where this lipid disorder is thought to be rare. This deletion in exon 2 removes an aspartic acid and a glycine from the first cysteine-rich ligand binding repeat of the LDLR, and impairs its transport but not lipoprotein binding in fibroblasts.[23] Frequent detection of a deleterious mutation can be due to consanguinity, recurrent mutational events, genetic drift, founder gene effect, multiple introduction of the mutation into a population or heterozygote advantage.

The 6-bp deletion identified originally in a homoallelic Xhosa FH homozygote,[23] and now also in a homozygous Pedi and three FH heterozygotes (Pedi and two Tswana's) on the same haplotype, have not (yet) been reported in other populations. These findings largely exclude the likelihood of a recurrent mutational event due to slipped mispairing or multiple entries of the deletion-mutation into the Black population. Detection of the deletion in different tribes suggests that it originated in Africa approximately 3000 years ago prior to tribal separation.[29] Although FH patients with the deletion may therefore be distantly related, family ties cannot at present explain its relatively high prevalence among Black FH patients. The apparently low prevalence of FH in South African Blacks and the large population size furthermore argue against a founder effect. It is, however, possible that the deletion-mutation was propagated and inherited within a small group of people who later evolved separately into different African tribes. Another

plausible explanation is that this deleterious deletion-mutation may be associated with a selective advantage in Africa. Already in 1990 Hobbs and co-workers[30] noted that the presence of several founder mutations in different South African population groups[4, 31] may be indicative of a Darwinian selection that favours the heterozygous state in this region of the world. Since the most likely selective agent in Africa would be infectious diseases, the finding that LDLR-deficient mice are protected against lethal endotoxemia and severe gram-negative infections[32] supports the likelihood of such an evolutionary selection mechanism conferring a survival advantage. In addition to binding and inactivating endotoxin, lipoproteins also bind certain viruses and inhibit their infectivity[33]

Although the family data presented in this study demonstrate that the -175g→t polymorphism residing in a *cis*-acting element in the LDLR promoter[34] does not cause the FH phenotype in affected individuals, further studies are warranted to investigate the likelihood that this variant may influence disease expression. The possibility that the significantly higher frequency of the -175g→t promoter polymorphism in South African Black FH patients compared to controls ($P<0.05$) is caused by linkage disequilibrium with another downstream mutation causing the FH phenotype, was excluded by haplotype studies demonstrating that the rare t allele was associated with different LDLR haplotypes. This allele furthermore co-segregated with missense mutation E387K in one family. These different chromosomal backgrounds may be the result of recombination events, reflecting the age of the -175g→t variant. Compared to Caucasians, Blacks are considered older in evolutionary terms[35] and can therefore be expected to have accumulated variation

over longer times. It is possible that the -175g→t polymorphism did not spread to other parts of the world, thereby explaining its apparent absence in Caucasian populations.[18, 36, this study] The African origin of the -175g→t variant was confirmed by detection of the rare t allele at a low frequency in control DNA samples obtained from Nigerians and African-Americans.[26] African Americans has originated mostly from the western African coast and arrived in North America between the 16th and 19th centuries.

One Sotho proband was heterozygous for a known splicing defect in intron 3 (313+1G→A) and for the R232W mutation in exon 5. In all the patients with mutation 313+1G→A studied to date, the splicing defect is associated with a clinical picture of severe hypercholesterolaemia and early CHD.[37, 38] Patient SH had a TC concentration of 13 mmol/l, but it is uncertain whether this high level is solely due to the 313+1G→A mutation or whether there is an additional effect of the downstream R232W mutation. Family studies could not rule out the possibility of a double mutation, but demonstrated that the splicing defect is the consequence of a *de novo* mutation. None of the family members of SH were hypercholesterolaemic, including his 72-year old father (LDLC 1.9 mmol/l), who was heterozygous for mutation R232W. This finding indicates that R232W does not affect LDLR function or, alternatively, that clinical expression of this missense mutation is altered by other genetic and/or environmental factors.

Although the identified missense mutations have not been characterised further, they are likely contributors to the FH phenotype in our patient sample, since all the codon changes involve conserved amino acids and were not detected in the

normal population. Screening for mutations causing FDB[16, 17, 39] resulted in the identification of two silent mutations T3540T and T3552T (data not shown) previously described in a Nigerian and African American subject, respectively.[17] Failure to identify disease-related mutations in all the patients studied may be due to limitations imposed by the screening techniques used, clinical misdiagnosis of FH, or mutations in other genes causing the ADH phenotype.[40, 41]

Both the Zulu and Pedi patients clinically diagnosed with homozygous FH presented with relatively low pretreatment TC levels (<15 mmol/l) for this severe condition[1] and neither have yet suffered from CHD. The relatively mild expression of homozygous FH in these subjects largely precludes an estimation of the prevalence of heterozygous FH in the South African Black population based on the prevalence of homozygous FH. Elevated plasma cholesterol levels causing FH in a family frequently remain undetected until the occurrence of coronary events or clinical signs indicative of FH is observed in one or more family members. This may particularly be the case in the South African Black population, as hypercholesterolaemics with lipid profiles compatible with the diagnosis of heterozygous FH frequently lack xanthomata characteristic of this condition.[42, this study] None of the FH heterozygotes with the relatively severe 6-bp deletion in exon 2[23] presented with CHD. These findings provide evidence that FH is probably underdiagnosed in the South African Black population, most likely as a consequence of altered expression of FH-related mutations. This may be due to interaction with other genetic and environmental factors, including a prudent diet.[11] Data provided by us and others[43-45] therefore suggest that clinical/biochemical criteria for the diagnosis

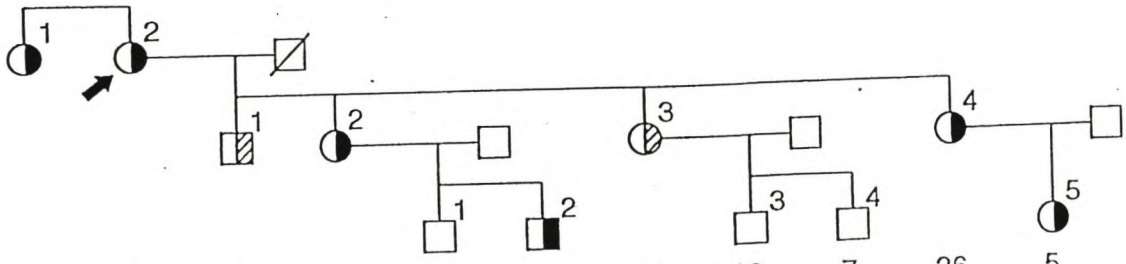
of FH need to be different by country/population and that DNA methods may assist in making a definitive disease diagnosis.

Table 1
Characteristics of African probands analysed for LDLR and apo B gene mutations

Index	Ancestry	Sex	Age	TC	TG	HDL	LDL	Clinical	LDLR gene sequence changes	Apo B gene sequence changes	Relatives Tested
				(mmol/l) [c]							
CM	Xhosa	F	52	8.5	2.7	1.5	5.8	CHD	R793Q		0
MX	Xhosa	M	50	10.8	2.0	0.9	9.0	Arc, Xan	-175g→t		0
AN	Swazi	F	58	10.1	0.9	1.3	8.4	Arc, Xan, CHD	D151H		0
AS	Swazi	M	49	8.0	1.0	2.1	5.4	Arc, CHD	P678L		0
AM[a]	Swazi/Zulu	F	56	8.3	1.5	1.9	5.7	Arc			11
CK[b]	Zulu	M	26	13.8	0.8	1.3	12.1	Arc, Xan	-175g→t		1
SH	Sotho	M	33	12.7	2.2	1.2	10.5	Arc, Xan	313+1G→A; R232W		4
RK	Sotho	M	58	10.7	2.3	1.0	8.6	CHD	R385Q		0
KN[b]	Pedi	F	32	14.9	0.8	1.4	13.1	Arc, Xan	6-bp del, 6-bp del		0
EF	Pedi	F	56	13.1	1.1	1.2	11.4	Arc, PVD, CHD	E387K; -175g→t; C347C	T3552T; T3540T	10
LP	Pedi	F	61	9.4	0.8	0.9	8.1	Arc, Xan	6-bp del		3
CN[a]	Pedi	F	57	7.4	2.6	0.9	5.3	Arc, PVD			6
RM[a]	Pedi/Tswana	F	54	10.8	0.4	1.3	9.3	Arc, ?Xan			3
LM[a]	Tswana	F	56	6.1	1.8	1.8	3.5	Arc, CHD	-175g→t	T3552T	0
RL	Tswana	F	30	9.3	0.8	1.7	7.2	Arc, Xan	6-bp del		0
CS	Tswana	F	47	7.9	0.7	1.7	5.9	Arc	6-bp del		0

The majority of mutations summarised in this table were included in a recent mutation update.[22]
Reference plasma cholesterol concentrations in the general Black population are given in ref. 24
TC, total cholesterol; TG, triglycerides, HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; CHD, coronary heart disease, PVD, peripheral vascular disease, Arc, arcus cornealis; Xan, xanthomata.
[a]Probable FH; [b]Clinical FH homozygotes; [c]Pretreatment concentrations, except for proband LM for whom pretreatment levels was not available

Figure 1 Pedigree of proband EF (arrow) clinically diagnosed with heterozygous FH. Clinical, biochemical and genetic data are provided for individuals of whom DNA samples were available. Those with elevated plasma cholesterol levels are indicated by dark- (mutation-positive for E387K) and shaded symbols. The presence (+) or absence (-) of LDLR gene mutations and recognition sites for SmaI, StuI, AvaII and NcoI are indicated.



Age (years)	62	57	38	35	15	13	31	12	7	26	5
TC (mmol/l)	9.3	13.1	5.8	10.5	2.8	6.1	7.4	3.5	5.2	8.6	7.3
LDLC (mmol/l)	5.9	11.4	2.8	8.8	1.8	4.8	5.2	1.9	4.0	7.2	6.2
HDLC (mmol/l)	3.0	1.2	2.4	1.1	0.8	0.8	1.4	1.3	0.9	1.0	0.8
TG (mmol/l)	0.9	1.1	1.3	1.5	0.4	1.1	1.7	1.6	0.6	0.8	0.7
Xanthomas	-	-	-	-	-	-	-	-	-	+	-
CVD/PVD	-	PVD	-	-	-	-	-	-	-	-	-
E387K	+ -	+ -	- -	+ -	- -	+ -	- -	- -	- -	+ -	+ -
-175G/T	+ -	+ -	+ -	+ +	+ -	+ -	- -	- -	- -	+ -	+ -
Sma I	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Stu I	+ +	+ +	+ +	+ +	+ +	+ +	+ -	+ +	+ +	+ -	+ +
Ava II	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Nco I	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

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References

1 Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The Metabolic Basis of Inherited Disease.* 7th ed. New York: McGraw-Hill, 1995; pp 1981-2030.

2 Innerarity TL, Weisgraber KH, Arnold KS. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. *Proc Natl Acad Sci USA* 1987;**84**:6919-6923.

3 Seftel HC, Baker SG, Sandler MP, Forman MB, Joffe BI, Mendelsohn D, Jenkins T, Mieny CJ. A host of hypercholesterolaemic homozygotes in South Africa. *British Medical Journal* 1980;**281**:633-636.

4 Kotze MJ, Langenhoven E, Warnich L, du Plessis L, Retief AE. The molecular basis and diagnosis of familial hypercholesterolemia in South African Afrikaners. *Ann Hum Genet* 1991;**55**:115-121.

5 Steyn K, Goldberg YP, Kotze MJ, Steyn M, Swanepoel AS, Fourie JM, Coetzee GA, Van der Westhuyzen DR. Estimation of the prevalence of familial hypercholesterolemia in a rural Afrikaner community by direct screening for three Afrikaner founder low density lipoprotein receptor gene mutations. *Hum Genet* 1996;**98**:478-484.

6 Boyce AN, Harrison WA. Legacy of the past: A history for std. IV. Second revised edition. The Rustica Press, Pty., Ltd., Wynberg Cape, pp 1-13.

7 Du Toit ED, MacGregor KJ, Taljaard DG, Oudshoorn M. HLA-A, B, C, DR and DQ polymorphisms in three South African population groups: South African

Negroes, Cape Coloureds and South African Caucasoids. *Tissue Antigens* 1987; **31**:109-25.

8 Bottema CDK, Ketterling RP, Yoon H-S, Sommer SS. The pattern of factor IX germ-line mutation in Asians is similar to that of Caucasians. *Am J Hum Genet* 1990;**47**:835-841.

9 Krawczak M, Cooper DN. Gene deletions causing human genetic disease: mechanisms of mutagenesis and the role of the local DNA sequence environment. *Hum Genet* 1991;**86**:425-441.

10 Kotze MJ, Thiart R, Loubser O, de Villiers JNP, Santos M, Vargas MA, Peeters AV. Mutation analysis reveals an insertional hotspot in exon 4 of the LDL receptor gene. *Hum Genet* 1996;**98**:476-478.

11 Vermaak WJH, Ubbink JB, Delport R, Becker PJ, Bissbort SH, Ungerer JPJ. Ethnic immunity to coronary heart disease? *Atherosclerosis* 1991;**89**:155-162.

12 Rubinsztein DC, Coetzee GA, van der Westhuyzen DR, Langenhoven E, Kotze MJ. Familial defective apolipoprotein B-100 is rare in hypercholesteroloemic South African Afrikaners, coloureds and Indians. *S Afr Med J* 1995;**85**:355-357.

13 Kotze MJ, Langenhoven E, Retief AE, Steyn K, Marais MP, Grobbelaar JJ, Oosthuizen CJ, Weich HF, Benade AJ. Haplotype associations of three DNA polymorphisms at the low-density lipoprotein receptor gene locus in familial hypercholesterolemia. *J Med Genet* 1987;**24**:750-755.

14 Friedewald WT, Levy FI, Fredrickson DS. Estimation of the concentration of the low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. *Clin Chem* 1972;**18**:499-509.

15 Kotze MJ, Theart L, Callis M, Peeters AV, Thiart R, Langenhoven E. Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low-density lipoprotein receptor gene mutations. *PCR Methods Applic* 1995;**4**:352-356.

16 Nissen H, Guldberg P, Hansen AB, Petersen NE, Horder M. Clinically applicable mutation screening in familial hypercholesterolemia. *Hum Mutat* 1996;**8**:168-177.

17 Pullinger CR, Gaffney D, Gutierrez MM, Malloy MJ, Schmaker VN, Packard CJ, Kane JP. The apolipoprotein B R3531C mutation: characteristics of 24 subjects from 9 kindreds. *J Lipid Res* 1999;**40**:318-327.

18 Jensen HK, Jensen LG, Hansen PS, Færgeman O, Gregersen N. High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. *Clin Chem* 1996;**42**:1140-1146.

19 Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* 1992;**1**:445-466.

20 Südhof TC, Goldstein JL, Brown MS, Russell DW. The LDL receptor gene: a mosaic of exons shared with different proteins. *Science* 1985;**228**:815-812.

21 Theart L, Kotze MJ, Langenhoven E, Loubser O, Peeters AV, Lintott CJ, Scott RS. Screening for mutations in exon 4 of the LDL receptor gene: identification of a new deletion mutation. *J Med Genet* 1995;**32**:379-382.

22 Varret M, Rabés JP, Thiart R, Kotze MJ, Baron H, Cenarro A, Descamps O, Ebhardt M, Hondelijn JC, Kostner GM, Miyake Y, Pocovi M, Schmidt H, Schuster H, Stuhrmann M, Yamamura T, Junien C, Beroud C, Boileau C. LDLR

database (second edition): new additions to the database and the software, and results of the first molecular analysis. *Nucl Acid Res* 1998;**26**:250-255.

23 Leitersdorf E, Hobbs HH, Fourie AM, Jacobs M, van der Westhuyzen DR, Coetzee GA. Deletion in the first cysteine-rich repeat of low density lipoprotein receptor impairs its transport but not lipoprotein binding in fibroblasts from a subject with familial hypercholesterolemia. *Proc Natl Acad Sci USA* 1988;**85**:7912-7916.

24 Steyn K, Kazenellenbogen JM, Lombard CJ, Bourne LT. Urbanization and the risk for chronic diseases of lifestyle in the Black population of the Cape Peninsula, South Africa. *J Cardiovasc Risk* 1997;**4**:135-142.

25 Ehrenborg E, Clee SM, Pimstone SN, Reymer PW, Benlian P, Hoogendijk CF, Davis HJ, Bissada N, Miao L, Gagne SE, Greenberg LJ, Henry R, Henderson H, Ordovas JM, Schaefer EJ, Kastelein JJ, Kotze MJ, Hayden MR. Ethnic variation and in vivo effects of the -93t→g promoter variant in the lipoprotein lipase gene. *Arterioscler Thromb Vasc Biol* 1997;**17**:2672-2678.

26 Hoogendijk CF. Analysis of regulatory mutations at the low-density lipoprotein receptor gene locus. University of Stellenbosch, MSc Thesis, 1999.

27 Blackett PR, Altmiller DH, Jelley D, Wilson DP. FH Tulsa-1 and -2: Two unique alleles for familial hypercholesterolemia presenting in an affected two-year-old African-American male. *Am J Med Genet* 1995; **59**:300-303.

28 Peeters AV. Analysis of the genetic contribution to the risk of cardiovascular disease in monogenic hypercholesterolemia. University of Stellenbosch, PhD thesis, 1997.

29 Guthrie M. Some developments in the prehistory of the Bantu origins. *J Afr History* 1962;**3**:273-282.

30 Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: Mutational analysis of a membrane protein. *Annu Rev Genet* 1990;**24**:133-170.

31 Meiner V, Landsberger D, Berkman N, Reshef A, Segal P, Seftel HC, van der Westhuyzen DR, Jeenah MS, Coetzee GA, Leitersdorf E. A common Lithuanian mutation causing familial hypercholesterolemia in Ashkenazi Jews. *Am J Hum Genet* 1991;**49**:443-449.

32 Netea MG, Demacker PNM, Kullberg BJ, Boerman OC, Verschueren I, Stalenhoef AF, van der Meer JW. Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. *J Clin Investig* 1996;**97**:1366-1372.

33 Feingold KR, Grunfeld C. Lipoproteins: are they important components of host defense? *Hepatology* 1997;**26**:1685-1686.

34 Mehta KD, Chang R, Underwood J, Wise J, Kumar A. Identification of a novel cis-acting element participating in maximal induction of the human low density lipoprotein receptor gene transcription in response to low cellular cholesterol levels. *J Biol Chem* 1996;**274**:33618-33622.

35 Von Haeseler A, Sajantila A, Pääbo S. The archaeology of the human genome. *Nat Genet* 1996;**14**:135-140.

36 Top B, Uitterlinden AG, van der Zee A, Kastelein JJ, Leuven JA, Havekes LM, Frants RR. Absence of mutations in the promoter region of the low-density lipoprotein receptor gene in a large number of familial hypercholesterolemia

patients revealed by denaturing gradient gel electrophoresis. *Hum Genet* 1992;**89**:561-565.

37 Sun XM, Patel DD, Bhatnagar D, Knight BL, Soutar AK. Characterization of a splice-site mutation in the gene for the LDL receptor associated with an unpredictably severe clinical phenotype in English patients with heterozygous FH. *Arterioscler Thromb Vasc Biol* 1995;**15**:219-227.

38 Jensen HK, Jensen LG, Hansen PS, Færgeman O, Gregersen N. Two point mutations (313+1G→A and 313+1G→T) in the splice donor site of intron 3 of the low-density lipoprotein receptor gene are associated with familial hypercholesterolemia. *Hum Mutat* 1996;**7**:269-271.

39 Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA* 1989;**86**:587-591.

40 Varret M, Rabès J-P, Saint-Jore B, Cenarro A, Marinoni JC, Civeira F, Devillers M, Krempf M, Coulon M, Thiart R, Kotze MJ, Schmidt H, Buzzi JC, Kostner GM, Bertolini S, Pocovi M, Rosa A, Farnier M, Martinez M, Junien C, Boileau. A third major locus for autosomal dominant hypercholesterolemia maps at 1p34.1-p32. *Am J Hum Genet* 1999;**64**:1378-1387.

41 Haddad L, Day IN, Hunt S, Williams RR, Humphries SE, Hopkins PN. Evidence for a third genetic locus causing familial hypercholesterolemia. A non-LDLR, non-apoB kindred. *J Lipid Res* 1999;**40**:1113-1122.

42 Marais AD, Berger GM. A diversity of genetic hyperlipoproteinaemias in Black patients. Experience at the lipid clinics at the Groote Schuur Hospital and the

Red Cross War Memorial Children's Hospital, Cape Town. *S Afr Med J* 1986;**70**:583-587.

43 Slimane MN, Pousse H, Maatoug F, Hammami M, Ben Farhat MH. Phenotypic expression of familial hypercholesterolemia in Central and Southern Tunisia. *Atherosclerosis* 1993;**104**:153-158.

44 Gagné C, Moorjani S, Torres AL, Brun D, Lupien P-J. Homozygous familial hypercholesterolemia. *Lancet* 1994;**343**:177.

45 Pimstone SN, Sun X-M, du Souich C, Frohlich JJ, Hayden MR, Soutar AK. Phenotypic variation in heterozygous familial hypercholesterolemia. A comparison of Chinese patients with the same or similar mutations in the LDL receptor gene in China or Canada. *Arterioscler Thromb Vasc Biol* 1998;**18**:309-315.