The genetics of familial hypercholesterolaemia and establishing familial hypercholesterolaemia genetic testing as a clinical diagnostic service.

Thesis submitted to fulfil the requirements for the degree
Doctor of Philosophy of the University of London
June 1999

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ABSTRACT

Familial hypercholesterolaemia (FH) is a monogenically inherited disorder of lipoprotein metabolism caused by a mutation in the low density lipoprotein receptor gene (LDLR). However in a few individuals, the defect lies in the gene for apolipoprotein B (APOB), the ligand for the LDL-receptor, and this is called familial defective apolipoprotein B-100 (FDB), while in others the receptor function is apparently normal and the defect must lie elsewhere. Mutation studies are the most practical way in which one can identify the cause of heterozygous hypercholesterolaemia.

A genetic diagnostic service for FH has been established and the various aspects of setting up are described, with unusual cases being reported. The mutations identified are described and mutation detection rates were calculated for groups of paediatric and adult probands from the UK. The feasibility of alternative mutation screening methods and the specificity and sensitivity of reducing the number of tests has been assessed from the results obtained over the last four years.

A quantitative fluorescent multiplex PCR screen was adapted to analyse LDLR rearrangements which would improve the genetic diagnosis of FH individuals. One assay based on exons 1, 8, 10, 12 and 16 were optimised and tested on known major rearrangements. A group of FH probands from the USA were then analysed with this multiplex assay. The inter and intra-assay variation were very wide, so a second method was designed to overcome these problems, universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR). The multiplex set developed analysed exons 3, 5, 8, 14, and 17 of LDLR, and the method could also be used to detect major rearrangements in other genes. The method was evaluated by conducting a trial on 15 reported deletions and duplications. Two groups of FH patients from the UK were screened with this UPQFM-PCR assay.

The influence of LDLR & APOB mutations on the cholesterol-lowering response of the HMG-CoA reductase inhibitor simvastatin was investigated in patients with heterozygous FH. Data suggest that there may be a difference in cholesterol-lowering between 'severe' and 'mild' LDLR mutations.

Future developments and transferring the findings into a clinical genetic service are discussed.
ACKNOWLEDGEMENTS

It would be a very difficult task to thank all those who have contributed and guided me during my thesis, but my appreciation goes to all who have made my life as a PhD student very enjoyable. I would like to start by thanking the John Pinto Foundation for funding my work, to Professor Steve Humphries and Dr Helen Middleton-Price for their continued support, encouragement and enthusiasm throughout. A special thanks to Professor Steve Humphries for his invaluable guidance and supervision. I would like to also thank all of the people I have had the pleasure to work with in the Clinical Molecular Genetics group at Great Ormond Street Hospital/Institute of Child Health and in the Cardiovascular Genetics group at University College London, especially the members of the FH group who I started out with, Lema Haddad, Ros Whittall, Ian Day and Villi Gudnason.

To my friends outside work, Jackie, Anna, Anjali who stood by me at all times, Camden Football Club and Boxercise for my weekly stress relief.

Finally to my family, to my sister, Jane, who helped me with hundreds of DNA extractions during her stint at ICH and to my parents, for their constant encouragement and continuous financial support throughout my many student years.
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<td>AFCAPS</td>
<td>Air Force Coronary Atherosclerosis Prevention Study</td>
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<tr>
<td>apoA</td>
<td>apolipoprotein A</td>
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<td>APOA</td>
<td>apolipoprotein A gene</td>
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<td>APS</td>
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<td>ARMS</td>
<td>amplification refractory mutation system</td>
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<tr>
<td>ASO</td>
<td>allele specific oligonucleotide</td>
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<td>BHA</td>
<td>British Hyperlipidaemia Association</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BMD</td>
<td>Becker muscular dystrophy</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>CABG</td>
<td>coronary artery bypass graft</td>
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<tr>
<td>CARE</td>
<td>Cholesterol and Recurrent Events trial</td>
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<td>Charing Cross Hospital</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
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<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane regulator</td>
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<td>CISS</td>
<td>chromosomal in situ suppression</td>
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<td>CMC</td>
<td>chemical mismatch cleavage</td>
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<td>cytomegalovirus</td>
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<td>Daltons</td>
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<td>denaturing gradient gel electrophoresis</td>
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<td>Duchenne muscular dystrophy</td>
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<td>DNA</td>
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<td>EAS</td>
<td>European Atherosclerosis Society</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
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<td>EMC</td>
<td>enzyme mismatch cleavage</td>
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<td>ethidium bromide</td>
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<tr>
<td>EQA</td>
<td>External Quality Assurance</td>
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<tr>
<td>FAH</td>
<td>fumarylacetoacetate hydrolase</td>
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<tr>
<td>FCHL</td>
<td>familial combined hyperlipidaemia</td>
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<td>FDB</td>
<td>familial defective apolipoprotein B100</td>
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<td>FH</td>
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<td>FISH</td>
<td>fluorescent in situ hybridisation</td>
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<td>HDL</td>
<td>high density lipoprotein</td>
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<td>HELP</td>
<td>Heparin Extracorporal LDL Precipitation</td>
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<td>HT1</td>
<td>heriditory tyrosinaemia type I</td>
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<td>HMG-CoA</td>
<td>B-hydroxy-B-methylglutaryl-coenzyme A</td>
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<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>l</td>
<td>litre (s)</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<td>LDL-c</td>
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<td>LDL-receptor gene</td>
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<td>Long-Term Intervention with Pravastatin in Ischaemic Disease trial</td>
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<td>Lp(a)</td>
<td>lipoprotein (a)</td>
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<td>LPL</td>
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<td>MADGE</td>
<td>microtitre array diagonal gel electrophoresis</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase</td>
</tr>
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<td>MEDPED</td>
<td>Make Early Diagnosis to Prevent Early Death</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>mg</td>
<td>milligram (s)</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre(s)</td>
</tr>
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<td>MRFIT</td>
<td>Multiple Risk Factor Intervention Trial study</td>
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<td>mRNA</td>
<td>messenger RNA</td>
</tr>
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<td>MTHFR</td>
<td>5,10-methylene tetrahydrofolate reductase gene</td>
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<td>MUFA</td>
<td>monounsaturated fatty acids</td>
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<td>NCEP</td>
<td>National Cholesterol Education Program</td>
</tr>
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<td>NHS</td>
<td>National Health Service</td>
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<td>NIDDIM</td>
<td>non-insulin-dependent diabetes mellitus</td>
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<td>NPHS</td>
<td>Northwick Park Heart Study</td>
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<td>NTBC</td>
<td>2-(2-nitro-4-trifluoro-methylbenzylol-1,3-cyclohexanedione)</td>
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<td>OLA</td>
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<td>ornithine transcarbamylase</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>plasminogen activator inhibitor-1</td>
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<td>pulsed field gel electrophoresis</td>
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<tr>
<td>PMD</td>
<td>programmable melting display</td>
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<tr>
<td>PROCAM</td>
<td>Prospective Cardiovascular Münster study</td>
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<td>PTCA</td>
<td>percutaneous transluminal coronary angioplasty</td>
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<td>PTT</td>
<td>protein truncation test</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
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<td>PWS</td>
<td>Prader Willi syndrome</td>
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<td>QFM</td>
<td>quantitative fluorescent multiplex</td>
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<tr>
<td>RSCA</td>
<td>Reference Strand Conformation Analysis</td>
</tr>
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<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RT-PCR</td>
<td>reverse transcriptase-PCR</td>
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<tr>
<td>SAFA</td>
<td>saturated fatty acids</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>SRE</td>
<td>sterol regulatory element</td>
</tr>
<tr>
<td>SREBP</td>
<td>SRE binding protein</td>
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<tr>
<td>SSCP</td>
<td>single stranded conformational polymorphism</td>
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<tr>
<td>STR</td>
<td>short tandem repeat</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus thermostable DNA polymerase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>TEMED</td>
<td>NNN’N’tetramethylethylenediamine</td>
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<tr>
<td>TexCAPS</td>
<td>Texas Coronary Atherosclerosis Prevention Study</td>
</tr>
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<td>TBE</td>
<td>Tris-borate buffer</td>
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<tr>
<td>TX</td>
<td>Tendon xanthoma</td>
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<tr>
<td>µg</td>
<td>microgram (s)</td>
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<tr>
<td>µl</td>
<td>microlitre (s)</td>
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<tr>
<td>UPQFM</td>
<td>universal primer quantitative fluorescent multiplex</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<td>UV</td>
<td>ultraviolet</td>
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XIX
Abbreviations of amino acids:

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<th>Three-letter abbreviation</th>
<th>One-letter abbreviation</th>
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<td>Asparagine</td>
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<td>N</td>
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<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
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<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
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<td>Glutamine</td>
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<td>Q</td>
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<td>Glutamic acid</td>
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<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
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<td>L</td>
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<td>Lysine</td>
<td>Lys</td>
<td>K</td>
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<td>Methionine</td>
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<td>W</td>
</tr>
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<td>Tyrosine</td>
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<td>Y</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
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Chapter 1: Introduction

1.1. Coronary heart disease
Cardiovascular disease is the main cause of death in the UK, accounting for nearly 300,000 deaths per year. As in the rest of the developed world, this represents approximately 26% of all deaths. It is the major cause of premature death in both sexes, although there are differences. Males are affected more often, one in three men are affected compared to one in four women, but disease also occurs at an earlier age. The origins of coronary heart disease (CHD) lie in the development of atherosclerosis, a process which may begin in childhood. Many common multifactorial diseases such as hypertension, hyperlipidaemias, diabetes and obesity contribute to the development of CHD. Together these cost the National Health Service (NHS) approximately £1.5 billion a year, most of which is spent on treatment and surgery and only 1% is spent on health promotion and prevention (Betteridge & Morrell, 1998).

Clinical investigations of CHD patients manifesting qualitative or quantitative abnormalities of the plasma lipoproteins have led to the current knowledge of lipid metabolism. Discoveries of major gene mutations affecting the apolipoproteins, the key enzymes that control lipid transport, and the cellular receptors that recognise specific apolipoproteins, have contributed greatly to the understanding of lipoprotein metabolism. Despite these advances the basis of population variation in lipoprotein concentrations remains poorly understood. Genetic and environmental factors interact in the development of CHD in humans. The genetic factors may be divided into two groups; firstly there are single gene defects, such as familial hypercholesterolaemia (FH), and secondly complex disorders where a combination of genetic variants in one or more genes combine to have a cumulative effect in predisposing an individual to CHD. Since FH is the primary research focus of this thesis, general aspects of CHD and risk factors will be related to FH where possible. The molecular cause of most monogenic disorders have been discovered but there are many factors which influence the severity of the phenotype. Often these factors can be altered, and may be activated/enhanced by particular events in the environment such as diet, smoking, stress and physical inactivity. Extensive research has been undertaken to elucidate the risk factors involved in the aetiology of atherosclerosis and CHD but little is known about the gene:gene or gene:environment interactions in multifactorial coronary disorders. Some associations have been identified but large, long-term epidemiological studies are required to dissect further factors.
1.2. Atherosclerosis

In recent years much has been learnt about the atherosclerotic process which can be divided into three main stages; the formation of the fatty streak, the fibrous plaque and finally the complicated lesion (reviewed by Stary et al, 1994; Libby et al, 1996; Allen, 1998). The initial injury to the endothelium primarily occurs at the branch points of arteries where specific adhesive glycoproteins appear on the surface of endothelial cells. Monocytes and T-lymphocytes attach to these cells, migrate between them and embed in the sub-endothelial space. All of these processes are influenced by growth regulatory molecules and chemoattractants released by the altered endothelium, the adherent leukocytes and smooth muscle cells (reviewed by Ross, 1993). The monocytes acquire the characteristics of macrophages, which engulf lipid and become lipid-laden foam cells. The foam cells, the T-cells and the smooth muscle cells form a fatty streak, the initial lesion of atherosclerosis. They can occur in the aorta as early as three years old and usually by the age of 15 in the coronary arteries. Fatty streaks can regress, slowing the atherosclerotic process, but the next stage is irreversible. The lesions continue to accumulate more cells, the macrophages scavenge more lipids and some of the foam cells migrate back into the bloodstream by pushing the endothelial cells apart. These areas may then become thrombogenic leading to the aggregation of platelets and thrombi formation (reviewed by Ross, 1999). The thrombi can release various growth regulators and cytokines leading to the formation of a fibrous plaque (reviewed by Libby et al, 1996). Calcification, necrosis, thrombosis and ulceration may cause the plaque to become a complicated lesion giving rise to the clinical symptoms of atherosclerosis, ischaemia and infarction from thrombosis and thromboembolism. One or more of the branches of the coronary artery may be narrowed to less than 25% of its original diameter. The major lipid constituent (45%) of this advanced lesion is cholesterol, derived almost entirely from the blood and not from local synthesis (reviewed by Allen, 1998).

Goldstein & Brown showed that the low density lipoprotein (LDL) taken up by monocytes was chemically modified and that the process appeared to be receptor-mediated. The receptor was named the scavenger receptor and it lacks down-regulatory mechanisms which would function to prevent overload when cellular cholesterol increases (Brown & Goldstein, 1983). Later it was shown by studying the effects of antioxidants in experimental atherosclerotic animals that the in vivo LDL modification was peroxidation (Steinberg et al, 1989). Antioxidants inhibit LDL modification, reduce LDL uptake into the arterial wall and this stems the atherosclerotic process. Oxidised LDL may contribute to atherosclerosis in many ways, direct cytotoxicity to arterial endothelium, stimulation of monocyte adhesion and monocyte chemotaxis and
interaction with the coagulation system through increased expression of thromboplastin and plasminogen activator inhibitor-1 (PAI-1) (Robbie et al, 1996). These effects promote the activation of the coagulation cascade (reviewed by Heinecke, 1998).

The susceptibility of LDL to oxidation may be explained by LDL structure. Four subclasses, divided according to their size, density and lipoprotein content, can be separated by density gradient ultracentrifugation (Krauss & Burke, 1982). In healthy subjects the most abundant LDL subclass is LDL-II although women have proportionately more of the larger, less dense LDL-I particles than men whilst men have more of the smaller, denser LDL-III particles. It is the LDL-III particles that have been strongly related to CHD (Austin et al, 1988) and possible explanations include the slower fractional catabolic rate of dense LDL and its increased susceptibility to oxidation.

\[\text{---}\]

### 1.3. Risk factors in coronary heart disease

Over the last decade many CHD risk factors have been determined which has probably contributed to the decline in CHD in the western world. Intervention against hypercholesterolaemia with view to the reduction of CHD morbidity and mortality has been shown to be effective. Gradually genetic and environmental risk factors are being identified through large epidemiological studies such as the Framingham Heart study and the USA Multiple Risk Factor Intervention study (MRFIT) (reviewed by LaRosa et al, 1990). These factors, summarised below in Table 1.1, include both modifiable and non-modifiable factors, with most having genetic and non-genetic components.

<table>
<thead>
<tr>
<th>Modifiable risk factors</th>
<th>Non-modifiable risks</th>
</tr>
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<tbody>
<tr>
<td>Hyperlipidaemia (especially high LDL-c, low HDL and high triglyceride, low HDL)</td>
<td>Age</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Sex</td>
</tr>
<tr>
<td>Diabetes mellitus and impaired glucose intolerance</td>
<td>Family history of CHD</td>
</tr>
<tr>
<td>Smoking</td>
<td>Personal history of CHD</td>
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<tr>
<td>Excess alcohol consumption</td>
<td></td>
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<tr>
<td>Physical inactivity</td>
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<tr>
<td>Coagulation factors</td>
<td></td>
</tr>
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<td>Psychosocial factors</td>
<td></td>
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</table>

Table 1.1: Modifiable and non-modifiable factors associated with increased CHD risk.

Risk factors seldom occur in isolation and tend to cluster in individuals. For example, individuals with low HDL and high triglyceride may also have truncal obesity,
hypertension and impaired glucose tolerance with hyperinsulinaemia, which collectively form the insulin resistance syndrome (Reaven 1988). The effect of the risk factors is often multiplicative but for several risk factors there are no clearly defined threshold levels at which increased risk begins.

1.3.1. Non-modifiable risk factors

1.3.1.1. Age

The relative risk of CHD declines with age evident in polygenic hypercholesterolaemia as well as FH, for example in the Multiple Risk Factor Intervention Trial (MRFIT) there was an ~8-fold increase in cholesterol in ages 35-39 years falling to 2.4-fold in men aged 55-57 (Stamler et al, 1986). The relative risk of fatal CHD is 100-fold greater in young heterozygous FH adults aged 20-39, although patients who survived through middle age appear to no longer to be at a substantially increased relative risk (Simon Broome Steering Committee, 1991 & 1999).

1.3.1.2. Gender

Males are more susceptible to CHD mortality than females (4:3 ratio) throughout the world and although the ratio decreases with age it does not disappear (Slack, 1969). The rate of increase of CHD rises equally in men and women after the menopause, but women still appear to lag ten years behind men in the presentation of CHD and this continues to beyond the age of 75. For women with FH, aged 20-39, despite treatment, the relative risk of a fatal coronary event was increased 125-fold and the annual coronary mortality was 0.17% whilst in men aged 20-39 the relative risk was increased 48-fold and the annual coronary mortality was 0.46% (Simon Broome Steering Committee, 1999). The annual coronary mortality for men and women aged 60-70 was 1.1% representing a significant excess in mortality for women (relative risk 2.6) but not for men (relative risk 1.1) (Simon Broome Steering Committee, 1999).

Examining other risk factors, women tend to have higher cholesterol levels, blood pressure, fibrinogen levels, are more obese and have more diabetes than men. Favourable factors include higher HDL levels throughout their life, lower triglyceride levels, less central obesity and a protective effect from oestrogens.

1.3.1.3. Family history of CHD

Family history reflects genetic and environment risk factors which are hard to differentiate. The Framingham heart study showed that even after excluding the clear contributions from patients with genetic hyperlipidaemias, a history of CHD in parents is associated with a 30% increased risk (LaRosa et al, 1990).
1.3.1.4. Personal history of CHD

Patients with a history of CHD are at increased risk of further events. One study over a 10-year period showed the risk of dying from CHD was increased more than 20 times in men with previous MI (Pekkanen et al, 1990). Although the personal history of CHD cannot be changed, modifiable risk factors, discussed later can slow the progression of atherosclerosis.

1.3.2. Modifiable risk factors

1.3.2.1. Total and LDL-cholesterol

Hypercholesterolaemia was one of the earliest identified risk factors for CHD (Kannel et al, 1971). For any individual cholesterol levels are determined by a combination of genetic and environmental components and are potentially modifiable. Clinical trials have demonstrated that cholesterol lowering will reduce the occurrence of new CHD events and CHD mortality. A 10% reduction in total cholesterol has been found to correspond to a 20% reduction in CHD risk even at low levels in an individual on a western diet (5.0-4.5mmol/l) (Smith et al, 1993). Lipid-lowering drugs (section 1.9) delay CHD and slow or halt the atherosclerotic process (Scandinavian Simvastatin Survival Study (4S), 1994).

1.3.2.2. High density lipoprotein

High density lipoprotein (HDL) was shown in the 1970's to be a powerful and independent predictor of CHD and this has been confirmed in all recent studies e.g. in the Framingham Heart study (Schaefer et al, 1994). The relationship is inverse, low levels of HDL being associated with increased risk of CHD. The relationship is particularly important in women. Low levels of HDL often reflect obesity, smoking, lack of exercise or impaired glucose tolerance but genetic influences may also be responsible. Levels below 0.9mmol/l in men and below 1.1mmol/l in women are negative risk factors whilst levels greater than 1.5mmol/l in men and greater than 1.7mmol/l in women are protective (Schaefer et al, 1994).

1.3.2.3. Triglycerides

Evidence for triglycerides as an independent risk factor has been hard to determine (reviewed by Gotto, 1998). Triglyceride levels are subject to greater measurement variability and patients must fast for 18 hours to obtain a reliable estimate. HDL shows a strong, inverse correlation with triglycerides and studies that take into account or adjust for HDL usually find no independent association for triglycerides. In the Framingham heart study, individuals with high triglyceride in the presence of low HDL seemed to be at increased CHD risk (Castelli, 1992). Evidence from clinical studies
such as the PROCAM (Prospective Cardiovascular Munster) study (Assmann et al, 1998) have linked hypertriglyceridaemia with potentially important atherogenic factors; intermediate density lipoprotein (IDL), small dense LDL and increased cholesteryl ester exchange. This may provide a greater understanding of atherogenesis and provide potential sites for therapeutic intervention (reviewed by Durrington, 1998; Sprecher, 1998).

1.3.2.4. Lipoprotein (a)
Elevated plasma lipoprotein (a) [Lp(a)] is an independent risk factor for the development of premature CHD (Bostom et al, 1996). In the Framingham heart study elevated Lp(a) had a similar attributable risk as a total cholesterol level of 6.2mmol/l or more, or an HDL level less than 0.9mmol/l (Bostom et al, 1996). Lp(a) is composed of lipid, apoB-100 and glycoprotein [apo(a)]. There is considerable sequence homology between apo(a) and its close neighbour on chromosome 6 (<50kb), plasminogen. Lp(a) contains one kringle 5-like domain, a serine protease domain and 12-51 tandem repeats resembling the fourth kringle of plasminogen (reviewed by Utermann, 1995). Several different electrophoretic isoforms of apo(a) have been reported, the size of which varies from 400,000-700,000 daltons, due to the number of kringle 4-like units (Lackner et al, 1993). Family studies showed that the apo(a) isoforms are inherited as an autosomal quantitative genetic trait (Utermann et al, 1987). The lower the number of repeats the higher the plasma Lp(a) levels (Boerwinkle et al, 1992). Variation at the APO(a) locus affects plasma Lp(a) levels but it only explains approximately 90% of the variability of this trait (Boerwinkle et al, 1992), thus additional genes and/or environmental factors may affect Lp(a) levels.

1.3.2.5. Homocysteine
Elevated plasma homocysteine is another independent risk factor for CHD (Boushey et al, 1995). Homocysteine in the plasma arises solely from the breakdown of the dietary amino acid methionine. Dietary homocysteine does not appear, under normal circumstances, to influence plasma homocysteine so the level of plasma homocysteine is rigorously controlled and kept within a narrow range in normal subjects. The plasma level of homocysteine is dependent on genetically regulated levels of essential enzymes and the dietary intake of their cofactors, folic acid, vitamin B6 and vitamin B12. Impaired renal function, increasing age and pharmacological agents (e.g. nitrous oxide, methotrexate) can also contribute to increased levels of homocysteine (reviewed by Duell & Malinow, 1997). The mechanism of the involvement of homocysteine in atherogenesis may include promotion of platelet activation and enhanced coagulability, increased smooth muscle cell proliferation, cytotoxicity, induction of endothelial
dysfunction, and stimulation of LDL oxidation (reviewed by Duell & Malinow, 1997). Folate supplementations and pharmacological doses of vitamin B12 may normalise and reduce homocysteine levels but further research is required to determine the efficacy of this intervention in reducing morbidity and mortality associated with atherosclerotic vascular disease (Malinow et al, 1997).

1.3.2.6. Angiotensin-converting enzyme
Circulating levels of angiotensin-converting enzyme (ACE) in humans are linked with an insertion (I)/deletion (D) polymorphism in the ACE gene: DD individuals have higher levels of ACE than either ID or II individuals. Controversy exists as to whether the ACE DD genotype increases the risk of CHD. Studies have suggested that the DD genotype is associated with increased plaque instability and possible mechanisms include interactions with neointimal formation, coronary artery spasm and coronary thrombosis (Salmani et al, 1996). ACE inhibitors are used in patients with heart failure and hypertension and may be useful in the prevention of MI and stroke (reviewed by Yusuf & Lonn, 1998).

1.3.2.7. Thrombogenic factors
The role of thrombogenic factors in the development of unstable angina, MI and sudden death was not clearly established until the early 1980's. The Northwick Park Heart Study (Meade et al, 1986) demonstrated a positive relationship between fibrinogen and CHD. High fibrinogen levels are most common in smokers (10% higher) and the rise is proportional to the number of cigarettes smoked. Factors VII (Meade et al, 1986; Junker et al, 1997) and VIII (Meade et al, 1986) and low fibrinolytic activity (Hamsten et al, 1985) have also been associated with CHD. Plasma fibrinogen is not routinely measured in CHD risk assessment but has been shown to increase blood viscosity and platelet aggregation which leads to plaque thrombosis (Ernst & Resch, 1993).

1.3.2.8. Hypertension
Hypertension has been established as one of the major independent risk factors for CHD (reviewed by Kannel, 1996; Schwartz & Sheps, 1999). The consequences of hypertension are stroke, heart disease leading to heart failure, renal vascular disease and peripheral vascular disease. Lipid risk factors coexist in the hypertensive individual more often than by chance even when confounding variables are taken into account. Hypertension is a continuous variable and the higher the level, the greater the cardiovascular risk. It is a reversible risk factor, although there is a strong genetic component, but in most cases it is treatable (MacMahon et al, 1986).
1.3.2.9. Diabetes
Three quarters of diabetics die from large vessel disease whilst the remaining quarter die from CHD which is probably due to the exaggerated effect of the conventional risk factors. Hypertriglyceridaemia and reduced HDL occur in patients with non-insulin-dependent diabetes mellitus (NIDDM) and they also have a preponderance of the small, dense atherogenic LDL subclass III (Tan et al, 1995).

1.3.2.10. Diet
The effect of diet is well known to have an important role in CHD risk and this is mediated through the effects of dietary fats on plasma lipids, especially LDL (reviewed by Mann et al, 1993). There are three main classes of fatty acids; saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA), and they vary in their influence on lipid levels. SAFA have the most significant effect on lipoproteins, raising total cholesterol, LDL-c and triglyceride and this has been associated with increased CHD risk in many studies (reviewed by Mann et al, 1993). In contrast to SAFA, MUFA and PUFA reduce total cholesterol, LDL-c and triglyceride and to a greater level with PUFA although HDL levels may fall slightly, whereas HDL levels either remain unchanged or slightly increased with MUFA (Lichtenstein et al, 1993). MUFAs incorporated into LDL have been shown to be resistant to oxidation in vitro, compared to PUFAs (reviewed by Hensrud & Heimburger, 1994). Compliance with a cholesterol lowering diet is commonly associated with a reduction in serum cholesterol of 10-15% (Lichtenstein et al, 1993). In all people, and in particular those with other risk factors, additional dietary measures may be beneficial. Omega-3-polyunsaturates, found in fish oils, have been shown to have a protective effect against CHD (reviewed by Connor & Connor, 1997). Blood pressure may be lowered by the reduction of sodium and potassium consumption as shown in the MRFIT study (Dyer et al, 1995). Antioxidants (vitamins C & E, β-carotene and flavonoids) found in fruit, vegetables, tea and red wine may have benefits in preventing CHD but results have been inconsistent (Pandey et al, 1995; Stamler et al, 1997a; Evans et al, 1998). However evidence suggests that a diet low in saturated fats, high in unsaturated fats, with lots of fruit, vegetables and fish reduces cardiovascular mortality risk (Wood et al, 1998).

1.3.2.11. Smoking
One sixth of the total deaths and one quarter of CHD deaths in the developed world are attributable to smoking (Betteridge & Morrell, 1998). Smoking represents the most important preventable cause of death in the developed world. Risk is increased in a near linear mode by the number of cigarettes smoked. Cigarette smoke induces endothelial cell changes as a result of injury and enhanced deposition of fibrinogen and
lipids leading to the progression of the atherosclerotic process. Smoking also appears to reduce HDL and increase triglycerides (Butowski et al, 1998). Stopping smoking can reduce CHD risk although variable results have been shown on the speed of the benefits (Wannamethee et al, 1995; Stamler et al, 1997b).

1.3.2.12. Alcohol
The effects of alcohol use on risk of CHD is expressed graphically as a U-shaped relationship. People who drink small amounts of alcohol and those who abstain have lower rates of CHD than those who drink heavily (Jackson et al, 1991). Alcohol produces a beneficial rise in HDL at a moderate intake and beyond this there is a rise in triglycerides and VLDL. Fibrinogen is reduced and there are beneficial influences on platelet aggregation and plasminogen activation. With increasing consumption the prevalence of hypertension does however increase (reviewed by Betteridge & Morrell, 1998).

1.3.2.13. Obesity
The relationship of body mass index (BMI) and the risk of mortality is J-shaped, thin people show a higher risk than normal weight individuals, but obese individuals show the highest risk (reviewed by Garrison et al, 1996). There are several forms of obesity which are often gender related. In males the central, truncal and android obesity effects hepatic lipid and insulin metabolism whilst central obesity in women confers significant CHD risk. Obesity is associated with hypertension, elevated cholesterol, triglycerides and reduced HDL and insulin insensitivity with increased glucose and insulin levels and increased rates of NIDDM (Reaven, 1988). When these factors have been taken into consideration, different conclusions have been drawn as to whether obesity is an independent risk factor or merely a marker for others. Whatever the result obesity is a modifiable risk factor.

1.3.2.14. Physical inactivity
It is hard to assess if lack of exercise is an independent risk factor because of the positive effects of exercise on other characteristics, as shown by the MRFIT study and the CARDIA (coronary artery risk development in young adults) study which investigated fitness in 1777 individuals aged 18-30 over a seven year period (Crow et al, 1986; Sternfield et al, 1999). Exercise causes a slight reduction in blood pressure, a reduction in weight, improved glucose tolerance and psychological and social benefits. HDL is increased and triglycerides are reduced, all of which would reduce risk of CHD (reviewed by Bijnen et al, 1994).
1.3.2.15. Psychosocial factors

The Whitehall I & II studies showed an inverse social gradient in mortality from CHD among British civil servants i.e. there were higher rates in workers of lower employment grade (Marmot et al, 1984, 1991, 1997). In the first Whitehall study, started in the late 1960's, clerical officers (low grade) had more than twice the mortality rate of administrators (high grade) (Marmot et al, 1984). Twenty years separates the two studies but the social class difference in morbidity remains and the prevalence of ischaemic heart disease has increased three-fold between the lowest and highest grades (Marmots al, 1991, 1997). An inverse association between employment grade and prevalence of angina, presence of ischaemia and symptoms of chronic bronchitis was also observed. Possible explanations for these differences include early-life environment (physical height) (Barker et al, 1989), behaviour (smoking, physical inactivity, eating habits) and social circumstances (housing tenure, car access) and how an individual perceived their work environment (opportunities to learn and develop skills and psychological work load). Type A behaviour (competitive, aggressive, time urgent) has been proposed to dispose to heart disease (Shekelle et al, 1983) but in the Whitehall II study this did not explain the pattern of lower status job-higher risk although the hostility component was more prevalent in those with lower job status (Marmot et al, 1991).

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1.4. Cholesterol

1.4.1. Structure

Together with phospholipid the unesterified form of cholesterol comprises the major component of cell membranes. Its presence helps to maintain membrane fluidity and therefore the barrier between cell and environment. Unesterified cholesterol is also important as a precursor of steroid hormones and of bile acids.

There are several different lipoprotein species found in plasma. A central core of cholesterol ester and triglyceride is surrounded by an outer monolayer of molecules such as free cholesterol, phospholipids and apoproteins which determine the particular lipoprotein (Shen, Scanu & Kezdy, 1977). The apoprotein component stabilises the lipoprotein structure and has ligand and cofactor functions in lipoprotein metabolism. ApoB-100 and apoE are necessary for the binding of lipoproteins to cellular receptors (Basu et al, 1976; Mahley et al, 1977; Mahley, 1988), whereas apoAI and CII are activators of enzymes important in lipoprotein metabolism (reviewed by Leroy, Dallongeville & Fruchart, 1995; Cooper, 1997). Lipoproteins are classified predominately according to their separation in the ultracentrifuge, dependent on the
hydrated density of different lipoproteins, from the large chylomicrons (mainly triglyceride and a small amount of cholesterol) through VLDL, IDL and LDL to the small HDL particle which is composed of cholesterol and a very small amount of triglyceride (Bosanquet, Bickerstaffe & Fraser, 1980).

1.4.2. Function
Lipoproteins serve to transport absorbed dietary fat and endogenously synthesised cholesterol and triglyceride. There are three main pathways of lipoprotein metabolism; the exogenous pathway for cholesterol absorbed from the intestine, the endogenous pathway for the cholesterol and triglyceride entering the bloodstream from the liver and non-intestinal organs, and reverse cholesterol transport of cholesterol from peripheral tissues to the liver for excretion.

The exogenous pathway (Fig 1.1) involves the cholesterol and triglyceride ingested (reviewed by Havel & Kane, 1995). In the typical western diet approximately 80-140g triglyceride and 0.5-1.5g cholesterol are consumed daily. The cholesterol and triglyceride is absorbed from the intestine, reesterified and combines with apoB-48 to form chylomicrons which are then secreted into the circulation via the lymph. Additional apoproteins are acquired, apoC’s and apoE, which are transferred from HDL. In the muscle and adipose tissue the major triglyceride component is hydrolysed to fatty acids and glycerol by lipoprotein lipase which is activated by apoCII. The fatty acids are then delivered to the muscle for energy or reesterified to glyceride and stored in adipose tissue, whilst the surface components, apoproteins and phospholipid, become redundant and transfer to HDL (reviewed by Barter & Rye, 1996).

The partially hydrolysed chylomicron or ‘remnant’ is removed by the liver via the low density lipoprotein receptor-related protein (LRP) or the chylomicron remnant receptor and is dependent on apoE. Inside the hepatocyte the remnant, particle is degraded and the lipid component is metabolised and used to form VLDL which is secreted into the circulation to distribute triglycerides to the peripheral tissues during periods between meals. VLDL particles are smaller than chylomicrons, but once secreted undergo the same alterations, including the acquisition of apolipoproteins and the progressive removal of triglycerides from their core by lipoprotein lipase. VLDL particles are rich in triglycerides and contain three apolipoproteins apoE, apoC’s and apoB100. During the conversion of VLDL to LDL, an intermediate particle is formed, IDL, which can be removed from the circulation by the LDL-receptor via apoE. IDL has a short lifespan (2-6 hours) and the intracellular fate of its lipids can be storage, recycling back to VLDL or destruction to bile acids. IDL particles not taken up by the liver remain in the
circulation and become LDL which has a longer lifespan (average 2.5 days) because of the lower affinity of apoB for the LDL-receptor (Mahley & Rall, 1995). The majority of the LDL particles in the blood are taken up by LDL-receptor in the liver (Bilheimer et al, 1978), and alterations in the functional activity of the LDL-receptor leads to a longer LDL half-life and thus elevated plasma cholesterol levels as seen in patients with FH.

Fig 1.1: The three main areas of lipoprotein metabolism; exogenous and endogenous lipoprotein pathways and reverse cholesterol transport. (Source: Havel & Kane, 1995).
LDL is the major cholesterol-rich lipoprotein, carrying approximately 70% of plasma cholesterol. LDL particles have a much lower triglyceride content than its precursor VLDL and only one apolipoprotein is present, apoB-100. Plasma LDL-cholesterol (LDL-c) concentration is carefully regulated by its synthesis and catabolism. An adult on a low cholesterol diet typically synthesises about 800mg of cholesterol per day.

The final pathway in lipoprotein metabolism is reverse cholesterol transport (Fig 1.1) whereby cholesterol surplus to cellular requirements is returned from the peripheral cells to the liver for excretion (reviewed by Barter & Rye, 1996). The free cholesterol is esterified by the plasma enzyme, lecithin cholesterol acyl transferase (LCAT) and enters the hydrophobic lipid core of the HDL particle. The cholesteryl esters on HDL are rapidly transferred to acceptor lipoproteins, VLDL and LDL, by cholesterol ester transfer protein (CETP).

1.5. The hyperlipidaemias
Hyperlipidaemias were assigned to five main classes by Fredrickson, Levy & Lees in 1967, on the basis of increased levels of VLDL, LDL, chylomicrons or a combination of these. Primary hyperlipidaemias are characterised by an elevated level of serum cholesterol (mainly LDL) and/or triglycerides. Secondary hyperlipidaemias are commonly caused by diseases such as Diabetes mellitus, hypothyroidism and certain drugs. Clinical characteristics may be specific to one form but there is considerable overlap with most biochemical and clinical findings. The goals set in the European Atherosclerosis Society (EAS) guidelines (Wood et al, 1998) for blood lipids are total cholesterol levels below 5mmol/l and LDL-c levels below 3mmol/l. Concentrations of HDL and triglyceride are not used as goals of therapy but HDL levels below 1mmol/l in men and fasting triglycerides above 2mmol/l are markers of increased coronary risk.

1.5.1. Chylomicronaemia (Type I and V)
Chylomicronaemia comprises two forms of hypertriglyceridaemia, fat induced (type I) and mixed (type V). The two are clinically distinguishable, the symptoms, abdominal colics, xanthomas and hepatosplenomegaly appear in childhood in type I and in adulthood in type V (see review Fojo & Brewer, 1992). Additional characteristics are found in type V, diminished glucose tolerance and hyperuricaemia, as it is a more complicated disorder associated with diabetes, other diseases and also with excessive alcohol consumption.

Type I is biochemically characterised by high triglycerides (>11.4mmol/l) with
moderately elevated cholesterol. The underlying biochemical defect in type I is a selective deficiency of either endothelial lipoprotein lipase (LPL) or apoCII (reviewed by Fojo & Brewer, 1992) resulting in the slow removal of triglycerides from the plasma. Type I has an autosomal recessive pattern of inheritance with an incidence of less than 1:1000000. Over 50 LPL mutations but only a few APOCII mutations have been reported to date (reviewed by Brunzell, 1995). Type I patients require a fat restricted diet to lower their triglycerides to below 4.5mmol/l.

Massive hypertriglyceridaemia (11.5-23mmol/l) and marked hypercholesterolaemia is present in type V. The defect in type V is unknown although the two genes which are defective in type I are not the cause (reviewed by Brunzell, 1995). This form occurs in genetically predisposed individuals and is induced by a high-calorie intake of fat and/or carbohydrates. It is transmitted as a polygenic disorder with an estimated incidence of 1:5000. Treatment in type V individuals includes drastic reduction of calorie intake, replacement of dietary fat by protein and medium chain fatty acids plus treatment with fibrates and nicotinic acid. Together these usually produce marked and long-term improvement but the administration of heparin and plasmapheresis is often required to reduce the risk of pancreatitis (reviewed by Brunzell, 1995).

1.5.2 Familial hypercholesterolaemia
Familial hypercholesterolaemia is described in detail in section 1.6.

1.5.3. Familial combined hyperlipidaemia
Familial combined hyperlipidaemia (FCHL) is probably the most common genetically determined disorder of lipoprotein metabolism associated with CHD, with a prevalence of 1-2%, and is estimated to cause 10-20% of premature CHD. Within a family, several members will have CHD, mild hyperlipidaemia and often obesity and hyperinsulinaemia, but the more severe clinical signs, such as xanthelasma (small xanthomas under the eyes) and corneal arcus (clouding of the cornea), are rare. It is characterised by elevated levels of LDL-c (>4.9mmol/l), triglycerides (>2.3mmol/l) or both. Different patterns of hyperlipidaemia are often found within a family which make diagnosis difficult when examining only one member in clinic.

Although a dominant mode of inheritance was originally proposed (Goldstein et al, 1973) recent studies have questioned this simple mode of inheritance (Xu et al, 1994; Wijsman et al, 1998), and the genetic basis of the disorder has not been completely determined. The first FCHL locus, 1q21-23, was recently reported from the isolated Finnish population (Pajukanta et al, 1998). This is near the gene for ApoAll but
defects in APOAII were found to be not involved (Pajukanta et al, 1998). The syntenic region in the mouse was also recently found to be involved in combined hyperlipidaemia (Castellani et al, 1998). Additional putative loci influencing FCHL in Finnish families have recently been identified (Pajukanta et al, 1999).

1.5. Familial dysbetalipoproteinaemia (Type III)
This metabolic disorder is also known as remnant hyperlipoproteinaemia and is relatively rare, with an incidence of 1 in 5-10000 (reviewed by Durrington & Morrell, 1998). The coronary risk to affected patients is very high, but once diagnosed, good results can be obtained by lipid lowering therapy. Patients usually present with xanthomas of varying severity and location, although palmar xanthomas are specific to this disorder. Vascular complications commonly occur in the fourth or fifth decade. Biochemical diagnosis is suggested if there is moderate to marked elevation of cholesterol (7-20mmol/l) and triglycerides (3.5-9mmol/l). 90% of familial dysbetalipoproteinaemia patients are homozygous for the E2 allele but the causative gene is unknown (Feussner et al, 1997).

1.5.5. Familial hypertriglyceridaemia (Type IV)
This is the most common of hyperlipoproteinaemias, characterised by increased concentrations of VLDL which can be induced by a high carbohydrate diet. It does not usually manifest until after the age of 30. Clinical signs include xanthomas, abdominal crises, abnormal glucose tolerance, obesity and often a family history of diabetes mellitus. Triglycerides are below 11.5mmol/l and cholesterol is within the normal range which only increases if there is marked increase in VLDL. Diagnosis is by the presence of hypertriglyceridaemia in several family members and the absence of other lipoprotein phenotypes in the family. Familial hypertriglyceridaemia is thought to be genetically heterogeneous, and various biochemical defects can lead to isolated hypertriglyceridaemia (discussed by Wijsman et al, 1998).

1.6. Familial hypercholesterolaemia
1.6.1. History of FH and the low density lipoprotein receptor
In the 1930's an association was found between hypercholesterolaemia, xanthomas and premature atherosclerosis (Muller, 1938; Thannhauser et al, 1938). Muller concluded that FH is transmitted as a single gene determined autosomal dominant trait and this was substantiated in the 1950's (Wilkinson et al, 1950; Adlersberg et al, 1955). In 1964, Khachadurian showed that two forms of FH existed in the Lebanese population, the heterozygous form and the severe homozygous form. The link between FH and the
increase in the concentration of LDL-c was found using analytical ultracentrifugation (Gofman et al, 1954; Fredrickson, Levy & Lees, 1967).

The major breakthrough was in 1973, by Brown & Goldstein. They discovered the LDL-receptor and showed that uptake of LDL by the LDL-receptor could inhibit cholesterol synthesis in fibroblasts but HDL uptake could not, and that the inhibitory effect was absent in fibroblasts in patients who had homozygous FH (Goldstein & Brown, 1973). They went on to shown that the human LDL-receptor was a cell surface glycoprotein that contains approximately two asparagine-linked (N-linked) oligosaccharide chains of the complex type and 18 serine/threonine linked (O-linked) oligosaccharide chains, two thirds of which are clustered on one region of the protein. They showed the LDL-receptor was responsible for cholesterol uptake into cells via receptor mediated endocytosis of cholesterol-rich lipoproteins secreted by the liver (Goldstein & Brown, 1979). There are two ligands for the LDL-receptor, apoB100 and apoE. ApoB100 is a 400kDa glycoprotein, the sole protein of LDL, whereas the 34kDa apoE is found in multiple copies in triglyceride-rich lipoproteins and their remnants such as IDL and a subclass of HDL. ApoE is able to bind with up to 20-fold greater affinity to the LDL-receptor than those containing only one copy of apoB (Mahley, 1988).

Once the LDL-receptor and its gene had been fully characterised (Sudhof et al, 1985), it was possible to show that mutations in the structural or promoter regions of the gene were responsible for many cases of FH (Hobbs et al, 1990).

1.6.2. Mode of inheritance and population prevalence
FH is inherited as an autosomal dominant trait (Khachadurian, 1964) and has been found in all studied populations. In genetically heterogeneous populations such as Western Europe and North America the estimated frequency of heterozygotes is ~1 in 500, based on the Hardy-Weinberg equation and on the frequency of homozygotes (reviewed by Goldstein, Hobbs & Brown, 1995), placing FH among the most common single gene disease known. The homozygous form is far less common, 1 in a million. The relatively high world-wide frequency of FH is probably due both to a high mutation rate and to the fact that the majority of heterozygotes survive beyond reproductive age. There may be a selective advantage of carrying an LDLR mutation as shown in LDLR knock-out mice, who are able to fight infection better (Netea et al, 1996).
1.6.3. Clinical characteristics of FH

FH is expressed from birth in most cases as increased plasma total and LDL-cholesterol levels. As shown in Table 1.2 LDL-c levels are 2-3 fold higher in FH heterozygotes due to the extended half-life of plasma LDL from a normal value of approximately 2.5 to 4.5 days. LDL-c levels in FH homozygotes are 4-6 fold above normal levels due to a half-life of up to 6 days. Also in FH subjects, VLDL levels are unchanged but HDL levels are slightly lower and IDL levels are increased (Kwiterovich, Fredrickson & Levy, 1974).

Table 1.2: The mean and range of plasma lipid concentrations in FH heterozygotes, homozygotes and normal individuals.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Total cholesterol (mmol/l)</th>
<th>LDL-c (mmol/l)</th>
<th>VLDL (mmol/l)</th>
<th>HDL (mmol/l)</th>
<th>IDL (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-30</td>
<td>19.4 (15.5-29.5)</td>
<td>16.1</td>
<td>0.5</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>30-40</td>
<td>8.8 (7.0-12.9)</td>
<td>7.4</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Normals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-40</td>
<td>5.2 (3.3-6.8)</td>
<td>3.2</td>
<td>0.5</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>0-19</td>
<td>4.0 (3.1-6.0)</td>
<td>2.5</td>
<td>0.3</td>
<td>1.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

FH values come from various sources whilst the normal values are for UK and US whites (Kwiterovich, Fredrickson & Levy, 1974; Myant et al, 1990). IDL values are from Soutar et al, 1982). Sex differences are omitted.

There are two forms of homozygous FH which are clinically indistinguishable. The first form is when two copies of one mutation are found (true homozygotes) and this is often observed in countries where a founder gene effect occurs or where consanguinity is common. The second form, compound heterozygotes, are when two different LDLR mutations are found on the two chromosomes. Both forms of homozygous FH patient have severe hypercholesterolaemia leading to deposition of LDL in skin, tendons and arterial walls. Skin xanthomas begin to appear in infancy and tendon xanthomas are usually present in the Achilles tendon by the age of ten, whereas they are normally observed in heterozygous FH individuals only from the age of 20 and upwards.

FH carries an increased risk of CHD (Simon Broome Steering Committee, 1991). Myocardial infarctions occur during the first decade of life in homozygotes and generally in the fourth or fifth decade in heterozygotes although sometimes as early as
the third decade. Coronary events can be delayed by medical intervention available today. Lipid-lowering drugs are administered to FH homozygotes but they usually only result in moderate decreases LDL-c. There are only two means of greatly reducing the plasma cholesterol, LDL-apheresis and liver transplantation (section 1.12). The eldest biochemically diagnosed homozygote was a 57-year old Japanese man who fathered one son, and angina was only first noted at the age of 52. The second eldest was also a Japanese man, aged 50 years old. Despite his longevity he had angina from the age of 20 and three MI’s from the age of 38 years. FH does not reduce reproductive fitness but homozygotes rarely reproduce, one exception is a 36-year old Japanese woman who was homozygous for a null allele and had four children (reviewed by Goldstein, Hobbs & Brown, 1995). An explanation for these exceptions could be the lower incidence of CHD in the Japanese population due to their dietary habits which are rich in fish oils (section 1.3.2.8).

1.6.4. Factors affecting the FH phenotype

LDLR mutations result in elevations of the plasma total and LDL-cholesterol but individuals with the same mutation show a wide range of cholesterol levels and onset of clinical characteristics. Genetic and environmental factors interact to modify the phenotype. The risk factors previously described (section 1.3) affect everyone, including FH individuals but genetic factors also explain some of the variation. A few of these have been identified from large epidemiological studies in healthy individuals and at-risk groups.

1.6.4.1. Apolipoprotein E genotype

ApoE plays a central role in the metabolism of cholesterol and triglycerides. It is found in chylomicrons, VLDL, their remnant particles and HDL. ApoE acts as the ligand for the uptake of these particles by the LDL-receptor (reviewed by de Knijff & Havekes, 1996). There are three major and over 30 rare apoE variants in humans. The three major isoforms are apoE2, apoE3 and apoE4 and they are encoded by three codominant alleles, APOE2, APOE3 and APOE4 respectively, found at a single gene locus, APOE, on chromosome 19q13.2. They differ by a single base causing an amino acid substitution of C112R or R158C (APOE2: C112, C158; APOE3:C112, R158; APOE4:R112, R158). In healthy individuals 5-15% of variation in plasma cholesterol levels can be attributed to this common polymorphism (Sing et al, 1985). In most populations individuals carrying the apoE2 isoform display high levels of triglyceride and low levels of total and LDL-cholesterol due to defective binding of the apoE2 to the hepatic lipoprotein receptors which suppresses LDL production. The opposite is found in carriers of the apoE4 protein which has a greater binding efficiency. These
associations with plasmid lipid levels are not true of all populations, as shown in the Japanese (Eto et al, 1989). Explanations for these differences between populations are unknown, although gender, ethnic origin, lifestyle and diet are candidate factors. Mouse models may allow the factors to be investigated, as large epidemiological studies would be required to dissect the variation in humans with careful planning to compensate for these factors. In 1993 (Corder et al, 1993), it was shown that the E4 allele might influence the expression of Alzheimer's disease (AD). Ethical consideration must be taken into genetic testing for the E2 allele in dyslipidaemias as an E4 allele can indicate a risk to Alzheimer's disease, thus the presence or absence of an E2 allele is reported rather than stating the exact APOE genotype (Post, 1996). At the present time the presence of an E4 allele is thought to indicate the age of onset of Alzheimer's disease in combination with other factors rather than be a direct risk factor (Meyer et al, 1998).

1.6.4.2. Lipoprotein (a)

In a study of 367 family members of 30 South African and 30 French-Canadian index patients with FH (Lingenhel et al, 1998), Lp(a) levels were three-fold higher in FH heterozygotes and there was a larger variation of Lp(a) concentrations. This elevation was not due to the higher frequency of apo(a) isoforms with smaller number of repeats but that Lp(a) levels were elevated for each APO(a) genotype examined (Lingenhel et al, 1998).

The effect of LDLR mutation on Lp(a) levels was thought to be due to Lp(a) containing apoB100. Hence LDLR defects might result in a delayed catabolism of Lp(a) in the same way as it does for LDL. The three-fold increase in Lp(a) levels is similar to the two-fold increase typically observed in LDL-c caused by LDLR mutations (Goldstein, Hobbs & Brown, 1995). Several other interpretations however, are more likely. The role of LDLR in Lp(a) catabolism has been a subject of continuous debate. In vitro binding studies have generated conflicting results. One study in transgenic mice for the human LDL-receptor (Hofmann et al, 1988) has suggested that LDL-receptor does contribute to LDL catabolism in vivo. In contrast, in vivo turnover studies of Lp(a) have found identical decay curves in FH subjects and healthy controls suggesting that the LDL-receptor is not involved in Lp(a) removal (Rader et al, 1995). Evidence supporting that the LDL-receptor does not clear Lp(a) also comes from drug studies, where HMG-CoA reductase inhibitors lower plasma LDL-c levels but do not affect Lp(a) levels (Thiery et al, 1988; Kostner et al, 1989).

The observations of higher Lp(a) levels in FH patients conflicts with in vivo turnover
studies and HMG-CoA reductase inhibitor effects. One proposed explanation was that
the LDLR mutation on Lp(a) may be indirect rather than direct and is on synthesis rather
than on catabolism. This is supported by kinetic studies of Lp(a) in FH patients after
LDL apheresis (Lasuncion et al, 1993).

The conflicting results in the literature regarding the influence of LDLR mutations on
Lp(a) levels is thought to be due to the difficulties in detecting a minor gene effect in the
presence of a major gene effect. The major gene, APO(a) determines >90% of the
variation of Lp(a) levels in Caucasians (Boerwinkle et al, 1992) so this must be
considered when searching for additional factors.

1.6.4.3. Apolipoprotein CIII genotype
The gene APOCIII lies in a cluster of three apolipoprotein genes AI-CIII-AIV on
chromosome 11q23. ApoCIII is a component of VLDL and HDL and influences the
metabolism of apoB-containing lipoproteins. ApoCIII inhibits LPL activity and
therefore reduces the hydrolysis of triglyceride-rich particles which accounts for the
raised levels of triglyceride (reviewed by Talmud & Humphries, 1997) and thus
increased CHD risk. A C>G variation at 3238 in the 3'UTR of APOCIII was found to
be associated with increased triglyceride levels. The polymorphism is in the 3'UTR so
it is unlikely to be of functional significance but may act as a marker of a functional
mutation that has yet to be found, even though the initial association was found 15
years ago (reviewed Talmud & Humphries, 1997).

1.6.4.4. MTHFR C677T polymorphism
Plasma homocysteine levels vary between individuals and elevated levels are associated
with CHD (Froost et al, 1995; Boushey et al, 1995). The determinants of plasma
homocysteine concentration are both environmental as well as genetic (Kang et al,
1996). Mutations in the cystathionine β-synthase gene and 5,10-methylene
tetrahydrofolate reductase (MTHFR) gene cause severe hyperhomocysteinaemia and the
recessive syndrome of homocystinuria in the homozygous state (reviewed by Fletcher
& Kessling, 1998). Biochemical studies of patients with CHD have identified a form
of MTHFR with 50% of control activity at 37°C and marked thermolability at 46°C,
now termed the thermolabile variant. A missense mutation C677T resulting in an
alanine to valine substitution at codon 114 codes for the thermolabile enzyme which is
found in ~35% of the general population (Gudnason et al, 1998). This variant has been
shown to be a major cause of mildly elevated plasma homocysteine levels (reviewed by
Fletcher & Kessling 1998). Some studies have shown that homocysteine levels are
only elevated in T677 homozygotes if their serum folate levels were below average.
(Jacques et al, 1996, Harmon et al, 1996) indicating that folate supplementations in combination with B6 and B12, may overcome the effect of the thermolabile variant (Malinow et al, 1997).

1.6.4.5. ACE I/D polymorphism
The ACE DD genotype has been suggested to increase the risk of CHD (reviewed by Bauters & Amouyel, 1998). The effect of ACE genotype was studied in 213 adult FH/FDB patients. The odds ratio associated with the DD genotype was 2.57 for MI and 2.21 for CHD so it is associated with an increased risk of MI and CHD (O'Malley, Maslen & Illingworth, 1998).

1.6.4.6. Other genetic factors
Despite these known genetic factors there are some families where differences in lipoprotein levels between subjects carrying the same LDLR mutation cannot be explained. The presence of a cholesterol-lowering gene has been suggested to explain why some heterozygous LDLR mutation carriers have shown normal cholesterol levels whilst non-carriers have had levels below the 5th percentile (Hobbs et al, 1990; Kotze et al, 1993; Sass et al, 1995). Various genes were analysed but they either did not segregate with the suppresser phenotype (Hobbs et al, 1990) or were found to segregate with APO(a) (Kotze et al, 1993) or APOE (Sass et al, 1995).

1.6.5. LDL-receptor protein
1.6.5.1. Synthesis
The LDL-receptor is synthesised in the rough endoplasmic reticulum (ER) as a precursor (Tolleshaug et al, 1982) that contains high-mannose N-linked carbohydrate chains and the core sugar, N-acetylgalactosamine, of the O-linked sugars (Cummings et al, 1983). The O-linked sugars are added before the mannose residues of the N-linked chains are reduced. The receptor precursor is approximately 120kDa but after 30 minutes of synthesis the LDL-receptor decreases in mobility on sodium dodecyl sulphate (SDS)-polyacrylamide gels (Tolleshaug et al, 1982). The molecular weight increases to 160kDa and this change occurs at the same time as the conversion of the high mannose N-linked oligosaccharide chains to the complex endoglycosidase H-resistant form (Cummings et al, 1983). Also each O-linked chain is elongated by the addition of one galactose and one or two sialic acid residues (Cummings et al, 1983). These changes in carbohydrate content cannot cause the large increase in molecular mass, therefore the increase must be due to conformational changes in the protein during the elongation of the O-linked sugars (Cummings et al, 1983; Davis et al, 1986).
The receptor mediated clearance of LDL is represented in Fig 1.2. LDL-receptors appear on the cell surface 45 minutes after synthesis and they gather in clathrin coated pits (Goldstein, Anderson & Brown, 1979). The coated pits invaginate to form coated endocytic vesicles and the clathrin coat quickly dissociates. Multiple endocytic vesicles fuse together to create endosomes. The pH of the endosome falls below 6.5 due to the adenosine triphosphate pump driving protons through the membrane, and the acid conditions cause the LDL particle to dissociate from the receptor (Helenius et al, 1983). The receptor then returns to the cell surface where it binds another lipoprotein particle and another round of endocytosis is initiated (Basu et al, 1981; Brown et al, 1983). Each LDL-receptor makes one cycle every 10 minutes whether it contains an LDL particle or not (Basu et al, 1981). The LDL dissociated from the receptor is delivered to the lysosome when the endosome and lysosome membranes fuse. In the lysosome the protein component of LDL is hydrolysed to amino acids (Goldstein & Brown, 1974) and the cholesteryl esters are hydrolysed by a lysosomal acid lipase (Goldstein et al, 1975).

1.6.5.2. Regulation of plasma cholesterol
SREBP's (sterol regulatory element binding protein) regulate transcription of genes involved in the cholesterol biosynthetic pathway and the fatty acid biosynthetic pathway. Two members of the family have been identified by cDNA cloning (Hua et al, 1993; Yokoyama et al, 1993), SREBP-1 and SREBP-2. These genes produce at least three proteins SREBP-1a and SREBP-1c (splicing variants) and SREBP-2. They have a similar structure, an NH2-terminal transcription factor domain, a middle hydrophobic region containing two transmembrane segments, and a COOH-terminal regulatory domain. The NH2-terminal domain must be released from the membrane so that it can enter the nucleus. This release has been studied most extensively for SREBP-2. The proteolytic release of the mature SREBP involves two sequential cleavages two sites (Sakai et al, 1996). In sterol depleted cells, cleavage occurs at site 1, which lies between the leucine and serine in the sequence RSCL/S in the lumenal loop (Duncan et al, 1997) and is absolutely dependent on the presence of a positively charged residue (R or L) at the fourth position. Cleavage at site 1 breaks the covalent bond between the two transmembrane domains of SREBP-2. The second cleavage occurs in the transmembrane domain and is not dependent on sterols. It only occurs after the first cleavage and requires the presence of the tetrapeptide, DRSR, which is immediately adjacent to the transmembrane domain (Sakai et al, 1996). Once released, the NH2-terminal mature form of SREBP enters the cytosol and translocates to the nucleus, where it activates the transcription of SREBP-responsive genes.
Defects at either of these sites result in phenotypic abnormalities when cells are deprived of cholesterol; cells fail to induce cholesterol biosynthesis enzymes such as HMG-CoA synthase and HMG-CoA reductase, fail to induce LDLR and other sterol repressed genes and require unsaturated fatty acids in addition to cholesterol for cell growth. Therefore the released cholesterol suppresses the transcription of HMG-CoA reductase, inhibiting the de novo synthesis of cholesterol and activating acyl CoA:cholesterol acyl transferase (ACAT) which facilitates the reesterification of cholesterol to cholesterol ester. Thus the LDL-receptor pathway is a closely regulated system by which cells acquire cholesterol and cellular cholesterol homeostasis is maintained.

1.6.5.3. Homology to other proteins
The LDL-receptor has great similarity with several other proteins. Parts of the EGF homology region are also found in blood clotting proteins (factor IX, factor X and protein C) whilst the cysteine-rich repeat unit of the ligand binding domain is found in complement component C9 (Sudhof et al, 1985; Stanley et al, 1985). In each of these proteins the shared sequences are encoded by complete exons. This indicates that the LDL-receptor protein is a member of at least two families (Sudhof et al, 1985).

Fig 1.2: Receptor mediated clearance of LDL (Source: Goldstein Hobbs & Brown, 1995).
1.6.6. Low density lipoprotein receptor gene

1.6.6.1. Structure

The human low density lipoprotein receptor gene (LDLR) was cloned in 1982 (Russell et al., 1983; Yamamoto et al., 1984) and characterised shortly thereafter (Sudhof et al., 1985). It is ~45kb long and is localised on the short arm of chromosome 19, bands p13.1-13.3 (Francke et al., 1984). It is divided into 18 exons and 17 introns corresponding to the five domains of the protein (Sudhof et al., 1985) (Fig 1.3).

The mRNA is 5.3kb long, 2.5kb represents the 3’ untranslated region encoded by the 3’ end of exon 18, while the remainder encodes a protein of 860 amino acids which is reduced to 839 amino acids in the mature form (Yamamoto et al., 1984). The first exon encodes a short untranslated 5’ region and 21 hydrophobic amino acids that comprise the signal sequence. Exons 2-6 encode the ligand binding domain of seven cysteine rich repeats each consisting of 40 amino acids. Exons 2, 3, 5 and 6 each contain one repeat, whereas the much larger exon 4 encodes three repeats. Exons 7 to 14 encode the 400 amino acid sequence, which has 35% homology with the epidermal growth factor (EGF) precursor (Sudhof et al., 1985). This domain contains a further three cysteine repeats, A, B and C, and each repeat is again encoded by a single exon, exons 7, 8 and 14 respectively. The A and B repeats are separated from the C repeat by five copies of a 40-60 amino acid repeat (encoded by exons 9-13) and each of these five repeats consists of a conserved motif, Tyr-Trp-Thr-Asp (Davis et al., 1987a). Exon 15 encodes a sugar binding domain of 58 amino acids rich in serine and threonine residues, many of which are the attachment sites for O-linked carbohydrate chains. Exon 16 and the 5’ end of exon 17 encode 22 hydrophobic amino acids which constitute the membrane spanning domain. The 3’ end of exon 17 and the 5’ end of exon 18 encodes the 50 amino acids forming the cytoplasmic domain. The cytoplasmic tail contains a tetrameric signal sequence, Asn-Pro-Val-Tyr, that targets the LDL-receptor to the coated pit and is conserved in LDL-receptors from six animal species (Chen et al., 1990).

The 5’ flanking region of LDLR contains the cis-acting DNA sequences necessary for promoter activity and sterol regulation (Osborne et al., 1987; Dawson et al., 1988; Goldstein & Brown, 1990; Smith et al., 1990). They are located in a sequence 200bp upstream of the transcription start site. There are three 16bp direct imperfect repeats, two TATA boxes and a cluster of mRNA initiation sites which are required for initiation of transcription at the correct nucleotide (Sudhof et al., 1987; Smith et al., 1990). All of these elements are essential for full promoter activity. Repeats one and three contain high affinity binding sites for Sp1 (Goldstein & Brown, 1990), an abundant positive
transcription factor, but they themselves are not sufficient for high-level expression and require the action of repeat two (Smith et al, 1990). Repeat two does not bind Sp1 but includes a 10bp sequence known as the sterol regulatory element (SRE-1) which is required for sterol mediated regulation (Smith et al, 1990) (section 1.6.5.3). This element enhances transcription when cellular sterols are low, and its effect is abolished when sterols accumulate within cells (Goldstein & Brown, 1990; Smith et al, 1990). A similar element is found in the genes for other proteins involved in cholesterol metabolism including HMG-CoA reductase and HMG CoA synthase (Osborne et al, 1987; Goldstein & Brown, 1990).

Fig 1.3: Model of the estimated arrangement of the five domains of the LDL-receptor.

1.6.6.2. Founder gene effects
A founder gene effect occurs when one or a few individuals carrying a mutation moves to an isolated community. As the population expands the number of individuals carrying the particular mutation increases over time without dilution by new mutations (rare) or by further immigration events, leading to a high frequency of the mutation in that area. Founder effects are present for many different diseases in several genetically distinct populations which have been isolated by geographical distance, ethnic group or
religion. For example the ΔF508 mutation in the Cystic fibrosis transmembrane regulator gene (CFTR), arose roughly 52,000 years ago, in a population genetically distinct from any present European group, and spread throughout Europe in chronologically distinct expansions, which are responsible for the different frequencies of ΔF508 in Europe (Morral et al., 1994). Two mutations, C4446T and 8765delAG in the respective breast cancer-susceptibility genes, BRCA1 and BRCA2, occur in 68% of breast cancer and breast/ovarian cancer families from Quebec (Tonin et al., 1998).

For FH several such founder gene effects have been reported. The current population of the Transvaal Province in South Africa stems from a few Dutch, German and French founder families, about 2000 individuals, who settled in the Cape in the 17th & 18th centuries. The fertility rate was high and the population grew dramatically to over 3 million (reviewed by Goldstein, Hobbs & Brown, 1995). The frequency of FH in this group is 1 in 100 compared to 1 in 500 in most European populations and three mutations, D206E, V408M, D154N are found at frequencies of ~65, ~20 and 5% respectively (Kotze et al., 1991). The Ashkenazi Jewish population of Johannesburg, South Africa, who descended from a genetically isolated population of ~40,000 Lithuanian Jews who emigrated to South Africa, have a frequency of FH of 1 in 67 (Seftel et al., 1989; Meiner et al., 1991). The deletion of codon G197 is present in 80% of Jewish patients and this is likely attributable to their origin (Seftel et al., 1989). In Lebanon the frequency of heterozygous C660X carriers is 1 in 170 (Lehrman et al., 1987a) which is due not only to a founder effect but also due to a high incidence of consanguinity. Other examples include the French-speaking population of the Canadian province of Quebec where five mutations make up a population prevalence of 1 in 270 (Leitersdorf et al., 1990), with one mutation, >15kb deletion from the 5' flanking region to intron 1, accounting for 60% of LDLR mutations (Hobbs et al., 1987). Finally in Finland, where FH occurs at the incidence of 1 in 500, the geographical isolation has lead to 26-58% of patients carrying a 9.5kb deletion of ex16-18 (Aalto-Setala et al., 1992).

1.6.6.3. LDLR mutations
To date there have been over 700 LDLR mutations characterised at a molecular level, (Wilson et al, 1998, http://www.ucl.ac.uk/fh) but only approximately 130 have had in vivo characterisation which has included LDL-binding and uptake, receptor biosynthesis, receptor transport and gene structure. Mutations can be divided into three categories, point mutations, deletions and insertions involving 1-25bp and major rearrangements.
1.6.6.3.1. Point mutations
Point mutations make up the largest class of mutations and they are summarised on the FH website (http://www.ucl.ac.uk/fh). Point mutations can exert their effect on the receptor protein as premature termination codons, single amino acid substitutions or splice site mutations. Point mutations involving the transition C to T at CpG dinucleotides are known to be a common cause of genetic polymorphism and disease causing mutations (Cooper & Youssoufian, 1988), and 16% of the first 200 reported LDLR point mutations had occurred at these sites (Hobbs et al, 1992).

1.6.6.3.2. Deletions/duplications/insertions of less than 25bp
A number of deletions or duplications of under 25 nucleotides have been observed and these are either in frame, and result in the deletion of one more amino acids, or cause a frameshift that results in a protein that is normal until the position of the frameshift and then includes a number of incorrect residues, usually less than twenty, before a premature stop codon occurs. This mutant protein sometimes can be detected in cells but it usually fails to accumulate due to instability at the mRNA level.

1.6.6.3.3. Major rearrangements
The third class of mutations are the major rearrangements, deletions or insertions, ranging from, 25bp to over 20kb (Fig 1.4). A minimum estimate of the prevalence of major rearrangements in patients from genetically heterogeneous populations is 5%, since a proportion of the subjects may not have a mutation in LDLR (Horsthemke et al, 1987; Hobbs et al, 1988; Langlois et al, 1988; Sun et al, 1992) and the majority of these are unique to that family. The frequency of major rearrangements was determined in the UK population by studying a sample of 200 patients with a clinical diagnosis of heterozygous (189) or homozygous (11) FH, attending lipid clinics in the London area. They were screened for the presence of LDLR rearrangements by Southern blotting of genomic DNA. Major rearrangements were found in 4.5% of FH cases which accounted for 10% of all the identified mutations, although mutations were only identified in ~45% of cases (Sun et al, 1992). This study confirmed the heterogeneity of FH in the UK population. In contrast, two deletions occur at a high frequency in Finland and Quebec, Canada due to founder gene effects; a 9.5kb deletion of exon 15-18 (FH-Helsinki) is found in ~60% of Finnish FH patients (Aalto-Setala et al, 1989) whilst a 15kb deletion of the promoter and exon 1 (FH-French Canadian-1) is found in 60% of French Canadian FH individuals (Hobbs et al, 1987).

Despite entire exons being deleted or duplicated, the remaining protein is often undisrupted since many of the splice junctions occur at the same position in the reading
Deletions are often due to the misalignment of Alu repeats. The Alu family of short interspersed repeated elements appears to be present in all mammals and are thought to make up about 5% of the human genome (900,000 copies) with an average spacing of 4kb (Hwu et al, 1986). Human Alu sequences are ~300bp in length and consist of two related sequences each between 120-150bp long, separated by an A-rich region. They are often flanked by AT rich direct repeats. The function of Alu sequences is unknown although Ullu & Tschudi, 1984, proposed that they may be degenerate forms of 7SL RNA which have then been reverse transcribed and integrated into the genome. Other reports have shown that Alu sequences are transcribed by RNA polymerase II or III (Kariya et al, 1987) and indeed the Alu sequences do contain an internal RNA polymerase promoter (Jelinek & Schmidt, 1982; Saffer & Thurston, 1989).

There are over 28 Alu sequences in LDLR. This gives an average distribution of one Alu sequence per 1.6kb of gene which is two fold more frequent than the average region of the genome. A high frequency is also found in the human C1 inhibitor (Carter et al, 1991) where there is a greater frequency of Alu recombination events; this was later also found to be true for LDLR. Alu sequences are found in introns 2 to 8 and intron 12 to 17 and the 3' untranslated region of LDLR (Yamamoto et al, 1984) and the majority of deletions are found in these regions. As a result of several Alu sequences in some introns, a rearranged chromosome may be deleted for the same exons but have different breakpoints, for example a 5kb deletion of exon 5 found in an American and a Danish FH patient was shown to have identical 3' but different 5' breakpoints (Rudiger et al, 1991b).

Alu sequences involved in deletions may be orientated in the same or opposite directions and situated a large distance apart. Several models have been suggested for the mechanism of such deletions. One mechanism is the mispairing and unequal crossing over between two Alu sequences that are in the same direction on different chromosome homologues. This mechanism may explain the small group of deletions located at the 5'end of LDLR which delete the promoter and transcription start site region (French Canadian-1, FH-Bologna-1 and FH-Siracusa), therefore preventing the transcription of the deletion-bearing allele (Hobbs et al, 1987; Lelli et al, 1991a; Garuti et al, 1996). An example of this mechanism in the formation of a duplication is shown in the 14kb duplication of exon 2 to exon 8 which is predicted to involve Alu elements located in introns 1 and 8 (Lehrman et al, 1987b).
Fig 1.4: Locations of published LDLR major rearrangements. Insertions are marked \( \triangledown \). The sizes of the deletions and insertions are indicated above the respective lines. Sizes are not drawn to scale.
A second mechanism in which a deletion could arise is as a result of a homologous recombination involving two Alu sequences on a single chromosome homologue (Lehrman et al, 1985). It is assumed that when the DNA strands are separated during DNA replication, a stem-loop structure is formed between the 5’ Alu sequence and the oppositely oriented 3’ Alu sequence. A staggered break is then introduced by an unknown mechanism and the fragment between the breaks is cleaved and destroyed. The single stranded ends of the break fold back on themselves and complementary residues would be made. During this process a single nucleotide may be needed to fill in a gap. The two Alu sequences would then be ligated together to form a continuous strand (Lehrman et al, 1985). This mechanism is thought to explain the 5kb deletion of exons 16 to 18 and the 5kb deletion of exons 13 to 15 observed in two FH patients (Lehrman et al, 1985 & 1986).

1.6.6.4. Functional mutation classes
LDLR mutations have been grouped into five classes based on biosynthetic and functional studies of fibroblast cell lines derived from skin biopsy specimens (Brown & Goldstein, 1986; Hobbs et al, 1990). These classifications are summarised in Fig 1.5 and discussed below, but some mutations produce proteins which can fall into more one class. For example, many transport-defective receptors are also binding-defective. Promoter and intronic mutations are functionally categorised and also classified separately.

1.6.6.4.1. Class 1 mutations: null alleles
Class 1 mutations fail to produce immunoprecipitable LDL-receptor protein, null alleles. The most frequent types are nonsense and frameshift mutations, and these are randomly distributed among the exons. They produce mRNA that is normal in size, but reduced in concentration (Hobbs et al, 1988). The lack of protein may be due to rapid turnover of mRNA or accelerated degradation of the truncated receptor protein. Other Class 1 alleles contain deletions that include the promoter region and no mRNA or protein is produced. The final type in this class is when mutations generate abnormally sized mRNAs, and these consist of termination mutations, splicing mutations, single nucleotide deletions and large deletions (Goldstein, Hobbs & Brown, 1995).
Fig 1.5: Functional classes of LDLR mutations.

<table>
<thead>
<tr>
<th>Class of mutation</th>
<th>Synthesis</th>
<th>Transport from ER to Golgi</th>
<th>Binding of LDL</th>
<th>Clustering in coated pits</th>
<th>No recycling</th>
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<tbody>
<tr>
<td>1</td>
<td>X</td>
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<td>✓</td>
<td>✓</td>
<td>X</td>
</tr>
</tbody>
</table>

Each class of LDLR mutation affects different regions of the gene and thus interferes with different steps in the process by which the receptor is synthesised, processed and translocated. (Based on figure from Goldstein & Brown, 1986 & Hobbs et al, 1990).

1.6.6.4.2. Class 2 mutations: transport defective alleles

Class 2 mutations are the most common LDLR mutations. The normal LDL-receptor is synthesised in the ER as a partially glycosylated precursor with approximate molecular weight 120kD that is then transported to the Golgi where the N- and O-linked sugars are processed to give the mature LDL-receptor protein of 160kD (Goldstein et al, 1985). Class 2 alleles encode proteins which are blocked between the ER and Golgi apparatus, either completely (class 2A) or partially (class 2B). Only the 120kD protein is seen in fibroblasts in the class 2A category. Two thirds of these mutations are
located in the ligand binding domain and the remaining third in the EGF precursor homology domain (reviewed by Goldstein, Hobbs & Brown, 1995). The transport defective alleles found in the ligand binding domain usually result from missense mutations or short in frame deletions leading to the misfolding of the protein. Some in frame insertions in this region have also been classified into this group such as insertion of amino acids 200-207 (Hobbs et al., 1992). A cluster of class 2 mutations occur in repeat 5 of the ligand binding domain (exon 4) which unlike the other repeats binds two ligands, apoB and apoE (Russell, Brown & Goldstein, 1989). Mutations in LDLR that produce proteins completely blocked in transport (class 2A) are mainly found in the EGF homology region, where the structure is easily disrupted by a single amino acid change (Goldstein, Hobbs & Brown, 1995).

1.6.6.4.3. Class 3 mutations: binding defective alleles
These mutant receptors are synthesised, transported to the cell surface but are unable to bind LDL normally. The mutations, in frame insertions or deletions, cluster in the exons of the ligand binding domain and in the cysteine-rich repeats of the EGF precursor homology domain. Each of the seven repeats act independently on LDL-binding. Repeat 1 (exon 2) seems to have no effect on LDL-binding (apoB) or IDL-binding (apoE). The other repeats are all important for LDL-binding but not IDL-binding with the exception of repeat 5 (exon 4) which binds both LDL and IDL (Russell et al., 1989). Thus the extent of the binding defect is determined by the repeat in which the mutation is found, such that a patient who can clear IDL efficiently (mutation in repeat 1, 2, 3, 4, 6, 7, A, B, C) may have lower plasma cholesterol levels than a patient whose defective receptor does not clear IDL and LDL efficiently (mutation in repeat 5). For example fibroblast cells carrying a deletion of repeat 6 (exon 5) (FH-Paris 1) produces a receptor that appears to bind apoE containing lipoproteins (β-VLDL) with normal affinity but fails to bind apoB-containing lipoproteins (LDL) (Hobbs et al., 1986).

1.6.6.4.4. Class 4 mutations: internalisation-defective alleles
The mutant receptors of class 4 transport to the cell surface, bind LDL normally but fail to cluster in clathrin coated pits and therefore cannot be internalised and carry bound LDL into the cell. There are two subclasses of class 4, mutations that only alter the cytoplasmic domain (class 4A) and mutations that involve two regions, the cytoplasmic domain and the membrane spanning region (class 4B) (Hobbs et al., 1990). The residue at codon 807 in the cytoplasmic domain must be occupied by a tyrosine, phenylalanine, or tryptophan (Davis et al., 1987b) which forms part of a 12bp sequence, Asp-Pro-Val-
Tyr, that targets the LDL-receptor to the coated pit allowing internalisation. The tyrosine is highly conserved across six species (Chen et al, 1990).

Class 4B produce truncated receptors that lack the membrane spanning domain and the cytoplasmic tail. Most of these protein molecules are secreted from the cell but 10% remain attached to the cell membrane, where LDL is bound but not internalised. Class 4B mutations include three deletions extending from intron 15 to exon 18 (Lehrman et al, 1985; Davis et al, 1986). Splicing did not remove the truncated intron 15 so translation continued into intron 15 creating 55 novel amino acids at the carboxyl-terminal end (Lehrman et al, 1985). This sequence did not have the Asp-Pro-Val-Tyr signal so the mutant receptors failed to be internalised.

1.6.6.4.5. Class 5 mutations: recycling-deficient alleles
Normally the LDL-receptor endocytoses the bound LDL and then the LDL and receptor dissociates. The receptor is recycled whilst the LDL is broken down into its constituents in the lysosome. LDL-receptors are recycled every 10 minutes with a lifespan of about 24 hours (i.e. 140 cycles) (Basu et al, 1981). Mutations in class 5 encode receptors that bind and internalise ligands in coated pits, but fail to release the ligands into the endosome and thus fail to recycle back to the cell surface. Class 5 mutations cluster in the 5' end of the EGF precursor homology domain, especially in the first Try-Trp-Thr-Asp repeat separating repeat B from C. This domain mediates acid-dependent dissociation of receptor and ligand in the endosome, which enables recycling to occur, and failure to do this results in the degradation of the receptor-ligand complex and the recycling process is halted (Davis et al, 1987a).

1.6.6.5. Promoter mutations
To date, ten naturally occurring mutations have been found in the transcriptional regulatory elements of LDLR and they all involve one of the two binding sites for the Sp1 transcription factor. DNA samples from 350 heterozygous FH patients were screened for promoter mutations and none were detected (Top et al, 1992) and only one has been detected in 791 FH individuals studied by London (Day et al, 1997b). A 3bp deletion, CTT, involving the -92C of the consensus sequence of repeat 1 (FH Pedi-2), which is adjacent to an Sp1 binding site, was found in a compound heterozygote who also carried a frameshift mutation at codon 37 in exon 2 (Hobbs et al, 1992). Functional assays performed on cultured fibroblasts from this homozygote revealed that the amount of LDL-binding, uptake and degradation rate was ~10% of normal (Hobbs et al, 1992; Peeters et al, 1998). Another promoter mutation, C to T substitution at position -43, co-segregated with hyperlipidaemia in six family members (Koivisto et al,
1994) and electrophoretic mobility shift and DNase-1 footprinting assays indicated that the point mutation abolished binding of Sp1 transcription factor to repeat three of the promoter. The transcriptional activity of the mutant LDL-receptor promoter was about 5% of the wild-type promoter, as judged by transfection in HeLa cells. Fibroblast studies in one of the affected individuals revealed a markedly reduced LDL-receptor mRNA concentration as well as reduction of binding, internalisation and degradation of ¹²⁵I-LDL to values below 50% of those in normal fibroblasts (Koivisto et al, 1994). In contrast, the T-45C mutation in the proximal Sp1 binding site in repeat 3 resulted in a reduced transcriptional activity of only 43% of normal and the binding affinity for Sp1 was only reduced not abolished (Sun et al, 1995). This may be explained by the substituted nucleotide occurring in base which is not strongly conserved in the Sp1 binding consensus sequence.

1.6.6.6. Splice site and intronic mutations
Many splice site mutations have been identified in the nucleotides -2 to +6 of the 5’ splice site and -6 to +2 in the 3’ splice site in LDLR (FH website). Base substitutions at the invariant 5’ splice donor GT and 3’ splice acceptor AG are likely to be pathogenic by disrupting splicing, but the other splice site nucleotides are less conserved (reviewed by Krawczak, Reiss & Cooper, 1990; Cooper & Krawczak, 1995). The pathogenicity of substitutions around the splice sites have to be confirmed, as several LDLR polymorphisms are found in these regions, e.g. G1060+10C, T1061-8C, C1359-29T (FH website). Co-segregation analysis, mRNA analysis (to show loss of splicing or the involvement of cryptic splice sites) or cell studies provide some confirmation.

Outside of these 16 intronic nucleotides there has been one LDLR mutation, 25bp upstream of the 5’ splice site of exon 10, shown to be pathogenic, the deletion of 9 bases and insertion of 5 unrelated bases (Webb et al, 1996). This mutation is found in the Lariat structure of the splicing mechanism. The mutation destroys the only consensus sequence for a splicing branch point in intron 9 and analysis of mRNA from cells from the patient showed that it results in the retention of intron 9 or, more rarely, in the use of cryptic splice sites in exon 10. Functional studies confirmed reduced LDL-receptor activity. It has only been found in one FH patient but has not been seen in any normal individuals, and these findings together indicate it is a mutation rather than a silent variant (Webb et al, 1996).

1.6.7. Haplotype studies
There are many LDLR mutations that have been reported in two or more unrelated individuals from the same or different ethnic background. Haplotype analysis is carried
out to determine whether it is more likely that the two individuals inherited the same mutant allele from a common ancestor or whether the mutation may have occurred twice. The majority of DNA polymorphisms used for haplotype analysis are non-pathogenic single base-pair changes that were generally found because they introduce or remove a restriction enzyme site, hence the terminology restriction fragment length polymorphism (RFLP). RFLP’s are distributed throughout the genome, approximately every 200-300bp (Botstein et al., 1980; Cooper et al., 1985) and 29 RFLP’s have been described in LDLR which are used for haplotype studies (FH website). Recently two highly informative microsatellite markers were described (Ashworth et al., 1995) which has greatly aided haplotype studies. A tetranucleotide repeat at the locus D19S394 is situated 150kb telomeric to LDLR locus (5’ end of the gene). The heterozygosity value is 0.90 and 18 alleles have been observed (Haddad et al., 1997). The other marker is a dinucleotide repeat, D19S221, which is situated 1Mb from the 3’ end of the gene. D19S221 has 11 allele sizes and heterozygosity of 0.89 (Haddad et al., 1997). The two flanking markers are in strong linkage disequilibrium (D19S394 stronger as expected by its smaller physical distance) with LDLR, as shown by the tracking of the R329X mutation over many generations in FH families from Southampton (Day et al., 1997a), the C163Y mutation in individuals from Glasgow (Lee et al., 1998) and four common mutations found in the Greek population, C152R, S265R, V408M and G528D (Traeger-Synodinos et al., 1998).

1.7. Other genetic causes of monogenic hypercholesterolaemia

1.7.1. Apolipoprotein B mutations

ApoB-100 is the major protein component of LDL and is responsible for the binding of these lipoproteins to the LDL-receptor. The gene for apoB-100 (APOB) is found on chromosome 2p23 (Law et al., 1985), contains 29 exons and 28 introns (Blackhart et al., 1986) and codes for an mRNA of 14121 nucleotides (Chen et al., 1986; Knott et al., 1986). Exon 26, which is 7572bp in length, codes for almost half of the apoB protein (Blackhart et al., 1986). Mutations in APOB, disrupt the binding of LDL to its receptor and results in familial defective apolipoprotein B-100 (FDB). Unlike FH, the majority of FDB cases are caused by a single mutation which results in the substitution of a glutamine for an arginine residue, R3500Q (Innearity et al., 1990). Two more mutations have been observed, R3500W (Gaffney et al., 1995) and R3531C (Pullinger et al., 1995). The R3531C mutation was found to give rise to hypercholesterolaemia but was associated with a milder phenotype than the other two APOB mutations (Pullinger et al., 1995). Extensive searches have not found any other mutations in APOB that cause defective receptor binding of LDL (Pullinger et al., 1999). In European and North

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American populations the frequency of the R3500Q mutation is 2-5% of hypercholesterolaemic individuals (reviewed by Myant, 1993), whilst the other two mutations are rarer, under 0.5% (Gaffney et al, 1995; Tybjaerg-Hansen et al, 1998) although the R3500W mutation was found in 2.4% of Asian hypercholesterolaemic subjects (Tai et al, 1998). The R3500Q mutation occurs at a frequency of 1 in 500 in the normal population.

The region critical for receptor binding was found following site directed mutagenesis and protein analysis. The region spans 30 bases from amino acids 3359 to 3369 (Boren et al, 1998). A model has been proposed (Boren et al, 1998) where the first 89% of apoB-100 enwraps the LDL particle like a belt and the carboxyl terminal 11% constitutes a bow that crosses over the belt. The crossing over brings the carboxyl tail of apoB-100 close to amino acid 3500 but when this residue is changed the interaction is abolished due to the resulting conformational change. Studies replacing different amino acids at residue 3500 demonstrated that it is the arginine that is critical for receptor binding activity and it is conserved in all species that have been sequenced (Boren et al, 1998).

1.7.2. FH3 gene
Monogenic inherited hypercholesterolaemia is most commonly caused by LDLR or APOB mutations but not all cases are accounted for (Lestavel-Delattre et al, 1994; Zuliani et al, 1995 & 1999; Haddad et al, in press). In the study by Haddad both genes were screened in families selected with a strict clinical diagnosis of FH. In five families where no mutation was identified, co-segregation analysis was carried out between the FH phenotype and LDLR, APOB and APOE, using highly polymorphic microsatellite markers and the ApoE isoform. Four of the five families showed no evidence for recombination between LDLR and the hypercholesterolaemia phenotype but in one large family there were numerous exclusions of co-segregation with LDLR, APOB and APOE. Thus another locus was responsible for the hypercholesterolaemia in this family and is termed FH3 (Haddad et al, in press). A genome scan is currently been undertaken in the USA. Further families are being sought but this involves a long process; excluding LDLR and APOB mutations in ‘definite’ FH samples and then tracing family members to enable co-segregation analysis.

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1.8. Mutation detection

1.8.1. Methods to detect known and common mutations

This first class of tests are very useful if a founder effect is present, e.g. ΔF508 in CFTR accounts for ~70% of CF chromosomes world-wide (Morral et al, 1994), or in the cases where a few common mutations are present. These methods are well established in clinical genetic diagnostic laboratories. In FDB, three APOB mutations have been found, R3500Q (Innearity et al, 1990), R3531C (Pullinger et al, 1993) and R3500W (Gaffney et al, 1995). The R3500Q mutation occurs in 2-5% of FH individuals (reviewed by Myant, 1993) whilst in the Caucasian population the other two mutations are much rarer (Gaffney et al, 1995; Tybjaerg-Hansen et al, 1998). The two rare mutations can be tested using restriction analysis. The R3500Q mutation does not create or destroy a restriction site so alternative ways have to be used: artificial restriction creation site PCR analysis, allele-specific oligonucleotide analysis or amplification refractory mutation system assay are all in common use. Each method has its advantages and disadvantages and these are discussed below.

1.8.1.1. Restriction enzyme analysis

The simplest way of detecting a known mutation is when the mutation creates or destroys a restriction enzyme site. Restriction analysis consists of digestion of PCR products with a restriction endonuclease, followed by electrophoresis. The two rare APOB mutations R3531C and R3500W create natural restriction sites with NsiI and NlaIII respectively (Pullinger et al, 1993, Gaffney et al, 1995). Throughput may be increased considerably by the use of microtitre array diagonal gel electrophoresis (MADGE) technology (Day & Humphries, 1994). PCRs and restriction enzyme digests are carried out in 96-well microtitre trays and then loaded, using a 8-channel pipette, onto a thin submersed acrylamide gel, which is laid in the 96-well format. (section 2.6.3).

A modification of this method allows this simple technique to be used when no natural restriction site exists and this is known as an artificial restriction creation (ARC) or 'forced' PCR assay. It utilises mutagenic site PCR, allowing restriction sites to be introduced or abolished by the modification of either or both the sense and antisense PCR primers (Haliossis et al, 1989). Various 'forced' assays have been designed for the R3500Q mutation. One assay, created by Mamotte & van Bockxmeer in 1993, involves two tests to avoid the possibility of false positive and negatives. In the first PCR the antisense primer contains a mismatch that generates a ScaI restriction site in the mutant allele. Any positives are then further tested with a second PCR, where the antisense primer contains a mismatch that generates a Sau96I cut site in the normal
allele. These methods can be automated and again adapted for a high-throughput analysis such as MADGE (Day et al., 1995a).

1.8.1.2. Allele-specific oligonucleotides
This method is a binding assay using pairs of oligonucleotides that are specific to a sequence that includes a variant. Oligonucleotides of 10-14 bases are synthesised, complementary to the wildtype and to the mutant alleles. An allele specific oligonucleotide (ASO) will only anneal to sequences that match perfectly, a single mismatch being sufficient to prevent hybridisation under appropriate conditions. The ASO's are end-labelled with $^{32}$P γATP or with chemiluminescence (Rihn et al., 1995).

The standard ASO assay involves dot blotting the PCR product onto a membrane, hybridising with the specific ASOs and then removing the non-specific hybridisation with increasing stringency washes. Detection is chemically or by autoradiography, depending on the labelling method.

1.8.1.3. Chip technology
A high throughput technology based on the ASO methodology is being developed by the biotechnology company, Affymetrix (Chee et al., 1996) after initial developments by Fodor et al., 1993 and Favor et al., 1995. DNA 'chip' technology involves hybridisation of a DNA or RNA strand(s) to an array of oligonucleotide probes designed to match a specific sequence. Oligonucleotides are generated in situ on a silicon surface by combining DNA synthesis with phosphoramidite reagents modified with photolabile 5' protecting groups. Through selective photoprotection of the 'chip' areas spatial synthesis is accomplished. The arrays are made up of all four nucleotides at each position of a gene, which combine to form multiple arrays of many thousand oligonucleotides which can be tested in a few hours. A specialised confocal microscope scans the multiple arrays for a fluorescent signal. The degree of complementarity between the oligonucleotides and the target sequence determines the identity and amount of the target sequence. DNA chips have not yet been independently evaluated in a diagnostic environment but there have been two pilot trials; one project involved screening the mitochondrial genome (Chee et al., 1996) and it was concluded that one technician could complete 50 mitochondrial genome sequences (i.e.~16,000bp) per day. The second pilot project was to detect BRCA1 mutations (Hacia et al., 1996); the sensitivity for mutation detection was 93% which is predicted to account for all the point, small deletion and duplication mutations in BRCA1, the remaining 7% being major rearrangements. This technique should eventually provide a reliable and efficient method to identify and detect DNA sequences but it requires technology and expensive equipment which is unlikely to be found, in the near future, in a standard laboratory.
1.8.1.4. Amplification refractory mutation system

ASO's may be used for allele-specific amplification of a variable locus and this is called the amplification refractory mutation system (ARMS). The site of interest is enzymatically amplified by PCR only if the particular allele-specific primer is present in the mixture. Oligonucleotides with a 3' mismatched residue will, under appropriate conditions, not function as primers in the PCR. A basic ARMS reaction contains three primers, e.g. one sense primer which can bind to 'normal' DNA, one sense primer which can bind to 'mutant' DNA and a common antisense primer. The 'normal' one is refractory to PCR on 'mutant' template DNA; the mutant sequence is refractory to PCR on 'normal' DNA. Most ARMS assays analyse two mutations (A, B) in two reactions, one contains an ARMS primer specific for the 'normal' allele at site A and a primer specific for the 'mutant' allele at site B whilst the second reaction contains an ARMS primer specific for 'mutant' allele at site A and a primer specific for the 'normal' allele at site B. The two reactions are visualised by electrophoresis on adjacent lanes on an agarose gel. A normal homozygote generates a PCR product from only the normal primer, the heterozygotes from both normal and mutant ARMS primers and a mutant homozygote from only the mutant primer. Thus the two ARMS strategy allows internal controls for normal and mutant sequences. An ARMS test has been used in the analysis of the R3500Q mutation in APOB (Rust et al, 1993) and commonly used to analyse four CFTR mutations (Ferrie et al, 1992). Each of the two reactions tests for both normal and mutant changes. A more recent assay tests for 12 CFTR mutations (Robertson et al, 1998) but since only one normal product is amplified, this increases the risk of false positives and amplification failure.

1.8.1.5. Oligo ligation assay

The oligo ligation assay (OLA) is based on a PCR reaction followed by oligonucleotide ligation. Firstly the fragment of the gene that contains the mutation site is amplified by PCR. Two ASO probes and a third common probe are hybridised to one strand of the PCR product and the 3' ends of the ASO are immediately adjacent to the 5' end of the common probe. This sets up a competitive hybridisation-ligation process between the three probes at each locus. DNA ligase joins the ASO probes that have annealed to DNA targets, distinguishing between complete and incomplete complementarity of the allelic probes to their target template. The common probe is labelled with a fluorescent dye and the allele specific probes are linked to pentaethyleneoxide (PEO) mobility modifying tails. Thus each ligation product can be identified by its electrophoretic mobility and fluorescent colour on an ABI 377 DNA Sequencer. An OLA has been designed for two diseases, FH (Baron et al, 1996) and CF (Brinson et al, 1997). In both cases multiple mutations are screened in one reaction by altering the number of
PEO mobility modifiers (up to 70) and the use of three fluorescent dyes. The maximum possible is 200 mutations screened simultaneously in one lane. The CF OLA screens 31 mutations and 89.9% of mutations are detected in the British Caucasian population (North Thames (East) Regional Clinical Molecular Genetics laboratory data), the highest detection rate for a single test. Twenty LDLR mutations and the R3500Q mutation (APOB) are now screened in the FH OLA allowing detection of the majority of mutations found in the population from Munich in Germany (Baron et al., 1996). From current data this OLA screen would only detect approximately 7% of mutations found in the heterogeneous UK population and 4% of this figure is accounted by one mutation, the R3500Q mutation.

The previous approaches are not suitable for genetic diagnosis of FH where a wide spectrum of mutations occur such as in LDLR where over 700 mutations have been found to date. In the UK the most common LDLR mutation only makes up a maximum of 1% of the total FH cases with three local exceptions explained by founder effects, E80K in UK (Wenham et al., 1998), R329X in Southampton (Day et al., 1997a) and C163Y in Glasgow (Lee et al., 1998) which account for a maximum of 10% of FH cases in these regions.

1.8.2. Methods to detect unknown mutations
There are two sub-divisions of mutation screening, divided according to the type of mutation. The first group is screening for point mutations, small deletions and insertions whilst the second group is screening for major gene rearrangements.

1.8.2.1. Screening for point mutations, small deletions and insertions
1.8.2.1.1. Protein truncation test
The protein truncation test (PTT) is a technique that detects mutations which lead to disruption of the open reading frame (ORF). The PTT technique involves a first round of reverse transcriptase PCR (RT-PCR), a second round of nested RT-PCR and then the products are mixed with a T7-coupled reticulocyte lysate. Transcription and protein translation occur in vitro and 35S-Methionine is incorporated as the label. The protein products are separated on a SDS-PAGE gel and after fixing and soaking in a scintillant, the gel is dried and autoradiographed. A lower molecular weight band is observed if a truncated polypeptide is present.

It has been used to screen for mutations in the dystrophin gene in Duchenne muscular dystrophy (DMD) (Gardner et al., 1995). In DMD the majority of deletions cause a shift in the reading frame (2/3 of all mutations) whilst the remaining 1/3 of mutations are
accounted by >70 minor mutations resulting in the premature termination of translation. RT-PCR/PTT allows the whole coding region to be screened, small structural rearrangements and splice-site mutations are detected by sizing the RT-PCR products, whereas point mutations are determined by the PTT (Gardner et al, 1995). This method is effective, relatively quick and not a particularly labour intensive method to screen for unknown translation terminating mutations. PTT is not a feasible option to test individuals for FH as only a small percentage of the known LDLR mutations alter the ORF and the majority of mutations would be undetected.

1.8.2.1.2. Chemical mismatch cleavage

Chemical mismatch cleavage (CMC) (Cotton, Rodrigues & Campbell, 1988) detects mismatched bases in hybrid duplexes formed between wildtype and mutant DNA strands. The principle is as follows. The PCR product derived from the patient's mutant gene is denatured and reannealed in a 10:1 ratio to radiolabelled homologous sequences from a normal individual. Mutations in the target strands give rise to mismatched C or T nucleotides in the heterodimer and these are then exposed either to hydroxylamine which modifies C residues or osmium tetroxide which acts on C and T bases. The sites of these mismatches are then cleaved using piperidine and the products are electrophoresed on a denaturing polyacrylamide gel and visualised by autoradiography.

CMC detects all types and classes of mutation but since G and A mismatches cannot be identified directly using the sense strand, the antisense strand would detect C and T mismatches, sense and antisense probes must both be used. However, CMC has been shown to have 100% detection sensitivity (Condie et al, 1993). A major disadvantage of this method is the hazardous nature of osmium tetroxide; modifications using potassium permanganate have now been established (CMGS presentation, Leeds 1997). CMC is also rather labour intensive and time consuming, but an advantage is that the size of the cleavage products can give some indication of the location of the mutation. Recently adaptation to fluorescence-based detection systems has changed both the methodology and analysis of CMC (reviewed by Ellis et al, 1998).

1.8.2.1.3. Single-strand conformation polymorphism analysis

The single-strand conformation polymorphism (SSCP) technique (Orita et al, 1989) can identify almost all sequence variations in a single strand of DNA between 150 and 200 nucleotides in length. Under non-denaturing conditions a single DNA strand will adopt a conformation which is unique to its base composition. The mobility of a fragment is dependent on its size, shape and surface charge density as well as the ambient
temperature and ionic strength. Various conditions may therefore be optimised to obtain a high detection rate of 80-90%. These include differing temperatures, differing gel agents (5 or 10% glycerol) and various buffering conditions (Leren et al, 1993a). The ‘classical’ protocol is a denatured sample and $^{32}$P labelling for maximal detection sensitivity. Safer alternatives, such as silver staining (Walzer et al, 1993) or fluorescent detection (Makino et al, 1992), have been established and they also give cleaner, less diffuse bands.

SSCP analysis has limitations; there is an optimal length of 150-250 bases to detect base changes in single strands (Sheffield et al, 1993). Hyashi et al, 1993, claimed a 97% detection rate using SSCP analysis of products of 100-300 nucleotide size but only a 67% rate on products of 300-450 nucleotides. Longer strands exhibit less conformational change by a single base substitution whilst shorter sequences are too short to produce any stable conformations. Heteroduplex bands are often seen on the gel as a useful by-product of the procedure. The major advantage of SSCP is its simplicity and the cheap set up costs, since no specialised primers or equipment is required, but the detection rate is compromised if only one electrophoresis condition is used.

1.8.2.1.4. Denaturing gradient gel electrophoresis
The basic principle of denaturing gradient gel electrophoresis (DGGE) (Cariello & Shopek, 1993) involves the migration of DNA duplexes through an increasing gradient of denaturant until they reach a position where the strands dissociate, and then slower migration occurs. The melting behaviour and electrophoretic migration of a double-stranded DNA molecule depends on its base composition. However DGGE is relatively insensitive to mutations which dissociate in the highest temperature melting domains and this has been overcome with the addition of a GC clamp (tagged onto the 5’ end of one or both primers) (Sheffield et al, 1989) which serves to stabilise the duplex and permits the detection of differences in the melting profile of the remainder of the fragment. Programmes such as MELT 87 are used to determine the theoretical melting profile for the amplicon and thus suitable denaturing gradient gel conditions. DGGE may detect greater than 90% of single base-pair substitutions and seems to be an efficient mutation detection technique (Moyret et al, 1994; Schwaab et al, 1997). However it is less frequently used compared to SSCP analysis; this is mainly due to the cost of specialised equipment and the additional primer cost (GC clamps). The set up time is lengthy and primer design is difficult although this has been eased by the Windows programmes designed by Manolis, 1997 (unpublished).
1.8.2.1.5. Temporal gradient gel electrophoresis
The principle of this technique is the same as that of DGGE but a temperature gradient is set up over a uniform denaturant gel, via a metal plate connected to a heating system at one edge and a cooling system at the other (Henco et al., 1994). Temporal gradient gel electrophoresis (TGGE) requires special apparatus and it has been used less than DGGE due to the difficulty of maintaining an accurate spatial temperature gradient.

1.8.2.1.6. Profile of oligonucleotide dissociation gel electrophoresis
The profile of oligonucleotide dissociation gel electrophoresis (PODGE) is similar to the ASO principle but electrophoresis is the means of separating bound from free oligonucleotide and the temperature is the wash stringency variable (Day et al., 1995b). An oligonucleotide is annealed to a DNA target sequence and its melting profile is determined as the temperature rises over a set range. An oligonucleotide that is a perfect match will remain bound for the longest time and will therefore dissociate last, whilst an oligonucleotide bound to a mutant heterozygote or homozygote will be less stable due to the mismatch and will dissociate first. The advantages of this method include visualisation of the full melting profile of the oligonucleotide, no predetermination of the melting temperature, no prior knowledge of the mutant sequence and multiplexing is feasible. However at present the method requires autoradiographic detection and has yet been used to screen for unknown mutations.

1.8.2.1.7. Programmable melting display analysis
The PODGE apparatus has been adapted for a new technique, programmable melting display (PMD) analysis where it is used in conjunction with the MADGE system allowing high-throughput mutation screening. Multiple MADGE gels can be submersed in buffer in the PODGE apparatus. Over 90 minutes, a temperature gradient is set up over a range of 8°C from the stage at which the DNA is in its native unwound form (63°C) to finishing 1-2°C above the completely dissociated form (71°C). Gels are visualised with ethidium bromide. The PMD technique was tested by analysing exon 3 of LDLR on 791 FH samples which had already been screened by SSCP analysis (Haddad et al., unpublished data). The PMD method identified all the mutations found by SSCP but also more mutations, so the method was effective. However the apparatus is currently not commercially available and the PCR primers require the same GC clamp modification, as DGGE. However a large advantage is that PMD allows a high throughput version of DGGE and TGGE.

1.8.2.1.8. Mass spectrometry
A mutation detection technique involving mass spectrometry is currently being
developed, matrix assisted laser desorption ionisation time of flight (MALDI-TOF) (Fitzgerald & Smith 1995, Shaler et al, 1995). Since each nucleotide has a different molecular mass, point mutations can be characterised by mass spectrometry based on differences in mass. At the present time this technique has only been used to sequence short sections of DNA due to the problems in making the DNA ‘fly’, but it is expected that sequences of greater than 100bp will be soon possible. Multiple genotyping assays are being developed. A disadvantage of the MALDI-TOF mass spectrometry machine is that it is an expensive piece of equipment although it is are now being used in many biochemistry laboratories.

1.8.2.1.9. DNA sequencing
All the previous mutation screening methods do not define the nature and location of the nucleotide changes. Usually DNA sequencing is used to characterise the mutations found by other methods but it may also be used as the primary and sole screening method.

There are two sequencing protocols, the Maxam & Gilbert chemical degradation or chemical cleavage method (Maxam & Gilbert, 1977) or the Sanger enzymatic chain termination method (Sanger, Nicklen & Coulson, 1977). In the Maxam & Gilbert method four different chemical reactions are used in a single tube, cleaving the DNA at a particular base (A, C, T, G) depending on the base type (purine or pyrimidine). Each molecule is cleaved once if the ideal conditions are used and all possible fragment lengths are generated by the four different reactions (Maxam & Gilbert, 1977). In the Sanger method an oligonucleotide primer initiates the synthesis of the new DNA strand from the template DNA at a single site. In contrast to the Maxam & Gilbert method four separate reactions are performed, each containing all four deoxynucleotides, and one of the four dideoxynucleotide analogues. Chain elongation is halted where the dideoxynucleotide analogue is incorporated resulting in different sized fragments each ending in a particular dideoxynucleotide (Sanger, Nicklen & Coulson, 1977). The fragments are resolved by size on polyacrylamide gels for both methods. Sanger sequencing has been adapted to run the products on an automated ABI DNA sequencer by utilising fluorescent technology, either fluorescent primers or fluorescent dideoxy chain terminators. Sequencing is commonly used to confirm and characterise nucleotide changes found by other methods but the costs, time and sensitivity associated with fluorescent sequencing restrict its use as a first approach to mutation detection.

1.8.2.2. Mutation screening for major rearrangements
Screening for major rearrangements is very important in the disorders DMD and Becker
muscular dystrophy (BMD) since ~72% of affected males have gross deletions (65%) or duplications (7%) of one or more exons of the dystrophin gene. The detection of deletions of specific exons in the dystrophin gene is facilitated in DMD/BMD patients by the presence of only one copy of the gene in males, which results in the complete loss of the corresponding PCR fragments. Detection of deletions in carrier females is not easy by multiplex PCR because deletions of exons on one X chromosome are masked by the normal X chromosome. Duplications in both sexes are masked by the extra copy. This is also the case in virtually all FH individuals, whether heterozygotes or compound heterozygotes, where the normal chromosome 19 masks the rearrangement. Many methods have been described to display the quantitative abnormalities in deletion and duplication in DMD carrier females who have one copy of the abnormal gene and one copy of the normal gene: aberrant sized junction fragments have been detected successfully by whole cosmid hybridisation, fluorescence in situ hybridisation, standard Southern analysis with cDNA probes and pulsed field gel electrophoresis. These methods could in theory, also be used to detect the major rearrangements in heterozygous FH individuals.

1.8.2.2.1. Whole cosmid hybridisation
Human genomic DNA is digested, electrophoresed and Southern blotted using standard procedures. Cosmid probe DNA is labelled by random priming and competitive hybridisation with normal DNA is then carried out (Blonden et al, 1989). Whole cosmid hybridisation is performed on standard DNA samples, but this test is restricted to specific regions which are fully characterised, and where overlapping cosmids are available.

1.8.2.2.2. Fluorescence in situ hybridisation
The fluorescence in situ hybridisation (FISH) of deletions is based on chromosomal in situ suppression (CISS) hybridisation with cosmid clones. Multiplex PCR of the dystrophin gene revealed a region, exon 45, which is preferentially deleted in DMD (Wapenaar et al, 1988). Three cosmid clones spanning the region around exon 45 were used to detect deletions on metaphase spreads from PHA-stimulated human lymphocytes (Ried et al, 1990). This approach has now been improved by the development of multi-colour in situ paints allowing simultaneous staining of multiple gene targets, but constraints still remain to the visualisation of the multiple probes in such a small area. FISH is appropriate for detecting deletions/duplications if there is a single deletion/duplication responsible for a large number of patients e.g. the >500kb duplication of the proteolipid protein gene (PLP) found in patients with Pelizaeus-Merzbacher (Woodward et al, 1998). In a conditions such as FH, however multiple
probes would be needed to localise a major rearrangement in an FH patient, since deletions and duplications are distributed throughout the gene.

1.8.2.2.3. Southern analysis

The standard current method of diagnosing major rearrangements in most disorders is Southern analysis (Southern, 1975). There are several approaches based on this technique but the basic form detects the formation of junction fragments using a combination of restriction enzymes and cDNA probes. For a complete analysis of the dystrophin gene up to seven different subcloned cDNA probes are used on at least two different restriction digestions of genomic DNA (Koenig et al, 1987) and more digests and a similar number of probes are required for LDLR analysis (Sun et al, 1992). False negatives may occur as junction fragments are not always produced and small deletions or duplications (<100bp) are not visible. This method is lengthy and a large number of blots are needed to determine the extent of the mutation; large quantities of DNA are therefore required. The detection rate can be increased by the use of pulsed field gel electrophoresis (PFGE) (den Dunnen et al, 1987) but a drawback to this technique is that fresh blood samples are required as white cells are separated from whole blood, washed and embedded in high quality agarose blocks. High molecular weight DNA is then prepared in situ by treating the blocks appropriately (Bustamante, Gurrieri & Smith, 1993).

Qualitative analyses are preferable to quantitative tests since the presence or absence of an aberrant fragment or hybridisation signal gives a definitive diagnosis of carrier status. An exception is germinal mosaicism where the mutation may be present in germine cells but not in somatic cells as seen in female carriers of DMD (Bakker et al, 1989).

Quantitative Southern blots can be carried out to detect a difference in LDLR dose, although in the case of FH they have only been used in duplication analysis (Bertolini et al, 1994). Quantitative analysis is based on dosage difference observed between the control loci and the deleted/duplicated loci. A constant amount of starting material is required and care is required at all steps to obtain blots of sufficiently good quality for reliable dosage analysis. Although deletions (normal:deletion ratio 2:1) may often be seen by eye, dosage densitometry is required for more accurate detection and for detection of duplications (normal: duplication ratio 2:3). Each sample should be analysed in duplicate with adequate controls.
1.8.2.2.4. PCR based methods

Quantitative analyses are technically demanding and time consuming. Fresh samples are required for most methods; PFGE, CISS, FISH and therefore cannot be applied in most cases where stored DNA is available. PCR based methods are increasingly used since they are quicker and more sensitive. At present there are three PCR techniques which could be applied: RT-PCR, long PCR and quantitative multiplex PCR.

1.8.2.2.4.1. Reverse transcriptase PCR

Amplification of ectopic transcripts, reverse transcriptase PCR (RT-PCR), can detect abnormal sized products indicative of either a genomic deletion or duplication, a splice-site mutation or a cryptic splice site (reviewed by Freeman et al, 1999). Overlapping nested sets of primers are used to amplify the region of interest using total RNA extracted from peripheral blood lymphocytes. Products are visualised directly on ethidium bromide stained agarose gels and further characterised by sequencing. To determine gross rearrangements in DMD/BMD carrier females ten overlapping reverse-transcription/nested PCRs were used to obtain DNA fragments spanning the entire coding region of the dystrophin gene (Roberts et al, 1991).

1.8.2.2.4.2. Multiplex PCR

With the knowledge that most deletions in DMD are clustered in two regions, examination of only a subset of the 79 exons is sufficient to detect a majority of deletions. Chamberlain et al, 1988 described a series of genomic PCR primers that amplify six exons deleted in many DMD/BMD patients. This multiplex PCR analysis allows rapid deletion detection on small quantities or suboptimal samples of genomic DNA. Additional exons were added to the multiplex which resulted in a deletion detection rate of about 80%. A further multiplex was then designed (Beggs et al, 1990) which, when combined with the original set, brought the detection rate to over 98%.

1.8.2.2.4.3. Long PCR

The most successful long PCR methods combine standard PCR buffer and thermal cycling profiles, with a two-polymerase system to provide optimal levels of both processive polymerase activity and proof-reading 3' to 5' exonuclease activity. The two thermostable enzymes are usually Taq and Pwo DNA polymerase. Fragments of up to 30kb can be amplified under optimal conditions (Cheng et al, 1994), although under 20kb is more common (Cohen, 1994). The entire LDLR, except for intron 1, can be amplified in two fragments of 16.1 and 20kb by long PCR (Rodningen & Leren, 1996). Novel major rearrangements can be detected by long PCR but the long PCR
sets are generally developed to detect specific mutations found in particular countries (Rodningen et al, 1996; Peeters et al, 1997).

1.8.2.2.4.4. Quantitative multiplex PCR
Quantitative multiplex PCR has advantages over the previous methods in that the exact exons deleted or duplicated can be determined without further analysis and rearrangements of less than 100bp are detectable. Additional advantages include the speed and the low quantity of starting material, which are particularly important for prenatal diagnosis.

The first quantitative multiplex PCR assays for DMD was reported by Abbs & Bobrow, 1992 and Ioannou et al, 1992. The assay designed by Ioannou et al, 1992 consisted of the two previously designed multiplex sets (Chamberlain et al, 1988; Beggs et al, 1990) but only 18 cycles of amplification were carried out with the incorporation of $^{32}$P-labelled dCTP. The products were electrophoresed on an agarose gel and quantified by X-ray densitometry.

Six years after the first multiplex PCR, accurate diagnosis of carriers of deletions and duplications in DMD and BMD was possible by quantitative fluorescent multiplex PCR analysis (Yau et al, 1996). Two fluorescent multiplex assays, a 5' and a 3' set, amplify 25 exons of the dystrophin gene. The PCR products are accurately sized and quantified by a fluorescent DNA sequencer after only 18 cycles of amplification with one of each exon primer labelled with the same fluorescent tag. The amount of product amplified from each exon in a multiplex is divided by that from each of the other exons, and this ratio is compared with those from control samples to obtain a series of dosage quotients. The number of copies of each exon is thus determined (Yau et al, 1996). The assays can be used to screen at risk female carrier relatives for a major rearrangement.

1.9. Treatment of adults with FH
Drug therapy reduces LDL-c concentrations in patients with hypercholesterolaemia (Grundy, 1988) and known CHD and this leads to a reduction in total mortality and in cardiovascular events without a concurrent increase in any non-cardiovascular causes of morbidity or mortality (Simon Broome Steering Committee, 1991 & 1999). Several treatments are used in the management of patients with FH and they either act on lowering plasma cholesterol, triglycerides or have an effect on both.
1.9.1. Drugs which lower plasma cholesterol alone in FH

1.9.1.1. Anion-exchange resins

Two resins, cholestyramine and colestipol, have been used clinically over the last 20 years. They are administered orally and bind to bile acids in exchange for chloride ions in the intestine which prevents bile acid reabsorption. Depletion of the bile acid pool promotes a compensatory increase in bile acid synthesis, a depletion in the hepatic pool of cholesterol, an increase in hepatic cholesterol biosynthesis and an increased expression of LDL-receptors on hepatocyte membranes. The increased LDLR expression increases LDL catabolism, leading to a decreased number of LDL particles in plasma and a decrease in LDL-c (20-30%) and apoB concentrations. A slight increase in HDL is seen but an elevation in plasma triglycerides also occurs.

Resins come in a granular formula and require mixing with water or fruit juice prior to ingestion. Compliance can be a problem as the resins are fairly unpalatable and also produce gastrointestinal side effects of bloating, flatulence and constipation. Tablets have been produced to overcome the major drawback of these agents (reviewed by Betteridge & Morrell, 1998).

1.9.1.2. Probucol

Probucol treatment results in a mean 10% reduction of LDL-c and an increase in HDL levels of 15-20% and triglyceride levels are not affected, although variable results are observed (Nestruck et al, 1987; Eto et al, 1990). Problems associated with this form of treatment include gastrointestinal upset. Probucol therapy can be justified as an adjunctive therapy as it can reduce the size of xanthomas in FH homozygotes (Baker et al, 1982; Feher et al, 1993).

1.9.1.3. Plant sterols

The long-term use of sitostanol-ester margarine as a substitute for part of normal dietary fat has favourable effects. Its effects have been investigated since the 1950’s with renewed interest over the last five years. Sitostanol is not absorbed and lowers serum cholesterol by changing dietary fatty acid composition to less saturated and richer in monoeneres and by causing cholesterol malabsorption (Gylling, Radhakrishnan & Miettinen, 1997). It does not appear to interfere with the absorption of fat soluble vitamins. The margarine was found to reduce total and LDL-cholesterol respectively by 10 and 14% in mildly hypercholesterolaemic individuals (Miettinen et al, 1995). The effect of sitostanol margarine was also investigated in postmenopausal women who have had an MI (Gylling, Radhakrishnan & Miettinen, 1997). Rapeseed oil margarine reduced serum total and LDL-cholesterol by 8 and 15% after 7 weeks but the addition
of sitostanol ester in the margarine resulted in a further 5% reduction on both forms of cholesterol. A second group of women who were currently on statin treatment were additionally given the sitostanol margarine for 12 weeks. Simvastatin monotherapy lowered total and LDL-cholesterol by 23% and 35% but the margarine resulted in a further reduction of 9% and 11% respectively (Gylling, Radhakrishnan & Miettinen, 1997).

Therefore the substitution of sitostanol ester margarine for a portion of normal dietary fat is suitable as a strategy to reduce serum cholesterol in the population. The margarine, marked as Benecol, has recently been made available in the UK and other European countries, so further studies will be undertaken in the future.

1.9.2. Drugs which lower plasma cholesterol and triglyceride in FH

1.9.2.1. HMG-CoA reductase inhibitors (statins)

These agents are specific competitive inhibitors of the microsomal enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) which catalyses the cholesterol synthesis rate-limiting conversion of HMG-CoA to mevalonate. The inhibition of hepatic cholesterol synthesis by these drugs stimulates the expression of LDL-receptors resulting in the increased uptake of LDL and thus a reduction in plasma LDL-c. LDL production is also reduced by the increased VLDL clearance and decreased VLDL production altering the size of the LDL particle produced to one which has a greater affinity for the LDL-receptor. As apolipoprotein B is a major component of LDL, its plasma concentration also falls (discussed by Raal et al, 1997).

A number of large clinical intervention trials have been conducted over the last decade, designed specifically to study the impact of lowering plasma cholesterol concentrations on cardiovascular events. These have examined the effectiveness of lipid-lowering therapy in primary prevention of CHD (Shepherd et al, 1995; Downs et al, 1998) and in secondary prevention of mortality in patients with established coronary disease (Pedersen et al, 1996, LIPID Study Group, 1998; Plehn et al, 1999).

The first large trial, the Scandinavian Simvastatin Survival Study (4S) (Pederson et al, 1996) involved 4444 patients, aged 35-70 years (82% men) with CHD (79% with previous MI and 21% with angina). The treatment goal was to decrease cholesterol levels from 5.5-8.0mmol/l to 3-5.2mmol/l by starting simvastatin treatment at a daily dose of 20mg and increasing to 40mg if needed (37% of cases). Patients were treated for 5.4 years and a 30% reduction in mortality was observed. A second statin trial included patients who had sustained an acute MI, the Cholesterol and Recurrent Events
(CARE) trial (Plehn et al, 1999). Pravastatin was given at a fixed daily dose of 40mg for 5 years to 4159 patients (80% men). Patient’s cholesterol levels were lower than in the 4S study, less than 6.2mmol/l but unlike the 4S study no dramatic decrease in mortality was observed, only 9%. In contrast to the CARE trial but in line with the 4S trial, the Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) study found a 33% decrease in mortality over six years (LIPID Study group, 1998). In this trial 9014 patients, who had suffered an acute MI or unstable angina, and whose cholesterol was in the range 4.0-7.0mmol/l were placed on pravastatin. From these three large studies it is clearly evident that patients with CHD and cholesterol levels of 5mmol/l and above will experience a benefit from statin therapy.

The second group of statin trials investigated the benefit of statin therapy in primary prevention. Two large studies have been completed, the West of Scotland Coronary Prevention Study (WOSCOPS) (Shepherd et al, 1995) and the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) (Downs et al, 1998). In WOSCOPS a daily dose of 40mg pravastatin was given for 4.9 years to 6595 moderately hypercholesterolaemic men (mean cholesterol 7.0mmol/l) aged 45-64 years. The relative risk of a first MI or death from CHD was reduced by 31% which was similar to the 4S trial despite the average level of CHD risk being a third less. Similar results were achieved in AFCAPS/TexCAPS (Downs et al, 1998) involving 6605 participants (85% men) with mean serum cholesterol levels of 5.5mmol/l who randomised to either lovastatin (20-40mg/day) or placebo.

Studies have been carried out on statin dosages which generally range from 10-80mg/day. High doses of simvastatin in homozygous FH patients was studied by Raal et al, 1997. In a controlled, double blind dose escalation study the efficacy of simvastatin at doses of 80 and 160mg/day (administered in three doses) was evaluated. Twelve patients were randomised to two groups, group one took 80mg/day simvastatin and group two took 40mg/day for nine weeks. The dose in group one was then increased to 160mg/day whilst group two remained on 40mg/day for an additional nine weeks. Group one achieved a reduction in LDL-c by 25% on 80mg/day and this was lowered by a further 31% on 160mg/day. LDL-c in group two was only reduced by 14% (Raal et al, 1997).

Another new HMG-CoA reductase inhibitor, atorvostatin, has been preclinically and clinically evaluated. Results from patients randomised in early efficacy and safety studies were pooled in one database (Bakker-Arkema et al, 1997). Hypercholesterolaemic patients (n=131) were treated with either 2.5, 5, 10, 20, 40 or
80mg/day of atorvostatin or placebo. A dose-dependent response in LDL-c reduction was observed from 22% at 2.5mg to 57% at 80mg/day and it was well tolerated with a safety profile identical to the other statins. An advantage of atorvostatin is that it seems to preferentially lower those lipid and lipoprotein components most elevated in each disease state, LDL-c in patients with FH, triglycerides and VLDL in patients with hypertriglyceridaemia and all three; LDL-c, VLDL and triglycerides in hyperchylomicronaemia.

The statins pravastatin, lovastatin and simvastatin are compounds of microbial origin, whereas fluvastatin and atorvostatin are synthetic compounds. These five statins are therapeutically used in a dose range of 10 to 80mg/day but a new statin has been synthesised, cerivastatin sodium, which demonstrated its efficacy at significantly lower dosages, 0.1-0.3mg (reviewed by Bischoff et al, 1998). Cerivastatin has a very high affinity for HMG-CoA reductase and a high affinity for the liver, the target for the drug action. Cerivastatin has been administered to over 2700 patients with primary hypercholesterolaemia and the data has been pooled to test the efficacy, safety and tolerability of this novel statin (Stein, 1998). Cerivastatin achieved significant dose-dependent reductions in LDL-c of between 14.2 and 36.1% (0.025-0.4mg/day), accompanied by significant reductions in total cholesterol and triglycerides, and an increase in HDL (Stein, 1998). The use of an ultra-low dose has the general potential in avoiding or minimising drug-drug interactions, which is of particular importance for patients in a multiple drug therapy. The studies have shown that at 1% of the doses of other statins, cerivastatin is a safe, well tolerated and highly effective HMG-CoA reductase inhibitor.

Statins have been shown to have a beneficial effect on the entire lipid profile of hypercholesterolaemic patients with significant dose-dependent reductions in total cholesterol and triglycerides together with modest increases in HDL. They are convenient to take and are generally well tolerated with few adverse effects. Statins have become the most successful drug treatment in lowering total plasma cholesterol over the last decade although the effects of HMG-CoA reductase inhibitors has not been investigated in placebo controlled trials in FH individuals as it is unethical to treat the group of patients with a placebo.

1.9.2.2. Combination drug therapy
Resins continue to have an important role to play in combination therapy, in that their effects are additive to those of the statins, fibrates and nicotinic acid. They do not contribute to any additional risk of serious side effects of the other drug. If LDL-c fails
to decrease sufficiently on monotherapy, normally statins, then combination therapy, commonly resin and statin, can often improve lipid levels, although LDL-apheresis may be an alternative (section 1.12.2).

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1.10. Treatment of paediatric individuals with FH
The US National Cholesterol Education Program (NCEP) Expert Panel on blood cholesterol levels in children and adolescents (1992) and the British Hyperlipidaemia Association (BHA) recommendations for screening for hyperlipidaemia in childhood (Neil, Rees & Taylor, 1996) are similar, although the NCEP criteria are more comprehensive whilst the BHA recommendations have a more selective approach. A diagnosis of FH may be given in children and adolescents if LDL-c concentrations exceed:

- 4.9mmol/l on diet therapy if there is no positive family history or less than two cardiovascular risk factors
- 4.0mmol/l when there is a positive family history of premature cardiovascular disease (occurring before 55 years old)
- 4.0mmol/l when two or more other cardiovascular risk factors are present in the child or adolescent after attempts to control them have failed

The use of lipid lowering drug treatment in children remains a matter of judgement for the specialist. Lipid levels are of major consideration but the gender difference in the long-term risk of atherosclerosis must also be taken into account, with females having a lower long-term risk. Other factors to consider include the age at which symptoms of coronary atherosclerosis have developed in other family members and the concentration of various risk factors (high Lp(a) and low HDL).

Published reports have evaluated the efficacy, side-effects and short-term safety of non-systemically acting (resins, sterols) and systemically acting drugs (statins, fibrates, nicotinic acid) in familial hypercholesterolaemic children. Studies have been limited, due to the risk of adverse effects, and there have been a lack of double-blind placebo-controlled treatment periods, and observing the same patients on all varieties of drug treatment. In general the children have had similar reductions in lipids as described in adults (Ducobu et al, 1992; Sinzinger et al, 1992; Stein et al, 1999).

Children have been treated with resins but now more children are now being treated with low doses of HMG-CoA reductase inhibitors, although on a named-patient basis in the UK. Growth and sexual development was not be affected by statin therapy

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Children are being treated prior to puberty and if no side effects are observed then therapy is continued through puberty and into adulthood. One study of simvastatin treatment in 16 heterozygous FH patients tested a mean dose of 16mg/dl (range 10-40mg/dl) over 2 years. Concentrations of total and LDL-cholesterol were reduced by 26% and 37% respectively, when treated with simvastatin and 22% and 28% respectively in the case of lovastatin. Both drugs were well tolerated during the treatment periods and children showed normal growth development (Ducobu et al., 1992). Another study evaluated the efficacy and safety of lovastatin over one year in boys aged 10-17 years with heterozygous FH, from Oregon. Six boys were treated with lovastatin and the remaining six were treated with a placebo. LDL-c concentrations were reduced by 32% in the six boys treated with 10mg/dl (4 weeks), 41% with 20mg/dl (4 weeks) and 45% with 40mg/dl lovastatin (28 weeks) whilst the cholesterol levels remained high in the placebo set. No significant clinical or biochemical side effects were observed during this study and no adverse effects on growth and maturation (Stein et al., 1999).

Recently the effect of the sitostanol ester margarine on serum lipids was investigated in a group of healthy Norwegian children who were already on a diet low in saturated fat (Tammi et al., 1998). The margarine reduced serum total and LDL-cholesterol by 5.2% and 7.3% respectively, which was smaller than the respective decrease in adults (Miettinen et al., 1995) or in children with FH (Becker et al., 1993) but they had lower baseline cholesterol levels.

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1.11. Factors affecting the efficacy of drug treatment

1.11.1. Drug treatment in response to the genetic variation

1.11.1.1. LDLR mutation

*In vitro* studies have shown that the LDL-receptor binding activity can vary from 0-50% of normal (Hobbs et al., 1992). A differential response to drug therapy in FH patients might be related to the residual receptor activity of the abnormal protein which may be determined by the particular mutation present. For example, carriers of ‘mild’ mutations where residual receptor activity is high (10-15% of normal) may have a greater lipid-lowering response than carriers of ‘severe’ alleles, where residual activity is zero and therefore no useful up-regulation would be expected by statin therapy. A few studies have tested this hypothesis, the majority in countries where a founder gene effect is present and where as a result many FH patients have the same LDLR defect (Leitersdorf et al., 1993; Jeenah et al., 1993; Couture et al., 1997; Kajinami et al., 1998) (section 6). However these patients are also likely to share other genes and
environmental factors which may contribute to their phenotype.

1.11.1.2. APOE genotype
Studies on the effect of the APOE polymorphism on the lipid lowering effect of statin treatment have reported mixed results (see section 6). ApoE isoforms are known to have an important influence on plasma LDL-c levels in the general population. E2 allele carriers tend to have higher triglyceride and lower cholesterol levels because the apoE2 protein binds poorly to the LDL-receptor, resulting in a suppressant effect on LDL production (section 1.6.4.1). In contrast, E4 carriers tend to have higher total and LDL-cholesterol due to a reduced LDL clearance as the apoE4 protein binds tightly to the receptor (Mahley, 1988).

1.11.1.3. LDL subfraction
FH individuals tend to have cholesterol-enriched LDL with a high cholesterol:protein ratio. Lipid lowering drugs affect the distribution of LDL subfractions and this may effect the variation in response (Shepherd et al, 1980, Bilheimer et al, 1983). The LDL subfraction profile is no different between FH individuals treated with simvastatin and normal individuals (section 6) (Gaw et al, 1993).

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1.12. Aggressive lipid lowering treatment
LDL-c levels can be lowered in many patients by drug therapy. However, there is a small group of patients that are resistant to diet and drugs. For these patients more radical measures such as partial ileal bypass, portocaval shunt, liver transplantation, plasma exchange (Thompson et al, 1975) and LDL-apheresis (Armstrong et al, 1983; Yokayama et al, 1985) are required to prevent early death from coronary disease. Most homozygous and a few severely affected heterozygous FH patients fall into this category.

1.12.1. Plasma exchange
Plasma exchange was first carried out in 1974 (Thompson et al, 1975). Weekly or biweekly exchanges of the patients plasma for normal plasma or albumin has led to improved survival and regression of coronary artery lesions and xanthomas. Thompson et al, 1985, studied five homozygotes who were treated every 2 weeks for an average of 8.4 years. Patients, on plasma exchange, survived an average of 5.5 years longer than their respective homozygous sibs, who had died without benefit of plasma exchange.
1.12.2. LDL-apheresis

There are two approaches of LDL-apheresis, affinity chromatography with dextran sulphate or antibodies to LDL and selective precipitation with heparin at low pH. The affinity chromatography columns remove apoB-containing lipoproteins including Lp(a) leaving HDL to return to the patient (Yokayama et al, 1985). The alternative method, Heparin Extracorporal LDL Precipitation (HELP), involves precipitation of LDL with heparin (Armstrong et al., 1983). LDL is precipitated from plasma by lowering the pH and removed by filters, the plasma pH is then readjusted and returned. The HELP system can reduce LDL-c levels by 55% and has additional benefits because of its ability to reduce fibrinogen and Lp(a) by approximately 60% (Park, Merz & Braun, 1998) which are risk factors in thrombosis (Thompson et al., 1995; Cremer et al., 1994) a key process in the pathogenesis of CHD.

LDL-apheresis is associated with a few side effects. Chills and flushing are observed after using antibody columns, whilst uncontrolled haemorrhage may occur immediately after dextran sulphate absorption. This is a result of a loss of coagulation factors and is a potential hazard but levels are restored within a day. In the short term, side effects are few and the long term usage indicates that it is a safe method. For example, a female with homozygous FH with an untreated cholesterol of 25mmol/l had been having plasma exchange for 16 years, and at the age of 42 was free of clinical symptoms of CHD (Leren et al., 1993b).

1.12.3. Liver transplantation

The liver contains 75% of the total LDL-receptors in the body because it requires large amounts of cholesterol for secretion into bile, for conversion to bile acids, and for the production of lipoproteins. Transplantation of a normal liver with normal receptors lowers LDL-c levels profoundly in homozygotes. The first case was performed in a six year old girl with homozygous FH (Starzl et al., 1984). She underwent combined liver-heart transplantation and her LDL-c decreased by 81% (Starzl et al., 1984; Bilheimer et al., 1984). Liver transplantation should be considered only for FH homozygotes who are unable to produce any functional LDL-receptors and do not respond to other forms of therapy.

1.12.4. Gene therapy

Homozygous FH is suitable for somatic-cell gene therapy because it is a lethal disorder that is refractory to diet and drug therapies. Measurement of serum lipids provides a convenient endpoint to evaluate response to therapy, and liver transplantation has been shown to correct the underlying dyslipidaemia indicating that hepatic reconstitution of
LDLR expression is sufficient for metabolic correction (Bilheimer et al., 1994). The FH phenotype also occurs in the Watanabe heritable hyperlipidaemic (WHHL) rabbit (Watanabe et al., 1985) and rhesus monkeys (Scanu et al., 1988). The WHHL rabbit has been used to demonstrate one ex vivo gene therapy strategy where genetically modified cultured hepatocytes, carrying an expressible copy of LDLR, were reinjected into the spleen and resulted in cell engraftment in 2-4% of liver cells. Serum cholesterol levels were reduced by approximately 30% and LDLR expression persisted for 2-4 months (Chowdhury et al., 1991). Unfortunately the treated WHHL rabbit still had five-fold higher LDL-c levels than normal rabbits, presumably due to the small percentage of hepatocytes expressing LDLR.

The ex vivo approach involved the removal of 15% of her liver which was perfused with collagenase to disaggregate the hepatocytes and then placed in a medium containing LDLR expressing recombinant retroviruses. The transduced cells were then re-infused into the portal circulation (Grossman et al., 1994). Approximately 20% of cells were predicted to express LDLR, and this resulted in a 17% reduction in LDL-c (13 to 8 mmol/l) which remained stable for 18 months. The treated homozygous FH patient carried two copies of the W66G mutation, which is phenotypically ‘mild’. This method allows the potential of up-regulation of the LDLR, yet the LDL-c reduction was small. The biological reason for the LDL-c reduction was unknown but may have been due to increased expression of the exogenously derived LDLR or the upregulation of the patient’s endogenous receptor activity (Grossman et al., 1994).

The pilot study was extended to a further four heterozygous FH patients ranging in age (7-41 years) (Grossman et al., 1995). Each patient tolerated the procedure and LDL-c levels were reduced in three of the four patients and significantly in two. The poor efficiency of reconstitution in one patient may have been due to the lower quality of the transduced hepatocytes compared to the other patients; they plated at a lower efficiency and were slow to expand in vitro (Grossman et al., 1995). Although ex vivo gene therapy with retroviruses has resulted in preliminary positive results there have been technical difficulties which include surgical complications with the partial hepatectomy, incomplete collagenase digestion and the low titre of viral transduction.

An alternative strategy is an in vivo approach, where recombinant adenoviruses are used as vectors. The approach has been effective in lowering plasma cholesterol levels in rabbit (Kozarsky et al., 1994) and mouse (Herz & Gerard, 1993; Ishibashi et al., 1993) models of FH. A recombinant replication-defective adenovirus vector containing an expressible LDLR cDNA was transfected into hepatocyte cultures. LDLR
expression occurred in 90% of hepatocytes and IDL uptake into the liver was increased (Herz & Gerard, 1993). However, in mice, humoral and cellular immune responses to LDLR developed (Ishibashi et al, 1993) so an alternative strategy of ectopic expression of VLDLR in the liver was tested. VLDLR is homologous to LDLR but has a different pattern of expression and ligand specificity (Kozarsky et al, 1996; Kobayashi et al, 1996). Infusion of replication defective adenoviruses containing mouse VLDLR cDNA corrected the dyslipidaemia in the FH mouse and prevented any immune responses to the transgene, leading to a more prolonged metabolic correction (Kozarsky et al, 1996; Kobayashi et al, 1996). A major limitation of adenoviral vectors is that they are not integrated into the host genome and the gene inserted is eventually lost upon repeated cell division. Administration of adenovirus time after time is not feasible as an immune response to the viral proteins occurs after the initial infusion. Further testing of this strategy for somatic gene therapy is currently being undertaken.

Recently a new model of liver gene therapy has been described which eliminates the problems of low efficiency gene transfer (Overturf et al, 1996). It was expected that a limited number of retroviral transduced cells would have the capacity to form an entire new liver if placed under the appropriate selective pressure. This method was first attempted in the lethal inborn error, hereditary tyrosinaemia type I (HT1), where the gene for the fumarylacetoacetate hydrolase (FAH) is defective (Overturf et al, 1996). Transplantation of hepatocytes from an FAH expressing donor into a HT1 mouse resulted in substantial repopulation of the liver due to clonal expansions of the initially seeded hepatocytes. Results were similar when retroviruses expressing FAH were directly infused into the portal vein. The selective pressure was imposed at the time of reconstitution by withdrawing the drug, NTBC (2-[2-nitro-4-trifluoro-methylbenzylo]-1,3-cyclohexanedione), which blocks tyrosine catabolism upstream of FAH so preventing hepatocyte metabolite build up. A trial is required in humans with HT1 and the encouraging results have brought new hopes to the prospect of liver-directed gene therapy in which the genetically corrected hepatocytes would have a selective advantage.

1.13. Genetic testing for FH

Genetic diagnosis is now possible in many disorders, in the clinically affected individual and in their asymptomatic or symptomatic relatives. The testing of asymptomatic individuals is common practice in medicine, identifying adults with high
blood pressure or diabetes or testing children’s visual impairment, with a view to disease prevention. All new-borns are routinely screened for some rare genetic disorders, phenylketonuria and hypothyroidism, as the disorders can be easily treated in the few that are found to be affected.

Studies have shown that the annual mortality rate of FH patients is greater (relative risk of annual coronary mortality was 4.8-7.2), than in normal individuals (Simon Broome Steering Committee, 1991). Treatment can reduce the risk of CAD by lowering the plasma levels of LDL-c. After six years of lipid lowering therapy the relative risk of annual coronary mortality had decreased to 1.1-2.6 in the Simon Broome study (Simon Broome Steering Committee, 1999). Developments in the treatment of hyperlipidaemias have led to an increasing need for systematic recognition and management of these disorders (EAS guidelines: Pyrola et al, 1994; Wood et al, 1998). During the same period technological advances in genetics has allowed the diagnostic testing of many disorders including FH.

1.13.1. Is FH genetic testing needed?
In many cases, the diagnosis of FH can be made on clinical presentation, xanthomata, very high cholesterol levels, and segregation analysis in family studies. However there are several drawbacks with diagnosing FH solely on clinical characteristics; the onset of some FH features only occur in adulthood, phenotypes overlap and family studies are complicated to perform. The identification of heterozygous FH on biochemical parameters is made complicated in children by the frequent overlap between affected and normal individuals has been shown to occur from birth (Kwiterovich, 1993). An unequivocal diagnosis cannot be given in 5-10% of children (Leonard et al, 1977). The overlap was also shown in a study in the late 80’s where an additional problem was observed. A proportion of young children who were at risk of FH initially presented with lipid levels within the normal range, but in some of these elevated levels then developed at a later stage (Kessling et al, 1990). A diagnosis on cholesterol levels alone is also more difficult to establish in boys than girls because they have lower cholesterol concentrations, but unfortunately it is men who carry the greater risk of CHD and in whom it is most important to make an early diagnosis in order to start lifestyle advice and treatment (Leonard et al, 1977). These findings were extended in a study of an Irish family where 15-20% of family members would have been incorrectly diagnosed with cholesterol testing alone (Ward et al, 1995).

Month-to-month variability of lipids and lipoproteins and apolipoproteins has been observed. In a study of 63 school children (Gidding et al, 1998), LDL-c, HDL, apoB
and apoAI levels showed variability for up to two weeks after an acute infection. Recent infection significantly lowered HDL and apoAI whilst LDL-c was shown to be slightly elevated. Thus total cholesterol, LDL-c and triglyceride levels could be falsely high after an acute infection and give a misdiagnosis.

Low cholesterol levels have also been known in LDLR mutation carriers (section 3) (Hobbs et al, 1989; Koivisto et al, 1992; Kotze et al, 1993; Sass et al, 1995) confirming that biochemical testing alone can result in misdiagnosis (Koivisto et al, 1992). Long term studies will establish if the cholesterol lowering effect is protective for life or if the mutation present will become expressed at a later stage.

Debate continues about whether absolute cholesterol levels or the number of raised cholesterol years is the major risk factor. Genetic testing of children would allow the early opportunity to reduce not only their cholesterol level but also the number of raised cholesterol years through dietary intervention and subsequently drug treatment. The long term outcome of early intervention will become apparent when the paediatric and adolescent cases, identified in the last decade and currently on treatment, reach the age where coronary events may occur.

The identification and characterisation of the mutation may also have relevance in the decision of which treatment an individual should be given. More aggressive treatment may be required in carriers of ‘severe’ LDLR mutations compared to carriers of ‘mild’ LDLR mutations and individuals carrying the R3500Q mutation (FDB) may respond well to small doses of the HMG-CoA reductase inhibitor, simvastatin (section 1.11).

1.13.2. Strategies for genetic testing

Several approaches to screening for hypercholesterolaemia have been suggested, ranging from population-based screening to selective cascade screening. In 1992 the NCEP considered the options of no screening, universal screening, screening when a parent has elevated cholesterol levels and screening when a positive family history of premature cardiovascular disease is present and settled on the last two criteria (selective screening). A selective screening strategy was chosen and the EAS and World Health Organisation (WHO) endorsed this in the same year.

1.13.2.1. Population based screening

Population-based screening focuses on individuals who fall broadly into the categories of increased risk, such as men aged 20-65 and women aged 40-65 years. Universal screening identifies a large number of children with polygenic hypercholesterolaemia
and a small number with single gene disorders. In addition to the expense of population-based screening, longitudinal studies have yet to determine the effect of lipid lowering therapy during childhood on the development and progression of coronary atherosclerosis and the psychosocial effects of identifying a child at risk. Also, there is the consideration of parental attitudes to childhood screening and in a study carried out in Oslo only 40% of parents of FH children agreed to lipid testing of their children (Tonstad, 1996). Thus more research would be needed if universal screening was to be implemented for hypercholesterolaemia.

1.13.2.2. Selective screening for FH individuals
Selective screening aims to identify patients who will benefit most from early treatment, i.e. those with marked elevation of cholesterol due to major inherited disorders of lipoprotein metabolism such as FH. FH (including FDB) satisfy three of the WHO criteria for a worthwhile screening project, the condition can be identified before clinical symptoms are observed, it can be accurately tested and effective treatment is now available. In FH individuals LDL-c levels are usually exceptionally high, are usually present from birth and will persist without treatment. They also may develop premature cardiovascular disease from the age of 20, so identifying these children is far more beneficial than that of finding children with polygenic hypercholesterolaemia. It is hard to find children with FH based on family history, and the effectiveness of the modern lipid lowering therapy means fewer parents of young children with FH will experience premature cardiovascular disease. Thus the disease must be clinically established among adults, facilitating diagnosis and treatment in the next generation. Although a DNA-based screening method allows unequivocal diagnosis of FH, this approach is limited by the mutational heterogeneity of the disease. A combination of cholesterol and genetic testing would be the ideal approach to detect high risk family members in most populations. Once the proband is identified there is a 1 in 2 chance of any first degree relative being affected and 1 in 4 chance for second degree relatives, as opposed to a much lower likelihood of case finding when screening a selected population. It would be even lower if the general population were screened, one positive case every 500 tested. Selective screening can be divided into three classes; screening in populations where a founder gene effect occurs, screening in populations where there are a limited number of mutations or good traceable family records (governmental or religious) and screening in heterogeneous populations.

1.13.2.2.1. Selective screening in populations where a founder gene is observed
The presence of a founder effect confers many advantages for diagnostic purposes. Molecular screening is simplified as a single assay can be designed to identify the
particular mutation or haplotype occurring at a high frequency. Greater success in DNA
detection (i.e. fewer false negatives) can more often be given which result in better
genetic counselling, prevention of cardiovascular complications and treatment. DNA
based FH screening has been applied successfully in South Africa (Kotze et al, 1992)
due to the fact that three founder related LDLR mutations cause FH in approximately
90% of Afrikaners (Kotze et al, 1991) and approximately 15% of FH in patients of
mixed ancestry (Kotze et al, 1995a). Studies have shown that there is a correlation
between phenotypic expression of heterozygous FH in Afrikaner adults and the severity
of the receptor defect (Kotze et al, 1993; Graddt van Roggen et al, 1995). Diagnostic
mutation screening in the South African paediatric population was recently evaluated
with the genotype/phenotype correlation studies being extended to a group of children
with FH (Kotze et al, 1998). Mean total cholesterol levels for the three founder-related
LDLR mutations, D206E, V408M and D154N, were similar, indicating that genetic and
environmental influences, apart from LDLR mutation, are more important in
determining lipid levels in heterozygous children than in adults (Kotze et al, 1998).
Cut-off points were assessed for the best discrimination levels between those with and
without founder-related Afrikaner mutations. Retrospective analysis of molecular and
cholesterol data showed that 7.7% would have been misdiagnosed as FH and 6.9%
would have been falsely classified as normal, based on cholesterol levels. DNA
analysis would therefore be more specific in this founder population (Kotze et al,
1998).

In the province of Quebec in Canada five LDLR mutations account for 76% of FH
cases, 60% of which is due to the 10kb deletion at the 5'end of the gene. Patients are
easily identified as they are live within a small geographical area and therefore close to a
lipid clinic and the molecular analysis is easier due to the low number of mutations
present in the population.

1.13.2.2.2. Selective screening in countries with good documentation
In Iceland genetic testing has been greatly aided by the presence of a founder gene
effect, well documented family records held by the church and a limited population size.
Nearly 60% of FH cases in Iceland are caused by a common mutation in the 5' part of
intron 4 of LDLR (Gudnason et al, 1997). 50% of families can be traced through
searching church records and they have been traced to a common ancestor in the 18th
century. By tracing back through generations and then down other branches of the
pedigree, 411 key individuals were identified, each representing a nuclear family.
Positive test results were found in 15% of individuals which relates to a positive
detection rate of 1 in every 6 tested; compared to the general population rate of 1 in 500.
The 80-fold enrichment, the limited population size and the willingness by the family members to participate in a screening programme makes Iceland an ideal country for systematic identification of carriers and thus more effective use of preventative medicine (Sigurdson et al, 1998).

Another population where family records have greatly aided tracing are the Mormons in Utah, USA. Tracing is made easier by three factors. Mormon families are large, they are situated within a small radius and they can recall both their family pedigree and medical records with ease (Williams et al, 1993).

1.13.2.2.3. Selective screening in heterogeneous populations

Selective screening in heterogeneous populations has been carried out and some examples are outlined below. A targeted approach to identifying high-risk patients was undertaken as an initial strategy for the prevention of coronary disease in British Columbia, Canada where high cholesterol levels (over 6.2mmol/l) are found in 17% of the population. Patients with FH are identified through lipid clinics and then first degree relatives are offered molecular genetic testing and subsequently tested for the presence of FH. Using this expansion from a family centred programme to a broad community approach, 50% of high risk individuals have been identified (Hayden et al, 1993).

Clinical diagnostic and molecular genetic testing in Germany is well structured. A combination of computer and telephone networks link up local medical centres to a central FH database (Schuster, personal communication). Nineteen mutations, including the R3500Q in APOB, were analysed in a new PCR based test, the FH OLA (Baron et al, 1996) (section 1.9.1.5). This commercial assay was specifically designed for the community of Munich in Germany and is of no diagnostic use in other countries since the spectrum of mutations is different.

A nation-wide family based screening programme was initiated in the Netherlands in 1993 with the aim to identify and treat patients with FH at the asymptomatic stage. First and second degree relatives of a proband were traced and screened by molecular analysis over two years. Index cases (n=1112) were tested and 405 were identified as FH patients (366 through the screening programme). 91 mutations account for over 80% of cases (Lombardi et al, 1998). Although there is a high level of heterogeneity in the Netherlands, four of the most common mutations showed a distinct geographical distribution, simplifying the genetic testing.
In the UK, a selective approach to FH testing would be the most practical approach involving rigorous clinical diagnosis including cholesterol testing and genetic tests. A DNA based test for FH has several advantages over clinical and biochemical diagnoses not only for the proband but also for relatives. As discussed previously the identification of the pathogenic mutation provides a tool in family tracing, and genetic tests can provide unequivocal results in nearly all cases. LDLR and APOB screening would be offered to the proband and if a mutation is identified, the family members could be counselled and offered genetic testing to offer either reassurance with a negative result or closer clinical monitoring with a positive diagnosis. Unfortunately if no molecular defect is identified, the family cannot be offered genetic testing but their cholesterol levels can be measured to identify whether hypercholesterolaemia is present.

In the UK, the family-based testing approach is more difficult to put into practice, families are spread all over the country and there is no general register to enable family tracing other than through the family themselves. Buccal sample kits have encouraged genetic testing since individuals are able to take their own sample and post it to the laboratory rather than having to visit their local GP to give a blood sample. Families are counselled and tested, and results are then given in writing by their lipidologist or GP. Individuals who have tested mutation-positive are invited to a lipid clinic for dietary, lifestyle and drug therapy advice.

There are two main problems with selective screening:

1. The family history obtained in clinical practice is a poor predictor of childhood hypercholesterolaemia. Parents of school-age children are often too young to have experienced cardiovascular disease. The proportion of children with grandparents who had a history of premature cardiovascular disease is higher but many families do not know their medical history very well. A history of parental hypercholesterolaemia is associated with significantly higher cholesterol levels in the children but this criterion has limited usefulness as shown in a Norwegian study where only 12 of 36 children with total cholesterol greater or equal to 6.2mmol/l had a parent with hypercholesterolaemia (Tonstad, 1996).

2. A larger than expected proportion of individuals will have cholesterol testing. The NCEP (NCEP, 1989 & 1992), EAS (Pyörölä et al, 1994 & Wood et al, 1998) and BHA guidelines (Neil, Rees & Taylor, 1996) determined the levels of the 95th percentiles for total cholesterol at all ages. A total cholesterol level of 5.2mmol/l is the cut-off for children aged 1 to 19 years. However this level maybe too high in some populations e.g. China (Chen et al, 1992) and too low in some others e.g. Finland (Tonstad, 1997). The NCEP predicted that 25% of all children would be targeted for screening according
to the guidelines (NCEP, 1992) but studies in Utah (Williams et al, 1992) and Norway (Tonstad, 1997) found that a higher proportion of the population, 36-40%, would be targeted for cholesterol screening if these criteria were applied. These differences may be explained by dietary, environmental and unknown risk factors. Thus care must be taken in deciding cut-off levels for entry into molecular screening programmes in different ethnic groups and in the different countries. Cholesterol testing may be possible in larger numbers of individuals but in the current state of molecular technology and the large cost of such genetic tests it is unfeasible to test these number of individuals.

1.14. Prenatal diagnosis

Heterozygous FH occurs at a frequency of 1 in 500 in the general population and the onset of coronary disease occurs in the second to fourth decade. Since heterozygous FH is a condition in which clinical management can delay onset considerably, there is unlikely to be a demand for prenatal diagnosis for heterozygous FH but homozygous FH causes severe hypercholesterolaemia with onset of coronary disease in childhood. Treatment in these individuals includes diet, drugs, LDL-apheresis and surgical procedures such as liver transplantation and maybe in the future, gene therapy, but the outlook is still poor. In cases where the parents both have FH, prenatal diagnosis, to detect the homozygous or compound heterozygous form of FH, may be offered. The first case was carried out in 1978 (Brown et al, 1978) by the molecular analysis of amniotic fluid cells during the second trimester. The fetus almost completely lacked LDL-receptors by direct measurement of binding, uptake and degradation of 125I-labelled LDL. The pregnancy was terminated at the 20th week and the diagnosis of homozygous FH was confirmed by elevated cholesterol in the aborted fetus (Brown et al, 1978). The diagnosis was confirmed in a further six cases following termination but since there was an overlap in receptor activities between homozygotes with detectable (5-30%) receptor activity and heterozygotes, the technique can only be offered to receptor negative homozygotes.

In 1985 prenatal diagnosis of FH was achieved at the 24th week of gestation by analysis of lipid values in a fetal blood sample (de Gennes et al, 1985). Although an invasive sampling procedure was used, the laboratory test was simpler and quicker than the analysis of LDL-receptor binding in amniotic cells. The fetus had abnormal values which were confirmed by measuring LDL-receptor activity on fibroblast cultures from a skin biopsy (de Gennes et al, 1985).
The first prenatal diagnosis for exclusion of homozygous FH by LDLR restriction fragment length polymorphism (RFLP) analysis on chorionic villus tissue was performed in 1993 (Coviello et al, 1993). Both parents were FH heterozygotes and they had two children, a healthy son and a son affected with homozygous FH. The RFLP's were informative in the family and the fetus was found to be unaffected. At birth the child's cholesterol level and LDL-receptor activity were both normal (Coviello et al, 1993).

Prenatal diagnosis by the identification of the pathogenic mutations in individuals provides unequivocal results. It is made easier in populations where a founder gene effect is observed. A prenatal diagnosis at the eighth week of gestation was reported in a Christian-Arab family, both parents of which carried the 'Lebanese' mutation (Reschef et al, 1992). A direct assay characterised the fetus to be a heterozygote and RFLP analysis showed the presence of a male parent marker which showed that the fetus inherited the mutation from its mother (Reschef et al, 1992). This technique is simple, rapid and can be carried out at an early stage of gestation (generally after the 10th week). However, since FH is genetically heterogeneous it can only be performed in population groups with molecularly defined LDLR mutations. A mutation is only characterised in ~50% of individuals in heterogeneous populations and RFLP analysis is not always informative but two recently identified microsatellite polymorphisms flanking LDLR have high heterozygosity indices and are very informative. They have been applied retrospectively in a nuclear kindred of a child with apparent homozygous FH (de Oliveira et al, 1998). The heterozygous parents were fully informative for each microsatellite marker and the homozygous probands enabled the identification of phase between the two mutations and the microsatellite. The prenatal diagnosis showed the potential of co-segregation using the microsatellite markers. The method is simple, can be carried out in the first trimester and the results can be obtained quickly. The risk of a false positive is that of two double recombinants between markers and the mutation.

Requests for prenatal diagnosis will be low, to individuals at risk of having a homozygous offspring since until now a homozygous birth has been the primary means of ascertainment of FH in families or where a founder gene effect occurs and individuals have been tested in a national screening program. Testing will be very rare in the UK as these situations are uncommon.
1.15. Aims of the project

The main aim of this project was the development of a diagnostic service for familial hypercholesterolaemia.

The specific aims were:
1. to set up a diagnostic service for FH;

2. to improve techniques for the mutation screening of diagnostic samples;

3. to develop an assay to detect LDLR major rearrangements;

4. to explore the influence of a mutation in LDLR or APOB on the lipid-lowering response in heterozygous FH individuals which may have implications when reporting mutation data.
Chapter 2: Materials & Methods

2.1. Biochemical analyses
Blood was collected in EDTA tubes and plasma was separated by centrifugation. Total cholesterol and triglycerides were measured on a Hitachi 747 enzymatic assay, HDL with a precipitation method (Burstein et al, 1970) and Lp(a) levels by an ELISA method (Labeur et al, 1992). Plasma LDL-c was calculated by the Friedewald formula (Friedewald et al, 1972).

2.2. DNA extraction
Each specimen was given a unique identification code as well as assigned a family number. Patient and sample details were logged into a bound book and entered into a database. The extraction procedure operated under UK External Quality Assurance (EQA) guidelines and internal laboratory standards. At the start of each extraction a list was made of the samples to be extracted. Each tube transfer was observed by a second person who verified the two tube labelling’s and then signed the checking form.

2.2.1. DNA extraction from blood by the ‘salting out’ method
Genomic DNA was isolated, from potassium EDTA anticoagulated whole blood which was either fresh or had been frozen at -20°C, by the ‘salting out’ (Nicolaides et al, 1990). This method involved cellular lysis with IEPAL (Sigma Chemicals, USA), nuclear lysis with nuclei lysis buffer, proteinase K digestion, followed by salt precipitation of residual cell debris and ethanol precipitation of DNA. All transfers were checked by a second person to eliminate any errors. The protocol is outlined below:
1. Blood was thawed. 5-10ml blood samples were transferred to a 50ml falcon tube.
2. Red blood cells were lysed by adding ice cold water to give a final volume of 50ml. Samples were inverted several times to mix.
3. Tubes were spun at 2500rpm for 20min at 4°C in an IEC CENTRA-7R centrifuge.
4. Supernatants were discarded and the nuclear pellet was then washed with 25ml of 0.1% (v/v) IEPAL (Sigma) to disrupt the nuclear cell membrane.
5. Samples were vortexed until the pellets were completely resuspended.
6. Tubes were centrifuged for a further 20min as described in step 3.
7. The supernatant, containing the membranes, was discarded and the intact nuclear pellet was lysed with 3ml of nuclei lysis buffer (10mM Tris-HCl, 400mM NaCl, 2mM Na₂-EDTA, stored at 4°C). The pellet was resuspended by vortexing.
8. 600μl proteinase K solution (2mg/ml of proteinase K (Sigma Chemicals, USA)
diluted in proteinase K buffer (2mM Na₂-EDTA, 1% (v/v) SDS, stored at 4°C) and 200μl of 10% (w/v) SDS were added and the samples were mixed gently by inversion and incubated at 60°C for 2 hours or overnight at 37°C.

9. 1ml of saturated ammonium acetate (9.6mol/l) was added to the digested samples to precipitate the protein. After shaking vigorously for 15s samples were allowed to stand at room temperature for 10min.

10. Tubes were centrifuged at 2500rpm at room temperature.

11. Supernatants were transferred to a separate tube and the DNA was precipitated by the addition of two volumes of absolute ethanol.

12. DNA was then spooled out with a blunt ended glass pasteur pipette and transferred to a 1.5ml centrifuge tube containing TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) thought to dissolve the DNA to a concentration of 250μg/ml. For low yield samples, spooling out precipitated DNA was not possible. Samples were therefore centrifuged at 2500rpm for 20min to recover the DNA pellet and dissolved in 50μl TE.

13. To dissolve the DNA, tubes were rotated at room temperature overnight.

14. 5μl of the dissolved DNA was diluted into 495μl dH₂O and the absorbance measured at 260nm (DNA) and 280nm (protein) using a Gene Quant spectrophotometer (Amersham Pharmacia Biotech, Herts, UK). DNA was made to a final concentration of 250μg/ml. The purity of the DNA was determined from the ratio of the absorbance at 260nm and 280nm; a ratio of 1.8-2.0 indicated a preparation free of protein.

15. Samples were divided into two tubes: one sample stored in the working laboratory whilst the second sample stored in another -20°C freezer in the basement, in case of fire, as recommended by EQA guidelines.

2.2.2. DNA extraction from buccal washes

1. Patients were supplied with a buccal sample kit consisting of a 20ml sterilin tube, a 10ml vial of 0.9% saline, an instruction form and a stamp addressed package for return. Patients were asked to take the saline into their mouth, swirl it around for ~30s and then place it into the sterilin.

2. On arrival at the laboratory the sample was transferred to a 50ml falcon tube and spun at 2500rpm (IEC CENTRA-7R) for 10min.

3. Supernatant was removed and the pellet was resuspended in 500μl of solution 1 (10mM NaCl, 10mM EDTA, pH7.5).

4. The mix was transferred to a 1.5ml eppendorf and centrifuged at 13,000rpm (3185g) for 15s (Eppendorf 5415C variable speed centrifuge).
5. The supernatant was discarded and the pellet was resuspended in 500μl of solution 2 (1M TrisCl pH7.5) and mixed by inversion.
6. The mix was spun again at 13,000rpm for 15s and the supernatant, containing the DNA, was transferred to a fresh tube.

2.3. Polymerase chain reaction (PCR)

2.3.1. All exons and promoter of LDLR - a high throughput system

One microtitre tray was used to amplify the promoter and all 18 exons and their associated splice sites (total of 22 PCR reactions) from the genomic DNA of four patients (Whittall et al, 1995). The 20μl PCR reaction contained 50mM KCl; 10mM Tris-HCl, pH8.3; 0.067% W-1; 1.5mM MgCl₂ (Gibco BRL, UK); 0.2mM dATP, dGTP, dTTP; 0.02mM dCTP; 0.05mCi (α³²P) dCTP (ICN Pharmaceuticals, UK); 0.2U Taq polymerase (Gibco BRL, UK); in addition to 62.5ng genomic DNA. Sense and antisense oligonucleotides (1.6pmol of each) were dried in the microtitre wells in the format shown in Fig 2.1. Oligonucleotide sequences are shown in Table 2.1.

Fig 2.1: Schematic plan of an all LDLR exon PCR (for SSCP) of four patients.

<table>
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<tr>
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<th>1</th>
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<th>10</th>
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<tbody>
<tr>
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<td>4⁸-1</td>
<td>6-1</td>
<td>10⁸-1</td>
<td>12-1</td>
<td>16-1</td>
<td>P-2</td>
<td>4⁸-2</td>
<td>6-2</td>
<td>10⁸-2</td>
<td>12-2</td>
<td>16-2</td>
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<tr>
<td>B</td>
<td>P-3</td>
<td>4⁹-3</td>
<td>6-3</td>
<td>10⁹-3</td>
<td>12-3</td>
<td>16-3</td>
<td>P-4</td>
<td>4⁹-4</td>
<td>6-4</td>
<td>10⁹-4</td>
<td>12-4</td>
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<td>11-4</td>
<td>15-4</td>
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PCR’s are arranged in a particular format to allow loading with an 8-channel multipipette. Exon primer sets (promoter - 18) were dried into the labelled wells. PCR mix was made up as a mastermix which was then divided between the four patient DNA samples (1, 2, 3, 4). 20μl of each DNA-PCR mix was then added to the appropriate wells and overlaid with 20μl mineral oil (Sigma Chemicals, USA). Amplifications were performed on an Omnimgene thermal cycler (Hybaid Ltd, UK) and cycle conditions were as follows: 95°C 5min, 57°C 1min, 72°C 1min for 1 cycle, 95°C 1min, 57°C 1min, 72°C 1min for 34 cycles with a final extension of 72°C 5min.
Table 2.1: Oligonucleotide sequences for the amplification of the LDLR exons.

<table>
<thead>
<tr>
<th>Region/exon</th>
<th>Oligonucleotide sequence (Sense, antisense, 5' to 3')</th>
<th>Size (bp)</th>
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<tr>
<td>Promoter</td>
<td>FH110 CAG CTC TTC ACC GGA GAC CC&lt;br&gt; FH130 ACC TGC TGT GTC CTA GCT GG</td>
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<tr>
<td>Exon 1</td>
<td>FH2 ACT CCT CCC CCT GCT AGA AAC CTC A&lt;br&gt; FH131 CTA TTC TGG CGC CTG GAG CAA GCC</td>
<td>238</td>
</tr>
<tr>
<td>Exon 2</td>
<td>FH112 TGT AGA GAC CCT TTC TCC TTT TCC&lt;br&gt; FH132 GCA TAT CAT GCC CAA AGG GG</td>
<td>183</td>
</tr>
<tr>
<td>Exon 3</td>
<td>FH113 TTC CTT TGA GTG AGA GTT CAA TCC&lt;br&gt; FH133 GAT AGG CTC AAT AGC AAA GGC AGG</td>
<td>196</td>
</tr>
<tr>
<td>Exon 4 (5'end)</td>
<td>FH51 (AAAGTCGAC)GCTGCTGCGCATCCATCCCTTG&lt;br&gt; FH52 GTTGGCAGCTGAGGTCGGG</td>
<td>242</td>
</tr>
<tr>
<td>Exon 4 (mid)</td>
<td>FH53 AGACGAGGCTCTCCCGCCGT&lt;br&gt; FH54 GAGCAGGGGTACTGTCC</td>
<td>180</td>
</tr>
<tr>
<td>Exon 4 (3'end)</td>
<td>FH55 CGACTGCGAAGATGGCTCG&lt;br&gt; FH169 GGAACCCAGGGACGAGGAGC</td>
<td>235</td>
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<tr>
<td>Exon 5</td>
<td>FH116 AGA AAA TCA ACA CAC TCT GTC CTG&lt;br&gt; FH136 GGA AAA CCA GAT GGC CAG CG</td>
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<tr>
<td>Exon 6</td>
<td>FH117 TCC TCC TTC TCC TCT TCT CTG GC&lt;br&gt; FH137 TCT GCA AGC CGC CTG CAC CG</td>
<td>179</td>
</tr>
<tr>
<td>Exon 7</td>
<td>FH118 GGC GAA GGG ATG GGT AGG GG&lt;br&gt; FH138 GTT GCC ATG TCA GGA AGC GC</td>
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<td>Exon 8</td>
<td>FH119 CAT TGG GGA AGA GCC TCC CC&lt;br&gt; FH27 CCA CCC GCC GCC TTC CGG TGC TCA C</td>
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<tr>
<td>Exon 9</td>
<td>FH9 TCC ATC GAC GGG TCC CCT CTG ACC C&lt;br&gt; FH28 AGC CCT CAT CTC ACC TGC GGG CCA A</td>
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<tr>
<td>Exon 10 (5'end)</td>
<td>FH10 AGA TGA GGG CTC CTG GTG CCA ACG C&lt;br&gt; FH29 GCC CTT GTT ATC CGC AAG AGA GAC A</td>
<td>202</td>
</tr>
<tr>
<td>Exon 10 (3'end)</td>
<td>FH11 GAT CCA CAG CAA CAT CTA CTG CAG C&lt;br&gt; FH141 AGC CCT CAG CTG GTG GGA TA</td>
<td>163</td>
</tr>
<tr>
<td>Exon 11</td>
<td>FH12 CAG CTA TTC TCC TCC TCT CCA CCC G&lt;br&gt; FH142 GCT GGG ACG GCT GTC CTG CG</td>
<td>171</td>
</tr>
<tr>
<td>Exon 12</td>
<td>FH13 GCA CGT GAC CTC TCC TTA TCC ACT T&lt;br&gt; FH32 CAC CTA AGT GCT TCG ATC TCG TAC G</td>
<td>211</td>
</tr>
<tr>
<td>Exon 13</td>
<td>FH124 GTC ATC TTC CTT GCT GCC TG&lt;br&gt; FH33 GTT TCC ACA AGG AGG TTT CAA GGT T</td>
<td>218</td>
</tr>
<tr>
<td>Exon 14</td>
<td>FH265 GAA TCT TCT GGT ATA GCT GAT&lt;br&gt; FH145 GCA GAG AGA GGC TCA GGA GG</td>
<td>288</td>
</tr>
<tr>
<td>Exon 15</td>
<td>FH16 GAA GGG CCT GCA GGC ACG TG GAG C&lt;br&gt; FH146 GTG TGG TGG CAG GCC CAG TCT TT</td>
<td>247</td>
</tr>
<tr>
<td>Exon 16</td>
<td>FH127 CCT TCC TTT AGA CCT GGG CC&lt;br&gt; FH147 CAT AGG GGG AGG CTG TGA CC</td>
<td>173</td>
</tr>
<tr>
<td>Exon 17</td>
<td>FH128 GGC TCT CTG GTC TCG GGC GC&lt;br&gt; FH148 GGC TCT GGC TTT CTA GAG AGG G</td>
<td>242</td>
</tr>
<tr>
<td>Exon 18</td>
<td>FH129 GCC TGT TTC CTG AGT GCT GG&lt;br&gt; FH149 TCT CAG GAA GGG TTC TGG GC</td>
<td>135</td>
</tr>
</tbody>
</table>

FH2-FH33 (Hobbs et al, 1990), FH51-52 (Gudnason et al, 1994); FH53-54 (Gudnason PhD thesis, 1995); FH55 & FH169 (Gudnason et al, 1993); FH110-149 (Leitersdorf et al, 1990). (AAAGTCGAC) has a SalI site at 5'end of FH51.
2.3.2. Single LDLR exons

PCR reaction (20µl) contained 50mM KCl; 10mM Tris-HCl, pH8.3; 0.067% W-1; 1.5mM MgCl₂ (Gibco BRL, UK); 0.2mM dATP, dCTP dGTP, dTTP; 0.2U Taq polymerase (Gibco BRL, UK); sense and antisense primers (4pmol); in addition to 62.5ng genomic DNA. Primers are found in Table 2.1 & 2.2. Samples were overlaid with mineral oil. Amplifications were performed on an Omnigene thermal cycler (Hybaid Ltd, UK) and cycle conditions were as follows: 95°C 5min, 57°C 1min, 72°C 1min for 1 cycle, 95°C 1min, 57°C 1min, 72°C 1min for 34 cycles with a final extension of 72°C 5min.

Table 2.2: Oligonucleotide sequences for PCR’s for sequencing & sequencing primers.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotide sequences (Sense &amp; antisense 5’-3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter &amp; 1</td>
<td>FHA GTC AGC TCT TCA CCG GAG ACC CAA ATA CA FH131 (Table 2.1)</td>
<td>414</td>
</tr>
<tr>
<td>3</td>
<td>FH4 TGA GTG ACA GTT CAA TCC TGT CTC TTC TG FH23 ATAGGCAAGGCGGGCAGACACCTCAC</td>
<td>180</td>
</tr>
<tr>
<td>4</td>
<td>Ex4F CGG CTA TAG AAT GGG CTG GTG TTG Ex4R TGT TGT TGG AAA TCC ACT TCG GCA</td>
<td>557</td>
</tr>
<tr>
<td>10</td>
<td>FH340 TTG GCC CGC AGT GAG ATG AGG FH141 (Table 2.1)</td>
<td>310</td>
</tr>
<tr>
<td>14</td>
<td>FH342 CTT CCA CAA CCT CAC CCA GCC A FH145 (Table 2.1)</td>
<td>380</td>
</tr>
</tbody>
</table>


2.3.3. Artificial restriction creation or ‘forced’ LDLR PCR assays

PCR conditions were identical to section 2.3.2 except W-1 was omitted. Oligonucleotide sequences are listed in Table 2.3.

Table 2.3: Oligonucleotide sequences for ‘forced’ LDLR PCR assays.

<table>
<thead>
<tr>
<th>Mutation (Exon)</th>
<th>Oligonucleotide sequences (Sense and antisense, 5’ to 3’)</th>
<th>Size (bp)</th>
<th>Enzyme (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D69G (3)</td>
<td>FH278 TGCATTTCCTCAGTTCTGGAGTTGTCG</td>
<td>47</td>
<td>TaqI +</td>
</tr>
<tr>
<td></td>
<td>FH279 GCCGTTGTGCACTGCAGTTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E80K (3)</td>
<td>FH198 GGCCAAGTGGACTGGCACAACGGCTG</td>
<td>69</td>
<td>TaqI -</td>
</tr>
<tr>
<td></td>
<td>FH23 (Table 2.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FH23, 198 (O’Dell et al, 1996), FH278, 279 (Whittall, unpublished). ‘Forced’ base is marked in bold. In the mutant allele a gain of site (+) and a loss of site (-) is indicated.
2.3.4. Intragenic LDLR polymorphisms or rare variants

Three PCR assays were designed for polymorphisms in exon 10, 11 and 12 where assays had not been previously described. PCR reactions were identical to the PCR’s in section 2.3.2 except for the ‘forced’ C1725T assay (section 2.3.3). Oligonucleotide sequences are found in Table 2.4.

Table 2.4: Oligonucleotide sequences for LDLR polymorphic and rare variant PCR’s.

<table>
<thead>
<tr>
<th>Exon/Intron</th>
<th>Poly/rare variant</th>
<th>Oligonucleotide sequences (Sense &amp; antisense, 5’ to 3’)</th>
<th>Size (bp)</th>
<th>Natural /forced assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 2C81T</td>
<td>FH112 &amp; FH132 (Table 2.1)</td>
<td>183 Sfnal (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 4G324T</td>
<td>FH51 &amp; FH52 (Table 2.1)</td>
<td>242 MaelIII (G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Int 7 G1060+10C</td>
<td>FH118 &amp; FH138 (Table 2.1)</td>
<td>236 SmaI (G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Int 7 T1061-8C</td>
<td>FH119 &amp; FH27 (Table 2.1)</td>
<td>197 MboI (T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 8G1171A</td>
<td>FH119 &amp; FH27 (Table 2.1)</td>
<td>197 Stul (G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Int 9 C1359-29T</td>
<td>FH340 &amp; FH141 (Table 2.1)</td>
<td>310 HhaI (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 G1413A</td>
<td>FH10 &amp; FH141 (Table 2.1)</td>
<td>202 BsmAII (A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 C1617T</td>
<td>FH12 &amp; FH142 (Table 2.1)</td>
<td>171 AccI (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 C1725T</td>
<td>FH 304:TGA AGT TTG GAG TCA ACC CAT TA &amp; FH32 (Table 2.1)</td>
<td>78 ‘Forced’ MseI (T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 C1773T</td>
<td>FH13 &amp; FH32 (Table 2.1)</td>
<td>211 HincII (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 C1958T</td>
<td>FH12 &amp; FH33 (Table 2.1)</td>
<td>218 AvelI (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 G2231A</td>
<td>FH16 &amp; FH146 (Table 2.1)</td>
<td>247 MspI (G)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

‘Forced’ base in C1725T PCR assay is marked in bold. FH10-33 (Hobbs et al, 1990), FH112-146 (Leitersdorf et al, 1990). Nucleotide which cuts the respective restriction enzyme is marked in brackets.

2.3.5. APOB analysis

2.3.5.1. PCR for allele specific oligonucleotide (ASO) assays of R3500Q & R3531C

The G to A at nucleic acid 10708 (R3500Q) and 10800 (R3531C) were detected by allele specific oligomelting. The PCR (20µl) reaction contained 50mMKCl; 10mM Tris-HCl, pHi8.3; 0.067% W-1 (Gibco BRL, UK); 2.0mM MgCl2; 0.2mM dATP, dGTP, dCTP, dTTP; 20ng of each primer (Table 2.5); 0.2U Taq polymerase (Gibco BRL, UK); in addition to 62.5ng genomic DNA. PCR cycling conditions were identical to LDLR PCR (section 2.2.1).
Table 2.5: Oligonucleotide sequences of APOB assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Oligonucleotide sequence (Sense &amp; antisense, 5' to 3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASO PCR</td>
<td>APOBc GGA GCA GTT GAC CAC AAG CTT AGC T</td>
<td>354</td>
</tr>
<tr>
<td></td>
<td>APOBd GGG TGG CTT TGC TTG TAT GTT CTC C</td>
<td></td>
</tr>
<tr>
<td>'Forced'</td>
<td>FH210 TCT CGG GAA TAT TCA GGA ACT ATT G</td>
<td>87</td>
</tr>
<tr>
<td>R3500Q</td>
<td>FH211 GCC CTG CAG CTT CAC TGA GGA C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FH212 GCC CTG CAG CTT CAC TGA GTA C</td>
<td></td>
</tr>
</tbody>
</table>

'Forced' base is marked in bold. FH210-212 (Mamotte & van Bockxmeer, 1993).

2.3.5.2. 'Forced' PCR assays for R3500Q

Testing for the presence of the mutation R3500Q involved two 'forced' PCR digests, as described by Mamotte & van Bockxmeer, 1993. A Sau96I restriction site was introduced into PCR products of the normal allele whilst a Scal restriction site was introduced into the mutant allele in another PCR. The 25μl PCR reaction contained 50mM KCl; 10mM Tris-HCl, pH8.3; 2mM MgCl2 (Gibco BRL, UK); 0.25% DMSO (Sigma Chemicals, UK); 0.2mM dATP, dCTP dGTP, dTTP; 0.2U Taq polymerase (Gibco BRL, UK); sense and antisense primers (4pmol); in addition to 100ng wet genomic DNA (i.e. DNA not dried into wells). Two PCR's were set up for each sample, using two primer sets, FH210-211 and FH210-212 (Table 2.5). Samples were overlaid with mineral oil and cycle conditions were as published (Mamotte & van Bockxmeer, 1993). PCR products were then digested according to the original method except half volumes were used and samples were electrophoresed on an EtBr stained 4% agarose gel (3:1 NuSieve).

2.3.5.3. PCR digest assay for R3531C

An NsiI restriction site occurs in the R3531C mutant allele (Pullinger et al, 1995). PCR's were set up according to the ASO PCR method (2.3.5.1), digested with 1 unit of NsiI and visualised on an EtBr stained 3% (2:1 NuSieve) agarose gel.

2.3.5.4. PCR digest assay for R3500W

A natural NlaIII restriction site was utilised to test for the R3500W mutation (Gaffney et al., 1995). The NlaIII restriction site was only induced in the PCR of mutant alleles, and is specific for the R3500W mutation. The PCR was identical to the ASO PCR method (2.3.5.1) and 1 unit of NlaIII was added directly to 10μl of PCR product. Products were electrophoresed on a 3% (2:1 NuSieve) agarose gel and visualised by EtBr staining.
2.3.6. **PCR digest assay for APOE polymorphism**

APOE genotyping (E2, E3, E4) was carried out by gene amplification, cleavage with HhaI restriction enzyme and visualised on an EtBr stained MADGE, as described by Bolla et al, 1995.

2.3.7. **Diplex PCR of microsatellite markers D19S394 and D19S221**

The 20µl PCR reaction contained 50mM KCl; 10mM Tris-HCl, pH8.3; 1.5mM MgCl₂ (Gibco BRL, UK); 0.2mM dATP, dCTP dGTP, dTTP; 0.1% Tween-20 (Sigma); 0.2U Taq polymerase (Gibco BRL, UK); 2pmol of each of the two sense and antisense primers (Table 2.6); in addition to 62.5ng genomic DNA. Samples were overlaid with mineral oil. Amplifications were performed on an Omigene thermal cycler (Hybaid Ltd, UK) and cycle conditions were as follows: 95°C 5min, 57°C 1min, 72°C 1min for 5 cycles and 95°C 1min, 57°C 1min, 72°C 1min for 25 cycles with a final extension of 72°C 5min.

Table 2.6: Oligonucleotide sequences to amplify microsatellites flanking LDLR.

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Oligonucleotide sequence (sense/antisense, 5'-3')</th>
<th>No. of alleles</th>
<th>Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D19S394</strong></td>
<td>6-FAM-AGA CTA CAG TGA GCT GTG GGTG TTC CTA ACT ACC AGG C</td>
<td>17</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>D19S221</strong></td>
<td>HEX-GCA AGA GAC TCT GAC TCA ACA AAA CAT GAG ATC AAT GGC ATG AAA</td>
<td>10</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Hz= heterozygosity index. D19S394 oligonucleotides (Day et al, 1997) and D19S221 oligonucleotides (Genome database and citations therein). 6-Fam or Hex fluorescent label attached to 5'end of sense oligonucleotides.

2.3.8. **PCR of PWS and CF triplexes**

Two triplex sets of markers, used in the laboratory, verified paternity or family identification of samples; one found on chromosome 15 in the critical region of the gene for Prader Willi Syndrome (PWS) (15q) and the other flanking CFTR locus on chromosome 7q.

The PWS triplex PCR reaction contained 50mM KCl; 10mM Tris-HCl, pH8.3; 1.5mM MgCl₂ (Gibco BRL, UK); 0.2mM dATP, dCTP dGTP, dTTP; 0.5U Taq polymerase (Gibco BRL, UK); 1µl of primer mix (25pmol/l of the three sense and antisense primers); in addition to 250ng genomic DNA. Sequences of the primers are found in Table 2.7. Samples were overlaid with mineral oil. Amplifications were performed on an GRI thermal cycler (GRI Ltd, UK) and cycle conditions were as follows: 94°C 5min
for 1 cycle of denaturation, 94°C 1min, 55°C 1min, 72°C 1min for 25 cycles and a final extension of 10min for 72°C.

Table 2.7: Oligonucleotide sequences for microsatellite markers for paternity and identification of samples in FH pedigrees.

<table>
<thead>
<tr>
<th>Marker name (Locus)</th>
<th>Oligonucleotide sequence (sense/antisense, 5'-3')</th>
<th>No. of alleles &amp; size (bp)</th>
<th>Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR4 (D15S11)</td>
<td>GAC ATG AAC AGA GGT AAA TTG GTG G Hex GCT CTC TAA GAT CAC TGG ATA GG</td>
<td>10 243-263bp</td>
<td>0.74</td>
</tr>
<tr>
<td>AFM299wb4 (D15S122)</td>
<td>Hex GAT AAT CAT GCC CCC CA CCC AGT ATC TGG CAC GTA G</td>
<td>9 143-159bp</td>
<td>0.77</td>
</tr>
<tr>
<td>AFM273yf9 (D15S128)</td>
<td>Hex GCT GTG TGT AAG TGT GTT TTA TAT C GCA AGC CAG TGG AGA G</td>
<td>7 193-209bp</td>
<td>0.78</td>
</tr>
<tr>
<td>AC8 (IVS8 (CA)n)</td>
<td>6-FAM TCT ATC TCA TGT TAA TGC TG GTT TCT AGA GGA CAT GAT C</td>
<td>12 181-205bp</td>
<td>0.67</td>
</tr>
<tr>
<td>AC17 (IVS17b(CA)n)</td>
<td>6-FAM TGT CAC CTC TTC ATA CTC AT AAA CTT ACC GAC AAG AGG AA</td>
<td>10 122-140bp</td>
<td>-</td>
</tr>
<tr>
<td>AT17 (IVS17b (TA)n)</td>
<td>6-FAM GAC AAT CTG TGT GCA TCG GCT GCA TTT AGG TTA TC</td>
<td>6 207-302bp</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Chromosome 15 fluorescent triplex (Christian et al, 1995). IVS8(CA)n (Morrai et al, 1991), IVS17b(CA)n and IVS17b(TA)n (Zielinski et al, 1991). The overall heterozygosity value (Hz) for the CF triplex was 0.95.

The CF triplex PCR reaction contained 50mM KCl; 10mM Tris-HCl, pH8.3; 1.5mM MgCl₂ (Gibco BRL, UK); 0.2mM dATP, dCTP dGTP, dTTP; 0.5U Taq polymerase (Gibco BRL, UK); 1μl of primer mix (5pmol of IR4, AFM299wb4 & AFM273yf9 sense and antisense primers except for the AFM299wb4 sense primer which was at 20pmol); in addition to 250ng genomic DNA. Primers sequences are found in Table 2.7. Samples were overlaid with mineral oil. Amplifications were performed on an GRI thermal cycler (GRI Ltd, UK) and the cycle conditions were as follows: 94°C 5min, 50°C 1min, 72°C 45s for 25 cycles and a final extension of 10min for 72°C.

2.3.9. AmpFISTR Profiler Plus assay

AmpFISTR Profiler Plus kit is a commercial DNA fingerprinting kit (Perkin Elmer-Applied Biosystems, UK) which can be used in forensics and paternity testing. Ten short tandem repeat (STR) markers were amplified and electrophoresed on a 5% long ranger 6.0M urea gel on an ABI DNA 377 Sequencer. Nine polymorphic loci are located on various autosomes the amelogenin loci, is found on the X-Y homologous
gene (Table 2.8). The kit includes three allelic ladders, one for each fluorescent dye, which are mixed and run together to give every possible allele for each of the ten loci. The allelic ladder mix was used to genotype individuals and a probability value of two samples being identical can be assigned if caucasian or American black.

Table 2.8: The STR loci used in the AmpFISTR kit.

<table>
<thead>
<tr>
<th>Locus designation</th>
<th>Chromosome location</th>
<th>Common Sequence motif</th>
<th>Size range (bp)*</th>
<th>Dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S1358</td>
<td>3p</td>
<td>TCTA (TCTG)n3 (TCTA)n</td>
<td>114-142</td>
<td>5-FAM</td>
</tr>
<tr>
<td>vWA</td>
<td>12p12-pter</td>
<td>TCTA (TCTG)n3 (TCTA)n</td>
<td>157-197</td>
<td>5-FAM</td>
</tr>
<tr>
<td>FGA</td>
<td>4q28</td>
<td>(TTTC)n3 TTCT (CTTT)n CTCC (TTCC)n2</td>
<td>219-267</td>
<td>5-FAM</td>
</tr>
<tr>
<td>Amelogenin</td>
<td>X:p22.1-22.3; Y:p11.2</td>
<td>-</td>
<td>107-113</td>
<td>JOE</td>
</tr>
<tr>
<td>D8S1179</td>
<td>8</td>
<td>(TCTR)n3</td>
<td>128-168</td>
<td>JOE</td>
</tr>
<tr>
<td>D21S11</td>
<td>21</td>
<td>(TCTA)n3 (TCTA)n [TCTA]n3 TA (TCTA)n3 TCA (TCTA)n2 TCCA TA] (TCTA)n</td>
<td>189-243</td>
<td>JOE</td>
</tr>
<tr>
<td>D18S51</td>
<td>18q21.3</td>
<td>(AGAA)n</td>
<td>273-341</td>
<td>JOE</td>
</tr>
<tr>
<td>D5S818</td>
<td>5q21-31</td>
<td>(AGAT)n</td>
<td>135-171</td>
<td>NED</td>
</tr>
<tr>
<td>D13S317</td>
<td>13q22-31</td>
<td>(GATA)n</td>
<td>206-234</td>
<td>NED</td>
</tr>
<tr>
<td>D7S820</td>
<td>7q</td>
<td>(GATA)n</td>
<td>258-294</td>
<td>NED</td>
</tr>
</tbody>
</table>

* The size range is the actual base pair size of sequenced alleles contained in AmpFISTR allelic ladders & include the 3' A nucleotide addition. 5-FAM (blue), JOE (green) and NED (yellow). The method was as in the provided protocol with a starting sample of 1-2.5ng of DNA.

Samples were run on a 36cm 5% Long Ranger 6.0M urea gel (Amresco, Ohio, USA). A 12cm gel may be used in family studies but no allele frequencies are given. Analysis was carried out using Genescan and an AmpFISTR Profiler Plus Genotyper macro.

2.3.10. Quantitative fluorescent multiplex PCR (QFM-PCR)

2.3.10.1. Quantitative fluorescent multiplex PCR (strategy 1)

Aliquots of genomic DNA were incubated at 55°C for 30 min to ensure it was completely dissolved and equal concentration throughout the tube. Concentrations of genomic DNA were measured by UV spectrophotometry. Half the aliquot was used to determine the concentration and the remainder was diluted to a working concentration of 25 ng/μl, to use as the PCR template (Yau et al 1996).

The 18 exons and the promoter region were divided into four PCR sets (Table 2.9), two labelled with the fluorescent dye, 6-Fam, and two with Hex.
Table 2.9: The four multiplex sets of the QFM-PCR strategy 1 of LDLR.

<table>
<thead>
<tr>
<th>Multiplex set</th>
<th>Exons</th>
<th>Dye (Lane number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-8-10-12-16-18</td>
<td>6-Fam (1)</td>
</tr>
<tr>
<td>2</td>
<td>3-5-14</td>
<td>Hex (1)</td>
</tr>
<tr>
<td>3</td>
<td>4-6-9-13-15</td>
<td>6-Fam (2)</td>
</tr>
<tr>
<td>4</td>
<td>P-2-7-11-17</td>
<td>Hex (2)</td>
</tr>
</tbody>
</table>

The 20μl PCR reaction contained 50mM KCl; 10mM Tris-HCl, pH8.3; 0.067% W-1 (Gibco BRL, UK); 1.5mM MgCl2; 0.2mM dATP, dCTP, dGTP, dTTP; 0.2U Taq polymerase (Gibco BRL, UK); in addition to 100ng genomic DNA. A combination of oligonucleotides were used with a fluorescent tag (6-Fam or Hex) attached at the 5’ end of the antisense primer (Table 2.10). An external control (i.e. non-LDLR amplicon) was incorporated into some multiplex sets. Primers for exon 6 of the dystrophin gene were available in the laboratory, but this gene is found on the X-chromosome so gender must be taken into consideration when analysing the ratios. 2pmol of each primer were added except for exon 12, 14 and 18 where 4pmol were added due to weaker amplification. Samples were overlaid with mineral oil. Amplifications were performed on an Omnipulse thermal cycler (Hybaid Ltd, UK). Cycle conditions were as follows: 95°C 5min, 57°C 1min, 72°C 1min for 1 cycle, 95°C 1min, 57°C 1min, 72°C 1min for 19 cycles with a final extension of 72°C 5min. Samples were loaded as above. The area under the exon peaks, labelled with the same fluorescent label, were compared between the test samples, normals and appropriate rearrangement controls.

Table 2.10: Oligonucleotide sequences for the QFM-PCR of LDLR (strategy 1).

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotide sequence (Sense &amp; antisense, 5'-3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FH2 &amp; FH141F (Table 2.1)</td>
<td>238</td>
</tr>
<tr>
<td>3</td>
<td>FH113 &amp; FH133Fx (Table 2.1)</td>
<td>196</td>
</tr>
<tr>
<td>5</td>
<td>FH116 &amp; FH136Fx (Table 2.1)</td>
<td>180</td>
</tr>
<tr>
<td>8</td>
<td>FH119 &amp; FH27F (Table 2.1)</td>
<td>197</td>
</tr>
<tr>
<td>10</td>
<td>FH10 &amp; FH141F (Table 2.1)</td>
<td>298</td>
</tr>
<tr>
<td>12</td>
<td>FH12 &amp; FH32F (Table 2.1)</td>
<td>211</td>
</tr>
<tr>
<td>14</td>
<td>FH125 &amp; FH145Fx (Table 2.1)</td>
<td>288</td>
</tr>
<tr>
<td>16</td>
<td>FH127 &amp; FH147F (Table 2.1)</td>
<td>173</td>
</tr>
<tr>
<td>18</td>
<td>FH129 &amp; FH149F (Table 2.1)</td>
<td>135</td>
</tr>
</tbody>
</table>

FH2-27 (Hobbs et al, 1992), FH113-149 (Leitersdorf et al, 1990) & FH169 (Gudnason et al, 1993). 6-Fam (F) or Hex label attached at the 5’end of antisense primers.
2.3.10.2. Universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR) (Strategy 2)

DNA preparation were as in section 2.3.10.1. Normal and appropriate deletion and duplication controls were included in all PCR sets. The 20μl P1 PCR reaction contained 1x AmpliTaq GOLD PCR buffer (50mM KCl; 10mM Tris-HCl, pH8.3) (PE-ABI, UK), 1.5mM MgCl₂ (PE-ABI, UK); 0.2U AmpliTaq GOLD polymerase (PE-ABI, UK); in addition to 100ng genomic DNA. Sense and antisense specific universal oligonucleotides (Table 2.11) were added at different amounts; exon 5 (3pmol), exons 3 and exon 17 (4pmol) and exons 8 and 14 (8pmol), to compensate for differential amplification due to size and efficiency of primer binding. A 6 nucleotide tag is located between the universal and unique sequences of the P1 primers which is used for DNA sequencing. Unlabelled universal sense and antisense primers (universal and tag) are efficient primers (CG at 3' end) for sequencing P2 products that are specifically amplified. This strategy allows ABI sequence reads of all the exon, splice sites and the unique primer sequence of the PCR product. The two P2 universal primers can also be used in fluorescent SSCP analysis if they are labelled with different fluorescent tags, thus the P1/P2 system reduces the number of primers requiring a fluorescent tag and increasing the use of these primers. Amplifications were performed on an Omnigene thermal cycler (Hybaid Ltd, UK) with a tube control sensor. Cycle conditions for the P1 PCR were as follows: 94°C 12min, 46°C 1min, 72°C 1min for 1 cycle, 94°C 1min, 46°C 1min, 72°C 1min for 9 cycles with a final extension of 72°C 5min. 2μl of the P1 product was immediately removed and added to the appropriate P2 reaction. P2 reagents were as for P1 except 4pmol of the sense and 6-Fam labelled antisense universal primers were added instead of the specific-universal primers. P2 PCR cycle conditions were as follows: 94°C 12min, 57°C 1min, 72°C 1min for 1 cycle, 94°C 1min, 57°C 1min, 72°C 1min for 19 cycles with a final extension of 72°C 5min.

2μl of each P2 product was mixed with 2μl of dextran sulphate formamide loading dye and 0.5μl of Rox 500 standard (PE-ABI, UK). Samples were denatured at 96°C for 2min, and 1.5μl of each sample was loaded onto a 12cm well-to-read 4.2% non-denaturing acrylamide gel on an ABI DNA 377 Sequencer. Samples were electrophoresed for 70min.
Table 2.11: Oligonucleotides for the UPQFM-PCR of LDLR (strategy 2).

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotide sequence (Sense &amp; antisense 5'-3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>F-TCC GTC TTA GCT GAG TGG CGT ATC CCC G R-AGG CAG AAT CGA CTC ACC GCT ATC CAC G</td>
<td>-</td>
</tr>
<tr>
<td>Univ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1 - 3</td>
<td>F-Univ-CC TTT GAG TGA CAG TTC AAT CCT G R-Univ-CC CAG GAC TCA GAT AGG CTC AAT A</td>
<td>261</td>
</tr>
<tr>
<td>P1 - 5</td>
<td>F-Univ-GA GAA AAT CAA CAC ACT CTG TCC TG R-Univ-GA AAA CCA GAT GGC CAG CG</td>
<td>239</td>
</tr>
<tr>
<td>P1 - 8</td>
<td>F-Univ-GT CTC TAG GCC ATT GGG GAA R-Univ-AC TGC TGC CTG CAA GGG GT</td>
<td>285</td>
</tr>
<tr>
<td>P1 - 14</td>
<td>F-Univ-GG AAT GTT CTG GAA ATT TCT GGA AT R-Univ-AC GGC GAC AGA AAC AAG GC</td>
<td>322</td>
</tr>
<tr>
<td>P1 - 17</td>
<td>F-Univ-GT GCC TCT CCC TAC AGT GCT CCT R-Univ-TC CAC GGT AGC TGT AGC CGT CCT GGT TGT</td>
<td>223</td>
</tr>
<tr>
<td>P2</td>
<td>F-TCC GTC TTA GCT GAG TGG CGT A R-6-Fam-AGG CAG AAT CGA CTC ACC GCT A</td>
<td>-</td>
</tr>
</tbody>
</table>

P1 universal primer sequence (marked in blue) is attached to the 5' end of a 6-base tag (marked in red) (described in text). Fragment sizes include universal-unique primer sequences. The universal primer sequence common between the P1 and P2 primers is marked in blue. 6-Fam is attached to the 5' end of the antisense P2 universal primer.

2.4. High throughput SSCP analysis

Several aspects of the SSCP method have been modified to increase the speed with which the technique can be used for mutation detection (Whittall et al, 1995).

2.4.1. Gel set-up

Two gels were poured between one large glass sequencing plate (33 x 42cm) and two smaller plates (33 x 39cm), so that two gels can be electrophoresed simultaneously on one apparatus. All three plates were washed thoroughly with detergent and wiped with 70% (v/v) industrial methylated spirits (IMS). The inside surfaces of the large plate and the outer small plate and both sides of the middle small plate were coated with Sigmacote (Sigma Chemicals, USA) and wiped with distilled water. Plates were arranged such that the larger plate, Sigmacote side facing upwards, was rested on a raised levelled platform. Spacers were placed up slightly from the bottom. The small plate, which is coated on both sides, was then placed on top. Two more spacers were laid in line with the large plate and the final small plate is placed on top, Sigmacote side facing inwards. This plate was aligned perfectly with the large plate, forming a straight levelled edge at the bottom. This causes a staggered gel edge effect at the top where the
gels would be poured. The three plates were clamped at the sides with bulldog clips. The bottom gel was poured first by injection between the middle and bottom glass plate with a syringe. The plates were tapped firmly to prevent formation of bubbles. After polymerisation (~30min) the second gel was poured. After 1 hour the gels were ready for use. Electrophoresis was carried out in a model S2 sequencing gel system (Gibco BRL, UK) in the coldroom. The chambers were filled with 1x TBE (0.09M Tris-borate, 0.001M EDTA pH8.0). Care must be taken to ensure that no air was trapped between the plates in the lower buffer chamber; any bubbles were released by suction with a syringe and needle.

Gel mix was made in the following way: 7.5% polyacrylamide non-denaturant gel (Severn Biotech. Ltd, UK; 49:1 acrylamide:bis-acrylamide) in 1x TBE buffer with 5% glycerol and 10mmol/l EDTA. For polymerisation 153µl of both 25% ammonium persulphate (APS) and NNN’N’ tetramethylethylenediamine (TEMED) were added to 100ml of gel mix and mixed thoroughly before gel pouring.

2.4.2. Gel loading

6µl of formamide dye (95% formamide, 10mmol/l EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) was added to a 96-well microtitre plate (Hybaid Omnigene, UK). PCR product (6µl) was added to this, the PCR/formamide dye mix was denatured at 96°C for 3min and snap chilled on ice. 5µl was then loaded using an 8-channel pipette (the spacing between each channel was compatible with the microtitre wells and the sharktooth combs). Samples were loaded onto the back gel (between the two small plates) and electrophoresed at 600V until the samples had completely entered the gel. Samples were then loaded onto the second gel (between the large and small plates) and run in as before. Combs were removed and electrophoresis was continued at 400V for ~22 hours or until the xylene cyanol had migrated 30cm. The double gel format allows a whole 96-well microtitre tray to be analysed.

A further level of high throughput SSCP analysis included double loading when a lot of patients were analysed for one exon. After the first loading, the sharktooth comb was removed and repositioned half a well across from its original position enabling the first and second loading bands to be distinguished more easily. The first and second loading single and double strands need to be non-overlapping if the gel is to be informative. Calculation of the timing of the second loading is deduced by the SSCP patterns from previous single loaded gels. Double loading means that a whole 96-well microtitre
array of samples may be analysed on one gel.

2.5. ABI gels for Genescan analysis (QFM-PCR & LDLR microsatellite analysis)

2.5.1. Gel set-up
Amplified fragments of fluorescently labelled products were electrophoresed and separated on an ABI DNA 377 Sequencer (PE-ABI, UK). Gels were prepared using standard 12cm well-to-read glass plates, 0.2mm spacers and 36-well sharktooth combs. 4.2% denaturing acrylamide gel was made (4.2% acrylamide (19:1 acrylamide:bis-acrylamide), urea (Amresco, Ohio, USA), deionised with Amberlite MB-1A (Sigma Chemicals, USA) and filtered through a 0.2µM Nalgene filter (Nalgene Nunc International, USA). Gels were made two hours in advance and run in accordance with the manufacturer's instructions.

2.5.2. Gel loading
A mix of the internal size standard Genescan Rox-500 (PE-ABI, UK) and loading buffer (dextran blue at 30mg/ml in a ratio of 5:1 (v/v) 25mmol/l EDTA (pH8.0) to deionised formamide) was made up (0.5:2µl respectively). Varying amounts of PCR product were added to the loading dye; 2µl of QFM-PCR products, 1µl of single exon PCR products, and 1µl of the flanking microsatellite PCR products. Samples were denatured at 96°C for 2min and immediately transferred to wet ice. A maximum of 2µl of PCR/loading mix were loaded; 1.5µl of QFM-PCR/loading mix, 1µl of single exon PCR/loading mix, and 1µl of the microsatellite PCR/loading mix. Samples were electrophoresed according to the pre-set machine settings, 1200V for 1.5 hours at a constant temperature of 51°C.

2.6. Gel electrophoresis
2.6.1. Polyacrylamide gel electrophoresis (PAGE)
Mutations caused by a small deletion or affect an enzyme restriction site and give rise to digestion fragments that differ by only a few bases, can be resolved better by electrophoresis in a polyacrylamide gel under non-denaturing conditions. These are carried out in a slab gel system (Atto AE-6200, GRI Ltd, UK). Two glass plates of (large: 16 x 16cm and small: 16 x 14cm) were washed with detergent and 70% (v/v) IMS. Gels were placed in the setting apparatus according to the manufacturer. The gel was prepared with ACCUGEL 40 (National Diagnostics, UK) to give a final
concentration of 7-20% according to the sizes of the fragments to be visualised. A standard gel is: 10% acrylamide, 1x TBE and polymerised by 200μl 10% APS and 34μl TEMED. Gels were removed from the setting apparatus and placed in the electrophoresis tank. PCR fragments were electrophoresed in 1x TBE for 30min to 1.5 hour and then stained with EtBr (section 2.7.1.1). For weak or very small PCR products the fragments were stained with silver (section 2.7.1.3).

2.6.2. Agarose gel electrophoresis
Agarose (Gibco BRL, UK) was melted in a microwave oven to a solution of the appropriate concentration (1-2% usually depending on the size of the PCR fragment or size of fragment separation. Smaller fragments require a higher concentration of agarose) in 1x TBE buffer (pH8.3). Restriction enzyme digestion products were generally run on a 3% (2:1 NuSieve agarose:agarose) or a 4% (3:1 NuSieve agarose:agarose) to enable greater resolution. The melted agarose was left to cool down slightly and poured into a gel tray and the appropriate comb inserted. Gels were run submerged in 1x TBE at 125V.

2.6.3. Microtitre array diagonal gel electrophoresis (MADGE)
The MADGE system (Day et al, 1995a) was developed to enable high throughput genotyping, eliminating many time-consuming steps in gel set-up, loading, band detection and analysis in traditional agarose or polyacrylamide gel electrophoresis. MADGE consists of an open-faced horizontal ‘swimming pool’ (100 x 150mm) 2mm deep, and wells in a microtitre format. Wells are at an angle so that the maximum track length is 26.5cm, sufficient for most PCR analysis (Fig 2.1).

2.6.3.1. Acrylamide MADGE
To prepare a MADGE gel, a piece of standard glass (160 x 100 x 2mm) was cleaned with detergent and 70% (v/v) industrial methylated spirits (IMS). One side was coated with sticky silane (0.5% (v/v)) glacial acetic acid, 0.5% (v/v) γ methacryloxypropyltrimethoxysilane (Sigma Chemicals, USA) and ethanol). This enables the gel to stick to the glass plate. The glass plate was then rinsed with dH₂O and dried. 7.5% MADGE gels (50ml) were prepared from a 30% stock polyacrylamide solution (19:1 acrylamide:bis-acrylamide) containing 1x TBE and dH₂O. Polymerisation was initiated by addition of 150μl of both 25% APS and TEMED. The gel components were thoroughly mixed and poured into the centre of the former (Fig 2.2) and a glass plate was put on top, silanised side face down. A weight (500ml
bottle) was placed on top to maintain pressure between glass and former teeth. After polymerisation, the glass plate (with attached gel) was prised from the former, any excess gel mix scraped off the edge of the plate and the gel either used immediately or stored in 1x TBE at 4°C for several weeks.

Formamide dye (5μl) was mixed with 5μl of PCR product and 5μl loaded directly onto the gel using a 8-channel multichannel pipette. Gels were typically run at 150V for 30min and the DNA visualised by staining with EtBr post-electrophoresis (section 2.7.1.1).

2.6.3.2. Agarose MADGE

Agarose is unable to adhere to glass so a plastic support (Sigma electrophoresis film, Sigma Chemicals, UK) was placed between the 0.8% agarose gel and the glass plate. 2μl loading buffer was mixed with 5μl of PCR product and loaded directly onto the gel using a 8-channel multichannel pipette. The gel was submerged in 1x E buffer (0.4M Tris, 0.12M NaOAc, 0.02M EDTA, pH7.7) and run at 150V for 30min. Products were stained with EtBr and visualised on a UV transilluminator. The plastic film fluoresces so it has to be removed to visualise the PCR products.

Fig 2.2: Schematic plan view of the wells of a MADGE gel.

Distance between adjacent wells is set for a standard 96-well microtitre plate. The wells are 2mm², the angle between the direction of electrophoresis and the 12-well rows of the array allow a track length of 26.5mm per well (Day et al, 1995a).
2.7. Detection of DNA

2.7.1. Detection of DNA on polyacrylamide gels

2.7.1.1. Ethidium bromide (EtBr)

Gels were post-electrophoresis stained with EtBr. This involved soaking gels in 1x TBE with 1mg/ml EtBr and shaking gently for a few minutes. To visualise the DNA bands, gels were put on a UV transilluminator and photographed.

2.7.1.2. Autoradiography

Gels were transferred to 3MM Whatmann chromatographic paper, dried and exposed to X-Omat film (Kodak, UK) at -70°C for 1-3 days before film development.

2.7.1.3. Silver staining

DNA electrophoresed on acrylamide gels can be visualised by silver staining. The gel must be agitated gently and kept submerged throughout the various staining stages. The protocol (Mountford R, 1996) is outlined below:

1. The gel was transferred to a plastic tray and fixed by incubating in an aqueous solution of 10% ethanol, 0.5% acetic acid for 3min. The fixing solution was discarded and the gel was re-incubated in fixer for a further 3min. The fixer was then removed.

2. The gel was incubated in 0.1% (w/v) silver nitrate (AgNO₃) (Sigma Chemicals, USA) for 15min. The silver ions penetrate the gel and bind to nucleic acid bases. AgNO₃ may be reused up to five times.

3. The gel was rinsed twice with dH₂O, quickly so as not to allow the staining solution to leach out.

4. Developer (1.5% (w/v) NaOH, 0.1% (v/v) formaldehyde (Sigma Chemicals, UK)) was added to the gel for 10-20min until bands developed sufficiently. Formaldehyde was only added just before this step so that it reduces the bound silver ions to metallic silver once in alkaline conditions.

5. The solution was discarded and the gel was incubated with a fixing agent (0.75% (w/v) sodium carbonate) for 10min.

6. Gels were stored between two sheets of cellophane in an EasyBreeze drying frame (Hoefer Scientific Instruments, USA) which allows a strong, thin, hard copy.

2.7.1.4. Fluorescent detection: Genescan & Genotyper

Fluorescent intensity of the internal size standard and PCR products were collected by a Power Macintosh computer. Following each run, sample lanes were checked for correct tracking, the pre-defined internal size standard bands were manually sized and the PCR fragment sizes determined using the Local Southern method (Southern, 1975). This method uses the reciprocal relationship between fragment length and mobility. For
each fragment, a curve is created by using three standard points, one above and one below the fragment. Another curve is created by looking at an additional set of three points, one below and two above the fragment. The two size values are averaged to determine the unknown fragment length. The Genescan output was analysed and processed using Genotype 1.1 & now 2.1 software which identified peaks in the desired size range and presents the data in a graphical format.

2.7.2. Detection of DNA on agarose gels
2.7.2.1. Ethidium bromide
DNA was detected with 0.5mg/ml EtBr by adding it to the melted agarose prior to casting. EtBr was also added to the 1x TBE electrophoresis buffer in the tank, to prevent buffer fronts. Gels were viewed on a UV transilluminator.

2.7.2.2. Chemiluminescent detection: ASO assay for R3500Q and R3531C of APOB
2.7.2.2.1. Gel electrophoresis and blotting
PCR products were run on a 0.8% agarose MADGE in 1x E buffer and visualised by EtBr staining. Filters were denatured in 1.5M NaCl, 0.5M NaOH for 25min at room temperature. The electrophoresed products were transferred to nylon membranes (Hyaid N+, Amersham International) by standard blotting procedures for 2 hours using 20x SSC (3M NaCl, 0.3M Tris sodium citrate, pH7.0). Filters were fixed by irradiating the filters for 3min in a UV crosslinker.

2.7.2.2.2. Dioxygenin 3'end labelling ASO's
Two pairs of ASO's were used to detect the R3500Q mutation, ASO1 (5'GCACACGGTGTTC-3') and its mutant allele ASO2 (5'-GCACACAGTCTTC-3'), and two for the R3531C mutation, ASO3 (5'CACTCCAACGCATA-3') and its mutant allele ASO4 (5'-CACTCCAATGCATA-3'). ASO's were 3'end-labelled with dioxygenin (Boehringer Mannheim DIG oligonucleotide 3'end labelling kit).

1. The following reagents were added to a microfuge tube, on ice, 100pmol of the ASO to be labelled: 4μl of 5x reaction buffer (1M potassium cacodylate, 125mM Tris-HCl, 1.25mg/ml BSA; pH6.6), 4μl of 25mM CoCl2, 1μl of 1mM digoxygenin-11-dUTP, 50U/μl terminal transferase in 200mM potassium cacodylate, 1mM EDTA, 200mM KCl, 0.2mg/ml BSA, 50% (v/v) glycerol; pH6.5. The reaction was incubated at 37°C for 15min and placed on ice.
2. 1μl of a 20mg/ml glycogen solution and 1μl of 200mM EDTA, pH8.0 was added.
3. Unincorporated nucleotides were removed by precipitating the labelled
oligonucleotide with 2.4μl of 4M LiCl and 75μl of ice-cold absolute ethanol, mixed
well and placed at -70°C for 30min or -20°C for 2h.
4. Samples were centrifuged at 13,000rpm (3185g) for 15min in an Eppendorf 5415C
variable speed centrifuge at 4°C. The ethanol was decanted and the pellet was washed
with 50μl of 70% cold ethanol.
5. Samples were centrifuged at 13,000rpm for 5min at 4°C, the 70% ethanol was
removed and the pellet was then dried.
6. The labelled ASO was then resuspended in 20μl of water and stored at -20°C.

2.7.2.2.3. Hybridisation & stringency washes
Filters were prehybridised at 65°C in Boehringer Mannhein recommended hybridisation
solution [5x SSC (1:4 dilution of 20x SSC - 3M NaCl, 300mM sodium citrate; pH7.0),
1.0% (w/v) blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS]. Hybridisation
with the DIG-labelled ASO probes was for 12h at 37°C in Boehringer Mannheim
chemiluminesence hybridisation solution.

Stringency washes at 37°C were as follows: 2x 20min at 0.5x SSC (1:40 dilution of
20x SSC), 0.1% SDS, 2x 20min at 0.1x SSC (1:200 dilution of 20x SSC), 0.1%
SDS. Normal R3500Q and R3531C filters and the mutant R3500Q filter had an
additional wash of 5x SSC, 0.1% SDS at 42°C and 40°C respectively.

2.7.2.2.4. Chemiluminescent detection
The hybridisation signals were detected by a chemiluminescent reaction using CSPD
(Boehringer Mannheim DIG Luminescent detection kit). All incubations were
performed at room temperature with agitation.
1. After post-hybridisation washes, the filter was equilibrated in Buffer 1 (100mM
maleic acid, 150mM NaCl; pH7.5) and 0.3% Tween-20 (Sigma) for 1min.
2. Filters were treated with blocking reagent to prevent non-specific binding of antibody
to the membrane. The filter was covered with buffer 2 (1% (w/v) blocking reagent in
buffer 1) and gently shaken for 30min. Meanwhile the antibody solution was prepared.
3. Buffer 2 was poured off and the membrane was incubated for 30min in antibody
solution (Anti-DIG-alkaline phosphate diluted 1:10,000 (75mU/ml) in buffer 2).
4. The antibody solution was discarded and the filter washed twice for 15min, buffer 1.
5. Buffer 1 was poured off and the filter was allowed to equilibrate in buffer 3 (100mM
Tris-HCl; pH9.5, 100mM NaCl, 50mM MgCl₂) for 2min.
6. The filter was placed in a hybridisation bag, making sure it remained wet. 2.5ml of
diluted CSPD (1:100 dilution of 25mM CSPD in buffer 3) was added for each filter.

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The filter was gently agitated for 5min.
7. CSPD solution was removed, the membrane was blotted on 3MM Whatman paper and sealed semi-dry in a plastic bag. The membrane was incubated at 37°C for 15min which enabled the alkaline phosphate chemiluminescent reaction to be at a steady state to allow short exposures to X-ray film.
8. Filters were exposed to X-ray film at room temperature for 1-2 hours, to record the chemiluminescent signal.

2.8. DNA Sequencing by fluorescent dye terminator technology
2.8.1. Purification of PCR products
Unincorporated nucleotides were removed from PCR products by placing the products through MicroSpin S-400 & S-300 columns (Amersham Pharmacia Biotech, UK) which contain Sephacryl S-400 or S-300 HR resin equilibrated in TE buffer. The S-300 columns were used for PCR fragments smaller than 200bp. The purification procedure was as described below:
1. The resin in the column was resuspended by vortexing.
2. The cap was loosened by a quarter turn and snapped off at the bottom.
3. The column was placed in a 1.5ml microcentrifuge tube and spun in an Eppendorf 5415C variable speed centrifuge at 3000 rpm (700g) for 1min.
4. The column was placed in a new labelled 1.5ml tube, after wiping the TE droplet from the base of the column. Sample was added to the top-centre of the resin without touching the resin.
5. The column was spun as before and the purified sample was collected in the bottom of the microcentrifuge tube.

2.8.2. Sequencing reaction
The dRhodamine terminator cycle sequencing chemistry kit was used (Perkin Elmer Applied Biosystems, UK). Two 10μl sequencing reactions were carried out for each exon, one containing the sense primer and the other containing the antisense primer (Table 2.1 and Table 2.2). To each 0.5ml tube 1-5μl of purified PCR product was added, depending on the strength of the product on a checker gel, but generally 5μl. 4μl of dye terminator premix and 1μl of a 2pmol/μl primer (serially dilution from stock concentration of 100pmol/μl to 10pmol/μl and finally to 2pmol/μl) was added to the purified PCR product and the final volume was made up to 10μl with sterile water. The mix was vortexed, centrifuged, overlaid with 20μl of mineral oil and placed on a pre-
heated (96°C) PCR machine. The PE-ABI DNA thermal cycler model 480 and the cycle conditions were as recommended by the manufacturers.

2.8.3. Purification of sequencing products

1. To the sequencing products 10μl of dH₂O was added, for easier handling volume.
2. The sequencing products were separated from the overlaying mineral oil by adding the entire contents onto a strip of paraffin film. The film was tilted which resulted in the sample sliding down the film, leaving the mineral oil on its way. The sample was then pipetted back into a fresh microcentrifuge tube.
3. 300μl of 96% ethanol and 10μl 3M sodium acetate pH 5.2 was added, vortexed briefly and spun at 13,000 rpm (3185g) for 20min.
4. Supernatant was discarded and 200μl 70% ethanol was added to the invisible pellet. The contents were once again vortexed briefly and spun as before.
5. Supernatant was removed and the tube was place on a PCR block for a few minutes at 80°C to remove all traces of ethanol. Pellets were either stored at room temperature for a day or -20°C for longer periods.

2.8.4. Preparation of sequencing gel and loading of samples

Formamide loading buffer (3μl) was added to samples (dextran sulphate at 30mg/ml in a ratio of 5:1 (v/v) 25mM EDTA (pH 8.0) to deionised 98% formamide, 10mmol/l EDTA), denatured for 2min at 96°C and immediately placed on ice. 2μl of the product was loaded onto a sequencing gel.

2.8.5. Sequence analysis

Sequence analysis was carried out using Sequencing analysis 2.0 (PE-ABI, UK).

2.9. Suppliers of materials and reagents

All oligonucleotides were supplied freeze dried from Gibco BRL (UK), Genosys Ltd (UK) or MWG Biotech (UK). All enzymes and their appropriate buffers were from New England Biolabs (UK) or Kramel Biotech (UK), unless stated. Unless otherwise stated all chemicals were obtained from BDH Ltd (UK).

2.10. FH patient selection

2.10.1 Diagnostic criteria for FH in adult samples from the UK

The criteria used for the diagnosis of FH in adults were those of the Simon Broome Steering Comittee, 1991. These are summarised below:
a. total cholesterol $> 7.5\text{mmol/l}$ or LDL-$c > 4.9\text{mmol/l}$ if over 16 years old  
b. TX in patient or in first or second degree relative  
c. family history of MI under age 60 in first degree relative or family history of MI under age 50 in second degree relative  
d. family history of total cholesterol $> 7.5\text{mmol/l}$ in first or second degree relative  

For a diagnosis of 'definite' FH both a + b must be present but for 'possible' FH both a + c or a + d must be observed.

2.10.2 Diagnostic criteria for FH in paediatric samples from the UK  
a. total cholesterol $> 6.0\text{mmol/l}$ or LDL-$c > 4.4\text{mmol/l}$ (rarely determined) if under 16 years old  
b. TX in first or second degree relative  
c. family history of MI under age 60 in first degree relative or family history of MI under age 50 in second degree relative  
d. family history of total cholesterol $> 7.5\text{mmol/l}$ in first or second degree relative  

For a diagnosis of 'definite' FH both a + b must be present but for 'possible' FH both a + c or a + d. FH is one of the few hyperlipidaemias to express from birth so if a child has hypercholesterolaemia then there is a high probability that it is FH. For this reason any child which may be suspected of having FH ('possible' FH) but does not meet the criteria in every way is still analysed for LDLR and APOB mutations.

2.11. Statistical analysis  

$\chi^2$ analysis was used to compare the frequency of discrete variables between groups.  

Data was analysed using SPSS 6.1.3 for Windows.
Chapter 3: Diagnostic genetic service for FH

Summary
A genetic diagnostic service for FH has been established over the last four years in the Clinical Molecular Genetics Laboratory at Great Ormond Street Hospital for Children NHS Trust (GOSH) London. In total there have been 325 referrals, 325 in total; 205 probands and 120 family members, which have come from a number of lipid clinics and from general practitioners.

SSCP analysis was used to screen for LDLR mutations. SSCP bands caused by common (non-pathogenic) DNA variations in the gene were identified by independent PCR tests. The sequence change causing any remaining variant bands were subsequently identified by fluorescent DNA sequencing. Mutations were confirmed by either repeating the sequencing reaction or by direct assay in cases where there was a gain or loss of a restriction site in the mutant allele. APOB mutations were detected by direct PCR assays. Mutations predicted to be pathogenic were found in 72 probands, 63 in LDLR and 9 in APOB. The mutation detection rate was 72% in paediatric probands, 43% in adults with a ‘definite’ FH diagnosis and 13% in adults with a ‘probable’ FH diagnosis. The loss of sensitivity of reducing the number of exons tested has been assessed from the results obtained and a molecular screening strategy is proposed.
3.1. Introduction
In the UK 110,000 people are estimated to have the heterozygous form of FH, approximately 10% of these have a clinical diagnosis whilst only 1% have a genetic diagnosis (MEDPED data). The majority will have an LDLR mutation but a small proportion will have an APOB mutation (Tybjaerg-Hansen et al, 1990) or a mutation in some other yet to be identified gene (Haddad et al, in press). Hypercholesterolaemia is a general feature in LDLR and APOB mutation carriers, but there is a great variation in age of onset and severity of clinical symptoms, although the phenotype generally runs true within a family (Heiberg et al, 1977).

Studies have shown that the mortality rate of FH patients is significantly greater than in normal individuals (Simon Broome Steering Committee, 1991 & 1999), and treatment can reduce the risks of CHD by lowering the plasma levels of LDL-c. The early identification of individuals at risk allows changes in lifestyle, starting with dietary intervention and followed by drug treatment and these measures can lead to a better long-term prognosis.

The FH phenotype is expressed at birth and can be detected early in childhood by significant elevations in plasma total and LDL-cholesterol levels. Screening of children for heterozygous FH using an LDL-c level is reasonably efficient in families with known FH. A study was carried out in 29 infants, at birth and at a follow-up examination, who were born to 23 couples who were either normal or were FH heterozygotes (Kwiterovich, 1993). Total and LDL-cholesterol levels were determined at both testing times (Fig 3.1). Sixteen of the 29 infants had elevated LDL-c in cord blood but three of these did not have a total cholesterol value above the 95th percentile for their age. Therefore at birth approximately 20% of affected FH individuals would be missed by using cord blood total cholesterol levels alone, although a more accurate picture would be determined by LDL-c levels. Follow-up examinations, using LDL-c levels, were carried out between one and two years of age in 19 of the infants, 12 judged to be FH heterozygotes and seven considered to be normal at birth. A more accurate diagnosis was given at this stage, each of the normal infants remained normal at follow-up but one infant with an initial diagnosis of FH now had cholesterol levels in the normal range. The discrepancy between the diagnosis at birth and at follow-up was explained by the child being the only one who had been on a strict low cholesterol, low saturated fat diet since birth. This false negative would not have occurred if a mutation had been identified.

For population screening of FH, even the LDL-c level is too non-specific as many
factors can influence the concentration of LDL (discussed in section 1.3).

Fig 3.1: Distribution of the natural logarithm (ln) of LDL-c (A) and total cholesterol (B) from 217 and 236 children respectively born to one normal and one FH heterozygote parent. Source: Kwiterovich, 1993.

Considerable overlap in cholesterol levels between affected and normal individuals has also been shown in older infants and children (Leonard et al, 1977; Kessling et al, 1993).
1990). 8.4% of normal children were predicted to have levels over 6.2mmol/l (false positive) whilst 14.5% of affected children would have levels under 6.2mmol/l (false negative) (Leonard et al, 1977). Similar results were shown for younger children (Kwiterovich, 1993). Thus in 5-10% of children a cholesterol test would not provide an unequivocal diagnosis and 15-20% of FH relatives would be misdiagnosed on cholesterol testing (Ward et al, 1996). If cholesterol tests were carried out in young children a proportion may initially present with lipid levels within the normal range but develop hypercholesterolaemia at a later stage (Kessling et al, 1990) and these would have been missed. Diagnosis on cholesterol testing alone is also more difficult to establish in boys than girls due to lower cholesterol concentrations, unfortunately it is the men who carry the greater risk of CHD and in whom it is important to make an early diagnosis in order to start lipid lowering treatment (Leonard et al, 1977).

3.1.1. Strategies for genetic testing
There are several approaches to screening for FH ranging from population-based screening to selective family-based testing (section 1.13.2.2). The method which would be the most economical and practical to establish in the UK is the selective family based testing approach, using a combination of cholesterol and genetic testing (Fig 3.2) (Humphries, Galton & Nicholls, 1997). The initial referral would either be from lipid clinics or general practices. Once a mutation has been identified in the proband it can be tracked in family studies, providing individuals with either reassurance in the case of a negative result or closer clinical management in the case of a positive diagnosis. The patient distributes information about FH genetic testing to their relatives and following their consent they are sent a sampling kit. Relative testing has been aided by carrying out the genetic tests on mouthwash (buccal) samples, which are returned to the laboratory by standard post. The test is non-invasive and can be carried out in their home. The results of the genetic tests are reported back to the GP or lipidologist and then to the patient in question.

3.1.2. Quality assurance scheme
The diagnostic laboratory, situated at GOSH, offers a genetic service for common genetic conditions in the region (North East Thames) and a supraregional genetic service for certain rare genetic conditions. Genetic testing of FH was set up in this laboratory, since it can then operate under the UK external quality assurance (EQA) scheme (http://www.cmgs.org). This scheme has been operating in the majority of diagnostic laboratories in the UK and there are guidelines governing procedures, from the receipt of the specimen to the issuing of the report. All DNA samples and
information is stored indefinitely allowing the testing of family members in years to come. Participation in the EQA scheme will become a condition for accreditation.

Fig 3.2: Flowchart to represent the clinical and molecular testing of patients with FH.

3.1.3. Aims
The aim was to develop a clinical molecular genetic service for FH, to improve mutation screening techniques and to evaluate FH molecular screening strategies.
3.2. Materials and Methods

3.2.1. Acquisition of samples
At the initial lipid clinic consultation, a family history was taken by the clinician with details entered on the pedigree, of known CHD history and age of onset, any known lipid levels; total cholesterol, LDL-c and triglyceride levels. Details of lipid levels and CHD history on family members was also noted. A diagnosis of FH was based on the consultation and from the results of the biochemical tests. Diagnosis was divided into three classes according to the presence or absence of tendon xanthomata (TX), either 'definite' FH (TX+), 'probable' FH (TX-) or 'possible' FH (does not fulfil all the criteria of 'definite' or 'probable' FH). If either the 'definite' or 'probable' criteria were fulfilled, a 5-10ml EDTA blood sample was collected from the proband and any first degree relatives. Samples and forms were sent to the Clinical Molecular Genetics Laboratory at GOSH, by standard post.

3.2.2. Molecular analysis

3.2.2.1. LDLR analysis
LDLR was screened by SSCP analysis (2.3.1). Potentially FH-causing SSCP band shifts were subsequently sequenced using fluorescent dye terminator sequencing (2.3.1, 2.3.2, 2.8). The identified mutation was confirmed by either repeating the sequencing reaction on another PCR reaction, a natural PCR-digest assay (2.3.1) or a 'forced' PCR assay (2.3.2). Family members were screened by direct assays.

A PCR based method has been developed to screen for major rearrangements of exons 3, 5, 8, 14, 17 of LDLR (described in section 4). The 'definite' FH cases where no SSCP was detected, were screened for major rearrangements.

3.2.2.2. APOB analysis
The R3500Q and R3531C mutations were initially screened by a chemiluminescent ASO method using the MADGE format (2.7.2.2) but now the R3500Q mutation was tested by two 'forced' PCR digest assays (Mamotte & van Bockxmeer, 1993) (2.3.5.2) and the rare APOB mutation, R3531C, was tested by a direct assay (2.3.5.3). The R3500W mutation (Gaffney et al., 1995) was not tested although it could be tested in patients of Asian background, since it has only been found in this ethnic group (Choong et al., 1997; Tai et al., 1998).

3.2.3.3. Identity analysis
In cases where paternity was important for results, family relationships were verified by the analysis of microsatellite markers with high heterozygosity indices. Two triplex
sets of microsatellites, around the loci for cystic fibrosis (chromosome 7) (Morral et al, 1991; Zielinski et al, 1991) and Prader-Willi syndrome (chromosome 15) (Christian et al, 1995), available in the laboratory, were initially used to determine identity (2.3.8). Heterozygosity values were very high and two of the three markers were always informative within a family, which is required for reporting. Recently a DNA fingerprinting kit became commercially available, AMPFISTR (Perkin Elmer Applied Biosystems, UK) (2.3.9). This kit contains nine microsatellite markers from various chromosomes plus a PCR based test to confirm the sex of the samples (amelogenin locus). This is now the method of choice.

3.2.2. Reports

Reports were issued on the data generated for each patient, whatever the outcome. Reports were written by the scientist who generated the data and then checked by a senior scientist.
3.3. Results

3.3.1. Referrals

Samples were received from lipid clinics across the UK (Table 3.1), although most came from the paediatric clinic at GOSH and the adult lipid clinic at Charing Cross Hospital (CCH). 325 samples, 205 probands and 120 family members, were collected over approximately four years. The monthly breakdown of samples received between January 1995 and October 1998, inclusive, is shown in Table 3.2.

Table 3.1: Summary of samples received for FH genetic testing in individuals with clinically diagnosed FH, between January 1995 and October 1998, inclusive.

<table>
<thead>
<tr>
<th>Referring hospital or GP</th>
<th>No. of probands</th>
<th>No. of family members</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Ormond Street Hospital</td>
<td>44</td>
<td>54</td>
</tr>
<tr>
<td>Charing Cross Hospital</td>
<td>139</td>
<td>45</td>
</tr>
<tr>
<td>Rayne Institute, London</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Middlesex Hospital, London</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>St George’s Hospital, London</td>
<td>2</td>
<td>0</td>
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<tr>
<td>Guy’s Hospital, London</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Conquest Hospital, Hastings</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Brighton Hospital</td>
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<td>0</td>
</tr>
<tr>
<td>Manchester Hospital</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Worthing &amp; Southlands Hospital</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Newham General Hospital</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Royal United Hospital, Bath</td>
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<td>2</td>
</tr>
<tr>
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</tr>
<tr>
<td>Germany</td>
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<td>3</td>
</tr>
<tr>
<td>USA (Columbia)</td>
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<td>0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>205</strong></td>
<td><strong>120</strong></td>
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Table 3.2: Monthly audit of FH samples received from Jan 1995 to Oct 1998, inclusive.

<table>
<thead>
<tr>
<th>Month &amp; Year</th>
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<th>No. of family members</th>
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</tr>
<tr>
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<td>2</td>
<td>GOSH</td>
</tr>
<tr>
<td>Aug 1995</td>
<td>1</td>
<td>2</td>
<td>Rayne Institute</td>
</tr>
<tr>
<td>Sept 1995</td>
<td>3</td>
<td>5</td>
<td>GOSH</td>
</tr>
<tr>
<td>Oct 1995</td>
<td>1</td>
<td>0</td>
<td>GOSH</td>
</tr>
<tr>
<td>Nov 1995</td>
<td>3</td>
<td>1</td>
<td>GOSH</td>
</tr>
<tr>
<td>Jan 1996</td>
<td>1</td>
<td>0</td>
<td>GOSH</td>
</tr>
<tr>
<td>Feb 1996</td>
<td>1</td>
<td>2</td>
<td>GOSH</td>
</tr>
<tr>
<td>Mar 1996</td>
<td>1</td>
<td>2</td>
<td>GOSH</td>
</tr>
<tr>
<td>Apr 1996</td>
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<td>0</td>
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</tr>
<tr>
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<td>GP, London</td>
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<tr>
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<tr>
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<tr>
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<td>Sept 1997</td>
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<td>Worthing &amp; Southlands Hosp</td>
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<tr>
<td>Oct 1997</td>
<td>3</td>
<td>0</td>
<td>GOSH</td>
</tr>
<tr>
<td>Dec 1997</td>
<td>0</td>
<td>0</td>
<td>St George’s Hospital, London</td>
</tr>
<tr>
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<td>1</td>
<td>2</td>
<td>GOSH</td>
</tr>
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<tr>
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<td>GOSH</td>
</tr>
<tr>
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<td>2</td>
<td>1</td>
<td>CCH</td>
</tr>
<tr>
<td>June 1998</td>
<td>1</td>
<td>2</td>
<td>GOSH</td>
</tr>
<tr>
<td>July 1998</td>
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<td>Airdale General Hospital</td>
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</tr>
<tr>
<td>Sept 1998</td>
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</tr>
<tr>
<td>Oct 1998</td>
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<td>0</td>
<td>CCH</td>
</tr>
<tr>
<td>Nov 1998</td>
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<td>GOSH</td>
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<tr>
<td>TOTAL</td>
<td>205</td>
<td>120</td>
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3.3.2. Molecular characterisation

All patients were screened for APOB R3500Q mutation by either chemiluminescent ASO analysis or two ‘forced’ PCR assays (Mamotte & van Bockxmeer, 1993) (Fig 3.3) which avoid the possibility of false positive and negatives.

The coding region and splice sites of LDLR were screened by SSCP analysis. In one microtitre tray 22 PCR reactions were carried out in four patients and then loaded onto a double gel (Fig 3.4). Any failures were repeated (Fig 3.5).

In LDLR there are a number of known polymorphisms (http://www.ucl.ac.uk/fh) and although most resulted in recognisable band shift patterns, in a clinical genetic diagnostic setting a confirmation assay was required to ensure that the band shifts were due to the polymorphism and not from FH-causing sequence changes. The SSCP pattern for exon 12 was complex (Fig 3.6) and it was difficult to distinguish mutations from the combination of two polymorphisms, C/T at 1725 (Yamakawa-Kobayashi et al, 1993) and C/T at 1773 (Leitersdorf & Hobbs, 1987). Many of the common polymorphisms alter natural restriction enzyme sites which can be exploited for rapid and easy genotyping. The G/A polymorphism in exon 10 at nucleotide 1413 (Warnich et al, 1992), the C/T polymorphism in exon 11 at nucleotide 1617 (Leren et al, 1992) and the C/T polymorphism at 1725 of exon 12 (Yamakawa-Kobayashi et al, 1993) had not been reported to alter natural restriction enzyme sites so ‘forced’ PCR digest assays were considered. For the first two sites it was found that the polymorphisms created sites for recently identified and now commercially available restriction enzymes, BsmAI (Fig 3.7a) and Acil (Fig 3.7b) respectively. For the third site, as no available enzyme was altered, an Msel site was introduced by ‘forced’ PCR using a mismatched primer (Haliassos et al, 1989) (Fig 3.6b).

Polymorphisms were excluded and any remaining SSCP band shifts were characterised by fluorescent sequencing on an ABI Sequencer (Fig 3.8). Sequence alterations were confirmed by a direct assay with a gain or loss of a natural enzyme site or by repeating the sequencing reaction on a different PCR product.

Mutations thought to be pathogenic were found in 72 probands (Table 3.3), 63 (87.5%) in LDLR and 9 (12.5%) in APOB. LDLR mutations included 39 missense (61.9%), 6 nonsense (9.5%), 3 splice junction (4.8%), 8 small deletions (12.7%), 3 small insertions (4.8%) and 4 major rearrangements (4.2%). Several non-pathogenic changes were also observed (Table 3.4).
Fig 3.3: Two 'forced' PCR assays detected the R3500Q mutation in APOB (Mamotte & van Bockxmeer,1993). The 'forced' Sau96I assay (lanes 2-6) introduced a Sau96I site into the normal allele whilst the 'forced' Scal assay (lanes 7-11) introduced a Scal site into the mutant allele. Fragments were separated on a 10% acrylamide gel stained with EtBr. Lane 1 & 10 - 50bp size marker, lanes 2 & 7 - homozygous normal, lanes 3, 4, 5 & 8, 9, 10 - heterozygous carriers of R3500Q, lanes 6 & 11 - blank controls for the Sau96I and Scal assays respectively.
Fig 3.4: Part of a double SSCP gel where four patients were analysed for all LDLR exons at one time. Patients 1, 2, 3 and 4 are shown for exons 11-17. Band shifts were observed in exons 12, 13, 14 and 15 and as heteroduplexes in exons 13 and 14 (lanes marked by an arrow). The exon 14 band shift and heteroduplex in patient 1 was characterised to be due to P664L. Band shifts in exons 12, 13 and 15 were due to C1725T & C1773T, C1958T and G2231A polymorphisms respectively.
Fig 3.5: SSCP gel to confirm band shifts detected in an all exon SSCP gel. SSCP gel of the 3' end of exon 4 which encompasses part of repeat 4 and repeat 5 of LDLR. ss - single strands, ds - double strands, h - heteroduplexes. The four lanes with band shifts or heteroduplexes are marked by arrows. Mutations were characterised by fluorescent dye terminator sequencing: 1, 4 - E207X, 2 - dG197 and 3 - C163Y.
Fig 3.6: Molecular analysis of LDLR exon 12.
A: SSCP gel of exon 12 - polymorphisms in exon 12 (C1725T & C1773T) created difficulties in identifying band shifts caused by mutations, especially when only 4 samples were analysed at one time. Single strands (ss) and double strands (ds) are marked on the gel.
B: 'Forced' Msel assay for C1725T polymorphism. An Msel site was introduced into the rare T allele (lanes 3, 5 & 7). Fragments were separated on a 10% polyacrylamide gel and stained with EtBr.
C: HincII assay for C1773T polymorphism. A HincII site was introduced into the C alleles (lanes 3, 4, 5, 6 & 8). Fragments were separated on an EtBr stained 4% (3:1 NuSieve) agarose gel.
Genotypes of the two assays are found above the respective lane (B & C).
Fig 3.7: Polymorphic variations of G1413A in exon 10 (A) and C1617T in exon 11 (B) of LDLR. The regions of interest were amplified, digested and separated on an EtBr stained 4% (3:1 NuSieve) agarose gel.

A: Lane 1 - 50bp size marker, lane 2 - undigested PCR, lanes 3 & 4 - G14131G, lanes 5 & 6 - G1413A, lanes 7 & 8 - A1413A, lane 10 - blank control.
B: lane 1 - 50bp size marker, lane 2 - T1617T, lanes 3 & 4 - C1617T, lanes 5 & 6 - C1617TC, lane 7 - blank control. Genotypes are also listed above the lanes.
A: Antisense sequence of nucleotides 687-677 of LDLR exon 4

E207X heterozygote: C>A at 682
T T C C T N G T C A G

Normal sequence: C at 682
T T C C T C G T C A G

B: Antisense sequence of nucleotides 659-649 of LDLR exon 4

dG197 heterozygote: del CCA at 652
G C C A T C A C A C

Normal: C C A T C A C A C
Mutant: T C A C A G C

Normal sequence at 653
G C C A C C C A A T C A C

Fig 3.8: ABI electrophoregrams of mutant and normal sequences of two LDLR mutations, a nonsense and a three base deletion. A: E207X in repeat 5 of LDLR (exon 4). B: dG197 in repeat 5 of LDLR (exon 4). Mutations are marked by a * although the software indicates the mutations as an N (the 3bp deletion was actually 3bp upstream from that determined by the software peak thresholds) and the corresponding position on the normal sequences are marked by a *. The highlighted box represents the manual method of determining what bases are deleted: the deletion was deduced by manually identifying the two peaks at each position subtracting the normal sequence and observing the difference between the mutant and normal sequence. (the sequence which is not underlined).
<table>
<thead>
<tr>
<th>LDLR exon or APOB</th>
<th>Region</th>
<th>Mutation</th>
<th>Nucleotide change</th>
<th>No. of probands (C/A)</th>
<th>Support for pathogenicity</th>
<th>Direct assay (Gain + Loss)</th>
<th>No. of relatives tested per proband (No. of negatives)</th>
<th>Nsil -</th>
<th>BsIl +</th>
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<tbody>
<tr>
<td>2</td>
<td>Repeat 1</td>
<td>del A at 118*</td>
<td>118 G&gt;A</td>
<td>1A</td>
<td>Null allele (frameshift)</td>
<td>Direct sequencing analysis</td>
<td>1C</td>
<td>6 (1-ve)</td>
<td>---------</td>
</tr>
<tr>
<td>2</td>
<td>Repeat 1</td>
<td>D26N*</td>
<td>139 T&gt;G</td>
<td>1C</td>
<td>Co-segregation analysis</td>
<td>Direct sequencing analysis</td>
<td>Screened 188 normal chromosomes. D26 conserved</td>
<td>2 (C/A)</td>
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<tr>
<td>2</td>
<td>Repeat 1</td>
<td>W66G</td>
<td>259 C&gt;G</td>
<td>2G</td>
<td>French Canadian founder</td>
<td>25-70% LDLR activity</td>
<td>Observed now in an Italian. C68R &amp; C68Y observed</td>
<td>1A</td>
<td>0.0</td>
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<tr>
<td>3</td>
<td>Repeat 2</td>
<td>C68W*</td>
<td>267 A&gt;G</td>
<td>2G</td>
<td>Observed previously</td>
<td>15-30% LDLR activity</td>
<td>D69 conserved</td>
<td>1A</td>
<td>2.0</td>
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Table 3.3: Characterisation of LDLR and APOB mutations detected in patients with FH from the UK over the last four years.
<table>
<thead>
<tr>
<th>LDLR exon or APOB</th>
<th>Region</th>
<th>Mutation</th>
<th>Base number</th>
<th>Nucleotide change</th>
<th>Support for pathogenicity</th>
<th>No. of probands (C/A)</th>
<th>No. of relatives tested per proband (No. of negatives)</th>
<th>Direct assay (Gain +/Loss - in mutant allele)</th>
</tr>
</thead>
</table>
| 3                | Repeat 2 | E80K     | 301         | G>A              | Manchester founder gene mutation.  
|                  |        |          |             |                  | CpG site.                2(A,C) | 0.4                                                 | ACR: TaqI -                            |
|                  |        |          |             |                  | Co-segregation analysis in paediatric proband family.  
|                  |        |          |             |                  | E80 in conserved DXSDE motif \(\*\).             |                                      |                                      |                                        |
| 4                | Repeat 3 | del 2, ins 2 at 324 (C88R) | 324 | dGT, insTC but could be rare variant, G>T & C88R on different chromosome | C88R (15-30% LDLR activity)  
|                  |        |          |             |                  | G>T variant & C88R only observed separately.  
|                  |        |          |             |                  | Screened 188 normal chromosomes.  
|                  |        |          |             |                  | T87 and C88 conserved\(\*\)             |                                      |                                      | Combination of MaelII, Tail & HaelII        |
| 4                | Repeat 3 | C88Y*    | 326         | G>A              | C88F & C88R observed.  
|                  |        |          |             |                  | Screened 188 normal chromosomes.  
|                  |        |          |             |                  | C88 conserved\(\*\)             |                                      |                                      | Rsal +                                   |
| 4                | Repeat 3 | del A at 353* | 353 | dA (FsD97)    | Null allele (frameshift).  
|                  |        |          |             |                  |                                      |                                      |                                      | Size on 36cm ABI gel                    |
| 4                | Repeat 4 | C146X    | 501         | C>A              | Null allele (termination).  
<p>|                  |        |          |             |                  |                                      |                                      |                                      | Ddel + (control cut site)                |</p>
<table>
<thead>
<tr>
<th>LDLR exon or APOB</th>
<th>Region</th>
<th>Mutation</th>
<th>Base number</th>
<th>Nucleotide change</th>
<th>Support for pathogenicity</th>
<th>No. of probands (C/A)</th>
<th>No. of relatives tested per proband (No. of negatives)</th>
<th>Direct assay (Gain +/-Loss - in mutant allele)</th>
</tr>
</thead>
</table>
| 4                 | Repeat 4 | S156L    | 530         | C>T              | • Observed worldwide.  
• <2% LDLR activity.  
• CpG site.  
• Co-segregation analysis in paediatric proband family.  
• S156 in conserved DXSDE motif **. | 1A                  | 1(1 -ve)                                              | -                                           |
| 4                 | Repeat 4 | C163Y    | 551         | G>A              | • Glaswegian founder gene mutation.  
• C163 conserved**. | 2(A,C)               | 0,5                                                    | ACR: Rsal -                              |
| 4                 | Repeat 5 | del 3 at 652 (dG197) | 652 | dGGT              | • Lithuanian founder gene mutation.  
• <2% LDLR activity.  
• G197 conserved**. | 3(A,A,C)               | 1,0,2                                                  | Size on 10% acrylamide or 12cm ABI gel                           |
| 4                 | Repeat 5 | D200G    | 662         | A>G              | • Observed worldwide.  
• D200 conserved**. | 1A                  | 4                                                      | MspI +                                     |
| 4                 | Repeat 5 | D206E    | 681         | C>G              | • South African founder gene mutation.  
• Co-segregation analysis in one paediatric proband family.  
• D206 in conserved DXSDE motif **. | 3(A,C,C)               | (0,1,12 (10 -ve))                                      | Dde + (control cut site)                  |
<table>
<thead>
<tr>
<th>LDLR exon or APOB</th>
<th>Region</th>
<th>Mutation</th>
<th>Base number</th>
<th>Nucleotide change</th>
<th>Support for pathogenicity</th>
<th>No. of probands (C/A)</th>
<th>No. of relatives tested per proband (No. of negatives)</th>
<th>Direct assay (Gain +/-Loss - in mutant allele)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Repeat 5</td>
<td>E207X</td>
<td>682</td>
<td>G&gt;T</td>
<td>• Nonsense mutation.</td>
<td>4(A,C,C,C)</td>
<td>0,1,1,2</td>
<td>Bfal +</td>
</tr>
<tr>
<td>5</td>
<td>Repeat 6</td>
<td>E237X*</td>
<td>772</td>
<td>G&gt;T</td>
<td>• Nonsense mutation.</td>
<td>1C</td>
<td>2 (1 -ve)</td>
<td>Ncil -</td>
</tr>
<tr>
<td>6</td>
<td>Repeat 7</td>
<td>D283E</td>
<td>912</td>
<td>C&gt;G</td>
<td>• Observed worldwide.</td>
<td>1A</td>
<td>0</td>
<td>BsmFI - (control cut site)</td>
</tr>
<tr>
<td></td>
<td>Repeat 7</td>
<td>Del 2 at 932</td>
<td>932</td>
<td>dAA (FsK290)</td>
<td>• Null allele (frameshift)</td>
<td>1A</td>
<td>0</td>
<td>Size on 12cm ABI gel</td>
</tr>
<tr>
<td>7</td>
<td>Repeat A</td>
<td>H306Y*</td>
<td>979</td>
<td>C&gt;T</td>
<td>• H306 conserved.</td>
<td>1A</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Repeat A</td>
<td>P320R*</td>
<td>1022</td>
<td>C&gt;G</td>
<td>• P320 conserved except in Xenopus I &amp; II (N) and banded cat shark (R).</td>
<td>1A</td>
<td>0</td>
<td>Acil +</td>
</tr>
<tr>
<td>8</td>
<td>Repeat B</td>
<td>Ins 4 at 1121</td>
<td>1121</td>
<td>Ins GGGT (FsG352)</td>
<td>• Null allele (frameshift).</td>
<td>2A</td>
<td>0,0</td>
<td>Size on 10% acrylamide or 12cm ABI gel</td>
</tr>
<tr>
<td>10</td>
<td>EGF Spacer</td>
<td>Ins 4 at 1374*</td>
<td>1374</td>
<td>Ins CAGA (FsA438)</td>
<td>• Null allele (frameshift).</td>
<td>1A</td>
<td>0</td>
<td>Size on 10% acrylamide or 12cm ABI gel</td>
</tr>
<tr>
<td>LDLR exon or APOB</td>
<td>Region</td>
<td>Mutation</td>
<td>Base number</td>
<td>Nucleotide change</td>
<td>Support for pathogenicity</td>
<td>No. of probands (C/A)</td>
<td>No. of relatives tested per proband (No. of negatives)</td>
<td>Direct assay (Gain +/-Loss - in mutant allele)</td>
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<td>---------------------------------------------</td>
</tr>
<tr>
<td>10</td>
<td>EGF</td>
<td>L458P*</td>
<td>1436</td>
<td>T&gt;C</td>
<td>Co-segregation analysis.</td>
<td>1A</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Spacer</td>
<td></td>
<td></td>
<td></td>
<td>L458 conserved*.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>EGF</td>
<td>D461H</td>
<td>1444</td>
<td>G&gt;C</td>
<td>D&gt;H substitution observed.</td>
<td>2A</td>
<td>0,0</td>
<td>MwoI +</td>
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<tr>
<td></td>
<td>Spacer</td>
<td></td>
<td></td>
<td></td>
<td>D461 not conserved*</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>EGF</td>
<td>D471H</td>
<td>1474</td>
<td>G&gt;C</td>
<td>D471N observed.</td>
<td>1A</td>
<td>0</td>
<td>Hinfl -</td>
</tr>
<tr>
<td></td>
<td>Spacer</td>
<td></td>
<td></td>
<td></td>
<td>Mutation in second YWTD</td>
<td></td>
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<td></td>
<td></td>
<td>conserved motif which is</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>repeated 5 times between</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>repeats B and C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>EGF</td>
<td>P505S</td>
<td>1537</td>
<td>C&gt;T</td>
<td>Observed previously.</td>
<td>1A</td>
<td>0</td>
<td>Hinfl +</td>
</tr>
<tr>
<td></td>
<td>Spacer</td>
<td></td>
<td></td>
<td></td>
<td>5-15% LDLR activity.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P505 conserved*.</td>
<td></td>
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<tr>
<td>11</td>
<td>EGF</td>
<td>G544A*</td>
<td>1694</td>
<td>G&gt;C</td>
<td>G544V observed.</td>
<td>1A</td>
<td>0</td>
<td>Srfal -</td>
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<tr>
<td></td>
<td>Spacer</td>
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<td></td>
<td></td>
<td>G544V &lt;2% LDLR activity.</td>
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<td></td>
<td></td>
<td>G544 conserved*.</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>EGF</td>
<td>del 5, ins A</td>
<td>1715</td>
<td>dGTGCG, insA (FsS551)</td>
<td>Null allele (frameshift).</td>
<td>1A</td>
<td>0</td>
<td>Size on 10% acrylamide or 12cm ABI gel</td>
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<tr>
<td></td>
<td>Spacer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>EGF</td>
<td>P587L*</td>
<td>1823</td>
<td>C&gt;T</td>
<td>P587L observed in another</td>
<td>1C</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Spacer</td>
<td></td>
<td></td>
<td></td>
<td>nationality.</td>
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<td></td>
<td>Co-segregation analysis.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>P587 conserved*.</td>
<td></td>
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<td></td>
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<td>LDLR exon or APOB</td>
<td>Region</td>
<td>Mutation</td>
<td>Base number</td>
<td>Nucleotide change</td>
<td>Support for pathogenicity</td>
<td>No. of probands (C/A)</td>
<td>No. of relatives tested per proband (No. of negatives)</td>
<td>Direct assay (Gain +/Loss - in mutant allele)</td>
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</tr>
<tr>
<td>13</td>
<td>EGF Spacer</td>
<td>W599C*</td>
<td>1860</td>
<td>T&gt;C</td>
<td>• W599R observed. • W599 in FWTD, the 5th YWTD motif.</td>
<td>1A</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>EGF Spacer</td>
<td>Ins C at 1944*</td>
<td>1944</td>
<td>InsC (FsP628)</td>
<td>• Null allele (frameshift)</td>
<td>1A</td>
<td>0</td>
<td>Size on 36cm ABI gel</td>
</tr>
<tr>
<td>14</td>
<td>Repeat C</td>
<td>C646F</td>
<td>2000</td>
<td>G&gt;T</td>
<td>• C646W &amp; C646X observed. • Mutation in NWCE motif, conserved in hamster, rabbit, rat, mouse, shark &amp; *Xenopus laevis.</td>
<td>1C</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Repeat C</td>
<td>C656R</td>
<td>2029</td>
<td>T&gt;C</td>
<td>• Observed previously. • 5-30% LDLR activity. • Mutation in NWCE motif, conserved in hamster, rabbit, rat, mouse, shark &amp; *Xenopus laevis.</td>
<td>1A</td>
<td>0</td>
<td>HaeIII +</td>
</tr>
<tr>
<td>14</td>
<td>Repeat C</td>
<td>C660S</td>
<td>2041</td>
<td>T&gt;A</td>
<td>• C660X and C660Y observed. • C660 conserved™.</td>
<td>2(A,C)</td>
<td>0,1</td>
<td>BsmFl - (control cut site)</td>
</tr>
<tr>
<td>14</td>
<td>Repeat C</td>
<td>P664L</td>
<td>2054</td>
<td>C&gt;T</td>
<td>• Observed worldwide. • &lt;2% LDLR activity. • CpG site. • P664 conserved™.</td>
<td>8(A,C,C, C,C, C,C)</td>
<td>0,1,1,1,1,1, 1,2,(1 -ve), 4 (2 Hmz)</td>
<td>PstI +</td>
</tr>
<tr>
<td>LDLR exon or APOB</td>
<td>Region</td>
<td>Mutation</td>
<td>Base number</td>
<td>Nucleotide change</td>
<td>Support for pathogenicity</td>
<td>No. of probands (C/A)</td>
<td>No. of relatives tested per proband (No. of negatives)</td>
<td>Direct assay (Gain +/-/Loss - in mutant allele)</td>
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</tr>
<tr>
<td>Intron 14</td>
<td>5' splice region</td>
<td>2140+1 G&gt;C</td>
<td>2140+1</td>
<td>G&gt;C</td>
<td>• G always observed at +1, replacement of G predicted to cause instability^x.</td>
<td>1A</td>
<td>0</td>
<td>Alul +</td>
</tr>
<tr>
<td>Intron 14</td>
<td>5' splice region</td>
<td>2140+5 G&gt;A</td>
<td>2140+5</td>
<td>G&gt;A</td>
<td>• G at +5 - frequency of 0.84, replacement of G predicted to cause instability^x.</td>
<td>1A</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Intron 16</td>
<td>5' splice donor</td>
<td>2389+1 G&gt;A*</td>
<td>2389+1</td>
<td>G&gt;A</td>
<td>• G always observed at +1, replacement of G predicted to cause instability^x.</td>
<td>1A</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>V 7-8</td>
<td>Repeat A &amp; B</td>
<td>4kb V ex 7-8*</td>
<td>-</td>
<td>Ex 7-8</td>
<td>• Major duplication.</td>
<td>1A</td>
<td>1</td>
<td>Quantitative fluorescent multiplex PCR</td>
</tr>
<tr>
<td>V 7-18</td>
<td>-</td>
<td>~20kb V ex 7-3'UTR</td>
<td>-</td>
<td>Ex 7-3'UTR</td>
<td>• Major duplication.</td>
<td>1C</td>
<td>0</td>
<td>Quantitative Fluorescent multiplex PCR</td>
</tr>
<tr>
<td>Δ 3-5/ V 8-17</td>
<td>-</td>
<td>min Δ ex 3-5/ V 8-17</td>
<td>-</td>
<td>Not fully characterised</td>
<td>• Major deletion or duplication.</td>
<td>1A</td>
<td>0</td>
<td>Quantitative Fluorescent multiplex PCR</td>
</tr>
<tr>
<td>V 8</td>
<td>Repeat B</td>
<td>V exon 8</td>
<td>-</td>
<td>Ex 8</td>
<td>• Major duplication.</td>
<td>1C</td>
<td>1</td>
<td>Quantitative fluorescent multiplex PCR</td>
</tr>
<tr>
<td>LDLR exon or APOB</td>
<td>Region</td>
<td>Mutation</td>
<td>Base number</td>
<td>Nucleotide change</td>
<td>Support for pathogenicity</td>
<td>No. of probands (C/A)</td>
<td>No. of relatives tested per proband (No. of negatives)</td>
<td>Direct assay (Gain +/-Loss - in mutant allele)</td>
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</tr>
<tr>
<td>APOB</td>
<td>Exon 26</td>
<td>R3500Q</td>
<td>10708</td>
<td>G&gt;A</td>
<td>In vivo studies and well established cause of FDB.</td>
<td>8(A,A,A, A,A,C, A,A)</td>
<td>0,0,0,0,0, 1(-ve),1,2</td>
<td>ACR: Sau96I -&amp; Scal +</td>
</tr>
<tr>
<td>APOB</td>
<td>Exon 26</td>
<td>R3531C</td>
<td>10800</td>
<td>C&gt;T</td>
<td>In vivo studies</td>
<td>1C</td>
<td>6 (4 -ve)</td>
<td>NsiI +</td>
</tr>
</tbody>
</table>

C = child probands and A = adult probands.

* Novel mutations found in these patients, either by myself or novel when identified in same patients by Day et al, 1997b.

* Mutations found by Sun et al, 1992, which were also referred to the service. The mutation has only been observed in these families.

**Sequences were conserved at the amino acid level in human (Yamamoto et al, 1984), rabbit (Yamamoto et al, 1986), rat (Lee et al, 1989), Xenopus laevis I & II (Mehta et al, 1991), hamster (Bishop et al, 1992), mouse (Hoffer et al, 1993) and banded cat shark (Mehta et al, 1996) LDLR cDNA sequences unless stated.

*DXSDE motif is a conserved cluster of negatively charged amino acids found at the C terminal of the repeats which are thought to bind to the basic amino acids of apoB and/or apo E.

*Replacement of G at +1 or G at +5 predicted to reduce significantly the stability of base pairing of the splice site with the complementary region of U1 snRNA. Binding to U1 snRNA is essential for the pre-mRNA to be folded correctly before cleavage and ligation can occur with the spliceosome. Consensus nucleotide frequencies at splice sites came from Table 9.1, Cooper & Krawczak, 1993.
Table 3.4: Rare variants and amino acid substitutions found in FH patients from the UK that are predicted to be non-pathogenic.

<table>
<thead>
<tr>
<th>LDLR Exon/Intron</th>
<th>Region</th>
<th>Mutation</th>
<th>Base number</th>
<th>Base change</th>
<th>Support of non-pathogenicity</th>
<th>No. of probands</th>
<th>No. of family members</th>
<th>Direct assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 14</td>
<td>Repeat C of EGF precursor homology domain</td>
<td>G654G*</td>
<td>2025</td>
<td>C&gt;T</td>
<td>• No amino acid substitution. • G654 conserved^ but nucleotide is not conserved: human - GGC&gt;GGT, rabbit - GGC, rat - GGT, Xenopus I &amp; II - GGG, hamster - GGT, mouse - GGT, banded cat shark - GGT.</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Intron 7</td>
<td>Intron 7</td>
<td>1061-8T&gt;C</td>
<td>1061-8</td>
<td>T&gt;C</td>
<td>• Inherited from non-FH parent &amp; on same haplotype as T705I (section 5.10). • Identified in individuals with normal cholesterol and in patients with other LDLR mutations (section 5.10).</td>
<td>1</td>
<td>1 (-ve)</td>
<td>MboII +</td>
</tr>
<tr>
<td>Exon 15</td>
<td>O-linked sugar domain</td>
<td>T705I</td>
<td>2177</td>
<td>C&gt;T</td>
<td>• See 1061-8T&gt;C variation. • T705 conserved^ except in mouse or banded cat shark.</td>
<td>1</td>
<td>1 (-ve)</td>
<td>ACR: Nsil +</td>
</tr>
<tr>
<td>Intron 16</td>
<td>Intron 16</td>
<td>2390-16 G&gt;A*</td>
<td>2390-16</td>
<td>G&gt;A</td>
<td>• L458P mutation tracked with FH phenotype in family but this variant did not.</td>
<td>1</td>
<td>2 (-ve)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Novel sequence changes.

\^Sequences were conserved at the amino acid level in humans (Yamamoto et al, 1984), rabbit (Yamamoto et al, 1986), rat (Lee et al, 1989), Xenopus laevis I & II (Mehta et al, 1991), hamster (Bishop et al, 1992), banded cat shark (Mehta et al, 1996) and mouse (Hoffer et al, 1993) LDLR cDNA sequence.
3.4. Discussion: Evaluation of service

3.4.1. Identifying pathogenic mutations

Mutations were detected in 72 FH probands, ranging from missense mutations to major deletions or duplications in LDLR and the R3500Q and R3531C mutations in APOB. All identified nucleotide alterations had to meet certain criteria to be classified as pathogenic. These criteria were similar to that described by Cotton & Scriver, 1998, but with the addition of technical points:

1. Technical criteria:
   a. Sequence change identified in a second PCR product and if possible a second sample from the patient, although not necessary if tests are carried out under EQA guidelines.

   External Quality Assurance guidelines:
   In a diagnostic laboratory various checking steps are incorporated in the molecular analysis procedures carried out. Thus for a disorder like CF or DMD the following checks are made. All tube transfers are ‘checked’ and for every procedure a list of samples are drawn up and this form has to be signed by the checker at each transfer. The samples were loaded onto a gel and the order of loading is written onto a gel sheet. The order is confirmed by a second person who then signs the gel sheet. When appropriate a picture of the gel is then placed on this gel sheet, a gel identification number is given and stored as a permanent record. As the various steps are checked a result can be reported from a single test result. Data is read once, checked by a second scientist and then entered, along with the gel identification number, onto a form containing all the data from a single family.

   DNA extraction of FH samples operated under EQA guidelines but no checks was undertaken for the molecular analysis, as all tests were carried out in microtitre trays. All SSCP band shifts caused by possible disease-causing mutations were characterised by sequencing a second PCR product using the original stock of DNA, and confirmed by sequencing or a direct assay on a third PCR product;

2. Genetic criteria:
   a. Nonsense, frameshift, large deletion, insertion or duplication, or splice site mutations are proof in themselves of being deleterious. Further analysis must be carried out for missense mutations which may be non-pathogenic variations;

   b. Previous independent occurrence of the mutation in an unrelated patient - although
caution must be taken as to how the pathogenicity was proven;

c. Absence of any other lesion in the coding region is suggestive evidence that a given base change is causative although some doubt may remain;

d. Co-segregation analysis - if the FH phenotype co-segregates with the mutation, and does not co-segregate in its absence, then the mutation is compatible with the phenotypic effect;

e. Failure to observe such a mutation in at least 100 normal chromosomes, however we use at least 188 normal chromosomes, i.e. one microtitre tray including one positive and negative control;

3. **Protein criteria:**

a. Occurrence of the missense mutation in a region of known structure or function - each of the five structural domains of the LDL-receptor are encoded by a defined set of exons (section 1.6.6.1). Cellular studies (reviewed by Goldstein, Hobbs & Brown, 1995) have shown the significance of each region, although less is known about the O-linked sugar domain, encoded by exon 15;

b. Occurrence of the missense mutation in an evolutionary conserved amino acid is likely to have greater functional importance, and mutations that alter conserved residues by replacement with amino acids of different physical character, are likely to affect the protein structure and function.

This was fulfilled by looking for the conservation of the amino acids over a number of species. The cDNA and protein sequence of the LDL-receptor has been characterised in the human (Yamamoto *et al*, 1984), cow (Russell *et al*, 1983), rabbit (Yamamoto *et al*, 1986), rat (Lee *et al*, 1989), hamster (Bishop *et al*, 1992), toad (*Xenopus laevis*) (Mehta *et al*, 1991), mouse (Hoffer *et al*, 1993) and most recently in the banded cat shark, *Chiloscyllium plagiosum* (Mehta *et al*, 1996).

All five functional domains of the LDL-receptor were assembled in their modern form more than 450 million years ago, as revealed from the cloning and sequencing of an LDL-receptor cDNA from the banded cat shark, *Chiloscyllium plagiosum* (Mehta *et al*, 1996). The shark LDL-receptor has the same overall architecture as the mammalian and amphibian counterparts. Overall the ligand binding, EGF precursor homology and
cytoplasmic domains are highly conserved between species with the cytoplasmic tail being the most conserved domain (Table 3.5). Between Xenopus and humans there are only six changes in 50 residues in the most conserved region, the cytoplasmic tail (Mehta et al., 1991a). Presumably there is some advantage to having these residues but this may not be readily detected in kinetic studies in cultured cells. The clustered O-linked sugar domain is not conserved in sequence but it is in structure with all species having many serine and threonine residues in this region. The signal peptide shows no sequence conservation. Thus if an amino acid is conserved across the species, altering it is more likely of functional significance.

Substitutions which do not alter an amino acid may still be pathogenic as shown by a G to an A transition in the alanine codon at position 344 in the gene for fibroblast growth factor 2, which introduces a cryptic splice donor site resulting in Crouzon syndrome (Li et al., 1995). Substitutions in the following regions may be pathogenic and therefore should be investigated for pathogenicity:

i. 5' and 3' splice sites outside the conserved nucleotides - may cause exon skipping, usually reduce the amount of mature mRNA generated and/or activate cryptic splice sites in the vicinity;

ii. Introns - activate cryptic splice sites, leading to the production of aberrant mRNA species;

iii. Lariat structure - affect the formation of the lariat structure involved in splicing;

iv. Branch-point sequences - may reduce the efficiency of mRNA splicing and may lead to the activation of cryptic splice sites.

4. Expression analysis:
Demonstration that a mutant protein produced in vitro possesses the same biochemical properties and characteristics as its in vivo counterpart (~130 of the 700 mutations known to date have been studied at the cellular level (FH website)). If the mutation is shown to result in a defect, in synthesis, transport, binding, clustering in coated pits or recycling, by an in vitro expression analysis all other criteria become redundant;

5. Gene therapy:
Reversal of the pathological phenotype in the patient/cultured cells by replacement of the mutant gene/protein with its wildtype counterpart.

The available data supporting the pathogenicity of each mutation is shown in Table 3.3. Previously undescribed missense mutations were predicted to be pathogenic if they
fulfilled certain criteria: \(1 + 2(c, d \text{ or } c+d \text{ & } e) + 3(a+b)\). If any expression analysis had been carried out on another substitution but at the same residue this may indicate the effect of the detected mutation.

Table 3.5: Conserved amino acid sequence of the LDL-receptor: comparison of sequences from multiple species with that of the human LDL-receptor.

<table>
<thead>
<tr>
<th>Ligand binding domain (2-292)</th>
<th>EGF precursor homology domain (293-693)</th>
<th>O-linked sugar domain (694-767)</th>
<th>Transmembrane domain (768-789)</th>
<th>Cytoplasmic domain (790-839)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>-</td>
<td>(\sim36)</td>
<td>(\sim60)</td>
<td>(\sim90)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>75</td>
<td>83</td>
<td>62</td>
<td>86</td>
</tr>
<tr>
<td>Rat</td>
<td>78</td>
<td>84</td>
<td>41</td>
<td>55</td>
</tr>
<tr>
<td>Mouse</td>
<td>76</td>
<td>83</td>
<td>-</td>
<td>48</td>
</tr>
<tr>
<td>Hamster</td>
<td>76</td>
<td>86</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>Toad (Xenopus laevis 1)</td>
<td>70</td>
<td>70</td>
<td>-</td>
<td>54</td>
</tr>
<tr>
<td>Toad (Xenopus laevis II)</td>
<td>69</td>
<td>70</td>
<td>-</td>
<td>58</td>
</tr>
<tr>
<td>Banded cat shark (Chiloscyllium plagiosum)</td>
<td>68</td>
<td>67</td>
<td>-</td>
<td>39</td>
</tr>
</tbody>
</table>

Data taken from Mehta et al, 1991a; Lee et al, 1989; Hoffer et al, 1993 and Mehta et al, 1996. \(^1\)Amino acids in the human LDL-receptor. \(^*\)Actual figures not available, but estimates from comparisons with other species determined. - No data available.

Despite these criteria care must be taken to ensure that the identified mutation is likely to be pathogenic, as shown when two mutations, E256K and I402K, were found on the same allele (Ekstrom et al, 1995). Both mutations adhered to the genetic criteria of pathogenicity. The E256K mutation altered a conserved amino acid in the EGF precursor homology domain although not in a cysteine-rich repeat, changed the amino acid from a negatively charged to a positively charged amino acid and it was not detected in over 200 normal subjects. The I402T mutation also encodes part of the EGF precursor homology domain and the amino acid changes from an aliphatic, hydrophobic isoleucine to a positively charged, hydrophobic threonine. It was only cellular studies, which aren't often practical, which showed which mutation was causing the phenotype. Cells only expressing the I402T mutation, or the combination of I402T and E256K mutations were seriously affected in mediating uptake and degradation of LDL. Contrary to initial predictions, the cells expressing only the E256K mutation showed
the same binding, uptake and degradation of $^{125}$I-labelled LDL as cells transfected with normal LDLR cDNA. These results suggest that the pathogenic mutation was I402T and that the E256K mutation is a rare sequence variation. This case demonstrates that despite these criteria, caution should be taken on making predictions about the pathogenicity of a mutation. The E256K substitution has been observed in the presence of a second mutation, W-18X (Cenarro et al, 1998) and in this case the null allele was predicted to be the mutation. Therefore the E256K substitution appears to be a non-pathogenic variant.

3.4.2. Mutation detection rates
The individuals tested in this study were ethnically diverse and the clinical diagnoses varied between lipid clinics. To evaluate detection rates the patients were divided according to their age; adult and paediatric cases, and adults were further into ‘definite’ (TX+) or ‘probable’ FH (TX-). In 99 patients examined by one clinician and described in section 6, the group with a diagnosis of ‘definite’ FH had an overall detection rate significantly higher than in the group with a ‘probable’ FH diagnosis (43% v 13%, p<0.01). In 36 paediatric/adolescent FH probands referred from GOSH and Middlesex Hospital the detection rate was 72% including major rearrangement analysis (discussed in section 4). Detection rates were significantly higher in paediatric cases compared to adult cases (72% v 43%, p<0.01). This may reflect the fact that FH is one of the few hyperlipidaemias expressed from birth rather than post puberty thus a child who has hypercholesterolaemia is very likely to have FH whereas in adults hypercholesterolaemia may be due to many other factors (see sections 1.3 & 1.5). These data suggest that to prevent unnecessary screening, clinical criteria must be adhered to strictly.

3.4.3. Mutation spectrum
Nucleotide substitutions are thought to either result from chemical (e.g. deamination of 5-methylcytosine, depurination), physical (e.g. slippage) or enzymatic (e.g. post replicative mismatch repair or exonucleolytic proofreading) mechanisms (reviewed by Cooper & Krawczak, 1993). The efficiency of all these processes is known to be sequence-dependent leading to the non-random distribution of point mutations.

3.4.3.1. ‘Hotspot’ LDLR exons
Overall the greatest number of LDLR mutations were found in exons 3 (9.5%), exon 4 (28.6%), exon 10 (7.9%) and exons 14 (22.2%); 46% of LDLR mutations were found in the ligand binding domain and 46% were found in the EGF precursor-like domain. In paediatric cases, mutations were only found in six exons, 2, 3, 4, 5, 12 & 14, which
encode two domains of the LDL-receptor protein, the ligand binding domain (46%) and the EGF precursor homology domain (38%) (Fig 3.9). Exons 4 and 14 accounted for 65% of these mutations and three mutations, D206E, E207X and P664L accounted for these high figures.

Mutations were not evenly distributed among the ligand binding domain. As reported previously (Gudnason et al, 1993; Hobbs et al, 1992), repeat 5 contains three times as many mutations as any of the other repeats. This suggests that repeat 5 occupies a crucial structural position so that any alteration in its sequence interferes with folding. The high detection rate for this repeat may also reflect patient referral selection bias as repeat 5 is the only cysteine-rich repeat which binds apoE and apoB (Russell, Brown & Goldstein, 1989) and which thus results in a more severe FH phenotype (Gudnason et al, 1993).

Exon 14 was the other region where a high number of mutations were identified in paediatric probands but this was also observed in adult probands (Table 3.6 & Fig 3.9). Exon 14 encodes the third growth factor repeat, repeat C, in the EGF precursor homology domain and the high frequency of mutations in this region is probably due to the functional significance of this domain. Point mutations causing single amino acid substitutions in the EGF precursor-like domain result in a protein which is poorly transported, if at all, to the cell surface. This region also allows the acid-dependent dissociation of the ligand from the receptor, and mutations in this region prevent this happening which results in the degradation of the receptor because it cannot recycle to the cell surface after the first round of endocytosis (Davis et al, 1987a). The P664L mutation accounted for seven of the nine exon 14 mutations in the paediatric cases and this mutation is one of the more 'common' LDLR mutations world-wide. This mutation has been found in many ethnic groups and haplotype analysis has shown that this mutation is likely to have occurred independently several times in patients with very different genetic backgrounds. The mutant precursor is processed more slowly to the mature form than normal, but the mature protein also has a lower binding affinity for LDL than the normal receptor (Knight et al, 1989; Soutar et al, 1989).
Fig 3.9: The spectrum of mutations across the exons of LDLR in 26 paediatric FH probands (A) and 46 adult (B) FH probands. The spectrum of different LDLR mutations (excluding major rearrangements) reported on the FH website (C).
As shown in Table 3.6, the number of exon 3, 4 and 14 mutations compared to other regions are significantly different between paediatric and adult cases (p<0.01). This difference is due to greater frequency of exon 14 mutations present in paediatric cases compared to adults (p<0.025). These regions may be more deleterious for receptor function so the children attending the lipid clinics may represent the more severely affected individuals. During adulthood, cholesterol levels increase in carriers of mutations in other exons and this is brought to the attention of GPs and lipidologists and thus detected here molecularly. Thus the spectrum of mutations detected in the paediatric cases is likely to be due to selection bias.

Table 3.6: Comparison of mutation regions between paediatric and adult FH cases.

<table>
<thead>
<tr>
<th>Mutation region comparison</th>
<th>Paediatric cases</th>
<th>Adult cases</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR v APOB</td>
<td>2/26</td>
<td>7/46</td>
<td>&lt;0.50</td>
</tr>
<tr>
<td>Ex3 v other mutations</td>
<td>2/26</td>
<td>4/46</td>
<td>&lt;0.90</td>
</tr>
<tr>
<td>Ex4 v other mutations</td>
<td>8/26</td>
<td>10/46</td>
<td>&lt;0.50</td>
</tr>
<tr>
<td>Ex14 v other mutations</td>
<td>9/26</td>
<td>5/46</td>
<td>&lt;0.025*</td>
</tr>
<tr>
<td>Ex3/4/14 v other mutations</td>
<td>19/26</td>
<td>19/46</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Deletion v other LDLR mutations</td>
<td>2/26</td>
<td>2/46</td>
<td>&lt;0.75</td>
</tr>
</tbody>
</table>

* Significant differences between paediatric and adult FH cases.

Of the 580 different LDLR point mutations currently reported (FH website) 11.3% are located at CpG sites, which is similar to the 15% reported in 1992 in one study of 157 FH homozygotes and 13 heterozygotes (Hobbs et al, 1992). The CpG dinucleotide is specifically associated with a high frequency of C>T and G>A transversions due to increased mutability of 5-methylcytosine by deamination to thymine (Duncan & Miller, 1980), together with a less than perfect repair mechanism for resulting G:T mismatches (discussed by Cooper & Krawczak, 1993).

3.4.3.2. Mutation depleted regions

No mutations were identified in the promoter, exons 1, 9, 15, 17 or 18 and only one mutation was identified in exon 5, 11 and 16 in the paediatric and adult cases. The number of unique mutations reported world-wide in exons 5, 9, 11, 17 are between 20-42 but only 10 promoter, 14 exon 1, 8 exon 15, 10 exon 16 and 1 exon 18 mutation has been observed (FH website). Not only are the number of unique mutations lower in these exons but also the frequency of these mutations (Hobbs et al, 1992; Day et al, 1997b). Explanations for the lack of mutations in particular exons could be either the failure to detect mutations in these exons for technical reasons, less mutations may
occur because the sequence has less CpG regions or by the fact that these regions may be less functionally important so that variations that occur are non-pathogenic.

In the GOSH Clinical Molecular Genetics Laboratory SSCP analysis is routinely used to analyse many genes. Detection rates range from 50% in the gene EYA1, in patients with Branchio-oto-renal syndrome (BOR) (Rickard et al, submitted) to 98% in the gene interleukin-2 receptor gamma chain (IL-2Rγ), in patients with X-linked severe combined immunodeficiency (XSCID) (Lester et al, unpublished data) and these differences in detection rates are unlikely to be explained by differences in SSCP efficiency but rather by the strictness of clinical and immunological data. The main alternative to SSCP is DGGE which has been shown to have higher detection rates in some disorders (Moyret et al, 1994; Schwaab et al, 1997). DGGE has been implemented to diagnose FH in Dutch (Lombardi et al, 1995), Swedish (Ekstrom et al, 1995) and Danish patients (Nissen et al, 1996). Standardised PCR and DGGE conditions have been established to screen for LDLR promoter region, all 18 LDLR exons and flanking intronic sequences (Lombardi et al, 1995). The detection rate in this study was 81% but the sample size was very small (n=32) and the clinical criteria were very strict, for example total cholesterol > 9.5mmol/l.

The PCR products of exons 1, 9, 15 and 17 are 238, 273, 247 and 242bp respectively and these are approaching the upper end of the resolution range for SSCP detection. Some mutations may have been undetected due to lack of separation as seen with the exon 10 G1413A polymorphism which is only sometimes observed with the 298bp exon 10 product but clearly observed with the 5' exon 10 product, 202bp. In contrast exon 14 missense mutations produce large shifts using the 288bp PCR product and the exon 15 G2231A polymorphism is easily seen. In an ongoing study, not described in this thesis, two exon 15 mutations were detected showing that mutations in exon 15 are detectable. The exon 18 PCR product is only 135bp in size so may be too small for SSCP analysis but it has not been possible to test this since no exon 18 positive control is available (only one exon 18 mutation has been reported).

Promoter and exon 1 mutations are rarely detected (Top et al, 1992; Hobbs et al, 1992; Day et al, 1997b), so the failure to detect any is probably an accurate result. The lack of mutations within these two regions is probably attributed to the small number of base pairs that are crucial for promoter function (<50) and exon 1 only encodes 21 amino acids of the signal sequence which is cleaved during translocation of the polypeptide chain into the lumen of the ER.
The other regions where no or few mutations were observed are described in functional importance below:

Exon 9 is found in the EGF precursor-like domain. Many mutations have been reported in this region but in particular in exon 7, 8 and 14 which encode the three growth factor repeats found in this domain. Exon 9 forms part of the spacer between repeats B and C and 42 different exon 9 mutations have been observed. One exon 9 mutation, V408M, occurs at a high frequency in South Africa due to a founder gene effect (Leitersdorf et al, 1989; Kotze et al, 1991). Thus the lack of exon 9 mutations in the adult group is either just an anomaly, a true result for UK individuals or due to the failure to detect them by the SSCP technique. Two exon 9 mutation controls have been detected by this SSCP method so this observation suggests that exon 9 mutations are rare in the UK population.

Exon 15 encodes a region rich in serine and threonine residues that are attachment sites for O-linked sugars. Few mutations that result in single amino acid substitutions have been observed in this domain and its function remains unclear (discussed in detail in section 5.10).

Exon 16 encodes part of the transmembrane domain. Only four point mutations have been observed in this domain in FH patients, probably because conservative changes would have little effect of function. However truncated receptor proteins that lack this region due to premature termination codons, introduced as a result of out of frame deletion, are secreted from the cell and cannot function in receptor-mediated endocytosis (Lehrman et al, 1987a). Four deletions have been observed and the other four exon 16 mutations are splice site mutations which are predicted to be pathogenic.

Exon 17 encodes 14 amino acids (776-789) of the membrane-spanning region that anchors the receptor at the cell surface. Five of the 19 exon 17 mutations occur in this region. The cytoplasmic tail is encoded by exon 17 (790-828) and exon 18 (829-839). Residue by residue site-directed mutagenesis of this cytoplasmic region and expression of the mutant cDNA species in cultured cells has defined four amino acid residues that are essential for directing the receptor-ligand complexes on the cell surface to cluster in coated pits (Davis et al, 1987b). Four point mutations have been observed in this internalisation signal, NPVY (FH website). It also emerged from the site-directed mutagenesis experiments that receptor proteins truncated after residue 812 function apparently normally in cultured cells. However, amino acids 812-39 of the receptor protein are highly conserved between species, suggesting that they do have a function.
Expression studies of mutant LDLR truncated at 812 revealed that residues 812-28 were necessary as a signal to direct the receptor to the basolateral surface of the hepatocytes (Yokode et al., 1992).

3.4.4. Screening strategies

Overall the greatest number of mutations were found in LDLR exons 3 (8.3%), exon 4 (25%), exon 10 (8.3%) and exons 14 (19.4%), in total accounting for 61% of mutations. 78% of the detected mutations would have been found if these four exons, the R3500Q mutation in APOB and LDLR major rearrangements were analysed. The distribution of mutations in these regions was similar when the samples were divided into adults and paediatric subjects (Table 3.7).

Table 3.7: The number of mutations in LDLR exons 3, 4, 10 & 14 initially identified by SSCP analysis, APOB mutations identified by direct PCR assays and major deletions/duplications identified by universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR) (exons 3, 5, 8, 14, 17) in mutation positive paediatric, adult, and paediatric and adults combined.

<table>
<thead>
<tr>
<th>Region</th>
<th>Paediatric probands</th>
<th>Adult probands</th>
<th>Overall probands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mutns</td>
<td>% of total mutns</td>
<td>No. of mutns</td>
</tr>
<tr>
<td>Exon 3</td>
<td>2</td>
<td>7.7</td>
<td>4</td>
</tr>
<tr>
<td>Exon 4</td>
<td>8</td>
<td>30.8</td>
<td>10</td>
</tr>
<tr>
<td>Exon 10</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Exon 14</td>
<td>9</td>
<td>34.6</td>
<td>5</td>
</tr>
<tr>
<td>APOB R3500Q</td>
<td>1</td>
<td>3.8</td>
<td>7</td>
</tr>
<tr>
<td>Del/dup</td>
<td>2</td>
<td>7.7</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>22/26</td>
<td>85</td>
<td>34/46</td>
</tr>
</tbody>
</table>

The current method of FH genetic testing involves screening all 18 exons and the promoter. This required evaluating as to whether this method was the most practical or whether a cascade screening system would be more effective and practical. Since the mutation spectrums were different between paediatric and adult cases two cascade screens are proposed.

A possible cascade for adult FH cases would involve three stages (Fig 3.10); SSCP analysis of LDLR exons 3, 4, 10 and 14 and APOB R3500Q mutation by a direct
assay, secondly testing nine exons where a few mutations have been identified (exons 2,5,6,7,8,11,12,13,16) and finally, screening for major rearrangements of LDLR by universal primer quantitative fluorescent multiplex PCR (section 4). If no mutation has been identified then exons 1, 9, 15, 17, 18 and the promoter could be screened but this final stage may not cost and time effective.

In paediatric FH cases, the first stage would involve screening exons 3, 4 and 14. The next two stages would be to test for the R3500Q mutation and for major rearrangements, in either order. If no mutation has been identified then the regions where a few mutations had been identified would be screened, exons 2,5,6,7,8,11,12,13,16. Finally if no mutation has been identified, exons 1, 9, 15, 17, 18 and the promoter, could be screened.

In both cascades, if after all stages no mutation was detected a negative report would be issued stating further tests may be offered in the future. Further exons may be screened for major rearrangements once the assays have been developed and LDLR exclusion mapping may become possible if lots of family members from several generations are obtained. Further tests may involve mutation screening of genes which are as yet unidentified. These include the ‘third’ gene (or more) which results in a phenotype identical to FH (Haddad et al, in press) and genes involved in FCHL, which is known to have different patterns of hyperlipidaemia within a family (as discussed in section 1.5.3).

In many disorders it is common practice to examine specific exons of a gene and sometimes in a sequential manner, e.g. CF, Marfan’s syndrome (Clinical Molecular Genetic Laboratory data), and to terminate mutation analysis when a pathogenic mutation is found. The actual pathogenic mutation may be missed since not all the coding sequence is analysed.
Fig 3.10: Screening cascades for FH genetic testing in paediatric and adult probands. The order was determined retrospectively and is based on the detection rates from the highest to lowest and the ease of the specific genetic test. The percentage of mutations identified at each screen are based on figures determined from the respective FH probands where a mutation was identified.

**Paediatric FH probands**

**Screen 1:**
LDLR ex3,4,14 by SSCP analysis  
→ 73.1%

**Screen 2:**
APOB R3500Q (R3500W in patients of Asian origin)  
→ 76.9%

**Screen 3:**
Deletions/duplications of ex3,5,8,14,17 by QFM-PCR  
→ 84.6%

**Screen 4:**
LDLR ex2,5,6,7,8,10,11,12,13,16 by SSCP analysis  
→ 96.1%

**Screen 5:**
LDLR promoter & ex1,9,15,17,18 by SSCP analysis  
→ 100%

**Adult FH probands**

**Screen 1:**
LDLR exons 3,4,10,14 by SSCP analysis  
→ 52.2%

**Screen 2:**
APOB R3500Q (R3500W in patients of Asian origin)  
→ 67.4%

**Screen 3:**
LDLR ex2,5,6,7,8,11,12,13,16 by SSCP analysis  
→ 95.7%

**Screen 4:**
Deletions/duplications of ex3,5,8,14,17 by QFM-PCR  
→ 100%

**Screen 5:**
LDLR promoter & ex1,9,15,17,18 by SSCP analysis

**Report:**
Negative result which does not exclude a diagnosis of FH.
Further tests may be offered in the future:
1. PCR multiplex sets for screening further exons for major rearrangements.
2. If a large multigeneration family then LDLR exclusion mapping may be offered.
3. Mutation analysis of new genes found to be involved in FH
3.4.5. De novo mutations

Three de novo LDLR mutations have been reported, an 18bp duplication at 678 or 681 in exon 4 (Kotze et al, 1995b), a deletion of exons 14-15 (Koivisto et al, 1997) and W-12R in exon 1 (Cassanelli et al, 1998). Although only three cases have been reported the possibility of de novo mutations cannot be excluded. Children who present with elevated cholesterol but have no family history of hypercholesterolaemia should still be screened for FH.

3.4.6. Two mutations on a single allele

Two mutations on a single allele has been observed in many disorders and the cases found in LDLR are shown in Table 3.8. In most disorders, one mutation was found to be causative, and the other modified the onset of severity of the disease (discussed by Jensen et al, 1997). The LDL-receptor was shown to be defective when the N543H and 2393del9 mutations were found together on the same allele but the receptor was not greatly impaired when only one of the mutations was present (Jensen et al, 1997). In other FH cases one of the two alleles has been shown or is predicted to be pathogenic (Kotze et al, 1997; Cennaro et al, 1998; Ekstrom, personal communication). If cascade screening was implemented then these double mutations on the same alleles may be missed. Care must be taken in predicting if a mutation is likely to be pathogenic by adherence to the criteria discussed in section 3.1.4.

<table>
<thead>
<tr>
<th>First mutation</th>
<th>Exon</th>
<th>Second mutation</th>
<th>Exon</th>
<th>Htz/Hmz</th>
<th>Ethnic origin</th>
<th>No. of probands</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-18X</td>
<td>1</td>
<td>E256K</td>
<td>6</td>
<td>Htz</td>
<td>Spanish</td>
<td>1</td>
</tr>
<tr>
<td>Q71E</td>
<td>3</td>
<td>313+1G&gt;C</td>
<td>int3</td>
<td>Htz</td>
<td>Spanish</td>
<td>1</td>
</tr>
<tr>
<td>C95R</td>
<td>4</td>
<td>D679E</td>
<td>14</td>
<td>Htz</td>
<td>Spanish</td>
<td>1</td>
</tr>
<tr>
<td>854ins6</td>
<td>4</td>
<td>657del5</td>
<td>4</td>
<td>Htz</td>
<td>German</td>
<td>1</td>
</tr>
<tr>
<td>E256K</td>
<td>6</td>
<td>I402T</td>
<td>9</td>
<td>Htz</td>
<td>Swedish</td>
<td>1*</td>
</tr>
<tr>
<td>C281Y</td>
<td>6</td>
<td>1706-10G&gt;A</td>
<td>11</td>
<td>Htz</td>
<td>Spanish</td>
<td>1</td>
</tr>
<tr>
<td>D333A</td>
<td>8</td>
<td>2140+5G&gt;A</td>
<td>14</td>
<td>Hmz</td>
<td>Austrian</td>
<td>1</td>
</tr>
<tr>
<td>1115delA</td>
<td>8</td>
<td>1115ins6</td>
<td>8</td>
<td>Htz</td>
<td>Japanese</td>
<td>2</td>
</tr>
<tr>
<td>Q363X</td>
<td>8</td>
<td>D365E</td>
<td>8</td>
<td>Htz</td>
<td>Cypriot</td>
<td>1</td>
</tr>
<tr>
<td>N543H</td>
<td>11</td>
<td>2393del9</td>
<td>17</td>
<td>Htz</td>
<td>Danish, Dutch</td>
<td>2/63, 10/184</td>
</tr>
<tr>
<td>A585T</td>
<td>12</td>
<td>G654S</td>
<td>14</td>
<td>Htz</td>
<td>Austrian</td>
<td>2/530</td>
</tr>
</tbody>
</table>

Table taken from Varret et al, 1998 except *Ekstrom, personal communication.
3.4.7. Non-pathogenic variants

Four substitutions, found in three probands, were determined to be non-pathogenic. Two substitutions were in the coding regions and two were in the introns. A C>T substitution at codon G654 (exon 14), did not result in an amino acid alteration and the particular nucleotide was not conserved across species, GGC in human (Yamamoto et al, 1984) and rabbits (Yamamoto et al, 1986), GGT in rat (Lee et al, 1989), hamster (Bishop et al, 1992), mouse (Hoffer et al, 1993) and banded cat shark (Mehta et al, 1996) and GGG in Xenopus LDLR I and II (Mehta et al, 1991). The other coding substitution, T705I, was found in exon 15 of LDLR on the same chromosome as an intron 7 variant, 1061-8T>C (Jensen et al, 1996). T705I had been reported in other families (Lombardi et al, 1995 & 1997) but no firm conclusions as to its pathogenesis had been made and this is discussed in detail in section 5.10. The fourth non-pathogenic substitution was found 16 bases from the intron 16 - exon 17 splice site. This was likely to be non-pathogenic as an exon 10 mutation, L458P, was also found in this individual and this mutation co-segregated with the FH phenotype.

3.4.8. LDLR exclusion mapping

This was not offered as part of the FH genetic testing but as a research tool in certain families. Cases are described in section 5 (cases 13 & 14).

3.4.9. Reports

Generally reports had a short summary of the clinical and genetic aspects of FH, reason for referral, a results table and details on the result (Fig 3.11 & Fig 3.12). As shown in Fig 3.11, positive reports were written according to the clinical referral (i.e. proband, family member) and to what mutation was identified. If available the following details should be included on the report:

1. Mutation details - nucleotide and amino acid characterisation;
2. Extent of DNA analysed - should specify the region and extent of DNA analysed (especially if only part of the gene was analysed);
3. Mutation and phenotype segregation details;
4. Conservation analysis (if missense mutation) - whether or not conserved amino acids are changed by the base change and also which species the conservation was compared with.
5. Analysis of 188 normal chromosomes (if missense mutation).

In cases where no abnormality was found, a report was issued within six months of referral, and normally less, stating that no mutation has been found in the coding region.
and splice sites of LDLR or the APOB mutations R3500Q and R3531C. A negative result does not exclude the individual from having FH since it is only possible to detect 43% of LDLR mutations in adults and 72% of mutations in paediatric cases, by current methodology. It is stated that further analysis is automatically carried out when new tests become available and if positive, these are reported (Fig 3.12).

3.5. Conclusion

Each FH proband from the UK usually has a unique mutation, in contrast to certain populations where founder gene effect is present. Consequently, in most populations, including the British, techniques that permit examination of certain exons or the entire coding region of LDLR have to be established for the genetic diagnosis of FH.

A pilot genetic diagnostic service for FH has been established at the GOSH Clinical Molecular Genetics Laboratory. The entire LDLR is screened by SSCP analysis and direct assays are used to test two/three APOB mutations. As with all genetic services, the tests are continually assessed and improvements will be made. From the mutations detected over the last four years a screening cascade has been proposed to reduce costs and workload. When the third gene is identified the screening cascade may include these analyses. Additional CHD factors may also be tested and improvements to the service will occur as new diagnostic tools are developed.

During the next 12 months, the genetic testing of FH is being transferred from a pilot scheme to a service which will operate under the NHS where samples may be referred from anywhere in the UK.

* * * * * * * * * * * * * * * * * *

Acknowledgements: Thanks to many people but particularly Lighta Godhini, Paul Cummings and Jane Heath for the isolation of the DNA. Thank you to Dr Helen Middleton-Price and Dr Maureen Boxer for checking all the reports.
Familial hypercholesterolaemia (FH) research report

INFORMATION
Familial hypercholesterolaemia is inherited as an autosomal dominant disorder. The majority of individuals with FH have a mutation in the low density lipoprotein receptor gene (LDLR) but a small proportion will have a mutation in the apolipoprotein B-100 gene (APOB). The current tests screen for small LDLR mutations and two APOB mutations, R3500Q and R3531C. 5-10% of mutations found in UK FH patients are large LDLR deletions or duplications and an assay is currently under development.

REASON FOR REFERRAL
X has elevated cholesterol and a family history of FH. FH genetic testing - LDLR & APOB mutation analysis.

RESULTS

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab no.</th>
<th>LDLR analysis</th>
<th>APOB R3500Q &amp; R3531C</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>-</td>
<td>E207X/N</td>
<td>Negative</td>
</tr>
</tbody>
</table>

REPORT
X has been found to be heterozygous for the E207X mutation in exon 4 of LDLR by SSCP analysis followed by DNA sequencing. The mutation was confirmed by a PCR-Bfai restriction enzyme assay. The mutation alters a G to a T at nucleotide 682 and it is predicted to result in the loss of a glutamic acid and the truncation of the protein at codon 207. This mutation is known to cause FH and has been frequently reported in cases of FH (FH website: http://www.ucl.ac.uk/fh). Thus the identification of an LDLR mutation confirms the diagnosis of FH.

Family members can now be offered genetic testing. A mouthwash sample would be sufficient for carrying out tests on family members. Please contact the laboratory for further details.

Reported by: Date:
Checked by: Date:

NB: Results depend on the samples being labelled correctly and, where relevant, family relationships being as indicated.

These results were generated as part of a research project.

I:\DNA Lab\FH reports\1999\Jan\X
Familial hypercholesterolaemia (FH) research report

INFORMATION
Familial hypercholesterolaemia is inherited as an autosomal dominant disorder. The majority of individuals with FH have a mutation in the low density lipoprotein receptor gene (LDLR) but a small proportion will have a mutation in the apolipoprotein B-100 gene (APOB). The current tests screen for small LDLR mutations and two APOB mutations, R3500Q and R3531C. 5-10% of mutations found in UK FH patients are large LDLR deletions or duplications and an assay is currently under development.

REASON FOR REFERRAL
Y has raised cholesterol and a family history of hypercholesterolaemia. FH genetic testing - mutation analysis of LDLR & APOB.

RESULTS

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab no.</th>
<th>SSCP analysis of LDLR gene (18 exons and promoter)</th>
<th>APOB R3500Q &amp; R3531C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td></td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

KEY: Negative = No abnormal results were observed

REPORT
Y was negative for LDLR mutation screen by SSCP analysis. From a sample of 101 adult FH patients approximately 40% have been identified as point mutations using SSCP of LDLR while approximately 5% have a deletion or duplication (Heath et al, Atherosclerosis 1999;143:41-54). Deletion analysis is, at present, being developed in this laboratory and Y will be screened for a deletion in due course.

A further 3% of FH probands have the R3500Q mutation and a few have R3531C mutation within APOB. Y was negative for the R3500Q and R3531C mutations.

Reported by: Date: 
Checked by: Date: 

NB: Results depend on the samples being labelled correctly and, where relevant, family relationships being as indicated.

These results were generated as part of a research project.

I:\DNA\Lab\FH reports\1999\Jan\Y
Chapter 4: Quantitative fluorescent multiplex PCR to detect LDLR major rearrangements

Summary
Quantitative fluorescent multiplex PCR (QFM-PCR) allows the identification of major gene rearrangements and minor rearrangements of 1bp or greater. The main advantages of this technique are that it avoids the problems associated with Southern blotting and that smaller rearrangements are detectable.

A QFM-PCR screen was adapted to analyse gene rearrangements of LDLR. The promoter and 18 exons were divided into four PCR sets, two labelled with one dye and two with another dye. One set based on exons 1, 8, 10, 12 and 16 was further developed and evaluated on a set of reported gene rearrangements and a series of test samples, a group of 95 adult FH probands from the USA. Four unreported major rearrangements and one minor rearrangement were detected. Inter and intra-assay variation was very wide, probably due to differential amplification regarding size. Despite the addition of multiple controls in each assay, many samples had to be repeated due to anomalies and false positives.

A second method was developed and designated universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR). This method was designed to minimise the problems found with the previous strategy. The multiplex set developed included exons 3, 5, 8, 14, and 17 of LDLR, and 86% of reported major rearrangements would be detectable by this assay. The method has been evaluated by conducting a trial on a set of 15 reported major rearrangements.

Two groups of UK patients with a clinical diagnosis of FH and where no mutation had been identified in LDLR or APOB (14 children and 42 adults), were screened for the presence of major LDLR rearrangements by this UPQFM-PCR assay. Three major rearrangements were detected (overall frequency in the two groups: 2.6%, n=116), two previously undescribed and a third case which, because of unavailability of DNA, was not fully characterised. A 4bp insertion was identified in a fourth patient.
4.1. Introduction

In the majority of FH patients a mutation in LDLR is responsible for the defect in LDL clearance. More than 700 LDLR mutations have been reported (Wilson et al, 1998; FH website: http://www.ucl.ac.uk/fh) and approximately 80 of these are major rearrangements, ranging from 25bp to over 20kb. Deletions and duplications account for approximately 5% of mutations found in FH patients from genetically heterogeneous populations (Hobbs et al, 1986; Horsthemke et al, 1987; Langlois et al, 1988; Sun et al, 1992).

4.1.2. Mechanisms

The analysis of previously reported deletions and duplications have indicated that there are preferential sites for major rearrangements in LDLR; exons 1 to 8 and exons 13 to 18 (Yamamoto et al, 1984). The central portion, exons 9 to 12, has only been implicated in a few rearrangements. This uneven distribution is thought to be due to the high number of Alu repeats clustering at both ends of the gene, 21 over 45kb (Kajinami et al, 1990), compared to other genes such as the β-globin cluster where only eight Alu sequences are present within 60kb (Hentorn, Smithies & Mager, 1990). Many LDLR major rearrangements have breakpoints that are located at these Alu sequences (Hobbs et al, 1986). Another gene which has a high number of repeats is the complement component 1 inhibitor (C1I) gene, where 17 Alu sequences are present within a 17kb region (180). Deletions and partial deletions of the C1I gene appear to account for 15-20% of the mutations that cause type 1 hereditary angioneurotic edema (Stoppa-Lyonnet et al, 1991) and a high proportion of these occur with Alu sequences. The presence of many Alu sequences in a gene does not always indicate a high level of rearrangements, such as the 66kb human growth hormone gene cluster (GH1) which contains 48 Alu sequences but do not appear to be the cause of the high frequency of clustered GH1 deletions causing familial growth hormone deficiency (Vnencak-Jones & Phillips, 1990), and the α-galactosidase A gene (GLA) which has 12 Alu sequences in a 12kb gene region (30% of GLA is Alu sequences) but deletions only accounted for 4% of mutations causing Fabry disease and not all involved Alu sequences (Kornreich et al, 1990).

Alu sequences consist of two tandem repeats referred to as the left arm (~132 nucleotides) and the right arm (~166 nucleotides), the latter being a duplication of the left arm with a 34 nucleotide insertion (reviewed by Schmid & Jelinek, 1982). Many of the characterised deletions and duplications have breakpoints in the left arm of Alu
sequences between the RNA polymerase III promoters A and B (Lehrman et al, 1987a). Thus the breakpoints do not occur randomly and it has been found that the left arm is transcribed more efficiently in vitro (Paolella et al, 1983). The A and B sequences between the left arm are likely to unwind or bend at some point during transcription by RNA polymerase III and its associated transcription factors, and it is possible that this unwinding promotes recombination (Lehrman et al, 1987a).

Deletions and duplications are thought to arise from one of two mechanisms involving Alu sequences. If the two Alu sequences are orientated in opposite directions then a double-stem-loop structure (intrachromosomal recombination) can be postulated (Lehrman et al, 1986) but if they are orientated in the same direction then a deletion or duplication may arise due to unequal crossing over after the mispairing of two Alu sequences on different chromosomes (interchromosomal recombination) (Hobbs et al, 1986). These mechanisms have also been shown in other disorders such as γ,β-thalassaemia (Ottolenghi & Giglioni, 1982; Jagadeeswaran et al, 1982; Vanin et al, 1983).

DNA rearrangements also occur where there is very little sequence homology and may be explained by chromosomal translocation, gene amplification, retrotransposition and the developmental rearrangement of immunoglobulin genes (reviewed by Cooper & Krawczak, 1993). Sequences which were originally remote from one another, are brought into close proximity through their attachment to chromosome scaffolding and this has been suggested to occur in the α and β-globin genes (Vanin et al, 1983). Another mechanism resulting in a gene rearrangement is gene fusion, arising from the deletion of the intervening sequence, and this is observed in several disorders such as red green colour blindness (Nathans et al, 1986) and glucocorticoid-suppressible hyperaldosteronism (Pascoe et al, 1992).

4.1.3. Methods of detection
Many methods have been described to display the deletions and duplications (section 1.8.2.2). All these techniques have disadvantages; they are either technically demanding, require fresh samples or are subject to error. The ability to perform deletion analysis by a PCR based technique should provide considerable advantages over traditional Southern blot based techniques, on account of rapidity and only requiring small quantities of starting material. There are two PCR based methods of detecting major rearrangements in autosomal disorders or in female carriers of X-linked
disorders, long PCR and quantitative multiplex PCR (section 1.8.2.2.4). Long PCR can detect novel major rearrangements and this technique has been used to detect particular LDLR mutations (Rodningen et al, 1996, Peeters et al, 1997). The entire LDLR (~45kb), except for intron 1, can be amplified in two fragments of 16.1 and 20kb (Rodningen et al, 1996) although deletions of single exons or smaller may be missed when comparing such large fragment sizes and inter-Alu deletions can occur artefactually during the PCR (Ji et al, 1994). The second method, quantitative multiplex PCR, can determine which exons are deleted or duplicated without further analysis and minor rearrangements, as small as 1bp, can be detected.

4.1.4. Quantitative multiplex PCR
Quantitative multiplex PCR has greatly improved with the recent developments in fluorescent technology and is now used to screen female relatives of Duchenne muscular dystrophy (DMD) individuals for deletions and duplications in the dystrophin gene (Yau et al, 1996) (section 1.9.2.2.4.4).

In 1995, a PCR method was described which simplifies the conditions for qualitative multiplex PCR (Shuber et al, 1995) (section 1.8.2.2.4.4). Specific primers were tagged at the 5' end with an unrelated 20-nucleotide universal primer sequence. Multiple genomic sequences were co-amplified under identical conditions and cycling parameters with very little optimisation of PCR conditions. An important factor in quantitative multiplex PCR is that comparable yields of the individual co-amplified products should be obtained and this was shown to occur by this method (Shuber et al, 1995).

A quantitative fluorescent multiplex PCR, to detect LDLR major rearrangements, was designed on this principle, and has been designated universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR). The UPQFM-PCR consisted of two phases of amplification, P1 and P2 and the principle is shown in Fig 4.1. Ten rounds of amplification of each exon in the multiplex set is carried out in the first PCR (P1), with specific primers tagged to newly designed sense and antisense universal primers. The P1 PCR is expected to have enhanced specificity and efficiency, conferred by the tagged primers due to the normalisation of the hybridisation kinetics. Even if this is not completely the case and the efficiency ratios for all amplicons in the reaction are not in unity and are still dependent on size and primer annealing efficiency, the reduction in the number of cycles will greatly reduce this problem. A small quantity of the P1 product is then transferred to a second PCR reaction (P2) where only one set of
primers, the universal sense and antisense primers, amplify all the multiplex exons, thus all exons should theoretically amplify consistently and with equal efficiency. A fluorescent dye is tagged at the 5' end of one of the universal primers resulting in the incorporation of fluorescence in all of the multiplex products in the P2 reaction. The products are then resolved and detected on an ABI DNA 377 Sequencer.

Fig 4.1: Universal primer quantitative fluorescent multiplex PCR strategy.

The first PCR reaction (P1) amplifies the various exons from unique primer sequences tagged to newly designed universal primers with a 6-base tag in-between (relevance described in text) whilst the second reaction (P2) amplifies the P1 products using the universal primers. One of the universal primers (antisense in this case) was labelled with a fluorescent dye which is incorporated into the PCR products. PCR products are electrophoresed on an ABI DNA Sequencer. Ratios of the exon peak areas against each other are determined and these are then compared to ratios obtained for normal, deletion and duplication controls.
A multiplex set was designed to screen for major rearrangements of LDLR involving exons 3, 5, 8, 14 and 17. Two groups of patients with a clinical diagnosis of FH and where no mutation had been identified in LDLR or APOB were screened by this assay.
4.2. Materials and Methods
4.2.1. Patient groups
4.2.1.1. Quantitative fluorescent multiplex PCR
A group of unrelated adult FH patients (n=96) from Portland, Oregan, USA were screened for major rearrangements of exons 1, 8, 10, 12 and 16 of LDLR. The clinical criteria were based on the National Cholesterol Education Program guidelines (NCEP 1989).

4.2.1.2. Universal primer quantitative multiplex PCR
Two groups of FH patients were screened for LDLR major rearrangements. The first group consisted of 76 adult probands, 42 of whom had no detected mutation in LDLR or APOB, who each had a diagnosis of ‘definite’ FH, i.e. untreated LDL-c > 4.9mmol/l and a family history of TX (Simon Broome Steering Committee, 1991). They were attending two lipid clinics in Charing Cross Hospital, London and King Edward V Hospital, Sussex and formed part of the genotype:simvastatin response study described in chapter 6. The second group was made up of 40 probands under the age of 16, 14 of whom had no mutation identified in LDLR or APOB, attending lipid clinics held at GOSH and Middlesex Hospital, London. The diagnosis was made on an initial total cholesterol level above 6.0mmol/l and a family history of hypercholesterolaemia and premature CHD.

4.2.2. Molecular analysis
4.2.2.1. Quantitative fluorescent multiplex PCR
Two methods of QFM-PCR, as described in sections 2.3.10.

4.2.2.2. DNA sequencing
Minor rearrangements were characterised by sequencing in both directions (2.8).

4.2.2.3. Data and statistical analysis
Data and statistical analysis was identical for both PCR strategies. The area under the peaks was generated by ABI Genescan and Genotyper software and then transferred into a Excel macro (Table 4.1). Peak area ratios were determined between each exon and the other four exons in the multiplex set. The mean ratio ±1 standard deviation (SD) and ±2SD of four normal controls were calculated. If the SD of the four normal controls was greater than 0.1 (i.e. >10%) due to the ratios from one control, then that control was omitted and the mean and SD’s were recalculated, but if it was due to more than one control then the analysis of the assay was aborted. Ratios of the test samples

140
were compared against the control mean ratios. Any ratio outside 2SD was dark shaded and any ratio outside 1SD was pale shaded. A deletion or a duplication was inferred if the ratio was within the confidence limits determined from the major rearrangement controls. Samples where ratios were found to be outside both normal and mutation confidence limits, and samples where a deletion or duplication was identified, were retested.

Table 4.1: Excel macro template of normal four normal controls and one sample.

<table>
<thead>
<tr>
<th>Normals</th>
<th>Peak areas of control</th>
<th>Exons</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Mean ±2SD</td>
<td>of exon peak area ratios</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ex5/5)</td>
<td>(ex5/8)</td>
<td>(ex5/14)</td>
<td>(ex5/17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ex8/8)</td>
<td>(ex8/14)</td>
<td>(ex8/17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ex14/14)</td>
<td>(ex14/17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ex17/17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B Areas</td>
<td>Ratio of peak areas</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Exons</td>
<td></td>
<td>ex3/3</td>
<td>ex3/5</td>
<td>ex3/8</td>
<td>ex3/14</td>
<td>ex3/17</td>
</tr>
<tr>
<td></td>
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<td>ex5/5</td>
<td>ex5/8</td>
<td>ex5/14</td>
<td>ex5/17</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ex8/8</td>
<td>ex8/14</td>
<td>ex8/17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ex14/14</td>
<td>ex14/17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ex17/17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3. Results

4.3.1. Quantitative fluorescent multiplex PCR (QFM-PCR) (strategy 1)

The aim was to develop four multiplex sets covering all the exons of LDLR. The first set included exons 1, 8, 10, 12, 16 and 18. Exons 12 and 18 were very weak compared to the other four exons which was likely due to the primer design as exon 18 was the smallest exon (135bp). The ratios of exons 1, 8, 10, 12 and 16 did not change when exon 18 dropped out or amplified well, but the ratio to exon 18 was very variable, thus it was omitted from the assay. A second set included exons 3, 5 and 14. Exon 14 continuously dropped out or was very reduced and this was probably mainly due to the primers chosen but may have been due to the differences in product size (ex14 - 288, exon 3 - 196 & exon 5 - 180bp). Ratios were very variable so the set was abandoned.

At 18 and 20 cycles the PCR set 1 (exons 1,8,10,12,16) was in the exponential phase but by 25 cycles the peaks had broadened and the plateau phase of the amplification had occurred (Fig 4.2). The number of cycles chosen was 20 as a sufficient amount of fluorescence was incorporated and the peaks were narrow.

The QFM-PCR assay was tested on seven known major rearrangements (Table 4.2) and examples of the Genotyper traces of the known gene rearrangements compared to normal controls are shown in Fig 4.3 & 4.4.

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>Characterised major rearrangement</th>
<th>Exons detected by QFM-PCR assay set</th>
<th>First reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH Bologna-1</td>
<td>Δ prom-exon 6</td>
<td>Δ exons 1</td>
<td>Lelli et al, 1991a</td>
</tr>
<tr>
<td>FH Pavia</td>
<td>Δ exons 2-12</td>
<td>Δ exons 8, 10, 12</td>
<td>Bertolini et al, 1992</td>
</tr>
<tr>
<td>FH Massa</td>
<td>Δ exons 3-10</td>
<td>Δ exons 8 &amp; 10</td>
<td>Bertolini et al, 1995</td>
</tr>
<tr>
<td>FH Genoa-1</td>
<td>Δ exon 11-12</td>
<td>Δ exon 12</td>
<td>Bertolini et al, 1995</td>
</tr>
<tr>
<td>FH Padova-2</td>
<td>Δ exons 16-17</td>
<td>Δ exon 16</td>
<td>Bertolini et al, 1995</td>
</tr>
<tr>
<td>FH Salerno</td>
<td>V exons 9-14</td>
<td>V exon 10 &amp; 12</td>
<td>Bertolini et al, 1994</td>
</tr>
<tr>
<td>FH Viterbo</td>
<td>V exons 16-17</td>
<td>V exon 16</td>
<td>Lelli et al, 1991a</td>
</tr>
</tbody>
</table>

Deletions are shown by Δ and duplications are shown by V.
Fig 4.2: Ratios of exons 1, 8, 10, 12 to exon 16 over an increasing number of cycles of amplification.
A: Genotype traces of 1 normal and the 4 major rearrangement controls. Trivial names and exon boundaries for the reported deletions are listed with the particular exons deleted in this multiplex set shown in brackets. Peak areas are indicated under the exon name and these are used to determine the dosage ratios in the excel macro. Deletions are marked with an asterisk. B: Dosage data for 4 normal controls and 4 major rearrangements. Ratios outside the normal range are shaded. Data outside but close to the confidence limits are placed in brackets.
Fig 4.4: Genotyper traces of QFM-PCR multiplex set 1: two normal controls (A, B) and two known major rearrangements

A: Genotyper traces of QFM-PCR. Trivial names and exon boundaries of 'test' exons are listed with the particular exons deleted/duplicated in this multiplex set shown in brackets. Peak areas are indicated under the exon name. Deviations from normal are marked with an asterisk.

B: Dosage data for 4 normal controls and the deletion and duplication controls. Ratios outside 2SD are shaded and indicate a rearrangement.

A

B

C

D

145
Despite controlling many steps in the PCR reaction; quantifying the DNA concentration, careful pipetting of reagents and using the same PCR machine, the reactions were very variable within an assay and between assays. Some assays produced clear cut results whilst others produced numerous false positives (Table 4.3). The major problem appeared to be differential amplification and this could have been due to the specificity of primers and to the product size that was being amplified. Many attempts were made to reduce these, including the quantification of the DNA after dissolving at 55°C; varying primer, dNTP, MgCl₂ concentrations and using tubes rather than plates, thus enabling the use of a tube sensor, which provides a more accurate temperature control, but the assay remained very variable.

Table 4.3: The success rate for obtaining a result on the first attempt, the level of false positives and the failure rate of QFM-PCR set 1 in 95 FH probands from the USA.

<table>
<thead>
<tr>
<th></th>
<th>First screen (% of total)</th>
<th>Further screens (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clear results</td>
<td>61 (64%)</td>
<td>-</td>
</tr>
<tr>
<td>No. of false positives or anomalies</td>
<td>3 (3%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>No. of complete failures</td>
<td>31 (33%)</td>
<td>24 (25%)</td>
</tr>
</tbody>
</table>

Peak areas were used to determine dosage ratios and from these the percentage reduction of the deleted or duplicated exons was determined (Fig 4.3 & 4.4). The mean reduction in peak area for deleted exons was 46±4% (n=4) whilst duplicated exons showed a mean increase of 146±14% (n=3) if each major rearrangement was counted once. If the mean reduction or increase were calculated for the same set of known controls but repeated in several assays the data was 49±8% (n=47) for deletions and 146±19 (n=19) for duplications.

The interassay variation was determined by calculating 1SD and 2SD of the ratios of several known controls against the mean normal ratios for each assay over a number of assays. Exon ratios outside 1SD accounted for 39% of cases whilst 12% were also outside 2SD. The mean exon ratio outside 1SD was 0.06 (0.01-0.23) and outside 2SD was 0.04 (0.01-0.13). Thus a large proportion of ratios lay outside 1SD and even outside 2SD. Intra-assay variation was tested by analysing one sample repeatedly in one assay and data is shown for three samples in two assays (Table 4.4). Intra-assay variation was at the greatest for control 5 and ratios to exon 12. Considerable interassay variation also occurred, as shown by control 1.
Table 4.4: Intra-assay variation (mean±1SD to exon 16) in 6 samples in 2 assays.

<table>
<thead>
<tr>
<th>Control</th>
<th>No. of repeats</th>
<th>Ex1/Ex16</th>
<th>Ex8/Ex16</th>
<th>Ex10/Ex16</th>
<th>Ex12/Ex16</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>8</td>
<td>0.99±0.04</td>
<td>0.77±0.02</td>
<td>1.21±0.06</td>
<td>0.61±0.02</td>
</tr>
<tr>
<td>C2</td>
<td>7</td>
<td>0.90±0.02</td>
<td>0.66±0.02</td>
<td>1.03±0.07</td>
<td>0.52±0.05</td>
</tr>
<tr>
<td>C3</td>
<td>8</td>
<td>0.89±0.02</td>
<td>0.69±0.04</td>
<td>1.01±0.09</td>
<td>0.48±0.04</td>
</tr>
<tr>
<td>C4</td>
<td>7</td>
<td>1.09±0.04</td>
<td>0.94±0.10</td>
<td>0.86±0.06</td>
<td>0.26±0.04</td>
</tr>
<tr>
<td>C5</td>
<td>7</td>
<td>1.33±0.11</td>
<td>1.30±0.16</td>
<td>0.95±0.09</td>
<td>0.20±0.20</td>
</tr>
<tr>
<td>C1</td>
<td>8</td>
<td>1.40±0.06</td>
<td>1.32±0.05</td>
<td>1.02±0.08</td>
<td>0.27±0.07</td>
</tr>
</tbody>
</table>

C1/C2/C3 were repeated 7-8 times in one assay whilst C4/C5/C1 were repeated 7-8 times in another assay. C1 was common to both assay.

As well as detecting major rearrangements by quantification, the QFM-PCR detected minor rearrangements by accurately sizing. Deletions or insertions of 1bp were detectable as mobility shifts of individual amplicons (peak splitting since heterozygotes) if 36cm well-to-read plates were used (~210mins). The smallest deletion or insertion detected was 2bp on the 12cm well-to-read plates which were routinely used for speed (70mins) and because all samples had been tested for minor deletions or insertions by SSCP analysis. A 4bp insertion was identified in exon 8 (Fig 4.5) and it was characterised, by fluorescent dye terminator sequencing, to be due to a duplication, GGGT at nucleotide 1120 which is predicted to cause a frameshift from glycine 353. This mutation has been described in several patients from several ethnic groups (FH Nashville) (FH website).

A group of 95 FH patients from the USA were tested for major rearrangements of LDLR using this assay to determine the frequency of major rearrangements. The percentage of false positives, the number of failures and the number of repeats was also examined to see if the assay was feasible as a diagnostic tool. Four patients carried major rearrangements of two unreported types (Table 4.5). QFM-PCR with diplex or triplex sets were used to define the boundaries of the major rearrangements, using appropriate controls.
Fig 4.5: Genotyper traces of a normal control (A) and a minor rearrangement of exon 8 (B) detected by the QFM-PCR set.

A

B

Peaks are labelled with the exon name and peak area. Peak size is not marked but a 4 bp insertion was identified in exon 8 (marked with an asterisk) which was due to a duplication, GGGT at 1120 (FsG353).
Table 4.5. LDLR major rearrangements detected in the US FH group.

<table>
<thead>
<tr>
<th>Patient</th>
<th>LDLR exons deleted or duplicated (QFM-PCR)</th>
<th>Characterisation of rearrangement (exon boundaries)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Δ ex8 &amp; 10</td>
<td>Δ ex6-10*</td>
</tr>
<tr>
<td>2*</td>
<td>Δ ex8 &amp; 10</td>
<td>Δ ex6-10*</td>
</tr>
<tr>
<td>3</td>
<td>V ex16 or Δ ex1-12</td>
<td>V ex14-17*</td>
</tr>
<tr>
<td>4*</td>
<td>V ex16 or Δ ex1-12</td>
<td>V ex14-17*</td>
</tr>
</tbody>
</table>

Exon boundaries of LDLR major rearrangements characterised by QFM-PCR as described in the text. *Rearrangements confirmed in affected relatives. From surname analysis patients 1 & 2 are thought to share a common ancestor and patients 3 & 4 are though to have a common ancestor. *Indicate previously unreported mutations.

Patient 1 & 2
In patients 1 & 2 a deletion of exons 8 and 10 was observed by this one multiplex set (Fig 4.6) and methodical characterisation of this major rearrangement is explained below and summarised in Fig 4.7. The universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR) confirmed that exon 8 was deleted and showed that exons 5 and 14 were not deleted. Characterisation was then by various diplex and triplex PCR reactions using the QFM-PCR principle. The first diplex quantified exons 7 and 17 and the data could be interpreted in two ways, either exon 7 was deleted or exon 17 was duplicated. From the QFM-PCR set 1 and the UPQFM-PCR set is was known that the major rearrangement was a deletion and this was now shown to include exon 7. The second diplex included exons 6 and 16. Exon 16 had already been shown to be normal by the QFM-PCR set 1 so this diplex was to test the deletion extended to exon 6. The dosages obtained could be interpreted either as a deletion of exon 6 or a duplication of exon 16, thus the deletion extended to exon 6. A diplex of exon 7 and 11 showed that exon 11 was normal. The limits of the deletion in patients 1 and 2 had been determined, exon 6 to 10 with the deletion breakpoints located in introns 6 and 10.

Patient 3 & 4
In patients 3 & 4 the QFM-PCR showed one of the test exons to be duplicated, exon 16 or exons 1,8,10 & 12 to be deleted (Fig 4.8). The confidence limits of deletions and duplications were not mutually exclusive so deletions could not be distinguished from deletions. Characterisation and confirmation of the mutation, by QFM-PCR, showed that exons 14 to 17 were duplicated (Fig 4.9).
Fig 4.6: A: Genotyper traces and Excel macro of QFM-PCR multiplex set 1 in normals and patients 1 and 2 from the USA.

A: Normal control

B: Normal control

C: Patient 1: deletion ex8 & 10

D: Patient 2: deletion ex8 & 10

A: Each peak is labelled with the exon name and peak area. Deletions are indicated by an asterisk. B: Section of dosage excel macro sheet (described in text) of 4 normal controls and patients 1 (C) & 2 (D). Ratios outside 2SD are shaded (dark) and anomalies shaded (pale). Patients 1 and 2 have a deletion of exons 8 & 10.
Fig 4.7: Characterisation of LDLR major rearrangement in patients 1 & 2 from the USA.

<table>
<thead>
<tr>
<th>Normal LDLR</th>
<th>5'-1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 - 12 - 13 - 14 - 15 - 16 - 17 - 18-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>QFM-PCR: (ex1,8,10,12,16,18) - 8,10,12 deleted</td>
<td></td>
</tr>
<tr>
<td>UPQFM-PCR: (ex3,5,8,14,17) - 8 deleted</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex7,17) - 7 deleted , 17 normal or 7 normal, 17 duplicated</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex6,16) - 6 deleted , 16 normal or 6 normal, 16 duplicated</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex6,9,13) - 6,9 deleted or 13 duplicated</td>
<td></td>
</tr>
<tr>
<td>Deletion of ex6-10 LDLR</td>
<td>5'-1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9 - R - 10 - 11 - 12 - 13 - 14 - 15 - 16 - 17 - 18-3'</td>
</tr>
</tbody>
</table>

QFM-PCR was used to identify the major rearrangement. UPQFM-PCR confirmed the major rearrangement. QFM-PCR diplex and triplex sets were used to determine the exon boundaries of the major rearrangements. Patients 1 & 2 were shown to have a deletion of exons 6-10. Deletions shown by Δ and normals shown by (N) indicate deduced data whilst solid symbols △ or N indicate data obtained from various PCR reactions. Results of the multiplexes can be deduced in several ways, as explained in the text, but the symbols show what actually occurred in patient 1 & 2.
Fig 4.8: A; Genotyper traces and Excel macro of QFM-PCR multiplex set 1 in normals and patients 3 and 4 from the USA.

A: Normal control

B: Patient 3: duplication ex16/deletion ex1-12 (duplication ex16)

C: Patient 4: duplication ex16/deletion ex1-12 (duplication ex16)

A: Each peak is labelled with the exon name and peak area. The major rearrangement is shown as a duplication, marked by an asterisk. B: Section of dosage excel macro sheet (described in text) of 4 normal controls and patients 3 (B) & 4 (C). Ratios outside 2SD are shaded. Patients 3 & 4 have a duplication of exon 16 (shaded cells) or a deletion of exons 1-12.
Fig 4.9: Characterisation of LDLR major rearrangement in patients 3 & 4 from the USA.

| Normal LDLR | 5'---2---3---4---5---6---7---8---9---10---11---12---13---14---15---16---17---18---3' |
| QFM-PCR: (ex1,8,10,12,16) - 16 duplicated or 1,8,10,12 deleted | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| UPQFM-PCR: (ex3,5,8,14,17) - 14,17 duplicated or 3,5,8 deleted | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| QFM-PCR: (ex6,18) - both normal, deleted or duplicated | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| QFM-PCR: (ex16,18) - 16 duplicated, 18 normal or 16 normal, 18 duplicated | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| QFM-PCR: (ex8,16,18) - 16 duplicated, 18 normal or 16 normal, 18 deleted | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| QFM-PCR: (ex5,14) - 14 duplicated, 5 normal or 14 normal, 5 deleted | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| QFM-PCR: (ex6,13) - 13 duplicated, 6 normal or 13 normal, 6 deleted | N-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N)-(N) |
| Duplication of ex13-17 LDLR | 5'---1---2---3---4---5---6---7---8---9---10---11---12---13---14---15---16---17---18---3' |

QFM-PCR was used to identify the major rearrangement. UPQFM-PCR confirmed the major rearrangement. QFM-PCR diplex and triplex sets were used to determine the exon boundaries of the major rearrangements. Patients 3 & 4 were shown to have a duplication of exons 7 to 18. Duplications shown by ▼ and normals shown by (N) indicate deduced data whilst solid symbols ▼ or N indicate data obtained from various PCR reactions. Results of the multiplexes can be deduced several ways, as explained in the text, but the symbols show what actually occurred in patients 3 & 4.
4.3.2. Universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR) (strategy 2)

UPQFM-PCR consisted of two PCR reactions. The minimum number of cycles was used in the first PCR reaction (P1). Five to nine cycles of P1 were tried but too little product was transferred to the second PCR reaction (P2) for sufficient fluorescence to be incorporated so 10 cycles was used. In many different assays the exponential phase of the P2 reaction was shown to occur up to 25 cycles of amplification. Four samples (three normal controls and one known deletion) were amplified seven times each. One of each control was removed after 15, 18, 20, 23, 25, 28 and 30 cycles. Samples were electrophoresed and dosage ratios were then calculated. The amount of fluorescence incorporated was undetectable at 15, low at 18 cycles and in a suitable detection range from 20 cycles. Ratios were similar for 20, 23 and 25 cycles but beyond this number the peaks deviated from the ratios found between 20 and 25 cycles. Although at 22 cycles the reaction was still in the exponential phase, the peak heights approached the upper limit of the linear scale of the ABI detector and the peaks began to broaden, preventing the resolution of deletions and duplications as small as 2bp. The number of cycles in the P2 reaction was chosen at 20 as the products were easily detectable and did not require dilution prior to loading.

The UPQFM-PCR assay was tested on 15 known major rearrangements and the results are shown in Table 4.6.

Examples of the Genotyper traces of four of the samples compared to two normal controls are shown in Fig 4.10, where differences in peak heights between normal, and deleted exons are clearly visible. Peak areas were used to determine dosage ratios and from these the reduction ratio of the deleted exons or the increased ratio of the duplicated exons was determined. The mean reduction in peak area for deleted exons was \(46\pm7\%\) (n=20) whilst duplicated exons showed a mean increase of \(146\pm5\%\) (n=3) although there was a lack of known duplications. Interassay variation was determined by calculating the SD of the ratios of a normal sample against the mean normal ratios for each assay over a number of assays; healthy Caucasian controls X and Y represent the two extremes. Control X was included in 12 assays. Ratios were determined for the peak areas of exons 3, 5, 8 and 17 compared to exon 14, thus a total of 48 exon ratios. Four ratios were outside 1SD by 1-3%. For control Y, 36 exon ratios were determined and a third of their ratios fell outside 1SD but again only by 1-3%. Thus the majority of ratios lay within 1SD and all were well within 2SD. Intra-assay variation was tested by analysing one sample repeatedly in one assay and ranged from 0.01 to 0.06 (n=13).
Table 4.6. Reported LDLR major rearrangements analysed by UPQFM-PCR.

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>Characterised major rearrangement</th>
<th>Exons detected by UPQFM-PCR</th>
<th>First reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH Bologna-1</td>
<td>Δ prom-exon 6</td>
<td>Δ exons 3 &amp; 5</td>
<td>Lelli et al, 1991a</td>
</tr>
<tr>
<td>FH Aarhus-4</td>
<td>Δ exons 2-5</td>
<td>Δ exons 3 &amp; 5</td>
<td>Jensen, 1997</td>
</tr>
<tr>
<td>FH Pavia</td>
<td>Δ exons 2-12</td>
<td>Δ exons 3, 5 &amp; 8</td>
<td>Bertolini et al, 1992</td>
</tr>
<tr>
<td>FH Aarhus-2</td>
<td>Δ exons 3-6</td>
<td>Δ exons 3 &amp; 5</td>
<td>Rudiger et al, 1991b</td>
</tr>
<tr>
<td>FH Massa</td>
<td>Δ exons 3-10</td>
<td>Δ exons 3, 5 &amp; 8</td>
<td>Bertolini et al, 1995</td>
</tr>
<tr>
<td>FH Aarhus-3</td>
<td>Δ exon 5</td>
<td>Δ exon 5</td>
<td>Rudiger et al, 1991b</td>
</tr>
<tr>
<td>FH Leiden-1</td>
<td>Δ exon 7-8</td>
<td>Δ exon 8</td>
<td>Russell et al, 1987</td>
</tr>
<tr>
<td>FH Macerata</td>
<td>Δ exons 13-14</td>
<td>Δ exon 14</td>
<td>Lelli et al, 1991b</td>
</tr>
<tr>
<td>FH Rome</td>
<td>Δ exons 13-14</td>
<td>Δ exon 14</td>
<td>Bertolini et al, 1995</td>
</tr>
<tr>
<td>FH Potenza</td>
<td>Δ exons 13-15</td>
<td>Δ exon 14</td>
<td>Lelli et al, 1993</td>
</tr>
<tr>
<td>FH Aarhus-1</td>
<td>Δ exon 17</td>
<td>Δ exon 17</td>
<td>Rudiger et al, 1991a</td>
</tr>
<tr>
<td>FH Salerno</td>
<td>V exons 9-14</td>
<td>V exon 14</td>
<td>Bertolini et al, 1994</td>
</tr>
<tr>
<td>FH Viterbo</td>
<td>V exons 16-17</td>
<td>V exon 17</td>
<td>Lelli et al, 1991a</td>
</tr>
</tbody>
</table>

Deletions are shown by Δ and duplications are shown by V.

As for QFM-PCR, minor rearrangements can be detected using UPQFM-PCR. A 4bp insertion was identified in exon 8 (Fig 4.11) and it was characterised, by fluorescent dye terminator sequencing, to be the same duplication, identified in the USA group, a GGGT at nucleotide 1120 (FsG353).

Two groups of FH patients were screened for major LDLR rearrangements, 76 adult and 42 paediatric patients from the UK. Three major rearrangements were detected. Patients A and C showed more than one test exon to be deleted or duplicated whilst patient B showed a duplication of a single test exon (Table 4.7). QFM-PCR was used
to define the boundaries of the major rearrangements. Multiplex sets of two or three exons were amplified on the DNA from the patient, four normals and relevant major rearrangement controls.

Table 4.7: LDLR major rearrangements detected in the two UK FH groups.

<table>
<thead>
<tr>
<th>Patient</th>
<th>FH group</th>
<th>LDLR exons deleted or duplicated (UPQFM-PCR)</th>
<th>Characterisation of rearrangement (exon boundaries)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adult</td>
<td>∨ ex8, 14 &amp; 17 or ∆ ex3 &amp; 5</td>
<td>∨ ex8-17 or ∆ ex3-5*</td>
</tr>
<tr>
<td>B*</td>
<td>Paediatric</td>
<td>∨ ex8</td>
<td>∨ ex8-10*</td>
</tr>
<tr>
<td>C*</td>
<td>Paediatric</td>
<td>∨ ex8, 14 &amp; 17 or ∆ ex3 &amp; 5</td>
<td>∨ ex7-18*</td>
</tr>
</tbody>
</table>

Exon boundaries of LDLR major rearrangements were characterised by QFM-PCR as described in the text. *Rearrangements confirmed in affected relatives. §No further characterisation could be carried out as only a minute amount of DNA was extracted from the child’s small blood sample taken. *Indicate previously unreported mutations.

**Patient A**

Patient A either carried a duplication of exons 8, 14 and 17 or a deletion of exons 3 and 5 but because of a lack of DNA no detailed characterisation could be carried out.

**Patient B**

Patient B showed one of the test exons to be duplicated, exon 8, by UPQFM-PCR. Characterisation and confirmation of the mutation, by QFM-PCR, showed that exons 8-10 were duplicated.

**Patient C**

Patient C had a similar pattern of peak areas to patient A, duplication for exons 8, 14 and 17 or a deletion of exons 3 and 5 (Fig 4.12). Details of the methodical characterisation of this major rearrangement is explained below and summarised in Fig 4.13. The first step, a diplex PCR of exons 6 and 16 confirmed the UPQFM-PCR result and showed that if a deletion had occurred it included exon 6. The second multiplex included exons 1, 6 and 10 and the result was that exons 1 and 6 were reduced compared with exon 10. Thus the mutation was either a deletion that extended from exons 1 to 6 or a duplication of exon 10. The next multiplex involved co-amplification of exons 7 and 17. A normal ratio pattern was observed thus one, two or three copies of exons 7 & 17 were present which can be explained by either both exons
are deleted, both are *neither* deleted nor duplicated (i.e. normal) or both are duplicated respectively. From the knowledge of the previous multiplex results it was deduced that exon 7 was either normal, deleted or duplicated and exon 17 was normal or duplicated. By logical elimination there were two feasible explanations, either exons 7-17 were normal or duplicated. Two multiplex sets with an external control were used to show that the rearrangement was a duplication. The external control was an exon of the dystrophin gene, which was not ideal as the gene is found on the X-chromosome, but it was readily available. The gender must therefore be considered in the ratio determination. The external locus was amplified with exon 16 which was thought to be duplicated and with exon 18 which would provide information indicating if it was included or excluded in the duplication. If the mutation was a duplication a 3:2 ratio would be expected between exon 16/18 and the external locus but if a 1:1 ratio was observed the exon was not involved. Both exons 16 and 18 were shown to be duplicated. Therefore patient C had a duplication of >20kb encompassing exons 7-18, starting in intron 7 and ending in either the 3' untranslated region of LDLR or downstream from LDLR.
Fig 4.10: Genotyper traces and Excel macro sheet of the UPQFM-PCR of normals and three deletions.

A: Normal control

B: FH Leiden: deletion ex7-8 (ex8)

C: FH Pavia: ex3-10 (ex3,5,8)

D: FH Rome: deletion ex13-14 (ex14)

A: Exon boundaries are shown for each reported deletion and the particular exons deleted are shown in brackets. Exon names and peak areas shown under the respective peaks. Deletions are indicated by an asterisk. B: Dosage data on four normal controls and three reported deletions. Details of the macro sheet are in the text.
Fig 4.11: Genotyper traces of a normal control (A) and a minor rearrangement of exon 8 (B) detected by the UPQFM-PCR set.

A: Normal control

<table>
<thead>
<tr>
<th>Exon</th>
<th>Peak Size</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>226.07</td>
<td>12228</td>
</tr>
<tr>
<td>3</td>
<td>262.15</td>
<td>8762</td>
</tr>
<tr>
<td>5</td>
<td>237.77</td>
<td>12826</td>
</tr>
<tr>
<td>8</td>
<td>292.48</td>
<td>10146</td>
</tr>
<tr>
<td>14</td>
<td>322.84</td>
<td>18677</td>
</tr>
</tbody>
</table>

B: 4bp insertion in exon 8

<table>
<thead>
<tr>
<th>Exon</th>
<th>Peak Size</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>225.72</td>
<td>6644</td>
</tr>
<tr>
<td>3</td>
<td>261.97</td>
<td>4779</td>
</tr>
<tr>
<td>5</td>
<td>237.51</td>
<td>7045</td>
</tr>
<tr>
<td>8</td>
<td>292.05</td>
<td>2529</td>
</tr>
<tr>
<td>8*</td>
<td>295.79</td>
<td>3091</td>
</tr>
<tr>
<td>14</td>
<td>322.66</td>
<td>9962</td>
</tr>
</tbody>
</table>

Peaks are labelled with the exon name, peak size and peak area. A 4bp insertion was identified in exon 8 (marked with an asterisk) which was due to a duplication, GGGT at 1120 (F5 G353).
Fig 4.12: Genotyper traces & Excel macros of UPQFM-PCR of a normal control & patient C (V).

A

Control 2

Patient C

B

<table>
<thead>
<tr>
<th>Normals</th>
<th>Peak areas of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons</td>
<td>C1</td>
</tr>
<tr>
<td>3</td>
<td>6758</td>
</tr>
<tr>
<td>5</td>
<td>10784</td>
</tr>
<tr>
<td>8</td>
<td>16976</td>
</tr>
<tr>
<td>14</td>
<td>16448</td>
</tr>
<tr>
<td>17</td>
<td>17610</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normals</th>
<th>Mean of exon peak area ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>0.56-0.72</td>
</tr>
<tr>
<td>5</td>
<td>0.48-1.00</td>
</tr>
<tr>
<td>8</td>
<td>0.59-1.13</td>
</tr>
<tr>
<td>14</td>
<td>0.86-1.12</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient C</th>
<th>Areas</th>
<th>Ratio of peak areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>3676</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>5761</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>11663</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>19030</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>18337</td>
<td>1</td>
</tr>
</tbody>
</table>

Results: Vex8,14,17/ Δ ex3,5

Shaded areas indicate duplicated exons whilst brackets indicate ratios which lie just outside +2SD but have been shown, by repeated assays, to be in the expected range - () indicate duplicated exon ratios whilst [ ] indicate normal exon ratios.
Fig 4.13: Characterisation of LDLR major rearrangement in patient C from the paediatric UK group.

<table>
<thead>
<tr>
<th>Type of Assay</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal LDLR</td>
<td></td>
</tr>
<tr>
<td>UPQFM-PCR: (ex3,5,8,14 &amp; 17) -</td>
<td></td>
</tr>
<tr>
<td>- 8,14 &amp; 17 duplicated or 3 &amp; 5</td>
<td></td>
</tr>
<tr>
<td>deleted</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex 6 &amp; 16) -</td>
<td></td>
</tr>
<tr>
<td>- 16 duplicated or 6 deleted</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex 1, 6 &amp; 10) -</td>
<td></td>
</tr>
<tr>
<td>- 10 duplicated or 1 &amp; 6 deleted</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex 7 &amp; 17) -</td>
<td></td>
</tr>
<tr>
<td>- normal ratios, 7 &amp; 17 normal,</td>
<td></td>
</tr>
<tr>
<td>duplicated or deleted</td>
<td></td>
</tr>
<tr>
<td>QFM-PCR: (ex16 &amp; external locus;</td>
<td></td>
</tr>
<tr>
<td>ex18 &amp; external locus) - 16 &amp; 18</td>
<td></td>
</tr>
<tr>
<td>duplicated</td>
<td></td>
</tr>
<tr>
<td>Duplication of ex7-18 LDLR</td>
<td></td>
</tr>
</tbody>
</table>

UPQFM-PCR was used to identify the major rearrangement. QFM-PCR was used to determine the exon boundaries of the major rearrangement. Patient C was shown to have a duplication of exons 7 to 18. Duplications shown by ▼ and normals shown by (N) indicate deduced data whilst solid symbols▼ or N indicate data obtained from various PCR reactions. Results of the multiplex sets could be deduced several ways, as explained in the text, but the symbols show what actually occurred in patient C.
4.4. Discussion

QFM-PCR is a rapid way of detecting major and minor rearrangements in any gene of interest. The method only requires small quantities of starting material which is then amplified, resolved by electrophoresis and quantified using fluorescence.

There are two major problems of quantitative multiplex PCR, inter- and intra-assay variation. Interassay variation has been found to occur due to incorrect concentration, poor purity and level of degradation of the DNA sample, which was also encountered by Chamberlain et al., 1988 and Yau et al., 1996. DNA must be diluted to a constant concentration of 25μg/ml. Lower concentrations either gave an accurate result or completely failed to amplify, but concentrations above this level produced abnormal peak patterns which virtually always resulted in the calling of an apparent non-contiguous deletion or duplication. Before tests were repeated the concentration was re-measured and a fresh dilution was made and this generally corrected the problem. A few samples continually failed to give an informative result and these were generally a result of the poor purity of the DNA but reprecipitation was found to eliminate most of the problems.

Variation was greatest with the first multiplex PCR strategy, QFM-PCR. Too many samples had to be repeated either due to variable ratios in the controls or non-contiguous deletions or duplications. The second strategy, UPQFM-PCR provided a more accurate means of major rearrangement detection. Interassay variation was minimised by ensuring PCR reagents were accurately made and dispensed. Variation was still observed, in particular when a new batch of buffer was used or when a new primer aliquot was added, despite being diluted as a large batch. Confidence limits (±2SD) for normal, deleted and duplicated exons still showed some overlap, but criteria for detecting the major rearrangements were set using appropriate controls which must always be used to validate each assay. Ratios were not able to discriminate between a deletion and a duplication but if these criteria are adhered to then this method provides a means of detecting major rearrangements in any gene.

Over 250 samples from a heterogeneous population have been analysed with this multiplex set. No rare variants were found in the nucleotides of the primer sequences, which could lead to a failure of amplification from one allele and thus give a false positive result. However, verification should be carried when a single exon appears deleted as practised with the DMD assay, using another method, such as QFM-PCR method where different primer sequences are used.
Additionally to detecting major rearrangements, deletions and insertions of greater than 1bp were detected using both strategies. A 4bp insertion in exon 8 was identified in two patients, one by each strategy. Characterisation showed the two insertions to be identical, a duplication, GGGT, at nucleotide 1120 resulting in a frameshift at codon 353. This duplication (FH Nashville) has been found in several FH patients and the LDL-receptor activity was found to be <2% of the normal, so is likely to be the pathogenic cause of FH in these two patients. Exon 8 appears to be a region where many 4bp deletions and duplications are observed due to the presence of four base repeats (FH website). Although mutation detection methods such as SSCP and DGGE followed by sequencing, can detect this class of mutations, QFM-PCR can be used to confirm the number of nucleotides involved. Relatives may be screened for the minor rearrangement either by only amplifying the specific exon or by carrying out the multiplex reaction.

4.4.1. Quantitative fluorescent multiplex PCR (strategy 1)
From data available on the FH website the proportion of reported LDLR rearrangements that would be detectable by this one multiplex assay (exons 1, 8, 10, 12, 16) were estimated to be 55%. The largest region not covered by this multiplex set is between exons 2 to 7 where 27% of reported mutations are found. A second region not analysed, exons 13 to 15, accounts for 11% of reported gene rearrangements.

95 FH probands from the USA were screened for LDLR major rearrangements by the QFM-PCR multiplex set. Two gene rearrangements, previously undescribed, were found in four patients. Analysis of their surnames showed that there were likely to be two common ancestors although it was unknown to the patients.

**Patient 1 & 2**
A deletion of exons 6-10 was identified in patients 1 & 2 by QFM-PCR set 1, confirmed and characterised by UPQFM-PCR and QFM-PCR. A guanine from exon 5 is spliced to GC of exon 11 creating the expected first exon 11 amino acid, glycine, and then the reading frame is maintained. This is predicted to result in an in frame deletion of 6.0-9.4kb spanning from intron 5 to intron 10, depending on the exact breakpoints. The deletion of exons 6-10 is similar to a deletion found in Finnish FH patients, FH Oula -1 (Aalta-Setala et al, 1992), which involves a 7.5kb deletion of exons 7-10. No 5' breakpoints have been observed in intron 5 but 3’ breakpoints have occurred in intron 5 as exon 5 is deleted in several FH individuals from France, (FH Paris-1) (Hobbs et al, 1986), England (FH London-2) (Horsthemke et al, 1987) and Denmark.
3' breakpoints in intron 10 have been observed in several deletions; FH Oula-1 (Aalta-Setala et al, 1992); FH Reykavik (Taylor et al, 1989) and FH Amsterdam-5, (MEDPED meeting 1998).

**Patient 3 & 4**

A duplication of exons 14 to exon 17 was detected and characterised by QFM-PCR and UPQFM-PCR in patients 3 and 4. This previously unreported duplication of intron 13 to 17 spans 12.3-15.2kb. This is the second case of a major rearrangement with the 5' breakpoint in intron 13 (Koivisto et al, 1997). Several deletions have 3' breakpoints in intron 17 (FH Padova-2) (Bertolini et al, 1995), (FH Tsukuba-2) (Yamakawa et al, 1989) and one reported duplication (FH Viterbo) (Lelli et al, 1991a). This exon 14-17 duplication is predicted to be the pathogenic cause of the patient's FH phenotype as the duplication results in a change in the reading frame with the formation of a termination codon two amino acids down from the exon 17/exon 14 splicing junction. This major rearrangement is one of the largest known LDLR duplications, spanning 12.3-15.2kb. The largest two rearrangements described are the 14kb duplication of exons 2-8 (FH St Louis) (Lehrman et al, 1987b) and the 10.5kb duplication of exons 9-14 (FH Salerno) (Bertolini et al, 1994).

Four gene rearrangements were detected in 95 FH probands although two are suspected to share a common ancestor. The frequency of LDLR rearrangements in this group was 2% excluding the two individuals sharing an ancestor. It was determined that this multiplex set would detect 55% of reported gene rearrangements, thus taking this into consideration the expected detection rate could be approximately 4%. It is unlikely that there is a founder gene effect present in Portland, Oregon, as it is a heterogeneous population so a common major rearrangement has probably not been missed. LDLR major rearrangement frequencies of 2-4% are similar to previous results (Horsthemke et al, 1987; Langlois et al, 1988).

4.4.2. *Universal primer quantitative fluorescent multiplex PCR (strategy 2)*

The proportion of reported LDLR rearrangements that would be detectable by this UPQFM-PCR assay is 86%. Two regions are not covered, promoter to exon 2 and exon 15. Deletions of the 5' flanking region to intron 1 or 2, have been observed in several patients and one particular mutation occurs at a high frequency, 60%, due to a founder gene effect in the French-Canadian population of Quebec (FH French Canadian-1) (Hobbs et al, 1987). The other region not covered is exon 15 which is deleted in the Japanese (FH Tonami) (Kajinami et al, 1988) and in Finnish (FH Espoo)
(Koivisto et al, 1993) FH populations. For these populations a specific UPQFM-PCR assay could be designed to encompass the regions deleted.

Using this method to screen LDLR in two groups of FH individuals, three major rearrangements were found and they are predicted to be the pathogenic cause of the FH phenotype.

**Patient A**
LDLR mutation in patient A was identified by UPQFM-PCR to be either a deletion of at least exons 3 and 5 or a duplication of at least exons 8 to 17 but due to a lack of DNA it was not fully characterised. Many reported deletions have started in the 5' flanking region and have ranged in size, from 6kb to over 25kb, ending in introns 1 to 6 (FH website). If the rearrangement in patient A is a >25kb deletion it may be similar to the deletions described in two patients, one from England and the other from Bologna, Italy (FH Bologna-1) (Lelli et al, 1991a; Sun et al, 1992). These two deletions encompass the 5' flanking region to intron 6 but have different 5' breakpoints. LDLR rearrangements have been shown often to be in frame, thus the two exons around the breakpoints are spliced together during RNA processing and translation of this mRNA is predicted to produce a smaller or larger receptor protein (Lehrman et al, 1987b; Bertolini et al, 1995; Chae et al, 1997). If it was a deletion this may be the case. If it was a duplication it may or may not include exon 18 and a portion of or the complete 3' UTR so a larger receptor may be produced, although the mRNA may be very unstable. Further work would be required to characterise this rearrangement and to deduce the effect of the mutation at the protein level.

**Patient B**
The mutation in patient B was a duplication spanning from intron 7 to 10 and is therefore 2.6-5.6kb depending on the exact intronic breakpoints. This duplication may have occurred due to misalignment of two Alu repeats in introns 7 and intron 10 of LDLR (Fig 4.14). No such duplication has been described to date and the rearrangements described previously have generally involved breakpoints in different introns. The duplication is predicted to be pathogenic as it results in a shift in the reading frame. The G at nucleotide 1586 (exon 10) is spliced to A at 1061 (exon 8) causing codon 333 to change from GAT (aspartic acid) to GGA (glycine). A termination codon is found three amino acids downstream resulting in the truncation of the protein.
Fig 4.14: Hypothesis of the mechanism for the presence of the duplication in patient B.

Unequal crossing over may have occurred between Alu sequences present in intron 7 and intron 10. Two copies of the normal receptor gene, one with exons indicated by squares and the other with exons indicated by circles, are aligned to illustrate the point of unequal crossing-over in patient B. The recombination event is predicted to yield two products, a duplication of exons 8-10 and a hypothetical product in which exons 8-10 are deleted.
Patient C
The rearrangement in patient C showed similar ratios to that of patient A, indicating either a deletion of exons 3-5 or a duplication of exons 8-17. Confirmation of a duplication was shown by a quantitative diplex reaction with an LDLR exon and a non-LDLR locus. Characterisation showed it to be a duplication of exons 7-18. This previously undescribed duplication spans at least 20kb from intron 6 to the 3’ untranslated region and may be larger. A proposed mechanism for the formation of this duplication could be the misalignment of Alu sequences resulting in unequal crossing over (Fig 4.15). The mutation is predicted to be pathogenic for the same reason as described for the duplication possibility in patient A.

In the adult group (n=76) one major rearrangement may have been previously detected (Sun et al, 1992) by this multiplex set. Major rearrangements accounted for a total of 1.3% of mutations (4bp duplication excluded). In the paediatric group (n=42) two unreported duplications were detected and no minor rearrangements were identified, thus the frequency of major rearrangements was 4.8%. The assay detects 86% of reported gene rearrangements thus the adult and paediatric rearrangement frequency rates may be estimated to be 1.5% and 5.5% respectively which is similar to that previously reported (Horsthemke et al, 1987; Langlois et al, 1988).
Fig 4.15: Hypothesis of the mechanism for the presence of the duplication in patient C.

Unequal crossing over may have occurred between Alu sequences present in intron 6 and the 3' untranslated region. Two copies of the normal receptor gene, one with exons indicated by squares and the other with exons indicated by circles, are aligned to illustrate the point of unequal crossing-over in patient C. The recombination event is predicted to yield two products, a duplication of exons 7-18 and a hypothetical product in which exons 7-18 are deleted.
The first eight exons encoding the LDL-receptor are in the same reading frame so deletions or duplications of exons in this region are in frame. If other regions are involved an out of frame gene rearrangement may occur, leading to a truncated receptor lacking several important domains. These may have a lower LDL-binding efficiency and are often unstable at the mRNA level so are rapidly degraded intracellularly (Hobbs et al, 1992). The gene rearrangements identified by the two QFM-PCR assays have been shown to be in frame or out of frame but have not been investigated at the functional level. It is unknown if more than one transcript occurs from the mutant allele as seen in many genes (Cooper, Krawczak & Antonarakis, 1995; Maquat, 1996) including LDLR (Bertolini et al, 1995; Webb et al, 1996; Nissen et al, 1997; Rodningen et al, 1998) or if the mutant allele is transcribed into mRNA or at what speed the receptor is processed. All that can be predicted is that the receptor proteins encoded by these mutant alleles will have a shortened or elongated protein.

As with single nucleotide mutations there is a great variability in phenotypic expression. Major rearrangements could be predicted to have a severe effect on the binding activity of LDL and β-VLDL but as shown by in vitro studies many gene rearrangements have milder effects (Table 4.8). An example was shown by site directed mutagenesis experiments of a deletion of the first repeat (exon 2) which was shown to have no effect on the binding or internalisation of LDL or β-VLDL or on the recycling of receptors in transfected mammalian cells (van Driel et al, 1987). Simultaneous deletion of exons 2 and 3 has resulted in a receptor which binds LDL 70% of the normal rate (Esser et al, 1988) suggesting that the first two repeats of the ligand binding domain are not necessary for LDL-receptor function. In the natural 10kb deletion of exons 2-3 (FH Tonami-2) (Kajinami et al, 1989) the major rearrangement was associated with lower cholesterol levels than those of typical FH patients. Two carriers had normal plasma cholesterol levels (Kajinami et al, 1989). The receptor activity of a true homozygote for this deletion was approximately 40% of normal binding (Mabuchi et al, 1990).

These results were confirmed by the ¹²⁵I-labelled LDL degradation assay (Rodningen et al, 1999). Cells from a Norwegian proband, homozygous for a deletion of exons 2 and 3 had a value for degradation of 0.3x that of normal cells whereas cells from homozygotes of null alleles gave values of 0.1. This indicated that the mutant receptor was able to bind, internalise and degrade LDL, so the patient would have a clinically mild phenotype. In contrast, heterozygous carriers had a severe phenotype with total cholesterol levels of 11-12mmol/l. These findings are in agreement with those of Sass
et al, 1995 who found that heterozygous FH French Canadians carrying a 5kb deletion of exons 2 and 3, had unexpectedly high values of total cholesterol. Thus heterozygotes for this deletion seem to be more severely affected than the homozygotes. A compound heterozygote was identified who inherited a splice acceptor mutation, 1359-1A>G from her father and the exon 2-3 deletion from her mother (Rodningen et al, 1999). Both parents had high cholesterol and their homozygous daughter was also severely affected with a total cholesterol of 32mmol/l at the age of one. Theoretically a compound heterozygote possessing one ‘mild’ and one ‘severe’ mutation would be expected to have a phenotype in between that of a true homozygote possessing a ‘mild’ mutation and a true homozygote possessing a ‘severe’ mutation but this was not the case. The deletion of exons 2-3 seemed to exert a dominant negative effect in the heterozygous mother but the splice site mutation showed a dominant effect over the deletion in the homozygous state, thus suggesting that two allelic receptor proteins do not function independently of each other (Rodningen et al, 1999). The LDL-receptor was proposed to function as a dimer and this could be used to explain these interactions although no conclusive evidence was ever found (Van Driel et al, 1987; Patel et al, 1993). A mild phenotype would then be expected in a true homozygote with homodimers made up of two mildly defective proteins, whereas a relatively severe phenotype would be expected in patients who are heterozygotes or compound heterozygotes, irrespective of the severity of the mutation. However the phenotypic variability may also be due to environmental or protective genetic factors. Therefore predictions as to the phenotype of a major rearrangement can only be made by comparing with major rearrangements which involve deletions or duplications of the same domains.
Table 4.8: LDL-binding efficiencies of characterised ‘severe’ and ‘mild’ LDLR rearrangements.

<table>
<thead>
<tr>
<th>'Severe' LDLR rearrangements</th>
<th>% of normal LDL-binding</th>
<th>'Mild' LDLR rearrangements</th>
<th>% of normal LDL-binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH St Louis (Vex2-8)</td>
<td>5-15%</td>
<td>FH Siracusa-3 (ΔP,ex1-2)</td>
<td>50%</td>
</tr>
<tr>
<td>FH Cape-Town (Δex7-8)</td>
<td>2-5%</td>
<td>FH Bologna-1 (ΔP,ex1-6)</td>
<td>40-50%</td>
</tr>
<tr>
<td>FH Baltimore (Δex17-18)</td>
<td>2-5%</td>
<td>FH Pavia (Δex2-12)</td>
<td>45-55%</td>
</tr>
<tr>
<td>FH Viterbo (Vex16-17)</td>
<td>20%</td>
<td>FH Massa (Δex3-10/3-12)</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Salerno (Vex9-14)</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Genoa-1 (Δex11-12)</td>
<td>46%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Catania (Δex12-18)</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Chieti/Macerata (Δex13-14)</td>
<td>43-50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Roma (Δex13-14)</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Espoo (Δex15)</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FH Padova-2 (Δex16-17)</td>
<td>45-60%</td>
</tr>
</tbody>
</table>

With regard to the future of this technique, the UPQFM-PCR is an effective method of detecting major and minor rearrangements and could be applied to any gene where deletions or duplications account for a significant proportion of mutations, e.g. steroid sulphatase gene (STS) in individuals with ichthyosis (Shapiro et al., 1989), proteolipid protein gene (PLP) in individuals with Pelizaeus-Merzbacher disease (Woodward et al., 1998), growth hormone 1 gene (GH1) in individuals with familial growth hormone deficiency (Vnenckak-Hones et al., 1990) and the ornithine transcarbamylase gene (OTC) in individuals with ornithine transcarbamylase deficiency (Segues et al., 1995).

Future developments include adding an unlinked control to this set which would distinguish deletions from duplications especially when the major rearrangement extends either from the promoter or to the last exon or if the entire gene was deleted or duplicated. It would also be useful to design a second assay to include areas not covered presently. The aim would be to develop a GC-rich panel of amplicons so that
the promoter, exon 1 and 2 can be amplified with an exon at the opposite end of the
gene, possibly exon 15, plus an unlinked locus. An alternative way would be to
include exon 1 and 15 in the established set which would increase the percentage of
detectable reported mutations to 92% and 94% respectively. The sensitivity rate would
then be similar to the rate of the dystrophin QFM-PCR assay where 98% of deletions
and 90% of duplications are detected (Chamberlain et al, 1990; Beggs et al, 1990; Yau

4.5. Conclusion
The dystrophin fluorescent multiplex assay is used routinely to screen for deletions and
duplications but due to the wide confidence intervals of peak area ratios, this LDLR
assay, described here, cannot be used as the only test for LDLR rearrangements, rather
as a scanning technique which should point to the small percentage (<5%) of FH
patients with major deletions or duplications. Identified deletions and duplications can
then be confirmed by another technique such as PCR across the breakpoint, long PCR
or small QFM-PCR sets. The main advantages are that UPQFM-PCR is less laborious
and time consuming than Southern blotting and allows analysis to be carried out on
samples where there is little DNA. The UPQFM-PCR approach should have a
generality for set up and primary scanning for major rearrangements in any gene.

Acknowledgements
I would like to thank Professor Stefano Bertolini, Dr Henrik Jensen and Dr Anne Soutar for supplying
DNA's of known major rearrangements and to Dr Stephen Scharf for his advice on multiplex PCR.
Chapter 5: FH case reports

5.1. Introduction

The family based genetic screening strategy for FH was started four years ago. Clinical recognition was gradual but new cases and family members of tested individuals are now referred. Many parents request testing of their children as early as possible, a few cases have even been analysed on cord blood samples. This early diagnosis can either provide assurance in the case of a negative result or allow early dietary and clinical management in the case of a positive result.

The majority of cases are either reported as positive or negative for an LDLR screen and APOB tests. Occasionally unusual results are found and these provide areas of research interest but can cause difficulties in reporting. Finally, if no mutation is identified in LDLR or APOB, two microsatellite markers flanking LDLR can be analysed. This can only be undertaken in particular cases where relatives are available and then only to exclude a diagnosis of FH. The criteria described in section 3.4.1 were used to assess the pathogenicity of the identified mutations and 14 cases are described in detail:

Case 1 - Exon 4 mutation, C163Y
Case 2 - Exon 4 mutation, E207X
Case 3 - Exon 14 mutation, P664L
Case 4 - Exon 4 mutation, D200G
Case 5 - Exon 2 mutation, dA at 118
Case 6 - Exon 3 mutation, E80K
Case 7 - Exon 4 mutation, D206E
Case 8 - Exon 12 mutation, P587L
Case 9 - Exon 14 homozygous mutation, P664L
Case 10 - Exon 15 T705I and intron 7 1061-8T>C variants
Case 11 - R3500Q mutation
Case 12 - R3531C mutation
Case 13 - No mutation identified in LDLR or APOB
Case 14 - No mutation identified in LDLR or APOB
5.2. Methods and Materials

5.2.1. Subjects for T705I and R3531C analysis
The patient sample consisted of 791 samples with a clinical diagnosis of 'definite', 'probable' and 'possible' FH according to the criteria set by the Simon Broome Steering Committee, 1991 (section 2.10.1).

The comparison group consisted of 2287 Caucasian men from the Park Heart Study (NPHS-II) (Miller et al, 1996). Exclusion criteria included non-Caucasian, a history of unstable angina or MI, regular medication with aspirin or anticoagulants, cerebrovascular disease, malignancy (except skin cancer other than melanoma), diseases exposing staff to risk of infection, mental disorder or other conditions precluding informed consent or regular attendance for examination (Miller et al, 1996).

5.2.2. Molecular analysis
An assay was designed for the T705I substitution where an NsiI restriction site was introduced into the rare C allele (I) by a mismatch in the sense primer (underlined): sense primer: 5'-CAG TGG CCA CCC AGG AGA CAT G.CA-3' and antisense primer: 5'-ATC TCC ACC GTG GTG AGC CCA-3'. PCR conditions were as described in section 2.3.3. NsiI (2U) was added directly to each PCR product. The products were separated on a 7.5% MADGE (section 2.6.3.1).

The I705 carriers were then analysed for the 1061-8C variation in intron 7 by a natural EarI restriction digest. Amplification of exon 8 with primers FH119 and FH27 (Table 2.1) and reaction conditions were as in section 2.3.2. A restriction site was lost in the rare C allele but a constant cut site exists for confirmation of digestion.

791 FH probands were screened for the R3531C mutation by an ASO assay (section 2.3.5 & 2.7.2.2).

5.2.3. Statistical analysis
A one way analysis of variance was used to compare the lipid levels between the I705 carriers and non-carriers. The data was analysed using STATA (Intercooled Stata 5.0).
5.3. Interesting and common case reports

5.3.1. Case 1 - C163Y LDLR mutation (Fig 5.1)

II.1 was attending a lipid clinic for raised total cholesterol and TX, characteristic of FH. The exon 4 mutation, C163Y, was initially detected by SSCP analysis and characterised by fluorescent sequencing. The G>A transition at nucleotide 551 was confirmed by a ‘forced’ PCR assay where a nucleotide change in the primer creates an Rsal restriction site in the normal allele (Lee et al, 1998). The loss of the restriction site indicates the C163Y mutation. A sample was also available from the patient’s father, who had a similar lipoprotein profile, and he was also found to be heterozygous for the mutation. Further family members were referred; III.1 & III.2 had total cholesterol levels above the 95th percentile for their ages (MEDPED data). Mutation analysis confirmed they were affected. The youngest child, III.3, had an aversion to needles so a buccal (mouthwash) sample was taken for genetic analysis. He was found to be affected based solely on molecular analysis and would now need careful monitoring especially as the total cholesterol levels are very high in his older siblings.

There were three lines for proof of pathogenicity of the C163Y mutation. Firstly the nucleotide substitution G to A at 551 occurs at cysteine 163 which is a conserved amino acid across many species, human (Yamamoto et al, 1984), rabbit (Yamamoto et al, 1986), rat (Lee et al, 1989), Xenopus laevis I & II (Mehta et al, 1991), hamster (Bishop et al, 1992), mouse (Hoffer et al, 1993) and banded cat shark (Mehta et al, 1996). Secondly the C163Y mutation occurs in the fourth cysteine rich repeat of exon 4 which functionally binds to apoB. Thirdly this mutation was originally found in an Irish FH patient (Ward & Graham, FH website) and has been found at a higher frequency in FH patients from Glasgow (Lee et al, 1998).

5.3.2. Case 2 - E207X LDLR mutation (Fig 5.2)

The proband II.1 attends the paediatric lipid clinic at GOSH. He has an elevated total cholesterol of 7.0mmol/l which is above the 95th percentile for his age (Sporik et al, 1991). His mother also had a total cholesterol level above the 95th percentile at 10mmol/l. A mutation was found at the 3' end of exon 4 of LDLR, by SSCP analysis. Fluorescent dye terminator sequencing showed that a guanine was substituted for a thymine, at nucleotide 682, and this is predicted to result in the premature termination of the protein at codon 207. The mutation can be confirmed by a natural restriction digest where the mutation introduces a Bfal site.
Fig 5.1: Case 1 - C163Y mutation. C163Y co-segregates with the FH phenotype indicated by filled symbols and the proband is marked by an arrow. The highest untreated total cholesterol (mmol/l), the presence or absence of TX in adults and the age of referral for molecular analysis are shown under the pedigree symbols. Each family member is directly above the respective lane on the gel. III.3 had a 50% risk of inheriting the mutation. No biochemical tests (NT) were carried out on III.3 as he had an aversion to needles so a buccal sample was taken for genetic analysis. C163Y is in exon 4 of LDLR which encodes the fourth cysteine-rich repeat. C163Y was detected by a 'forced' PCR digest where an Rsal restriction site was introduced into the normal allele. Fragments were separated on a 10% polyacrylamide gel and visualised by silver staining. Lane 1 - 50bp size marker, lanes 2 - C163Y, lane 3 - C163Y, lane 4 - negative for C163Y, lane 5 - C163Y, lane 6 - C163Y, lane 7 - C163Y, lane 8 - negative for C163Y, lane 9 - C163Y mutation control and lane 10 - blank control. III.3 was shown to carry the C163Y mutation.
Fig 5.2: Case 2 - E207X mutation. E207X in exon 4 of LDLR co-segregated with the FH phenotype, shown by the filled symbols. The proband is marked by an arrow, the highest untreated total cholesterol (mmol/l) and the age of referral for molecular analysis are shown under the pedigree symbols. A BfAl restriction site was introduced into the mutant allele and the digested PCR products were visualised on a 3% (2:1 NuSieve) agarose gel stained with EtBr. Each family member is directly above the respective lane on the gel. Lane 1- 50bp size marker, lane 2 - E207X, lane 3 - E207X, lane 4 - negative for E207X, lanes 5 - negative E207X control, lane 6 - E207X positive control and lane 7 - blank control. II.1 was shown to carry the E207X mutation.
5.3.3. Case 3 - P664L LDLR mutation (Fig 5.3)

The proband, II.1, had a total cholesterol level of 4.4mmol/l, which is approximately at the 30th percentile for a 7 year old (Sporik et al, 1991). His mother and father had cholesterol levels of 9.0 and 5.6mmol/l respectively. The mother had been referred to a lipid clinic after a MI at the age of 29. She was clinically diagnosed as ‘possible’ FH and placed on a daily dose of 20mg simvastatin, lowering her levels to 6.1mmol/l. II.1 had normal cholesterol levels but there is an overlap between affected and normal levels in children (Leonard et al, 1977) so a molecular diagnosis could confirm or exclude FH if a mutation was shown to be present. LDLR was screened by SSCP analysis and an abnormal band shift was detected in exon 14, which was characterised to be due to the substitution of a cytosine for a thymine, at nucleotide 2054. The transition is predicted to result in the replacement of a proline for a leucine at codon 664 (P664L).

Proline 664 is found in the third growth factor repeat of the LDL-receptor, repeat C, and this codon is conserved across species; human (Yamamoto et al, 1984), rabbit (Yamamoto et al, 1986), rat (Lee et al, 1989), Xenopus laevis I & II (Mehta et al, 1991), hamster (Bishop et al, 1992), mouse (Hoffer et al, 1993) and banded cat shark (Mehta et al, 1996). The mutation was first identified in four hypercholesterolaemic patients from London (King-Underwood et al, 1991) and after further screening was found to be relatively common in the hypercholesterolaemic population, 3% in London (Tybjaerg-Hansen et al, 1990) and now world-wide (FH website).

The mutation can be tested by a PCR digest, where a natural PstI restriction site is introduced into the mutant allele. The family wanted their youngest child aged 5 years, to be tested for the mutation. The child’s cholesterol level was 3.1mmol/l, which is very low (20th percentile) (Sporik et al, 1991), but it was an advisable decision as her brother tested positive and had normal cholesterol levels. A buccal sample kit was posted to her home and she tested negative for the P664L mutation. One year later the couple had their third child and requested FH genetic testing to be undertaken as early as possible. The direct assay was carried out on DNA extracted from cord blood and the baby tested negative. The youngest two children do not have to have regular cholesterol monitoring whilst their elder brother should and may require treatment in later life.

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Fig 5.3: Case 3 - P664L mutation. P664L in exon 14 of LDLR. The filled symbols indicate a clinical diagnosis of FH and the proband is marked by an arrow. The highest untreated total cholesterol levels (mmol/l) (NT - cholesterol not tested) and the age of referral for molecular analysis are shown under the pedigree symbols. Genetic analysis was carried out on a cord blood sample in II.3. The mutation created a PstI restriction site and the PCR digest products were visualised on an EtBr stained 3% (2:1 NuSieve) agarose gel. Each family member is directly above the respective lane on the gel. Lane 1 - 50bp size marker, lane 2 - negative for P664L, lane 3 - P664L, lane 3 - P664L, lane 4 - negative for P664L, lane 6 - negative for P664L and lane 7 - blank control. II.1 was shown to carry the P664L mutation but II.2 and II.3 tested negative.
5.3.4. Case 4 - D200G LDLR mutation (Fig 5.4)

II.2, II.3 and II.5 had elevated plasma total cholesterol, TX and a family history of hypercholesterolaemia and were undergoing lipid-lowering treatment. They were referred for molecular analysis and an LDLR mutation was identified, D200G, where an adenine was replaced by a guanine at nucleotide 662 of exon 4. Aspartic acid at codon 200 occurs in cysteine-rich repeat 5 of the ligand binding domain and is conserved across species, human (Yamamoto et al, 1984), rabbit (Yamamoto et al, 1986), rat (Lee et al, 1989), Xenopus laevis I & II (Mehta et al, 1991), hamster (Bishop et al, 1992), mouse (Hoffer et al, 1993) and banded cat shark (Mehta et al, 1996). Repeat 5 is the only repeat which binds apoB and apoE containing lipoproteins (Russell, Brown & Goldstein, 1989) and mutations in this region have been found to have a more severe phenotype (Gudnason et al, 1993).

The mutation can be tested by a simple PCR MspI digest where a gain of a site occurs in the mutant allele. The mutation was confirmed in II.4. A buccal sample was received from a 32-year old daughter of II.2 who had a total cholesterol of ~5mmol/l (60-70 percentile for age) (MEDPED data), indicating she did not have a clinical diagnosis of FH. Molecular analysis revealed a surprising result, she carried the D200G mutation. Possible reasons for her low cholesterol may include a low fat diet or the presence of lipid-lowering factors. In the Netherlands, carriers of LDLR or APOB mutations have been observed to have total cholesterol levels below the 95th percentile at the age of 60+, although almost all are above the 75th percentile (Defesche, personal communication). The daughter will now require closer clinical management to monitor her cholesterol, as elevations may occur in the future. Therefore genetic analysis was not only important to the daughter but also to her family where mutation testing will be provide a more accurate means of diagnosing FH.

5.3.5. Case 5 - dA at 118 LDLR mutation (Fig 5.5)

Four members were attending a lipid clinic (I.1, II.2, II.3 and III.1) for FH, TX were present in all four. Mutation analysis, SSCP followed by sequencing, identified a novel one base deletion of an adenine at nucleotide 118 of exon 2. This is predicted to result in a frameshift at isoleucine 19 until a premature termination codon occurs. The null allele was confirmed by a natural restriction PCR assay which utilised the loss of an NsiI site in the mutant allele. All four were shown to carry the mutation, thus confirming the diagnosis of FH. Buccal samples were later received from III.2, III.3, III.4. Two had cholesterol levels above the 95th percentile for their age, whilst no cholesterol tests had been carried out in the youngest child. The mutation was
confirmed in the eldest two (III.2 & III.3) and III.4 was found to carry the mutation. Therefore the young boy does now have to attend the lipid clinic on a regular basis.

5.3.6. Case 6 - E80K LDLR mutation (Fig 5.6)
A clinical diagnosis of ‘definite’ FH was given to II.2 at the lipid clinic held at Charing Cross Hospital. Mutation analysis identified a G>A substitution at nucleotide 301 (E80K) in exon 3 of LDLR. Exon 3 encodes the second cysteine-rich repeat of the ligand binding domain and the mutation occurs in the conserved cluster of negatively charged amino acids, DXSDE, found at the carboxy terminus of the repeats which are thought to bind to the basic amino acids of apoB. The E80K mutation has been found to occur in 10% of FH patients from Manchester, UK, (Webb et al, 1992). A ‘forced’ PCR test was designed to incorporate a TaqI restriction site in the normal allele, thus the loss of the site indicates an E80K carrier (O'Dell et al, 1996). The mutation was confirmed to have been inherited from her mother. Family members II.3 and III.1 were referred with II.2 and they also tested positive. II.3 brought her daughter, III.2, to the paediatric lipid clinic at GOSH, knowing the child was at risk of having FH. Samples were taken for genetic and biochemical tests. She was found to carry the mutation and her cholesterol was above the 95th percentile for her age (Sporik et al, 1991).

5.3.7. Case 7 - D206E LDLR mutation (Fig 5.7)
The proband, III.11, was referred to GOSH because her cholesterol level was high (~95th percentile for her age) (Sporik et al, 1991) and her father had hypercholesterolaemia. An exon 4 mutation was detected in both individuals, resulting in a substitution of a cytosine for a guanine at nucleotide 681, which is predicted to cause the amino acid change of an aspartic acid to glutamic acid at codon 206. The mutation occurs in the conserved DXSDE motif in repeat 5, which binds both apoB and apoE containing lipoproteins. A simple test can detect the presence of the mutation with the introduction of a DdeI restriction site in mutation carriers. The grandfather of the proband, I.2, had 19 siblings and many died of MI's in their mid 40's so other family members requested genetic testing. Buccal sample kits were sent to many locations in London and the UK. The direct assay was carried out and results were given to the individuals by their GP. All members tested negative except for a male aged 60-years, who knew he had the heart disease risk factor and was already on lipid-lowering treatment. He is the oldest living family member with the mutation. The reason for the low frequency of mutation carriers was because the majority of the male mutation carriers died in their early 40's from coronary related events and did not have children. Also a detailed family history was only taken subsequently to mutation testing and
many of the individuals came from another marriage where neither the father or mother carried the mutation so they only had a population risk of FH. The negative results brought relief to family members and the proband can be closely monitored.

5.3.8. Case 8 - P587L mutation in exon 12 (Fig 5.8)

A 10-year old boy, II.2, presented at the paediatric clinic at GOSH with an untreated plasma cholesterol of 8.1mmol/l (above the 95th percentile for age) (Sporik et al, 1991). His father had died of a MI at the age of 42 and his sister also had hypercholesterolaemia (Fig 5.8a). The boy was referred for genetic analyses two years later when the research project commenced. DNA was only available from the mother and son. No abnormal SSCP band shifts were observed in any exon in mother or son except in the case of exon 12, where a complex pattern was seen (Fig 5.8b). The two exon polymorphisms, C1725T (Yamakawa-Kobayashi et al, 1993) and C1773T (Leitersdorf & Hobbs, 1987), were analysed by the designed ‘forced’ MseI assay and the natural HincII assay (Leitersdorf & Hobbs, 1987) and the two individuals were found to share the same genotype, C/C at 1725 and T/T at 1773. Thus the extra SSCP band, marked by an arrow, may be due to the presence of an additional sequence variation in exon 12 present in the affected son and absent in the mother. Sequencing analysis (Fig 5.8c) revealed a C>T nucleotide substitution at 1823 in II.2, which I.2 did not carry. This base change was predicted to result in a previously reported mutation, a leucine for a proline substitution at codon 587. Although there is no formal proof that this substitution is FH-causing there are three lines of evidence to suggest its pathogenicity. The C1823T nucleotide substitution was not observed in 100 normal individuals and has been mutation observed in another FH subject. Identical amino acid substitutions have been found to cause FH (FH website) and proline 587, in repeat C, is conserved across the following species; human (Yamamoto et al, 1984), rabbit (Yamamoto et al, 1986), rat (Lee et al, 1989), Xenopus laevis I & II (Mehta et al, 1991), hamster (Bishop et al, 1992), mouse (Hoffer et al, 1993) and banded cat shark (Mehta et al, 1996) indicating its functional importance. The hypercholesterolaemic phenotype was predicted to have been inherited from his father (despite the mother having a high cholesterol level) and the mother would therefore not be expected to carry the mutation. Thus the usefulness of the polymorphic assays in combination with the mutation screening helped to identify the pathogenic mutation in this family. A sample was later received from the proband’s sibling (II.1) who was confirmed to carry the PS87L mutation, thus the mutation co-segregated with the hyperlipidaemia in the family. Recently cord blood was received from the baby of II.1, who was found to be negative for the PS87L mutation.
Fig 5.4: Case 4 - D200G mutation. D200G in exon 4 of LDLR co-segregated with the FH phenotype, indicated by the filled symbols. The highest untreated total cholesterol (mmol/l), the presence or absence of TX in adults and the age of referral for molecular analysis are shown under the pedigree symbols. NT - no cholesterol test results available. II.2, II.3 and II.4 were referred together. A MspI restriction site was introduced into the mutant allele. PCR digest products were visualised on an EtBr stained 3% agarose gel. Each family member is directly above the respective lane on the gel. Lane 1 - 50bp size marker, lanes 2, 3, 4 & 5 - D200G, lane 6 - negative for D200G, lane 7 - D200G and lane 8 - blank control. III.1 was shown to carry the D200G mutation.
Fig 5.5: Case 5: dA at 118 mutation. Novel one base deletion of nucleotide A118 in exon 2 of LDLR co-segregated with the FH phenotype (filled symbols). Samples from I.1, II.2, II.3 and III.1 were referred for mutation analysis at the same time. The highest untreated total cholesterol (mmol/l) (NT - cholesterol not tested), the presence of TX in the adults (NT - TX not checked in paediatric cases) and the age of referral for molecular analysis are stated under the pedigree symbols. The deletion was detected by the loss of an Nsil restriction enzyme site in the mutant allele. PCR digest products were visualised on an EtBr stained 3% agarose gel. Each family member is directly above the respective lane on the gel. Lane 1 - 50bp size marker, lanes 3, 4, 5, 7, 8, 9 & 10 were heterozygous for dA at 118 whilst lanes 2 & 6 were negative for dA at 118, lane 11 - blank control. III.4 (lane 10) was found to carry the deletion and thus has FH.
Fig 5.6: Case 6 - E80K mutation. E80K in exon 3 of LDLR co-segregated with hyperlipidaemia shown by the filled symbols. The proband III.1 is marked by an arrow, the highest untreated total cholesterol (mmol/l) (NT - cholesterol no tested), the presence or absence of TX in adults and the age of referral for molecular analysis are shown under the pedigree symbols. A buccal sample was analysed for III.1. The mutation was detected by a 'forced' PCR digest where a TaqI restriction enzyme site was introduced into the normal allele. Fragments were separated on a 10% polyacrylamide gel and visualised by silver staining. Each family member is directly above the respective lane on the gel. Lane 1 - 50bp size marker, lanes 3, 4, 5, 7 & 8 - E80K, lane 2, 6 & 9 - negative for E80K, lane10 - blank control and lane 11 - 10bp size marker. M - E80K mutant allele fragment, N - normal allele fragment, PD - primer-dimer artefact.
Fig 5.7: Case 7: D206E mutation. D206E of exon 4 of LDLR was found in proband III.11, marked by an arrow. Filled symbols indicated hypercholesterolaemia and ? indicated no cholesterol results available. Family members sent buccal samples for D206E mutation testing. The pedigree was received later, thus some members were tested but only had a population risk of inheriting an LDLR mutation. M/N - heterozygous for D206E, N/N - negative for D206E, ** individuals with a 50% chance of carrying D206E (FH), * individuals with a population risk of FH (1/500) and and even lower risk of carrying D206E. Risks were determined assuming I.3 carried the D206E mutation as she was known to have CHD.
Fig 5.8: Case 8 - Molecular analysis of LDLR.
A: Filled symbols represent subjects with hyperlipidaemia and the proband is marked by an arrow. Untreated cholesterol levels (mmol/l), clinical history and age of referral for molecular analysis are shown under the pedigree symbols. Mutation details (M - P587L, N - negative for P587L) and the exon 12 polymorphism genotypes, C1725T ('forced' Msel assay) and C1773T (HincII assay), are represented on the pedigree.
B: SSCP gel of exon 12 with the II.2 in lane 16 and I.2 in lane 22. The SSCP band suggestive of an additional sequence variation is shown by the arrow.
C: Two ABI electrophoregrams of part of exon 12 show the presence and absence of the P587L mutation in the son (II.2) and mother (I.2) respectively. The P587L mutation is due to a C>T substitution at 1823 and is indicated as an N on the sequence.
5.3.9. Case 9 - Homozygous for P664L in exon 14 (Fig 5.9)

Three children in this family attended a lipid clinic on a regular basis. At the time of referral for molecular analysis the two eldest children were currently 12 and 13-years old with total cholesterol levels of 22.4mmol/l and 19.8mmol/l respectively, which are in the range of homozygous FH. They currently undergo biweekly LDL-apheresis. The parents have cholesterol levels in the heterozygous FH range and were later found to be first cousins. The third child, referred at the age of 2, had a total cholesterol level of 7.6mmol/l which is above the 95th percentile for age (Sporik et al, 1991) (Fig 5.9a). The requests was to determine if II.3 was a heterozygote with high cholesterol or a homozygote with low cholesterol.

FH homozygotes occur at a frequency of 1 in a million. Compound heterozygotes (two different LDLR mutations) are rarer than true homozygotes (two copies of one LDLR mutation) which usually arise from consanguineous relationships or in populations where a founder gene effect is observed. No family history was provided by the clinician except the family were of Asian background suggesting that the relationship may a consanguineous one so the presence of two copies of one mutation was expected in the homozygous individuals. LDLR flanking markers (D19S394 and D19S221) were analysed in the family and the haplotype associated with FH was deduced (Fig 5.9a). As these markers have high heterozygosity, any individual homozygous at this haplotype is likely to be a true homozygote whilst two haplotypes indicates compound heterozygosity. The two homozygotes carried two copies of this haplotype, thus true FH homozygotes, whilst the third child only had one copy, so she was a heterozygote with high cholesterol. It was assumed that the (GAAG)n repeat marker had probably slipped one repeat from 259 to 255 ABI mobility units in II.1 (marked in blue on Fig 5.9a). One homozygote and one heterozygote were analysed for mutations in LDLR by SSCP analysis. No abnormal band shifts were present in the homozygote but a heteroduplex was seen in exon 14 of the heterozygote (Fig 5.9b). The SSCP was recognisable and predicted to be due to the P664L mutation which can be tested by a natural PstI PCR assay. All five family members were tested (Fig 5.9c). The two homozygotes carry two copies whilst the two parents and their third child carry one copy.

On reflection no SSCP shifts were observed in the single strands of exon 14. Generally two bands are observed and if a mutation is present one single strand band is shifted. Homozygotes are predicted to have no normal single strands but two mutant single strands but this did not seem to be the case. SSCP analysis is very unpredictable.
and the molecular analysis of this case provided a valuable lesson in the analysis of FH homozygotes and indicates that a homozygous sample should be accompanied by a heterozygous sample for analysis. A mutation was identified showing that a defect in LDLR was responsible for the FH characteristics and this provided an unequivocal diagnosis of FH in the family. Based on the independent results determined by the two flanking LDLR markers and the mutation analysis, II.3 was deduced to be a FH heterozygote. Prenatal diagnosis would be possible if requested, by using these flanking LDLR markers or unequivocally by P664L mutation analysis, but this is unlikely due to the ages of the parents.

In the future, a step prior to mutation analysis may help predict the status of an FH homozygote. True homozygotes or compound heterozygotes can be distinguished by the analysis of flanking LDLR markers in the patient (and family members if available). If the patient is found to be a true homozygote, SSCP analysis should be carried out on the sample mixed with normal control DNA, so as to induce the formation of heteroduplexes.

5.3.10. Case 10 - T705I in exon 15 (Fig 5.10)

In the family shown (Fig 5.10), the index patient, III.1, aged 5, was referred from GOSH. She had an untreated total cholesterol of 4.2mmol/l which is at the 40th percentile for age (Sporik et al, 1991), although she was taking a lipid restricted diet. This value is within the normal range suggesting she may not have FH, although there is considerable overlap in cholesterol levels between normal and affected children (Leonard et al, 1977; Kwiterovich et al, 1993) and in some cases elevated levels may develop later in life (Kessling et al, 1990). Her mother, who is 34-years, presented with hypercholesterolaemia (8mmol/l) and xanthelasma and her grandfather had a MI at 39-years and died at 55. A clinical diagnosis of 'possible' FH was given and the index case had a 50% chance of inheriting the defective allele.

In the index case (Fig 5.10) no mutation was identified by SSCP analysis and UPQFM-PCR, in LDLR (point or major rearrangement) or in APOB by direct assays. Mutation screening was then carried out on a sample from her hypercholesterolaemic mother, in case a mutation was present in the mother which the daughter had not inherited. Two SSCP band shifts were identified (excluding band shifts caused by known polymorphisms) and characterised to be due to the C>T transition at nucleotide 2177 in exon 15 and T>C at 1061-8 in intron 7. The variants were confirmed to be present in the mother and absent in the daughter by the respective digests, NsiI and EarI (Fig
5.10), suggesting the daughter was at low risk of FH. This conclusion depends on the strength of the inference that 1705 and/or 1061-8C are pathogenic or whether the mother may be a carrier for another unidentified mutation that had been inherited by the daughter. In the light of these findings the effect of the 1705 and 1061-8C variants needed to be assessed.

To explore the functional significance of the substitution a high throughput genotyping MADGE method was developed and used to screen 2287 healthy Caucasian men from the Northwick Park Heart Study (NPHS-II) (Meade et al, 1986). As shown in Table 5.1, 30 carriers were found, 28 of which also had the intron 7 variant 1061-8C. Thus the carrier frequency of the 1705 variant was 1.3%. The intron 7 variant was analysed in 200 men from the NPHS-II study and no additional intron-7 carriers were found. Therefore the carrier frequency of the intron 7 variant was calculated to be ~1.2%. No statistically significant differences were observed between the mean total cholesterol and triglyceride levels of the 1705 carriers and the non-carriers (Table 5.1). Therefore the two variants, 1705 or 1061-8C are unlikely to be affecting the function of the LDL-receptor and the detection in subjects with FH is a consequence of their allele frequency in the general population.

Table 5.1: Characteristics (mean ± SD) of 1705 carriers and non-carriers in 2287 ‘healthy’ Caucasian men from NPHS-II.

<table>
<thead>
<tr>
<th>T705I group</th>
<th>TT</th>
<th>TI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.1±0.15</td>
<td>55.0±1.30</td>
<td>0.09</td>
</tr>
<tr>
<td>(n=2256)</td>
<td>(n=30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.4±0.15</td>
<td>26.8±1.20</td>
<td>0.56</td>
</tr>
<tr>
<td>(n=2255)</td>
<td>(n=30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.75±1.02</td>
<td>5.66±1.08</td>
<td>0.60</td>
</tr>
<tr>
<td>(n=2241)</td>
<td>(n=30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.81*</td>
<td>1.62*</td>
<td>0.27</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(1.77-1.85)</td>
<td>(1.35-1.96)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=2242)</td>
<td>(n=30)</td>
<td></td>
</tr>
</tbody>
</table>

* Geometric means and 95% confidence limits as triglycerides were loge x for the analysis.

Published data are inconsistent as to whether the exon 15 C>T substitution at 2177, which alters the amino acid at codon 705 from a threonine to a isoleucine, is affecting LDL-receptor function. In a study from the Netherlands 100 normolipidaemic controls
were screened by DGGE and sequencing and the 1705 variant was found in two individuals, one heterozygous carrier and one homozygote (Lombardi et al, 1997). Therefore the carrier frequency of the 1705 allele was 2% which is very similar to the estimate in the larger group of UK men, 1.3%. All but two 1705 carriers from the NPHS-II group carried the 1061-8C variation in intron 7, which has been associated with this exon 15 variation (Jensen et al, 1996). The 1061-8C variant was screened in 200 men from the NPSH-II group and the variant was only detected in the 1705 carriers thus the carrier frequency was estimated to be ~1.2% in the general population.

Taken together these data strongly suggest that the 1705 variant is not having a major effect on LDL-receptor function. It is the second ‘non-functional’ variant described to date, with only the A370T being previously known. T370 occurs at a frequency of 6% in the UK (Taylor et al, 1988) and is associated with, at most, only a modest effect on plasma lipid levels. Cell studies have not detected a significant impairment of LDL-receptor function of the T370 substitution (Gudnason et al, 1995b).

The T705I substitution in the coding region of LDLR was thought to be one of the defective alleles in a compound heterozygote whilst the second mutation remained undetected (Hobbs et al, 1992). It is reasonable to assume from the Dutch and our data that both defects in this homozygote had not been identified. The second case occurred in a 40-year old man who had a very high total cholesterol of 17.78mmol/l (Lombardi et al, 1995 & 1997). A splice site mutation (313+1G>A) was inherited from his hypercholesterolaemic mother whilst the 1705 variant came from his normolipidaemic father. One possible explanation is that the 1705 variant is only expressed when another LDLR defect is present, as shown by the double mutants N543H and 2393del9 (Jensen et al, 1997), but two of the probands younger siblings also had slightly elevated cholesterol yet did not share an LDLR haplotype with their normocholesterolaemic sibling, i.e. they did not carry either LDLR substitution. Thus another variation in LDLR or in another gene may be responsible for the hypercholesterolaemia in these two siblings and would also explain the high cholesterol level in the proband. In another report (Arca et al, 1998) the 1705 substitution co-segregated with the hypercholesterolaemia phenotype, but again this could be explained by the presence of an unidentified mutation which may or may not be in linkage disequilibrium with the 1705 variation.

Exon 15 consists of 171 nucleotides which encodes 57 amino acids, of which 18 are threonine or serine residues (Yamamoto et al, 1984; Davis et al, 1986) and most of the
O-linked sugars of the LDL-receptor are attached to these threonine and serine residues (Davis et al, 1986). A similar region is also conserved in the LDL-receptors of other mammals (Davis et al, 1986). The functional role of this domain was investigated using site directed mutagenesis, where a portion of exon 15 was deleted and then expressed by transfection into fibroblast cell lines (Davis et al, 1986). The mutated cDNA coded for a receptor protein which was functionally indistinguishable from the normal receptor. Individuals carrying a similar natural deletion of exon 15 (FH-Espoo) have LDL-c levels which are relatively low and a mild form of FH (Koivisto et al, 1993). Thus a major rearrangement is actually a mild mutation, suggesting that mutations in this region may only have a mild effect on receptor function and therefore on lipid levels. Only six point mutations or single base deletions have been described in exon 15 (FH website); two point mutations resulting in a stop codon, a minor deletion predicted to result in frameshift and a splice donor site mutation, and all of these are highly likely to be pathogenic. In addition to the T705I substitution, two missense mutations have been reported, T721I (Nauck et al, 1997) and R723Q (Sun et al, 1997 & 1998). LDL-binding studies showed that the Q723 mutation had 70% of normal activity and is therefore a mild LDLR mutation. No details are known about the T721 mutation. It may be functional in that an O-linked sugar may attach at this site but it may be non-pathogenic as with T705 which involves the same amino acid substitution.

Thus from the available data on the reported exon 15 mutations, missense mutations and FH-Espoo, they appear to have a mild effect on the LDL-receptor protein. Ideally, cellular studies should be carried out on all novel missense mutations but this is often not feasible. However at the very least 100 normal individuals should be screened for any novel missense mutation to determine frequency and if any carriers detected, association studies should be performed. In particular, care should be taken in reporting missense mutations identified in exon 15 of LDLR as FH-causing, as they appear to have a modest effect on LDL-receptor function. In the family studied a report was issued stating that two LDLR variants were identified in the mother but no mutation was found in the mother or daughter.
Fig 5.9: Case 9 - P664L mutation co-segregated with the FH phenotype.
A: The black and grey symbols indicate a clinical diagnosis of homozygous and heterozygous FH respectively and the highest untreated total cholesterol values (mmol/l) are shown under the pedigree symbols. Microsatellite markers flanking LDLR are shown on the pedigree and the marriage to be a consanguineous relationship, thus II.1 and II.2 are true homozygotes and II.3 a heterozygote.
B: SSCP analysis of exon 14 of LDLR (ss - single strands, ds - double strands). P664L heteroduplex (h) was observed but no shifts were observed in the single strands (lanes 2, 3, 5, 6, 7). The heteroduplex was only observed in the homozygotes when mixed with normal DNA (lanes 2 & 5). Family members are stated above the gel.
C: P664L of exon 14 of LDLR, detectable by the introduction of a PstI restriction site in the mutant allele (M). Fragments were separated on a 4% (3:1 NuSieve) agarose gel, stained with EtBr. Family members are stated above the gel. Lanes 1 - 100bp size marker, lane 2 - uncut PCR, lane 3 & 5 - II.1 & II.2 P664L homozygotes, lanes 4, 6 & 7 - I.1, I.2 & II.3 P664L heterozygotes, lane 8 - P664L negative, lane 9 - P664L positive control & lane 10 - blank control. Molecular analysis showed III.3 to be an FH heterozygote. M - mutant allele fragment, N- normal allele fragment.
Fig 5.10: Case 10: T705I and 1061-8T>C variants. No mutation was found in LDLR or APOB but rare variants in LDLR exon 15 (1705) and intron 7 (1061-8C) were found in II.2 but not in III.1, thus not co-segregating with the hyperlipidaemia, shown by the filled symbols. A sample from the father, II.1 and grandfather, I.1, was not available. The proband is marked by an arrow, the highest untreated total cholesterol (mmol/l) and the age of referral for molecular analysis are shown under the pedigree symbols.

A: T705I substitution was detected by an 'forced' PCR assay, where an Nsil restriction site is introduced into the rare T allele (1705). Digested fragments were electrophoresed on a 4% (3:1) NuSieve agarose gel and stained with EtBr. Lane 1 - 50bp size marker, lane 2 - III.1: normal T705, lane 3 - II.2: 1705 carrier.

B: 1061-8T>C variation was detected by a natural PCR digest assay where the rare variant C loses an Earl site, although there is an constant digestion site. Digested fragments were electrophoresed on a 10% acrylamide gel and stained with EtBr. Lane 1 - 1kb size marker, lane 2 - homozygous 1061-8T, lane 3 - III.1: homozygous 1061-8T, lane 4 - II.2: 1061-8T>C carrier and lane 5 - 1061-8T>C carrier.
5.3.11. Case 11 - R3500Q mutation (Fig 5.11)

The paediatric proband had a total cholesterol level of 6.7mmol/l which is above the 95th percentile for a boy aged 5. There was a family history of hypercholesterolaemia, his mother and grandfather had died of an MI aged 28 and 55-years respectively. The proband was screened for mutations in LDLR and APOB. No mutation in LDLR was identified but he was found to be heterozygous for the R3500Q mutation in APOB and this was reported. Subsequently his 27-year old aunt was referred for genetic testing and was found to not carry the R3500Q mutation. The 18 exons and the promoter of LDLR were screened in the aunt and no mutations were detected. Thus the R3500Q mutation did not co-segregate with the hypercholesterolaemia in this family and this caused a diagnostic dilemma which could be explained in several ways:

1. II.2 carried an LDLR or a 'third' gene mutation and an APOB mutation, her son, III.1 had only inherited the R3500Q mutation and her sister, II.3 only carried the unidentified LDLR or 'third' gene mutation;

2. The R3500Q mutation was inherited from the proband's father, II.1, who was unavailable and LDLR defect in the maternal side had not been identified;

3. The clinical diagnosis in the proband's mother and her sister was not FH and the defect was due to another gene or factor.

Whatever has occurred, an APOB mutation was identified in the proband which is likely to result in raised cholesterol at some point in his life but it may or may not result in CHD (Tylbaerg-Hansen & Humphries, 1992). He may also carry another mutation or CHD factor but this has not been identified or studied.

In this family FH genetic testing was able to determine one cause of the hyperlipidaemia in the proband but his risk may be higher as the R3500Q mutation did not co-segregate with the hyperlipidaemia in the aunt. Family members are often not available, in paediatric cases a sample is often available from the hypercholesterolaemic parent but it is rare in adult cases, therefore co-segregation of the mutation with the FH phenotype is often not possible. Proof of co-segregation may be particularly important when APOB mutations are identified in children who have borderline cholesterol levels, as the mutation in APOB may have been inherited from the parent who has normal or slightly elevated cholesterol rather than from the hypercholesterolaemic parent.

It is now evident that the R3500Q mutation has a significant effect on raising LDL-c levels, both in children, adolescents and adults of both sexes but it is variable in expression (reviewed by Tybjaerg-Hansen & Humphries, 1992). In the first study (Innearity et al, 1990), 41 R3500Q heterozygotes were identified from four cities across
Canada and the USA, with only one proband having corneal arcus and TX and this subject was from Montreal where the mean cholesterol is higher in the normal population than in the three other US cities. In contrast eight of ten R3500Q carriers from a hyperlipidaemic population from the UK and Scandinavia had corneal arcus and xanthomas (Tybjaerg-Hansen et al, 1990; Corsini et al, 1991) and they identified an FDB proband whose affected father had an MI at age 38. The presence of TX and premature CHD was also found to occur in 26% and 22% respectively in a larger study of 54 adult FDB individuals (Rauh et al, 1992).

From the FDB families reported in detail, the penetrance of the R3500Q mutation is comparable with that of LDLR mutations. In the study reported by Innearity et al, 1990, 28/30 heterozygous relatives of eight probands had hypercholesterolaemia, thus a penetrance of 93%. In the three families reported by Schuster et al, 1990, 9/10 heterozygous relatives were found to be hypercholesterolaemic so a penetrance of 90% and in the study by Myant et al, 1991, 15/18 heterozygous relatives of five probands had cholesterol levels above the 95th percentile, a penetrance of 83%. It has been observed that cholesterol levels in FDB homozygotes are similar to FDB heterozygotes unlike the major differences between FH heterozygotes and homozygotes (Funcke et al, 1992; Myant, 1993). A similar situation was also found in individuals who had FH and FDB where their cholesterol levels were in the range found in FH heterozygotes (Rauh et al, 1991), although cholesterol levels and clinical features in one family who carried the R3500Q mutation, the D206E and V408M LDLR mutations, were found to be intermediate in severity between heterozygous and homozygous FH (Rubinsztein et al, 1993). Thus it is clear that FDB can give rise to premature coronary atherosclerosis but from the described studies (Innearity et al, 1990; Schuster et al, 1990; Myant et al, 1991) and more recent studies (Tybjaerg-Hansen & Humphries, 1992; Gallagher & Myant, 1993; Miserez & Keller, 1995; Hansen et al, 1997) FDB has been shown to be more variable in expression than the classic FH.

5.3.12. Case 12 - R3531C mutation (Fig 5.12)

Two siblings, III.1 and III.2, were attending a paediatric clinic for high levels of cholesterol, approximately 5.7mmol/l at the age of ten and nine respectively. Hypercholesterolaemia was inherited from the maternal line and was present in their mother, their two uncles and their grandmother (Fig 5.12). No mutation was identified in LDLR but a rare C to T transition at nucleotide 10800 (R3531C) in APOB was found in the two siblings. The R3531C mutation was subsequently tested in their relatives and it was not present in any individual from their maternal side but it was inherited
from their father. The mother (II.2) was screened for mutations in LDLR in case the children did not inherit the mutation which the mother carries, but no mutation was identified. Microsatellite markers flanking LDLR were analysed in the family and the genotypes are shown in Fig 5.12. A common LDLR haplotype co-segregated with all hypercholesterolaemic individuals from the mother's family, herself, her two brothers and her mother which may be explained by an unidentified defect in LDLR or purely by the probability of chance (LOD = 1.02) (Fig 5.12). The hypercholesterolaemia in the children may be due to the R3531C mutation and the hypercholesterolaemia present in the maternal line may be due to another genetic factor which is not expressed until older. II.4 had a different D19S221 genotype that was not present in his father or mother. This may have been due to slippage of the dinucleotide marker by two repeat units (Scholotterer C & Tautz D, 1992), non-paternity, although there was a common D19S394 haplotype with father and son or a recombination event in I.1, although the FH phenotype co-segregated with the marked haplotype. Therefore the two young children should be closely monitored for the effects of the R3531C mutation but also in case a hyperlipidaemic phenotype starts to be expressed due to a factor inherited from their mother.

The R3531C mutation was originally found in a female of Celtic and native American ancestry (Pullinger et al, 1995) who had a total cholesterol level of 8.87mmol/l and pronounced peripheral vascular disease. 1560 individuals were subsequently screened and one further unrelated 59-year old Italian male was found to have the R3531C mutation (Pullinger et al, 1995). From these two families eight individuals were found to carry this mutation and the age and sex adjusted LDL-c was 4.4±0.3mmol/l which was 1.16mmol/l higher than unaffected family members. As shown in Table 5.1 the R3531C mutation was found to have an LDL-binding affinity of ~63% of the normal whilst the R3500Q mutation had a binding affinity of ~36% (Pullinger et al, 1995). Recently the same group reported findings of nine R3531C probands and 15 affected relatives from Glasgow and San Francisco (Pullinger et al, 1999). LDL-c from R3531C heterozygotes, compared to normal LDL-c, had 49% of the binding affinity which was slightly lower but similar to their previous findings (Table 5.1).

A MADGE form of ASO analysis was applied to screen 791 FH samples for the R3531C mutation. No R3531C carriers were found, thus the mutation is very rare in the UK population.

The R3531C mutation is suggested to result in a milder phenotype than R3500Q.
carriers and even milder than LDLR mutation carriers (Pullinger et al, 1999) but variable expression, modulated by environmental and genetic factors was observed in these families (Pullinger et al, 1995, 1999). Other groups have now identified this mutation in patients with CHD (Ludwig et al, 1997), with a history of atherosclerosis (Rabes et al, 1997) and with severe hypercholesterolaemia (Wenham et al, 1997).

Table 5.1: Dual-label fibroblast binding assay in normals & R3531C mutation carriers.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number of people tested</th>
<th>Total cholesterol*</th>
<th>% LDL-binding affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls (Study 1)</td>
<td>11</td>
<td>4.40±0.28</td>
<td>96</td>
</tr>
<tr>
<td>Normal controls (Study 2)</td>
<td>7</td>
<td>5.23±0.26</td>
<td>104 (n=7)</td>
</tr>
<tr>
<td>Heterozygotes R3531C (Study 1)</td>
<td>24</td>
<td>6.21±0.36</td>
<td>60 (n=4)</td>
</tr>
<tr>
<td>Heterozygotes R3531C (Study 2)</td>
<td>4</td>
<td>5.28±0.26</td>
<td>49</td>
</tr>
<tr>
<td>Unaffected relatives R3531</td>
<td>8</td>
<td>4.78±0.31</td>
<td>87</td>
</tr>
<tr>
<td>(Study 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaffected relatives R3531</td>
<td>18</td>
<td>4.69±0.16</td>
<td></td>
</tr>
<tr>
<td>(Study 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygotes R3500Q (Study 1)</td>
<td>6</td>
<td>6.85±0.91</td>
<td>34</td>
</tr>
<tr>
<td>Heterozygotes R3500Q (Study 2)</td>
<td>3</td>
<td></td>
<td>37 (n=3)</td>
</tr>
</tbody>
</table>

* Plasma cholesterol values (mmol/l) ± SEM were adjusted for age (to 25 years) and gender. Table determined from data in Pullinger et al, 1995 & 1999.

The combined data (Pullinger et al, 1995 & 1999) identified 11 cases out of 4130 hyperlipidaemic individuals, a frequency of one in 375. Four haplotypes have been associated with the R3531C mutation, one which appears to be of Scottish, Irish origin (Pullinger et al, 1999). This evidence, given that recombination events in this region of APOB have not been detectable (Dunning et al, 1992), points to multiple independent R3531C mutations.
Fig 5.11: Case 11 - R3500Q mutation of APOB. Filled symbols represent subjects with hyperlipidaemia and the proband is shown by an arrow. Untreated total cholesterol levels (mmol/l), age of death from MI and age of referral for genetic analyses is stated under the pedigree symbols. The mutation was detected by two 'forced' PCR digests with the restriction enzymes, Sau96I and Scal. Fragments were separated on a 10% acrylamide gel stained with EtBr. The order of samples is shown above the gel. Lane 1 - 50bp size marker and lane 12 - 10bp size marker. Lanes 2-6 - 'forced' Sau 96I assay: lane 2 - II.3 - normal for R3500Q, lanes 3 & 4 - III.1 - R3500Q, lane 5 - R3500Q positive and lane 6 - blank control. Lanes 7-11 - 'forced' Scal assay; lane 7 - II.3 - normal for R3500Q, lanes 8 & 9 - III.1 - R3500Q, lane 10 - R3500Q positive and lane 11 - blank control. M - mutant allele, N - normal allele.
Fig 5.12: Case 12 - R3531C mutation of APOB. Filled symbols represent subjects with hyperlipidaemia and the probands are marked by an arrow. Untreated total cholesterol levels (mmol/l) and age of referral for genetic analyses are stated under the pedigree symbols. An Nsil restriction site was introduced into the variant allele and the fragments were separated on an EtBr stained 4% (3:1) NuSieve gel. Each family member is directly above the respective lane on the gel. Lane 1 - 100bp size marker, lane 2 - C3531 variant, lane 3 - C3531, lane 4 - C3531, lane 5 - R3531, lane 6 - R3531, lane 7 - R3531, lane 8 - no R3531C variant and lane 9 - blank control. The R3531C variant did not cosegregate with the hyperlipidaemia. No mutation was identified in LDLR. Microsatellites flanking LDLR were analysed and the various haplotypes are shown in different colours with inferred haplotypes in brackets. An LDLR haplotype co-segregated with the hyperlipidaemia (marked in red). The D19S221 genotype underlined in II.4 indicates a different allele which is explained in the text.
5.3.13 Case 13 - LDLR microsatellite analysis (Fig 5.13)

Hypercholesterolaemia and TX were present in several members of the family studied in case 13 (Fig 5.13).

Fig 5.13: Case 13 - Microsatellite analysis of LDLR.

Filled symbols represent subjects with the hyperlipidaemia. The highest untreated total cholesterol (mmol/l), the presence or absence of TX and the age of referral for molecular analysis are stated under the pedigree symbols. No mutation was identified in the family, thus microsatellites flanking LDLR and LDLR intragenic polymorphisms were analysed. The haplotype, indicated in red, co-segregated with the FH phenotype in this family. Microsatellite markers around the CFTR locus confirmed relationships (data not shown).

All tested negative for the three APOB mutations, R3500Q, R3531C and R3500W, and no LDLR mutation was identified, although only exons 3, 5, 8, 14 and 17 have been analysed for major rearrangements. Thus at the present time no further mutation
analysis can be carried out in this family, but linkage analysis was possible as there were samples from several members and from two generations (Day et al. 1997a, Haddad et al., 1997). Two microsatellite markers were analysed, D19S394 [(GAAG)n] which is approximately 150kb from the 5’ end of LDLR and D19S221 [(CA)n] which is 1Mb from the 3’ end of LDLR. Genotypes for three intragenic polymorphisms, the C81T in exon 2, the G1060-10C in exon 7 and the G1413A in exon 10 were also determined, primarily to eliminate polymorphic band shifts found by SSCP analysis. A haplotype co-segregated with the hypercholesterolaemic phenotype (marked in red on Fig 5.13) (LOD = 0.9). III.2 had elevated cholesterol but no TX and there was doubt about whether the patient had the same phenotype as the other family members. If an unidentified mutation in LDLR causes the hypercholesterolaemia phenotype, III.2 does not have FH since he did not carry the affected haplotype.

5.3.14. Case 14 - LDLR microsatellite analysis (Fig 5.14)

Two siblings presented at the GOSH paediatric lipid clinic with elevated cholesterol above the 95th percentile for age and sex (Sporik et al., 1991). II.1 had an initial value of 7.0mmol/l and II.2 had a cholesterol value of 8.0mmol/l indicating they had the same phenotype which was inherited from their father (Fig 5.14). The children went on a low cholesterol diet and returned six months later for cholesterol tests, 5.0 and 7.0mmol/l respectively. A third reading, a year after the initial consultation their cholesterol levels were 4.0 (25th percentile) and 9.1mmol/l (above 95th percentile) respectively. At the 6 month visit samples were taken from the two siblings for genetic testing. LDLR was screened and the R3500Q mutation in APOB was tested. The only abnormal band shift detected by SSCP analysis was in exon 8 which also contains a common polymorphism at nucleotide 1171 (A/G). The band shift was investigated for the polymorphism by a StuI digest. One sibling (II.1) was homozygous for the StuI polymorphism and the other (II.2) was homozygous for the absence of the StuI polymorphism indicating there was not a common chromosome 19 between them. Microsatellite studies, D19S394 and D19S221, were then analysed to confirm this result and indeed the two siblings did not share a chromosome 19 and thus LDLR (Fig 5.14). Thus if LDLR is responsible for the hypercholesterolaemia in this family then one child has FH and one does not. The reduction in total cholesterol in II.1 (above 95th to 25th percentile for age) (Sporik et al., 1991), purely by diet, indicates that she does not have FH and that her elevated cholesterol was from a high fat diet. Her brother’s cholesterol has remained elevated (above 95th percentile for age) (Sporik et al., 1991) and he may have FH. Thus LDLR flanking markers were unexpectedly helpful in this family despite only having samples from two siblings.
Fig 5.14: Case 14 - LDLR microsatellite analysis.

Filled symbols represent subjects with hyperlipidaemia and three total cholesterol readings, taken over one year and the age of referral for genetic analysis are stated under the pedigree symbols. The first readings were untreated, the second and third readings were after 6 and 12 months on a low fat diet. No mutation was identified in the family but microsatellite analysis was studied despite only having the two siblings. The two siblings did not share an LDLR marker between them thus it was predicted that II.2 was affected and II.1 was unaffected assuming LDLR is responsible for the FH phenotype in the father.

5.4. Conclusion

The value of genetic testing in FH is shown in cases 1, 3, 5, 8, 9 and 14 where children had normal or slightly elevated cholesterol levels or where no biochemical tests were analysed. An unequivocal diagnosis was also made in a 32-year old woman (case 4), who unexpectedly tested positive for a pathogenic sequence variant despite a normal cholesterol level. Unequivocal results were given to many individuals and in the future more relatives of these probands can be offered genetic testing. These results enable patients to make informed decisions with regard to their future.

Acknowledgements: DNA was isolated by many people but particularly Lighta Godhini, Paul Cummings and Jane Heath. Genotyping the control group from the NPHSII was carried out by Ros Whittall. Statistical analysis of the T705I data was carried out by Sarah Bujac.
Chapter 6: Influence of LDLR & APOB mutations on the cholesterol-lowering response of the HMG-CoA reductase inhibitor simvastatin in patients with heterozygous FH

Summary
In a genetically heterogeneous group of 109 patients with a clinical diagnosis of heterozygous FH, the influence of gender, APOE genotype and the type of molecular defect in LDLR or APOB on the reduction of plasma LDL-c levels to treatment with a HMG-CoA reductase inhibitor (simvastatin) were studied. Response was determined as the percentage fall in LDL-c from untreated levels and as the proportion of patients where LDL-c levels fell below 4.9 or 4.1 mmol/l. Of the patients, 86 individuals had a diagnosis of 'definite' FH (TX+) and these individuals presented with a significantly higher untreated total and LDL-cholesterol compared to the 23 individuals who had a diagnosis of 'probable' FH (TX-) (8.14±0.19 v 6.81±0.25, p=0.001). Overall, simvastatin doses of 10, 20 or 40mg/day resulted in a significant fall of LDL-c of 28%, 39% and 49% but at all doses those with TX had significantly higher LDL-c levels than those without, and significantly fewer TX+ patients achieved LDL-c levels below 4.9 and 4.1 mmol/l than the TX- group (p<0.05 at each dose). In the TX+ group the response to treatment was of similar magnitude in men and women and in patients with different APOE genotypes. In the 'probable' FH probands only three mutations were identified (detection rate 13%), one LDLR and two APOB, a detection rate significantly lower (p=0.02) than in the 'definite' FH probands where 28 mutations were detected (detection rate 37%). In the TX+ patients where no mutation was detected, treatment resulted in a greater proportion achieving LDL-c levels below 4.9 and 4.1 mmol/l compared to those with any LDLR mutation, this difference was close to significance at the 4.9 mmol/l threshold at 10mg/day (41 v 13%, p=0.058). For the 14 patients with an LDLR mutation that was predicted to be 'severe', fewer achieved LDL-c levels below 4.9 or 4.1 mmol/l at each dosage compared to the 16 individuals with 'mild' mutations, and this difference was statistically significant at the maximal dosage of 40mg/day (p=0.018).

Thus although characterisation of the molecular defect in FH patients may not be relevant to their immediate clinical management, those with a particular mutation may need more aggressive lipid lowering treatment to reach LDL-c levels recommended to reduce the risk of CHD.
6.1. Introduction

FH is usually caused by a mutation in LDLR. More than 700 LDLR mutations have been reported (http://www.ucl.ac.uk/fh) and different types of mutation result in different residual functional LDL-receptor activity, varying from <2-55% (Sun et al, 1997). However an LDLR mutation is not found in all patients with a clinical diagnosis of FH (Hobbs et al, 1992; Day et al, 1997b; Sun et al, 1997), and furthermore, in approximately 3% of the UK patients a defect has been detected in the APOB (Tybjaerg-Hansen et al, 1990).

The major medications used in the treatment of FH are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). This class of agents (statins) inhibits the rate-limiting step of cholesterol synthesis in the liver, which as a consequence results in an increase in hepatic LDL-receptor activity and thus a decrease in the level of plasma LDL-c. A wide inter-individual variation in treatment responses to drug treatment in terms of LDL-c -lowering have been observed in a number of studies (Ojala et al, 1990; de Knijff et al, 1990). This variation can be partially explained by various environmental and genetic factors that affect the action of a drug in each individual.

6.1.1. Effect of LDLR or APOB mutations

A differential response to drug therapy between FH patients might occur if the residual receptor activity of the abnormal protein differed as a result of the particular mutation present. For example, carriers of ‘mild’ mutations where residual receptor activity is high (10-15% of normal) may have a greater lipid-lowering response than carriers of ‘severe’ mutations, where residual activity is zero and therefore no useful up-regulation can be obtained by statin therapy.

In FH patients from Israel three common mutations occur; two missense mutations producing defective receptors (D147H and dG197) and a frameshift mutation (C660X), all of which had virtually no residual LDL-receptor activity. The lowest mean baseline and fluvastatin treated LDL-c levels was observed in C660X carriers and the post-treatment LDL-c percentage reduction was significantly greater in these individuals compared to carriers of the two missense mutations (Leitersdorf et al, 1993). An hypothesis has been set out to explain these observations: mutated mRNA that remains stable (mRNA positive) is translated into polypeptides which dysfunction during processing, transport, or at the site of LDL-receptor action. During up-regulation of the expression of the LDL-receptor, these mutant receptor proteins are hypothesised to
interfere with the function of the normal LDL-receptor protein along its intracellular metabolic pathway or its biological action, thus potentially reducing the cholesterol-lowering response to simvastatin therapy (Leitersdorf et al., 1993). Mutations that result in stop codons (mRNA negative) cause the translation of the mRNA to prematurely halt, followed by rapid degradation of the mutated mRNA and the truncated protein. Thus these null mutations cannot interfere with the normal LDL-receptor. It has been proposed that D147H and dG197 mutant receptors interfere with the processing and/or transport of normal protein or that they interact with the normal receptor during treatment of heterozygous individuals, preventing their cholesterol levels from being lowered to the same extent.

A further study investigated the effect of statin treatment in patients with two mutations found at a high frequency in South Africa. In individuals carrying the V408M or D206E mutations, where the residual LDL-receptor activity is reported to be <2% and 20% respectively (Fourie et al., 1988; Leitersdorf et al., 1989a), the pre-treated mean LDL-c levels was (non-significantly) higher in the V408M individuals whilst the post treatment values were identical in patients with either mutations (Jeenah et al., 1993). Thus a substantially greater fall in plasma LDL-c, in response to simvastatin, occurred in V408M carriers (Jeenah et al., 1993).

Two recent papers reported further investigations into the influence of LDLR genotype and on response to drug treatment in heterozygous carriers from heterogeneous populations. In 116 heterozygous FH individuals from the Netherlands, 27 were analysed at the molecular level, giving 13 mRNA positive (RNA detected) mutations and 14 mRNA negative (RNA not detected) mutations. Effects of simvastatin treatment (20mg/day dosage) on the mRNA level were studied in the two groupings of patients rather than by specific mutation (Sijbrands et al., 1998). The magnitude of the response was found to be similar in both groups although the mRNA negative carriers had higher pre-treated LDL-c levels, lower levels of HDL and significantly higher frequency of TX than in the mRNA positive carriers (Sijbrands et al., 1998). In this case there was no evidence for interference of mutant receptor on normal receptor function and similar LDL-c lowering response was seen among carriers of a large variety of both mRNA positive and mRNA negative mutations.

In the second study of 42 heterozygous FH cases (Sun et al., 1998), patients were subdivided according to the type of molecular defect.
1. ‘Severe’ mutations (n=12) predicted to produce little or no functional LDL-receptor protein
2. ‘Mild’ mutations (n=6) mutations in regions of the protein which have been found to be less functionally important
3. Mutations in the EGF precursor-like domain (n=5) and do not result in the accumulation of detectable amounts of the precursor form of the protein
4. APOB R3500Q carriers (n=5)
5. No mutation was identified (n=14).

Patients with ‘severe’ mutations had higher LDL-c before and during treatment with simvastatin and bile acid sequestrants than carriers of ‘mild’ mutations. The maximum inducible LDL-receptor activity in cultured lymphoblasts was found to be inversely correlated with LDL-c before and during treatment in patients with LDLR mutations but not in individuals with no detectable mutation (Sun et al, 1998).

In the late 80’s and early 90’s the LDL-receptor was proposed to function as a dimer although no conclusive evidence was ever found (van Driel et al, 1987; Patel et al, 1993). Van Driel et al, 1987, showed that a proportion of LDL-receptors were close enough in the membrane to be linked by bi-functional cross-linking agents but it was not known if this association played any part in determining the binding properties of the receptor. LDL-binding appeared to have been affected by some degree of aggregation of receptors in the cell membrane in two instances (Gavignon et al, 1988; Knight et al, 1989). Cross-linking of the LDL-receptor was further investigated and it was shown to occur at 4°C but less efficiently at 37°C (Patel et al, 1993). Suggestions were that LDL-binding can be modulated by changes in the alignment of receptors on the cell surface and thus it is possible for mutations in regions not normally involved in binding to affect binding by disrupting the normal interaction between receptors (Patel et al, 1993). No further work on the dimer hypothesis has been reported.

Homozygous FH individuals are also treated with statins although theoretically they should not be able to show a lipid-lowering response. Successful treatment of homozygous FH with statins was initially shown in patients with receptor-defective mutations and the cholesterol-lowering effect of the drug was ascribed to the residual LDL-receptor activity (Bakker et al, 1991; Kriek et al, 1992; Gudnason et al, 1995a). However in a recent study an FH homozygote had a reduction in serum cholesterol by 30% with simvastatin and by a further 11% with probucol and nicotinic acid (Feyer et al, 1993). The patient in question had two copies of a mutation in exon 11 at codon 540 resulting in a premature stop codon. No detectable LDL-receptor activity was
found and thus the reduction of LDL-c could not have occurred as a result of increased LDL-receptor mediated clearance. Suggestions were that either the synthetic rate of LDL had decreased or that there was increased clearance through non-LDL-receptor mediated pathways (Feyer et al, 1993).

6.1.2. Other factors influencing drug treatment in FH individuals
The site of action for statins is the LDL-receptor so any factor which exerts its effect on this may influence the drug effect, such as APOE genotype (Ojala et al, 1991; de Knijff et al, 1990; O'Malley & Illingworth, 1990) or LDLR mutation (Leitersdorf et al, 1993; Jeenah et al, 1993; Karyam et al, 1994; Sun et al, 1998). Many studies have investigated these factors whilst trying to maintain confounding factors such as diet and other environment as constant as possible.

6.1.2.1. APOE genotype
The polymorphism at the apolipoprotein E locus is one of the major factors affecting the variability of serum lipid levels in normal populations (Davignon et al, 1988). The E4 allele is associated with high, and the E2 allele with low total and LDL-cholesterol levels (Utermann et al, 1979; Robertson & Cummings, 1985; Sing & Davignon, 1985). Theoretically, the response to statin could be impaired in subjects who carry the E4 allele as apoE4 containing proteins are cleared more effectively by the liver than those with other apoE isoforms, resulting in increased levels of hepatic cholesterol and thus suppression of HMG-CoA reductase (Weintraub et al, 1987). Thus HMG CoA reductase could already be inhibited in these subjects, resulting in lower response to statins. On the other hand the efficiency of cholesterol absorption is reported to be greater in subjects who carry the E4 allele than in those who carry the E2 allele (Kesaniemi et al, 1987). Since lovastatin inhibits cholesterol absorption (Miettinen, 1991; Nielsen et al, 1993) statins may be more effective in E4 carriers.

Many studies have investigated the influence of the apoE phenotype with treatment in individuals with FH (Nestruck et al, 1987; de Knijff et al, 1990; O'Malley & Illingworth, 1990; Ojala et al, 1991; Carmen et al, 1993; Berglund et al, 1993). Two studies (Nestruck et al, 1987; Eto et al, 1990) reported a significantly better effect of probucol among a group of FH patients who carried an E4 allele but the opposite response has also been observed (Manttari et al, 1991). Early reports suggested that variations at APOE isoform had an influence on the response to statins (Davignon et al, 1988; O'Malley & Illingworth, 1990, de Knijff et al, 1990). Davignon et al, 1988 found a marked difference in effect of lovastatin treatment between two siblings with
FH, the E4 carrier sib responded much better than the other sib, who was E3/E3 (21% v 10% LDL-c reduction respectively). In contrast, O’Malley & Illingworth, 1990 & de Knijff et al, 1990 did not find any significant relationship between APOE polymorphism and the lowering of total and LDL-cholesterol in FH subjects in response to lovastatin and simvastatin treatment respectively. There was a trend for E4 carriers to respond less effectively to lovastatin therapy with respect to total and LDL-cholesterol, 31.7% reduction in LDL-c compared to those carrying E3, 40.7% or E2, 46.5% but more effectively in reducing plasma triglycerides (O’Malley & Illingworth, 1990). In contrast, a Swedish study of FH individuals found there to be no relationship between APOE genotype and the cholesterol lowering response to pravastatin or cholestyramine medication (Carmena et al, 1993). This result may be due to the decreased frequency of the E2 allele in Scandinavia compared with the rest of Europe and the USA or due to the high plasma LDL-c levels in these patients although, no effect was also seen in individuals from the normal population who had above average cholesterol levels (Carmena et al, 1993).

When the subjects were studied according to their gender there were once again mixed results with APOE genotype and LDL-c-lowering response. De Knijff et al, 1990, reported a greater response (9%) in LDL-c reduction in E3/E3 females treated with simvastatin than in E3/E3 males. A similar response was also observed with lovastatin treatment in E4 female carriers (10% greater lowering) than in male carriers (Carmena et al, 1993). By contrast, no sex related differences in response were observed in several studies (O’Malley & Illingworth, 1990; Ojala et al, 1991). It is possible that this discrepancy could be due to significant differences between the sexes for baseline and post-treatment HDL but in another study the baseline and post-treatment HDL levels were different but the response to HDL levels on simvastatin treatment was the same in both sexes (O’Malley & Illingworth, 1990; Ojala et al, 1991).

These results indicate that despite the well established effect of different apoE phenotypes on total and LDL-cholesterol levels in healthy subjects, the ApoE isoform does not contribute significantly to the variation in concentration of either total or LDL-cholesterol seen in FH patients nor the variation seen in cholesterol lowering with drug treatment.

6.1.2.2. LDL subfraction

FH individuals tend to have LDL with a high cholesterol: protein ratio that returns to normal with treatment. Lipid-lowering drugs affect the distribution of LDL
subfractions and this may effect the variation in response (Shepherd et al, 1980; Bilheimer et al, 1983). A study following the changes in the LDL subfraction profile with simvastatin showed there to be no difference between two groups, FH and normal individuals. Triglycerides had the greatest influence on the LDL subfraction distribution both before and after statin therapy; as plasma triglyceride increased, the concentration of the smallest LDL (LDL-3) also increased and the opposite was also seen. Lipid-lowering drugs act on all subfractions by reducing the cholesterol:protein ratio. This was confirmed in moderately hypercholesterolaemic patients where LDL subfractions fell, and all apoB containing lipoproteins exhibited a reduction in their cholesteryl ester content. Enhanced clearance of cholesterol-rich particles occur (VLDL-2 and IDL) which causes a reduced flux of apoB into LDL thus causing a fall in LDL-c levels (Gaw et al, 1993).

These genotype: drug response studies suggest that the response shown by an individual with FH to cholesterol-lowering therapy may be determined to some extent by their specific LDLR mutation and possibly by APOE genotype but they identify no clear relationship between the type or class of mutation (missense, null etc.) and degree of response, apart from the FH regression study where only 23 patients with a known LDLR defect were evaluated (Sun et al, 1998). We have therefore carried out a retrospective study to evaluate and confirm the response to simvastatin in a genetically heterogeneous group of 109 patients with a clinical diagnosis of ‘definite’ or ‘probable’ FH. Investigations into the involvement of four possible factors in determining the degree of LDL-c-lowering in response to statin therapy, namely the presence or absence of TX, gender, APOE genotype, presence of LDLR or APOB mutation. For this last analysis TX+ individuals carrying an LDLR mutation were further classified into carriers of ‘severe’ or ‘mild’ mutations based on the predicted consequence of the detected mutation (Hobbs et al, 1992; Sun et al, 1997; FH website). These subgroups were then compared to TX+ individuals where no mutation had been identified. Effectiveness was assessed by calculating the percentage fall in LDL-c levels on treatment compared to untreated levels and also by determining the proportion of patients at each treatment dosage in whom LDL-c levels fell below 4.9mmol/l or 4.1mmol/l. These values were chosen because they are the recommended intervention levels for hyperlipidaemic subjects without other risk factors (4.9mmol/l), or with other CHD risk factors (4.1mmol/l) (Grundy et al, 1988; Pyorala et al, 1994).
6.2. Materials & Methods

6.2.1. Patient group
109 FH patients (101 probands) attending lipid clinics at Charing Cross Hospital, London and King Edward V Hospital, Sussex were recruited and examined by one consultant. 86 individuals (76 probands) had a diagnosis of ‘definite’ FH, i.e. untreated LDL-c > 4.9mmol/l and TX in the patient or first degree relative. 23 patients were classified as ‘probable’ FH, i.e., LDL-c > 4.9mmol/l and a family history of hypercholesterolaemia (Simon Broome Steering Committee, 1991). Smoking habit was determined from patient notes and subjects defined as either a smoker (current or ex-smoker) or not a smoker. Hypertension was defined as systolic and diastolic levels of greater than 150 and 90mm Hg respectively and patients were designated on the evidence of a MI, coronary artery bypass graft (CABG), percutaneous transluminal coronary angioplasty (PTCA) or a positive coronary angiography.

58 patients were placed on 10mg/day simvastatin. 33/58 were subsequently increased to 20mg/day with the addition of a further 43 patients (total of 76 individuals) on a daily dose of 20mg. A total of 49 individuals were given a 40mg/day dose, two had been given only 10mg previously, 18 had been given 10mg and 20mg previously, 23 on 20mg and six individuals were initiated on the maximal dose of 40mg after previous use of another statin. Patients were treated for not less than three months at each dosage.

6.2.2. Biochemical analyses
Lipids were measured according to the methods described in section 2.1.

6.2.3. Molecular analyses
DNA isolation and molecular analysis of LDLR and APOB was as described in sections 2.1.1 and 2.3 respectively. APOE genotyping was according to section 2.3.6.

6.2.4. Statistical analysis
Reductions in plasma lipid levels were expressed as a percentage of untreated baseline levels, defined as \[\frac{\text{baseline} - \text{treatment}}{\text{baseline levels}} \times 100\]. \(\chi^2\) analysis was used to compare the frequency of discrete variables between groups. Pearson’s correlation coefficients were determined. The Hardy-Weinberg value was calculated to test genetic equilibrium with respect to the distribution of the APOE genotype. The comparison group for APOE genotype frequencies consisted of 476 men and women from the county of Hertfordshire, England who were born between 1911 and 1930 and were available for the investigation (Henry et al, 1997). Significance was at \(p<0.05\).
6.3. Results

As shown in Table 6.1 the 56 male and 53 female FH patients had similar mean age, BMI and baseline plasma lipid levels, and there was a similar proportion of current or ex-smokers, patients with hypertension, TX and evidence of CHD.

Table 6.1: Characteristics of 56 male and 53 female FH patients on simvastatin treatment.

<table>
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<tr>
<th>Trait</th>
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<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>56</td>
<td>53</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.2±1.55</td>
<td>49.8±1.89</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25±0.44</td>
<td>24±0.51</td>
</tr>
<tr>
<td>Smoking</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>16%</td>
<td>13%</td>
</tr>
<tr>
<td>Tendon xanthomata</td>
<td>84%</td>
<td>74%</td>
</tr>
<tr>
<td>CHD</td>
<td>35%</td>
<td>38%</td>
</tr>
<tr>
<td>Baseline total cholesterol</td>
<td>9.65±0.24</td>
<td>10.12±0.23</td>
</tr>
<tr>
<td>Baseline LDL-c</td>
<td>7.70±0.24</td>
<td>7.97±0.23</td>
</tr>
<tr>
<td>Baseline HDL</td>
<td>1.22±0.14*</td>
<td>1.39±0.06</td>
</tr>
<tr>
<td>Baseline triglyceride</td>
<td>1.88±0.13</td>
<td>1.68±0.12</td>
</tr>
<tr>
<td>Lp(a) median &amp; range(mg/dl)</td>
<td>45 (0-176)</td>
<td>37 (1-200)</td>
</tr>
<tr>
<td>LDL-c at 10mg simvastatin</td>
<td>5.69±0.34</td>
<td>5.36±0.28</td>
</tr>
<tr>
<td>LDL-c at 20mg simvastatin</td>
<td>4.75±0.17</td>
<td>4.84±0.26</td>
</tr>
<tr>
<td>LDL-c at 40mg simvastatin</td>
<td>4.31±0.22</td>
<td>4.30±0.25</td>
</tr>
<tr>
<td>No. below 4.9mmol/l at 40mg</td>
<td>66%</td>
<td>49%</td>
</tr>
<tr>
<td>No. below 4.1mmol/l at 40mg</td>
<td>26%</td>
<td>44%</td>
</tr>
<tr>
<td>No. of LDLR mutations</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>No. of APOB mutations (R3500Q)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>No. of individuals with no</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td>mutation detected in LDLR/APOB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE E2/E3 genotype</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>APOE E3/E3 genotype</td>
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<tr>
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<td>13</td>
</tr>
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<td>1</td>
</tr>
<tr>
<td>APOE E4/E4 genotype</td>
<td>3</td>
<td>2</td>
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</tbody>
</table>

Cholesterol values expressed as mean ± SEM (mmol/l). *Indicates a significant difference between males and females at p<0.05.
Of the patients, 86 (76 probands) had TX and were therefore classified as 'definite' FH, while 23 TX- unrelated patients had a clinical diagnosis of 'probable' FH. In the TX+ group a molecular defect was found in 30 patients, with three individuals having the APOB mutation, R3500Q, whilst only three patients had detected mutations in the 'probable' FH group, one in the LDLR (a null mutation in exon 13, 1944 Ins C) and two R3500Q mutations. Overall, the LDLR mutation detection rate in probands was 26% (26/99), however the detection rates was significantly different (p=0.02) between the 'definite' (TX+) probands (37%) and 'probable' FH (TX-) probands (13%). In the TX+ group no gender difference was observed in the detection rate, 37% in male probands (n=41) and 34% in female probands (n=35) (p=0.97).

The mutation spectrum (Table 6.2) showed the expected high frequency of mutations in exon 4 of the LDLR (23% of LDLR mutations) (Gudnason et al, 1993), which encodes part of the ligand binding domain. In all, 22 missense (two splice), five small deletions, two small insertions, one small rearrangement and one large duplication was detected. Eight mutations were novel to this study.

6.3.1. Response to simvastatin
Patients were placed on simvastatin at a dosage of either 10, 20 or 40mg/day. All patients showed a response to simvastatin therapy, 95% achieved a reduction in LDL-c to 20-40% of the baseline value, with 69% of patients achieving an LDL-c level of under 4.9mmol/l, 39% under 4.1mmol/l and 7% under 3.2mmol/l. Mean HDL levels increased by 7-9% at increasing dosage whilst triglyceride levels decreased by 6-25%. However no statistically significant differences were observed for HDL, triglycerides or Lp(a) in response to treatment (Table 6.3) and these parameters were not considered in subsequent comparisons.

6.3.2. Effect of the presence of TX on baseline lipid levels and response to simvastatin
As shown in Table 6.3 the mean baseline LDL-c was significantly higher in the TX+ group (p=0.014) and continued to be higher at each simvastatin dose (p=0.001, p=0.003, p=0.001 for 10, 20, 40mg/day respectively) (Table 6.4). At all doses the proportion of treated TX+ patients achieving LDL-c levels below 4.9mmol/l or 4.1mmol/l was lower than in the group of TX- patients, and these differences in the proportion of patients of achieving acceptable LDL-c levels were statistically significant at all doses. However the overall percentage reduction in cholesterol was identical between groups at each simvastatin dose, with mean LDL-c reductions of ~30%, ~41% and ~51% (Fig 6.1A).
Table 6.2: Clinical characteristics, lipoprotein profiles and molecular characterisation of 30 FH patients with TX and one FH patient without TX and a defined LDLR mutation.

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<tr>
<th>ID</th>
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<th>Sex</th>
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<th>MI</th>
<th>CABG</th>
<th>PTCA</th>
<th>+CA</th>
<th>BChol</th>
<th>BLDL</th>
<th>BHDLD</th>
<th>BTG</th>
<th>LDL10</th>
<th>LDL20</th>
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<th>APOE</th>
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<td>0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>NT</td>
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<td>11.4</td>
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<td>2.12</td>
<td>8.78</td>
<td>8.46</td>
<td>6.11</td>
<td>33</td>
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<tr>
<td>2</td>
<td>118delA*</td>
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<td>Y</td>
<td>NT</td>
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<tr>
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<td>8.70</td>
<td>6.69</td>
<td>0.90</td>
<td>2.43</td>
<td>5.08</td>
<td>-</td>
<td>24</td>
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</tr>
<tr>
<td>21</td>
<td>D461H</td>
<td>48</td>
<td>M</td>
<td>27.8</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>11.4</td>
<td>9.78</td>
<td>1.07</td>
<td>1.20</td>
<td>-</td>
<td>4.94</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>D471H*</td>
<td>55</td>
<td>F</td>
<td>30.0</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>11.6</td>
<td>9.47</td>
<td>0.95</td>
<td>2.59</td>
<td>-</td>
<td>6.05</td>
<td>34</td>
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<tr>
<td>23</td>
<td>P505S</td>
<td>55</td>
<td>F</td>
<td>23.0</td>
<td>1</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>NT</td>
<td>13.3</td>
<td>10.6</td>
<td>2.30</td>
<td>0.90</td>
<td>-</td>
<td>6.26</td>
<td>4.62</td>
<td>34</td>
</tr>
<tr>
<td>24</td>
<td>1716dGTGGC/insA*</td>
<td>29</td>
<td>F</td>
<td>17.3</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>NT</td>
<td>10.8</td>
<td>8.68</td>
<td>1.80</td>
<td>0.70</td>
<td>-</td>
<td>5.71</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>25</td>
<td>1802insA*</td>
<td>35</td>
<td>F</td>
<td>19.0</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>NT</td>
<td>8.10</td>
<td>6.76</td>
<td>1.03</td>
<td>0.67</td>
<td>-</td>
<td>3.54</td>
<td>-</td>
<td>33</td>
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<td>26</td>
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<td>51</td>
<td>M</td>
<td>22.8</td>
<td>1</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>10.1</td>
<td>8.20</td>
<td>1.19</td>
<td>1.40</td>
<td>5.94</td>
<td>4.47</td>
<td>2.93</td>
<td>33</td>
</tr>
<tr>
<td>27</td>
<td>C656R</td>
<td>49</td>
<td>F</td>
<td>23.9</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>11.8</td>
<td>9.94</td>
<td>1.36</td>
<td>1.10</td>
<td>-</td>
<td>5.72</td>
<td>5.06</td>
<td>33</td>
</tr>
<tr>
<td>28</td>
<td>IVS14+5G&gt;A*</td>
<td>50</td>
<td>M</td>
<td>28.4</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>NT</td>
<td>8.30</td>
<td>6.20</td>
<td>1.00</td>
<td>2.40</td>
<td>4.10</td>
<td>3.64</td>
<td>-</td>
<td>34</td>
</tr>
<tr>
<td>29</td>
<td>IVS16+1G&gt;A</td>
<td>52</td>
<td>M</td>
<td>30.1</td>
<td>1</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>NT</td>
<td>11.6</td>
<td>9.42</td>
<td>0.90</td>
<td>2.80</td>
<td>-</td>
<td>4.71</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Dup exon 7-8</td>
<td>67</td>
<td>M</td>
<td>24.7</td>
<td>1</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>9.60</td>
<td>7.61</td>
<td>1.40</td>
<td>1.30</td>
<td>-</td>
<td>2.94</td>
<td>-</td>
<td>34</td>
</tr>
</tbody>
</table>

Note: Smoking habit (SH) was classified as: 0 - never, 1 - ex, 2 - current. MI, CABG, PTCA are classified as yes (Y) or no (N). If a coronary angiography (CA) had been undertaken it is noted as +CA. BChol, BLDL, BHDLD, BTG, LDL10, LDL20, LDL40, APOE are all in mmol/L. Cholesterol values (mmol/l).
Table 6.3: Characteristics of 86 TX+ and 23 TX- heterozygous FH patients on simvastatin treatment. Cholesterol values expressed as mean ± SEM (mmol/l).

<table>
<thead>
<tr>
<th>Trait</th>
<th>TX+</th>
<th>TX-</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (No. of probands)</td>
<td>86 (76)</td>
<td>23 (23)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>47.5±1.40</td>
<td>51.8±2.33</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25±0.39</td>
<td>24±0.63</td>
<td></td>
</tr>
<tr>
<td>Baseline cholesterol</td>
<td>10.16±0.19</td>
<td>8.82±0.27</td>
<td>0.0009*</td>
</tr>
<tr>
<td>Baseline LDL-c</td>
<td>8.10±0.19</td>
<td>6.81±0.25</td>
<td>0.014*</td>
</tr>
<tr>
<td>Baseline HDL</td>
<td>1.32±0.10</td>
<td>1.58±0.07</td>
<td></td>
</tr>
<tr>
<td>Baseline triglycerides</td>
<td>1.81±0.10</td>
<td>1.68±0.20</td>
<td></td>
</tr>
<tr>
<td>Lp(a) median &amp; range (mg/dl)</td>
<td>24 (0.5-200)</td>
<td>40 (4-132)</td>
<td></td>
</tr>
<tr>
<td>No. of LDLR mutations</td>
<td>30 (25)</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>No. of APOB30500Q mutations</td>
<td>3 (3)</td>
<td>2 (2)</td>
<td>0.013*</td>
</tr>
<tr>
<td>No. of no mutations detected in LDLR/APOB</td>
<td>53 (48)</td>
<td>20 (20)</td>
<td></td>
</tr>
<tr>
<td>APOE E2/E3 genotype</td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>APOE E3/E3 genotype</td>
<td>41</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>APOE E3/E4 genotype</td>
<td>26</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>APOE E2/E4 genotype</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>APOE E4/E4 genotype</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Only significant values are stated, at the p<0.05 level.

6.3.3. Effect of gender on the response to simvastatin
As shown in Table 7.4, in the TX+ group there was no significant differences between men and women in the size of the lipid-lowering response to statin treatment or in the number of subjects achieving LDL-c levels below the 4.9 or 4.1 mmol/l thresholds at any dose (final treatment values for 4.9 mmol/l: M=66% v F=49%, p=0.50 and at 4.1 mmol/l: M=26% v F=44%, p=0.08). Similarly no gender difference in response was observed in the group as a whole (Table 7.1).

6.3.4. Effect of APOE alleles on baseline lipid levels and on the response to simvastatin
In the whole group the frequency of genotypes was as expected from Hardy-Weinberg proportions. The allele frequencies of APOE polymorphism in this group of FH patients was E2:0.06, E3:0.72, E4:0.22 which was significantly different from the healthy UK population (p<0.01) due to the higher frequency of the E4 allele. There was no difference in the distribution of APOE alleles in the group of patients with or without TX (Table 6.3) or in those with or without a detected mutation (data not shown).
Due to small sample size, baseline lipids and response to statins was only compared between E3/E3 and E3/E4 & E4/E4 individuals. As shown in Table 6.4, in the TX+ group the presence of the allele E4 was associated with 8.5% lower LDL-c at baseline and 7.2% lower levels on the 40mg/day dose, although these difference were not statistically significant (p=0.09 and p=0.40 respectively). Compared to the E3/E3 group, those with the E4 allele had significantly lower levels of LDL-c at 10mg/day (20.8%, p=0.02) and at 20mg/day (18.6%, p=0.01) when compared to E3/E3 individuals, however the percentage reduction was not significantly different at any treatment dose (overall reduction achieved E3/E3 v E3/E4, 49% v 47%, p=0.63), nor was the proportion of E4 patients achieving the 4.1mmol/l level greater than that of the E3/E3 group at all doses of simvastatin (data not shown).

### 6.3.5. Effect of LDLR mutation on baseline lipid levels and on the response to simvastatin in TX+ patients

Due to the small number of patients with the R3500Q mutation in APOB, no detailed analysis of their response to statin treatment was carried out, although the mean percentage reduction in LDL-c levels achieved by this group was similar to that seen in the other two groups, LDLR mutation and no mutation identified. In individuals with FDB it was observed that the lowest dose, 10mg/day, provided a mean reduction in LDL-c of 20-40%, whereas higher doses were required to achieve this level of reduction in the other two groups. The proportion of patients in each group achieving the 4.9mmol/l and 4.1mmol/l levels was greatest in APOB, intermediate in the no detected mutation and lowest in LDLR mutation group.

Baseline lipid levels and response to treatment was compared in the TX+ group who had either no mutation detected or any LDLR mutation detected. Detailed characteristics of each of the 30 TX+ patients (and the one TX- patient) who carried an identified LDLR mutation are presented in Table 6.2. As shown in Table 6.4 the mean baseline LDL-c was 7.4% higher in the group of individuals with any identified LDLR mutation, although this difference did not reach statistical significance. This relative difference in mean LDL-c was also seen at all drug treatment points, but the mean percentage reduction with each dose was similar for those with or without a detected LDLR mutation. The proportion of patients in each group achieving the 4.9 and 4.1mmol/l levels were greater in the no detected mutation group compared to the LDLR mutation group, although none of these differences achieved statistical significance.

The 30 TX+ FH individuals carrying an identified LDLR mutation were subdivided into patients carrying ‘severe’ or ‘mild’ mutations. ‘Severe’ mutations were classified either
as exon 4 repeat 5 mutations or null mutations (n=14) whilst ‘mild’ mutations were all other LDLR mutations combined (n=16). As shown in Table 6.5 and Fig 6.1B, compared to those with no detected mutation, mean LDL-c were 7.6% higher in those with a ‘severe’ mutation but these differences were not statistically significant. This ranking was similar at all treatment points, and at the 40mg dose mean LDL-c levels in the ‘severe’ group were significantly higher than in the other two groups of patients (by ANOVA p=0.02). The proportion of ‘severe’ patients achieving the 4.9mmol/l or 4.1mmol/l levels was less than for the ‘mild’ patients at all dosages, and this difference was statistically significant at 40mg/day for the 4.1mmol/l threshold (‘severe’: 0% v ‘mild’: 70%, p=0.018) (Table 6.5 & Fig 6.2). When patients were grouped according to exon 4 repeat 5 and exon 4 null mutations (n=7) versus all other LDLR mutations (n=23) no difference in baseline levels or in statin response between groups was observed (data not shown).
Table 6.4: Mean ± SEM total and LDL-cholesterol (mmol/l) at baseline and after simvastatin treatment and the proportion achieving LDL-c levels below 4.9 & 4.1mmol/l in 109 FH patients with and without TX, in 47 TX+ males and 39 TX+ females, and in 76 TX+ FH patients who are either APOE3 or E4 allele carriers.

<table>
<thead>
<tr>
<th></th>
<th>TX+</th>
<th>No. below 4.9/4.1 mmol/l (%)</th>
<th>TX-</th>
<th>No. below 4.9/4.1 mmol/l (%)</th>
<th>TX+ males</th>
<th>No. below 4.9/4.1 mmol/l (%)</th>
<th>TX+ females</th>
<th>No. below 4.9/4.1 mmol/l (%)</th>
<th>Presence of E4 allele</th>
<th>No. below 4.9/4.1 mmol/l (%)</th>
<th>Absence of E4 allele</th>
<th>No. below 4.9/4.1 mmol/l (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male: Female</td>
<td>47:39</td>
<td>-</td>
<td>9:14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20:12</td>
<td>21:23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baseline total chol</td>
<td>10.18±0.19* (n=86)</td>
<td>-</td>
<td>8.82±0.27  (n=23)</td>
<td>-</td>
<td>9.93±0.26  (n=47)</td>
<td>10.4±0.27 (n=39)</td>
<td>-</td>
<td>9.68±0.26  (n=32)</td>
<td>-</td>
<td>10.4±0.28 (n=44)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total chol at 40mg/day</td>
<td>6.38±0.17* (n=40)</td>
<td>-</td>
<td>5.15±0.24  (n=9)</td>
<td>-</td>
<td>6.18±0.23  (n=24)</td>
<td>6.69±0.28 (n=16)</td>
<td>-</td>
<td>6.15±0.34  (n=14)</td>
<td>-</td>
<td>6.64±0.22 (n=21)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Baseline LDL-c</td>
<td>8.14±0.19* (n=86)</td>
<td>-</td>
<td>6.81±0.25  (n=23)</td>
<td>-</td>
<td>7.99±0.26  (n=47)</td>
<td>8.24±0.27 (n=39)</td>
<td>-</td>
<td>7.63±0.28  (n=32)</td>
<td>-</td>
<td>8.34±0.28 (n=44)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LDL-c at 10mg/day</td>
<td>5.76±0.25* (n=46)</td>
<td>15(32%)** 6(28%)</td>
<td>4.51±0.27  (n=12)</td>
<td>8(67%)</td>
<td>5.99±0.34  (n=22)</td>
<td>7(32%)</td>
<td>5.54±0.38  (n=24)</td>
<td>8(33%)</td>
<td>4.81±0.28** (n=15)</td>
<td>8(53%)**</td>
<td>5.99±0.35 (n=24)</td>
<td>7(29%)**</td>
</tr>
<tr>
<td>LDL-c at 20mg/day</td>
<td>4.99±0.17* (n=60)</td>
<td>32(58%)** 17(28%)**</td>
<td>3.92±0.15  (n=14)</td>
<td>13(93%)</td>
<td>4.95±0.18  (n=37)</td>
<td>19(51%)</td>
<td>10(37%)</td>
<td>5.10±0.32  (n=23)</td>
<td>12(52%)</td>
<td>4.30±0.28** (n=19)</td>
<td>14(74%)</td>
<td>5.24±0.21 (n=34)</td>
</tr>
<tr>
<td>LDL-c at 40mg/day</td>
<td>4.55±0.17* (n=40)</td>
<td>26(58%)** 15(38%)**</td>
<td>3.23±0.19  (n=9)</td>
<td>9(100%)</td>
<td>4.46±0.23  (n=24)</td>
<td>16(67%)</td>
<td>8(33%)</td>
<td>4.68±0.28  (n=16)</td>
<td>10(63%)</td>
<td>4.39±0.34  (n=14)</td>
<td>11(79%)</td>
<td>4.73±0.23 (n=21)</td>
</tr>
</tbody>
</table>

*Indicates a significant difference between TX+ and TX- subjects at p<0.005.  **Indicates a significant difference between TX+ and TX- subjects at p<0.05.
Table 6.5: Comparison of mean ± SEM total and LDL-cholesterol levels (mmol/l) at baseline and at simvastatin doses of 10, 20, 40mg/day in TX+ patients who either have a ‘severe’ LDLR mutation (exon 4 repeat 5 or null mutation) or a ‘mild’ LDLR mutation (LDLR mutation other than exon 4 repeat 5 or a null mutation).

<table>
<thead>
<tr>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>'Severe' LDLR mutations</th>
<th>'Mild' LDLR mutations</th>
<th>No. below 4.9 or 4.1mmol/l (%)</th>
<th>No mutation in LDLR or APOB</th>
<th>No. below 4.9 or 4.1mmol/l (%)</th>
<th>Any LDLR mutation</th>
<th>No. below 4.9 or 4.1mmol/l (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male: Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline total chol</td>
<td>9:6</td>
<td>-</td>
<td>7:8</td>
<td>-</td>
<td>29:24</td>
<td>-</td>
<td>16:14</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td></td>
<td>(n=16)</td>
<td></td>
<td>(n=53)</td>
<td></td>
<td>(n=30)</td>
</tr>
<tr>
<td>Total chol at 40mg/day</td>
<td>10.44±0.46</td>
<td>10.51±0.40</td>
<td>-</td>
<td>10.1±0.25</td>
<td>-</td>
<td>10.5±0.30</td>
<td>-</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td>(n=11)</td>
<td></td>
<td></td>
<td>(n=22)</td>
<td></td>
<td>(n=17)</td>
</tr>
<tr>
<td>Baseline LDL-c</td>
<td>8.36±0.51</td>
<td>8.53±0.42</td>
<td>-</td>
<td>7.95±0.25</td>
<td>-</td>
<td>8.54±0.31</td>
<td>-</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td>(n=16)</td>
<td></td>
<td></td>
<td>(n=53)</td>
<td></td>
<td>(n=30)</td>
</tr>
<tr>
<td>LDL-c at 10mg/day</td>
<td>6.58±0.52</td>
<td>0 (0%)</td>
<td>5.67±0.58</td>
<td>2 (29%)</td>
<td>5.53±0.34</td>
<td>12 (41%)</td>
<td>6.29±0.40</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td>(n=7)</td>
<td></td>
<td>2 (29%)</td>
<td>(n=29)</td>
<td></td>
<td>(n=15)</td>
</tr>
<tr>
<td>LDL-c at 20mg/day</td>
<td>5.51±0.49</td>
<td>3 (27%)</td>
<td>4.99±0.30</td>
<td>8 (62%)</td>
<td>4.86±0.21</td>
<td>19 (54%)</td>
<td>5.22±0.28</td>
</tr>
<tr>
<td>(n=11)</td>
<td></td>
<td>(n=13)</td>
<td></td>
<td>4 (31%)</td>
<td>(n=35)</td>
<td></td>
<td>(n=24)</td>
</tr>
<tr>
<td>LDL-c at 40mg/day</td>
<td>5.54±0.30*</td>
<td>2 (33%)</td>
<td>4.38±0.28</td>
<td>7 (64%)</td>
<td>4.38±0.25</td>
<td>16 (73%)</td>
<td>4.79±0.24</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td>(n=11)</td>
<td></td>
<td>5 (45%)</td>
<td>(n=22)</td>
<td></td>
<td>(n=17)</td>
</tr>
</tbody>
</table>

*Indicates significance between two groups at p<0.02.
Fig 6.1: Dose response curves of LDL-c in 109 patients with or without TX (A) and with an LDLR mutation, an APOB mutation or no mutation detected in either gene (B). The percentage reduction in LDL-c at the maximal dose of 40mg/day is shown for each data set.

A

B
Fig 6.2: Dose response curves of LDL-c levels in TX+ patients, with a ‘severe’ or ‘mild’ LDLR mutation. The LDL-c target point of 4.1mmol/l is indicated with a dashed line and the number of individuals reaching the target level are represented as a proportion of the total number of each class. A: TX+ patients with ‘severe’ LDLR mutation (n=14). B: TX+ patients with a ‘mild’ LDLR mutation (n=16). *Indicates a significance between the two groups at p<0.05.
6.4. Discussion

In this study the possible involvement of four different factors in determining the degree of LDL-c-lowering in response to statin therapy was investigated and these were gender, presence/absence of TX, APOE genotype, and type of genetic defect (LDLR/APOB) present. Current consensus guidelines (Grundy et al, 1988; Pyrola et al, 1994) propose that the goal of statin treatment in a hypercholesterolaemic patient is to reduce LDL-c to 4.9mmol/l if no other risk factors are present and to 4.1mmol/l in their presence, and less than 3.2mmol/l in those with CHD, with the necessary increase in dosage to achieve this for each patient (Grundy et al, 1988). Other, lower, levels could also be proposed and may be more desirable for FH patients, but these thresholds were used here for analytical comparison. The clinically-important aspect of treatment is therefore the proportion of patients achieving the desired LDL-c level at any particular level of statin treatment. Thus, for factors associated with higher untreated LDL-c, the percentage fall in LDL-c with a particular dose of treatment may be different, but this does not mean that response is materially different or clinically relevant.

Results from trials investigating the effect of lowering LDL-c on reducing CHD have suggested that the percentage reduction in LDL-c may be as good a predictor of reduction in cardiovascular risk as is the absolute cholesterol level achieved (Thompson et al, 1995; Jackson, 1997). This hypothesis is unproved in patients with FH, who are at high risk from elevated LDL-c but in whom incidence of other risk factors such as hypertension, diabetes or smoking, is typically not increased. However it is not ethical to carry out placebo-randomised control studies in this group of patients to test the hypothesis. The concept of percentage reduction is particularly important in those patients with FH who have CHD, in whom the target level of LDL-c of <3.2mmol/l is difficult to achieve, even with highly effective drug therapy (Nawrocki et al, 1995; Marais et al, 1997; Jones et al, 1998). Coronary angiographic studies have suggested that regression of atherosclerosis does not occur until a 45-55% reduction in LDL-c is achieved. However in patients with FH, lesion progression can be reduced at smaller percentage reduction levels in LDL-c levels (Thompson, 1997). This suggests that other mechanisms such as the stabilisation of atherosclerotic plaque, effect on smooth muscle cell proliferation, restoration of endothelial function, reduction in platelet activity and alteration in macrophage-induced cytokines may also be important in the prevention of cardiac events (reviewed by Vaughan, Murphy & Buckley, 1996; Rosenson & Tangney 1998). Recent analysis of the number of CHD deaths occurring in FH patients on the Simon Broome register show that compared with the high event rate (overall 8-fold increased risk) in the period 1986-1991 (Simon Broome Steering Committee, 1991) there has been a significant reduction (p=0.08) in coronary events.
over the last five years (now 3.5-fold increased risk) since statins became commonly available (Simon Broome Steering Committee, 1999). These observational results are in keeping with the hypothesis that a 20–30% reduction in LDL-c in patients with high LDL-c achieves a greater reduction in mortality than in those with lower baseline levels (Assmann et al., 1997).

In this group of FH patients, simvastatin treatment reduced LDL-c in all individuals and 75/109 reached levels of 4.9mmol/l and 42/109 achieved 4.1mmol/l or below. The target level of 3.2mmol/l was reached in 8/109 individuals, two at a dose of 20mg/day and the remaining six at 40mg/day. Although numbers were small there were no obvious differences in any characteristics between these patients and the other 101 individuals, 50% were TX+, 63% had suffered an MI and 25% had a mutation in the LDLR (one ‘severe’ & one ‘mild’). The effect of simvastatin on the percentage lowering of LDL-c was similar for the groups of patients classified as ‘definite’ FH (TX+) or ‘probable’ FH (TX-), although baseline untreated LDL-c values were 1.3mmol/l higher in the ‘definite’ FH individuals. However, the final mean LDL-c values on treatment remained 1.3mmol/l higher in the TX+ patients, and significantly fewer of the TX+ group achieved levels below 4.9mmol/l or 4.1mmol/l compared to the TX- group. The similarity in lipid-lowering observed with increase in dose of statin in the TX+ and TX- patients suggests that there are no major differences in the characteristics of these two groups (whether genetic or environmental) that influence reduction. However, whether the failure in the TX+ group to achieve a greater reduction in LDL-c levels will result in more future coronary events will require investigation in a longitudinal study.

APOE polymorphism is known to have an important influence on plasma LDL-c levels in the general population (Sing et al., 1985) and was examined as a genetic factor likely to determine baseline lipid levels and response to treatment. The observation in the sample of a higher frequency of the E4 allele in FH patients compared to the general population confirms reports of others (Duly et al., 1997). Several studies have suggested that APOE genotype does not alter the response to statins (O'Malley et al., 1990; de Knijff et al., 1990), although a non-significant lower response in E4 carriers treated with lovastatin has been reported compared to E3 carriers (Carmena et al., 1993). The present study found no significant difference in response to statins in E4 carriers compared to E3 carriers.

The mutation detection rate was 3-fold higher in the TX+ individuals compared with the TX- group which confirms the diagnostic usefulness of TX for the disorder. Only two
R3500Q mutations and one LDLR mutation were found in the TX-group. Molecular analysis of the 86 ‘definite’ FH patients (76 probands) resulted in a mutation detection rate that was lower than but not dissimilar from other studies of genetically heterogeneous populations (Whittall et al, 1995; Day et al, 1997b; Sun et al, 1997). This low rate may be due to the insensitivity of the SSCP technique, as well as the fact that large deletions or insertions were not examined in all patients, nor were introns or the 3' untranslated region. Others have also shown that some patients with a clinical diagnosis of heterozygous FH do not have defective LDL-receptor activity in their cultured cells, and that other inherited defects may therefore be responsible for the hypercholesterolaemia (Lestavel-Delattre et al, 1994) although the binding assay is not 100% accurate (Nissen et al, 1998). LDL-receptor activity was not analysed in this group so this possibility cannot be excluded. In the individuals where no molecular defect had been identified, co-segregation analysis of LDLR would be possible where extended families were available (Day et al, 1997a; Haddad et al, 1997) to determine whether or not the LDLR locus is likely to be the cause of the FH, or provide evidence for whether another gene is defective and causing clinical FH (Lestavel-Delattre et al, 1994; Zuliani et al, 1995 & 1999; Haddad et al, in press).

Overall, the frequency of FDB was 5%, similar to the incidence previously reported for hypercholesterolaemic patients in the UK (Tybjaerg-Hansen et al, 1990), three with TX and two without. As seen in other studies (Tybjaerg-Hansen et al, 1990; Innearity et al 1990), these FDB patients had slightly lower mean untreated plasma LDL-c levels than those with a detected LDLR mutation (6.95 v 8.32mmol/l, p=0.07), but because of the small number of patients available no statistical analysis of response was carried out in this group. The percentage reduction in LDL-c levels was similar, to that of non-FDB individuals, as seen by Maher et al, 1991, but it appeared that unlike the non-FDB individuals lower doses of simvastatin were sufficient to reach the target cholesterol levels. This may be partly due to increased receptor mediated hepatic removal of mutant and normal precursors of LDL, using apoE as the recognition particle. In a retrospective study the response to simvastatin and cholestyramine was compared in 11 FDB patients with that in 11 FH heterozygotes shown to not carry the R3500Q mutation (Mayer et al, 1991). In both groups the mean plasma LDL-c levels fell by ~30% with the maximum reduction being 50% which occurred in an FDB and an FH individual. Similar results (19-43% LDL-c reductions) have also been observed in FDB patients treated with a daily dose of 40mg simvastatin (Illingworth et al, 1992).

For LDLR, the detection rate was 3-fold higher in the TX+ individuals compared with the TX- group which confirms the diagnostic usefulness of TX for the disorder. Only
one patient with an LDLR mutation was found in the TX- group, and this raises the possibility that a greater proportion of these 'probable' FH patients may not in fact have FH, but may have either a different genetic cause of the familial hyperlipidaemia such as recently suggested in one family from Utah (Haddad et al, in press), or they may have primarily 'non-genetic' cause of the hyperlipidaemia despite having a strong family history. The region of the gene where the greatest number of mutations was found was exon 4 as previously described (Gudnason et al, 1993). The 3' part of exon 4 codes for repeat 5, the apoB/apoE ligand binding domain of the LDL-receptor, and mutations in this region have been found to have a more severe phenotype (Gudnason et al, 1994). Thus in the TX+ group, patients were classified into three groups to examine baseline cholesterol levels and drug response; a 'severe' or a 'mild' LDLR mutation or no mutation detected (in either LDLR or APOB). Subgroups were classified by the severity of the LDLR mutation determined from published or predicted binding affinities to apoB and apoE containing lipoproteins (Hobbs et al, 1992; Sun et al, 1997; FH website). 'Severe' mutations, null mutations and mutations in repeat 5 of exon 4, were those predicted to have <2% residual LDL-receptor activity and no apoE ligand binding ability, i.e. no VLDL or IDL-binding. 'Mild' LDLR mutations were those predicted to have some residual LDL-receptor active, and/or where the ability to bind apoE containing lipoproteins would not be impaired. One intronic mutation was identified 5 bases downstream of the exon-intron boundary and this was characterised as 'mild', although no functional studies were undertaken to confirm the pathogenic nature. Although the groups of patients had virtually identical baseline LDL-c, the LDL-c in those with the 'severe' exon 4 repeat 5 or null mutations did not decrease with treatment to the same extent as in the patients with the 'mild' mutations. Thus although the small sample number of patients with a detected mutation in this genetically heterogeneous group limits the power of the study, the data suggest that statins had a greater effect on the 'mild' mutation group, which is compatible with the hypothesis that overall, these mutations had a higher level of LDL-receptor residual activity that may be up-regulated (Gudnason et al, 1994). These observations confirm the data from the FH regression study, where LDLR mutations in 23 patients were also subdivided by predicted severity (Sun et al, 1998). Baseline LDL-c was highest in the individuals with 'severe' mutations, intermediate in those with 'mild' mutations, and lowest in the no identified mutation group, and as found in our study, the treatment levels also followed the same molecular defect severity gradient.

A larger world-wide study is currently taking place, the IMPRES FH Study, where the effect of the genotype on the response to the lipid lowering effect of simvastatin is being investigated in a large number of individuals from heterogeneous populations and
6.5. Conclusion
A mutation was identified in 36 individuals, 31 LDLR and 5 APOB. An LDLR mutation was detected in 30 TX+ patients, 14 were classified as 'severe' mutations and 16 as 'mild' mutations. On the basis of MI, CABG, PTCA or positive coronary angiography 5/14 carriers of 'severe' LDLR mutations had CHD compared to 7/16 carriers of 'mild' LDLR mutation. Thus no strong relationship was demonstrated between severity of mutation and presence of CHD, which is not surprising given the multifactorial nature of CHD, and variation in patient management. Other factors influencing drug treatment studies of individuals with identical LDLR mutations have concluded that the severity of the clinical phenotype and response to drug therapy is not solely determined by the nature of the mutation (Ferrieres et al, 1995). The challenge to the clinician is to treat effectively those patients with FH who are most at risk of developing premature CHD. In this respect, it is not necessarily the genetic diagnosis, or the type of mutation, be it 'severe' or 'mild' which is important, but the ability to assess the impact of other risk factors and to detect early atherosclerosis. In the future the refining of risk by using possible phenotype or genotype information may therefore be important.

Acknowledgements
Manjeet Bolla is thanked for APOE genotyping.
Future

During the next year FH genetic testing will be transferred from a research project to a clinical service supported by the NHS. Genetic testing of FH individuals requires assessing. Cascade screening may be introduced but also different techniques need to be applied. The current method of screening is by radioactive high-throughput SSCP analysis but this needs to be replaced by a safer method. The technique must be able to take large numbers so an automated method may be suitable.

The Clinical Molecular Genetics Laboratory now has a PCR robot and LI-COR machine. The PCR robot can be programmed to dispense any reagents, place trays on the PCR machine, carry out a PCR reaction and add loading buffer, ready for electrophoresis on the LI-COR. The LI-COR is a fluorescent-based gel system, similar to the ABI DNA Sequencer. One technique, Reference Strand Conformation Analysis (RCSA) has been designed to work on this machine. This method is principally fluorescent heteroduplex analysis. PCR products are electrophoresed on a long thin gel and data is sent to a computer. A gel image is produced with data recorded on the distance travelled by each band. Software to store these band distances is currently under development, so that a variant band can be recognised as being caused by a known mutation. Direct assays can then be used to confirm the mutation so eliminating the sequencing step. The company evaluated the technique by analysing over 500 CFTR mutations and in the Clinical Molecular Genetics Laboratory it is currently being tested for mutations in fibrillin 2.

In the long-term, chip technology may be the standard method of mutation detection. Arrays are made up of all four nucleotides at each position of a gene, which combine to form multiple arrays of many thousand oligonucleotides which can be tested in a few hours. This technique should provide a reliable and efficient method to identify and detect DNA sequences but it requires technology and expensive equipment which is unlikely to be found, in the near future, in a standard research or diagnostic laboratory.

All these methods detect point mutations, small deletions and duplications but fail to identify major rearrangements. Most major rearrangements are detected by Southern analysis but universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR) could be utilised. It has been evaluated for major rearrangements for five LDLR exons. An external locus needs to be incorporated into the current assay and additional
exons could be added to increase the number of exons analysed or further sets could be
designed.

In paediatric cases, the mutation detection rate was 72%, so 28% of mutations are
unidentified. Evidence for a third gene, which results in a phenotype identical to FH,
has been found in one family and there may be more. Genome scanning projects are
attempts to identify the genes which cause familial combined hyperlipidaemia
(FCHL). Some of the adults clinically diagnosed as having FH, may have FCHL,
since different patterns of hyperlipidaemia are often found within a family. Therefore
screening the genes for FCHL in these adults may be an option.

There are also many CHD modifying factors, mostly determined by biochemical tests,
e.g. Lp(a), HDL, fibrinogen, or single genotypes, e.g. ApoE isoform by APOE
genotyping, deletion/insertion polymorphism in ACE, C677T variant in MTHFR. Risk
analysis of an FH individual should incorporate some of these modifying factors.

In the UK, 110,000 people are estimated to have the heterozygous form of FH but only
approximately 10% of these are aware of their diagnosis and only 1% have a genetic
diagnosis. It would be better to diagnose individuals before their first MI, and with the
lipid lowering drugs available today their risk of MI could be reduced. Many tests,
both biochemical and genetic, are carried out on each FH subject and the genetic tests
are very expensive so family tracing needs to be promoted. At present, there is a lack
of preventative medicine, with few genetic nurses and none to trace relatives of FH
probands. In the long-term it would be cost effective to trace at risk individuals and
such a system already operates in Iceland and in the Netherlands. Thus as molecular
genetic analysis expands so should the genetic counselling infrastructure.
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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

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Received 16 April 1998; received in revised form 3 August 1998; accepted 8 October 1998

Abstract

In a genetically heterogeneous group of 109 patients with a clinical diagnosis of heterozygous familial hypercholesterolaemia (FH), the influence of gender, apolipoprotein (apo) E genotype and the type of molecular defect in the LDL-receptor (LDLR) gene on the reduction of plasma LDL-cholesterol levels to treatment with a HMG-CoA reductase inhibitor (simvastatin) were studied. Response was determined as the percentage fall in LDL-cholesterol from untreated levels and as the proportion of patients where levels fell below 4.9 or 4.1 mmol/l. Of the patients, 86 individuals had tendon xanthomata (TX + ) and a diagnosis of 'definite' FH and these individuals presented with a significantly higher untreated LDL-cholesterol compared to the 23 individuals who did not have xanthomas (TX – ) and a diagnosis of 'probable' FH (8.14 ± 0.19 vs. 6.81 ± 0.25, P = 0.001). Overall, HMG-CoA reductase inhibitor doses of 10, 20 or 40 mg/day resulted in a significant fall of LDL-cholesterol levels of 29, 39 and 49%, but at all doses those with TX had significantly higher levels than those without, and significantly fewer TX + patients achieved LDL-cholesterol levels below 4.9 or 4.1 mmol/l than the TX – group (P < 0.05 at each dose). In the TX + group the response to treatment was of similar magnitude in men and women and in patients with different apoE genotype. In the 'probable' FH probands only three mutations were identified (detection rate 13%), one in the LDLR gene and two in the APOB gene, a detection rate significantly lower (P = 0.02) than in the 'definite' FH probands where 28 mutations were detected (detection rate 37%). In the TX + patients where no mutation was detected, treatment resulted in a greater proportion achieving LDL-cholesterol levels below 4.9 and 4.1 mmol/l compared to those with any LDLR mutation, this difference was close to statistical significance at the 4.9 mmol/l threshold at 10 mg/day (41 vs. 13%, P = 0.058). For the 14 patients with an LDLR mutation that was predicted to be 'severe', fewer achieved LDL-cholesterol levels below 4.9 or 4.1 mmol/l at each dosage compared to the 16 individuals with 'mild' mutations, and this difference was statistically significant at the maximal dosage of 40 mg/day (P = 0.018). Thus although characterisation of the molecular defect in FH patients may not be relevant to their immediate clinical management, those with a particular mutation may need more aggressive lipid-lowering treatment to reach LDL-cholesterol levels recommended to reduce the risk of coronary heart disease (CHD). © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cholesterol; Familial hypercholesterolaemia (FH); LDLR mutation; Simvastatin; Tendon xanthoma (TX)
1. Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant inherited disorder of lipoprotein metabolism. It is characterised by hypercholesterolaemia due to elevated plasma low density lipoprotein (LDL) levels, tendon xanthomas (TX), premature coronary heart disease (CHD) and a family history of one or more of these. FH is defined as being caused by a defect in LDL clearance due to a mutation in the low density lipoprotein receptor gene (LDLR). More than 500 mutations at the LDLR have been reported, [1-3], http://www.ucl.ac.uk/fh and different types of mutation result in different residual functional LDL-receptor activity, varying from <2 to 55% [4]. However an LDLR mutation is not found in all patients with a clinical diagnosis of FH [1], and furthermore, in approximately 3% of the UK patients a defect has been detected in the apolipoprotein B-100 gene (APOB), the ligand for the LDL-receptor [5]. This disorder has been designated familial defective apolipoprotein B-100 (FDB)[6].

The major medications used in the treatment of familial hypercholesterolaemia are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) which inhibit the rate-limiting step of cholesterol synthesis in the liver, resulting in an increase in hepatic LDL-receptor activity and thus a decrease in the level of plasma LDL-cholesterol. A differential response to drug therapy between FH patients might occur if the residual receptor activity of the abnormal protein differed as a result of the particular mutation present. For example, carriers of 'mild' mutations where residual receptor activity is high (10-15% of normal) may have a greater lipid-lowering response than 'severe' alleles, where residual activity is zero and therefore no useful up regulation can be obtained by statin therapy.

A few studies have tested this hypothesis, the majority in countries where a founder gene effect is present and where as a result many FH patients have the same LDLR defect [7-10]. These patients are also likely to share other genes and environmental factors which may contribute to their phenotype. Two papers recently reported further investigation into the influence of the LDLR genotype and their response to lipid-lowering drug therapy. In one study the lipid-lowering response was compared in heterozygous carriers of mRNA positive and mRNA negative LDLR mutations, and the magnitude of the response was found to be similar [11]. In another study of 42 heterozygous FH cases, patients with 'severe' mutations had higher LDL-cholesterol before and during treatment with simvastatin and bile acid sequestrants than carriers of 'mild' mutations. The maximum inducible LDL-receptor activity in cultured lymphoblasts was found to be inversely correlated with LDL-cholesterol before and during treatment in patients with LDLR mutations but not in individuals with no detectable mutation [12].

These studies suggest that the response to cholesterol lowering therapy of FH may be determined to some extent by their specific LDLR mutation but they identify no clear relationship between the type or class of mutation (missense, null etc.) and degree of response, apart from the FH regression study which evaluated response in 23 patients with a known LDLR defect [12]. We have therefore carried out a retrospective study to evaluate and confirm the response to simvastatin in a genetically heterogeneous group of 109 patients with a clinical diagnosis of 'definite' or 'probable' FH. We have investigated the possible involvement of four different factors in determining the degree of LDL-cholesterol lowering in response to statin therapy, namely the presence or absence of TX, gender, APOE genotype, and type of LDLR mutation present. For this last analysis TX+ patients were classified as having a 'mild' or 'severe' type of LDLR mutation based on the predicted consequence of the detected mutation [1,4] and these were compared with TX+ patients where no mutation had been identified in either the LDLR or APOB gene. Effectiveness of treatment was assessed by calculating the percentage fall in LDL-cholesterol levels on treatment compared to untreated levels and also by determining the proportion of patients at each treatment dosage in whom LDL-cholesterol levels fell below 4.9 or 4.1 mmol/l. These values were chosen because they are the recommended intervention levels for hyperlipidaemic subjects without other risk factors (4.9 mmol/l), or with other CHD risk factors (4.1 mmol/l) [13,14].

2. Materials & Methods

2.1. Patient group

109 FH patients (101 probands) attending two lipid clinics in the South of England were recruited, all having been examined by one of us (MS). Eighty-six individuals (76 probands) had a diagnosis of 'definite' FH, i.e. untreated LDL-cholesterol greater than 4.9 mmol/l and tendon xanthoma (TX) in the patient or a first degree relative. 23 patients were classified as 'probable' FH, i.e. LDL-cholesterol greater than 4.9 mmol/l and a family history of hypercholesterolaemia and no TX detected [15]. Smoking habit was determined from patient notes and subjects defined as either a smoker (current or ex-smoker), or not a smoker. Hypertension was defined as systolic and diastolic levels of greater than 150 and 90 mm Hg respectively and patients were designated CHD positive on the evidence of a myocardial infarct, coronary artery bypass graft (CABG), per-
cutaneous transmullinal coronary angioplasty (PTCA) or positive coronary angiography. Fifty-eight patients were placed on 10 mg/day simvastatin. 33/58 were subsequently increased to 20 mg/day with the addition of another 43 patients (providing a total of 76 individuals) on a daily dose of 20 mg. A total of 49 individuals were given a 40 mg/day dose, two had been given only 10 mg previously, 18 had been given 10 and 20 mg previously, 23 on 20 mg and six individuals were initiated on the maximal dose of 40 mg after previous use of another statin. Patients were treated for not less than 3 months at each dosage.

2.2. Biochemical analyses

A fasted blood sample was collected in EDTA tubes and plasma was separated by centrifugation. Total cholesterol and triglycerides were measured on a Hitachi 747 enzymatic assay, high density lipoprotein (HDL) levels by an ELISA method [17]. Plasma levels of LDL-cholesterol were calculated by the Friedewald formula [18].

2.3. Molecular analyses

2.3.1. Isolation of DNA

Genomic DNA was isolated from frozen whole blood, using salt and ethanol precipitation [19].

2.3.2. LDLR analysis

The promoter and all 18 exons and their associated splice sites were amplified from patient genomic DNA by PCR using a microtitre tray format and analysed for single strand conformation polymorphisms (SSCP) [20]. Primer sequences are available on request from KH (kheath@hgmp.mrc.ac.uk). The 20 µl PCR reaction contained 50 mM KCl; 10 mM Tris–HCl, pH 8.3; 0.067% W-1 (Gibco BRL, UK); 1.5 mM MgCl2; 0.2 mM dATP, dGTP, dTTP, 0.02 mM dCTP; 0.05 mCi (32P)dCTP; 20 ng of each primer and 0.2 U of Taq polymerase (Gibco BRL, UK); in addition to 62.5 ng genomic DNA. PCR conditions were identical to the LDLR PCR. The amplified DNA was electrophoresed on a 0.8% agarose microtitre array diagonal gel electrophoresis (MADGE) [21] using a plastic support (Sigma electrophoresis film). The products were stained with ethidium bromide and visualised on a UV transilluminator, thus confirming amplification. The electrophoresed products were transferred to nylon membranes (Hybaid N+, Amersham International) and fixed by irradiating for 3 min in a UV crosslinker. Filters were pre-hybridised at 65°C and hybridised with the allele-specific oligonucleotide (ASO) probes for 12 h at 37°C in Boehringer–Mannheim chemiluminescence hybridisation solution according to the Boehringer–Mannheim Chemiluminescence kit. Two pairs of ASO’s were used for the R3500Q mutation, ASO1 (5'-GCA-CACGGTGTTC-3') and its mutant allele ASO2 (5'-GCACACAGTCCTC-3'), for the R3531C mutation, ASO3 (5'-CACTCCAACGCA-3') and its mutant allele ASO4 (5'-CACTCAAATGCA-3'). ASO’s were 3’-end-labelled with dioxygen according to the Boehringer-Mannheim Chemiluminescence labelling kit. Stringency washes at 37°C were as follows: 2 x 20 min at 0.5 x SSC, 0.1% SDS, 2 x 20 min at 0.1 x SSC, 0.1% SDS. The two normal R3500Q and R3531C filters and the mutant R3500Q filter had an additional wash of 5 x SSC, 0.1% SDS at 42°C and 40°C respectively. After washing, the filters were incubated at 37°C for 15 min and exposed to X-ray film Kodak X-Omat for 1 h and 2–6 h at room temperature.

APOB3500Q ‘dropouts’ and positive samples were then tested or confirmed using a two-test strategy for R3500Q. The test involves two artificial restriction site PCR digests [24]. A Sau96I restriction site is introduced in the mutant allele in another PCR. The 20 µl reaction was as for the ASO PCR except 80 ng of genomic DNA was added. Two
PCR sets were set up with two primer sets, FH210–211 (ARG primer set) and FH210–212 (GLN primer set) and conditions were as published [22]. The PCR products were then digested according to the original method except half volumes were used and the samples were electrophoresed on an ethidium bromide stained 4% agarose gel (3:1 NuSieve).

For APOB3531C, PCR dropouts were re-tested by a PCR digest using a natural Nsil restriction site for the R3531C [23]. A natural Nlalll restriction site was utilized to test for the R3500W mutation [24]. In both cases the mutant strand has an additional cut site. PCR's were set up according to the ASO PCR method, digested with the respective enzyme and visualised on an ethidium bromide stained 3% agarose gel.

2.3.4. APOE genotyping

APOE genotyping (E2, E3, E4) was carried out by gene amplification and cleavage with HhaI restriction enzyme [25].

2.4. Statistical analysis

The reductions in plasma lipid and lipoprotein levels were expressed as a percentage of untreated baseline levels, defined as [(baseline-treatment/baseline levels) × 100]. χ² analysis was used to compare the frequency of discrete variables between groups. Pearson’s correlation coefficients were determined. The Hardy–Weinberg value was calculated to test genetic equilibrium with respect to the distribution of the APOE genotype. The control group for APOE genotype consisted of 476 men and women from the county of Hertfordshire, England who were born between 1911 and 1930 and were available for the investigation [26]. Significance was tested at the P < 0.05 level. The data was analysed using SPSS 6.1.3 for Windows.

3. Results

Characteristics of the 56 male and 53 female FH patients are shown in Table 1. The men and women had similar mean age, BMI and baseline plasma lipid levels, and there was a similar proportion of current or ex-smokers, and patients with hypertension, tendon xanthomas (TX) and evidence of CHD.

Of the patients, 86 (76 unrelated patients) had TX and were therefore classified as ‘definite’ FH, while 23 (TX –) unrelated patients had a clinical diagnosis of ‘probable’ FH. In the TX + group a molecular defect in the LDLR gene was found in 30 patients, with three individuals having the APOB mutation, R3500Q, whilst only three patients had detected mutations in the ‘probable’ FH group, one in the LDLR (a null mutation in exon 13, 1944InsC) and two in the APOB gene (R3500Q). Overall the LDLR mutation detection rate in probands was 26% (26/99), however the detection rates were significantly different (P = 0.02) between the ‘definite’ (TX +) probands (detection rate of 37%) and ‘probable’ FH (TX –) probands (detection rate of 13%). In the TX + group no gender difference was observed in the detection rate, 37% in male probands (n = 41) and 34% in female probands (n = 35) (P = 0.97).

The mutation spectrum (Table 2) showed the expected high frequency of mutations in exon 4 of the LDLR [27], which encodes a part of the ligand binding domain. In all, 22 missense (two splice), five small deletions, two small insertions, one small rearrangement and one large deletion were detected. Eight mutations were novel to this study.

3.1. Response to simvastatin

Patients were placed on simvastatin at a dosage of either 10, 20 or 40 mg/day. All patients responded to simvastatin, 95% achieved a reduction in LDL-cholesterol to 20–40% of the baseline value, with 75% of patients achieving LDL-cholesterol level of under 4.9 mmol/l and 42% under 4.1 mmol/l. Mean HDL-cholesterol levels increased by between 7 and 9% at increasing dosage whilst triglyceride levels decreased by 6–25%. However no statistically significant differences were observed for HDL-cholesterol, triglycerides or Lp(a) levels in response to treatment and these parameters were not considered in subsequent comparisons.

Table 1

<table>
<thead>
<tr>
<th>Trait</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>56</td>
<td>53</td>
</tr>
<tr>
<td>Age (year)</td>
<td>47.2 ± 1.55</td>
<td>49.8 ± 1.89</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 0.44</td>
<td>24 ± 0.51</td>
</tr>
<tr>
<td>Current smoking</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>16%</td>
<td>13%</td>
</tr>
<tr>
<td>CHD</td>
<td>35%</td>
<td>38%</td>
</tr>
<tr>
<td>Baseline total cholesterol (mmol/l)</td>
<td>9.65 ± 0.24</td>
<td>10.12 ± 0.23</td>
</tr>
<tr>
<td>Baseline LDL-cholesterol (mmol/l)</td>
<td>7.70 ± 0.24</td>
<td>7.97 ± 0.23</td>
</tr>
<tr>
<td>Baseline HDL-cholesterol (mmol/l)</td>
<td>1.22 ± 0.14</td>
<td>1.39 ± 0.06</td>
</tr>
<tr>
<td>Baseline triglyceride (mmol/l)</td>
<td>1.88 ± 0.13</td>
<td>1.68 ± 0.12</td>
</tr>
<tr>
<td>Lp(a) median &amp; range (mg/dl)</td>
<td>45 (0–176)</td>
<td>37 (1–200)</td>
</tr>
<tr>
<td>No.of LDLR mutations</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>No.of APOB mutations</td>
<td>2</td>
<td>3</td>
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<tr>
<td>No.of individuals with no mutation</td>
<td>38</td>
<td>35</td>
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<tr>
<td>detected in LDLR or APOB</td>
<td></td>
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<tr>
<td>APOE E2E3 genotype</td>
<td>6</td>
<td>4</td>
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<tr>
<td>APOE E3E3 genotype</td>
<td>24</td>
<td>29</td>
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<tr>
<td>APOE E3E4 genotype</td>
<td>20</td>
<td>13</td>
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<tr>
<td>APOE E2E4 genotype</td>
<td>1</td>
<td>1</td>
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<tr>
<td>APOE E4E4 genotype</td>
<td>3</td>
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</table>

* Cholesterol values are expressed as mean ± SEM.
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Table 2
Clinical characteristics, lipoprotein profiles and molecular characterisation of 30 patients with ‘definite’ and one patient with ‘probable’ heterozygous familial hypercholesterolaemia and a defin

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3.2. Effect of the presence of TX on the baseline lipid levels and response to simvastatin

Untreated levels of HDL, triglycerides and Lp(a) were similar in TX+ and TX− individuals (data not shown). However, as shown in Table 3 the mean baseline LDL-cholesterol was significantly higher in the TX+ group (P = 0.0001). Both the mean total and LDL-cholesterol continued to be higher in the TX+ group at each simvastatin dose (P = 0.001, P = 0.003, P = 0.001 for 10, 20, and 40 mg/day respectively). At all doses the proportion of treated TX+ patients achieving LDL-cholesterol levels below 4.9 or 4.1 mmol/l was lower than in the group of TX− patients, and these differences in the proportion of patients of achieving acceptable LDL-cholesterol levels were statistically significant at all doses. However the overall percentage reduction in cholesterol was identical between groups at each simvastatin dose, with mean LDL-cholesterol reductions of ~30%, ~41% and ~51% being observed in each group, and this is shown in Fig. 1A.

3.3. Effect of gender on the response to simvastatin

As shown in Table 3, in the TX+ group there was no significant difference between men and women in the size of the lipid-lowering response to statin treatment or in the number of subjects achieving LDL-cholesterol levels below the 4.9 or 4.1 mmol/l thresholds at any dose (final treatment values for 4.9 mmol/l:...
Table 3
Mean (+SEM) LDL-cholesterol at baseline and after simvastatin treatment and the proportion achieving LDL-cholesterol levels below 4.9 and 4.1 mmol/l in 109 FH patients with and without tendon xanthomata (TX), in 47 TX+ males and 39 TX− females, and in 76 TX+ FH patients who are either APOE3 or E4 allele carriers

<table>
<thead>
<tr>
<th>TX positive</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>TX negative</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>TX+ males</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>TX+ females</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>Presence of E4 allele</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>Absence of E4 allele</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male: female</td>
<td>47:39</td>
<td>9:14</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20:12</td>
<td>–</td>
<td>21:23</td>
<td>–</td>
</tr>
<tr>
<td>Baseline total chol (mmol/l)</td>
<td>10.18 ± 0.19* (n = 86)</td>
<td>8.82 ± 0.27 (n = 23)</td>
<td>9.93 ± 0.26 (n = 47)</td>
<td>10.4 ± 0.27 (n = 39)</td>
<td>9.68 ± 0.26 (n = 32)</td>
<td>10.40 ± 0.28 (n = 44)</td>
<td>6.38 ± 0.17* (n = 40)</td>
<td>5.15 ± 0.24 (n = 9)</td>
<td>6.18 ± 0.23 (n = 24)</td>
<td>6.69 ± 0.28 (n = 16)</td>
<td>6.15 ± 0.34 (n = 21)</td>
</tr>
<tr>
<td>Final total chol (mmol/l) 40mg/day</td>
<td>8.14 ± 0.19* (n = 86)</td>
<td>6.81 ± 0.25 (n = 23)</td>
<td>7.99 ± 0.26 (n = 47)</td>
<td>8.24 ± 0.27 (n = 39)</td>
<td>7.63 ± 0.28 (n = 32)</td>
<td>8.34 ± 0.28 (n = 44)</td>
<td>5.76 ± 0.25* 15(52%)** 6(28%) (n = 46)</td>
<td>4.51 ± 0.27 (n = 12)</td>
<td>8(67%) (n = 22)</td>
<td>5.99 ± 0.34 (n = 33)</td>
<td>7(32%) 2(29%) 5.54 ± 0.38 (n = 24)</td>
</tr>
<tr>
<td>LDL-cholesterol at simvastatin 10mg/day</td>
<td>4.99 ± 0.17* 32(58%)** (n = 60)</td>
<td>3.89 ± 0.15 (n = 14)</td>
<td>13(93%) (n = 37)</td>
<td>19(51%) (n = 23)</td>
<td>12(52%) (n = 23)</td>
<td>14(74%)** (n = 19)</td>
<td>5.24 ± 0.21 (n = 34)</td>
<td>20mg/day</td>
<td>4.55 ± 0.17* 26(58%)** (n = 40)</td>
<td>3.23 ± 0.19 (n = 9)</td>
<td>9(100%) (n = 24)</td>
</tr>
</tbody>
</table>

* Indicates a significant difference between TX positive and TX negative subjects at P<0.005.
** Indicates a significant difference between TX positive and TX negative subjects at P<0.05.
3.4. Effect of APOE alleles on baseline lipid levels and on the response to simvastatin

In the whole group of FH patients, the frequency of genotypes was as expected from Hardy–Weinberg proportions. The allele frequencies of the APOE polymorphism was E2:0.06, E3:0.72, E4:0.22 which was significantly different from that reported in a healthy UK population sample (P < 0.01), due to the higher frequency of the E4 allele. There was no difference in the distribution of APOE alleles in the group of patients with or without TX or in those with or with no detected mutation (data not shown).

Due to small sample size, baseline lipids and response to statins was only compared between E3/E3 and E3/E4 & E4/E4 individuals. As shown in Table 3, in the TX + group the presence of the allele E4 was associated with 8.5% lower LDL-cholesterol at baseline and 7.2% lower levels on the 40 mg/day dose, although these differences were not statistically significant (P = 0.09 and P = 0.40 respectively). Compared to the E3/E3 group, those with the apo E4 allele had significantly lower levels at 10 mg/day (20.8%, P = 0.02) and at 20 mg/day (18.6%, P = 0.01) when compared to E3/E3 individuals, however the percentage reduction was not significantly different at any treatment dose (overall reduction achieved E3/E3 vs. E3/E4 & E4/E4, 49% vs. 47%, P = 0.63), nor was the proportion of E4 patients achieving the 4.1 mmol/l level greater than that of the E3/E3 group at all doses of simvastatin (data not shown).

3.5. Effect of LDLR mutation on baseline lipid levels and on the response to simvastatin in TX + patients

Due to the small number of patients with the apo B3500Q mutation, no detailed analysis of their response to statin treatment was carried out, although the mean percentage reduction in LDL-cholesterol levels achieved by this group was similar to that seen in the group overall (data not shown). Baseline lipid levels and response to treatment was compared in the TX + group who had either no mutation detected or any LDLR mutation detected. Detailed characteristics of each of the 30 TX + patients (and the one TX — patient) who carried an identified LDLR mutation are presented in Table 2. There were no significant differences in mean levels of HDL, triglycerides or Lp(a) between patients who had any LDLR mutation or no mutation detected (data not shown). However, as shown in Table 4 the mean baseline LDL-cholesterol level was 7.4% higher in the group of individuals with any identified LDLR mutation, although this difference did not reach statistical significance. This relative difference in mean LDL-cholesterol levels was also seen at all drug treatment points. However, the mean percentage reduction with each dose was similar for those with or without a detected LDLR mutation. The proportion of patients in each group achieving the 4.9 and 4.1 mmol/l levels were greater in the no detected mutation group compared to the LDLR mutation group, although none of these differences achieved statistical significance.

The 30 definite FH individuals carrying an identified LDLR mutation were subdivided into patients carrying 'severe' or 'mild' mutations. 'Severe' mutations were classified either as exon 4 repeat 5 mutations or null mutations (n = 14) whilst 'mild' mutations were all other LDLR mutations combined (n = 16). As shown in Table 4 and Fig. 1B, compared to those with no detected mutation, mean LDL-cholesterol levels were 7.6% higher in those with a 'severe' mutation, and 7.3% higher lower in those with a 'mild' mutation but these differences were not statistically significant. This ranking was similar at all treatment points, and at the 40 mg dose mean LDL-cholesterol levels in the 'severe' group were significantly higher than in the other two groups of patients (by ANOVA P = 0.02). The proportion of ‘severe’ patients achieving the 4.9 or 4.1 mmol/l levels was less than for the ‘mild’ patients at all dosages, and this difference was statistically significant at 40 mg/day for the 4.1 mmol/l threshold (‘severe’: 0% vs. ‘mild’: 70%, P = 0.018) (Table 4 and Fig. 2). When patients were grouped according to exon 4 repeat 5 and exon 4 null mutations (n = 7) versus all other LDLR mutations (n = 23), no difference in baseline levels or in statin response between groups was observed (data not shown).

4. Discussion

In this study we have investigated the possible involvement of four different factors in determining the degree of LDL-cholesterol lowering in response to statin therapy, namely presence/absence of TX, gender, APOE genotype, and type of LDLR mutation present. The current consensus guidelines [13,14] propose that the goal of statin treatment in a hypercholesterolaemic patient is to reduce LDL-cholesterol to 4.9 mmol/l if no other risk factors are present and to 4.1 mmol/l in their presence, and less than 3.2 mmol/l in those with CHD, with the necessary increase in dosage to achieve this for each patient [13]. Other, lower, levels could also be proposed and may be more desirable for FH patients, but we have taken these thresholds for analytical comparison. The clinically important aspect of treatment is therefore the proportion of patients achieving the de-
Table 4
Comparison of mean (±SEM) LDL-cholesterol levels at baseline and at simvastatin doses of 10, 20, 40 mg/day in TX+ patients who either have a 'severe' LDLR mutation (exon 4 repeat 5 or a null mutation) or a 'mild' LDLR mutation (LDLR mutation other than exon 4 repeat 5 or a null mutation)

<table>
<thead>
<tr>
<th></th>
<th>'Severe' LDLR mutations</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>'Mild' LDLR mutations</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>No. mutation in the LDLR or APOB</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
<th>Any LDLR mutation</th>
<th>No. below 4.9 or 4.1 mmol/l (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male: female</td>
<td></td>
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</tr>
<tr>
<td>Baseline total cholesterol (mmol/l)</td>
<td>10.44 ± 0.46 (n = 14)</td>
<td>7.8 (n = 14)</td>
<td>10.51 ± 0.40 (n = 16)</td>
<td>29.24 (n = 53)</td>
<td>16.14 (n = 30)</td>
<td></td>
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<tr>
<td>Final total cholesterol at 40 mg/day simvastatin (mmol/l)</td>
<td>7.22 ± 0.33 (n = 6)</td>
<td>6.39 ± 0.32 (n = 11)</td>
<td>6.17 ± 0.24 (n = 22)</td>
<td></td>
<td>6.68 ± 0.25 (n = 53)</td>
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<tr>
<td>Baseline LDL-cholesterol (mmol/l)</td>
<td>8.56 ± 0.48 (n = 14)</td>
<td>8.53 ± 0.42 (n = 16)</td>
<td>7.95 ± 0.25 (n = 53)</td>
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<td>8.54 ± 0.31 (n = 30)</td>
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<tr>
<td>LDL-chol at 10 mg/day simvastatin (mmol/l)</td>
<td>6.83 ± 0.52 (n = 8)</td>
<td>0(0%) 0(0%)</td>
<td>5.67 ± 0.58 (n = 7)</td>
<td>2(29%) 2(29%)</td>
<td>5.53 ± 0.34 (n = 29)</td>
<td>12(41%) 4(14%)</td>
<td>6.29 ± 0.40 (n = 15)</td>
<td>2(13%) 2(13%)</td>
</tr>
<tr>
<td>LDL-chol at 20 mg/day simvastatin (mmol/l)</td>
<td>5.51 ± 0.49 (n = 11)</td>
<td>3(27%) 3(27%)</td>
<td>4.99 ± 0.30 (n = 13)</td>
<td>8(62%) 4(31%)</td>
<td>4.86 ± 0.21 (n = 35)</td>
<td>19(54%) 10(29%)</td>
<td>5.22 ± 0.28 (n = 24)</td>
<td>11(46%) 7(29%)</td>
</tr>
<tr>
<td>LDL-chol at 40 mg/day simvastatin (mmol/l)</td>
<td>5.54 ± 0.30* (n = 6)</td>
<td>2(33%) 0(0%)*</td>
<td>4.38 ± 0.28 (n = 11)</td>
<td>7(64%) 5(45%)</td>
<td>4.38 ± 0.25 (n = 22)</td>
<td>16(73%) 10(45%)</td>
<td>4.79 ± 0.24 (n = 17)</td>
<td>9(53%) 5(29%)</td>
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* Indicates significance between the two groups at P < 0.02.
Fig. 2. Dose response curves of LDL-cholesterol in 'definite' heterozygous FH patients (TX+). The LDL-cholesterol target point of 4.1 mmol/l is indicated with a dashed line and the number of individuals reaching the target level are represented as a proportion of the total number of each class. A: TX+ patients with 'severe' (n = 14) LDLR mutation. B: TX+ patients with a 'mild' (n = 16) LDLR mutation.

sired LDL-cholesterol level at any particular level of statin treatment. Thus, for factors associated with higher untreated LDL-cholesterol, the percentage fall in LDL-cholesterol with a particular dose of treatment may be different, but this does not mean that response is materially different or clinically relevant.

The results from trials investigating the effect of lowering LDL-cholesterol on reducing CHD have suggested that the percentage reduction in LDL-cholesterol may be as good a predictor of reduction in cardiovascular risk as is the absolute cholesterol level achieved [28,29]. This hypothesis is unproven in patients with FH, who are at high risk from elevated LDL-cholesterol but in whom incidence of other risk factors such as hypertension, diabetes or smoking, is typically not increased. However it is not ethical to carry out placebo-randomised control studies in this group of patients to test the hypothesis. The concept of percentage reduction is particularly important in those patients with FH who have CHD, in whom the target level of
LDL-cholesterol of <3.2 mmol/l is difficult to achieve, even with highly effective drug therapy [30-32]. Coronary angiographic studies have suggested that regression of atherosclerosis does not occur until a 45–55% reduction in LDL-cholesterol is achieved. However in patients with FH, lesion progression can be reduced at smaller percentage reduction of LDL-cholesterol levels [33]. This suggests that other mechanisms such as the stabilisation of atherosclerotic plaque, effect on smooth muscle cell proliferation, restoration of endothelial function, reduction in platelet activity and alteration in macrophage-induced cytokines may also be important in the prevention of lesion progression and reduction of cardiac events [34]. Recent analysis of the number of CHD deaths occurring in patients from the Simon Broome register of patients with FH in the UK show that compared with the high event rate in the period 1986–1991 [15] there has been a marked reduction in coronary events over the last 5 years since statins became commonly available [35]. These observational results are in keeping with the hypothesis that a 20–30% reduction in LDL-cholesterol in patients with high LDL-cholesterol achieves a greater reduction in mortality than in those with lower baseline levels [36].

In this group of FH patients, simvastatin treatment reduced LDL-cholesterol in all individuals, 75/109 reached levels of 4.9 mmol/l and 42/109 achieved 4.1 mmol/l or below. The effect of simvastatin on the percentage lowering of LDL-cholesterol was similar for the groups of patients classified as ‘definite’ FH (TX+) or ‘probable’ FH (TX—), although baseline untreated LDL-cholesterol values were 1.3 mmol/l higher in the ‘definite’ FH individuals. However, the final mean LDL-cholesterol values on treatment remained 1.3 mmol/l higher in the TX+ patients, and significantly fewer of the TX+ group achieved levels below 4.9 or 4.1 mmol/l compared to the TX— group. The similarity in lipid-lowering observed with increasing dose of statin in the TX+ and TX— patients suggests that there are no major differences in the characteristics of these two groups (whether genetic or environmental) that influence response. However, whether the failure in the TX+ group to achieve a greater reduction in LDL-cholesterol levels will result in more future coronary events will require investigation in a longitudinal study.

The APOE polymorphism is known to have an important influence on plasma and LDL-cholesterol levels in the general population [37] and was examined as a genetic factor likely to determine baseline lipid levels and response to treatment. The observation in the sample of a higher frequency of the E4 allele in FH patients compared to the general population confirms reports of others [38]. Several studies have suggested that APOE genotype does not alter the response to statins [39,40], although a non-significant lower response in E4 carriers treated with lovastatin has been reported compared to E3 carriers [41]. The present study found no significant difference in response to statins in E4 carriers compared to E3 carriers.

The molecular analysis of the 86 ‘definite’ FH patients (76 probands) resulted in a mutation detection rate that was lower than but not dissimilar from other studies of genetically heterogeneous populations [2,4,20]. This low rate may be due to the insensitivity of the SSCP technique, as well as the fact that large deletions or insertions were not examined in all patients, nor were introns or the 3' untranslated region. Others have also shown that some patients with clinical heterozygous FH do not have defective LDL-receptor activity in their cultured cells, and that other inherited defects may therefore be responsible for the hypercholesterolaemia [42]. We have not analysed LDL-receptor activity in any of the patients so cannot exclude this possibility. In the individuals where no molecular defect had been identified, co-segregation analysis of the LDLR would be possible where extended families were available [43,44], to determine whether or not the LDLR locus is likely to be the cause of the FH, or provide evidence for whether another gene is defective and causing clinical FH [45].

Overall, the frequency of FDB was 5%, similar to the incidence previously reported for hypercholesterolaemic patients in the United Kingdom [5], three with TX and two without. As seen in other studies [5,6], these FDB patients had slightly lower mean untreated plasma LDL-cholesterol levels than those with a detected LDLR mutation (6.95 vs. 8.32 mmol/l, P = 0.07), but because of the small number of patients available no analysis of response was carried out in this group. For the LDLR gene, the detection rate was 3-fold higher in the TX+ individuals compared with the TX— group which confirms the diagnostic usefulness of TX for the disorder. Only one patient with an LDLR mutation was found in the TX— group, and this raises the possibility that a greater proportion of these ‘probable’ FH patients may not in fact have FH, but may have either a different genetic cause of the familial hyperlipidaemia (such as recently suggested in one family from Utah [45], or they may have primarily ‘non-genetic’ causes of the hyperlipidaemia despite having a strong family history.

The region of the gene where the greatest number of mutations was found was exon 4 as previously described [27]. The 3' part of exon 4 codes for repeat 5, the apoB/apoE ligand binding domain of the LDL-receptor, and mutations in this region have been found to have a more severe phenotype [46]. Thus in the TX+ group, patients were classified into three groups to examine baseline cholesterol levels and drug response; a ‘severe’ or a ‘mild’ LDLR mutation or no mutation detected (in either the LDLR or APOB gene). The
subgroups were classified by the severity of the LDLR mutation determined from published or predicted binding affinities to apoB and apoE containing lipoproteins [1]. 'Severe' mutations, null mutations and mutations in repeat 5 of exon 4, were those predicted to have <2% residual LDL-receptor activity and no apoE ligand binding ability, i.e. no VLDL or IDL binding. 'Mild' LDLR mutations were those predicted to have some residual LDL-receptor activity, and/or where the ability to bind apoE containing lipoproteins would not be impaired. One intronic mutation was identified five bases downstream of the exon–intron boundary and this was characterised as 'mild', although no functional studies were undertaken to confirm the pathogenic nature. Although the groups of patients had virtually identical baseline LDL-cholesterol, the LDL-cholesterol in those with the 'severe' exon 4 repeat 5 or null mutations did not decrease with treatment to the same extent as in the patients with the 'mild' mutations. Thus, although the small sample number of patients with a detected mutation in this genetically heterogeneous group limits the power of the study, the data suggest that statins had a greater effect on the 'mild' mutation group, which is compatible with the hypothesis that overall, these mutations have a higher level of LDL-receptor residual activity that may be upregulated [46]. These observations confirm the data from the recently published FH regression study, where LDLR mutations in 23 patients were also subdivided by predicted severity [12]. The baseline LDL-cholesterol was highest in individuals with 'severe' mutations, intermediate in those with 'mild' mutations, and lowest in the no identified mutation group, and as found in our study, the treatment levels also followed the same molecular defect severity gradient.

Genetic analysis for any disorder is most relevant for identification of relatives who also carry the mutation so that monitoring, lifestyle advice and therapy when appropriate can be targeted most effectively. Molecular analysis is particularly advantageous in young children with FH, where the total cholesterol values of affected and unaffected children may overlap [47,48], so that both false positive and false negative diagnosis can occur. By contrast, mutation testing in relatives gives unequivocal results, and early systematic diagnosis may be helpful for their families [49]. In this group of UK FH patients the mutation detection rate was almost 3-fold higher in individuals with tendon xanthomata and this suggests that the most cost-effective strategy for mutation screening would be to focus genetic testing on these subjects.

In this study response was similar in men and women and in patients with different apoE genotype, but patients with 'definite' FH carrying a mutation which is predicted to severely disrupt LDL-receptor function (mutations in repeat 5 in exon 4 and null mutations) were more likely to require treatment at a high dosage to achieve the same level of LDL-cholesterol compared to patients with a 'mild' mutation or patients with no detected mutation. In 16 of the group of 30 patients in whom a mutation was detected, the mutation was designated as likely to be 'severe'. Five of these 16 had CHD confirmed on the basis of MI, CABG, PTCA or positive coronary angiography out of the total of 15 who had confirmed CHD in the entire mutation detected group. Thus no strong relationship was demonstrated between 'severity' of mutation and presence of CHD, which is not surprising given the multifactorial nature of CHD, and variation in patient management. The challenge to the clinician is to treat effectively those patients with FH who are most at risk of developing premature CHD. In this respect, it is not necessarily the genetic diagnosis, or the type of mutation, be it 'severe' or 'mild' which is important, but the ability to assess the impact of other risk factors and to detect early atherosclerosis. It is unlikely that trial evidence on which to base a desirable target for LDL-cholesterol concentration or percentage reduction will become available for FH patients, and thus refining of risk by using other possible phenotype or genotype information is important.

Acknowledgements

The authors would like to thank: Manjeet Bolla for the APOE genotyping at the Icelandic Heart Association supported by the Icelandic Council of Science, and Dr Mike Seed and Rosie Seed for their help in collating the patient data. The patients studied are registered with the Simon Broome Familial Hypercholesterolaemia Register. Karen Heath is a PhD student sponsored by the John Pinto Foundation, Villi Gudnason and Steve Humphries are recipients of British Heart Foundation grants (RG/95007 and PG/95189). A grant from Merck Sharpe & Dohme provided support for consumables.

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Letter to the Editor

Rapid detection of polymorphisms in exons 10, 11 and 12 of the low density lipoprotein receptor gene (LDLR) and their use in a clinical genetic diagnostic setting

To the Editor:

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder caused by mutations in the low density lipoprotein receptor gene (LDLR) (1). It is characterised by hypercholesterolaemia, tendon xanthomas (TX), premature coronary heart disease and a family history of one or more of these. DNA tests for relative tracing are feasible and useful (2), especially in paediatric cases where there is an overlap between cholesterol levels in FH and normal subjects (3). We have established a clinical genetic diagnostic service for FH (2) and have been using single strand conformation polymorphism (SSCP) for rapid mutation screening of the LDLR (4). The method gives a detection rate in adult FH probands of ~40% (5) and in child FH probands of ~70% (Heath and Humphries, unpublished data).

In the UK, a quality assurance scheme operates in clinical diagnostic laboratories. For a diagnostic report to be issued, a restriction enzyme polymerase chain reaction (PCR) assay or sequencing in both directions must be carried out to distinguish SSCP band shifts caused by polymorphisms from mutations. We set out to design rapid PCR assays for the G1413A, C1617T and C1725T polymorphisms in exons 10, 11 and 12 of the LDLR, respectively (details on FH website: http://www.ucl.ac.uk/fh, (6)). Recently identified and now commercially available, restriction enzyme sites are created by the exon 10 and 11 polymorphisms, BsmAI and AciI respectively, but no available enzyme site is altered for the exon 12 polymorphism; thus, an MseI site was introduced by 'forced' PCR (7).

Results and discussion

The frequency of the three polymorphisms was determined in 100 normal UK subjects. The exon 10 polymorphism was found to be relatively common in this population (Freq A = 0.31, 95% CI 0.29–0.49) but the exon 11 (Freq T = 0.08, 95% CI 0.039–0.11) and exon 12 polymorphisms (Freq T = 0.10, CI 0.06–0.14) were rare.

The utility of these assays in a clinical genetic diagnostic setting is demonstrated. A 10-year-old boy presented at a paediatric lipid clinic with an untreated plasma cholesterol of 8.1 mmol/l. His father had died of a myocardial infarction at the age of 42 years (cholesterol unknown) and his sister has hypercholesterolaemia (8.0 mmol/l) (Fig. 1a). A diagnosis of possible FH was given, as no TX were visible. The only difference in SSCP band shifts between the two samples, the son and his unaffected mother, was found in exon 12 (Fig. 1b). The SSCP pattern of exon 12 is especially complex because of the presence of two polymorphisms, C1725T and C1773T (HincII, FH website), which makes it difficult to distinguish between band shifts caused by mutations from polymorphisms. This difficulty was resolved by determining the C1773T and C1725T genotypes with the HincII and ‘forced’ MseI assays (Fig. 1a). The two subjects were found to have the same genotypes, thus suggesting that the extra SSCP band, in the affected son only, may be due to the presence of an exon 12 mutation. Sequencing analysis revealed a C > T nucleotide change at 1823 in II.1 (Fig. 1c) which the mother did not carry. This base change is predicted to result in the substitution of a proline for a leucine substitution at codon 587. Although we have no formal proof that this substitution is FH-causing, it has recently been reported in another patient with FH (A Peeters, FH website) and proline-leucine substitutions have been found to cause FH (FH website).

Thus in combination with SSCP mutation screening, the three polymorphism assays will be useful in the clinical genetic diagnostic service for FH.

Karen E Heath
Steve E Humphries
Letter to the Editor

Fig. 1. Mutation analyses of the LDLR and APOB genes in a family with FH are as described (4, 5). Filled symbols represent subjects with hyperlipidaemia. a) The cholesterol levels, the exon 12 genotypes, C1725T (‘forced’ MseI assay) and C1773T (HincII assay) are presented on the pedigree. The PCR conditions for the polymorphism assays are as published (5) except W-1 was omitted in the ‘forced’ exon 12 PCR. Primer sequences are on the FH website and the ‘forced’ antisense primer for exon 12 is 5’-TGAATTTGAGGTCAAACCATT-3’ (‘forced’ base marked in bold). The digested PCR products are separated on an Et-Br stained 4% (3:1 NuSieve) agarose gel (exons 10 and 11) or a silver-stained 10% polyacrylamide gel (exon 12); b) the SSCP gel of exon 12 is shown for 18 different subjects, with the family members indicated and the SSCP band suggestive of an additional sequence variation is shown by the arrow; c) two ABI electrophoregrams of part of exon 12 show the presence and absence of the P587L mutation in the son (II.1); and mother (I.2), respectively. The P587L mutation is due to a C > T alteration at 1823 and is indicated as an N.

Acknowledgements
We thank Professor James Leonard who referred this patient and Dr Maureen Boxer for helpful comments on the manuscript. The work was supported by grants from the John Pinto Foundation and British Heart Foundation (RG/95 007).

References


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The low-density lipoprotein receptor (LDLR) is expressed in many tissues and plays a crucial role in the metabolism of cholesterol, mediating the transport of cholesterol into cells. Defects in the LDLR give rise to familial hypercholesterolemia (FH), which is characterized clinically by elevated levels of plasma cholesterol, tendon xanthomata, and early-onset coronary heart disease. FH is an autosomal dominant disorder affecting about 1 in 500 persons in the population with heterozygous mutations. Homozygote individuals are found at a frequency of 1:10^6 in most populations. In geographically or culturally isolated populations, FH is found at much higher frequency; often, certain mutations dominate because of founder effects, e.g., in French Canadians, Afrikaners, Christian Lebanese, Druze, and Ashkenazi Jews.

The gene is located on chromosome 19 (p13.1 to p13.3), spanning 45 kb and comprising 18 exons and 17 introns that code for a mature protein of 839 amino acids. To date, >400 mutations have been identified in the LDLR gene. To facilitate the collection of information on these mutations and provide a core resource for information on the LDLR gene, we set up a mutation database on the World Wide Web (WWW). This database contains a variety of fields of information about each mutation. At present, there are >400 mutations in the database; we expect the database to grow and provide a core resource to enable further research, such as genotype and/or phenotype analysis.

The database can be accessed free of charge via personal computer with WWW access and a web browser software package; the universal resource locator is http://www.ucl.ac.uk/fh. On accessing the database, the user is immediately taken to an introductory screen giving hypertext-linked access to other areas of the database. The database consists of 2 core elements, a sequence file, and a mutation list (see Figure 1). The annotated sequence file is a feature of this database that is not found on many of the other mutation databases on the WWW. It consists of the full coding sequence for each of the 18 exons of the gene and some of the sequence of the 17 introns of the gene. This sequence file exists in 2 formats essentially based on the same structure of sense and antisense nucleotide base sequence, numbered according to the method of Sudhof et al, with the corresponding amino acid shown for each codon given in single amino acid code. In addition, this file has some information on functional regions in the protein and on polymerase chain reaction primers used to amplify regions of the gene in our laboratory. We hope that these will be of use to other workers in this field.

Mutations in the gene are represented by an asterisk and numbering system above the mutation site in the gene. This system allows the mutations to be catalogued and identified in the mutation list. In the second sequence map, each mutation number and asterisk is hypertext-linked to information on the mutation. Simply clicking on the mutation, using a computer mouse, displays the information on that mutation. The hypertext link is created by the curator after new mutation data has been received and reviewed.

Information on each mutation is provided in a variety of fields: nucleotide base change and number, amino acid change and number, the country in which the mutation is found, any geographic region within this country, and the author plus citation, if available. In addition, there is a comments field for more information about the mutation. This mutation list is searchable in 1 or more of the information fields provided for each mutation. For example, typing "W66G" in the "Amino Acid Change" field brings up information on the mutation, including its systematic name (c.259T>G) according to the accepted nomenclature of Antonarakis. The mutation can be identified by searching under "French Canadian" or under any of the "authors" or in the "nationalities" field. If >1 field is used, the stringency of the search is increased. This mutation list does not contain large deletions or insertions found in the gene; these are represented separately in tabular form, with relevant information on each deletion or insertion given with the same fields as for the point mutations.

New mutations can be submitted to the database electronically. The information submitted is held until it can be reviewed appropriately, and then the new mutations are added to the database. In the submission
form, submitters are asked to provide information in a variety of fields—name, contact address, and so on—and they can also ask for the mutation to be withheld from the database for a period of time to enable publication. We envisaged that in the future, a citation from the database will be sufficient reference for a mutation. The submission form asks for a description of the mutation and the techniques used to identify and validate it, including confirmation on a second polymerase chain reaction. Finally, regional and geographic data, the ethnic origin of the patient, and phenotypic and haplotypic information can be submitted. It is not essential that information be provided in all fields.

The database’s curators can be contacted through the web site. There are also hypertext links to other databases that may be of interest to the users, such as the Genome Database, Online Mendelian Inheritance in Man (OMIM), the Mutation Database Web Site (Melbourne, Australia), and the Human Gene Mutation Database (Cardiff, Wales, United Kingdom). This database can thus act as an electronic journal for publications of mutations in the LDLR gene, with potential for both refereed and unrefereed data to be handled and be available on the WWW. Current plans are for occasional hard-copy publication in an appropriate journal.

We believe that the database will be of use to those studying familial hypercholesterolemia to allow collection and collation of new mutations in the gene and to those who need access to information on mutations already found in the gene. It will allow researchers who have identified the same mutation in different patients to set up collaborative studies to investigate the effect of the mutation on clinically important phenotypes, such as plasma cholesterol and age of onset of coronary artery disease. The database will also be a model for databases in other genes where mutation informa-
1705 variant in the low-density lipoprotein receptor gene has no effect on plasma cholesterol levels.

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Short title: LDLR 1705 variant has no effect on cholesterol levels.

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Abstract

An exon 15 C>T substitution at 2177 of LDLR alters threonine at 705 to isoleucine and has been identified both in individuals with familial hypercholesterolaemia (FH) and in subjects with plasma cholesterol levels in the normal range. To explore the functional significance of the substitution a high throughput genotyping MADGE method was developed and used to screen 2287 healthy Caucasian men from the Northwick Park Heart Study (NPHS-II). Thirty heterozygous carriers were identified, a carrier frequency of 1.3%. No significant differences in plasma total cholesterol or triglycerides were observed between 1705 carriers and non-carriers. The majority (n=28) shared an LDLR haplotype, defined by the presence of the 1061-8C variant in intron 7. In 200 individuals from the NPHS-II no additional individuals were shown to carry the intron 7 variant, thus the carrier frequency was 1.2%. Therefore the two variants, 1705 or 1061-8C are unlikely to be affecting the function of the LDL-receptor and the detection in subjects with FH is a consequence of their allele frequency in the general population.

Key words

Low-density lipoprotein receptor gene (LDLR), mutation, T705I, variant.
Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant inherited lipoprotein disorder characterised by elevated plasma low-density lipoprotein (LDL) levels, xanthomas, premature coronary heart disease and a family history of one or more of these. Homozygous FH occurs in 1 in a million individuals and they are severely affected whilst heterozygotes are moderately affected and occur at a frequency of 1 in 500 in genetically heterogeneous populations. FH is caused by a mutation in the LDL-receptor gene (LDLR) and over 700 mutations have been reported [1, http://www.ucl.ac.uk/fh]. Among these a missense mutation, T705I in exon 15 (FH Paris-9), was originally reported in a compound heterozygote ('homozygous' FH subject) of French-American origin [2] but has now been observed in several heterozygotes who also carry another mutation in the coding region of the LDL-receptor protein [3, 4, 5]. The presence of the I705 variant has also been reported in two normcholesterolaemic subjects in the heterozygous and homozygous form, which led to the suggestion that the T705I change is a non-FH causing variation [6]. The most recent report of this variation was in a Spanish family where the hypercholesterolaemia segregated with the I705 substitution and no other mutation was identified [7]. Possible explanations for these contradictory findings have been that the exon 15 variant is only pathogenic when another environmental or genetic factor is present, or that in some subjects the I705 variant is in linkage disequilibrium with a second as yet unidentified causative mutation.

We have set up a clinical genetic diagnostic service for FH [8] and the I705 variant was identified in an individual with a clinical diagnosis of possible FH who was referred for FH genetic testing. To investigate the pathogenicity of the amino acid substitution at codon 705 we have determined the frequency of the I705 variant in 2287 healthy UK men and examined the effect of this variant on plasma lipid levels.
A one way analysis of variance was used to compare the lipid levels between the I705 carriers and non-carriers. The data was analysed using STATA (Intercooled Stata 5.0).
Results

In the family shown, the index patient, aged 5, was referred from Great Ormond Street Hospital for Children, London. She had an untreated total cholesterol of 4.2 mmol/l which is at the 40th percentile for age [18], although she was taking a lipid restricted diet. Her mother, who is 34 years, presented with hypercholesterolaemia (8 mmol/l) and xanthelasma and her grandfather had a myocardial infarction (MI) at 39 years and died at 55. In the index case (Fig 1A) no mutation was identified, by SSCP analysis and UPQFM-PCR, in LDLR (point or major rearrangement) or in the apolipoprotein B-100 gene (APOB) by direct assays. Mutation screening was then carried out on a sample from her hypercholesterolaemic mother, in case a mutation was present in the mother which the daughter had not inherited. Two SSCP band shifts were identified (excluding band shifts caused by known polymorphisms) and characterised to be due to the C>T transition at nucleotide 2177 in exon 15 and T>C at 1061-8 in intron 7. The variants were confirmed to be present in the mother and absent in the daughter by the respective digests, Nsil and Earl (Fig 1 B & C).

To determine the frequency of the I705 variant a microtitre array diagonal gel electrophoresis (MADGE) method was developed and 2287 healthy Caucasian men were screened from the NPHS-II. As shown in Table 1, 30 carriers were found, 28 of which also had the intron 7 variant 1061-8C. Thus the carrier frequency of the I705 variant was 1.3%. The intron 7 variant was analysed in 200 men from the NPHS-II study and no additional intron-7 carriers were found. Therefore the carrier frequency of the intron 7 variant was calculated to be ~1.2%. No statistically significant differences were observed between the mean total cholesterol and triglyceride levels of the I705 carriers and the non-carriers (Table 1).
Discussion

The index patient had an untreated total cholesterol at the 40th percentile for her age [18], although she was taking a lipid restricted diet. This value is within the normal range suggesting she may not have FH, although there is considerable overlap in cholesterol levels between normal and affected children [19, 20] and in some cases elevated levels may develop later in life [21]. A clinical diagnosis of 'possible' FH was given and the index case had a 50% chance of inheriting the defective allele. In this family, the only sequence alterations were I705 and 1061-8C, identified in the mother but not in the daughter suggesting the daughter was at low risk of FH. This conclusion depends on the strength of the inference that I705 and/or 1061-8C are pathogenic or whether the mother may be a carrier for another unidentified mutation that had been inherited by the daughter. In the light of these findings the effect of the I705 and 1061-8C variants needed to be assessed.

Published data are inconsistent as to whether the exon 15 C>T substitution at 2177, which alters the amino acid at codon 705 from a threonine to a isoleucine, is affecting LDL-receptor function. In a study from the Netherlands 100 normolipidaemic controls were screened by DGGE and sequencing and the I705 variant was found in two individuals, one heterozygous carrier and one homozygote [3]. Therefore the carrier frequency of the I705 allele was 2% which is very similar to the estimate in the larger group of UK men, 1.3%. All but two I705 carriers from the NPHS-II group carried the 1061-8C variation in intron 7, which has been associated with this exon 15 variation [4]. The 1061-8C variant was screened in 200 men from the NPSH-II group and the variant was only detected in the 1705 carriers thus the carrier frequency was estimated to be ~1.2% in the general population.

Taken together these data strongly suggest that the I705 variant is not having a major effect on LDL-receptor function. It is the second ‘non-functional’ variant described to date, with only the A370T being previously known. T370 occurs at a frequency of 6% in the UK [22] and is associated with, at most, only a modest effect on plasma lipid levels. Cell studies have not detected a significant impairment of LDL-receptor function of the T370 substitution [23].
The T705I substitution in the coding region of LDLR was thought to be one of the defective alleles in a compound heterozygote whilst the second mutation remained undetected [2]. It is reasonable to assume from the Dutch and our data that both defects in this homozygote had not been identified. The second case occurred in a 40-year old man who had a very high total cholesterol of 17.78mmol/l [3, 6]. A splice site mutation (313+1G>A) was inherited from his hypercholesterolaemic mother whilst the I705 variant came from his normolipidaemic father. One possible explanation is that the I705 variant is only expressed when another LDLR defect is present, but two of the probands younger siblings also had slightly elevated cholesterol yet did not share an LDLR haplotype with their normocholesterolaemic sibling, i.e. they did not carry either LDLR substitution. Thus another variation in LDLR or in another gene may be responsible for the hypercholesterolaemia in these two siblings and would also explain the high cholesterol level in the proband. In another report [7] the I705 substitution co-segregated with the hypercholesterolaemia phenotype, but again this could be explained by the presence of an unidentified mutation which may or may not be in linkage disequilibrium with the I705 variation.

Exon 15 consists of 171 nucleotides which encodes 57 amino acids, of which 18 are threonine or serine residues [24, 25] and most of the O-linked sugars of the LDL-receptor are attached to these threonine and serine residues [25]. A similar region is also conserved in the LDL-receptors of other mammals [25]. The functional role of this domain was investigated by Davis and coworkers, 1986, using site directed mutagenesis, where a portion of exon 15 was deleted and then expressed by transfection into fibroblast cell lines. The mutated cDNA coded for a receptor protein which was functionally indistinguishable from the normal receptor [25]. Individuals carrying a similar natural deletion of exon 15 (FH-Espoo) have LDL-cholesterol levels which are relatively low and a mild form of FH [26]. Thus a major rearrangement is actually a mild mutation, suggesting that mutations in this region may only have a mild effect on receptor function and therefore on lipid levels. Only six point mutations or single base deletions have been described in exon 15 [http://www.ucl.ac.uk/fh]; two point mutations resulting in a
stop codon, a minor deletion predicted to result in frameshift and a splice donor site mutation, and all of these are highly likely to be pathogenic. In addition to the T705I substitution, two missense mutations have been reported, T721I [27] and R723Q [28, 29]. LDL-binding studies showed that the Q723 mutation had 70% of normal activity and is therefore a mild LDLR mutation. No details are known about the I721 mutation. It may be functional in that an O-linked sugar may attach at this site but it may be non-pathogenic as with T705 which involves the same amino acid substitution.

Thus from the available data on the reported exon 15 mutations, missense mutations and FH-Espoo, they appear to have a mild effect on the LDL-receptor protein. Ideally, cellular studies should be carried out on all novel missense mutations but this is often not feasible. However at the very least 100 normal individuals should be screened for any novel missense mutation to determine frequency and if any carriers detected, association studies should be performed. In particular, care should be taken in reporting missense mutations identified in exon 15 of LDLR as FH-causing, as they appear to have a modest effect on LDL-receptor function.

Acknowledgements

We are grateful to Dr Isabel Smith for access to information on the family. Karen Heath is a PhD student sponsored by the John Pinto Foundation, and financial support is from British Heart Foundation grants (RG95007 & RG93008).
References


29. Sun X-M, Neuwirth C, Patel DD, Knight BL, Soutar AK with the Familial hypercholesterolaemia regression study group. Influence of genotype at the low density lipoprotein (LDL) receptor gene locus on the clinical phenotype and response to lipid-lowering
Table 1: Characteristics (mean ± SD) of I705 carriers and non-carriers in 2287 ‘healthy’ Caucasian men from NPHS-II. * Geometric means and 95% confidence limits as triglycerides were log x for the analysis.

<table>
<thead>
<tr>
<th>T705I group</th>
<th>TT</th>
<th>TI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56.1±0.15 (n=2256)</td>
<td>55.0±1.30 (n=30)</td>
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<tr>
<td>BMI</td>
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<tr>
<td>Total cholesterol</td>
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<td>5.66±1.08 (n=30)</td>
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</tr>
<tr>
<td>Triglycerides</td>
<td>1.81* (1.77-1.85)</td>
<td>1.62* (1.35-1.96)</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Fig 1: No mutation was found in LDLR or APOB but rare variants in LDLR exon 15 (I705) and intron 7 (1061-8C) were found in II.2 but not in III.1, thus not tracking with the hyperlipidaemia, shown by the filled symbols. A sample from the father, II.1, was not available. The proband is marked by an arrow, the highest untreated total cholesterol (mmol/l) and the age of referral for molecular analysis are shown under the pedigree symbols.

A: T705I substitution was detected by an artificial restriction PCR assay, where an NsiI restriction site is introduced into the rare T allele (I705). Digested fragments were electrophoresed on a 4% (3:1) NuSieve agarose gel and stained with EtBr. Lane 1 - 50bp size marker, lane 2 - III.1: normal T705 and lane 3 - II.2: I705 carrier.

B: 1061-8T>C variation was detected by a natural PCR digest assay where the rare variant C loses an Earl site, although there is an additional site for a digestion control. Digested fragments were electrophoresed on a 10% acrylamide gel and stained with EtBr. Lane 1 - 1kb size marker, lane 2 - homozygous 1061-8T, lane 3 - III.1: homozygous 1061-8T, lane 4 - II.2: 1061-8T>C carrier and lane 5 - 1061-8T>C carrier.
I

II

III

1st MI 39 yrs
died 55 yrs

xanthelasma
elevated cholesterol
34 yrs
1705/1705
1061-8C/1061-8T

4.2 mmol/l
5 yrs
T705/T705
1061-8T/1061-8T

A

III.1 II.2

- 139 (C)
- 115 (T)

150
100
50

1 2 3

B

III.1 II.2

- 183 (C)
- 153 (T)

197
152
142

1 2 3 4 5
Letter to the Editors

Rapid testing for three mutations causing familial defective apolipoprotein B100 in 562 patients with familial hypercholesterolaemia

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Revised 7 February 1996; accepted 26 February 1996

Keywords: Familial defective apoB100; Familial hypercholesterolaemia; apoB Mutation testing

Dear Editors,

Apolipoprotein B, as ligand for the low density lipoprotein (LDL)-receptor, plays a central role in the catabolism of LDL. Familial defective apolipoprotein (apo) B100 (FDB) is a term used to define mutations in the apoB gene that directly reduce low density lipoprotein receptor (LDL-R) binding. To date three mutations falling into this category have been identified; apoB R3500Q [1], apoB R3531C [2] and apoB R3500W [3]. All three mutations occur at CpG sites at either Arginine3500 (CGG) or Arginine3531 (CGC). In the case of the two apoB 3500 mutations, both possible transitions (C→T and G→A) occur. ApoB R3500Q, the first identified FDB mutation, has been extensively studied (see reviews by Tybjaerg-Hansen and Humphries [4] and Myant [5]. The estimated frequency of the mutation ranges from 1/500 [6] to 1/700 [7] in the UK and Germany, and as high as 1/250 in Switzerland [8] but undetected in Finland [9]. A striking feature of apoB R3500Q, is that the mutation almost always occurs on a rare apoB haplotype and is found widely distributed across Europe, the US and in Australia, which strongly suggests that carriers of the mutation are of common descent [4] and that the mutation occurred many generations ago [5]. Although the mutation has a wide range of penetrance, carriers of apoB R3500Q may have clinical features indistinguishable from familial hypercholesterolaemia (FH), namely hypercholesterolaemia, a family history of coronary heart disease, and/or xanthomas and corneal arcus. ApoB R3500Q occurs at a frequency of approximately 3% amongst FH patients attending lipid clinics.
Thus, in a heterogenous population such as the UK, apoB R3500Q represents one of the most common single gene mutations, including LDLR mutations, associated with an FH phenotype.

ApoB R3531C was identified and characterised in an American study in two individuals, one of Celtic/Native American origin and the second of Italian descent and was found on two different apoB haplotypes [2]. ApoB R3500W was found in two hypercholesterolaemic patients in Scotland, one of Caucasian origin, the other Asian [3]. In both carrier families the mutation was associated with hypercholesterolaemia in adulthood [3]. Once again, the mutation occurred on two different apoB haplotypes, thus suggesting that in both studies, in all four cases, these mutation were of independent origin.

In order to address the question whether carriers of apoB R3531C and apoB R3500W also express FH clinical characteristics, and whether routine testing for these mutations, in addition to apoB R3500Q, in mutation identification in FH family studies would be expedient, we tested 562 patients with a clinical diagnosis of FH, for all three FDB mutations. These FH patients represent a subset of patients previously assembled for LDL-R mutation characterization [10]. Briefly, we utilised a cheap, rapid single polymerase chain reaction (PCR) method to screen for the three FDB mutations, using high throughput techniques developed in our laboratory using microtitre plate arrays, with pre-applied dried template DNA [11] and small scale simplified set-up polymerase chain reaction (PCR). This greatly enhances mutation testing. The PCR used amplified the appropriate region of the apoB gene spanning nucleotide 10531–10885. ApoB R3500Q was identified by dot blotting and 32P dCTP labelled allele specific oligonucleotides (ASOs) as previously described [6]. ApoB R3500W mutation introduces a NlaIII restriction site, and apoB R3531C mutation introduces an NsiI restriction site. Fragments were resolved by the use of 5% microtitre array diagonal gel electrophoresis (MADGE) (GenetiX, Dorset, UK) [12] and visualised with ethidium bromide. In all three cases positive control analyses of patients identified in previous studies, were included. (ApoB R3531C heterozygote DNA was a kind gift from Dr. Clive Pullinger, San Francisco, CA).

In agreement with previous studies, 17/562 carriers of apoB R3500Q were identified, i.e. at a frequency of 3% (95% C.I. 2.0–4.0%). However, neither apoB R3531C nor apoB R3500W were detected in the sample (< 1/562).

As well as occurring at lower general population frequencies, the absence of R3500W and R3531C from this group of FH patients could be due to a number of reasons. ApoB R3531C appears to be associated with a less severe hypercholesterolaemia and in receptor binding assays apoB R3531C-LDL has residual binding of 63% compared with control LDL on a fibroblast receptor-binding assay, while by comparison apoB R3500Q has 36% residual binding [2]. Therefore patients with FDB due to apoB R3531C might not present with an FH phenotype. By contrast, the effect of the apoB R3500W appeared to be similar to that of apoB R3500Q using the LDL-growth promotion assay on U937 cells, which have no endogenous LDL synthesis, and cell growth in the presence of LDL from carriers of either apoB R3500Q or R3500W was reduced by 50% compared to control LDL [3].

The amino acid change at this site, whether glutamine or tryptophan seems to have the same deleterious effect on LDL-R binding and uptake. The two described independent cases of apoB R3500W, and similarly the two cases of apoB R3531C, all occur on different haplotypes, indicating that each pair of carriers is not of common descent and that no allele has increased in frequency due to population expansion. Apo B R3500W was originally identified in patients from Glasgow, Scotland, but we did not detect the mutation in the 36 FH patients who were recruited in Edinburgh. There were however 2/36 carriers of apoB R3500Q in this Scottish sample. Thus apoB R3500Q remains the only known FDB mutation that is both common in the UK and is associated with an extreme enough hypercholesterolaemic phenotype to cause classical FH and remain worth testing for. Despite rapid testing techniques, it does not seem of practical value to test for apoB R3531C or apoB R3500W for FH mutation identification.
Acknowledgements

We acknowledge the support of the British Heart Foundation – RG16 (PJT, SEH) and a BHF Intermediate Fellowship and the Sir Halley Stewart Trust to INMD and the Greater Glasgow Health Board – RSG/END/9293/z (DG). We would also like to acknowledge our clinical colleagues whose patients were included in this study, Dr. G. Thompson, Dr. M. Seed, Dr. J. Betteridge, Dr. R. Williams and Prof. M. Oliver, and the patients for their co-operation.

References


SHORT COMMUNICATION

THE USE OF A HIGHLY INFORMATIVE CA REPEAT POLYMORPHISM WITHIN THE ABETALIPOPROTEINAEMIA LOCUS (4q22–24)

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Received 11 March 1997
Revised 20 May 1997
Accepted 27 May 1997

SUMMARY

Abetalipoproteinaemia is a rare autosomal-recessive disorder caused by a defect in the large subunit of the microsomal triglyceride transfer protein (MTP) which is required for the assembly and secretion of apolipoprotein B-containing lipoproteins. We report here the use of a polymorphic CA dinucleotide repeat in intron 10, MTPIVS10, of the large subunit of the human MTP protein in the analysis of a pregnancy in a consanguineous family, in which abetalipoproteinaemia was suspected, although prenatal diagnosis was subsequently refused. The mutation in the family has been identified as a novel four-nucleotide insertion/duplication of exon 17 between nucleotides 2349 and 2350 of the cDNA sequence of the MTP gene. However, the marker, MTPIVS10, can be used as an alternative to the time-consuming mutation detection techniques. © 1997 by John Wiley & Sons, Ltd.

No. of Figures: 3. No. of Tables: 1. No. of References: 15.

KEY WORDS: abetalipoproteinaemia; CA repeat; microsomal triglyceride transfer protein; mutation; prenatal diagnosis

INTRODUCTION

Abetalipoproteinaemia (ABL) is a rare autosomal-recessive disorder that is characterized by a virtual absence of apolipoprotein B (apoB)-containing lipoproteins and by low plasma concentrations of triglyceride (TG) and cholesterol (Kane and Havel, 1989). ABL is caused by a defect in the assembly and secretion of plasma lipoproteins, very low density lipoproteins (VLDLs) in the liver and chylomicrons in the intestine (Wetterau et al., 1992). Its presentation is clinically heterogeneous. It may present in infancy with failure to thrive and the lack of fat-soluble vitamins can cause neurological impairment, in adults, if untreated, resulting in retinitis pigmentosa, spinocerebellar degeneration with ataxia, and acanthocytosis of erythrocytes. Vitamin E supplementation prevents the neurological syndrome (Muller et al.,
Microsomal triglyceride transfer protein (MTP) is required for the assembly and secretion of apoB-containing lipoproteins (Sharp et al., 1993; Shoulders et al., 1993), catalysing the transport of TG, cholesteryl ester, and phosphatidylcholine between phospholipid surfaces. MTP is a heterodimer consisting of the multifunctional enzyme protein disulphide isomerase (PDI) and a larger unique subunit of 97 kD (Sharp et al., 1993; Shoulders et al., 1993). The gene encoding the large subunit of human MTP is found on human chromosome 4q22-24, contains 18 exons, and spans approximately 55-60 kb (Sharp et al., 1994; Shoulders et al., 1994; Narcisi et al., 1995).

In abetalipoproteinaemic subjects reported to date, frameshift or splice-site mutations in the gene encoding the 97 kD subunit have been found, which disrupt normal production of the protein (Sharp et al., 1993; Shoulders et al., 1993). Twelve mutations have been identified which result in truncated proteins (Shoulders et al., 1994; Narcisi et al., 1995; Ricci et al., 1995) and recently the first missense mutation was characterized (Rehberg et al., 1996). Mutation screening using techniques such as SSCP (Orita et al., 1989) and DGGE (Myers et al., 1985) are labour-intensive, time-consuming, and not 100 per cent sensitive, and not all mutations may be detected by such methods. An imperfect CA repeat, MTPIVS10, is found in intron 10 of the human large subunit of MTP (Sharp et al., 1994) (Fig. 1) with a high heterozygosity index. The combination of four polymorphic sites, Q95H, I128T, Q244E, H297Q, and the CA repeat differentiated 16 haplotypes within the MTP locus (Narcisi et al., 1995). In this study, we have further characterized the heterozygosity index of the CA repeat and the use of this single polymorphic marker provides a rapid, accurate tool for prenatal diagnosis and identity testing, and in genome-wide linkage screening programmes.

CASE REPORT

The proband (II.1) is the first child of healthy Asian parents who are first cousins (Fig. 2). She was born in Pakistan at full term, weighing 2.04 kg. From the age of 6 months, she had episodes of diarrhoea together with skin and chest infections. Shortly after arriving in the U.K. at the age of 3 years and 9 months, she presented with growth failure and developmental delay. On examination, she was small (<third centile), underweight (<third centile), and microcephalic (<third centile). She had some mild abdominal distension. Knee and ankle tendon reflexes were absent.

Investigations were as follows: acanthocytes on blood film; plasma cholesterol 0.9 mmol/l; absence of pre-β and β-lipoproteins on lipoprotein electrophoresis; absent sensory nerve conduction; and marked accumulation of fat in the mucosal cells on intestinal biopsy—findings that are consistent with the diagnosis of abetalipoproteinaemia.

Developmental assessment showed that she was significantly delayed, with language and cognitive development at the 2 to 2.5-year level. Other skills were more mature, at least at the 3-year level. She was treated with a very low fat diet and supplements of vitamins E and K. The diarrhoea has stopped and she has gained weight rapidly but there has been less improvement in her developmental delay.

The mother presented at 14/52 gestation, requesting genetic counselling and enquiring about possible prenatal testing. The informativeness of the family was tested using the MTP intron 10 CA repeat.

MATERIALS AND METHODS

PCR amplification of DNA

A 150 bp fragment of intron 10 was amplified by the polymerase chain reaction (PCR) (Saiki et al., 1983), whilst reducing dietary fat is an important treatment for the other clinical symptoms.

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1989) using the forward primer 5'-TCC ACA GGA TTC ATA ACC-3' and the reverse primer 5'-TTC TCC ACT CTT CCC CAT-3' (personal communication, Dr Richard Gregg) (Fig. 1). The 30 µl PCR reaction contained 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 0.001 per cent gelatin; 0.067 per cent W-1 (Gibco BRL, Paisley, U.K.); 1.5 mM MgCl₂; 0.2 mM dATP, dGTP, dTTP; 0.02 mM dCTP; 0.75 mCi [³²P]dCTP; 50 ng of each primer; and 0.3 U of Taq polymerase (Gibco BRL, Paisley, U.K.), in addition to 250 ng of genomic DNA. Samples were overlaid with mineral oil. The amplifications were performed on an Omnigene thermal cycler (Hybaid Ltd, U.K.) and the conditions for the CA repeat were as follows: one cycle of denaturation of 95°C for 5 min; 35 cycles including 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and one cycle of final extension of 72°C for 5 min.

Gel electrophoresis

The products were run on a 6 per cent denaturing gel. Five microlitres of the PCR mixture was mixed with 5 µl of formamide loading buffer (95 per cent formamide, 20 mM EDTA, 0.05 per cent bromophenol blue, 0.05 per cent xylene cyanol FF). The sample was denatured for 3 min and immediately placed on ice. Samples (5 µl) were loaded onto a 6 per cent polyacrylamide gel (Sequagel-6, National Diagnostics, U.S.A.) in a sequencing apparatus Model S2 (Gibco BRL, Paisley, U.K.) with 0.09 M Tris-borate, 0.02 M EDTA buffer (pH 8.3) and run for 1.5 h at 65 W, 1.5 kV, 200 mA. Gels were dried and exposed to Kodak X-Omat AR film for 24 h at −70°C.

Fluorescent method

DNA samples from 361 normal males were analysed for the intron 10 dinucleotide repeat marker using a non-radioactive method. The reverse primer was of the same sequence as that stated previously, except that it was fluorescently labelled with N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) linked to the 5’ end by an aminohexyl link. Alleles were separated by electrophoresis on a 6 per cent denaturing gel (Sequagel-6, National Diagnostics, U.S.A.), which was carried out on an ABI 373A DNA Sequencer. The fluorescent data were collected using Genescan® and Genotyper® software, which allows accurate sizing of the amplified fragments using the mobilities of known size standards (Rox 350, Applied Biosystems).

DNA sequencing

The promoter, 189 bp of 5’ flanking sequences, and all 18 exons and their associated splice sites were amplified from patient genomic DNA by PCR and were sequenced (Narcisi et al., 1995). The PCR conditions and the DNA sequences of the oligonucleotides used to amplify and sequence the individual exons are available on request from CCS. PCR products were bound to streptavidin-coated magnetic beads as recommended by the manufacturer (Dynal), denatured, and sequenced according to the Applied Biosystems PRISM Sequenase Terminator Single-stranded DNA sequencing kit.

RESULTS AND DISCUSSION

Frequency of MTPIVS10 CA alleles in the normal population

The frequency of MTPIVS10 alleles was estimated by typing 361 healthy individuals from a heterogeneous population from South-East England. Fifteen variations to the structure (CA₄AA(CA)₃GA(CA)₄TA(CA)₄ACA were observed with five additional alleles possible within the repeat range (Table I). The maximal heterozygosity was 0.85 with an observed heterozygosity of 0.68. The nucleotide sequence of the most common allele, allele 4, is shown in Fig. 1, with a frequency of 0.26; the rarest alleles were 1 and 6, with frequencies of 0.001.

Results of family DNA analysis

The frequency of at-risk couples who are estimated to be fully informative for the marker is 47 per cent. Forty-three per cent remaining at-risk couples will be partially informative (i.e., one parent is heterozygous for the marker and the other is homozygous) and only the diagnosis of ABL, not carrier status, would be feasible. Thus, only 10 per cent of at-risk couples would be unable to be offered prenatal diagnosis, using this marker.

Partial informativeness was observed in family A, where three alleles were present; I.1 and I.2 were heterozygous for the intronic CA repeat marker, with one allele, allele 4, in common. This allele segregated with the ABL gene and was
A 150 bp region of intron 10 of the large subunit of the human MTP gene encoding a CA dinucleotide microsatellite was PCR-amplified. Fifteen variations to the structure were identified. The allele sizes are given in ABI mobility units, which approximate to base pairs. Occurrence equals the overall number of alleles with a particular repeat size, out of a total of 722 alleles, and the frequency represents the percentage of the total for each variation.

<table>
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<th>Allele</th>
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The mutation results in the translation of six spurious amino acids, DCGNNN, before a termination codon is found, causing the truncation of the protein of 789 amino acids. I.1 was heterozygous for the mutation, confirming the mutation. Sequencing also eliminated the possibility of a recombinational event between the mutation and the intron 10 polymorphism, thus confirming the diagnosis.

**CONCLUSION**

The polymorphic CA dinucleotide repeat in intron 10 of the large subunit of the human MTP protein, MTPIVS10, can be used as an informative marker for the prenatal diagnosis of families at risk of the autosomal-recessive disorder abetalipoproteinaemia. Previously seven to eight alleles had been found with the intronic marker and a total of 722 alleles.
A novel mutation was subsequently localized, an insertion/duplication of four nucleotides in exon 17, between nucleotides 2346 and 2347 of the cDNA sequence (Shoulders et al., 1993), confirming the diagnosis in the family. To date, this is the first report of a mutation in exon 17 of the MTP gene resulting in a truncated protein predicted to be 789 amino acids. Mutations reported to date have been generally unique to the family, so the entire MTP gene needs to be screened and subsequently abnormal gel patterns sequenced. Time-consuming mutational analysis combined with the delay of waiting until childbirth for lipid analysis to be performed highlights the usefulness of the CA dinucleotide repeat marker in the 97 kD MTP subunit, in cases when rapid testing is required in a family with abetalipoproteinemia.

ACKNOWLEDGEMENTS

We would like to thank Professor R. Winter, who referred this patient for analysis. This work was supported by grants from the British Heart Foundation.

REFERENCES


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