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FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100
CAPE TOWN EXPERIENCE

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ABSTRACT

Elevation of Low Density Lipoprotein (LDL) in Familial hypercholesterolaemia (FH) as a clinical diagnosis can be due to mutations in the LDLR, apo B or PCSK9. It has been suggested that familial binding defective B100 is biochemically less severe than the other 2 causes of FH. Whilst the prevalence of FDB may be as high as 1:700 in several European countries and some parts of America, but it may be absent in some countries. Since the discovery of the R3500Q mutation in apo B, few other mutations have been discovered. The Cape Town experience of genotypic diagnosis of FDB was analysed for a description of the genetic causes, clinical manifestations and for comparison with the commonest LDL receptor mutation (D206E) in our clinic with defective functional status.

Groote Schuur Hospital had 37 patients with the heterozygous familial hypercholesterolemia phenotype proven to be FDB. FDB accounts for only 0.5% of the entire FH population referred to the lipid clinic. FDB accounted for 3.9% of the FH phenotype patients that have been genotyped in the clinic. Thirty-four patients had the classic R3500Q mutation, from different kindreds and varied ancestral origins. One patient was diagnosed with: R3500W, R3531C, H3543Y. Screening for mutations in 40 indigenous black South Africans with primary hypercholesterolemia for FDB mutations in exon 26 and 29 of apoB excluded FDB in this ethnic group.

Thirty-three patients with FDB cohort were compared with 53 patients with LDLR D206E mutation matched for age, gender, BMI and triglycerides. The two groups were similar when compared for physical signs, which were not present in all cases. Both cohorts exhibited premature coronary atherosclerosis but the onset between these two groups was statistically significantly different, with the onset of about ten years later in FDB.

The trend for higher total cholesterol and LDLC in the LDLR D206E cohort reflected the underlying distribution difference in LDL particle size favouring small dense LDL in FDB. The treatment was limited by hospital policies but the response to statin therapy was similar for both cohorts.

This analysis showed that FDB is a serious monogenic disorder of lipoprotein metabolism that presents clinically with the heterozygous FH phenotype and has a few subtle differences from the FH caused by LDLR mutations. The mutations in apoB affect mainly patients who have white or mixed ancestry.
ACKNOWLEDGEMENTS

I am grateful to the hard-working Groote Schuur lipid clinic, lipid laboratory and the lipid staff for their dedication and record keeping. This project would not have been successful without them. I must specifically thank Dr Dirk Blom, Dr Jean Firth and Professor A.D. Marais for the comprehensive notes and good record keeping. Sister Jennifer Ross helped greatly with the finding of the folders. Sister Rose Jooste also helped with the patient file collection. Mrs. Sheena Jones introduced me to some laboratory work and was very resourceful. My indebtedness goes to the lipid clinic patients for agreeing to research and providing the information.

I am particularly thankful to have worked with Professor A.D. Marais for the entire project and time spent at the clinic. I thank him for his kindness and tremendous academic support. He introduced me to the insightful world of research and writing. He was extremely patient, very knowledgeable and has broader insight about medicine and life in general. He sacrificed his time in educating me about lipid metabolism and has introduced and assisted me to academic reading and writing to produce this thesis. His enthusiasm and spirit of endurance influenced me greatly.

My gratitude thus goes to the entire lipidology staff for their immaculate work under difficult circumstances.
Abbreviation list

ACAT= acyl-co enzyme A Cholesterol Acyl Transferase
Apo= Apolipoprotein:
CE= Cholesterol ester:
CETP= Cholesterol Ester Transfer Protein:
CM= Chylomicron;
EGF= Epidermal Growth Factor;
ER= Endoplasmic Reticulum;
FCH= Familial Combined Hyperlipidaemia;
FDB= Familial Defective Apolipoprotein B;
FH= Familial Hypercholesterolaemia;
HL= Hepatic Lipase;
HDL= High Density Lipoprotein;
HMGCoA= 3-Hydroxymethylglutaryl Coenzyme A;
LCAT= Lecithin Cholesterol Acyltransferase;
LDL= Low Density Lipoprotein;
LDLR= Low Density Lipoprotein Receptor;
LPL= Lipoprotein Lipase;
MTP= Microsomal Triacylglycerol Transfer Protein;
NARC1= Neutral Apoptosis Regulated Convertase 1;
PCR= Polymerase Chain Reaction
PCSK9= Proprotein Convertase Subtilin/kexin type 9;
SREBP= Sterol Regulatory Element Binding Protein;
TC= Total Cholesterol;
TG= Triglyceride (Triacylglycerol); 
VLDL= Very Low Density Lipoprotein
DECLARATION

I, Bonginkosi Mahala, hereby declare that the research described herein was performed by me with the assistance as indicated in acknowledgements. The dissertation was written by me and reviewed by my supervisor. Neither the whole thesis or any part thereof has been or is being or will be submitted by me for any degree at this or any other university.

I give permission to the university to reproduce the whole or any part of this manuscript for research purposes.

…………………………………..                      ………………………………
Bonginkosi Mahala                                                             Date
Chapter 1
Literature review
1.1 INTRODUCTION

The heterozygous familial hypercholesterolemia (FH) phenotype can be defined as an inherited disorder raising LDL concentration significantly higher than the usual population range and clinically characterised by a personal or family history of premature ischaemic heart disease in the affected subject or relatives and lipid deposition as tendon xanthomata. This phenotype can vary from the heterozygous to the homozygous forms whilst an intermediate form is also encountered as a transition. The cardinal features and gene dose effect are described in table 1.1. Occasionally other genes such as truncated apo B disorders may mask the phenotype.

There are at present five different genes known to be responsible for this clinical phenotype viz. Low Density Lipoprotein Receptor mutations (LDLR), apoB mutations, Proprotein Convertase Subtilin/Kexin type 9 (PCSK9), ARH and ABCG5G8. The LDLR was viewed as the cause of FH as outlined by Nobel Prize Winners Brown and Goldstein in 1985, until familial binding defective B100 and other proteins were described.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Heterozygous</th>
<th>Homozygous</th>
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<tbody>
<tr>
<td></td>
<td>Tendon xanthomata</td>
<td>Tendon xanthomata</td>
</tr>
<tr>
<td></td>
<td>Coronary disease &gt;25 years</td>
<td>Coronary disease &lt; 25 years</td>
</tr>
<tr>
<td></td>
<td>5mmol/l &lt; LDLC&lt; 12 mmol/L</td>
<td>LDLC &gt; 12 mmol/L</td>
</tr>
<tr>
<td>Genetic disorder</td>
<td>LDL receptor: 1 allele</td>
<td>LDL receptor both alleles</td>
</tr>
<tr>
<td></td>
<td>ApoB: 1 or 2 alleles</td>
<td>No gene dose effect</td>
</tr>
<tr>
<td></td>
<td>PCSK9: 1 allele</td>
<td>Not yet described</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ARH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Phytosterolaemia)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(CTX)</td>
</tr>
</tbody>
</table>

Table 1.1 FH phenotypes and their genetic causes
1.2 DISORDERS INCREASING LDLC CONCENTRATION

A brief review will be given for each of these entities before lipoprotein metabolism and apoB are reviewed. The details of LDLR and PCSK9 mutations will not be discussed in this review but comparisons will be made between FDB, LDLR and PCSK9 mutations where relevant.

Familial Defective Binding (FDB)

FDB can be defined as a group of autosomal dominantly inherited mutations in apoB-100 that adversely affect its binding to the low density lipoprotein receptor (LDLR) receptor.

The classical form is by a single base change in the apoB gene, located in exon 26 on chromosome 2, resulting in an arginine to glutamine substitution at the codon for amino acid 3500 of apoB100 (R3500Q). Only a few more mutations are known today, 7 in exon 26 and 2 in exon 29, see table 1.2 below.

<table>
<thead>
<tr>
<th>Exon 26</th>
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<tbody>
<tr>
<td>Mutation</td>
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<tr>
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<td>Benn</td>
</tr>
<tr>
<td>R3480W</td>
<td>Boren</td>
</tr>
<tr>
<td>R3500L</td>
<td>Gaffney</td>
</tr>
<tr>
<td>R3500Q</td>
<td>Innerarity</td>
</tr>
<tr>
<td>R3500W</td>
<td>Gaffney</td>
</tr>
<tr>
<td>R3531C</td>
<td>Wenham</td>
</tr>
<tr>
<td>H3543Y</td>
<td>Soufi</td>
</tr>
</tbody>
</table>

Table 1.2. Reported FDB Mutations, the investigator reporting them and the year of report LDL receptor mutations
The most common cause of the FH phenotype is a mutation in the LDLR. The gene is located in chromosome 19. FH is inherited in an autosomal dominant fashion. The frequency of FH globally is estimated at 1/500 persons,\(^7\) with founder effects in some populations occurring as frequently as 1/70 persons\(^12\). There are now more than a thousand mutations known in LDLR. (www.correlagan.com/fields/other/reviews/LDLR_APOB_CRLGOw.PDF) and www.primarycare.ox.ac.uk/vascular/research/Simon_Broome date accessed 10 November 2008.

**Proprotein Convertase subtilin /Kexin type 9 (PCSK9):** the gene responsible is on chromosome 1 (PCSK9). This gene encodes for the neural apoptosis regulated convertase 1 (NARC1). PCSK9 is involved in the degradation of the LDLR; mutations that cause a gain of function render LDLR activity low.

**ARH:** The disorder is due to mutations in the adaptor protein for endocytosis of the LDLR. A very specific interaction between the cytoplasmic tail of the LDL receptor (NPVY) with the phosphotyrosine-binding domain of ARH is defective, failing to link LDL receptor to clathrin and the adaptor protein-2. The result is such that clearance of LDLC into the liver is severely defective and results in homozygous FH phenotype.

**ABCG5G8:** Phytosterolaemia is due to over-absorption of plant sterols from enterocytes, as well as an under-excretion from the hepatocytes into the bile as a result of disruptions of ABC transporters G5 and G8. It has a variable LDL hypercholesterolaemia but is diagnosed by the high plant sterol concentration in plasma. Cerebrotendinous Xanthomatosis (CTX) is due to mutations in cholesterol 27 hydroxylase, an essential step in cholesterol oxidation for bile acid synthesis. The LDLC is not raised but the xanthomata resemble the homozygous FH phenotype. Additionally, the nervous system is involved in a leukodystrophy and lenses of the eye develop cataracts.

A brief review of lipid metabolism, LDL receptor and ApoB and lipoprotein metabolism will be revised here before FDB is discussed as a clinical entity.
1.3 LIPID AND LIPOPROTEIN METABOLISM

Lipids are water-insoluble organic compounds that can be very simplistically classified as fatty acids (and their esters; Triacylglycerol, phospholipids and other complex liquids) and sterols. They are of cardinal importance for the cell and are transported in blood as lipoproteins where they associate with apoproteins, enzymes and other proteins. A brief review will be done here to relate lipids, lipoproteins and the metabolism of lipoproteins relevant to FDB.

1.3.1 Phospholipids (PL)

These are integral components of all cell membranes, forming a lipid bilayer in which the polar groups are oriented towards the aqueous environments on either side of the cell membrane. In addition to its membrane requirements the phospholipids are synthesised in the liver for bile and lipoprotein synthesis.

PL’s are divided into two main groups depending on whether they contain a glycerol or a sphingosyl backbone. i.e. glycerophosphates and sphingophospholipids. The sphingophospholipids, as the name suggests, contain sphingosine or a related amino alcohol. The most common examples are phosphorylcholine ester of an N-acylsphingosine (sphingomyelin), the most important lipid in nervous tissues. Though present on LP, sphingophospholipids are not generally measured or evaluated for their impact on Lipoprotein and cellular metabolism.

There are various types of glycerophospholipids: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol being the most commonly encountered. Phosphatidylcholine is the major phospholipid and predominates in LP where it forms a shell around the insoluble neutral lipids in the core of the LP. Phospholipid contains the phosphate group together with choline (hydrophilic) and two fatty acids on the rest of the glycerol backbone and these non-polar fatty acids are directed to the interior of the lipoprotein particle due to their hydrophobic nature. The phospholipids are essential in the structure of LP as they provide an interface between non-polar lipids and the aqueous environment in which LP are suspended. Additionally they accommodate apoproteins. The unsaturated fatty acids within the phospholipids
may undergo oxidation, a process that is implicated in atherosclerosis. Indeed, lysophospholipids and especially oxovaleroyl phosphotidyl choline impart atherogenic properties to LP.

1.3.2 Triglycerides (TG)
The lipid stores of animals and almost all plants are triglycerides. They represent the most efficient way to store energy (38kJ/g) as compared to carbohydrate and protein (both 19kJ/g). The esterification of small fatty acids protects cells against the detergent action of fatty acids and the adverse impact on glycolysis (Randle effect). Dietary lipid is digested to fatty acids and these are re-esterified for transport in chylomicrons from the enterocyte. In a parallel fashion, the liver may receive new esterified fatty acids from adipocytes, synthesise it de novo or may digest TG imported as remnant lipoproteins to export TG again in LP or store them intracellularly in droplets. In animals and humans, the TGs are stored in the adipose tissue but during metabolic stress a significant amount may build up in the liver, heart and skeletal muscle.

1.3.3 Cholesterol

Cholesterol is a sterol that may exist in the esterified or free form. It is an essential component of cell membranes. All nucleated cells can synthesise cholesterol, and mature cells with a high demand for cholesterol may continue to synthesise it to satisfy their requirements. These are hepatocytes (bile and lipoprotein production), enterocytes (lipoprotein production) and adrenocytes (corticosteroid production). Cells in the nervous system and the skin cannot obtain cholesterol from lipoprotein and thus may also have ongoing synthesis of cholesterol as well.

Cholesterol is produced at the end of a pathway that also provides isoprenes (used for the prenylation of proteins), Ubiquinone (mitochondrial energy) and dolichol (glycosylation of proteins). The rate-limiting enzyme in this pathway is HMG-CoA reductase. This enzyme converts 3-hydroxy-3-methyl CoA to mevalonic acid and can be inhibited by statins.

During cell growth cholesterol is assimilated. The cell can synthesise cholesterol de novo, can import cholesterol through specialized receptors such as the LDL and related receptors and can use cholesterol after cleavage of stored cholesterol ester by the action of the neutral cholesterol hydrolase. The regulation of cholesterol synthesis and the importation by the LDL receptors occurs
by sensing the amount of cholesterol in the membranes. The depletion of cholesterol in the cell will up-regulate the transcription of both HMG-CoA reductase and LDL receptors through a complex mechanism. In addition to more of this protein being expressed when a need for cholesterol is detected, HMG-CoA undergoes rapid changes in its activity by virtue of phosphorylation and dephosphorylation.

The up-regulation of cholesterol synthesis and the importation in the time of need is made possible by an interaction of a regulatory protein with the promoters of the genes for the LDL receptor, HMG-CoA reductase and HMG-CoA synthase in a complex system.

Cholesterol importation into the cell involves a high affinity, saturable process by means of the LDL receptor and by means of a low affinity process of passive association with the cell membrane and internalization. LDL receptor-related protein (LRP) may import lipoprotein into the cell from lipoprotein remnants especially in the liver. The LDL receptor binds apo E that allows remnants of TG-rich LP to be taken up, and apo B that allows LDL to be taken up. It should be noted that apo B on LP produced by the liver is only in the appropriate conformation for binding to LDLR in the final, smallest LP (LDL) whereas apo B48 made in enterocytes lack the receptor-binding domain.

1.3.4 Lipoproteins

Lipoproteins, composed of lipid and apoproteins, are a means of transporting insoluble lipids, chiefly (TG), (CE) and cholesterol.

The lipoproteins generally have a spherical shape but vary significantly in size density, constituent lipids and apoproteins. Greater lipid content makes for larger diameters and lower density mainly by TG content. The shell contains polar lipids (phospholipids, cholesterol) and apoproteins while the core contains the neutral lipids (TG and CE). The enterocytes and hepatocytes are the sites of assembly for the TG-rich lipoproteins, using ApoB as the critical protein for binding lipid in nascent lipoproteins. The TG-rich lipoproteins secreted by the enterocytes are called Chylomicrons (CM) and those secreted by the hepatocytes are termed Very Low Density
Lipoproteins (VLDL). Each CM contains one ApoB 48 molecule and each VLDL has one ApoB 100 molecule. The secretion of lipoproteins containing Apo B follows the pathway identified by other proteins in constitutive secretory cells.

The biogenesis of lipoproteins is complex and some details remain controversial. The assembly of nascent VLDL particles proceeds in two steps. Firstly a nascent TG-rich particle is generated at the luminal aspect of the rough endoplasmic reticulum (ER) that contains a very small fraction of the core lipids found in nascent VLDL as isolated from Golgi cisternae. Secondly, the lipid content is expanded in a pre-Golgi compartment.

The effective accomplishment of the first step requires MTP that assembles in a heterodimer with protein disulfide isomerase. MTP is a protein of 97 kDa expressed primarily in the cells that secrete lipoproteins containing ApoB. The critical role that MTP plays is illustrated by the observation that mutations of MTP are associated with abetalipoproteinaemia: a condition in which LP containing apo B is absent. MTP can transfer TG as well as CE, and to some extent PL, between lipid vesicles. MTP is also thought to promote the acquisition of non-polar lipids during and after the completion of translation of ApoB. A substantial fraction of ApoB is degraded prior to secretion by two types of degradation processes. Firstly, by proteosomes in the cytosol following ubiquitinisation. Secondly, intraluminal ER proteases. ApoB degradation is thought also to occur post translationally, depending on availability of lipid to produce secretion-competent particles. The details are not clear but could explain an important mechanism to control ApoB secretion. Additionally, apo B is degraded when lipid hydroperoxides target nascent VLDL for autophagy. The crucial role of apo B in lipoprotein synthesis is demonstrated by the observation that truncations lead to low levels of apo B-containing lipoproteins or even abetalipoproteinaemia.

The lipoproteins have different metabolic fates according to their apoprotein complements. The apoproteins include ApoAI, AII, AIV, CI, CII, CIII and E. When the TG-rich LP enters the circulation there is redistribution of apoproteins and High Density Lipoprotein (HDL) supplies
these to TG-rich lipoproteins. The circulating TG-rich lipoproteins attach to the proteoglycans in the vascular beds of the muscle and adipose tissue. Lipoprotein lipase (LPL), present on the proteoglycans, is activated by ApoC-II and inhibited by ApoC-III. Triglycerides are hydrolysed by the activated LPL to release fatty acids that will diffuse into the tissue or be bound to albumin. This process results in a smaller core and redundant shell around the core buds off with ApoA-I to form HDL.

Chylomicrons are synthesized by the small intestine and are found in the systematic circulation postprandially. From the enterocytes they enter the lymphatic drainage into the blood stream via the thoracic duct. The chylomicrons contain mostly TG, cholesterol and CE and are the vehicle of transportation for dietary fat to the liver and peripheral tissues. In addition to the obligatory apo B, each new CM will contain apoproteins A-I, A-IV and acquires apoproteins C-ii and E by the transfer from HDL particles in the blood stream. The Apo C-II activates LPL when CM binds to the vascular bed proteoglycans in the adipose tissues and skeletal muscle. Lipoprotein lipase removes much of the TG from the chylomicron. Chylomicron remnants, still containing most of the original dietary cholesterol, are removed in the liver by virtue of Apo E binding the LDLR and LRP as well as other binding structures.

Very-low-density lipoproteins (VLDL) are synthesized continuously by the liver and contain most of the body’s endogenously synthesised TG and a smaller quantity of cholesterol. The VLDL is the most important fuel during prolonged fasting. Apoprotein B-100 is an essential component of the VLDL. Apo CII and E are incorporated later into VLDL by the transfer from the HDL particles. The TGs are removed progressively by the lipoprotein lipase leaving IDL particles. Apo B is not in the appropriate conformation to act as ligand for the LDLR when on VLDL or IDL.

Intermediate-density lipoprotein particles are generally the remnants of VLDL after the action of LPL. Parts of apo B-100 and all of Apo E are on the surface of this molecule. IDL particles bind to
the LDL receptors through Apo E and are then removed from the circulation. Some of the IDL particles are further metabolised by hepatic lipase to LDL.

**Low-density lipoproteins** are the main carriers of cholesterol in the human plasma. They deliver cholesterol to the liver and peripheral cells when these cells express LDLR. The LDL particle has one copy of apo-B100, which is almost the only protein present in LDL. Trace amounts of Apo E and other apoproteins can be found in the LDL fraction. Apo B is the ligand for the LDL receptor and mediates clearance of LDL. Once bound to the receptor, the receptor-ligand complex invaginates into the cell in the clathrin-coated pits that fuse to liposomes. During acidification, the LDLR and LDL particles dissociate with the former recycling and the latter being degraded (in the lysosome).

Plasma LDL concentration is linked to atherosclerosis by epidemiological studies and by the observation of premature ischaemic heart disease in severe disturbance of LDL metabolism, such as FH. The LDL particle can be retained in the wall of the vasculature, and its oxidation is thought to attract macrophages and promote inflammation.

The LDL also forms part of Lp (a) lipoprotein as a result of linkage of Apo(a) to the Apo B100 with a single disulphide bond involving C4057 and C4326 of the respective proteins, with amino acids 4330 – 4397 of apo B playing an important role in the formation of Lp(a) as well as a lysine-rich region 4372 –4392. Raised Lp(a) concentration is associated with increased cardiovascular disease. Although Lp(a) is raised in patients with LDLR mutations its concentration is chiefly dependant on production and not on the clearance through LDLR. The sulphhydril bond for forming Lp(a) is contained also in apo B with mutations reported for FDB but there is little information about Lp(a) in FDB. If clearance of Lp(a) is through other mechanisms than apo B binding then the Lp(a) should not be much affected by FDB.

**High Density Lipoproteins** are small protein and phospholipid-rich lipoproteins and are under intensive research for their roles in reverse cholesterol transport, inflammation and immune response. Nascent HDL particles are produced in both the liver and intestine. They are disc-shaped
and contain Apo A-i. They mature by addition of PL, and proteins: Apo E and C from chylomicrons and VLDL particles in the circulation. Through the activation and action of LCAT, FC is esterified to FA on PC and the particle becomes spherical as a result of the accumulation of neutral lipid in its core. The HDL transports cholesterol away from the peripheral tissue. Reverse transport happens indirectly by transfer of CE from HDL to VLDL in the circulation by CETP or directly when HDL delivers cholesterol to the liver and steroid-synthetic tissues through interaction with cells.

1.3.5 LDL receptor and its mutations

The LDL receptor is a transmembrane protein encoded on chromosome 19 and comprises 18 exons in 48kb of mRNA. The total protein size is 843 amino acids. The protein can be divided into 5 parts i.e. ligand binding domain (292 amino acids, from exon 2 to 6), epidermal growth factor precursor homologous domain (400 amino acids, from exon 7 to 14) a carbohydrate rich domain (58 amino acids, exon 15), the transmembrane domain (22 amino acids, exon 16), and the cytoplasmic tail (50 amino acids). The protein is initially translated to form a 120-kDa precursor that is modified at the Golgi apparatus to 160 kDa and then transported on to the cell surface to the clathrin-coated pits from where it is internalized into the cell and recycled.

The amino-terminus of the LDLR is situated extracellularly and has 7 repeats of 40 amino acids each. Cysteine is prominent in the binding domain. This region is the binding site for apoproteins on lipoproteins. Binding requires calcium and involves interaction with negatively charged amino acids on the ligand. The 5th repeat is necessary for binding to Apo E whilst repeats 2 to 7 are for binding to the large ligand, ApoB. Upon maturation the LDLR is sorted and traffics to the basolateral surface of the cell. The receptor can now bind to the ligand and form a lipoprotein-receptor complex that is internalised through the clathrin-coated pit, with the assistance of the adaptor proteins. The cytoplasmic tail signals internalisation. In the endosomal system the LDL receptor dissociates from the ligand on the lipoprotein during the acidification; the epidermal growth factor like domain is important for this
function. The lipoprotein proceeds to the lysosome for enzymatic degradation into its constituent cholesterol, fatty acids and amino acids. The receptor is recycled approximately 150 times.

Defects in the LDL receptor are divided into six classes depending on the impact of the mutation on the presence of mRNA, receptor maturation in cells, disparity between the LDL and the immunoglobulin binding on the cell surface, internalisation, and degradation and trafficking. The net effect of mutations may be that LDL receptor mutations may have some residual function (receptor defective mutations) or no function at all (null mutations with less than 2% of binding activity in true homozygotes cell culture studies).

<table>
<thead>
<tr>
<th>Nature of defect</th>
<th>Class 1</th>
<th>Class 2</th>
<th>Class 3</th>
<th>Class 4</th>
<th>Class 5</th>
<th>Class 6</th>
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</thead>
<tbody>
<tr>
<td>Promoter mutations, no detectable mRNA</td>
<td>Retardation of the maturation by glycosylation,</td>
<td>Normal maturation present on the surface</td>
<td>Mature and occupies surface but cannot be internalised.</td>
<td>Increased receptor degradation typically failed dissociation.</td>
<td>Cytoplasmic tail.</td>
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</tr>
<tr>
<td>Activation of gene fails</td>
<td>Depending on complete or incomplete arrest of maturation (Class2a) or completely (class2b)</td>
<td>LDL binding is defective</td>
<td>Cytoplasmic tail mutation.</td>
<td>Receptor does not recycle to the cell surface</td>
<td>Does not localise to basolateral surface of polarized cells.</td>
<td></td>
</tr>
<tr>
<td>Null mutations</td>
<td>Defective or null mutations</td>
<td>Defective mutations</td>
<td>Null mutation</td>
<td>Null mutation</td>
<td>Null mutation</td>
<td></td>
</tr>
<tr>
<td>D206E</td>
<td>V408M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3 Classification of LDLR mutations.
In this thesis more attention is paid to the ligand mutations rather than the LDL receptor mutation, therefore LDLR mutations will not be discussed in further detail. A comparison between a defective LDLR mutation such as D206E and FDB is clearly of interest as both are partially functioning proteins involved in the ligand-receptor interaction for clearance of LDL.

1.3.6 Apolipoprotein B

ApoB is a large molecular mass hydrophobic protein that is permanently associated with lipoproteins, serving as a scaffold for assembly of lipid for LP transporting triglyceride. It is also a ligand for low-density lipoprotein to the cognate (LDL) receptor.

This protein was called ApoB as it is found in the beta and prebeta bands on the electrophoresis. In 1980 it was recognized that there are two primary physiologic forms of ApoB in human plasma and thoracic duct. The full size protein (Apolipoprotein B100) is synthesized in the liver and the spliced version, at nucleotide 6666, produces B48 in the gut. It is encoded on chromosome 2 and has 29 exons of which the largest is exon 26. The protein’s molecular weight is 549 kDa whereas that of Apo B48 is 264kDa. Excess apo B is degraded when no lipid is available to produce lipoprotein. After synthesis apo B avoids degradation by receiving neutral lipid by transfer process mediated by Microsomal Triacylglycerol Transfer Protein (MTP).

The apoprotein B binding region is rich in positively charged amino acids and interacts with the binding domains of the LDLR when the conformation is appropriate on LDL. It should be noted that apo B is chiefly deeply embedded in LDL with some protrusion of the protein at the N-terminus Fig 1.1. A ribbon structure has been proposed for the interaction that enables the confirmation for binding to the LDLR. Fig 1.2. This involves interactions between tryptophan 4369 and arginine R3500Q, arginine 3531C and arginine 3480.

Impaired clearance of defective ApoB relative to normal ApoB species, results in enrichment of LDL to about 70% with FDB apo B. Apo E remains functional ligand in FDB. Thus, LDLR permits the normal entry of cholesterol into the liver from the remnants of VLDL and CM.
there may be great similarities between defects in apo B as the ligand for the LDL and defects in the 
LDL receptor, some subtle differences can be expected to be normal in FDB; it may be abnormal in 
LDLR defects. Up-regulation of the LDLR during growth may have less impact on LDL catabolism 
in LDLR defective subjects compared to FDB subjects for reasons of improving IDL uptake as well 
as having more functional receptors for LDL uptake. However, circulating LDL will be enriched 
with the ligand defective form to about 70% and may be enriched with longer residence time. 
Certainly, in a homozygote with FDB, small dense LDL was demonstrated. It is not clear whether 
the small LDL is due to the formation of compositionally different lipoproteins or whether 
exchange with TG by CETP and subsequently TG hydrolysis by HL accounts for the small LDL 
described.
Figure 1.1 Diagram of the structure of ApoB.

The shaded circles represent cysteine, open circles – carbohydrate and amino acids numbers are indicated for fragments I to V. (Yang ML, 1990).
Fig 1.2 Model of LDL receptor binding. Normal receptor binding in apoB100 depends on an interaction between arginine 3500 and tryptophan 4369 (R3500-W4369). Mutation of the arginine (FDB mutation) or the tryptophan (FDB-like mutation) disrupts receptor binding. The R3500-W4369 interaction is essential for the correct folding of the carboxyl terminus of apoB100 to permit normal interaction between LDL and its receptor, but this interaction is not as favourable for receptor binding as removing the carboxyl tail. LDL with apoB97 has normal receptor binding, whereas LDL with apoB95 lacks a carboxyl tail and therefore has enhanced receptor binding. Tryptophan 4369 interacts not only with arginine 3500, but also arginine 3480 and arginine 3531. Site B (i.e. residues 3359-3369) is the receptor site.  

(Boren J, 2001).
1.4 Familial Defective apolipoprotein B-100 (FDB)

Familial defective binding ApoB100 can be defined as a group of autosomal dominantly inherited mutations in apoB-100 that adversely affect its binding to the low density lipoprotein receptor (LDLR). The originally described mutation R3500Q remains the most commonly encountered cause of FDB. The estimated frequency in the United Kingdom, Netherlands and Germany is 1/600-700 but there are founder effects in Switzerland and Rheine-Main area of Germany with the prevalence estimated to be 1/72 to 1/114. Several other mutations are known today to cause the FDB see table 1.1. The R3500W, a mutation at codon 3500 causing arginine to tryptophan substitution was described by Gaffney et al in 1995 in Scotland as a cause of LDL hypercholesterolaemia. R3531C is a more recently found mutation (1995) by single stranded confirmation polymorphism (SSCP) analysis in 2 kindreds that were of Celtic and Native American ancestry and Italian ancestry.

The Marburg mutation, apo-B-100 H3543Y is also a new mutation (2004). The change of CAC at 3543 to TAC results in amino acid change from histidine to tyrosine. The original discovery of 3 German patients was subsequently followed by a larger study of more than 3300 patients. These studies suggest that this is the most probable Apo-B mutation in Germany.

1.4.1 Historical background of FDB

The original model for familial hypercholesterolaemia was defective clearance due to impaired LDLR activity. LDL from such patients has normal clearance in healthy normal controls. Vega et al identified that there was slowed clearance of donor LDL by receptor-competent recipients, together with impaired clearance of the LDL to receptors on the normal human fibroblasts (Vega at el 1986). These were the first observations supporting the existence of functional ligand defects in LDL by determining the removal from plasma of human plasma LDLC from normal subjects and patients with heritable LDLC increases, Vega and Grundy. In 1989 Soria et al identified the
mutation in these individuals that results in the change of amino acid at position 3500 from arginine to glutamine. This classical R3500Q mutation has been suggested to come from a common ancestor in Europe about 6000 years ago. Myant et al studied 200 unrelated heterozygote FDB (R3500Q) in North America and Western Europe. Almost all the individuals were Caucasian except for one Chinese man. Haplotyping at the Apo B locus on chromosome 2 showed the mutation to be present on the chromosome with the same rare haplotype in almost all the probands. Various factors might have influenced movements in European populations e.g. wars, pilgrimages and voluntary migrations in the past 1000 years. Ludwig has also suggested that R3500Q came from the same ancestral chromosome and there was no evidence for recurrent mutation.

1.4.2 Founder Effects

The founder effect can be described as the phenomenon of a higher than anticipated prevalence of a mutant gene in a population, typically with one or only a few mutations in the given gene. It results from the confinement of a new or small population to a geographical location by migration of relatively small numbers or cultural isolation. Such founder effects are known to exist for the LDLR in Afrikaners, French Canadians and Christian Lebanese. Whilst the prevalence of FH that is usually assigned to LDLR mutations has been estimated to be 1/500 in most populations, it is estimated at 1/75 to 1/100 for Afrikaners and French Canadians respectively.

Data for FDB is not as complete as for the LDLR mutations. The prevalence of FDB has been reported to be about 1/500 to 1/700 in US, Canada and some parts of Europe. Detailed haplotype analyses of mutant ApoB alleles from many FDB individuals suggest that this is a founder type mutation in Caucasians. In some countries e.g. in Switzerland, the mutation has been found to be as common as 1/240. The frequency of such a mutation in different populations is likely to be governed by founder effects and random genetic drift and should vary greatly. FDB has not been detected in Fins, Japanese or Israelis. One family of Afrikaners was described who had members
with LDLR and/or apo B R3500Q mutations. FDB has otherwise not been documented in South Africa.

1.4.3 Diagnosis

The diagnosis of FDB requires the recognition of heterozygous FH phenotype by the clinician after which genotyping or functional testing is required to link the diagnosis to apoB. Whilst at the clinical level the FH phenotype signifies a serious condition, and an exact diagnosis does not influence the treatment, the confirmation of the diagnosis at a genetic level enhances screening of relatives and influences genetic counseling.

1.4.3.1 Clinical diagnosis

The FH phenotype comprises a family history of premature ischaemic heart disease, LDL hypercholesterolaemia and tendon xanthomata.

Patients are asked for the manifestations of atherosclerosis in the form of ischaemic heart disease, peripheral vascular disease, transient ischaemic attacks and strokes. Sudden death may also be suggestive of premature ischaemic heart disease. The family history of premature coronary artery disease is helpful in identification of an autosomal dominant mode of inheritance and the patient’s ancestry may suggest the molecular defect if there is a link to a population with a founder effect. In patients with suspected FH an annotated family tree is most informative to demonstrate the heritable nature of the premature ischaemic heart disease and/or hypercholesterolaemia. The dietary history and the detection of additional factors for atherosclerosis are important for management.

The physical examination of the patient with suspected FH yields valuable information that strengthens the diagnosis of FH. Tendon xanthomata and the arcus cornealis are viewed as good physical signs for familial hypercholesterolaemia but are not invariably present and may occur for other reasons. The occurrence of tendon xanthomata and arcus cornealis increases with age. Xanthelasma is not a sensitive physical sign for hypercholesterolaemia, as it can occur in patients
with normal lipid profiles. Arcus cornealis can occur in elderly without significant lipid problems and tendon xanthomata can occur in other lipid abnormalities e.g. dysbetalipoproteinemia. The physical signs are nevertheless very helpful in the diagnosis of familial hypercholesterolaemia. However, one is likely to miss a significant amount of FH patients if one is to rely only on these physical signs alone with LDL hypercholesterolaemia.

**Arcus cornealis:** This term has been given to the visible line partially or completely following the periphery of the cornea with a small clear space (of Vogt) separating it from the limbus. The superior and inferior limbal areas are the warmer regions of the cornea due to the increased vascularity and it is thought that there is increased capillary flow close to these areas resulting in increased delivery and deposition of the lipoproteins. The prevalence of arcus cornealis in older persons varies between 20 and 35% in the general population and can vary with ethnicity. Black males have the highest frequency. The lipid Research Clinics Programs Prevalence Study found that arcus cornealis and Xanthelasma were highly correlated, especially in young people. These were both associated with high levels of plasma LDL cholesterol.

**Xanthelasma palpebrum** appears as single or multiple soft velvety yellow-orange papules or plaques on the eyelids presenting initially at the medial canthus of the eye and spreading laterally. The plaque is embedded in the corium and the overlying epidermis is thin and may be wrinkled. The prevalence increases with age. It is more commonly found in women than men. The recognition of xanthelasma should alert the physician to presence of dyslipidaemia and the plasma lipid analysis is suggested.

**Tendon Xanthomata** are localised infiltrates of lipid with the histologic hallmark of foam cells in tendons. The foam cells are tissue macrophages that have phagocytosed lipoproteins that have entered the tissues from the circulation. It is likely that LP undergoes oxidation to become ligand for receptors that are constitutively expressed in macrophages, which thus import cholesterol even though the cell may have more than it needs. The lipids found in foam cells are esterified cholesterol but occasionally other sterols and triglycerides accumulate in significant amounts.
Tendon Xanthomata are firm enlarged nodules that are attached to or form an integral part of the tendon. The tendon is covered by normal skin that can easily be moved over the nodule. Xanthomata commonly involve the Achilles tendons, and less commonly the extensor tendons of the digits. Periosteal xanthomata may occur on tibial tuberosities and the elbows. Achilles tendon xanthomata in the presence of raised cholesterol level with normal triglycerides are virtually diagnostic of FH.

Figure 1.3 Clinical signs of familial hypercholesterolaemia, pictures by D Marais

Top left: arcus cornealis, Bottom left: Achilles tendons
Top right: Extensor tendon xanthomata
Bottom right: Cutaneous, subcutaneous and tendon xanthomata in the hands of a patient with homozygous FH.
1.4.4 DIFFERENTIAL DIAGNOSIS

At the clinical level, especially at the initial consultation, an exact diagnosis may not be possible. After considering disorders that may cause secondary dyslipidaemia of LDL (with or without minor changes in VLDL), other primary causes need consideration. The recognition of the complete heterozygous FH phenotype restricts the causal diagnosis to three genetic entities and genotyping is required to identify the exact cause. The differential diagnosis can be broadly divided into primary and secondary causes.

1.4.4.1 Secondary dyslipidaemias

It is prudent to exclude the secondary causes of hyperlipidaemia because they may need treatment in their own right and the dyslipidaemia may resolve on treating the cause. Secondary dyslipidaemias that should be considered in the differential diagnosis of FH are tabulated in table 1.4. LpX is included as it may be falsely attributed to LDL in the Friedewald equation that is still in use at Groote Schuur Hospital. The two most common disorders giving rise to severe LDL hypercholesterolaemia are nephrotic syndrome and hypothyroidism.

<table>
<thead>
<tr>
<th>Condition</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>↑</td>
<td>↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
<td>↑</td>
<td>↑↑</td>
<td>─</td>
</tr>
<tr>
<td>Cholestasis</td>
<td>─</td>
<td>↑↑ (LpX)</td>
<td>↓</td>
</tr>
<tr>
<td>Hepatocellular disease</td>
<td>↑</td>
<td>─↑</td>
<td>↓</td>
</tr>
<tr>
<td>Analbuminaemia</td>
<td>─</td>
<td>↑↑</td>
<td>─</td>
</tr>
<tr>
<td>Severe</td>
<td>─</td>
<td>─↑</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

| Severe hypergammaglobulinaemia   | ─    | ─↑  | ↑↑  |

Table 1.4 Causes of secondary hyperlipidaemia

LpX=lipoprotein X
1.4.4.2 Primary hypercholesterolaemias

Having excluded the secondary causes of hyperlipidaemia, the differential diagnosis of primary hypercholesterolaemia is still wide. The heterozygous form of FDB is indistinguishable from that resulting from low-density lipoprotein receptor mutations and that resulting from neural apoptosis regulated convertase 1 coded by the Proprotein convertase subtilin / Kexin 9 gene. Genetic testing is the only reliable and practical way to discriminate between LDLR mutations, FDB and PCSK9.
<table>
<thead>
<tr>
<th>Gene</th>
<th>FAMILIAL HYPERCHOLESTEROLAEMIA</th>
<th>FCH</th>
<th>POLYGENIC</th>
<th>DYSBETA</th>
<th>PHYTO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>19</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inheritance</td>
<td>Dominant</td>
<td>Dominant</td>
<td>Dominant</td>
<td>Variable</td>
<td>Recessive</td>
</tr>
<tr>
<td>Mutations</td>
<td>&gt;10000</td>
<td>9</td>
<td>3</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Founder Population</td>
<td>South Africa, Lebanon, Gujarat, French, Canadian, Lithuania</td>
<td>Swiss, Germany</td>
<td>Norway, UK, USA, and France</td>
<td>Netherlands, ApoE Leiden, ApoE K146Q Xhosa (ATXB), ApoE R145C</td>
<td></td>
</tr>
<tr>
<td>Prevalence</td>
<td>1/70 to 1/500</td>
<td>1/210 to 1/500</td>
<td>?</td>
<td>1/100</td>
<td>Unknown</td>
</tr>
<tr>
<td>Achilles tendon xanthomata</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Arcus cornealis</td>
<td>Common</td>
<td>Common</td>
<td>Common</td>
<td>Background</td>
<td>Background</td>
</tr>
<tr>
<td>LDLc</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓ (Friedewald value ↑)</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>N</td>
<td>N</td>
<td>N or ↑</td>
<td>N or ↑</td>
<td>N</td>
</tr>
<tr>
<td>Other sterols</td>
<td>No’</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Phytocholesterol</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.5 Differential Diagnosis for patients with Familial Hypercholesterolaemia
Not all subjects with mutations in LDLR, apoB or PCSK9 will display the full clinical phenotype on which to proceed with genetic workup. Hence the differential diagnosis of severe LDL hypercholesterolaemia is important to consider carefully. Table 1.5 compares the FH phenotype with other dyslipoproteinaemia.

Although extremely rare, other causes of xanthomatous disease must be considered especially in the absence of family history of premature coronary artery disease. Two other conditions are known to cause xanthomata and variable degrees of hyperlipidaemia: phytosterolaemia and cerebro-tendinous xanthomatosis. Both of these disorders have been recorded in the lipid clinic.

1.4.4.3 Familial Combined Hyperlipidaemia (FCH)

FCH is believed to be a common disorder but its cause is unknown and it may represent a central problem of lipoprotein overproduction that is modified by age and minor variations in other genes. The family displays a dominant inheritance of dyslipidaemic of various patterns and the condition is often milder in children. Some family members display elevation of LDL, some of VLDL + LDL and some have chiefly increased VLDL. The condition is believed to be devoid of tendon xanthomata. Within this diagnosis, there maybe subjects with LDLR mutations.

1.4.4.4 Dysbetalipoproteinemia (Type III hyperlipoproteinaemia)

Patients with type III hyperlipoproteinaemia have elevated concentrations of plasma cholesterol and triglycerides due to the accumulation of remnants of TG-rich lipoproteins when there is a lack of functional apo E. The clinical features of the disorder are varied. Many type III subjects have cutaneous xanthomas, particularly tubo-eruptive or tuberous xanthomas, and infiltration of the palmar creases. These patients have a high incidence of premature coronary and peripheral atherosclerosis. The primary molecular defect in most patients with dysbeta lipoproteinaemia is the presence of a mutant form of apo E (apo E-2) that differs from normal apo E (apo E-3) by only a single amino acid substitution (cysteine for arginine at residue 158). The apo E-2 variant binds poorly to LDL receptors. Dysbetalipoproteinaemia due to this cause is associated with a recessive mode of
inheritance that typically also requires a metabolic stress for the phenotype to emerge. Other rarer forms of mutant apo E causing dysbeta appear to be associated with dominant inheritance but still display a delayed penetrance. In the dominant form of the disorder patients possessing a single allele for one of the rare variants of apo E still may not have the overt hyperlipidaemia from birth but typically change in adulthood. The diagnostic hallmark of this disorder is the presence of apo E that is defective in binding to lipoprotein receptors. The condition enriches VLDL with cholesterol and in almost all cases raises plasma apo E concentration.

1.4.4.5 Phytosterolaemia (sitosterolemia) and cerebrotendinous xanthomatosis

These rare recessive disorders are clinically characterised by tendon and tuberous xanthomata and the former has a strong predisposition to premature coronary atherosclerosis. In phytosterolaemia cholesterol maybe elevated to the same values of heterozygous FH but the onset of xanthomata is usually in the first decade and skin xanthelasma are prominent but the condition is recessive and parents have normal levels or modest elevation of cholesterol as expected in the normal population. Increased serum cholesterol levels are found along with high concentrations of phytosterols: β-sitosterol, campesterol, stigmasterol and others. The defect is in the ABC transporters G5 and G8, which are expressed in the enterocyte and hepatocyte so that plant sterols can be excreted.

Cerebrotendinous xanthomatosis (CTX) is differentiated from phytosterolaemia by involvement of ocular lenses and CNS. CTX is a rare recessive disorder due to cholesterol 27hydroxylase (CYP 27) deficiency. There is accumulation of cholestanol and cholesterol in most tissues, especially in tendons forming xanthomas and in the brain. This disorder is clinically characterised by progressive dementia, cerebellar ataxia, tuberous and tendon xanthomas, cataracts and some increased risk of atherosclerosis but relatively normal plasma cholesterol concentration. More than 20 different mutations have been identified in patients with CTX. In CTX plasma cholesterol concentration is normal to mildly elevated.
1.4.5 Routine laboratory investigation

A fasting lipid profile is arguably indicated at least once in all young adults to detect unsuspected FH subjects but certainly is indicated in subjects with a personal or family history of premature ischaemic heart disease or physical signs suggestive of dyslipidaemia. The plasma full lipid profile comprises triglycerides, total cholesterol, HDLC and LDLC. The total cholesterol of more than 7.5mmol/L is suggestive of serious risk for cardiovascular disease and FH should be considered as the diagnosis.

Other biochemical tests are necessary for the exclusion of secondary hyperlipidaemias. A renal function test (plasma urea and creatinine) will rule out significant kidney disease as a cause of the lipid abnormalities. Although renal impairment is associated with a Frederickson IIb dyslipidaemia it may not always be so severe that monogenic disorders such as FCH and FH should be considered. It is also important to do urine dipsticks and urinary protein quantification as nephrotic syndrome may cause severe elevation of LDLC in the range of FH without raising urea and creatinine concentration. Thyroid stimulating hormone assay is necessary to screen for hypothyroidism. The clinical signs of hypothyroidism may not always be present with thyroid disease. Determination of serum albumin could reveal nephrotic syndrome or analbuminaemia. However rare, the latter needs to be considered in the context of FH. The plasma bilirubin along with the ALP and GGT could detect cholestatic liver disease that raises plasma cholesterol and the Friedwald calculation for LDLC may be misleading in this setting. The liver enzymes (AST, ALT) should be done because of the statin therapy that will be initiated, may derange these. The fasting blood glucose should be done to exclude diabetes mellitus. Uric acid levels should be done, as hyperuricaemia and gout are common conditions that may be aggravated by thiazide diuretics and aspirin.

1.4.6 DNA analysis and genotyping

The clinical suspicion of the Familial Defective Binding should be followed by a definitive diagnostic procedure. The most practicable diagnostic test is genotyping. LDL clearance or uptake of LDL into the cells represents alternative approaches to the diagnosis. Enhanced reactivity of the ligand defective LDL with monoclonal antibody MB47, known to react with an epitope in the region of ApoB sequence involved in binding to the LDLR provides a
strong evidence for structural abnormality in ApoB but may not detect all mutations. Though these procedures are definitive they are not practical for clinical application.

Genotyping is accurate and cost effective because only a few mutations have been reported to cause FDB. Mutations that impair the ligand activity towards the LDL (Apo B, E) receptor have been reported only from exons 26 and 29. Overall, there are 7 mutations described in these 2 exons see table 1.2. Earlier methods sought mutations individually but newer techniques such as high resolution melting (HRM) can detect reliably all duplexes in an amplicon.

The introduction of high resolution thermal melting (denaturation) of DNA by a fluorescent dye for the presence of double strands has made the detection of any heteroduplex reliable but has the disadvantage of failing to detect true homozygous subjects. This can be remedied by spiking the DNA sample with wild type DNA. Hybridization with allele-specific oligonucleotide probes is an alternative approach to genetic diagnosis. Mutations that introduce restriction sites or amplification with restriction sites, amplification refractory mutation systems and single stranded DNA mobility shift analysis can also be used.

Measurement of receptor binding of LDL or uptake into cells would test ApoB in a broader way than the genotyping and could still be applied after genotyping of exons 26 and 29 has been done.

1.4.6 CARDIOVASCULAR COMPLICATIONS OF FDB

Atherosclerosis is poorly understood, but is clearly a multifactorial disease in which, especially in FH, LDL hypercholesterolaemia plays a strong role. There is usually diffuse vessel disease from large to small arteries. Atherosclerosis has dire consequences in the form of ischaemic heart disease, neurological complications such as transient ischaemic attacks and stroke, peripheral vascular disease and death. However, in FH the coronary arteries bear the brunt of the disease.
**Ischaemic heart disease** is the single largest cause of death in the developed world. The most serious complication of raised LDLC is premature ischaemic heart disease.

Slack, Beaumont Stone et al have given overwhelming evidence that FH is associated with severely increased risk for premature coronary artery disease. Slack et al found that the mean age to develop coronary artery disease for men was 43 years and 53 years for women. The risk was 5% at 30 years and 51% by 50 years and increased to 85% by 60 years of age.

**Peripheral vascular disease:** Atherosclerotic disease can manifest as claudication. Slack, Stone, Beaumont et al also reported an increased risk of intermittent claudication amongst FH individuals by 8-16%. The severity increases with age.

**Stroke:** The risk of stroke due to hypercholesterolaemia is controversial especially in non-FH patients. But placebo-controlled studies have shown reduction of strokes in patients on statin therapy. An analysis of 1031 patients at the Groote Schuur Hospital Lipid Clinic revealed that only a small proportion of subjects with FH also had strokes.

**Other rare complications:** Achilles tendonitis or tenosynovitis can occur and can be painful and debilitating. Aneurysms and dissection are not associated with FH.

**Psychosocial:** Knowing about a condition with dire consequences may have a profound psychological impact on an individual. The knowledge seemed not to cause long term distress in a study done by Croyle, on psychological effects of screening for disease prevention and detection. However, nowadays FH is an illness whose natural history should be dramatically improved by preventative and interventional treatment. Indeed, the Simon Broome Register of FH in the UK has already shown a significant reduction in risk of 76%, within two decades of introducing statins and a lesser period of achieving ideal LDL concentration.
1.4.7 MANAGEMENT OF FDB

Atherosclerosis is not well understood and is clearly multifactorial. It is thus appropriate to identify and address all known risk factors for atherosclerosis. The risk factors are often classified as immutable (age, gender and monogenic disorders), environmental or lifestyle (obesity, smoking, inactivity, fatty diet etc). Novel risk factors include hyperhomocystenaemia, Lp(a), PLA2 and newer genes linked through genome wide association studies.

“The prevention of atherosclerosis presents a long term challenge to all health care professionals and to public health care policy. Health professionals should strive to help patients to optimize their risk factor profile before atherosclerotic disease is manifest”. Various models have been used in an attempt to predict an individual’s risk of developing ischaemic heart disease. Including the Framingham data from North America but these are not generalisable to all individuals or populations and underestimate the risk in FH.

The NECP guidelines and joint European guidelines recommend aggressive LDLC lowering to less than 2.6 and 3.0mmol/L respectively in high and very high-risk states. Patients with FH have much higher LDLC concentrations and these goals might be difficult to meet. The encouraging news is that atherosclerosis regression can be seen if LDLC lowering is sufficient. Nissen suggests > 75% change. Target concentrations of LDL <1.8 mmol/L have been suggested in very high-risk settings. The management of FDB therefore requires comprehensive multidisciplinary approach to enhance the outcome to the best possible one. The risk factors for IHD burden should be addressed individually and collectively.

1.4.7.1 Lifestyle modification

**Dietary modification:** Lower intake of cholesterol is associated with lower risks of coronary heart disease and of all cause mortality by epidemiologic studies. Dietary triglyceride and especially saturated fat also enhance risk. Several diet studies were done and have shown good outcomes from improved low cholesterol diet though these were not
done in FH. (Indo-Mediterranean diet, Lyon diet Heart study National cholesterol education programme). Brown and Goldstein first described how dietary cholesterol suppresses the LDL receptor activity by the liver, leading to LDL hypercholesterolaemia. On a low fat diet the LDLR is up regulated in the liver. Even in FH due to LDLR mutations, increased LDLR expression will also increase. The removal of normal apo B and to some extent, the removal of defective apo B will also increase to some extent. Similarly, greater expression of LDLR in subjects with PCSK9 will also lower LDLC concentration. The aim of diet modification is to lower the LDL by means of up-regulation of the LDL receptors of FH heterozygotes to enhance the clearance of the LDLC. Groote Schuur Hospital Lipid Clinic has showed that the LDLC concentration can be lowered by about 1.5mmol/L on average by intensive dietary counseling.

Since the elevation of LDLC in FH remains greater than the best diet response can achieve, dietary modification alone is not sufficient for FH patients to get the cholesterol to target and drug treatment is required.

Modest alcohol consumption reduces the risk of ischaemic heart disease and should be limited to 10 to 20 grams per day for men and 10 grams per day for women on general principles to avoid its other risks.

**Smoking avoidance:** Cigarette smoking is a major risk factor to the development of fatal myocardial infarction. Much education has been done on the effects of smoking and its risk factors. In South Africa cigarette packets clearly indicate the risk of myocardial infarction but the public does not appear to heed this warning.

Myocardial infarction is increased by six-fold in women and by three-fold in men who smoke about 20 cigarettes compared with non-smokers, without FH being present. An analysis at Groote Schuur Hospital revealed that smoking was strongly associated with premature ischaemic heart disease in FH. Cessation of smoking has been associated with the reversal of the risk. The benefit extends to those with established coronary heart disease as well.
The long-term (19-year) follow-up demonstrates that patients who continue to smoke have an 82% mortality compared to 37% of those who stop in the general population. 

**Exercise and cardiac rehabilitation:** Regular physical exercise is associated with half the risk of future cardiac events in a cohort that examined subjects without FH. The benefits are in part associated with the improvement of blood pressure and lipid profile. 

Physical activity should be strongly recommended in patients with FH even though there is no evidence of benefit in this condition. Exercise may also assist in earlier diagnosis of angina pectoris. 

**Body mass reduction:** overweight patients and obese patients (BMI>30kg/m²) are at increased risk of coronary vascular disease. The estimate is about 5% deaths from CAD in men and 6% of such death in women who are obese but this is not studied in FH. The adverse effect of obesity is more pronounced when the fat is concentrated on the abdomen, known as central obesity. The waist dimension or high waist: hip ratio can identify central obesity. 

**Hypertension** is a traditional risk factor for coronary artery disease. Both systolic and diastolic hypertensions are associated with increased risk for atherosclerosis. There are no specific trials of antihypertensive treatment for the primary prevention of atherosclerosis, although evidence favours certain classes of drugs for secondary prevention such as β-blockers and ACE inhibitors with demonstrable secondary preventative benefits. While there are no studies testing the impact of hypertension on the development of atherosclerosis in FH, or even whether a lower value such as applies to diabetes management would be of benefit, it makes intuitive sense to be on the lookout for hypertension and to treat this early with lipid neutral medication.
**Diabetes Mellitus:** abnormal glucose tolerance or raised fasting glucose is associated with vascular disease even in the absence of significant dyslipidaemia. Diabetes mellitus substantially increases the risk for Atherosclerosis by two to four-fold in men and even higher in women. Diabetes not only increases the risk for atherosclerosis but also magnifies the effect of other risk factors for atherosclerosis e.g. hypertension, raises cholesterol levels and obesity.

1.4.7.2 Drug therapy of FH

The available lipid lowering drugs are divided into the following classes of drugs:

1. Statins (HMG CoA Reductase inhibitors)
2. The Bile Acid Sequestrants
3. Nicotinic Acid
4. Cholesterol Absorption Inhibitors
5. The Fibrates

**The HMG-CoA inhibitors:** This group of drugs (statins) has been shown to reduce the cardiovascular events in primary and secondary prevention of atherosclerosis but no special clinical outcome studies against placebo have been undertaken in FH as the risk of FH is very high and it has been accepted that placebo-controlled studies would not be ethical. Statins have become the first line of treatment for familial hypercholesterolaemia because they have been proven to be the most useful in LDL reduction. “The advent of this group of drugs holds promise that the prognosis of FH can be reversed to the background risk of the population”. In heterozygous adult patients with FH, life long treatment with lipid lowering drugs is indicated, because these drugs slow down progression of atherosclerosis as judged by coronary angiography. HMG-CoA-reductase inhibitors are the drugs of first choice in the lipid lowering therapy.

The statins vary in their power of reduction of cholesterol but behave similarly on doubling their doses, by further decrements of 6%. Equivalent doses for a 30% reduction are rosuvastatin 2.5 mg/d, atorvastatin 5mg/d, simvastatin10mg/d, lovastatin 20 mg/d, fluvastatin 40 mg/d and pravastatin 80 mg/d. The maximum doses of atorvastatin (80 mg/d)
and rosuvastatin afford the best current reduction of LDL C by about 54%. Nevertheless, on 80 mg of rosuvastatin, only 25% of FH reached the LDL C target. Accepting an untreated LDL C of 6 mmol/l it is clear that maximum treatment will barely achieve the recommended LDL C of 3 mmol/l for primary prevention.

Simvastatin can reduce LDL cholesterol by 30 to 40% in most hypercholesterolaemic patients. The ASAP study has shown that aggressive LDL cholesterol reduction by atorvastatin was accompanied by regression of carotid intima media thickness in patients with FH while Simvastatin with lesser LDL C reduction did not. The ASAP study concluded that aggressive lipid lowering is indicated, beneficial and safe in patients with FH. Although the ASAP study used carotid IMT as end point, the study is relevant as IMT is increasingly acknowledged as a reliable measure to predict future events and the clinical relevance of progression of IMT as a marker for cardiovascular disease is beyond doubt. Small dense LDL C are reduced in concentration in heterozygous FH on statin therapy and this may have additional anti-atherogenic effects by reducing not only LDL C but also a small dense LDL C and remnant-particle cholesterol.

Statins have been proven to work for the FDB patients. 12 patients were treated with Lovastatin 20 mg/d the LDL C decreased by 21.5%, and decreased by 32% on 40 mg of lovastatin.

**Fibrates:** A cross over study between pravastatin and gemfibrozil by Hansen demonstrated that pravastatin reduced LDL C and apo B by 20-25%. The addition of fibrates managed to reduce cholesterol and LDL C by 4-6%. The triglycerides were lowered by 25% and raised HDL C by 11%. Fibrates are thus ineffective as monotherapy against LDL hypecholesterolaemia. Nevertheless they may offer benefit in subjects with mildly raised TG and or low HDL C.

**Bile Acid Sequestrants:** These are non-absorbable anion exchange resins that bind bile salts irreversibly, resulting in depletion of bile acid pool. The end result is greater cholesterol conversion to bile acids, which in turn up-regulates the LDL R to maintain
cholesterol levels within the liver, thus lowering plasma cholesterol levels. These agents might be difficult to take because of the gastro-intestinal side effects. Schmidt conducted a study in 1993, which showed that cholestyramine and colestipol reduce LDLC in FH patients. In this study LDLC fell by 32% in FDB patients and by 21% in LDLR mutation patients. The results indicated FDB and LDLR mutation hypercholesterolaemic patients respond to bile acid sequestrants. When mono-therapy with atorvastatin is insufficient to treat severely hypercholesterolaemic patients, colestipol acts to reinforce the action of statins.

**Nicotinic Acid (Niacin):** Nicotinic acid in pharmacological doses reduces the concentration of plasma triglycerides, LDLC and raises HDLC. It also lowers Lp(a). Its mechanism of action is probably mostly by reduction of VLDL secretion by the liver. The effects of nicotinic acid was tested on FDB patients and compared with the LDLR mutation patients. The LDLC concentration was reduced by 24% in the 4 FDB patients and 14% in the LDLR patients. This indicates that nicotinic acid is effective in the treatment for FDB patients.

**Cholesterol Absorption Inhibitors:** Ezetimibe is an azetidinone derivative that acts as an inhibitor of intestinal absorption of cholesterol. Ezetimibe localises at the brush border of the small intestine and decreases cholesterol uptake into the enterocytes. This apparently does not affect absorption of other soluble food nutrients and related compounds such as fatty acids, bile acids, progesterone, ethynyl estradiol and soluble vitamins. It inhibits the absorption of cholesterol and plant sterols by inhibiting the passage of dietary and biliary cholesterol across the enterocyte through interference with uptake by the Niemann Pick C1-like protein. Ezetimibe is not sufficient as monotherapy: the expected LDLC reduction is 18% with the registered dose of 10 mg. Its application with a statin in the enhance study did not show any significant difference in changes in intima-media thickness though LDLC lowering was enhanced. Combined therapy with ezetimibe and statin provides an incremental reduction in LDLC levels of 12-19%.

**Combination therapy:** The ENHANCE study has compared simvastatin with or without ezetimibe in FH. The LDLC was much reduced in the combined treatment group. Combined therapy did not result in a significant difference in changes in intima-media thickness (IMT)
as compared with simvastatin alone but this may be attributable to previous treatment and relatively normal values of IMT.

Kawashiri et al has shown that the addition of colestemide will act to reinforce the reaction of statins when monotherapy is insufficient to treat severely hypercholesterolaemic patients as in heterozygous FH. The effect of monotherapy on LDLC is a reduction of approximately 15% but in combination with statin, it is reported at approximately 23%.

**Plasmapheresis:** This has been shown to reduce the LDLC in patients with FDB. This expensive and invasive procedure is less often used since the advent of the highly effective statin therapy has made treatment highly effective.

**FDB compared with other causes of FH phenotype**

There has been some controversy about the severity of FDB versus LDLR mutations but from a practical clinical point of view they should be viewed as indistinguishable on usual clinical and biochemical assessment.

FDB seems to be biochemically less severe in children. This could be explained by increased LDLR activity during growth with the addition of much more LDLR activity than in FH subjects with LDLR mutations. In homozygous FDB, it would appear that there is no significant gene dose effect. Compensatory up-regulation of LDLR can be expected to ameliorate homozygotes with FDB because the greater internalisation of LDL will prevent the formation of LDL. There are differences in FH due to the LDLR mutations depending on the net functional impart that the mutations have on the LDLR activity. Null mutations result in higher LDLC concentration. Kotze reported that the V408M mutation has a worse biochemical phenotype than the D206E mutation is responsible for the majority of FH in the Afrikaner. Since the more common defective LDLR mutations account for the bulk of FH, it is important to compare FDB with FH due to a defective mutation such as D206E.

Theoretically the statins act mainly by increasing high affinity receptor mediated catabolism of LDL. In FH the fall in plasma LDL levels due to statin therapy has been shown to be almost entirely due to increased catabolism of LDL by stimulation of the LDL receptor pathway, with no consistent change in LDL production. In FDB heterozygotes about 70% of the total circulating LDL-C is defective and only 30% is normal. The binding affinity of the defective LDL to the LDL receptor is less than 5% of normal. The similarity in response has not been explained fully, as it only means that the enhanced catabolism of LDLR pathway brought about by the statins only enhances absorption of only 30% of LDL
in FDB and 100% in FH heterozygotes. This suggests that some other mechanism produces the similar response to statin therapy seen in the two conditions. In FDB, the uptake of apo-
E containing remnant particles (VLDL and IDL) is not impaired. The upregulation of LDL receptors by the statins may enhance the clearance of apo-E containing remnants containing both normal and defective apoB before they are converted to LDL. Increased clearance of remnants would also lead to decreased LDL production of both normal and defective LDL by the liver in FDB subjects, as suggested by Rubinsztein et al. In support of the latter mechanism, homozygotes have been shown to have normal plasma remnant concentrations and respond well to statin therapy with pronounced decreases in total cholesterol and LDL-C levels, in contrast to FH homozygotes that are usually resistant to statin therapy.

Rubinsztein et al have shown similar response to therapy in patients with LDLR defects, FDB, and patients with both LDLR defects and FDB. This suggested that the clearance of apo-E containing defective remnants by the statins in FDB is as effective as upregulation of LDL receptor activity in LDL receptor defects.

To date there has been no report of homozygous PCSK9. PCSK9 appears to have higher LDL-C concentration and this translates into a lower likelihood of achieving targets with statin treatment.

In conclusion FDB is a serious disorder that results in the FH phenotype with its attendant premature atherosclerosis. It is amenable to statin therapy and like FH, due to LDLR mutations should have a remarkably better prognosis provided it is diagnosed and treated timeously. More so than with LDLR mutations, a genetic diagnosis will assist in the identification of younger subjects with FDB.
Chapter 2

METHODS

This section will cover the methodology of the clinic, the laboratory and that of the analysis of information on FDB. The current database for the Groote Schuur Hospital Lipid Clinic covers experience over the past 24 years. It is computerised with initial clinical and laboratory parameters and contains diagnostic and research information. This database is used to study the causes of the FH phenotype.

The Lipid clinic is a referral clinic for patients with severe dyslipidaemias: plasma triglycerides of more than $\geq 5$ mmol/L, and/or total cholesterol of $\geq 7.5$ mmol/L. Patients with premature coronary artery disease and those with physical signs of dyslipidaemia or adverse effects of pharmacologic treatment are also accepted.

There are separate databases for non-routine investigations including electrophoresis and DNA diagnosis. The full-standardised operating procedures are attached as appendices. The methods will be summarised briefly.

2.1 Clinical Documents

Detailed clinical assessments of patients have been done by a small number of experienced specialists, one of whom has worked at this clinic for more than 24 years. New patients were admitted to the clinic only by these experienced clinicians. A standardised format was introduced in 1985 where after it was adapted and retained since the early 1990’s.

**Admission Form 1:** summarises the patient’s identity, reason for referral, symptoms of atherosclerosis, past medical history, medication, dietary habits and lifestyle habits. On the reverse side of this form is the detailed family genogram as far back as possible. Special attention is given to the region from which the grand parents came, their ethnic origin and surnames. This is illustrated in fig 2.1
Admission Form 2: summarises the anthropometry, physical examination and blood chemistry. It emphasises the physical signs of dyslipidaemia and cardiovascular system. The physical signs and chemistry are captured in the database fig 2.2

Admission Form 3: comprises the comprehensive risk assessment, clinical diagnoses and management plan. The diagnosis reached here is captured on the database. See fig 2.3

Consent form for DNA: was approved by the UCT ethics and research committee in the format specified for human genetic investigations. DNA is kept for diagnosis and research in consenting patients. This is illustrated in fig 2.4

Follow-up Form 1 and 2: summarise the laboratory lipid profile together with medication and follow-up text respectively. For brevity, and ease of reference each page summarises 5 visits. All the documents are made in duplicate for the lipid laboratory and Groote Schuur folder. This is illustrated in fig 2.5 and fig 2.6
Figure 2.1 Admission Form 1 for Lipid Clinic
Figure 2.2 Admission Form 2 for Lipid Clinic
### GSH LIPID CLINIC ADMISSION FORM (3)

#### PATIENT

#### RISKS ASSESSMENT

<table>
<thead>
<tr>
<th>TRIGLYCERIDE</th>
<th>FAMILY HIST</th>
<th>HYPERTENSION</th>
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<tbody>
<tr>
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<td>SMOKING</td>
<td>DIABETES MELL</td>
</tr>
<tr>
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<td>EXERCISE</td>
<td>RENAL DISEASE</td>
</tr>
<tr>
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<td>DIET</td>
<td>HYPERURICAEMIA</td>
</tr>
<tr>
<td>App (a)</td>
<td>OBESEITY</td>
<td></td>
</tr>
</tbody>
</table>

#### CLINICAL ASSESSMENT

| LIPID DISORDER | FREDRICKSON TYPE: |

#### OTHER CLINICAL PROBLEMS

1. 
2. 
3. 
4. 
5. 
6. 
7. 
8.

#### FAMILY STUDIES

#### MANAGEMENT

#### D.I.E.T

#### OTHER

#### DRUGS

#### FOLLOW-UP

#### COMPUTERISED | DICTATED

---

**Figure 2.3 Admission Form 3 for Lipid Clinic**
CONSENT FOR DIAGNOSTIC & RESEARCH INVESTIGATION

Please read carefully and feel free to ask any questions you may have. If you agree, place a check (√) in the relevant block, if you disagree, delete the relevant clause.

I, ........................................................................................................... hereby consent to the removal of
blood ☐, skin ☐, or other tissue (specify) .............................................. from myself and/or
........................................................................................................... to whom I am the (relationship)
...........................................................................................................

The purpose of the investigation is to perform
☐ diagnostic tests for dyslipidaemia and/or other metabolic disease that may predispose to atherosclerosis
☐ research investigations currently and in future, subject to the conditions of the institutions above and the
ethics review that govern the research at these institutions. This may mean the investigation for alternative
inherited defects to explain my condition, or other factors which may have a bearing on the outcome of my
condition, or for testing of any other gene that may be under research.

The result(s) of the analysis carried out on the sample(s) will be made known to me, via my doctor, in
accordance with the relevant protocol if and when available. The doctor(s) to be informed:

...........................................................................................................

Additionally, I authorise that the results be made known to the following family member(s):

...........................................................................................................

After due explanation by ........................................................................, I understand that

(1) Conventional procedures and techniques are employed by trained staff to obtain and process the samples
and that consequently the risk to my health is minimal.
(2) Confidentiality and anonymity of the sample(s) are respected by coding the samples with numbers. My
written permission is required for release of identifiable information to any other party.
(3) Precise diagnoses may not always be possible because of limited knowledge about the cause(s) of the
condition(s) under investigation because current understanding and technology are imperfect. The research
may thus be of no direct benefit to me but the samples may provide general insight into disease and health.
(4) The cost involved for me will be declared and no tests for which payment is required will be undertaken
without prior consultation. Costs may paid by research budgets or will be according to scheduled tariffs.
(5) No commercial claims can be made on developments from these investigations by myself or my family.
(6) Permission to investigate may be withdrawn at any time by me, without prejudice to my future medical
care by the lipid clinic staff.

Signed .................................................. Date ....................................
Witnessed by (Signatures) .................................................................

Printed Names & Dates ........................................................................

Admsn5DNA.wpd

Figure 2.4 DNA consent form for Lipid Clinic
Figure 2.5 Follow-up form 1 chemistry and drug sheet
<table>
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</tr>
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</tr>
<tr>
<td>April 1</td>
<td>Jane Smith</td>
</tr>
<tr>
<td>May 1</td>
<td>Richard Lee</td>
</tr>
<tr>
<td>June 1</td>
<td>Susan Brown</td>
</tr>
</tbody>
</table>

Figure 2.6 Follow-up form 2. Treatment boxes correspond with visits on follow-up form 1.
2.2 Routine laboratory investigations

A comprehensive range of Routine laboratory chemistry tests is requested at admission to the clinic. Patients are instructed to fast for more than 8 hours before venesection and to abstain from alcohol for this test.

The venesection is done with vacuum tubes selected for selection of serum or plasma. The patient is seated after a rest and the blood is immediately sent to the routine chemical pathology laboratory. These tests are limited by budget constraints but will detect most secondary dyslipidaemias and describe baseline values.

In the case of new patients, the blood tests are:

A. The Lipid profile: TG, TC, HDLC and the LDLC were obtained by Friedewald calculation. The apo A1, apo B, Lp(a) levels were also measured.
B. Renal function: urea and creatinine.
C. Liver function tests: bilirubin, AST, ALT, GGT, ALP and albumin.
D. Endocrine and metabolic test: TSH, fasting glucose and uric acid.
E. Muscle enzyme: CK

Conventional enzymatic spectrophotometric assays are used for auto analysers by the National Health Laboratory Services (NHLS). In most cases new patients also had an agarase gel electrophoresis.

At follow-up the fasting TG, TC, HDLC and LDLC are determined as well as liver, renal or endocrine tests that were abnormal at admission. Further tests are determined according to the clinical setting.

TEST PRINCIPLES

Triglycerides test principle [Roche]: The patient’s serum sample is added to the R1 (buffer /4-chlorophenol/enzymes) and the reaction starts.

The triglycerides in presence of 3 water molecules react with the lipoprotein lipase (LPL) to form glycerol and three fatty acids. The glycerol reacts with the ATP and the glycerol kinase (GK) and magnesium to form glycerol-3- phosphate and ADP. The glycerol -3-phosphate
reacts with the oxygen and glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H₂O₂).

The H₂O₂ produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff (Trinder endpoint reaction). The colour intensity is directly proportional to the concentration of cholesterol and can be determined photometrically.

**Total cholesterol test principle [Roche]:** Cholesterol is determined enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esters and water react under the catalytic action of cholesterol esterase to form free cholesterol and free fatty acids. The cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide created forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity is directly proportional to the concentration of cholesterol and can be determined photometrically.

**HDLC test principle [Roche]:** The cholesterol concentration of HDLC is also determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino group (approximately 40%). The HDLC esters are hydrolysed by the PEG-cholesterol esterase to form free HDLC and free fatty acids. The free HDLC is then oxidised by PEG-cholesterol oxidase to form Δ⁴-cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HDSA [sodium N-(2-hydroxy-3-sulfopropyl)-3.5-dimethoxyaniline] to form a purple blue dye. The colour intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically.

**The Friedewald equation for the calculation of LDLC:** The designated comparison method for the determination of LDLC, using ultracentrifugation and precipitation, known as "ß-quantification", is cumbersome and time-consuming and requires expensive instrumentation and trained personnel. The Friedewald equation (LDLC = total cholesterol - HDLC - [triglycerides (in mmol/L)/2.17 or triglycerides (in mg/dL)/5]), the most frequently used method for the calculation of LDLC, assumes that VLDL particles maintain nearly
constant cholesterol: triglyceride ratio. However, this assumption is invalid in the presence of chylomicronemia and increased VLDL or intermediate-density lipoprotein particles and cannot be used if the triglyceride is >4.5 mmol/l.

**Test principle for lipoprotein (a) [Roche]:** This is an immunoturbidimetric assay. Addition of anti-lipoprotein (a) antibodies to the patient’s sample reacts with lipoprotein (a) to form an antigen/antibody complex, which is determined turbidimetrically after agglutination.

**Test principle for Apolipoprotein A [Roche]:** This is also an immunoturbidimetric test. The anti-apolipoprotein A antibodies react with the antigen in the sample to form antigen/antibody complexes, which, following agglutination, can be measured turbimetrically.

**Test principle for Apolipoprotein B [Roche]:** Anti-apolipoprotein B antibodies react with the antigen in the sample to form antigen/antibody complexes which, following agglutination, are measured turbimetrically.

**2.3 GGE:** Since 1991 there has been an interest in lipoprotein particle size characteristics. Electrophoresis is one of the methods that can be used for sub-classification of LDL. The UCT lipid laboratory developed an in-house gradient gel electrophoresis method, using polyacrylamide gels (Blom and Marais).

Briefly lipoproteins are pre-stained with Sudan Black before running overnight in a 2-8% gradient in a glycine buffer at pH 8.3. The categorisation of LDL is into 5 sizes. Since there are no rigorously definable calibration standards, a large and a small LDL sample is selected from a range of particle sizes and is carried from gel to gel to enhance uniformity of reporting.

**2.4 Polymerase chain reaction (PCR) method for FDB and LDLR mutations.**

**2.4.1. Familial Defective ApoB 100:** This assay determines if the R3500Q and R3500W mutation causing binding defective B100 is present. The detailed method is appended. (14). It is combined with the ARMS (Amplification Refractory Mutation System) of V408M to
confirm that amplification has occurred during the assay. The principle is that the mutant DNA fragment forms a heteroduplex with a larger size that can be seen on the gel.

2.4.2. **High-resolution melting (HRM):** This new and robust method for PCR product analysis is capable of detecting sequence changes creating heteroduplexes with greater reliability than gels. In principle the amplicon is double-stranded and Evagreen fluoresces when bound to dsDNA. Annealing is perfect in subjects who have two identical strands but imperfect in heteroduplexes. The bonds holding heteroduplexes together are thus weaker than in homoduplexes. Heating thus causes denaturation earlier in heteroduplexes and the lower melting point is evident by the loss of fluorescence. The method according to the manufacturer see appendix.

2.4.3. **Assay for LDLR D206E mutation:** Exon 4 is amplified and then digested with the restriction enzyme Ddel for Afrikaner-1 (FH-1) or MboII for Afrikaner-2 (FH-2). The oligomers are added for fragment expansion. The positive controls and test DNA are run and overlayed with mineral oil. The PCR cycles are according to the procedure. In FH-1 20uL of product is cut and diluted and is left at 37°C overnight. In FH-1, the mutation creates another Ddel cutting site in the 87bp fragment. Ddel thus cuts the normal PCR product in 2 fragments: 133bp and 87 bp. The mutant is cut further making fragments 64 bp and 23 bp in size. The 23 bp fragment usually runs off the gel.

2.5 **DNA sequencing of Amplicons that displayed heteroduplex behaviour were sequenced.**

DNA sequencing was performed at the central Analytical Facility at the University of Stellenbosch using the Big Dye Terminator technique and applied Biosystems AB3730 XL capillary sequencer. According to the manufacture’s protocol Academics.sun.ac.za/saf/about.htm.

2.6 **Data management**

The information for these patients was entered in a commercially available database (Paradox V9). The following information was entered: genotype, name, date of birth, gender, kindred, date at presentation, smoking habits, presence or absence of atherosclerosis
as ischaemic heart disease; peripheral vascular disease, TIA and stroke, hypertension, diabetes and additional illness. Anthropometric measures and physical examinations were also done: Height, mass, body mass index (BMI), waist measurement, hip measurement, waist/hip ratio (WHR), the presence of xanthelasma, tendon xanthomata, and arcus cornealis. Presentation and best lipid profiles were entered on the limited dose of statin therapy due to hospital policies i.e. triglycerides, total cholesterol, HDLC, LDLC, Apo A1, Apo B and Lp (a) levels, gradient gel electrophoretogram (GGE) and LDL species.

2.7 Analytical Procedure and statistical calculations

For this study, the information on FDB was taken from the Lipid Clinic database (Corel/Paradox V9). First a search was done on the DNA results to identify subjects with FDB and LDLR D206E mutations. To compare FDB and D206E for differences in lipoprotein profile and clinical aspects, each FDB subject was matched with a similar subject with the D206E mutation in the LDLR with respect to gender, age BMI and TG. Then chemical information was entered from the main clinic database and FH database, expanded with additional information from folders.

For statistical analysis selected information was transferred from paradox database to a spreadsheet (Quattro pro) for calculations where after the numerical data was transferred to Graph Pad Prism. Parametric or nonparametric comparisons were done as appropriate. Specific tests are indicated with results. Categorical data were analysed using contingency tables.
CHAPTER THREE

RESULTS

3.1 INTRODUCTION

In this chapter the results are presented for subjects with FDB and are contrasted with matched subjects for the D206E mutation in the LDLR, which represents an LDLR-defective heterozygous FH phenotype.

3.2 Characterization of FH phenotype

At the time of this analysis, after 22 years of academic orientation, the clinic had on record 7574 patients referred with a wide variety of lipid disorders. The clinical diagnosis of the heterozygous Familial hypercholesterolaemia (FH) phenotype was on record for 1627 (22%). FH is thus one of the most common diagnostic entities amongst persons referred with severe dyslipidaemia. Of the 1627, nine hundred and thirty three (57%) had a genotypic diagnosis for the FH phenotype. The detection of LDLR mutations is by a strategy of first excluding founder mutations according to genetic background, followed by some exon-by-exon sequencing which is still incomplete. The LDLR mutations identified by December 2008 are listed in table 3.1. Amongst those with known LDLR genotypes there were 357 individuals (21.9%) with the mutation referred to as Afrikaner 1 (LDLR D206E). This is the most common LDL receptor mutation in white Afrikaans-speaking persons (Afrikaners) and people of mixed ancestry in South Africa. Only one patient and her two siblings were found to have a PCSK9 mutation but there had not been a systematic search for mutations in PCSK9. This was the “French” mutation S127R (resulting in serine to threonine substitution), detected by collaboration with an overseas research unit.

3.3 Detection of FDB

Patients with the FH phenotype but in whom the (limited) search for LDLR mutations was unsuccessful were studied for FDB. Kindreds with FDB and their mutations are summarised
in table 3.2. Initially this was done for exon 26 only by gel electrophoresis for heteroduplex detection but later high resolution thermal melting was introduced to comprehensively detect mutations in exons 26 and 29. The systematic search for Familial Defective Binding Apolipoprotein B100 (FDB) in the FH phenotype revealed 37 patients (0.5%) with mutations associated with the defective ligand status of Apo B100. There were 16 kindreds, 11 (69%) were of mixed ancestry, 3 (19%) were British immigrants, 2 (12.5%) were white South Africans, and 1 was a Sicilian immigrant. In some instances relatives were attending the clinic without the family link being known, whilst other relatives of patients with confirmed FDB who had abnormal cholesterol were tested for the Apo B mutations. This increased the patient numbers but to avoid distortion, kindreds with their mutations and ethnic origin are depicted in table 3.2. Most patients had the classic R3500Q mutation (n=34, or 92%). The other three patients each had other previously reported mutations i.e. R3500W, R3531C and H3543Y. Four family members of a patient with R3500Q who came for screening of cholesterol were discovered to have the mutation chose to be treated elsewhere. Thus no detailed clinical data were available for analysis on all identified subjects with FDB. Only 33 patients’ records were available for in-depth analysis. FDB is thus present in the Western Cape Province of SA, in various population groups. These findings about FDB are not consistent with the findings by Rubensztein 1995, where FDB was absent as a cause of hypercholesterolaemia in Cape Town. This report came from a research laboratory that had not systematically studied patients with the FH phenotype.

A search was undertaken for mutations in exons 26 and 29 that could cause FDB in 48 black patients at the clinic who had monogenic hypercholesterolaemia as outlined in methods (chapter two). Of these, 4 had the full heterozygous FH phenotype. There were no FDB mutations found in this group to have caused the hypercholesterolaemia. Four patients had Apo E2/2 indicating the need for differentiation of severe dyslipidaemia by special investigation for diagnostic, prognostic, therapeutic and possibly genetic counseling purposes. Two patients had R145C that is a recognised cause of dysbeta lipoproteinaemia. During the work-up for FDB, 4 “innocent” or synonymous mutations were detected by high resolution melting. These were GAG to GAA at codon 3466 coding for the same amino acid (glutamic acid), ACG to ACA at codon 3540 coding for the same amino acid (threonine), ACC to ACT at codon 3552 coding for threonine and CTC to CTT at codon 3559 coding for leucine.
It can therefore be concluded that FDB due to mutations in exons 26 or 29 is rare in the local black population, or at least in the Xhosa population which is the predominant black population in the Cape.

<table>
<thead>
<tr>
<th>GENE, LOCUS</th>
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<th>Nr</th>
<th>COMMENTS</th>
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<td>R57C</td>
<td>GSH</td>
<td>3</td>
<td>Indian</td>
</tr>
<tr>
<td></td>
<td>R60C</td>
<td>GSH2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E80K</td>
<td>Lancashire</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LDLR Ex4</td>
<td>Del TC at 369</td>
<td>GSH3</td>
<td>5</td>
<td>Black</td>
</tr>
<tr>
<td></td>
<td>C146Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D154N</td>
<td>Afrikaner3</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D157V</td>
<td>(novel)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Del 197</td>
<td>Lithuani</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D200G</td>
<td>Padova-1</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ins201-206</td>
<td>Padua-4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DelAC AC206Fs</td>
<td>GSH4</td>
<td>7</td>
<td>Indian</td>
</tr>
<tr>
<td></td>
<td>D203A</td>
<td>Afrikaner1</td>
<td>359</td>
<td>Afrikaner, Brit, Col</td>
</tr>
<tr>
<td></td>
<td>D206E</td>
<td>French-Canadian 3</td>
<td>8</td>
<td>Indian, Brit</td>
</tr>
<tr>
<td></td>
<td>E207K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDLR Ex5</td>
<td>Q233P</td>
<td>Reggio-Emilia 2</td>
<td>1</td>
<td>Afrikaner</td>
</tr>
<tr>
<td>LDLR Ex6</td>
<td>E256K</td>
<td>Amsterdam</td>
<td>1</td>
<td>Malay</td>
</tr>
<tr>
<td></td>
<td>E267K</td>
<td></td>
<td>3</td>
<td>Malay</td>
</tr>
<tr>
<td></td>
<td>S283L</td>
<td></td>
<td>4</td>
<td>Afrikaner, Dutch</td>
</tr>
<tr>
<td></td>
<td>C292Y</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Del AA931</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LDLR Ex7</td>
<td>G322S</td>
<td>Picardie-3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C329X</td>
<td>Fossum</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Mutation</td>
<td>Frequency</td>
<td>Population</td>
<td>Notes</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>-----------</td>
<td>------------</td>
<td>-------</td>
</tr>
<tr>
<td>LDLR Ex8</td>
<td>del A 1085 C343R C356Y Q357X G361V C371X C371Y</td>
<td>7</td>
<td>2</td>
<td>Dutch immigrant</td>
</tr>
<tr>
<td>LDLR Ex9</td>
<td>A378T F382S N384K R385Q L401P V408M</td>
<td>2</td>
<td>16</td>
<td>Black, Dutch</td>
</tr>
<tr>
<td>LDLR Ex10</td>
<td>D461N</td>
<td>2</td>
<td></td>
<td>Afrikaner</td>
</tr>
<tr>
<td>LDLR Ex12</td>
<td>170-1 G&gt;A</td>
<td>1</td>
<td></td>
<td>Brit</td>
</tr>
<tr>
<td>LDLR Ex13</td>
<td>del Ex13-14</td>
<td>1</td>
<td></td>
<td>Coloured (Knysna)</td>
</tr>
<tr>
<td>LDLR Ex14</td>
<td>W645X C660X P664L</td>
<td>1</td>
<td></td>
<td>Lebanese, Gujarat</td>
</tr>
<tr>
<td>PCSK9 Ex2</td>
<td>S127R</td>
<td>3</td>
<td></td>
<td>Coloured</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>899</td>
<td></td>
<td>Phenotypic FH 1627</td>
</tr>
</tbody>
</table>

**Table 3.1.** Genotypic diagnosis of heterozygous FH phenotype at the Division of Lipidology of Groote Schuur Hospital and University of Cape Town by December 2008.
Table 3.2. Kindreds with Apo B-100 mutations in exons 26 and 29 detected by heteroduplex analysis

<table>
<thead>
<tr>
<th>MUTATION</th>
<th>KINDRED</th>
<th>COMMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3500Q</td>
<td>A (3)</td>
<td>British immigrants</td>
</tr>
<tr>
<td>R3500Q</td>
<td>B (3)</td>
<td>British immigrants</td>
</tr>
<tr>
<td>R3500Q</td>
<td>C (2)</td>
<td>White English speaking</td>
</tr>
<tr>
<td>R3500Q</td>
<td>D (3)</td>
<td>White ancestry</td>
</tr>
<tr>
<td>R3500Q</td>
<td>E (3)</td>
<td>White ancestry (Irish, Afrikaner)</td>
</tr>
<tr>
<td>R3500Q</td>
<td>F (2)</td>
<td>Mixed ancestry (Uniondale)</td>
</tr>
<tr>
<td>R3500Q</td>
<td>G (1)</td>
<td>Mixed ancestry (Uniondale)</td>
</tr>
<tr>
<td>R3500Q</td>
<td>H (1)</td>
<td>Mixed ancestry (Malaysian)</td>
</tr>
<tr>
<td>R3500Q</td>
<td>I (10)</td>
<td>Mixed ancestry</td>
</tr>
<tr>
<td>R3500Q</td>
<td>J (1)</td>
<td>Mixed ancestry</td>
</tr>
<tr>
<td>R3500Q</td>
<td>K (1)</td>
<td>Mixed ancestry</td>
</tr>
<tr>
<td>R3500Q</td>
<td>L (1)</td>
<td>Possible mixed ancestry</td>
</tr>
<tr>
<td>R3500Q</td>
<td>M (2)</td>
<td>Mixed ancestry</td>
</tr>
<tr>
<td>R3500W</td>
<td>N (1)</td>
<td>Sicilian immigrant</td>
</tr>
<tr>
<td>R3531C</td>
<td>O (2)</td>
<td>British immigrant</td>
</tr>
<tr>
<td>H3543Y</td>
<td>P (1)</td>
<td>Mixed ancestry</td>
</tr>
</tbody>
</table>

3.4 CLINICAL FINDINGS AND ANTHROPOMETRY

This section describes the clinical features of the subjects with FDB as well as subjects with LDLR D206E to contrast FDB with the commonest cause for heterozygous FH phenotype due to LDLR mutations. The LDLR D206E mutation is commonly found in white and people of mixed ancestry in South Africa. This makes for easy selection of patients to compare so that differences in the phenotype due to LDLR mutations (see table 3.1) and Apo B mutations can be discerned. To allow for the most important influences on risk and
LP profiles the FDB subjects were matched with LDLR mutant FH subjects for age, gender, BMI and triglycerides. The findings are summarised in table 3.3.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FDB (34)</th>
<th>LDLR D206E (53)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>F= 16</td>
<td>F= 27</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>M= 21</td>
<td>M= 26</td>
<td></td>
</tr>
<tr>
<td>Presentation age</td>
<td>50.6 ± 17.96</td>
<td>44.2 ± 17.72</td>
<td>0.11</td>
</tr>
<tr>
<td>Follow up years</td>
<td>6.3 ± 6.36</td>
<td>6.9 ± 7.01</td>
<td>0.725</td>
</tr>
<tr>
<td>AC</td>
<td>Y=13 N=16 U=4</td>
<td>Y=17 N=20 U=16</td>
<td>0.975</td>
</tr>
<tr>
<td>Xma</td>
<td>Y=15 N=13 U=5</td>
<td>Y=23 N=15 U=15</td>
<td>0.625</td>
</tr>
<tr>
<td>Xanthelasma</td>
<td>Y=5 N= 25 U=3</td>
<td>Y=4 N=47 U=2</td>
<td>0.2799</td>
</tr>
<tr>
<td>Mass (Kg)</td>
<td>71.5 ± 16.03</td>
<td>73.2 ± 16.83</td>
<td>0.64</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164 ± 10.44</td>
<td>170 ± 11.83</td>
<td>0.22</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>26.2 ± 5.3</td>
<td>25.07 ± 4.25</td>
<td>0.32</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>88 ± 13.74</td>
<td>85 ± 14.78</td>
<td>0.38</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>100.1 ± 7.53</td>
<td>100.4 ± 10.07</td>
<td>0.88</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.88 ± 0.11</td>
<td>0.85 ± 0.1</td>
<td>0.16</td>
</tr>
<tr>
<td>CAD (AP, MI)</td>
<td>9</td>
<td>19</td>
<td>1.0</td>
</tr>
<tr>
<td>Onset age of CAD in years</td>
<td>55.0 ± 10.25</td>
<td>46.0 ± 12.40</td>
<td>0.044</td>
</tr>
<tr>
<td>CVA/ TIA</td>
<td>1</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>PVD</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>HPT</td>
<td>Y=14 N=19</td>
<td>Y=12 N=39</td>
<td>0.09</td>
</tr>
<tr>
<td>DM</td>
<td>Y=5 N=28</td>
<td>Y=0 N= 51</td>
<td>0.008</td>
</tr>
<tr>
<td>Smoking</td>
<td>Y=7 N=24 U=2</td>
<td>Y=23 N=28 U=2</td>
<td>0.058</td>
</tr>
</tbody>
</table>

Table 3.3. The clinical features of FDB patients compared with those of patients with LDLR D206E mutation matched for age, gender, BMI and TG concentration.

F=female, M=male, N=no, Y=yes, U=unknown, AC=arcus cornealis, Xma=xanthomata, Xsma=xanthelasma, Kg=kilogram, m=meters, cm=centimeters, BMI=body mass index, CAD=coronary artery disease, AP=angina pectoris, MI=myocardial infarction, CVA=cerebrovascular accident, TIA=transient ischaemic attack, PVD=peripheral vascular disease, HPT=hypertension, DM=diabetes mellitus.
3.4.1 At presentation

The detailed clinical analysis in table 3.3 is for the 16 females and 21 males with FDB. Matching for age, body mass index and plasma triglycerides identified twenty-seven females and twenty-six males in the LDLR D206E cohort. Although the proportion of the male: female is not identical, this difference was not statistically significant \( P=0.5 \). The youngest age at presentation for FDB was 12 years; the oldest patient was 82 years old. The average age at presentation was \( 50.6 \pm 17.96 \) years. The youngest age at presentation for LDLR D206E cohort was 10 years. The oldest patient was 77 years, with the average age at presentation of \( 44.2 \pm 17.72 \) years. There was no statistically significant difference in the presentation age \( P=0.1 \). The average follow-up in years for the FDB was \( 6.3 \pm 6.4 \) years. The average follow-up for the LDLR D206E patients was similar, \( 6.9 \pm 7.01 \) years with \( P=0.725 \). This table indicates that the two cohorts were quite similar and thus suitable for comparison of differences in the phenotype.

3.4.2 Physical signs and anthropometric measurements

Xanthomata and arcus cornealis are viewed as good physical signs for making a specific diagnosis of FH. Xanthelasma is neither a sensitive nor a specific physical sign for hypercholesterolaemia. These physical signs are not a prerequisite for the diagnosis to be subjected as being FH, and are not invariable to patients with FH. Xanthelasma can occur in patients with normal lipid profiles, arcus cornealis can occur in elderly without significant lipid problems and tendon xanthomata can occur in other lipid abnormalities. One is likely to miss a significant number of patients with FH if one is to use the strict physical criteria for the FH phenotype \(^{34}\) but it is of interest whether at the bedside and/or with routine investigations there may be a difference between FDB and LDLR mutations to guide genotyping investigations efficiently to an exact diagnosis.

**Arcus cornealis**

In the FDB cohort 13 (39%) patients had arcus cornealis, whereas in 16 (49%) subjects this physical sign was absent. In 4 (12%) patients there was no record of this physical finding leading to the suspicion that this sign was absent but since this could not be verified it is
interpreted as unknown. However, if it was absent in this subgroup then 20 (60%) of the subjects did not have arcus cornealis, and this sign is rather unreliable.

In the LDLR D206E cohort there were 17 (32%) subjects with arcus and 20 (38%) subjects without this physical sign. Amongst the total 53 subjects 16 (30%) had no recording of this physical sign. Although this suggests that the sign was likely absent, these subjects were omitted for the same reason as for the FDB cohort. Accepting the unknown as being absent, the percentage without arcus is also high at 68% and unreliable, especially in older people.

The comparison reveals that for those who had documentation for the presence or absence of this physical sign there was no statistically significant difference (P=0.975, Fishers exact) between FDB and LDLR D206E.

When the cumulative prevalence for arcus cornealis was calculated for the FDB subjects this physical sign appeared only after the 3rd decade of life. By the 6th decade cumulative prevalence of arcus cornealis was approximately 36% but rose to 42% the next two decades. This is described in table 3.4 and illustrated in fig 3.1.

For the LDLR D206E subjects the cumulative prevalence for arcus cornealis was initially higher than for the FDB cohort. The arcus was present in 2% of this population from the 3rd decade of life. The figures rose to 13% by the 4th decade of life and 25% by the 5th decade. By the 6th decade 34% had arcus cornealis, increasing to 40 and 49% in the following decades. (Figure3.1). Although the numbers are small, the difference in cumulative prevalence of arcus cornealis suggests that arcus develops earlier in subjects with the LDLR mutation. The later development of arcus may be a reflection of the lower LDL concentration in FDB before adulthood.
<table>
<thead>
<tr>
<th>Age in decades (years)</th>
<th>FDB</th>
<th>LDLR D206E</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-20</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>21-30</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>31-40</td>
<td>0%</td>
<td>13%</td>
</tr>
<tr>
<td>41-50</td>
<td>6%</td>
<td>25%</td>
</tr>
<tr>
<td>51-60</td>
<td>9%</td>
<td>34%</td>
</tr>
<tr>
<td>61-70</td>
<td>36%</td>
<td>40%</td>
</tr>
<tr>
<td>71-80</td>
<td>42%</td>
<td>49%</td>
</tr>
</tbody>
</table>

Table 3.4. Cumulative prevalence of arcus cornealis at presentation in patients with heterozygous FH phenotype due to FDB and LDLR D206E mutations.

![Graph showing cumulative prevalence of arcus cornealis](image_url)

Figure 3.1 Cumulative prevalence of arcus cornealis in the heterozygous FH phenotype due to FDB and LDLR mutations.
Xanthelasma

In the FDB cohort there were 5 (16%) patients with documented xanthelasma while in 25 (78%) this sign was absent and 3 (9%) there was no documentation. In the LDLR D206E mutation 4 (8%) patients had xanthelasma and in only 2 (3%) this sign is not documented. There was no statistically significant difference in the prevalence of xanthelasma in FDB and LDLR D206E mutations (P= 0.28, Chi squared). Based on its low prevalence in the FH phenotype, xanthelasma should not be regarded as a reliable physical sign to suggest FH but must nevertheless prompt a plasma lipid tests when present in any person seen for any reason.

Tendon Xanthomata

Achilles tendons were systemically palpated and recorded by a small number of experienced physicians. No funds were available for X-ray, xerography, CT scans or ultrasound to provide a more objective evaluation that would also have a better power of discrimination between normal and abnormal tendons.

In the FDB cohort 15 (46%) subjects had this physical sign on clinical examination at presentation, in 13 (39%) subjects this sign was not found, and in 5 (15%) subjects there was no record of this physical finding. Although it is likely that this physical sign was absent and was consequently not recorded, these subjects were not included in the analysis. The 15/28 (54%) individuals’ maybe an overestimate of the presence of xanthomata, it is more likely that 15/33 or 46% is the correct average figure though age is a strong requirement for developing this sign.

In the LDLR D206E mutation cohort there were 23 (43%) subjects who had Achilles tendon xanthomata present while in 15 (28%) subjects this sign was absent. In 15 (28%) there was no documentation of this physical sign.

For those who had documentation of this physical sign (28 FDB subjects and 38 LDLR D206E subject) there was no statistically significant difference in the prevalence of tendon xanthomata between FDB and LDLR D206E heterozygous FH phenotypes (P=0.625, Chi
squared). However, for the same reason as for FDB the more likely correct general prevalence of tendon xanthomata is 57%.

When the cumulative prevalence of Achilles tendon xanthomata was calculated for the FDB subjects this physical sign was absent up to the 4th decade of life. The prevalence increases steadily to almost 50% by the 8th decade but the small numbers probably make this evaluation unreliable (figure 3.2)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>FDB</th>
<th>LDLR D206E</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-20</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>21-30</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>31-40</td>
<td>9%</td>
<td>11%</td>
</tr>
<tr>
<td>41-50</td>
<td>18%</td>
<td>21%</td>
</tr>
<tr>
<td>51-60</td>
<td>21%</td>
<td>32%</td>
</tr>
<tr>
<td>61-70</td>
<td>46%</td>
<td>38%</td>
</tr>
<tr>
<td>71-80</td>
<td>49%</td>
<td>43%</td>
</tr>
</tbody>
</table>

Table 3.5. Cumulative prevalence of tendon xanthomata at presentation in patients with FDB and LDLR D206E.
3.5 Anthropometry

The anthropometric data in this analysis were from the first consultation visit. Patients attending the lipid clinic for the first time underwent height, mass, waist circumference, and hip circumference measurements in a standardised fashion. The data is summarised in table 3.2.

**Body mass (Kg):** The average body mass for the FDB cohort was 71.5 ± 16.80 Kg. The average body mass for the LDLR D206E cohort was 73.2 ± 16.86 Kg. There was no statistically significant difference between the 2 groups (P=0.64). This is expected as these 2 cohorts were matched for the body mass index.

**Height (cm):** The average height for the FDB cohort was 164 ± 10.4 cm. The average height for the LDLR D206E cohort was 170 ± 11.8 cm. There was a statistically significant difference between the 2 cohorts (P=0.022, Mann Whitney). This is almost certainly a chance finding that is to be lost likely with a larger number in the FDB cohort, as there is no obvious reason why these subjects should be shorter than those with hypercholesterolaemia due to LDLR mutations.

Figure 3.2 Cumulative prevalence of tendon xanthomata.

![Graph: Cumulative prevalence of tendon xanthomata.](image)
Body mass index (BMI): The average BMI for the FDB cohort was $26.2 \pm 5.29 \text{ Kg/m}^2$. The average BMI for the LDLR D206E cohort was $25.07 \pm 4.25 \text{ Kg/m}^2$. These averages were not statistically significantly different between the two cohorts ($P=0.32$, Mann Whitney). This is expected as the FDB subjects were matched with LDLR D206E subjects for the BMI.

Waist circumference: The average waist circumference for the FDB cohort was 88.0 cm with a wide standard deviation of 13.74 cm. The average waist circumference for the LDLR D206E cohort was 85.0 cm, also with a wide standard deviation of 14.78 cm. There was no statistically significant difference between the two cohorts ($P=0.38$, Mann Whitney).

Hip circumference: The average hip circumference in cm for the FDB cohort was 100.1 ± 7.53 cm. The average hip circumference for LDLR D206E was 100.4 ± 10.07 cm. There was no statistically significant difference between the two cohorts ($P=0.88$, Mann Whitney).

Waist/Hip ratio (WHR): The average WHR for the FDB cohort was 0.88 with a narrow standard deviation 0.11. The average WHR for LDLR D206E cohort was 0.85 also with a narrow standard deviation 0.11. These averages were not statistically significantly different ($P=0.16$, Mann Whitney).

3.6 CARDIOVASCULAR COMPLICATIONS AND OTHER DISORDERS

The FH phenotype is renowned for premature complications of atherosclerosis mainly as coronary artery disease. It is clearly of interest to examine the type of atherosclerotic manifestation that occurred in these two cohorts and to compare the severity. These findings are summarised in table 3.2.

Ischaemic heart disease

Ischaemic heart disease is a spectrum of clinical syndromes ranging from chronic stable angina, through unstable angina to myocardial infarction and sudden death.
In the FDB cohort there were 9 (27%) patients with established coronary artery disease. In the LDLR D206E cohort there were 19 (36%) patients with established coronary artery disease. There was no statistically significant difference in the prevalence of CAD between the two cohorts (P=0.10, Chi Squared).

The onset age of coronary artery disease was 55.0 ± 10.25 years and 46.0 ± 12.4 years for FDB and LDLR D206E cohorts respectively. This difference is statistically significant (P=0.044, Fisher’s exact). Thus it appears that, though ischaemic heart disease is ultimately equally common in both cohorts, the onset is almost a decade later in the FDB.

The ischaemic heart disease for both cohorts manifested as chronic stable angina pectoris, acute coronary syndrome (unstable angina to transmural myocardial infarction) and chronic left ventricular failure secondary to the ischaemic myocardium. It is not practicable to analyse these small numbers for differences between the two cohorts.

**Cerebrovascular disease and Peripheral Arterial Disease**

There was only one patient from both cohorts with stroke. Though these small numbers do not lend themselves to statistical analysis, it is interesting to note that cerebrovascular disease is so uncommon in subjects with such a high propensity for coronary disease.

There were two patients with peripheral arterial disease in the FDB cohort and three patients from the LDLR D206E cohort. These numbers are too small for statistical analysis but they follow the pattern of the low prevalence of CVD compared with CAD.

**Deaths**

The follow-up was imperfect. No contact was attempted with patients or family where the original address and phone number failed to locate the patient.

The deaths that were recorded were as follows: For the FDB cohort, 2 patients were known to have died. In one patient the cause of death was not recorded. He was in his eighth decade of life. The other patient died in her eighth decade of life from intracerebral bleeding. For the LDLR D206E mutation, three patients died. In two patients the cause of death is not
documented. The other patient died at the age 48 years from cardiorespiratory arrest, probably related to myocardial infarction.

**OTHER DISORDERS**

**Hypertension:** In the FDB cohort there were 14 (42%) patients with hypertension and 19 (58%) patients without hypertension. In the LDLR D206E cohort there were 12 (23%) patients with hypertension, 39 (74%) without hypertension. In two (4%) no documentation of this disease was found on records. There was a trend for more hypertension in the FDB cohort with (P=0.09, Chi Squared). This trend is unlikely to be an intrinsic difference between the two disorders but could have favoured the development of atherosclerosis in the FDB cohort.

**Diabetes:** In the FDB cohort, 5 (15%) patients were diabetic patients and 28 (85%) non-diabetic patients. For the LDLR D206E cohort there were no diabetic patients in 2 (4%) there was no record of this disease on the records. There was a statistically significant difference (P=0.008, Fisher’s exact). Thus having matched for age, gender, BMI and TG did introduce a bias in the prevalence of diabetes between the two groups. Again, the presence of diabetes would likely aggravate atherosclerosis in the FDB cohort. There is no reason to suspect that mutant apo B is responsible for diabetes mellitus. There are no detailed studies to determine whether ethnicity will explain the different prevalences of diabetes between LDLR and FDB cohorts.

**Smoking:** For the FDB cohort there were 7(22%) patients who were smokers and 24 (75%) patients who were non-smokers and for 2 (6%) subjects there was no documented history about smoking habit. In the LDLR D206E mutation there were 23 (43%) patients who were smoking at presentation while 28 (53%) patients were non-smokers and in 2 (4%) there was no documentation of the smoking habit. Though there was a trend to suggest that the LDLR D206E mutation cohort smoked more than the FDB cohort, this was not strictly statistically significantly different (P=0.0582, Fisher’s exact). This might be a bias in favour of atherosclerosis in the LDLR D206E cohort.
3.7 BIOCHEMICAL INVESTIGATIONS AT PRESENTATION

Fasting TG, TC and HDLC were done at the first visit to the lipid clinic in all subjects whereas Apo Ai, B and Lp (a) measurements were only introduced later along with LDL particle size determination and genotyping of LDLR mutations and Apo B. Not all these investigations were available for patients in the untreated state. These findings are summarised in Table 3.6.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FDB (n)</th>
<th>D206E (n)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG mmol/L</td>
<td>1.2 ± 0.5 (32)</td>
<td>1.2 ± 0.76 (53)</td>
<td>0.61</td>
</tr>
<tr>
<td>TC mmol/L</td>
<td>7.9 ± 1.5 (32)</td>
<td>8.4 ± 1.62 (53)</td>
<td>0.099</td>
</tr>
<tr>
<td>HDLC mmol/L</td>
<td>1.3 ± 0.5 (32)</td>
<td>1.3 ± 0.42 (53)</td>
<td>0.68</td>
</tr>
<tr>
<td>LDLC mmol/L</td>
<td>5.9 ± 1.4 (32)</td>
<td>6.6 ± 1.7 (52)</td>
<td>0.069</td>
</tr>
<tr>
<td>Apo Ai g/L</td>
<td>1.3 ± 0.44 (24)</td>
<td>1.2 ± 0.32 (35)</td>
<td>0.400</td>
</tr>
<tr>
<td>Apo B g/L</td>
<td>1.5 ± 0.25 (24)</td>
<td>1.5 ± 0.30 (35)</td>
<td>0.55</td>
</tr>
<tr>
<td>Lp(a) (Log transformed) g/L</td>
<td>0.59 ± 0.35 (25)</td>
<td>0.42 ± 0.25 (35)</td>
<td>0.062</td>
</tr>
<tr>
<td>LDLC/apoB mmol/g</td>
<td>4.096 ± 0.89</td>
<td>4.59 ± 1.17</td>
<td>0.039</td>
</tr>
<tr>
<td>LDLsp non-diabetic</td>
<td>5:3:10:2:0</td>
<td>13:13:4:2:0</td>
<td>0.02</td>
</tr>
<tr>
<td>LDLsp L to S</td>
<td>10:12:2</td>
<td>26:4:2</td>
<td>0.0062</td>
</tr>
</tbody>
</table>

Table 3.6. Biochemical features of FDB compared with those of matched subjects for LDLR D206E mutation at presentation.

TG= triglycerides, TC= total cholesterol, HDLC= high-density lipoprotein in cholesterol, LDLC= low density lipoprotein cholesterol, Apo apolipoprotein (Ai; B) Lp (a). LDLsp= low-density lipoprotein species, lipoprotein species from large to small. n=actual number of patients that were counted.
Biochemical results

Triglycerides: The average fasting plasma triglycerides for the FDB cohort was 1.2 ± 0.5 mmol/L whilst for the LDLR D206E cohort was 1.2 ± 0.76 mmol/L. These two groups were matched for triglycerides concentration as stated before. (P=0.6, Fishers exact)

Total cholesterol: The average cholesterol for the FDB cohort was 7.9 ± 1.5 mmol/L. The average total cholesterol for the LDLR D206E was 8.4 ± 1.6 mmol/L. Although there is a trend towards higher TC for the LDLR D206E patients, this was not statistically significantly different (P=0.099, Fishers exact). This may enhance atherosclerosis in the LDLR mutation group, as the difference is likely in LDLC.

High Density Lipoprotein cholesterol (HDLC): The average HDL cholesterol at presentation was 1.3 ± 0.5 mmol/L for the FDB cohort. The average HDL cholesterol at presentation for the LDLR D206E was also 1.3 ± 0.41 mmol/L. There was no statistically significant difference (P=0.68, Fishers exact). This suggests that reverse cholesterol transport and other properties of HDL are unlikely to differ significantly.

Low Density Lipoprotein (LDLC): The average LDLC for the FDB cohort was 5.9 ± 1.4 mmol/L. The average LDLC for the LDLR D206E cohort was 6.6 ± 1.6 mmol/L. The results suggest that LDLC is higher in the LDLR D206E mutation but since P=0.07, it is not strictly considered to be statistically significantly different. This finding is also paralleled in TC (see above) and could influence atherosclerosis unfavourably to enhance atherosclerosis in the LDLR cohort of FH subjects.

Apolipoprotein Ai (Apo Ai): The average for Apo Ai concentration in the FDB cohort was 1.3 ± 0.44 g/L and that of the LDLR D206E cohort was 1.2 ± 0.32 g/L. There was no statistically significant difference between the two cohorts (P=0.40, Fishers exact).

Apolipoprotein B (Apo B): The average Apo B concentration for the FDB and LDLR D206E cohorts were the same, 1.5 ± 0.3 g/L, and thus not statistically different between the 2 groups (P=0.55, Fishers exact). Having had a trend towards higher TC and LDLC the near-similarity of apo B concentration between the two cohorts suggests a difference in LDL particle sizes, compatible with small LDL containing less cholesterol per apo B in FDB.
**LDLC/apo B ratio**: Small dense low-density lipoprotein (LDL) particles are associated with coronary disease. It is well known that the size and the density of plasma lipoproteins are related to their chemical composition and in particular to their protein to lipid ratio. Apo B-100 is the major protein present in LDL particles and most of the plasma total Apo B is found in the LDL. Earlier work done by Tallis in 1995 showed that the calculated LDLC/apoB ratio is not helpful in predicting the LDL particle size. Most studies have used acrylamide electrophoresis or NMR to study LDL size, avoiding the confounding influence of remnants on the cholesterol/apo B ratio.

In the FDB cohort the average LDLC/apoB ratio was $4.096 \pm 0.89$ mmol/g or (2161 mol/mol). In the LDLR D206E cohort the average LDLC/apoB ratio was $4.591 \pm 1.17$ mmol/g (2418 mol/mol). There was a statistically significant difference between the two cohorts ($P=0.039$, Mann Whitney). This suggests that there is smaller, denser LDL in FDB, presumably because the LDLC circulates much longer and has more time to be modulated by CETP and HL. This would explain how the particles become smaller and denser in the FDB patients even though the other factors that influence LDL size were matched especially age, TG concentration and obesity as reflected in BMI and WHR. These results are contrary to the Tallis findings, presumably because the much higher concentration of LDL in FH diminishes the impact of other lipoprotein in the calculation.

**Low Density Lipoprotein Species**

LDL particle size is influenced by the concentration of plasma triglycerides, hepatic lipase activity, cholesterol ester transfer protein activity and lipoprotein lipase activity. Small dense LDL species are associated with high plasma triglycerides, VLDL, IDL and low HDL levels. LDL particle size is also influenced by other conditions like age, obesity, physical inactivity, gender and diabetes.

Evaluation of LDL subclasses has shown that the small LDL species are associated with an increase risk of myocardial infarction independent of sex, age or weight. This has not been studied in FH cohort.
Non-denaturing gradient gel electrophoresis can discriminate LDL particle size directly, irrespective of the content of cholesterol or apo B in other lipoproteins. These analyses were undertaken before the use of statins. Although statins lower the concentration of LDL, the dominant LDL species identified by the GGE method in the lipid laboratory is not affected (unpublished data). The LDL species arranged in size from the biggest to the smallest, are designated in the categories A, AI, I, IB, B. This nomenclature in which the largest species is referred to as A and the smallest as B to conform to other electrophoretic gel methods which usually discriminate between two sizes. But intermediate (I) species can be identified as well as transitional species AI, IB in the in-house method, making for better analysis of LDL speciation.

There was a statistically significant difference between the distributions of LDL sizes in the two groups (P=0.015). Since diabetes is frequently associated with small LDL species, the diabetic subjects’ results were excluded in a separate analysis. There was a significant difference between the two cohorts even when the diabetics are excluded (P=0.0062, Fishers exact test). The data sets were also simplified to large, intermediate and small and such classification also revealed a significant difference in LDL size between the two cohorts (P=0.0062).

**Factors influencing the LDL species**

The factors that could still influence LDL particle size results are summarised in table 3.7. Because of the small numbers the species were only divided into three main categories, large (A+AI), intermediate (I) and the small species (IB+B) for better statistical analysis.

**The LDL species and the triglycerides:** The circulating TG is the most important factor influencing the LDL species size, with increased TG levels resulting in smaller LDL particle size.

The average TG for the large LDL species (A+AI) for the FDB cohort was 0.99 ± 0.33 mmol/L. The average TG for the large LDL species for the LDLR D206E cohort was 1.06 ± 0.41 mmol/L. There was no statistically significant difference between the two cohorts (P=0.75, Mann Whitney). The average TG for the intermediate LDL species (I) for the FBD cohort was 1.29 ± 0.51 mmol/L. The average TG for the intermediate LDL species was 1.13 ± 0.49 mmol/L for the LDLR D206E group. There was no statistically significant difference
between the cohorts (P=0.67, Mann Whitney). There were very few patients with the small LDL species and were therefore not statistically analysed. There was no statistically significant difference when the two cohorts were combined and compared for the TG influence across the LDL size (P=0.285, Kruskal Wallis Test). The LDL species was thus not significantly different in relation to TG in the relatively normal ranges encountered in this analysis.

The LDL species and the BMI: As body mass index increases the LDL particles become smaller and therefore denser. The average BMI for the patients with large LDL species (A+AI) in the FDB cohort was 26.56 ± 4.46 Kg/m$^2$. The average BMI for the patients with the large LDL species in the LDLR D206E cohort was 23.92 ± 3.64 Kg/m$^2$. There was no statistically significant difference between the two cohorts (P=0.133, Mann Whitney).

The average BMI for the patients with the intermediate LDL species in the FDB cohort was 27.93 ± 5 Kg/m$^2$. The average BMI for the patients with the intermediate LDL species in the LDLR D206E cohort was 25.33 ± 2.08 Kg/m$^2$. There was no statistical significant different between the two cohorts (P=0.52, Mann Whitney). There were small numbers of patients with the small LDL species and were not analysed statistically. When the patients were combined in the two cohorts and the species were compared from small to large there was a trend to suggest that the species become smaller as the BMI increases, though not statistically significantly different (P=0.086, Kruskal Wallis Test). The LDL species is thus no different in relation to bias between the two cohorts though the BMI does influence LDL size in the expected fashion in the whole cohort.

The LDL species and the WHR: The average WHR for the FDB cohort with the large LDL species (A+AI) was 0.85 ± 0.09. The average WHR for the LDLR D206E cohort with the large species was 0.82 ± 0.08. There was no statically significant difference between the two cohorts (P=0.27, Mann Whitney). The average WHR for the FDB cohort with the intermediate LDL species was 0.96 ± 0.1. The average WHR for the LDLR D206E cohort with the intermediate species was 0.85 ± 0.05. There was no statistical significant difference between the two cohorts (P=0.12, Mann Whitney). There were very few patients with the small species (IB+B) and were no statistically analysed. When the two cohorts were combined and the LDL species compared from large to small, this was statistically
significantly different (P= 0.022, Kruskal Wallis Test) favouring the increase in the WHR results in the small and denser LDL species.

**Age and LDL species:** The LDL particles become smaller and dense with the increasing age. The patients were analysed for age to see if it were true for the cohorts. There was no statistically significant difference between the two cohorts when the patients were analysed for age and the LDL species size. The P value for the large species (A+AI) was 0.57 and the P value for the intermediate species was 0.76. The two cohorts were matched for age.

**Gender and the LDL species:** There was statistically significant difference when the LDL species were compared from large to small for the female gender (P= 0.049, Chi squared). There was no statistical significant difference between the LDL species and the male gender (P=0.17) this is probably due to the small number of patients in the two cohorts skewing the results.

**Diabetes mellitus and the LDL species:** Diabetes mellitus is associated with the smaller LDL species. There were very few patients who had diabetes and this makes statistics to be skewed.

Only five patients in the FDB cohort had diabetes and none in the LDLR D206E cohort had the disease. There were only four patients in the FDB cohort who had the LDL species measured and these were compared with the non-diabetic in the FDB cohort as well and there was no significant difference between the two sub-population (P=0.5, Chi squared). None of the diabetic subjects had small species demonstrating the complex pathogenesis of small, dense LDL.
<table>
<thead>
<tr>
<th></th>
<th>FDB (n)</th>
<th>LDLR D206E (n)</th>
<th>P value</th>
<th>Both cohorts</th>
<th>Both cohorts’ P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI (A+AI)</strong></td>
<td>26.56±4.46 (10)</td>
<td>23.92±3.64 (26)</td>
<td>P=0.133</td>
<td>24.66±4.0 (36)</td>
<td>P=0.086</td>
</tr>
<tr>
<td><strong>BMI (I)</strong></td>
<td>27.93±5.83 (12)</td>
<td>25.33±2.08 (3)</td>
<td>P=0.52</td>
<td>27.4±5.33 (15)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI IB+B</strong></td>
<td>26.79±9.77 (2)</td>
<td>29.95±3.47 (2)</td>
<td>None</td>
<td>28.37±6.26 (4)</td>
<td></td>
</tr>
<tr>
<td><strong>TG (A+AI)</strong></td>
<td>0.99±0.33 (11)</td>
<td>1.06±0.41 (26)</td>
<td>P=0.75</td>
<td>1.04±0.38 (37)</td>
<td></td>
</tr>
<tr>
<td><strong>TG (I)</strong></td>
<td>1.29±0.51 (12)</td>
<td>1.13±0.49 (4)</td>
<td>P=0.67</td>
<td>1.25±0.5 (16)</td>
<td></td>
</tr>
<tr>
<td><strong>TG IB+B</strong></td>
<td>1.63±1.38 (2)</td>
<td>0.95±0.78 (2)</td>
<td>None</td>
<td>1.29±0.99 (4)</td>
<td></td>
</tr>
<tr>
<td><strong>WHR (A+AI)</strong></td>
<td>0.85±0.09 (11)</td>
<td>0.82±0.08 (21)</td>
<td>P=0.27</td>
<td>0.83±0.09 (32)</td>
<td>P=0.022</td>
</tr>
<tr>
<td><strong>WHR (I)</strong></td>
<td>0.96±0.1 (7)</td>
<td>0.85±0.05 (3)</td>
<td>P=0.12</td>
<td>0.93±0.1 (10)</td>
<td></td>
</tr>
<tr>
<td><strong>WHR IB+B</strong></td>
<td>0.98±0.0 (1)</td>
<td>0.85±0.01 (2)</td>
<td>None</td>
<td>0.89±0.08 (3)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.7(a) LDL species** as related to BMI, TG, WHR Where F=female, M=male, BMI=body mass index, TG=triglycerides, WHR=waist hip ratio. A=large LDL species, AI=intermediate large LDL species, I=intermediate LDL species, IB=intermediate small LDL species B=small LDL species n=actual number of patients counted, P=predictive value.
| Age (A+AI) | FDB: 46.40±17.65 (10) | LDLR D206E: 44.38±17.75 (26) | P value: 0.57 |
| Age (I) | FDB: 53.25±14.77 (12) | LDLR D206E: 54.75±16.70 (4) | P value: 0.76 |
| Age (IB+B) | FDB: 58.5±7.78 (2) | LDLR D206E: 16.00±4.24 (2) | P value: None |
| F (A+AI) | 5 | 15 | P value: 0.049 |
| F (I) | 6 | 2 |
| F (IB+B) | 1 | 1 |
| M (A+AI) | 6 | 11 | P value: 0.17 |
| M (I) | 6 | 2 |
| M (IB+B) | 1 | 1 |
| A+AI | DM: 2 | Non-DM: 9 | P value: 0.5 |
| I | DM: 2 | Non-DM: 10 |
| IB+B | DM: 0 | Non-DM: 2 |

Table 3.7 (b) LDL species as related to Age, Gender and Diabetes. Where DM= diabetes mellitus, A+AI= large LDL species, I= intermediate and IB+B= small LDL species.

Lipoprotein (a) [Lp(a)]

The average Lp (a) concentration for the FDB cohort was 0.59 ± 0.35g/L and that for the LDLR D206E cohort was 0.42 ± 0.25 g/L. There is a trend to suggest that the Lp (a) is higher in the FDB cohort but is not statistically significant (P=0.062, Mann Whitney). The statistics was performed after log transformation.

3.8 BEST BIOCHEMICAL RESULTS ON TREATMENT

The provincial government of the Western Cape severely restricted the doses and range of medication that could be used for the severe disorders seen at the clinic. Although
adjunctive treatment with cholestyramine was available this was not often used as it required the financially poor patients to travel unaffordable distances to the teaching hospital at monthly intervals and the agent is also not well tolerated and could also interfere with other medication. Ezetimibe is not available. Within these limitations, the response to treatment was reviewed and summarised in table 3.7.

<table>
<thead>
<tr>
<th>Best</th>
<th>FDB (n)</th>
<th>LDLR D206E (n)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose mg</td>
<td>28.9 ±28.5 (n=25)</td>
<td>22.7 ±26.2 (n=32)</td>
<td>0.4</td>
</tr>
<tr>
<td>TG mmol/L</td>
<td>0.82 ±0.36(23)</td>
<td>0.7 ±0.32(38)</td>
<td>0.29</td>
</tr>
<tr>
<td>TC mmol/L</td>
<td>5.6 ±1.25(23)</td>
<td>5.8 ±1.35(38)</td>
<td>0.628</td>
</tr>
<tr>
<td>HDLC mmol/L</td>
<td>1.6 ±0.55(23)</td>
<td>1.5 ±0.44(38)</td>
<td>0.50</td>
</tr>
<tr>
<td>LDLC mmol/L</td>
<td>3.7 ±1.04(23)</td>
<td>3.9 ±1.24(38)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Percentage% change

| TG | -28% ±25% | -29% ±26% | 0.81    |
| TC | -34% ±12% | -29% ±18% | 0.24    |
| HDLC | 56% ±10% | 17% ±22% | 0.14    |
| LDLC | -42% ±14% | -39% ±24% | 0.45    |

Table 3.8 The best biochemical response of subjects with FDB compared with those with LDLR D206E mutation on statin therapy analysed as equivalent dose values with atorvastatin.

**Statin dose:** All the statin dosages were corrected for Atorvastatin equivalence. Ten mg of Atorvastatin is assumed to be equal to twenty mg Simvastatin or forty mg Pravastatin or eighty mg Fluvastatin. Notes were scrutinized for the best control and appropriate doses.
The average dose for the treated FDB cohort was 28.9 ± 28.5mg. The average dose for the LDLR D206E cohort was 22.7 ± 26.2mg. There was no statistically significant difference between the two cohorts (P=0.4, Fishers exact test) as far as the limited and variable prescription was done by clinicians. There were only 23 patients on treatment for the FDB and 38 patients were on treatment for the LDLR D206E cohort, partly because of their age at presentation, lack of statins in the 1980s and early 1990s and loss to follow-up.

**Best triglycerides (Best TG).** The average best TG for the FDB cohort was 0.82 ± 0.36mmol/L with the percentage reduction of total triglycerides of 28% ± 25%. The average best TG for the LDLR D206E cohort was 0.72 ± 0.32mmol/L, with a percentage reduction of total triglycerides of 29% ± 26%. There was no significant difference between the two cohorts for the TG concentration in mmol/L and (P= 0.29) for percentage reduction (P=0.81). The reduction in TG is consistent with the previous reports on the use of statins in hypercholesterolaemia.

**Best total cholesterol (Best TC):** The average best TC for the FDB cohort was 5.6 ± 1.25mmol/L and that of LDLR D206E cohort was similar at 5.8 ± 1.35mmol/L. There was no statistically significant difference between the two cohorts P =0.63 for the best achieved TC. The average percent reduction for the TC in the FDB cohort was 34% ± 12% and the average reduction percentage for the LDLR D206E cohort was 29% ± 18%. There was no statistically significant difference in percentage change either between the two cohorts (P=0.24).

**Best Low Density Lipoprotein Cholesterol (Best LDLC):** The average best LDLC for the FDB cohort was 3.7mmol/L ± 1.1mmol/L and the best LDLC for the LDLR D206E cohort was 3.9mmol/L ± 1.2mmol/L. There was no statistically significant difference between the two groups (P=0.47 Fisher’s exact test).

The average percentage reduction of LDLC between the two cohorts was similar: 42 ± 14% and 39 ± 24% for FDB and LDLR D206E cohorts respectively (P=0.45). These responses clearly do not achieve current LDLC targets for the primary or secondary prevention of coronary artery disease, signifying the need for maximum doses of the most powerful statins (atorvastatin and rosvastatin) as well as adjunct therapy with Ezetimibe as a cholesterol absorption inhibitor.
Best High Density Lipoprotein Cholesterol (Best HDLC)

The remarkable average increase of HDLC though not statistically significant was unexpected and if real, could not be explained simply. The average best HDLC for the FDB cohort was 1.6 ± 0.55 mmol/L. The average best HDLC for the LDLR D206E 1.5 ± 0.44 mmol/L. There was no statistically significant difference between these values with (P=0.5).
CHAPTER FOUR

DISCUSSION

4.1 INTRODUCTION

Although FDB is a well-recognised disorder, there is a paucity of information about it internationally and even more so, locally in South Africa. The aim of this study was to collate and analyse information on patients identified to have FDB, to explore subjects with the heterozygous FH phenotype for new mutations in exon 26 and 29 of apo B and to compare these with defective LDLR subjects matched for parameters influencing lipoprotein concentration and risk.

This retrospective study of the Cape Town experience on Familial defective Apolipoprotein B-100 (FDB) is a small cohort study of the patients confirmed with this genetic disorder as a cause of the familial hypercholesterolaemia phenotype. The phenotype was found to be very similar to that due to the D206E mutation in the LDL receptor known locally as FH Afrikaner 1, that represents the defective type rather than null type of LDLR mutations. The null mutations, though less common in local experience are accepted as having a worse phenotype.

Since patients were generally referred and accepted simply based on lipid concentrations and not on physical signs or family history so that it is unlikely that there is a bias in selecting between the 2 causes of FH phenotype unless there is a higher prevalence of lipid concentration below the referral criteria. It is possible that pedigree tracing of subjects with milder phenotypes of FDB that are not evaluated as heterozygous FH, could detect more kindreds with heritable hyperlipidaemia. Such a detailed study would contribute to the knowledge on FDB but is not affordable at this clinic. There could well be subjects with FDB within the dyslipidaemia generally attributed to FCH where TG concentration is also elevated and LDLR or apo B mutations are viewed as being less likely. The lack of detection of FDB in the black cohort when lower TC cut-point was set is suggestive that FDB will not be prevalent at lower TC concentration though this sample was small and had a different ethnic selection.
4.2 FH phenotype.

The phenotypic diagnosis of FH is useful as it reflects a high risk of vascular disease common to all its causal mechanisms but subtle distinctions were found in the phenotype that could be useful for targeting DNA analysis, which is important for counselling for procreation.

Familial hypercholesterolaemia is a common condition found in 1 in every 500 people. In founder communities this condition could be found in 1 to every 75. Given that the patient population that requires referral for management of hypercholesterolaemia of > 7.5 mmol/L to the GSH lipid clinic is approximately 3 million, the expected number of FH subjects at a prevalence of 1/500, is at least 6000. This suggests that less than 1/3 has been referred. There is thus likely a low suspicion for this disease. This needs to be improved in both under and postgraduate training to ensure more systematic diagnosis and referral of subjects with a severe disorder from all communities. There was a likely selection bias in considering severe hypercholesterolaemia for treatment, since patients were mostly white or mixed ancestry and few blacks were encountered in this analysis whilst many more would be expected based on the prevalence of 1/500.

The LDLR mutations have heterozygous and homozygous phenotype and both these phenotypes have been encountered at the clinic. In the FDB cohort only the heterozygous FH phenotype was considered and proven genetically. In none of the study did the suspicion arise for the homozygous situation that could be suggested by subjects with premature IHD in both ancestral lines of the index cases. Heteroduplex analysis would miss true homozygotes for the FDB but would detect compound heterozygotes. Based on a few reports, the combination of heterozygous LDLR mutation status complexed with heterozygous FDB gives an intermediate phenotype between heterozygous and homozygous FH. Heterozygous PCSK9 complexed with heterozygous LDLR is also expected to possibly give a rise to intermediate or homozygous phenotype. Heterozygous PCSK9 complexed with heterozygous FDB may also be expected to possibly give a rise to intermediate phenotype between heterozygous and homozygous FH. However, none of the patients displayed an intermediate phenotype.
4.3 FDB findings for the region.

4.3.1 Occurrence at the clinic.

FDB is not common in the Familial hypercholesterolaemia (FH) phenotype encountered at the Groote Schuur Hospital, Cape Town. In fact, it comprises of 0.5% of cases or 16 kindreds out of a total of 1627 subjects with the phenotype. Previous studies suggested the under-diagnosis of FH (RR) in the region. FDB due to mutations in exon 26 and 29 of apo B is present in Western Cape. The heterozygous FH phenotype represents 22% of referrals of which 2.3% is due to FDB, accounting for 0.5% of all referrals. The predominant mutation is R3500Q but R3500W and R3531C and H3543Y are also present. The prevalence of FDB at the clinic does not necessary translate to the general population though a selection bias is not expected on clinical criteria. However awareness of dyslipidaemia may lead to greater identification of dyslipidaemia in some groups of the population or medical practitioners may have a bias in testing cholesterol. No founder effect for the known mutations has been documented locally but the number of immigrants with the disorder is interesting and could be explored if there are large systemic studies to identify dyslipidaemia. It is conceivable that FDB might be under-diagnosed so if there is a larger proportion of subjects with a milder phenotype, especially when younger subjects are screened.

A clinical diagnosis is of a phenotype and can be genetically heterogeneous and the genetic cause may have special implications for heritability, treatment or prognosis. Genotypic diagnosis has become easier to do and is cost effective especially with screening by High Resolution Melting (HRM). This makes it easier to have a proper genotypic diagnosis for research purposes, proper family counseling and treatment thereof.

The classical R3500Q mutation was found in South Africans of British ancestry, white English speaking South Africans, Afrikaners and people of mixed race. There were 14 kindreds with the classic R3500Q mutation. Of these, 11% was of mixed ancestry. The other mixed ancestral kindred with R3500Q had Malaysian ancestry; it is possible that this gene came from Malaysia as this has been described before. Two other mixed ancestral families had Irish ancestry; the Irish might have introduced the gene to these kindreds, as this gene is believed to have originated in Europe from a common ancestor many centuries ago. Two
kindreds with the classic R3500Q mutation were British descendants. It is not documented where in the United Kingdom these two kindreds came from. The gene is also found in the white Afrikaans kindred and is presumed to have come from a Scottish or Irish maternal grandmother as suggested by the family tree but the grandmother had already died. The white Afrikaans-speaking community, renowned for the founder gene for the low-density lipoprotein receptor defects now also needs consideration of FDB when such a patient presents with the FH phenotype. A careful pedigree should make the appropriate genotyping more effective. It has not been extensively investigated if FDB occurred, in the Afrikaners derived from early settlers in South Africa that included Germans, Dutch and French Huguenot ancestry. If this had been introduced then there might have been more Afrikaners with this diagnosis to date, especially since the causes of FH in the Afrikaner have been extensively investigated. This family with a typical Afrikaner surname is the only one in 16 kindreds with FDB.

There was only one patient with the R3531C mutation, she was of Sicilian ancestry and had a son with hypercholesterolaemia in America. This mutation has been reported earlier in America in individuals of Italian ancestry. Other migrations to the USA from Italy may possibly have caused a founder effect for this mutation from Sicily or elsewhere in Italy. The phenotype is very similar to R3500Q.

There was one patient with R3500W. He was of mixed ancestry but did not know much about his family genogram. Gaffney has reported this mutation in Scotland as a cause of hypercholesterolemia.

H3543Y is relatively new mutation and has been found in German patients who had hypercholesterolaemia and who were undergoing angiographic study for atherosclerosis. Only one patient in our study with this mutation was identified.

The mutations reported in exons 29 in association with FDB were not found in any of the patients at the Groote Schuur lipid clinic. Haplotyping at the Apo B locus on chromosome 2 would be interesting to do in patients with classic R3500Q to add further evidence that the South African patients stemmed from the common ancestor in Europe about 6000 years ago.
4.3.2. The search in patients of black ancestry

Mutations in exon 26 and 29 of apo B were not found in the few phenotypic FH subjects in the clinic and expanding the search to milder hypercholesterolemia still did not detect these mutations. Forty-eight patients with monogenic hypercholesterolaemia of which four had heterozygous FH phenotype were identified by a cholesterol cut-off level of 6.5mmol/L. None of the known mutations to FDB was detected in exons 26 and 29 new mutations were detected as the cause of hypercholesterolaemia in this group. The results of sequencing a few heteroduplex patterns revealed innocent mutations, where the codon is different but still codes for the same amino acid. Thus, FDB would seem to be absent or extremely rare in the black South Africans. A search of the upper quintile of cholesterol in a large cohort is needed to validate this finding. From the analysis of apo B in its binding to the LDLR it seems that only a limited region would confer low affinity for the LDLR and it is unlikely that mutations in other regions of apo B will cause FDB. Since the black population could be genetically very different and could thus have FDB relating to mutations in exons other than 26 and 29, a functional study of LDL intake could be a more effective approach to identify FDB, followed by exon for exon sequencing. It should be encouraged that cholesterol be checked routinely at primary care level in young adults and referral of patients with abnormal levels in this ethnic group, especially where patients are at risk for vascular disease by virtue of diabetes and/or hypertension.

The absence of FDB in the small number of blacks with the FH phenotype is remarkable and persisted when tests were extended to those who had less severe LDL hypercholesterolaemia. This would suggest that there is low prevalence of FDB in the black population. Although a defective function from mutations in other exons than 26 and 29 could still be present. Even more unlikely is the other possible explanation for the absence of FDB in exon 26 and 29: true homozygocity for FDB.

4.3.3 Clinical Recognition

4.3.3.1 Physical Signs
The clinical findings of subjects with FDB and their similarity to at least the common LDL receptor defective FH phenotype indicate that the concept of a heterozygous FH phenotype
is worth retaining as a means to identify three genes that produce a similar clinical entity. Xanthelasma is a poor physical sign, being present in only 16% of FDB subjects. Arcus cornealis was found in 39% of patients but may be absent until the 4th decade. A Tendon Xanthoma is a valuable physical sign in the diagnosis of middle-aged subjects with FH and was present in 46% of FDB subjects. Familial hypercholesteroleamia might be missed if one is to solely depend on this sign.

4.3.3.2 Complications
Across the wide age range seen at the clinic, twenty seven percent of patients in the FDB cohort had established coronary artery disease and at a similarly high prevalence to LDLR mutations though a little later.

4.4 Comparison of FDB with of LDLR D206E FH phenotype.

4.4.1 Similarities
Familial defective apolipoprotein B-100 is clinically indistinguishable from LDLR defective FH. Both cohorts presented at similar age and had similar physical signs: the tendon xanthomata and arcus cornealis. Both cohorts have premature coronary artery disease at a high proportion and little other manifestations of atherosclerosis.

4.4.2 Differences between FDB and LDLR mutation

Arcus cornealis: Arcus appeared at a younger age in the LDLR D206E cohort. The cumulative arcus cornealis was 13% by the 4th decade of life in the LDLR D206E cohort. This number had doubled by the 6th decade of life by 34%. The FDB subjects only caught up with the high prevalence in the 7th decade of life. The difference between the two cohorts in the early decades might be explained by observation that FDB is biochemically less severe early in life.

Ischaemic Heart Disease onset: Although both cohorts had a frequency of premature coronary artery disease, the age of onset of ischaemic heart disease was statistically significantly different between the two cohorts P=0.044. The mean age of onset for the IHD in the FDB cohort was 55 ± 10.25 years compared with LDLR D206E 46 ± 12.4 years. This
difference may be attributed to FDB being biochemically less severe earlier in life and the borderline lower LDLC concentration. The higher prevalence of hypertension and diabetes mellitus may have lessened the difference between the 2 causes of FH. The LDLR D206E cohort smoked more as a trend (P=0.0582) and this could have aggravated the risk in this cohort.

**LDL**

Apo B concentration was identical in the two cohorts but there was a trend for higher LDLC concentration and higher cholesterol/apo B in LDLR mutant subjects to suggest that FDB has smaller particles. The LDL species distribution for the FDB cohort was statistically significantly different from the LDLR D206E cohort. This was after matching for other influences on LDL size: TG, BMI, age, gender and after diabetic subjects were excluded. This difference in the LDL particle size suggests metabolic differences between FDB and LDLR defective FH. LDL particles in the FDB cohort bind defectively and become enriched by allowing for longer circulation of these LDL therefore prolonged action of CETP and hepatic lipase rendering the species to be smaller and hence denser. It is however also possible that there may be a difference in lipoprotein synthesis as some nascent lipoproteins are bound by LDL receptors in the hepatocyte. If apo B is a ligand, such particles will be secreted in the FDB rather than being degraded. Smaller LDL with longer circulating time could pose a higher risk of oxidation and glycation that are both expected to increase atherosclerosis.

The LDL particle size in females was statistically significantly different (P= 0.049), in favour of large species. Obesity also affects the LDL species, this was statistically significantly different (0.022), when the LDLR D206E and FDB cohort were combined and the LDL species were compared from large to small. This probably shows that WHR is a better indication of obesity than the BMI as the change was more dramatic.

**Response to treatment**

The treatment response between the two cohorts was very similar on equal amounts of statin dose. The total cholesterol decreased by 34% and 29% in the FDB and LDLR D206E cohorts respectively. The LDLC was reduced in a similar fashion in both cohorts. The mean
reduction for the FDB cohort was 3.7mmol/L and the average reduction for the LDLR D206E cohort was 3.9mmol/L. The average percentage reduction for the FDB cohort was 42% and the average percentage reduction for the LDLR D20E cohort was 39% P=0.45. These LDL values do not meet the target for the prevention of atherosclerosis, where values as small as 2.5mmol/L or less are favoured.

**Other differences**

This study involved relatively small numbers and might thus have missed some other differences between FDB and LDLR mutation heterozygous FH phenotype. Since there is a suggestion for taller subjects in FDB, it is of particular interest to study offspring of patients with FDB and LDLR FH to determine whether growth is different due to difference in delivery to growing cells, whether lipoproteins differ in concentration and size and whether CIMT thickening occurs at similar rates.

**4.5 Lessons learnt from the study**

1. FDB occurs in South Africans of white and mixed ancestry but is not yet documented in black South Africans nor in South Africans of Indian ancestry. In clinical practice, FDB needs consideration in patients with the heterozygous FH phenotype especially those of British ancestry and patients of mixed ancestry but even Afrikaners may have this diagnosis.

   From an academic point of view it should be sought in a large population study and preferably with a functional method that lands itself to efficient screening for ligand-defective LDL. Fluorescently labelled LDL could be used in uptake studies with normal fibroblast culture in microtitre plates.

2. The phenotype of FDB is similar to LDLR D206E with somewhat later onset of ischaemic heart disease but similar presentation. This is consistent with the report of lower concentration in children, possible delay in developing arcus in this study.

3. Mutations in apo B are varied. The predominant mutation causing FDB is R3500Q but R3500W, R3531C and H3543Y are present as well. Other mutations were found but they did not change amino acids.
4. Response to treatment is similar between FDB and LDLR D206E with an obvious requirement for maximum dose treatment. Regrettably the lipid clinic is not authorised to treat patients with FH optimally.

5. While LDLC concentration is borderline lower, Apo B concentration is identical. LDL species differ by being smaller in the FDB subjects.

6. It is possible that FDB is milder in young persons when compared with receptor-defective LDLR FH, giving rise to lower LDLC concentration as reported in the literature, possibly better/taller growth found in this study, and delaying the onset of premature IHD. However, the smaller LDL species may result in a catch-up in atheroma and physical signs, so that later in life no difference is found.

4.6 Recommendations

1. An increased awareness of the FH phenotype at primary, secondary and tertiary health care level cannot be over-emphasised, as the disease carries serious CV risk implications and the early diagnosis and treatment alters the natural history of the disease for the patient and the family. Various ways can be used to improve awareness. These include an emphasis of FH at under-graduate and post-graduate level of education. Additionally publications of this current study and hopefully more studies of similar nature locally will heighten awareness. Involvement of national and international registry will obtain more information. Public education through media and organisations such as the Heart and Stroke Foundation can improve awareness.

2. Expert centers, regionally if not nationally, need to evaluate severe dyslipidaemia by referral criteria resembling those of GSH lipid clinic, and strategic genotyping. Pedigree tracing is important to identify subjects at risk by cascade testing. This would be easier if there would be allocation of budget for the lipid clinic to have more staff members at the tertiary centers and at the clinical and laboratory level. This would allow efficient patient care, facilitate research and provide more answers to questions that will arise.
3. Owing to the lack of information of FH in previously disadvantaged population groups and the need for tracing as many FH subjects as possible, a register of FH to study the disorder of FH in South Africa is advocated. The utility of a register is Registry exemplified by the Simon Broome in the UK.

4. It is disappointing that a treatable condition was not supported to achieve recommended targets. Better treatment with full dose of statin and additional therapy e.g. Ezetimibe if needed, will bring down the LDLC concentrations to target as recommended in most guidelines for the primary and secondary prevention of IHD. Governmental and private healthcare sectors ought to be more aware of the severity of FH and the benefits if earlier and more aggressive treatment.

5. Diagnostic strategies should be derived for genotyping FH. Excluding LDLR mutations that commonly occur before HRM of exon 26 and 29 is reasonable unless more specific information can guide the investigation. If concerned about homozygous FDB- being missed, a careful clinical evaluation may preempt wild type spiking. Functional screening by fluorescent LDL up-take before looking at other exons in apo B could be a strategy to identify FDB.

6. The impact of FDB and/or small LDL species should be studied in more detail with attention to lipoproteins capillary, endothelial/cell and macrophage effects, relating to atherosclerosis. The production of apo B in VLDL by a turnover studies that can discriminate between normal and abnormal apo B as well as clearance of apo B from Lp (a) remain of interest. Lipid peroxidation is also of interest in the population of lipoproteins with normal and abnormal apo B.

7. Surrogate markers of atherosclerosis such as IMT comparisons between childhood FDB and LDLR FH could give more detailed information about the differential effects of FDB and LDLR mutation in FH especially early in life.

8. The phenotypic approach as recommended to identify serious monogenic defects has clinical applicability and genotyping will be used to detect the casual mutation.


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APPENDIX
TABLE OF CONTENTS:

The standard operating procedures in the lipidology division of Groote Schuur Hospital University of Cape Town are included, to describe in detail how the methods were performed.

1. Parzer’s rapid method for genomic DNA from blood

2. Non-denaturing gradient gel electrophoresis (GGE) for lipoproteins

3. Detection FH1, FH3, and Lithuanian mutations in exon 4

4. Detection of FH Afrikaner-2 and Familial defective Apolipoprotein B-100 ARMS assay (Amplification refractory mutation system)

5. High-resolution melting (HRM) analysis for the detection of Familial Ligand Defective Apolipoprotein B-100 mutations.
PARZERS RAPID METHOD FOR GENOMIC DNA FROM BLOOD

GENERAL:

This rapid method is for extracting genomic DNA from whole blood or other biologic material and is suitable for polymerase chain reactions and southern blotting. It has a good yield (about 250ug from 5ml whole blood) and good 260/280 absorbance ratios. On agarase gel electrophoresis the DNA appears undergraded. In our laboratory there is often a failure to do PCR and this can be corrected by a denaturation step.

MATERIALS:

Vacutainer tubes with anticoagulant. Waterbath.

CELL LYSIS BUFFER at pH=8

WASH BUFFER. NaCl 10mM, EDTA10mM, also pH=8

SARKOSYL SOLN. 20%Na laurylsarcosinate. Sigma L5125. 50g=$10

AMM ACETATE SOLN, 7.5M. Sigma A1542. 100g=$8.50

PROTEINASE K SOLN 10mg/ml.

GUANIDINE HCl 6.0M.

TE BUFFER. Tris HCl 10mM and EDTA 1mM at pH.

Icecold ethanol.

PROCEDURE.

The directions are for 5ml frozen blood. Thaw rapidly at 37°C and mix with 50ml cells lysis buffer. Incubate for 15mins on ice before centrifuging at 6800G for 10min at 4 °C. Decant the supernatent.

Thoroughly resuspend the pellet in wash buffer. Spin at 6300G for 10min at 4°C. Siphon off the supernatent.

To the almost white pellet add the following in the order stipulated. Sarkosyl solution 350ul, ammonium acetate solution 250ul, guanidine solution 3.5ml and then 125ul proteinase K. incubate at 60°C until clear (10-15min). Cool to 0°C.

Precipitate the DNA by adding 10ml ice-cold ethanol. The DNA is spooled off with a hooked glass rod. Wipe the excess alcohol on the rod off, gently.
Dissolve the DNA in TE buffer for 1 hr at 37°C. The material is usually incompletely soluble but can clarified by a brief centrifugation (15 secs) to precipitate the insoluble material. Store at -20°C.

DENATURATION. To 10ul of the DNA in TE buffer, add 10ul of 0.1M NaOH. Boil in waterbath or heating block for 10min. Snap cool on ice. Add 2.5ul of 1M Tris at pH 8.0.

NON-DENATURING GRADIENT GEL ELECTROPHORESIS
(GGE) FOR LIPOPROTEINS.

GENERAL

The technique of non-denaturing gradient acrylamide gel electrophoresis allows separation of lipoproteins (LP) by size. The LP may be demonstrated by staining lipid or protein. Pre-staining the sample with a lipid stain allows specific visualisation of lipoproteins in the original glass sandwich while protein staining has to be done after removing the gel and is only specific for large, apolipoprotein B-containing lipoproteins. Separation of CM and VLDL is difficult because of the size of these LP and these triglyceride-rich lipoproteins are seen as broad size ranges. The IDL range is also not so distinctly separated. Gels may be selected to demonstrate LpB series (LDL) and LpA series (HDL). A 2-16% gradient is usually recommended for LDL and for HDL, 4-30% [1] but our lab uses a mini-gel of 2-8% acrylamide gradient for LpB, and a 4-18% for LpA.

Qualitative differences in LDL may be important. The first relation between smaller denser LDL and heart disease was shown by Melissa Austin [4], who recognised two types of LDL. Dormans [5] suggested that 3 LDL subtypes could be identified by either ultracentrifugation or GGE, although the latter method could on occasion identify 5 bands. Tashiro [6] found a “midband”, which is probably Lp(a), that predicts heart disease in FH subjects. Similarly, HDL3 and HDL2 subtypes may influence atherosclerosis. The GGE and HDL ultracentrifuge subtypes have not been compared yet in our lab. In this system, small dense LDL can be demonstrated in the lowest quintiles of triglyceride, waist/hip ratio, body mass index and fasting glucose concentration. However, small dense LDL is seen usually at a triglyceride concentration of >1.7mmol/L, almost always at TG >2.5mmol/L. Similarly, a waist/hip ratio of >1, BMI >30 or glucose >7mmol/L is highly associated or with small dense LDL. It has been shown that a TG/HDLc ratio, in molar terms, of >1.33 is also strongly predictive of small dense LDL [15]. The system is also good for diagnosing dysbetalipoproteinemia [14].

Samples may be plasma or serum or isolated lipoproteins. Samples should immediately be placed on ice and processed as soon as possible, but have been satisfactory for LDL characterisation up to 1 week when stored at 4 Celsius, either stained immediately or after a delay. The samples can be frozen at -20celsius for a few weeks, and for several (>12) weeks at -80. There is a factor in the LPDS that can increase the size of LP on incubation. It is heat labile and dialysable but is neither CETP nor PLTP.
Samples from animals with cloven hooves may carry foot and mouth disease virus. Samples can be sterilised by heating to 56°C for 30 mins. This has been tested and shown not to affect human samples. After processing the same samples, should be placed at pH <6 or >9.

**MATERIALS**

SUDAN BLACK STAIN. 1% sudan black is added to ethylene glycol. Filter the solution. It keeps for several weeks.

SATURATED SUCROSE with a spatula tip of bromophenol blue.

SOLUTIONS FOR SDS-PAGE as declared in the laboratory methods but omits SDS from all. Fresh acrylamide solution is better for clarity of separation. Depending on the brand and batch of acrylamide slight adjustments may need to be made for optimal performance, adjusting the denser solution to 7 to 8%.

NILE RED STAIN. Dissolve 1mg in 100ul of dimethylsulphoxide as a stock solution (50X), then dilute by adding 2ul to 98ul of DMSO for use on GGE. Add 10 ul to 50ul of plasma. This stain with similar sensitivity to sudan black but requires UV light exposure for visualising the lipoproteins.

Minigel apparatus and powerpack in cold room or refrigerator.

**PROCEDURE LDL FOR (LpB) GEL**

Cast a 2-8% polyacrylamide gel and a 3% stacking gel with a minimal but definite layer of gel between the bottom of the wells and the separation gel. Mostly 2 gels are run in the system, with 15 lanes per gel. Label the glass plates. (The stacking gel may be coloured by a small amount of phenol red to make loading easier.)

To 100ul of plasma add 50ul of lipid stain, mix and incubate for a minimum of 1 hour at 4 Celsius. Spin for 20min at 10000G. Mix an equal volume of supernatant with saturated sucrose and load approximately 12ul per well.

Prefocus the gel at 20V for 30mins. This is ideal but not essential. Run the gel at 130V for 18-24 hours. The progress is visible! Remove the glass sandwich from the stand, dry with a paper tissue.

Recording of the gel. Write the gel identification on the dry glass plate. Mark the point at which
the separation and stacking gels meet by placing a dot next to the spacer. The gel is now placed face-down on a photocopy machine and covered with white paper before a photocopy is taken. Afterwards, photograph the gel if necessary. The gel can also be scanned in the Hoefer densitometric scanner while in the glass plates, or it may be recorded on the Biorad Geldoc videocamera. Hereafter it is dried on filter paper or cellophane.

**PROCEDURE HDL (LpA) GEL**

Cast a 4-18% gradient gel and a 3% stacking gel. Prepare the plasma sample with sudan black in ethylene glycol as for LDLGEL but load directly, 16ul/lane.

Prefocus the gel at 20V for 30mins, run at 130V for 4 hours. The progress is visible! It is analysed in the same way as the LDLGEL.

**INTERPRETATION OF LpB GGE**

**REPORTING.** The gel is inspected without knowledge of clinical or biochemical detail. Comment should be made about material in the stacking gel as occasionally there may be significant precipitate in the stacking gel, either from granules of sudan black that were not removed by the spin, or from lipoprotein aggregates. The origin (top or least dense part) of the separation gel reflects the largest particles. The minigel is about 60mm long. In our system the stain reflecting lipoproteins in the LDL density range, is from approximately the junction of the top 2/3 with the bottom 1/3. The chief LDL-related bands are in the bottom 1/6 of the stained area.

**TERMINOLOGY.** The gel is described by simple terminology avoiding terms inferring separation by density. Since the gel was developed to look at “LDL”, the zone between the origin and the LDL being referred to as "mid" and staining in this region was consequently referred to as M, which was later modified according to an earlier or later observation to M-early, or M-late to describe particles that were respectively larger or smaller. Subsequently it became clear that chylomicron-like particles tended to remain at the origin of the separation gel, and hence was termed O. At the “LDL” range, the largest particle is designated "A" while some intermediate "I" bands may be discerned or the "B" band as the smallest (densest) LDL which has been associated with a higher risk of ischaemic heart disease in FCH and with hypertriglyceridaemia. These common species of A and B appear to agree with the prevalences of A and B reported in most studies although they have not been specifically prepared. Subsequently it became clear that even further size distinctions can be made, now abbreviated to A-early, A/I, I/B and B-past. Sometimes a band between A and M-late could be discerned, and referred to as pre-A. This band specifically disappears on adding a reducing agent, proving it is Lp (a). A small letter is used to designate that the band stains faintly, and a capital letter is used to demonstrate dominant bands. A sharply focussed band in the M-early range is seen in cholestatic jaundice, and is referred to as M-focussed.
Describing particles from large to small on this gel would thus be: O, M-early (Me), M-focus (Mf, in Me range usually), M-late (Ml), pre-A, A-early (Ae), A, A/I, I, I/B, B, B-past (Bp). Chylomicrons correspond to O, VLDL1 (Sf 60-400) to Me, LpX to Mf, VLDL2 (Sf 20-60) to Ml, pre-A is Lp(a), Ae to Bp are all species of LDL.

**RELATION TO DYSLIPIDAEMIAS AND FREDRICKSON TYPES.** All hypertriglyceridaemias give rise to staining in the M region, but LpX which represents a hypercholesterolaemia, will also have staining in the M region. In type I and IV and V there is intense staining of the M region, often with something visible near the origin and the LDL bands are often only slightly visible, if not absent. In type III dyslipoproteinaemia the band peaks in the M range, with minimal LDL banding and there may be some staining at the origin.

Hypercholesterolaemias will stain mainly the LDL range with some entry into the Ml region if IDL is present. The LDL-A pattern is seen in most normal persons and hypercholesterolaemias, including FH, in which there is no hypertriglyceridaemia. Thus in normal and Fredrickson IIa one expects large species of LDL. In type IIb it would appear that almost always there are the in mid-portions, particularly me, indicating an excess of VLDL-like LP. The LDL particle size in X-linked ichthyosis is large, so that agarase gel electrophoresis to demonstrate fast-migrating beta-lipoprotein is still preferable to prove this diagnosis.

The situation of Lp(a) is preA. LpX is seen in the M zone, usually as a sharp band and the distinct impression is that it is detected more sensitively than in agarose electrophoresis. LpX often gives the sudan black a greenish tinge.

The colour of the lipoproteins can vary considerably from brown to blue, and the sharpness of the bands is much better with fresh acrylamide. Lipid staining of isolated lipoproteins is also more intense than the staining in whole plasma.

**CALIBRATION OF LpB GGE.** Currently there is no standardisation for assays of small or dense LDL. Small, dense LDL can be demonstrated by density (ultracentrifugation), or size (non-denaturing polyacrylamide gradient gel electrophoresis (GGE) or size exclusion chromatography such as FPLC). The reported size of LDL varies, with a slightly broader range reported [11] between 21 and 29 nm and the acrylamide gradients can vary [10]. Nuclear magnetic resonance can detect lipoproteins by unique emission signals according to their mass, allowing the discrimination of up to 15 lipoproteins [11] with estimates for LDL at 19 to 22nm. Ultracentrifugation uses a few ml of fresh plasma and permits compositional analysis but takes hours to days and gravitational forces may cause the loss of some apoproteins. Typically, three fractions are separated [12]: LDL-I at 1.025-1.034g/ml, LDL-II at 1.034-1.044g/ml, and LDL-III 1.044 - 1.060g/ml. The concentration can be reported as lipoprotein mass or protein or cholesterol.
It has not been possible to calibrate the system accurately so that the diameter of the particles can be properly calculated. Currently we readily identify A and B patterns amongst the 15 lanes and use one of each LDL type to carry across from gel to gel to bring some standardisation about. Lanes 7 and 8 contain an A and B species that are repeated used from gel to gel for a few weeks, but mixing them is not a good idea as some changes in size can occur. The observation is also that small LDL can enlarge with time. Of course gels that are prepared individually for each run are not exactly reproducible. However, it has been found that the classification is extremely reproducible on repeat samples.

Latex beads tended to clump and cause “ladders” and were thus not useful. We have generally accepted that LDL particles will have the typically published diameters and that there is a broad size range for all other particles. There is even disagreement in diameters for LDL from different laboratories using size markers, and also with other methods of determining lipoprotein particle size. This makes accurate diameter determinations less useful. The gel is primarily used in our laboratory to describe patterns of major dyslipoproteinæmia involving apolipoprotein B-containing lipoproteins with particular reference to dysbetalipoproteinæmia and lipoprotein X, and to determine LDL particle size categories and whether there are significant changes in these as a result of interventions.

Retardation factors. The system has been standardised against ultracentrifugally prepared VLDL1 (Sf 60-400), VLDL2 (Sf 20-60), IDL (Sf 12-20) and LDL (Sf 0-12). Taking the B particle as Rs=1.0, the following Rs values are typically found for V1: 0.2-0.45. For V2 the Rs is 0.45-0.7; for IDL it is 0.7-0.85 and for LDL A-B it will be 0.85-1.0 and about 1.05 for B-post.

Diagnostic aspects of LDL GGE. Hypertriglyceridaemias have M bands, and usually B or Bposts. In chylomicronæmia there is usually no LDL band. Mixed hyperlipidaemias also have M bands. Dysbetalipoproteinæmia has a preponderance of Ml band with very little or no LDL bands, although in some instances the pattern may change to only a LDL band in this condition upon successful therapy. If there is a lapse of dietary and drug compliance, the lipoproteins may increase in size. Most hypercholesterolaemias have A bands, sometimes I bands and occasionally there is a B band; the B usually being associated with a M or m. This pattern is almost the norm when the fasting triglyceride concentration is >1.8mM/L or waist hip ratio is more than 0.95. It is also prevalent in diabetics, but more so in men. PreA is seen variably and generally correlates with an apo(a) concentration of >50mg/dl, and some size variation can be seen. There is a distinct impression that LDL particles distribution distribute more strongly to a single species compared with ultracentrifugally derived subfractions. In a population survey, it was found that about 2% of adults have no visible LDL bands, and about 2 % have 2 distinct bands, sometimes of equal intensity. Occasionally there may be more than 2 bands visible in the LDL zone. Interventions that modulate triglyceride concentration, will usually alter LDL size at between 2 and 3 weeks. An interesting pattern of smearing through the preA region into the LDL region has not been explored.

Interpretation of the LpA GGE
REPORTING. This is done blindly and after selecting from the many lanes, likely equivalents of HDL 2 and 3 species. The bands tend to be poorly staining and broad, and only occasionally can speciation be distinguished in the two regions. The LDL bands at the top may be classifiable as A or B but are often inadequately separated for comment.

TERMINOLOGY OF "HDL GEL". Particles relating to HDL2b and HDL2a as well as HDL3a, HDL3b, HDL3c are described in the review by Silverman [7]. In our system we find mostly 2 peaks, one with Mr of 135kD and another at about 165kD. The former appears to be HDL3 and the latter HDL2. In some patients there is a smaller size lipid-staining peak, at about 115kD. In some instances larger particles are seen in hyperalphalipoproteinaemia; in one patient discrete bands at 209, 229, 269, 300 and 365kD.

Current practice is to assume that the common species of smaller size is HDL3, the larger is assumed to be HDL2. The description is thus of the intensities being dominant in either one of the two bands or equivalent. This agrees remarkably well with the area under the curve and the peak intensities on our gel scanner. Occasionally there may be small species of lipoproteins, to which the label HDL4 has been given, or larger species to which the label HDL1 has been given. These can range to a size close to the LDL band. Typically the Me, B pattern of the Lp GGE has only a 3 band on the LpA GGE.

STANDARDISATION OF PARTICLE SIZE. This has not been satisfactorily performed yet. Usually the pattern can be described by comparison with the other 14 lanes on the same run. Latex beads of defined diameter do not provide a single neat band on the LDL or HDL system, while on the HDL system the rainbow markers do not give neat bands when undenatured. Protein staining on our system would be confusing especially in the HDL range as there are many proteins at these sizes.

The utility of using haemoglobin is under investigation. Hb has a molecular mass of 64 458 and binds to haptoglobin (Hp) which has 2 binding sites for Hb. Hp is present in most individuals but rarely the Hp 0-0 status or anhaptoglobinemia is found. The molecule is a tetrad of two \( \alpha \) and \( \beta \) units. The \( \alpha \) units can be \( \alpha^1 \) or \( \alpha^2 \) and slow and fast migrating forms of \( \alpha^1 \) are known. Several phenotypes of Hp are known: Hp 1-1 (\( \alpha^1\alpha^1\beta_2 \)) with mass of 80dk, Hp1-2 (\( \alpha^1\alpha^2\beta_2 \)) with a mass of 120kd and (\( \alpha^2\alpha^2\beta_2 \)) of 160kd. The Hp 1-2 and 2-2 phenotypes can dimerise to 200 and 400kd forms. Assuming one Hb to be bound per molecule, red bands should be seen on the GGE at 65, 145, 185, 225, 265 and 465kd. It is difficult to prepare suitable plasma samples to act as distinct markers, but sometimes haemolysis leads to these bands being visible in the HDL gels.

An unusual ladder of proteins has been observed between HDL and LDL on the HDL gels from some subjects in the coronary care unit. The calculated molecular mass seems to escalate approximately 300kd intervals up to 2.5 million molecular mass.
APPLICATIONS

FLUORESCENT STAINING. The preliminary observations suggest that pre-staining the plasma lipoproteins with Nile red is as sensitive as Sudan Black staining for the LpB and LpA gels, but some optimisation is still required. There is intense staining at about 70kd on the LpA system which is not seen with a gel containing only albumin.

COOMASSIE STAINING. After the gel is run, it can be removed and placed in Coomassie staining solution according to lab protocols. If the plasma had not been pre-stained with Sudan Black, the LDL band is faint and possibly the preA band is more visible. Prestaining with Sudan Black enhances the Coomassie staining. Protein bands are visible below the LDL zone, at the edge of the gel and represent macroglobulins.

SILVER STAINING. This method becomes very sensitive for detecting protein and a decent band of LDL is seen with about 4ul of plasma in the lane. There is little protein visible in the lipoprotein range. Sudan Black enhances silver staining in a similar way that it enhances Coomassie staining and makes the entire range of lipoproteins from LDL to IDL stain very darkly. Acetone exposure of the gel for 30 minutes sensitises the unprestained LDL to silver.

WESTERN BLOT. This allows transfer to nitrocellulose and probing for apoproteins. The transfer of apoB is slow owing to its molecular mass and can be enhanced by adding SDS if lipids are not desired in further studies.

ANALYSIS ON GELDOC

The Biorad Geldoc apparatus allows convenient graphic recording of gels stained with visible dyes or of light emitted from fluorescent stains upon UV illumination. The system includes a personal computer, a videocamera mounted in a photographic black chamber with illumination facility, and a dedicated printer for images. The graphics files are saved in C: under directories created for the staff, and in file names that can trace the gel efficiently. The graphics file stored can be used to print images and to analyse selected portions of images directly on the screen or by transferring the data to other software as x, y coordinates. This latter process affords better analysis and interpretation, especially through Graphpad prism. The University of Cape Town Information Technology has been unable to link the Windows NT system to the network and the files have to be manually transferred by stiffy disk from the Geldoc to other personal computers. This also poses problems for back-up.
CAPTURING THE GEL.

Switch on the computer, the video camera and the printer. The computer will prompt signing on as the administrator and the password is MONALISA (case-sensitive).

Select Multi-analyst Shortcut from the programme icons by a double click. The display will have toolbars available but no image will be seen until one is created or recalled from stored files. To create a new image, go to File and select New. The videocamera view will now be on the screen.

Dry the gel with a tissue and make certain there are no avoidable marks on the gel. It is a good idea to place a dot against the left and right spacers so that the junction of separation and stacking gels can be marked for reference during analysis.

Go to Set-up. Enter the appropriate light source (white light for GGE with Sudan Black). Prepare the photographic chamber. Note that a white transforming plate is placed over the UV source for analysing gels stained with dyes absorbing in visible wavelengths. Place the gels appropriately on the white plate or on the glass for UV analysis. Close the chamber. Adjust the aperture, focus, and zoom of the camera.

Make certain that the background density is uniform (best on analytical setting at the switch on the bottom front of the camera box). Once set up, the image needs to be captured. Select Capture button at bottom left corner. This button may be masked by the lower edge of the screen, in which case click on the edge and drag it lower to display this button. Additionally click on View and remove the toolbar to allow the capture button to come into view. The capture process will prompt the file saving data: In the Save As windows, select the “Work” folder, double click on the appropriate “Name” folder and enter the appropriate Subfolder and file name. Enter the file name and save as a *.bif file.

Switch off the video camera (both the UV source and camera). To exit, select File, exit. To switch off the computer, go to Start, select shutdown and machine will close the programme and declare when it is safe to switch off manually.

PRINTING THE IMAGE.

This can be done from the video camera while the gel is being recorded, or from a saved image. In both cases the picture will include the surrounds of the gels unless there is appropriate cropping.
With the programme activated, select the saved image: go to File, select Open, select Work, follow directory through Folders and then select the appropriate File. The image will be displayed, but should be maximised for better viewing. Now select from the toolbar the open square to perform cropping so that the gel only is displayed, by clicking in one corner and dragging the mouse to enclose the area of interest. Take the mouse’s cursor to the Image button and select Crop.

Now return to File, select Videoprint. Now press the button attached to the printer.

ANALYSING THE GEL.

The password for the Multianalyst is case sensitive: MONALISA. With the Multi-Analyst software activated, select the file from the appropriate subdirectory. The image will be displayed on the screen, best maximised. Select lanes to be analysed with care. The toolbar contains a button with a folded image for single lane selection. Click on this and then click the mouse to the appropriate point in the gel, typically at the left top of the lane to be analysed, and drag the mouse to the right and bottom of the portion of interest. Select the display in profile to view the lane as a densitometric scan, with optical density on the vertical axis and cm migration on the horizontal axis. If several lanes are to be analysed, use the “Selector” button which will place a “+” on the gel after which pressing “F” on the vertical toolbar will find lanes automatically. Select sensitivity required, usually at mid-point. The bands thus selected are demarcated in red. The profile can be viewed by pressing “View Profile” on the horizontal toolbar. To include all the selected lanes, go to “View” on main toolbar and select “Show all lanes”.

With the scan profile image box on the screen the saving can proceed. From the File Menu select Export to Excel and select Active Window. Now open Excel and scroll far right across to columns until reaching the columns containing numerical values and labelled “cm” and “OD” for migration (x axis) and optical density (y axis). Select, copy and paste these 2 columns into a new Excel worksheet. This must now be saved as a text file (*.txt), onto a stiffy disk. On closing down the Multi-Analyst file, the process will request saving the changes (analyses and manipulations). Do not save these changes as they will alter the original gel record.

Activate Graphpad Prism, go to Files and select Import. Select the *.txt file on the stiffy drive or directory and proceed to import. Prepare a profile tracing by making a graph from the data sheet, best displaying the image as a line without the approximately 250 data points. The baseline correction can be done according to a personally selected value by using the Analyze and then Transform selections, or by going to Remove Baseline. The data may also be pruned to the appropriate rows by selecting Analzye and Prune.

The lane is analysed with retardation standardised to the given B distance for the particular run from which it is derived. Retardation is standardised according to the B band, Rs(B) = 1.0. Peak
particle size can be described as Rs. The profile should be analysed for AUC with the baseline subtracted.

ANALYSIS ON HOEFER SCANNER

The first densitometric scanner for GGE scanned about 1000 points as x, y coordinates at absorption of 595nm as it was originally designed for Coomassie stained SDS-PAGE. It was found suitable for Sudan Black and thus was used for capturing data. DJB developed the method below for capturing the data and processing it in Graphpad Prism.

Locate the scan desired on the stiffy disks, bearing in mind that each scan is a single lane. Open the file using Notepad. Choose Edit and select all data. Select Edit again and do Copy command.

Open Prism and prepare a datasheet which will receive the y coordinates. Paste the data to the Y column. Create a serially numbered column in the X-axis starting at point 1 and using increments of 1 (column dialogue box is found under Change). This procedure will create a graph.

Manipulate the data via Analyse and Transform command to Prune Rows. Exclude the first 100 points according to judgement about the start of the separation gel. Optionally, prune the rows again, by averaging 4 consecutive points. Now copy the y-data to a new data sheet in which X starts at zero in a series.

To convert the data to retardation factors, create a data sheet that will span from 0 to 1.1. Compare the marker and other lanes in the photocopy of the gel for A and B markers and ascertain the Rf values from these lanes. The LDL₃ is taken as Rf=1.0. Using the transform function on all the x points, the function is X/K where K is the value identified to mark Rf=1.0.

FUTURE DEVELOPMENTS

It is my belief that the LpB system is useful for studying LP phenotypes in the common hyperlipidaemias and also in normal to moderately disturbed metabolism. It is a simple way of confirming dense species of LDL and for looking for unusually large species of HDL. It should be possible to reproduce it on larger gel systems but electrophoresis time may become impractically long and may generate a lot of heat. The system should lend itself to transfer to membranes and immunodecoration to demonstrate qualitative differences in lipoproteins. In larger gels it may be possible to elute adequate amounts of material for sensitive methods to determine lipid (e.g. fluorimetric) and protein (e.g. immunoassays) contents. A combination of the two gels could be attempted as published from WV Brown’s unit [13].
The separation of HDL into pre- and forms can be done in 2 dimensional electrophoresis in which the first dimension is in 0.1% agarose gels and the second dimension is in 2-30% non-denaturing acrylamide gels [16].

REFERENCES:


DETECTION OF FH AFRIKANER-2 AND FAMILIAL DEFECTIVE APO B-100

ARMS ASSAY (Amplification Refractory Mutation System)

GENERAL

This assay determines whether the DNA contains the exon 9 mutation V408M (Afrikaner FH2) in the LDL receptor, and/or whether there is binding defective B100 from the mutation R3500W or R3500Q, and possibly R3531C. The Afrikaner 2 mutation constitutes about 20% of FH in Afrikaners and confers a biochemically worse disease, while familial binding defective B100 is responsible for about 1% of the xanthomatous hypercholesterolaemia phenotype at the UCT Lipid Clinic.

The ARMS PCR method is used to detect the FH Afrikaner-2 mutation while the same tube also contains oligonucleotides that amplify the apo B gene which serves as an internal control for the PCR. Additionally, a normal allele can be amplified as indicated below for 9.6 (G87) instead of the mutant primer 9.7 (G88).
The five oligos used in this assay are:

AB-1 (G84) 24mer: 5’-GGAGCAGTTGACCACAAGCTTAGC-3’
AB-2 (G85) 24mer: 5’-CAGGGTGGCTTTGCTTGTATGTTC-3’
9.5 (G86) 25mer: 5’-GCTCACCTGCAGATCATTCTCTGGG-3’
9.7 (G88) 25mer: 5’-AGCCTCATCCCCAACCTGAGGACCA-3’
9.6 (G87) 25mer: 5’-AGCCTCATCCCCAACCTGAGGACCG-3’

PROCEDURE:

Premix (50uls) x1 assay
dd water 38,0 uls
10x buffer (with MgCl2) 5,0 uls (1.5mmol/L)
dNTPs (2mM) 5,0 uls (200uM)
AB-1 (50 pmol/ul) 0,1 uls (5 pmol)
AB-2 (50 pmol/ul) 0,1 uls (5 pmol)
9.5 (50 pmol/ul) 0,2 uls (10 pmol) distal primer
9.7 (25 pmol/ul) 0,4 uls (10 pmol) mutant primer
Taq polymerase 0,2 uls(1 unit)
DNA (denatured) 1,0 uls

Run positive controls. Overlay with 2 drops of mineral oil. PCR cycles: 30 cycles 95 C for 35 secs, 60 C for 1 min, 72 C for 1 min. 1 cycle: 72 C for 10 mins. (Programme 14)

FH Afrikaner 2

Run 20 uls of product (plus 3 uls sucrose loading buffer) on a 12% acrylamide gel.

Only DNA with a mutant allele is amplified using the deliberately destabilised primer 9.7. Homozygosity or heterozygosity for this mutation can be confirmed by PCR with the common distal primer (9.5) and the normal allele specific primer (9.6). Product is formed in the heterozygote and not in the homozygote.
The apo B primers amplify a 345 bp product which acts as an internal control for the PCR in the absence of product for the normal allele of exon 9 of the LDL receptor.

**Familial Binding Defective B**

10 uls of PCR product (plus 2 ul sequencing loading buffer) is analysed on a long denaturing 10% (1% crosslinked) acrylamide gel.

- 9 g urea dissolved in 41.4 mls water
- 15 ml stock acrylamide
- 3.6 mls 10x TBE (0.6x)
- 0.4 mls 10% amps
- 40 uls TEMED
- 40% stock acrylamide
- 80g acrylamide+0.8g BIS in 200ml water

The gel is run at 350V between multiple loadings of PCR product and 200V overnight until the xylene cyanol band reaches the bottom of the gel. Stain with ethidium bromide and document the findings by a polaroid photograph, colour slide or Geldoc instrument.

Heteroduplex formation between normal and mutant alleles allows detection of mutations. The size of the heteroduplex can discriminate between the R3500W and R3500Q mutations.

Heteroduplex formation of PCR

Product in assay for familial binding defective apoB in 3 successive loadings of the gel. Double bands indicate the heteroduplex at position 4 in the first row, positions 4 and 5 in
the middle row and positions 1 and 5
the top row.

REFERENCES


DETECTION FH1, FH3, AND LITHUANIAN MUTATIONS IN EXON 4

GENERAL

This assay is designed to detect three relatively common mutations in South Africans with the familial hypercholesterolaemia (FH) phenotype. All three mutations are in exon 4, which is part of the ligand binding domain of the LDL receptor.

The prevalence of FH is about 1 in 75 for white Afrikaners and the 3 Afrikaner defects account for 75 to 100% of the phenotype, depending on the Cape Town or Johannesburg studies. The ratio of
the mutations is 3:2:1 for Afrikaner FH1, 2 and 3. The prevalence of FH in the Jewish communities is about 1 in 100, and is almost entirely due to the Lithuanian mutation. The Afrikaner and Lithuanian mutations have been demonstrated in the mixed ancestry (coloured) community in the Cape but together account for less than 25% of all FH phenotypes. The Afrikaner variants of FH seem to occur in the same proportion as in the whites.

Exon 4 is amplified and then digested with restriction enzyme DdeI for Afrikaner FH1 or MboII for Afrikaner FH2. The Lithuanian mutation is detected by heteroduplex formation of the exon 4 fragment in a glycerol SSCP gel. The oligomers for fragment expansion are: D1 (G80) 25 mer: 5' CCCCAGCTGTGGGCCTGCACAACG 3’ and D2 (G81) 20 mer: 5’CGCCACATACGCAGTTTCC 3’.

**PROCEDURE**

- **Premix** (50 uls) x1 assay
- dd Water 38.0 ul
- 10 x Buffer 5.0 ul (1.5 mmol/L)
- dNTPs (2mM) 2.5 ul (100 uM)
- D1 (20 pmol/ul) 0.25 ul (5 pmol)
- D2 (20 pmol/ul) 0.25 ul (5 pmol)
- Taq polymerase (5 units/ul) 0.2 ul (1 unit)
- DNA (rapid prep) 5.0 ul (genomic 1 ul + 4 ul H2O)

Run positive controls. Overlay with 2 drops of mineral oil. PCR cycles: 35 cycles: 95 C for 35 secs, 60C for 3 mins. 1 cycle: 95 C for 35 secs, 60 C for 6 mins. (Programme 4).

**FH1.** Cut 20ul of product with 1ul dil Dde1 (diluted 1+1, 5 ul H2O) at 37 C overnight. Spin. Add 3ul sucrose loading buffer. Load on 12% acrylamide gel. Stain with ethidium bromide.

**FH3.** Cut 10ul of product with 3 units MboII (0.5 ul stock + 3.5 ul H2O) @ 37 C overnight. Spin. Add 2ul sucrose loading buffer and load on 6% acrylamide gel. Stain with ethidium bromide.
**Lithuanian** Load 10μl of product + 2μl loading buffer on 6% acrylamide gel. Stain with ethidium bromide.

**Interpretation**

D1/D2 amplifies a fragment 220 bp containing a Dde1 cutting site. The FH Afrikaner 1 mutation creates another Dde1 cutting site in the 87 bp fragment. Dde1 thus cuts the normal PCR product in two fragments, 133 bp and 87 bp. The mutant is cut further making fragments 64 bp and 23 bp in size. The 23 bp fragment usually runs off the gel.

<table>
<thead>
<tr>
<th>Normal</th>
<th>133 bp</th>
<th>87 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>133 bp</td>
<td>64 bp</td>
</tr>
<tr>
<td></td>
<td>23 bp</td>
<td></td>
</tr>
<tr>
<td>Uncut hom het normal</td>
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</tbody>
</table>

Also present in the normal amplified fragment is an Mbo11 cutting site which is abolished by the FH Afrikaner 3 mutation.

<table>
<thead>
<tr>
<th>Normal</th>
<th>180</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Uncut normal het ho</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

21
The Lithuanian mutation forms a heteroduplex

**Figures below:** Left panel shows PCR result with DdeI for mutation D206E (AfrFH1) with fragments for heterozygote, homozygote and normal subjects (24Oct01). Middle panel demonstrates cut fragments with MboII: lane 1 uncut, lane 2 & 6 heterozygotes, and normal in lanes 3 to 5. The panel on the right shows the full pattern on heteroduplex for the Lithuanian mutation.

**REFERENCES**


High-resolution melting (HRM) analysis the detection of Familial defective apolipoprotein B-100 mutations
The patient’s DNA is extracted by proteinase kappa and ethanol separation from whole blood as described by Parzer. The sample was diluted with Tris-EDTA buffer to lower salt concentration as high salt concentration interferes with High Resolution Melting.

PCR amplification:

Two sets of primers are used to amplify exons in the apo B gene:

For exon 26:

<table>
<thead>
<tr>
<th>Primer Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’-AGGAGCAGTTGACCACAAGC-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’TTTGCCATGGAGTTCCA-3’</td>
</tr>
</tbody>
</table>

For exon 29:

<table>
<thead>
<tr>
<th>Primer Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’GAAGCTTCTCAAGAGTTACAG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GTCCTTAAGAGCAACTAACAG-3’</td>
</tr>
</tbody>
</table>

Each PCR mixture contains 1X the evergreen dye (Biotium), 2 mmol/L MgCl₂, 200µmol/L of deoxyribonucleotide phosphate, 0.125 mol/L each primer, 10 ng of DNA, 250g/ml bovine serum albumin (BSA) (Promega), 1U perpetual Taq DNA polymerase (Vivantis), 1x PCR buffer A (Vivantis), and H₂O to 10 and spun down in a Roche LightCycler capillary. Reaction for exon 26 is overlaid with ~2 µL of mineral oil to improve accuracy of melting curves. Amplification is carried out by initially denaturing at 95°C for 30s, followed by 50 cycles of 95°C for 3s, 60°C for 10s, and 72°C for 15s. A final extension step of 2min is performed at 72°C and the products are allowed to cool to room temperature.

Melting analysis and DNA sequencing:

DNA melting is carried between 75°C and 95°C and between 70°C and 90°C for exon 29 using HRM-1TM high resolution melter (Idaho Technology Inc). The analysis of the melting curves is performed using the HRM-1TM analysis provided by the manufacturer. PCR products exhibiting an aberrant melting curve are sequenced using BigDye terminator V3.1 chemistry (Applied Biosystems) in order to identify and sequence the change.

References: