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# Polyacrylamide Gradient Gel Electrophoresis for the Diagnosis of Dysbetalipoproteinaemia ( Type III Hyperlipidaemia )

A thesis submitted by Dirk Jacobus Blom in fulfilment of the requirements of Part III  
of the degree Master of Medicine in Medicine

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## Abbreviations

Apolipoprotein	Apo-
Area under Curve	AUC
Beta-migrating VLDL	$\beta$ -VLDL
Chinese Hamster Ovary cells	CHO cells
Cholesterol in LDL as measured with dextran sulphate precipitation method	LDL-C <sub>DS</sub>
Free Fatty Acid	FFA
Gradient Gel Electrophoresis	GGE
Heparan Sulfate Proteoglycans	HSPG
Hepatic Lipase	HL
High Density Lipoprotein	HDL
Intermediate Density Lipoprotein	IDL
Lipoprotein (a)	Lp (a)
Lipoprotein Lipase	LPL
Low Density Lipoprotein	LDL
Low Density Lipoprotein receptor-related protein	LRP
Magnesium Chloride	MgCl <sub>2</sub>
Plasma Triglycerides	TG
Ratio of Cholesterol in VLDL to Plasma Triglycerides	$C_{VLDL}/TG_{Plasma}$
Ratio of Cholesterol to Triglycerides within VLDL	$C_{VLDL}/TG_{VLDL}$
Receptor-associated Protein	RAP
Relative Molecular Mass	M <sub>r</sub>
Retardation factor	Rf
Total Cholesterol	TC
Triglyceride in LDL as measured with dextran sulphate precipitation method	LDL-TG <sub>DS</sub>
Very Low Density Lipoprotein	VLDL

## Chapter 1

### **Introduction**

Dysbetalipoproteinaemia is an uncommon (incidence approximately one to five per five thousand population) (1) genetic disorder of lipoprotein metabolism. This disorder is synonymous with Type III hyperlipidaemia, familial dysbetalipoproteinaemia, broad-beta disease or remnant removal disease. The disorder is best defined as an abnormal accumulation of remnants of triglyceride-rich lipoproteins. It is characterised by a mixed hyperlipidaemia, in which both cholesterol and triglycerides are elevated, often in a 2:1 molar ratio. This ratio however is very variable and is neither sufficiently sensitive or specific to be of diagnostic utility. In almost all cases ApoE is dysfunctional but a similar lipoprotein phenotype may be seen with hepatic lipase deficiency.

The investigation of dysbetalipoproteinaemia has provided us with unique insights into the metabolism of triglyceride-rich-lipoproteins and their remnants. Research into dysbetalipoproteinaemia led to the discovery and characterisation of Apolipoprotein E (ApoE) and the unravelling of its role in the transport, lipolysis and hepatic uptake of chylomicron-remnants and Very Low Density Lipoprotein(VLDL)-remnants. Clinically the occurrence of premature atherosclerosis in about a third to half of patients with this disease is of great relevance, especially as this dyslipoproteinaemia is most responsive to dietary and drug manipulation.(1) Early and accurate diagnosis is therefore of the utmost importance, especially as the hyperlipidaemic phenotype usually only manifests around the age of thirty in males and is followed by the development of significant atherosclerotic complications around ten to fifteen years later. This contrasts with patients who have familial hypercholesterolaemia and who are hyperlipidaemic from birth. Males with familial hypercholesterolaemia on average have their first

myocardial infarction at age forty-three(2).

Diagnosis of dysbetalipoproteinaemia has always been difficult as the characteristic palmar xanthomata which allow a confident clinical diagnosis only occur in about 20% of patients.(3) Electrophoretic methods (generally the presence of a broad  $\beta$ -band on agarose gel electrophoresis has been taken as indicative of the disorder )have not been sufficiently sensitive or specific for diagnostic purposes or have required ultracentrifugation of specimens and subsequent electrophoresis of the fractions obtained. Electrophoresis of ultracentrifugally obtained fractions is regarded as indicative of dysbetalipoproteinaemia if it demonstrates lipoproteins with density less than 1.0006 g/ml which migrate with reduced mobility in agarose or paper electrophoretic systems. These lipoproteins migrate similarly to Low Density Lipoproteins ( LDL) and are termed  $\beta$ -VLDL. (4)A subjective interpretation of electrophoretograms is however still required.

Accurate phenotypic diagnosis of this disorder has therefore generally relied on the quantitative chemical analysis of ultracentrifugally isolated VLDL. Analysis of VLDL chemical composition is not widely available due to the expense of acquiring ultracentrifuges and the highly-skilled staff necessary to perform the analyses. Genotypic diagnosis is somewhat more widely available, especially testing for the E2/E2 genotype which is the commonest underlying genotype in dysbetalipoproteinaemia. Testing for the rarer autosomal dominant mutations of ApoE is restricted to a small number of centres only.

In this study I will review dysbetalipoproteinaemia briefly and then describe and evaluate GGE as a new diagnostic technique for this disorder.

## Chapter 2

### **The Dysbetalipoproteinaemic Phenotype**

**Historically**, the first patients with dysbetalipoproteinaemia were described by Gofman (5) in 1952. He termed the condition " xanthoma tuberosum " - describing the xanthomatous lesions of the skin found overlying the extensor tendons as well as the palmar crease xanthomas seen in this condition. He also demonstrated by analytical ultracentrifugation that these patients had increased lipoproteins in the flotation range of small VLDL and IDL (  $S_f$  12-20 ). In 1967 Fredrickson published his classification of hyperlipidaemias based on paper electrophoretic patterns(4) and it became clear that " xanthoma tuberosum " and the condition termed Type III hyperlipidaemia by Fredrickson were in fact one and the same.

Several case series have been published describing the **clinical phenotype** (3;6;7). Data obtained from a retrospective review of all cases of dysbetalipoproteinaemia diagnosed at the Lipid Clinic, Groote Schuur Hospital/ University of Cape Town are provided for comparison. Patients were included in this review if they fulfilled one of the following diagnostic criteria: A ratio of cholesterol in VLDL/triglycerides in VLDL larger or equal to 0.42 ( by mass ), or a ratio of cholesterol in VLDL/triglycerides in plasma larger or equal to 0.30( by mass) or a mixed hyperlipidaemia with a genotype that is known to be associated with dysbetalipoproteinaemia ( in our clinic the relevant genotypes would be E2/E2 or E145 Arginine to Cysteine mutation (  $E_{R145C}$  )).

Patients may present to the lipid clinic in any one of several ways. Unfortunately most are seen when they present with complications of vascular disease and are subsequently found to be hyperlipidaemic. Some patients come to attention because of eruptive or tubo-eruptive xanthoma ( often via the Dermatology Department). In some hyperlipidaemia is incidentally discovered during routine screening tests or when a specimen taken for some other reason is noted to be lipaemic. A few

patients present with pancreatitis initially and are found to be hypertriglyceridaemic. Occasional patients have been found during investigation of families known to carry dominant ApoE mutations. The GSH/UCT experience of 105 cases is depicted in Table 2.1.

**Table 2.1**

Indications for referral of patients to Lipid Clinic

Reason for referral	Number seen ( Percent )
Secondary prevention( previous vascular event )	45 ( 43% )
Cutaneous Xanthoma	12 ( 11.5% )
Pancreatitis	4 ( 3.8 % )
Family screening	3 (2.8%)
Incidental Hyperlipidaemia	39 (37% )
No data available	2 ( 1.9 % )
N=105	

In 1975 Fredrickson (7) collected a case series of dysbetalipoproteinaemic patients in which 13/37 ( 35% ) patients were seen for xanthoma while 10/37 were referred for hyperlipidaemia alone. The remaining patients had both xanthoma and hyperlipidaemia (7/37) or vascular disease alone ( 3/37), with an initially unsuspected lipid abnormality. Four patients had either xanthoma or hyperlipidaemia in association with vascular disease.

There is a marked difference in the age of presentation between male and female patients(1;3;7;8).The mean age of presentation for men in Fredrickson's series (7) was 39.8 years while that for women was 49.1 years. The ages reported in a composite of four case series ( Men 39 years Women 49 years )are very similar(3)

In our cohort the overall mean age at presentation was 48.8 years ( SD 11.1 with a range from six to eighty). Males presented at a mean age of 46.33 ( SD 10.57 with a range from six to 65 ). Females presented at a mean age of 51.6 ( SD 11.4 with a range of 22 to 80). The ages at presentation differ significantly (  $p=0.015$  by Student's t-test ) between the sexes. Overall later presentation to our Lipid Clinic compared to other series possibly relates to difficulties accessing health care in South Africa, with patients having less routine biochemical testing or screening. In addition there was often a fairly long delay ( occasionally several years ) from the time the hyperlipidaemia was first discovered to the time that referral to the Lipid Clinic was instituted.

The pathognomonic **clinical sign** of dysbetalipoproteinaemia is the finding of " xanthoma striata palmaris " or palmar crease xanthoma(4;5). This yellowish infiltration of the palmar creases can be quite subtle and is often not initially noted, if not looked for specifically. The palmar crease xanthoma may also be noted by patients but ignored or dismissed by their medical attendants. The reported incidence of this clinical sign varies quite dramatically, depending on the criteria used to select patients for further specialized diagnostic studies. The reported incidence in the literature ranges from 23 -72 % (3) In our clinic 20% of patients had palmar crease xanthoma at the time of initial presentation. The xanthoma often regress completely with effective treatment of the hyperlipidaemia.

We saw cutaneous xanthoma(tubo-eruptive and eruptive ) in 18% of our patients while 12% had tendon (almost exclusively Achilles tendon ) xanthoma. The reported incidence of cutaneous xanthoma is as high as 70% in some series with tendon xanthoma being found on average in 20% of patients across various case series. Cutaneous xanthoma are more common than tendon xanthoma in all reported case series. The large variability in the reported incidence of the clinical manifestations of this disorder again relate to the way in which patients are referred to Lipid Clinics ( only if they have clinical abnormalities or after routine biochemical screening ) and

which patients subsequently have more detailed investigations to prove or disprove the diagnosis of dysbetalipoproteinaemia. If predominantly patients with clinical signs are investigated further, this obviously will skew the frequency with which clinical manifestations are reported. Most of our patients did not have any clinical manifestations of the disorder and the diagnosis was only made on further laboratory evaluation of their mixed hyperlipidaemia.

The **biochemical phenotype** is usually that of a severe mixed hyperlipidaemia. Mean cholesterol on presentation was 12.02mmol/l ( Range 4.8-34.30 ) in our study and 11.76 mmol/l in Fredrickson's study(7). Mean triglycerides were 8.37 mmol/l ( Range 1.6-63.2) and 7.89 mmol/l respectively. The ratio of triglycerides to cholesterol at presentation in our series ranged in molar terms from 0.1311 to 2.531 with a mean ratio of 0.62. This approximates the unitary ratio of triglycerides to cholesterol described when expressing both the above in mass terms.(4;7;8)

Regarding genotyping data is available for 100 of 105 patients in the review of the UCT/GSH experience.

## **Table 2.2**

### **Genotypes of Dysbetalipoproteinaemic Patients**

Genotype	Number
E2/E2	49
E146 mutation (Lys→Glu)	1
E145 mutation ( Arg →Cys)	22 ( 3 homozygotes )
Neither E2/E2 nor E145 mutation	26
Neither E2/E2 and E145 not tested	2

Dysbetalipoproteinaemia may be the consequence of identical mutations in both apoE alleles, such mutations ( eg apoE2) are referred to here as recessive. ApoE mutations that cause dysbetalipoproteinaemia in the presence of only one abnormal

apoE allele are defined as dominant.

Notable is the high frequency of the dominant E145 ( Arg→Cys ) mutation in our patients as well as the number of patients who do not have ApoE2/E2 or E145 but express the biochemical phenotype of dysbetalipoproteinaemia with as yet unexplored mutations in ApoE. Clearly further exploration of these patients with sequencing of their ε alleles is indicated to elucidate the underlying genetic problem.

The **clinical consequences** of a mixed hyperlipidaemia due to remnant accumulation are serious: 46% of our patients having clinical features of IHD (based on a history of angina, previous myocardial infarction, Coronary Artery Bypass Graft or Percutaneous Transluminal Coronary Angioplasty ) at any time. From presentation to March 2001 an additional 10% of patients developed features of IHD. Eighteen percent of patients had a history of claudication or surgery for peripheral vascular disease. These figures correlate reasonably well with the incidence of 21% (1) and 11-29 % (3) for peripheral vascular disease reported elsewhere. Peripheral vascular disease is much more commonly seen in patients with dysbetalipoproteinaemia than in other patients with disorders such as familial hypercholesterolaemia, where coronary artery disease occurs with comparable frequency but peripheral vascular disease is unusual. The reasons for the predilection of patients with dysbetalipoproteinaemia towards atherosclerosis in the peripheral vessels are not well understood.

Diabetes, hypothyroidism and gout are often found in association with dysbetalipoproteinaemia. The first two conditions are often also factors that precipitate or aggravate hyperlipidaemia. In our series 5/105 patients had documented biochemical hypothyroidism at presentation and showed marked improvement in lipid values on supplementation with thyroxine. Diabetes was extremely prevalent with 34% of our patients being diagnosed as diabetics. The

incidence of diabetes was not influenced by the genotype, if known. This figure is higher than the 4-11 % prevalence quoted elsewhere(1;3;7). In these studies a high incidence of glucose intolerance (up to 50% ) is also reported and part of the difference between our local figures and the previously published series may well be explicable by the lowered glucose criteria that currently apply for the diagnosis of diabetes.

A marked response to dietary and drug therapy is characteristic for patients with dysbetalipoproteinaemia. In our patients the maximal response (= Presenting lipids - best ever lipids on follow-up )was a mean reduction in cholesterol of 7.08 mmol/l. The maximal response achieved in a patient was a reduction in cholesterol of 31.5 mmol/l. Triglycerides were reduced by a mean of 5.9 mmol/l with the maximum reduction in a single patient being 55.6 mmol/l. Lipid values are very labile in these patients and it is quite common to see the cholesterol increase from levels around 4mmol/l to around 15 mmol/l with dietary indiscretion or non-compliance with medication.

## Chapter 3

### Dysbetalipoproteinaemia: Definition and Pathophysiology

#### 3.1 Definition

Dysbetalipoproteinaemia is defined by the presence of abnormal lipoproteins in the density range less than 1.006 g/ml when lipoproteins are separated by ultracentrifugation. Normally only chylomicrons and VLDL are found in this density range. Chylomicrons remain at the origin during paper or agarose gel electrophoresis while VLDL migrate to the pre- $\beta$  position(3;4). The abnormal population of lipoproteins found in dysbetalipoproteinaemia migrates in the  $\beta$ -position on paper or agarose gel electrophoresis. This is the position in which Low Density Lipoproteins ( LDL ) would be found normally and the abnormal lipoproteins were subsequently named " $\beta$ -VLDL " (9). An alternative designation is "floating beta lipoproteins"(4;7)to indicate the presence of lipoproteins with beta mobility that have " floated " to the low-density supernatant during ultracentrifugation. These lipoproteins were found to have an abnormal chemical composition when compared with normal pre-beta VLDL (10-12). They are enriched in cholesterol ( specifically cholesterol esters ) and relatively depleted of triglycerides. Two ratios describing the chemical composition of ultracentrifugally isolated VLDL have become the standard by which the presence of beta-VLDL is defined and the diagnosis of dysbetalipoproteinaemia subsequently made(13-16). These ratios and their derivation will be further discussed in the section concerning diagnosis of this disorder In addition, although total protein content is similar in beta-VLDL and pre-beta VLDL, the apolipoprotein composition is strikingly different. Beta VLDL contains less of the apolipoprotein C group while apolipoprotein E content is increased both in relative and absolute terms.(12;17)

The lipoproteins making up the abnormal beta VLDL originate both from hepatic and intestinal sources as demonstrated by the presence of both forms of ApoB amongst this population of lipoproteins.(12;18;19) In contrast to all other apolipoproteins ApoB

remains with the particle for its entire lifespan. ApoB48 is synthesized exclusively in the intestine in humans and is a marker for chylomicrons while ApoB100 is synthesized by the liver and secreted with VLDL. ApoB48 is the N-terminal half of ApoB100 and results from editing of mRNA in the intestinal cell pre transcription. This editing introduces a novel stop codon and the result is early termination of transcription and protein synthesis.(20)

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### 3.2 Pathophysiology of Dysbetalipoproteinaemia-Metabolism Of Chylomicrons and VLDL particles

To better understand the pathophysiology of dysbetalipoproteinaemia it is necessary to briefly review the metabolism of chylomicrons and VLDL with particular emphasis on pathways that are disrupted or altered in patients with dysbetalipoproteinaemia. Comprehensive reviews of the subject can be found in the following sources(1-3;21).

Triglycerides in the diet are hydrolysed by luminal lipases in the intestine and the resulting glycerol, free fatty acids ( FFA ), mono- and diglycerides are taken up by the enterocytes. Subsequent re-esterification of these constituents in the enterocyte yields triglycerides which together with other lipids and protein are assembled in the endoplasmic reticulum to form the core of chylomicrons. The apolipoprotein constituents of chylomicrons, before they are secreted, are ApoB48 as well as ApoAI and apoAIV. After transport to the Golgi apparatus the nascent chylomicrons are secreted by exocytosis Chylomicrons are initially secreted into lymphatic spaces and subsequently enter the plasma compartment via the thoracic duct. Here the chylomicrons acquire additional apolipoproteins which transfer to the chylomicrons from HDL. Amongst these apolipoproteins are the ApoC proteins as well as ApoE .

Circulating chylomicrons are then further metabolised by the action of lipoprotein lipase. This enzyme is found bound to glycosaminoglycans ( heparan sulphate ) on the luminal side of capillary endothelial cells, particularly in the vascular beds of muscle and adipose tissue. The action of lipoprotein lipase results in the hydrolysis of triglycerides and the generation of FFA and glycerol. Chylomicrons therefore lose some of their triglycerides and become relatively more enriched in cholesterol. The chylomicron remnants that are formed by the action of lipoprotein lipase, and to a lesser degree hepatic lipase, undergo conformational changes and subsequently altered affinities for apolipoproteins result in the loss of ApoA and ApoC to HDL. The further metabolic fate of chylomicron remnants will be discussed together with that of

VLDL remnants.

VLDL are synthesized in hepatocytes and provide a pathway for export of triglycerides and cholesterol from hepatocytes. The triglycerides in VLDL are derived from uptake of FFA circulating in plasma and re-esterification to triglycerides, endogenous synthesis in the liver of triglycerides or they can be derived from triglycerides delivered by chylomicron remnants(2). VLDL contain ApoB100 as their only ApoB protein, as well as ApoE and ApoC proteins in small amounts. More of the latter proteins are subsequently transferred to the VLDL from HDL in plasma. VLDL are metabolised similarly to chylomicrons by lipoprotein lipase, although the rate of hydrolysis of VLDL triglycerides is slower than that of chylomicron triglycerides. For chylomicrons the average residence time in plasma is about five to ten minutes, while it is 15 to 60 minutes for VLDL triglycerides. This is thought to relate to the smaller size of VLDL particles and the resulting inability to bind as many lipoprotein lipase molecules as chylomicrons. The action of lipoprotein lipase results in the formation of VLDL -remnants. These remnants are relatively enriched in cholesterol compared to the originally secreted VLDL. The size of the remnants depends on the initial size of the VLDL particle when secreted, smaller particles yield smaller remnants. Some of these smaller remnants will be isolated as Intermediate Density Lipoproteins ( IDL ) by ultracentrifugation. Small remnants can bind fewer ApoE molecules than larger remnants and are therefore cleared less efficiently than larger remnants.

Quantitatively about half of VLDL-remnants will be taken up by the liver and cleared while the other half will lose all apolipoproteins except ApoB100 to form Low Density Lipoproteins ( LDL ) with a density of 1.019-1.063 g/ml. The formation of LDL entails further lipolysis of VLDL remnants, mainly by hepatic lipase. LDL has a long residence in plasma ( about three days ) and is taken up into tissues ( with the liver contributing to the bulk of uptake ) via the LDL receptor or alternatively oxidized LDL can be taken up by the macrophage scavenger receptor.

### 3.3 Metabolism of VLDL and Chylomicron Remnants

For comprehensive and up to date reviews of the subject see references(21;22). Remnant lipoproteins are cleared from the plasma by the liver. ApoE is the apolipoprotein that is primarily responsible for mediating this process and is present in both VLDL- and chylomicron-remnants. It is necessary to understand the physiology of remnant clearance, to understand how ApoE mutations impact on this process and the dysbetalipoproteinaemic phenotype then develops.

Chylomicron remnants enter the space of Disse through the fenestrated sinusoidal epithelium. The epithelium acts as a biological filter, as it only allows the smaller chylomicron remnants to enter and excludes larger chylomicrons that have not yet had most of their triglyceride load lipolysed.(23) VLDL remnants are small enough to enter the space of Disse freely. The space of Disse is rich in heparan sulphate proteoglycans (HSPG ) which are bound to cell surfaces. (24) In addition there is a high concentration of ApoE (25)and hepatic lipase(26), these are both secreted by hepatocytes.(1;3)Furthermore remnant lipoproteins carry LPL into the space of Disse. The role of LPL in remnant metabolism has not been fully established, recent work suggests that under physiological conditions it is not of major relevance. (22)

**Hepatic lipase ( HL )** plays an important role in the binding and uptake of remnants in addition to its lipolytic role.(27) Important insights into its role are derived from the study of rare patients with hepatic lipase deficiency as well as animals or cells transfected with either normal or mutant hepatic lipase. Hepatic lipase normally hydrolyses both phospholipids and triglycerides in IDL, remnant lipoproteins as well as in large buoyant LDL and HDL. The resultant lipoproteins are smaller and denser than before lipolysis. Patients with hepatic lipase deficiency( of which there are about twelve described in the world literature(27) ) present with elevated levels of total cholesterol and triglyceride. Analysis of lipoprotein fractions shows increased triglyceride in all lipoprotein fractions while increased cholesterol is found mainly in

the VLDL, IDL and LDL fractions.(27;28) LDL is considerably larger than normal and enriched in triglycerides in these patients, who also often have demonstrable  $\beta$ -VLDL in their plasma.

Using rat hepatoma cell lines Mahley and coworkers(29) were able to demonstrate that these cells had increased binding affinity for and uptake of labelled canine chylomicron remnants and  $\beta$ -VLDL when transfected with, and expressing the human hepatic lipase gene. When the same cell line was transfected with mutant forms of hepatic lipase(29) interesting findings were made. One mutant HL bound normally to hepatic cells but lacked catalytic activity while another mutant had decreased binding to hepatic cells with preserved catalytic activity. Overexpressing catalytically inactive HL mutant led to increased uptake of remnants compared to baseline, but was reduced when compared to cells transfected with wild type hepatic lipase. Hepatic lipase mutants that had decreased binding to cells but normal catalytic activity also reduced uptake of remnants into cells when compared to cells transfected with wild type hepatic lipase. It is therefore clear that binding of HL to the hepatocyte is important for its action and that not all the enhanced uptake is mediated by the catalytic activity of the enzyme. This observation was recently confirmed to be correct in humans as well.(30) This is demonstrated by the description of three patients with hepatic lipase deficiency who have no demonstrable catalytic activity. Two of these patients produced no measurable HL protein, while one patient produced some detectable HL protein ( about 20% of normal ). This patient had triglyceride enrichment of all his lipoprotein like the other patients, but did not show the marked cholesterol enrichment in VLDL and IDL as displayed by the other two patients. The presence of a small amount of HL protein therefore seems to significantly influence remnant catabolism independently of its catalytic activity. HL seems to serve as a " bridge ", facilitating binding of ApoE enriched lipoproteins to their receptors.(31)

The receptors responsible for the uptake of remnants into the hepatocytes are the LDL receptor, LRP and HSPG(21;22;27). HSPG and LRP seem to act synergistically

in the uptake of remnants, but there is also evidence that HSPG may mediate direct uptake of remnants on its own(32). In a series of experiments (33-35) the roles of these receptors in and their relative contribution to remnant uptake have been studied.

The LDL receptor can take up remnants(36) due to its ability to bind ApoE and ApoB100. ApoE found in VLDL does not readily bind to the LDL receptor but loss of ApoC lipoproteins from VLDL, as is the case in VLDL remnants, results in ApoE 's binding domains becoming exposed due to conformational alterations within the lipoprotein. The binding affinity of VLDL remnants to the LDL receptor is much higher than that of LDL itself, partially accounting for the shorter plasma half-life of VLDL remnants when compared to LDL. The LDL receptor was the first receptor found to be capable of taking up remnants. However experiments with LDL receptor null mice (37)demonstrated that these mice have an increase in plasma total cholesterol but this is mainly due to an increase in LDL. There is no significant accumulation of remnants in these animals and they therefore must have an alternative pathway or pathways by which remnants can be metabolised. One of these alternative metabolic routes is the uptake of remnants via the LDL-receptor related protein (LRP ).

LRP has been shown to mediate the uptake of ApoE-rich lipoproteins. This receptor is multifunctional and also has HL, LPL and activated  $\alpha_2$ -macroglobulin as ligands. The role played by HSPG in the functioning of LRP is demonstrated by experiments in cultured cells(38) ( Chinese Hamster Ovary -CHO ). In a series of experiments VLDL remnants were enriched with ApoE to mimic the conditions found in the space of Disse and their binding affinity to the cultured cells determined. Enriching VLDL remnants with ApoE increased binding affinity about twelve-fold. Treating cells with heparinase ( to remove HSPG )abolished nearly all of this enhanced binding affinity. Remaining binding affinity was very similar to that found using non-enriched remnants. Blocking the LDL receptor with an antibody prevented most of the binding found with non-enriched remnants but only about 10% of the binding seen when ApoE enrichment had been performed. These results suggest that the LDL receptor

shows basal binding and that most of the enhanced binding with ApoE enrichment is mediated via HSPG. Mutated CHO cells incapable of synthesizing HSPG did not show enhanced binding when ApoE enrichment was performed, although the LRP receptor was capable of binding activated  $\alpha$ 2-macroglobulin normally. LRP binding on its own is therefore not sufficient to mediate ApoE enhanced binding to the cell surface in the absence of HSPG. Binding studies in fibroblasts homozygous and heterozygous for LRP mutations demonstrated that HSPG alone can mediate the binding and uptake of remnants(32). LRP-null fibroblasts bind ApoE enriched particles with higher affinity than when ApoE is not present and this enhanced binding is abolished when heparinase is added.

Remnants are therefore taken up into the liver via the LDL receptor, LRP and HSPG. A great deal of work has been done examining the relative contribution that each of these receptors makes to remnant clearance.(33;34;36;37;39). Most of this work has been done in transgenic and knockout animals, in which the genes for one or several of these receptors have been "knocked out" and the animal subsequently cannot synthesize the receptor protein. As LRP is required for normal embryonic development in mice, it has not been possible to directly generate LRP receptor-null mice. This problem has been overcome in two ways.

LDL is synthesized in the cytosol and then transported to the cell surface in association with a protein termed receptor-associated protein ( RAP ). One of the functions of this protein is to act as a molecular chaperone, guiding the receptor to the cell membrane.(40) Intravenous injection of RAP inhibits LRP and HSPG function, presumably because it occupies the binding sites on LRP.(41) In transgenic animals an additional RAP expression construct can be introduced, leading to overexpression of RAP. In animals overexpressing RAP only, there were small increases in LDL levels, similar to the increases seen when the RAP gene is knocked out and LDL receptor expression on hepatocytes reduced by 75%. Concomitant overexpression of RAP and knockout of LDL receptor genes however leads to a

Up to now the clearance of remnants has been considered in general, without attempting to differentiate between remnants of intestinal and hepatic origin. These remnants are obviously differentiated by the ApoB protein they carry in humans. In mice both the intestine and liver produce ApoB48, about 70% of hepatic ApoB transcripts in mice code for ApoB48. In a series of experiments (42) with mice that can only produce one ApoB species, it was shown that in " ApoB48 only mice " plasma cholesterol and ApoB48 levels increase about two to threefold if an additional LDL null receptor mutation is introduced. This reflects the ability of the ApoE contained in ApoB48 particles to bind to the LDL receptor. However when RAP was overexpressed the increase in lipid levels was very much more marked. In " ApoB100 only mice " overexpression of RAP did not significantly increase the levels of ApoB100 containing lipoproteins. These findings suggest that in mice ApoB48 is mainly taken up through the LRP/HSPG pathway but can utilise the LDL receptor as well. The LDL receptor in mice seems to be responsible for clearing the majority of ApoB100 containing particles.

Once remnants are endocytosed further catabolism occurs in the lysosomes and the remnant components can enter various further metabolic pathways -ie for cholesterol there could be excretion with bile, incorporation in cell membranes etc ...

## Chapter 4

### 4.1 ApoE and Dysbetalipoproteinaemia

**Apolipoprotein E:** It is important to briefly review information regarding ApoE ( with particular reference to its role in lipoprotein transport ) so as to be able to understand the nomenclature of the isoforms of ApoE and how mutations of this protein influence remnant metabolism. For a comprehensive review of ApoE refer to reference (43)

ApoE is protein with a relative molecular mass ( $M_r$ ) of 34000. It is made up of 299 amino acids and is particularly rich in arginine, hence its earliest designation as " arginine-rich apolipoprotein ". It is one of the apolipoproteins that can be found in chylomicrons, VLDL and HDL and their remnants. In normal plasma free of chylomicrons, the distribution by mass of ApoE is approximately equal between HDL and VLDL, with an average total plasma concentration of around 50 mg/l. The gene for ApoE lies on chromosome 19 and is closely linked to the gene for ApoC-I. (43)

ApoE is a polymorphic protein with three major isoforms described in humans. These are called Apo- E2, E3 and E4. The original nomenclature evolved from the differing mobility of the isoforms during isoelectrical gel focussing.(44) The different ApoE phenotypes result from the combination of three alleles that differ from each other at two codons. The alleles are designated  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$ . There are therefore six possible genotypes resulting from the combination of these alleles ( 3 homozygous and 3 heterozygous ). Further variability( secondary polymorphism ) is introduced by glycosylation ( sialylation ) of some ApoE molecules at residue 194 ( threonine ). Table 4.1 shows the prevalence of the various phenotypes in a European population (43). There are fairly marked racial differences in the prevalence of the various phenotypes, for instance ApoE3/E3 is more common in Asian populations than European populations and certain tribal groups in Papua New Guinea have a very high prevalence of the Apo E4/E4 phenotype.(1)

**Table 4.1**

Distribution of ApoE genotypes in an European population

E2/2	1%
E3/E3	60%
E4/4	4%
E2/3	12%
E2/4	2%
E3/4	23%

No of subjects: 1031 (43)

The main alleles differ from each other by single base pair substitutions. As the  $\epsilon 3$  allele is the commonest, it is considered the wild type. The ancestral allele is however most likely the  $\epsilon 4$  allele as this occurs in nearly all animals including primates such as the baboon.(1)

ApoE2    112   CYS   158 CYS  
ApoE3    112   CYS   158 ARG  
ApoE4    112   ARG   158 ARG

CYS=Cysteine ARG= Arginine
-------------------------------

The region of ApoE that binds to the LDL receptor lies between residues 136-150.(21) Dominant mutations that affect ApoE binding to receptors are usually found in this region. ApoE2 has altered receptor binding as the presence of CYS at residue 158 disrupts a salt bridge that normally forms between ARG 158 and ASP 154. This results in ASP 154 forming an alternative salt bridge with ARG 150 which disrupts the positive potential of the receptor binding region. This results in impaired binding, mainly to the LDL receptor in the case of ApoE2.

ApoE is mainly synthesized in the liver. About two thirds to three quarters of plasma

levels is accounted for by hepatic synthesis. The brain is second to the liver in the overall amount of ApoE synthesized. Other cell types that have been shown to have the capacity to synthesize ApoE include macrophages, smooth muscle cells and astrocytes. (43)

The commonest molecular cause of dysbetalipoproteinaemia is the homozygous presence of the E2 isoform of apoE. Other rarer causes are point mutations in ApoE that usually result in a dominant pattern of phenotypic expression. In addition a variant with a seven amino acid duplication of residues 121-127 has been described (ApoE Leiden) (45). The absence of ApoE also results in the expression of the dysbetalipoproteinaemic phenotype. (46;47) The phenotype in ApoE deficiency seems to be more severe with an earlier onset (occurring for instance in premenopausal females) and with more marked cholesterol enrichment of remnants. In addition hypertriglyceridaemia is not prominent in these patients, the mean triglyceride in four described patients being 2.5 mmol/l with a mean cholesterol of 13.7 mmol/l. Xanthomata have also been described in unusual locations, such as on the ear, in these patients. In addition to ApoE deficiency, antibodies to ApoE can occasionally be generated and result in the appearance of the dysbetalipoproteinaemic phenotype. This has been described in several case reports where the association of multiple myeloma and hyperlipidaemia characterised by palmar xanthoma has been reported. (48;49)

When there is hepatic lipase deficiency beta-VLDL is found in serum in the setting of a mixed hyperlipidaemia. This therefore also is phenotypic dysbetalipoproteinaemia although the molecular mechanism is different, as no direct abnormality of ApoE is present.

In the further discussions the focus will be on ApoE mutations and how they cause dysbetalipoproteinaemia, as they account for the vast majority of cases. The following table provides a brief overview of some described ApoE mutations and their receptor binding affinities if known. The table has been compiled from references (1;21)

**Table 4.2****ApoE mutations**

Mutation	LDL receptor	HSPG/LRP	Comment
E2 Arg 158 - Cys	< 2%	~ 50%	Homozygosity required for phenotypic expression
Arg 136 → Ser	Moderate	-----	Dominant expression ,found in Spanish population
Arg 136 → Cys	-----	-----	Variable phenotypic expression
Arg 142 → Leu	-----	-----	
Arg 142 → Cys	Low ( 20-502% )	Very low < 5%	Severe hyperlipidaemia with 100% penetrance
Arg145 → Cys	Moderate	Low	Makes up 25% of cases in local GSH experience, homozygotes described, dominant expression
Lys 146 → Gln	Moderate	-----	
Lys 146 → Glu	Low	Very low	High degree of penetrance
Lys 146 → Asn, Arg 147 → Trp	-----	-----	Onset of hyperlipidaemia at young age
Duplication residues 121-127	Low	Very low	Known as ApoE Leiden,100% penetrance young age of onset

**4.2 ApoE2/E2 and the Causation of the Dysbetalipoproteinaemic Phenotype**

The pathogenesis of dysbetalipoproteinaemia relates mainly to the E2/E2 genotype, differences exist between this common genotype and the other ApoE mutations and will be highlighted when relevant.

It is important to note that hyperlipidaemia only develops in a small proportion ( less than 10%) of people who are homozygous for the E2/E2 genotype. Paradoxically most carriers of E2/E2 are normolipidaemic or often even hypocholesterolaemic.(1) They do however have detectable beta-VLDL and this state could therefore be described as normolipidaemic dysbetalipoproteinaemia (**dysbetanormolipoproteinaemia**) with the hyperlipidaemic phenotype being termed **dysbetahyperlipoproteinaemia**.

Patients with the E2/E2 genotype become hyperlipidaemic in the presence of a second factor that 'stresses' the limited metabolic capacity for remnants and leads to their subsequent accumulation. One of the possible "second hits" could be a condition that results in the overproduction of VLDL. Increasing VLDL production leads to greater generation of vldl-remnants, which may overwhelm the limited metabolic capacity. An experiment with transgenic mice that express human E2/E2 shows that these mice have slightly reduced plasma cholesterol levels with normal triglycerides and slightly increased remnant levels. When these transgenic mice are crossbred with mice expressing human ApoB, the resulting double transgenic animals have significantly decreased plasma LDL cholesterol, mimicking the situation seen in most humans with the E2/E2 phenotype (50). If the E2/E2 mice are crossbred with mice overexpressing human ApoB the resultant double transgenic mice become markedly hyperlipidaemic with low LDL cholesterol and with marked remnant accumulation.(51)

A somewhat analogous situation in humans would be the co-inheritance of the E2/E2 genotype and the genetic predisposition to Familial Combined Hyperlipidaemia ( FCH ) resulting in the overproduction of ApoB containing lipoproteins.(51) Crossing E2/E2 mice with LDL receptor null mice also resulted in hyperlipidaemia(51) and this could partially explain how hypothyroidism acts as a precipitating factor, as it decreases LDL receptor expression. In a large study from Canada(52) 14 patients with E2/E2 genotypes and familial hypercholesterolaemia ( all with known LDL receptor mutations ) were identified and compared with a control groups of patients who also had FH but the E3/E3 genotype. The second control group contained patients with the E2/E2 genotype but without LDL receptor mutations. The patients carrying both defects shared clinical characteristics of FH and dysbetalipoproteinaemia, while their incidence of vascular disease was intermediate between the purely dysbetalipoproteinaemic and FH subjects. Co-expression of a LDL-receptor mutation and the E2/E2 genotype therefore does not have a simple additive effect, but results in complex modulation of individual gene expression. In humans, heterozygous mutations of LDL receptors on their own have

thus far not been shown to be sufficient to trigger the expression of dysbetalipoproteinaemia in predisposed subjects. A further mechanism, apart from increased VLDL production or impaired LDL receptor function, that can result in the expression of the dysbetalipoproteinaemic phenotype is impairment of lipase action, as can be seen for instance in the setting renal failure. (21)

Clinical conditions that can lead to the expression of the hyperlipidaemic phenotype therefore include diseases such as diabetes, renal failure and hypothyroidism. Obesity and alcohol excess are often relevant, as is the occurrence of menopause in female subjects with a subsequent decrease in oestrogen levels. Drugs that impair lipolysis or stimulate VLDL production may also be important. A topical illustration of the effect drugs can have, is provided by the description of a patient with HIV who developed a severe mixed hyperlipidaemia (total cholesterol 29.3 mmol/l and triglycerides 27.5 mmol/l), which was confirmed to be secondary to remnant accumulation, when he was treated with a protease inhibitor.(53) This patient was homozygous for E2/E2. As HIV protease shares some sequence homology with LRP this receptor may be inhibited in its function by protease inhibitor drugs.

The reasons for a lower than expected LDL level in dysbetalipoproteinaemic patients need exploration. Several explanations are possible. If VLDL is not efficiently converted into LDL, then LDL formation will be decreased with a consequential drop in plasma LDL levels. Alternatively decreased delivery of cholesterol to the liver by remnants could result in secondary up-regulation of LDL receptors and a subsequent drop in LDL levels. The third scenario imaginable is that VLDL-remnants (with defective ApoE and normal ApoB100) compete poorly with LDL (ApoB100 only) at the LDL receptor and the LDL is consequentially better cleared.

To resolve this question LDL receptor null mice were crossed with transgenic mice expressing various levels of human ApoE2(52). The LDL receptor null mice without human ApoE2 had elevated LDL cholesterol, as was to be expected. As the level of ApoE2 expression increased, LDL cholesterol levels decreased. This demonstrated

that at least in a mouse model the hypolipidaemic effect of ApoE2 is not reliant on the presence of the LDL receptor. In the same mice as ApoE2 increased and LDL cholesterol fell, triglycerides rose markedly. ApoE2 therefore does seem to impair lipolytic processing of VLDL by both lipoprotein lipase and hepatic lipase. This impaired lipolysis leads to decreased production of LDL and low levels of LDL cholesterol. Impairment of LPL activity by ApoE2 results from displacement of ApoCII (a vital co-factor for LPL activity) from VLDL.(52) ApoCII levels are decreased in VLDL with ApoE2 and adding extra ApoCII to these VLDL increases LPL mediated lipolysis about threefold. In addition to mediating receptor binding and uptake of remnants ApoE also activates hepatic lipase. ApoE2 is a much weaker activator of hepatic lipase than ApoE3 or ApoE4 (54). Again the end result is impaired processing of remnant particles, leading to low LDL cholesterol and hypertriglyceridaemia and hypercholesterolaemia secondary to remnant accumulation.

ApoE levels account for about 20-40% of the variation in VLDL and triglyceride levels in the normal population, independent of ApoE phenotype(55). In rat hepatic cells transfection with human ApoE3 and expression at high levels results in the overproduction of VLDL.(56) High levels of ApoE2 as found in patients with dysbetalipoproteinaemia may therefore exacerbate the dyslipidaemia by further promoting hepatic VLDL production.

It now also becomes clear why hypertriglyceridaemia is not marked in patients with ApoE deficiency. These patients have a profound defect in the binding and internalization of remnants, as all the pathways previously described ( LDL receptor, LRP, HSPG ) are reliant on ApoE as a ligand. However they do not have high levels of ApoE and subsequently lipolytic processing of remnants by LPL is not impaired and there is no VLDL overproduction. This scenario results in the formation of the very markedly cholesterol enriched remnants found in this condition ( VLDL cholesterol/ Plasma triglyceride by mass -mean 1.09 ) (47) but there is little

accumulation of triglyceride.

In summary, it has been indicated how remnants are normally catabolized and cleared from plasma and how accumulation of ApoE2 impairs lipolysis by inhibiting LPL and incompletely activating HL. In addition, high levels of ApoE increase VLDL production. The importance of a "second hit" in the pathogenesis of dysbeta**hyper**lipoproteinaemia in the E2/E2 homozygote has also been illustrated. These are conditions that result in overproduction of VLDL, impair lipolytic processing or decrease LDL receptor numbers.

There are, however, still some important points to address. In patients homozygous for E2/E2 the hyperlipidaemia can become manifest in males in late adolescence to early adulthood while it would be distinctly unusual to see hyperlipidaemia in a premenopausal female. This finding strongly points towards **oestrogen** having an important role to play in the modulation of lipid levels. Experiments with transgenic rabbits(57) expressing high levels of human ApoE2 showed that lipid levels were significantly lower in female than male rabbits. Ovariectomy of the female rabbits and oestrogen treatment of the male rabbits reversed this situation. The activities of LPL and HL are both significantly increased by oestrogen, as is expression of lipid receptors. In a human turnover study (58) a postmenopausal female with dysbeta**lipoproteinaemia** (palmar crease xanthoma, mixed hyperlipidaemia and  $\beta$ -VLDL present) was injected with radio-iodinated VLDL obtained from a patient with "endogenous" hyperlipidaemia or a Type IV pattern. Clearance of radio-activity from VLDL was very slow, with slow appearance of radio-activity in IDL and slow clearance of activity from there as well. In a period of 48 hours very little activity was seen in LDL. Treatment with oestrogen supplementation ( ethinyloestradiol 1 $\mu$ g/kg/day) resulted in complete correction of the hyperlipidaemia. A repeat turnover study showed that VLDL production had increased by 60%, but radio-activity cleared very much faster from the VLDL. Radio-activity appeared earlier in IDL and cleared faster from this compartment. Significant radio-activity was now also seen to appear in LDL. Catabolism of VLDL was therefore accelerated markedly and this was

sufficient to compensate for the increased VLDL production secondary to oestrogens, resulting in overall lower lipid values.

The second point to address is why the  $\epsilon 2$  allele needs to be present in the homozygous form for the genetic background to permit the development of the phenotype while the other known ApoE mutations are dominant and very often have a high penetrance as well. Adding to the confusion is that the LDL receptor binding of the dominant mutations is higher than that of ApoE2 ( see Table 4.2 ), yet they exert a more profound effect on remnant metabolism.

One of the explanations for this finding is the differential binding capacities of the various forms of ApoE to the HSPG/LRP pathway. This binding is relatively normal for E2 but severely defective in the other ApoE mutations. These mutations can therefore bind with reasonable efficiency to the LDL receptor but cannot use the alternative HSPG/LRP pathway(21). LDL binding on its own is not sufficient to allow for clearance of remnants and hyperlipidaemia can develop even in the absence of precipitating factors. ( Blocking the LDL receptor by antibody only reduced ApoE-enriched VLDL remnant binding by 10%, showing the importance of the HSPG/LRP pathway(38) )In the patient with ApoE2 the HSPG/LRP pathway can “ cope “ with the normal load of remnants till the system is “ stressed “ by environmental or hormonal factors and overt hyperlipidaemia develops.

Another factor responsible for the dominant expression of some ApoE mutations is that certain forms of ApoE preferentially associate with certain lipoproteins. ApoE2 has a preference for HDL whereas other mutated forms of ApoE have a preference for VLDL. This is illustrated by the fact that ApoE Leiden is found in VLDL in a mutant to normal ratio of up to 7:1.(45) This results in more ApoCII being displaced from VLDL and lipolysis is impaired even further.

It is therefore clear that remnant metabolism is complex and that multiple pathways interact with each other. The phenotype of dysbetalipoproteinaemia therefore is

inherently variable in its expression, causation and age of manifestation. The challenge is to diagnose the condition accurately and then to try to understand which pathogenetic mechanism has triggered the accumulation of remnants, so that therapy can be targeted towards the root cause.

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## Chapter 5

### **Diagnosis of Dysbetalipoproteinaemia**

#### 5.1 Literature review

The definitive diagnosis of dysbetalipoproteinaemia remains challenging. There is currently still not complete agreement as to what the diagnostic standards should be and which investigation should be regarded as the gold standard(1;3;7;13;59). All studies comparing the diagnostic efficiency of various tests suffer from the problem of a **non-uniform gold standard** and it is therefore difficult to make direct comparisons between the various tests.

In this section the evolution of the strategies employed to diagnose dysbetalipoproteinaemia will be traced, to better understand the status quo regarding diagnosis. Note that all chemical ratios reflecting lipoprotein composition are expressed as mass and not molar ratios. The reason for this is that all these ratios were initially expressed in mass terms and have generally not been converted to molar terms for routine usage. The ratios can be easily converted to molar ratios by multiplying the cholesterol concentration in mg/dl by 38.7 and the triglyceride concentration by 88.5. If ratios are given in molar terms in the text, this will be explicitly stated.

The demonstration of remnant accumulation, or alternatively termed  $\beta$ -VLDL, by either electrophoretic or chemical methods remains one of the cornerstones of diagnosis ( especially since dysbetalipoproteinaemia has been defined as a mixed hyperlipidaemia with the accumulation of  $\beta$ -VLDL ). Genotyping has become the second cornerstone upon which diagnosis rests.

Fredrickson(4) first described the different patterns seen on paper ( and subsequently agarose gel ) electrophoresis of lipoproteins, which today still form the

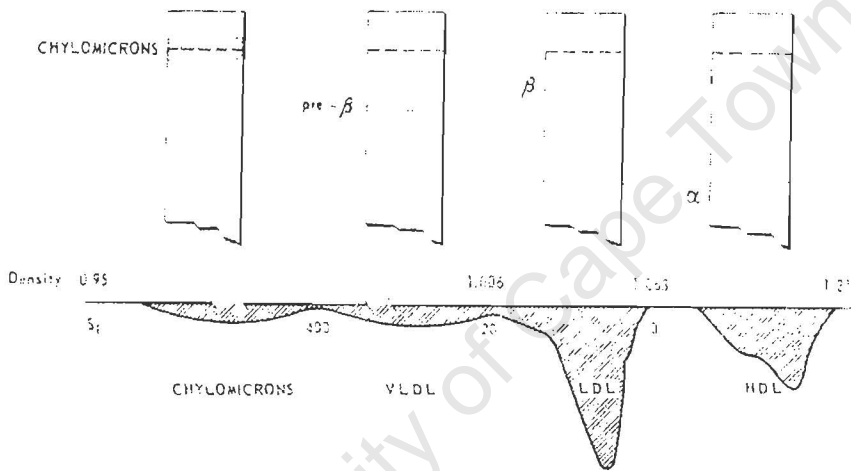
basis for the WHO classification of hyperlipidaemias.

The following graphic illustrates the naming of electrophoretic bands and their corresponding ultracentrifugal fractions. Source (60)

**Fig 5.1**

Ultracentrifugal Fractions and Paper Electrophoresis

THE PLASMA LIPOPROTEIN SPECTRUM AS SEGREGATED BY PAPER ELECTROPHORESIS (ABOVE) AND BY THE ULTRACENTRIFUGE (BELOW) IN WHICH  $S_f$  OR FLOTATION RATES ARE INVERSELY RELATED TO DENSITY



The following table defines the five patterns described during electrophoresis that are the basis for the WHO classification of dyslipidaemia.

**Table 5.2**

TABLE 1  
THE MAJOR ABNORMAL LIPOPROTEIN PATTERNS <sup>a</sup>  
AND THEIR TYPE NUMBERS

Type	Chylo- microns	LDL ( $\beta$ -lp)	VLDL (pre- $\beta$ -lp)	Floating $\beta$ -lipopro- teins <sup>b</sup>
I	+	-	-	-
IIa	-	+	-	-
IIb	-	+	+	-
III	-	+	+	+
IV	-	-	+	-
V	+	-	+	-

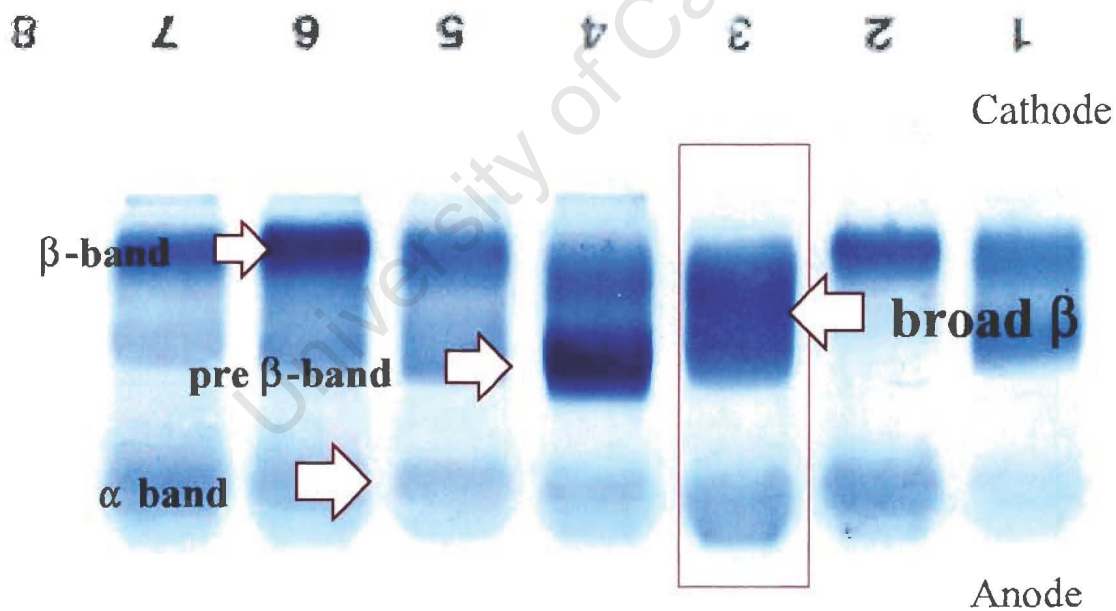
<sup>a</sup> + indicates which lipoprotein "family" (families) occurs in concentration above "normal" in the different abnormal patterns.  
<sup>b</sup> Also known as "broad  $\beta$ -lipoproteins".

The initial abnormality described was the "broad beta band" seen on electrophoresis of whole plasma.(4) This is a broad, often intensely staining band that commences in the usual  $\beta$  zone but then extends continuously into the pre- $\beta$  region. The usual "shoulder" or less intense staining region between the beta and the pre-beta bands is not seen. Although the presence of a broad-beta band on electrophoresis can give diagnostically useful clues, it is not sensitive enough to be used on its own as a diagnostic test. In a study (61)evaluating the use of polyacrylamide gel electrophoresis in the diagnosis of dysbetalipoproteinaemia only

50/118 (42.37% ) samples were found to have a broad  $\beta$ -band on paper electrophoresis. These samples originated from 27 patients who had  $\beta$ -VLDL on paper electrophoresis ( which was considered to be the gold standard for diagnosis at that time ) and were therefore diagnosed as having dysbetalipoproteinaemia. Relying on electrophoresis of plasma alone, at least half of dysbetalipoproteinaemic patients will not be correctly diagnosed. In addition it is not always easy to confidently distinguish a broad  $\beta$ -band from a IIb or type IV pattern as the ' shoulder ' between the bands in these patterns is occasionally not very marked either. This results in inter- and intra-observer variability, impairing the specificity of this test. An illustration of a lipoprotein electrophoresis in an agarose gel stained with Sudan Black is provided. The lane with the broad-beta band is highlighted.

**Fig 5.2**

Example of agarose gel electrophoresis with broad beta band



Patients with liver disease on Lipoprotein may on occasions also have an electrophoretic pattern similar to the broad-beta pattern.

Following on the descriptions of the electrophoretic patterns obtained with whole plasma ultracentrifugally separated subfractions of plasma were also electrophoresed and the mobilities of the various subfractions described. In patients with dysbetalipoproteinaemia, when lipoproteins with a density of less than 1.006 g/ml are electrophoresed on paper or agarose gels the very low density lipoproteins have  $\beta$ -mobility instead of the expected pre- $\beta$  mobility.(4) This finding has become to be regarded as the diagnostic hallmark of dysbetalipoproteinaemia by many and is often used as the " gold -standard " investigation when evaluating new diagnostic modalities.(16;62-65). The use of  $\beta$ -VLDL as a diagnostic standard is not without problems as it requires the qualitative interpretation of gels and an assignment of an " all or nothing " value. Problems arise in aged plasma where electrophoretic mobility may have changed or when only very faint bands are seen in the beta area. In addition the beta band of plasma and that of LDL generated by ultracentrifugation may have slightly differing mobilities, complicating interpretation even further.(13)

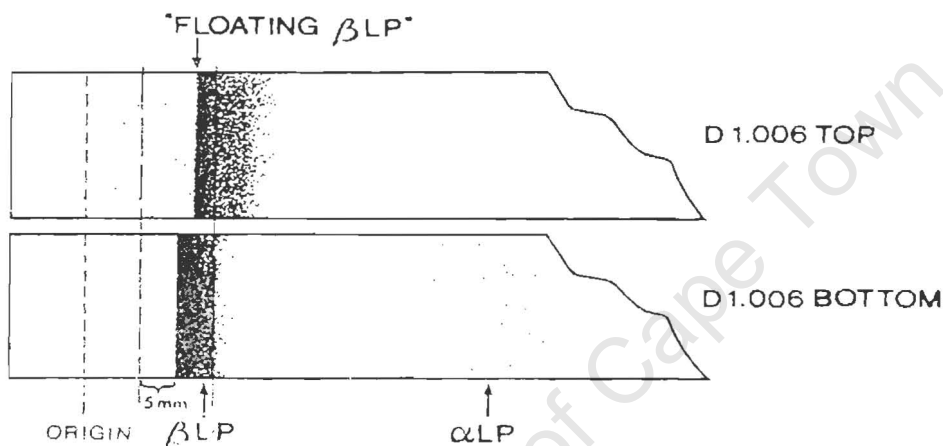
In 1975 Fredrickson published a paper(14) reviewing the diagnosis of dysbetalipoproteinaemia and comparing the electrophoretic assessment of  $\beta$ -migrating VLDL with the chemical assessment of VLDL composition ( in this case a ratio of VLDL cholesterol(here calculated as total cholesterol -infranatant  $d > 1.006$  g/ml cholesterol ) divided by plasma triglycerides was derived ). In this paper the definition used at that time for assigning the presence of  $\beta$ -VLDL is also illustrated. Plasma, ultracentrifugally generated supranatant ( density  $< 1.006$  g/ml ) and infranatant ( density  $> 1.006$  g/ml ) were electrophoresed side by side on paper strips and stained with Oil Red O. A judgement of " floating beta lipoproteins " or  $\beta$ -VLDL was made if the supranatant contained a lipoprotein band whose trailing edge lay in the zone that began 5 mm behind the trailing edge of the  $\beta$ -lipoprotein band in the infranatant fraction and that ended at the leading edge of this band. A subjective interpretation of the strip is however still required and incomplete concordance was

found between two independent observers.

The following provides an illustration of the method used to assign the presence of  $\beta$ -VLDL

### **Fig 5.3**

#### Definition of $\beta$ -VLDL



**Figure 1.** Diagram illustrating the definition of "floating beta" lipoproteins used in this study. Paper electrophoresis is performed on the supernatant (D 1.006 top) and infranatant (D 1.006 bottom) fractions obtained by preparative ultracentrifugation of plasma without adjustment of density (5). The strips are stained with a lipid-binding dye (oil-red-O), dried, and the origins aligned. The low-density lipoproteins ( $\beta$ LP) and high-density lipoproteins ( $\alpha$ LP) are found in the bottom fractions. Normally very low-density (pre-beta) lipoproteins are present in the top fraction and migrate such that their trailing edge is beyond the leading edge of the  $\beta$ LP band. The presence of a band in the top fraction that begins between the indicated lines is considered to be floating beta.

In addition  $\beta$ -VLDL was inconsistently present in the plasma of some patients with only 88% of patients having two concordant sequential analyses. In this study Fredrickson found chemical analysis of VLDL to better discriminate a unique subpopulation of VLDL enriched abnormally in cholesterol, than the presence of  $\beta$ -VLDL and suggested the adoption of this ratio as a diagnostic criteria for dysbetalipoproteinaemia.

Further efforts were made to refine **electrophoretic methods** in the diagnosis of dysbetalipoproteinaemia with particular emphasis on developing methods that would not require the use of ultracentrifugation.

In 1970 Seidel(66) developed a technique combining electrophoretic and immunological methods to diagnose dysbetalipoproteinaemia. Antibodies to  $\beta$ -lipoproteins were obtained commercially and an antibody was raised in rabbits to Lipoprotein X. ( LpX ) Previous work at that time had shown that VLDL contained an apoprotein (at that stage only defined as - non ApoA and non ApoB ) which was also found in LpX. The authors therefore performed immunoelectrophoresis using whole plasma as well as the supernatant and infranant fractions obtained after ultracentrifugation at density 1.006g/ml. They were able to demonstrate that precipitation with anti-LpX occurred in whole plasma in a precipitation line orientated towards the anode. This line was also present in the isolated supernatant but not in the infranant. Next they demonstrated that combining anti- $\beta$  lipoprotein and anti-LpX antibodies on the same gel, only patients with dysbetalipoproteinaemia showed precipitation with anti-LpX in the pre- $\beta$  and  $\beta$  lipoprotein area. Patients with Type IV hyperlipidaemia would for instance show a precipitation line against anti- $\beta$  lipoprotein in the pre- $\beta$  and  $\beta$ -areas, while there would only be precipitation against anti-LpX in the pre- $\beta$  area. In this small study only nine patients with dysbetalipoproteinaemia(defined by the presence of beta VLDL ) were evaluated and

the above described pattern was found in all patients. Again there is the problem of subjective interpretation of gels, deciding how far the precipitation line with LpX extends and whether it involves the  $\beta$  area or not. In addition the antibodies against LpX were not standardized and it was not known what they were reacting against. The antibodies were not obtainable on a large scale and there was no evaluation of different batches of antibodies either. Patients with cholestatic liver disease and LpX would also have been called false positive in this assay.

In 1970 Fredrickson(67) reported on the use of starch block electrophoresis in the investigation of patients with dysbetalipoproteinaemia. Electrophoresis is performed in a large block ( 50\*20\*1 cm ) prepared from purified potato starch. Whole plasma as well as ultracentrifugally subfractionated plasma was submitted to starch block electrophoresis. After electrophoresis the block is cut transversely into 1 cm segments and the lipoproteins are eluted from the starch and their chemical composition analysed. A profile can then be compiled plotting migration distance against lipid or protein concentrations. In normal patients VLDL was found to migrate uniformly in the same position as the serum  $\alpha_2$ -globulins and was therefore labelled as  $\alpha_2$ -VLDL. In patients with dysbetalipoproteinaemia there were two distinct populations of VLDL.  $\alpha_2$ -VLDL was found as in the controls but in addition a new species of VLDL was found. This was labelled  $\beta$ -VLDL as it migrated in the position of LDL . The migration distance of ultracentrifugally isolated LDL was determined by staining the gel with labelled antibodies to LDL. The authors showed that the relative proportions of the species to each other were influenced by diet: high carbohydrate feeding would increase the relative amount of  $\alpha_2$ -VLDL, while starvation or heparin infusion had the opposite effect. They then already correctly suggested that the  $\beta$ -VLDL fraction may be the metabolic end-product of the  $\alpha_2$ -VLDL. In 1974 Hazzard and Bierman(68) were unable to replicate these earlier findings of two discrete VLDL species in starch block electrophoresis, instead demonstrating a continuum of species. They were able to demonstrate that dietary feeding with carbohydrates increased the proportion of faster migrating ( triglyceride rich ) VLDL species while treating patients with Heparin ( and thereby accelerating lipolysis ) increased the

amount of slower migrating cholesterol rich VLDL. They therefore also concluded that slower migrating, cholesterol rich VLDL represented the remnants of the initially secreted triglyceride rich VLDL. With the inconsistencies in the reports regarding VLDL species identified in starch block electrophoresis and the technically demanding nature of the procedure, it never became established in the routine diagnostic process for dysbetalipoproteinaemia.

In 1972 Masket et al(61) described the use of polyacrylamide gel electrophoresis as a diagnostic tool for dysbetalipoproteinaemia. They prepared gels with a uniform (ie not gradient gels) 3% concentration of polyacrylamide in which lipoproteins prestained with Sudan Black were electrophoresed. Polyacrylamide gels separate particles dependent mainly on their size. A clear separation of chylomicrons (remained in loading gel), VLDL (found in interface of stacking and separating gel), LDL (migrated 0.5 to 0.7 cm in separating gel) and HDL (furthest migration in separating gel) could be obtained in this system. A distinct pattern was recognised in patients with dysbetalipoproteinaemia (defined here by the presence of  $\beta$  migrating VLDL) - the absence of a band migrating in the normal LDL position. Only five of 118 samples of patients with dysbetalipoproteinaemia had a band identifiable in the LDL region. The combination of a  $\beta$  migrating band on paper electrophoresis and absent LDL band on polyacrylamide gel electrophoresis was therefore found in 113/118 samples of patients with dysbetalipoproteinaemia, with a similar combination only being found in 3/228 samples from patients with other forms of hyperlipidaemia. These three had very high triglyceride concentrations while the five patients with dysbetalipoproteinaemia showing a LDL band on polyacrylamide electrophoresis had very well controlled lipid values.

In 1973 Seidel et al(69) described a new electrophoretic technique admitting that their previously described immunological technique(66) was not practicable for routine use on a large scale. This technique relies on the selective precipitation of VLDL in agarose gel by  $MgCl_2$  and Heparin. Immersing the gel in dextran sulphate with  $CaCl_2$  will subsequently precipitate all lipoproteins, which can alternatively also

be stained with a stain such as Oil Red O. By subjecting ultracentrifugally fractionated plasma to this procedure Seidel was able to demonstrate that in the concentrations used by him  $MgCl_2$ /Heparin only precipitated lipoproteins in the fraction of plasma with  $d < 1.0006$  g/ml. If plasma is electrophoresed and subsequently treated with  $MgCl_2$ /Heparin the position of the precipitated band can be marked and after subsequent further precipitation of all lipoproteins or staining with Oil Red its position can be determined relative to the other bands seen. Subsequent to this Seidel showed that patients with dysbetalipoproteinaemia had lipoproteins precipitable by  $MgCl_2$ /Heparin that migrated in the  $\beta$ -area. During the development of this technique it was noted that the ionic environment as well as the concentrations of magnesium and heparin were of great importance to achieve optimal precipitation of beta-VLDL. VLDL from patients with Type IV hyperlipidaemia, who have high levels of VLDL, was at times poorly precipitated.

The technique of selective precipitation of VLDL bands by  $MgCl_2$ /Heparin was further developed by Wikinski in 1991. (70) The authors again initially performed agarose gel electrophoresis of lipoproteins on microscope slides ( each specimen was electrophoresed in quadruplicate ) and then added  $MgCl_2$ /Heparin. The position of the precipitated band was marked and the slide then washed several times in sodium barbital buffer and then re-electrophoresed in a second step. During the second electrophoresis the precipitated VLDL or  $\beta$ -VLDL did not migrate any further ( having been denatured in the precipitation step ) but the LDL migrated further towards the anode. The gels were subsequently fixed and visually inspected. The initially precipitated band was then cut from the gel with a blade and cholesterol was assayed after butanol extraction by means of the Zak reaction. Depending on the intensity of the precipitated band in the gels, the bands from one to three of the gels were cut out and used to obtain sufficient material for analysis.

The authors also added known quantities of ultracentrifugally isolated IDL to serum samples and assessed the recovery after electrophoresis and precipitation. They were able to recover IDL cholesterol with a co-efficient of variation ( CV ) of 10.1 %

for 15 aliquots of the same serum. The correlation co-efficient between the IDL cholesterol measured electrophoretically and by ultracentrifugation in 30 samples was  $r=0.96$ . In seven patients with dysbetalipoproteinaemia (with the method of diagnosis not well defined in the article) the cholesterol concentration in IDL as measured after electrophoresis, precipitation and extraction was significantly higher than in all other control patients with dyslipidaemia. Some of the problems with this method again include poor separation of bands, especially after the second electrophoretic step if the washes preceding electrophoresis had not been adequate. In addition potential sources of error are introduced in the mechanical cutting of bands from the gel, especially if the bands are very close to each other. Each sample needs to be electrophoresed in quadruplicate so that sufficient material is available for analysis if the precipitated VLDL band is faint. The subsequent extraction and analysis of cholesterol is also relatively labour intensive.

In 1974 Godolphin(71) described a method of diagnosing dysbetalipoproteinaemia that relies on isoelectric focussing of plasma in a 3% polyacrylamide gel and staining for lipoproteins with Sudan Black. A group of 16 patients with dysbetalipoproteinaemia (again defined by the presence of  $\beta$ -VLDL) was examined and 13 displayed what seemed to be a characteristic dense band at an average isoelectric point of 5.44. This particular pattern was not seen in samples from either healthy volunteers or patients with other types of hyperlipidaemia. The isoelectric focussing technique of plasma is however technically fairly demanding and cumbersome and never gained great popularity for the diagnosis of dysbetalipoproteinaemia. Isoelectric focussing was however still to play an extremely important role in the delineation of the various ApoE polymorphisms.

In 1977 Papadopoulos(65) described a modified agarose-gel electrophoresis system that was promising in the diagnosis of patients with dysbetalipoproteinaemia. The previously described polyacrylamide systems (61;71) were at that stage quite complex technically and cumbersome to perform. In the newly described system

lipoproteins are rapidly ( time =12 min ) electrophoresed in a pure agarose gel on microscope slides. In 20 patients with dysbetalipoproteinaemia ( defined here by a ratio of Cholesterol in VLDL to plasma triglyceride greater than 0.30 ) the unique finding was the presence of two distinct  $\beta$ -migrating lipoprotein bands ( "  $\beta$ -lipoprotein doublet " ). The gels in addition contained a pre- $\beta$  band as well as a  $\alpha$ -band. When plasma was separated by ultracentrifugation into supernatant of density less than 1.006 g/ml and corresponding infranatant and again electrophoresed the infranatant contained the  $\alpha$ -band and one of the  $\beta$ -bands. The supernatant contained the one  $\beta$ -band ( with greater mobility ) and the pre- $\beta$  band. The one  $\beta$ -band therefore by definition is a "floating  $\beta$ -band " or  $\beta$ -VLDL. In 22 other patients who did not have a ratio of cholesterol in VLDL to plasma triglycerides greater than 0.30 this pattern was not found. This technique, although appealing, also does have multiple problems. The separation of the two  $\beta$ -bands was often quite small and aging of plasma readily obscured the separation completely. In addition small variations in technique often resulted in indistinct bands and technically unsatisfactory gels.

In 1978 Chew (72) described a single patient with dysbetalipoproteinaemia ( diagnosed clinically on the basis of palmar crease xanthoma and a mixed hyperlipidaemia with total cholesterol of 14 mmol/l and triglycerides of 7.3 mmol/l ) whose plasma was subjected to acrylamide gradient gel electrophoresis. The acrylamide gradient ranged from 1.4 % to 7%. This patient is unusual in that she was a 26-year-old premenopausal female with hyperlipidaemia and angina sufficiently severe enough to require coronary artery bypass grafting. She may well have had a one of the dominant ApoE mutations that causes early onset of hyperlipidaemia. In this patient in the untreated state, several dense bands were seen just below the " VLDL zone ". These bands probably represented the remnants characteristic of this condition. In addition little material was seen in the region where LDL should normally be visible.

In 1975 Shepherd (73;74)described the use of agarose columns to diagnose

dysbetalipoproteinaemia. Agarose gel columns separate lipoproteins according to size ( " molecular sieve chromatography " ) with the largest lipoproteins travelling through the column fastest. Shepherd separated lipoproteins from the other plasma proteins by ultracentrifugation of plasma with density adjusted to 1.225 g/ml. After concentrating the lipoproteins in a small volume  $I_{125}$  labelled purified LDL was added as a marker. The lipoproteins were loaded on a 50 cm agarose column and eluted with a Tris buffer. Eluent fractions were collected every four minutes. Fractions were assayed for radioactivity and the optical density at 280 nm determined as an index of lipoprotein presence. In control subjects three peaks on the optical density measurement were identified corresponding to VLDL, LDL and HDL ( in this order ). The radioactive LDL marker was found in the same fractions as the endogenous LDL. In patients with dysbetalipoproteinaemia ( defined by the presence of  $\beta$ -VLDL ) VLDL and LDL peaks were not well separated. The result was a single broad peak in the fractions normally containing the VLDL and LDL peaks. In addition little or no endogenous LDL was found in the fractions containing the radioactive LDL marker. This pattern was found not to be markedly affected by either diet or drug therapy and was also readily distinguishable from the patterns seen in other patients with a mixed hyperlipidaemia. This method is therefore appealing as the presence of excess remnants is directly demonstrated by the accumulation of lipoproteins of intermediate size between VLDL and LDL particles. In addition there are very few particles corresponding in size to the exogenous LDL marker present. Unfortunately the technique is not easy to perform- requiring ultracentrifugation, the use of columns and analysis of multiple specimens from the eluted fractions. It is therefore perhaps not surprising that this technique has never established itself in the routine diagnosis of dysbetalipoproteinaemia.

The following graphic illustrates the eluent profile of a patient with dysbetalipoproteinaemia and that of a normal control patient.

**Fig 5.4**

Agarose Column Elution Profiles of Normal and Dysbetalipoproteinaemic Subject

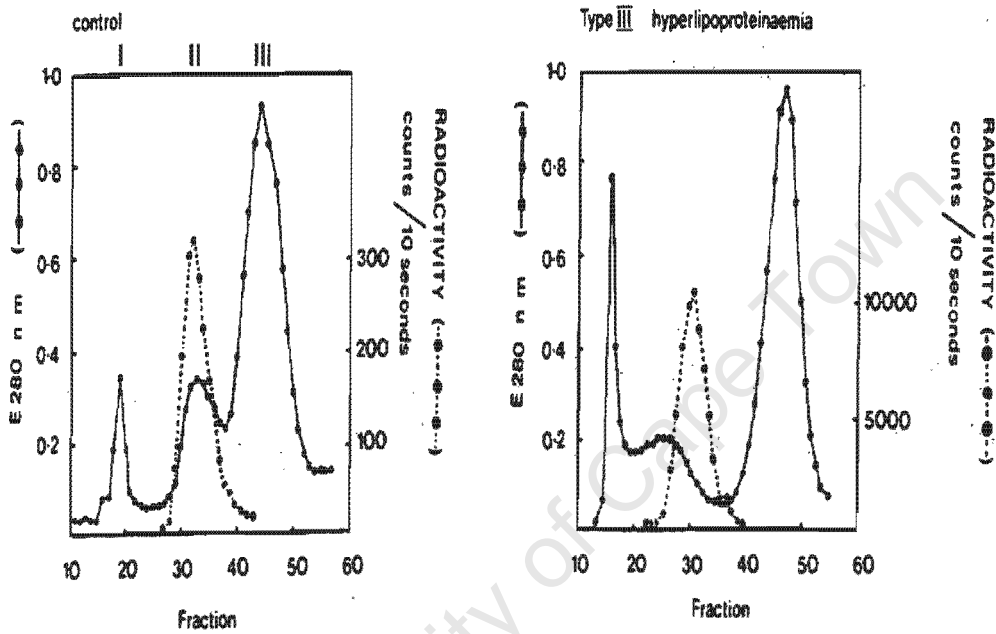


Fig. 1. Agarose column elution profile of lipoproteins from control and type III hyperlipoproteinaemic plasma. Fasting plasma from patient A.M. and a control subject were separated according to the procedure described in Methods. The agarose column elution profile with superimposed LDL marker peak is shown. The plasma from the control subject (age 21 y) contained 2360 mg/liter cholesterol and 973 mg/liter triglyceride

All electrophoretic methods described thus far rely on the demonstration of lipoproteins that have abnormal chemical and electrical properties and therefore migrate at altered speed in electrophoretic media ( at the speed of LDL into the  $\beta$ -position ) but have a density that is lower than that of the LDL with which they migrate. Chemical analysis of these lipoproteins would therefore hopefully offer a way to diagnose dysbetalipoproteinaemia that did not require subjective interpretation of gels and all the problems associated with electrophoretic techniques. As the  $\beta$ -VLDL are found at a density less than 1.006 g/ml the logical step was to compare this fraction's chemical composition with that of normal controls as well as other patients with a diagnosis of hyperlipidaemia. **Chemical analysis of VLDL composition** yields quantitative results, allowing numeric diagnostic criteria to be set.

In 1972 Hazzard and Bierman(15) were the first to systematically analyse the chemical composition of the various fractions of ultracentrifugally separated plasma. Their work was based on previous observations that chylomicrons isolated from **fasting** patients with dysbetalipoproteinaemia, disease being defined by the presence of  $\beta$ -VLDL, had abnormal chemical composition(75). These chylomicrons were relatively poor in triglycerides and rich in cholesterol with a ratio of triglyceride /cholesterol of less than 3.0. Chylomicrons analysed at the time of peak alimentary lipaemia had a normal composition. In addition other preliminary studies ((76;77) analysing VLDL from patients with dysbetalipoproteinaemia had shown the VLDL to be abnormally enriched in cholesterol.

In their study(15) Hazzard and Bierman analysed plasma from a total of 44 subjects. Nine of these subjects had dysbetalipoproteinaemia defined by the presence of  $\beta$ -VLDL on agarose gel electrophoresis **and** the presence of fasting chylomicronaemia as determined with polyvinylpyrrolidone flocculation. The other patients either had normal lipid values ( n=10 ), IIa pattern ( n=5 ), IV pattern ( n=14 ) or a type V pattern ( n=5 ). They ultracentrifuged plasma overlaid with saline of density 1.006g/ml overnight and measured cholesterol and triglycerides directly in the supernatant

fraction. The ratio of Cholesterol in VLDL ( abbreviated  $C_{VLDL}$  here ) and triglycerides in VLDL ( abbreviated as  $TG_{VLDL}$  ) was determined. This ratio was significantly higher in patients with dysbetalipoproteinaemia ( mean ratio of 0.60 ) than in the other patients ( mean ratio for normals 0.35, IIa 0.39, IV 0.27, V 0.25 ). Using a cutoff of 0.42 there were no false negatives, but five patients would have falsely been labelled as having dysbetalipoproteinaemia. These false positive patients included four normolipidaemic controls and one patient with a IIa pattern. This ratio is therefore of less use when the triglyceride concentrations are normal ( here a level of less than 1.58 mmol/l was regarded as normal ) as VLDL in general tends to become more cholesterol enriched with falling triglyceride values. This is also reflected in the significantly lower mean ratios observed in the hypertriglyceridaemic type IV and Type V patients. Problematic in this study was that patients with IIb patterns were specifically excluded from the analysis. These are, however, patients in whom the diagnosis of dysbetalipoproteinaemia may initially be considered. In addition patients with dysbetalipoproteinaemia when tested on repeated occasions had varying ratios, that were on occasions diagnostically negative and subsequently positive. Understanding the pathophysiology of dysbetalipoproteinaemia it becomes clear that the composition of remnants will be influenced by multiple factors including diet, drugs, diabetes etc... If there is overproduction of VLDL, as may occur in diabetes or with carbohydrate loading, VLDL will be richer in triglyceride and the ratio derived will subsequently be lower. The determination of a fasting ratio therefore only provides an isolated snapshot of the dynamic process of remnant metabolism and clearance.

In 1974 Mishkel(16) analysed plasma from 19 patients with dysbetalipoproteinaemia ( patients were required to have  $\beta$ -VLDL on at least three separate occasions ) and 100 controls with plasma cholesterol of more than 6.71 mmol/l and a cholesterol to triglyceride ratio of between 0.7 to 1.3 by mass. These patients were therefore specifically selected to have high triglycerides, that could raise the suspicion of dysbetalipoproteinaemia. Ultracentrifugation of plasma that had been overlaid with saline of density 1.006 g/ml was performed and the supernatant and infranatant

were analysed. In this study the 19 patients with dysbetalipoproteinaemia had a total of 191 ultracentrifugations performed and 119 ultracentrifugations were performed on control patients. A ratio of  $C_{VLDL} / TG_{VLDL}$  of more than 0.35 is suggested as diagnostic of dysbetalipoproteinaemia if the following criteria are met:

- TG not greater than 5.08 mmol/l ( as chylomicrons may then be present and although dysbetalipoproteinaemic chylomicrons are enriched in cholesterol when compared to normal chylomicrons they will still lower the ratio )
- Cholesterol of more than 5.16 mmol/l.

Patients with dysbetalipoproteinaemia had a mean  $C_{VLDL} / TG_{VLDL}$  ratio of 0.50 ( Standard deviation 0.13 ) while in the controls the mean ratio was 0.29 with a standard deviation of 0.09. (13) The second ratio analysed in this study was that of  $C_{VLDL}$  divided by plasma triglyceride ( abbreviated as  $TG_{Plasma}$  ). The authors found a ratio of more than 0.25 in 174 of 188 analyses of patients with dysbetalipoproteinaemia ( 14 false negative ) while there were 16 false positives in 119 control samples. The mean ratio in dysbetalipoproteinaemic patients was 0.35 ( standard deviation 0.10 ) with controls having a mean ratio of 0.20 ( standard deviation 0.05 ). When both ratios were combined only eight samples would have been considered false positive while more than 90% of patients with dysbetalipoproteinaemia had both samples in the positive range. Again most problems occurred at the extremes of lipid values where most false positive and negative ratios were found.

In 1975 Fredrickson(14) took a somewhat different approach towards developing a diagnostic standard for dysbetalipoproteinaemia based on ultracentrifugation. A study population was selected from patients previously seen at their clinics. These were patients that had had complete lipoprotein analyses done by ultracentrifugation previously and met the following criteria:

- Age greater than 19 years

- Initial plasma triglycerides more than 2.15 mmol/l
- Record of at least two complete lipoprotein analyses
- At least one first degree relative with hyperlipidaemia ( cholesterol or triglyceride more than 95% centile age adjusted limits ) -the recessive genetic nature of the E2/E2 homozygous state was not known at this stage.

182 patients were found and their records evaluated. Of these patients 176 had analyses done while their triglycerides were between 2.15 and 11.3 mmol/l and were further evaluated. In total 2225 analyses were available, or a mean of 12.6 per patient. The presence of  $\beta$ -VLDL was diagnosed if two consecutive ultracentrifugations with subsequent electrophoresis were positive. If the first two samples were not concordant ( only 159/182 of the patients were concordant ) the next analysis decided the assigned status. Ratios of  $C_{VLDL}$  to  $TG_{Plasma}$  were calculated from the previously derived lipoprotein analysis data. A mean of all ratios measured for a patient was derived, this was therefore usually a composite of values obtained in the untreated and treated stage. Of importance is that  $C_{VLDL}$  was not measured directly but calculated as the difference between plasma cholesterol and cholesterol in the infranatant fraction with density  $> 1.006$  g/ml.

Fredrickson subsequently compiled a frequency distribution plot of all mean  $C_{VLDL}/TG_{Plasma}$  ratios. This plot showed a skewed distribution to the right with a shoulder at a ratio of 0.3. Using a cut-off value of 0.3 therefore discriminates a population of patients, who have abnormal chemical composition of VLDL compared to hypertriglyceridaemic controls. These patients were accordingly defined as having dysbetalipoproteinaemia irrespective of whether  $\beta$ -VLDL was present on electrophoresis or not. Further analysis of the data shows that if plasma triglycerides are more than 1.7 a ratio between 0.25 and 0.29 should be considered as suspicious for dysbetalipoproteinaemia. If the ratio was less than 0.25 the statistical chance of having a ratio more than 0.30 in a hundred subsequent analyses is less than one in a hundred. Beta VLDL was found throughout the range

of the ratio's distribution, but was more frequently present in patients with higher ratios. In this study it was a worse discriminator than the chemical ratio and Fredrickson suggested it should be superseded by chemical analysis of the VLDL.

The frequency distribution plot obtained by Fredrickson looks as follows

### **Fig 5.5**

#### **Frequency Distribution of $\beta$ -VLDL according to Ultracentrifugal Ratios**

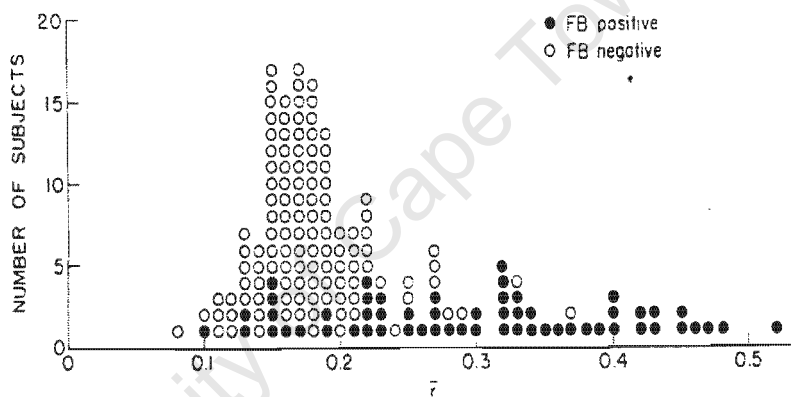


Figure 4. Distribution of the average ratios ( $\bar{r}$ ) in the 175 members of the study population for whom this value could be calculated.

FB indicates the presence of "floating beta" or  $\beta$ -VLDL

In 1975 Vessby (78) published the results of a study done in Uppsala. During health screening of 50 year old males 172 patients were identified that had cholesterol and or triglyceride concentrations greater than or equal to the 80% population centile. These patients all had full lipoprotein analysis by sequential ultracentrifugation and their VLDL was electrophoresed to determine the presence of  $\beta$ -VLDL. Patients were defined as dysbetalipoproteinaemic if they had  $\beta$ -VLDL. Twelve such patients were identified. Comparison was made between their lipoprotein analyses, those of

the other hyperlipidaemic subjects and those of 50 normolipidaemic control patients of the same age. Vessby suggested that a " III index " be calculated for patients and established a normal range for this index as well. It was suggested that patients with an " III index " of more than 98% of controls,  $C_{VLDL} / TG_{Plasma}$  ratio of more than 95% of controls and floating beta be labelled as having dysbetalipoproteinaemia. The " III index " incorporates the fact that patients with dysbetalipoproteinaemia have an abnormally low cholesterol to triglyceride ratio in LDL. It is calculated as follows. Cholesterol concentration is expressed as mg/dl while triglycerides are expressed as mmol/l.

$$\text{III index} = \frac{\text{Cholesterol / Triglyceride in VLDL}}{\text{Cholesterol / Triglyceride in LDL}} * 10$$

The " III index " has however not become established in the diagnosis of dysbetalipoproteinaemia. It was established using an entirely male reference population and seems to add little specificity or sensitivity compared to the use of indices reflecting VLDL chemical composition alone.

In 1976 Albers and Hazzard(13) compared most of the diagnostic techniques that were in use at that time. For their analysis they selected 198 plasma samples from 43 subjects that had  $\beta$ -VLDL. A control group of patients was selected that were  $\beta$ -VLDL negative. This control group was specifically weighted to include patients with hypertriglyceridaemia.

All patients had ultracentrifugation of plasma and chemical analysis of the fractions recovered (  $d > 1.006$  g/ml and  $d < 1.006$  g/ml ). In addition electrophoresis of plasma and the subfractions was performed on agarose slides. Agarose electrophoresis with magnesium-chloride/heparin precipitation as described by Seidel(69) was also performed. Selected samples had polyacrylamide gel electrophoresis performed as described by Masket.(61) Ratios calculated were:

1. Ratio of VLDL cholesterol derived from subtraction of infranantant cholesterol from total cholesterol/ plasma triglyceride
2. Ratio of directly measured VLDL cholesterol /plasma triglyceride
3. Ratio of directly measured VLDL cholesterol/ VLDL triglycerides

These ratios were plotted separately for the groups of  $\beta$ -VLDL positive and negative plasmas and a linear regression line and equation was determined, describing the relationship between the ratio plotted on the Y-axis and the plasma triglycerides plotted on the X-axis. As is to be expected the ratios became smaller as triglycerides increased. For further calculations only plasma samples with a triglyceride concentration between 1.7 and 11.3 mmol/l were considered, as the coefficient of variation of repeated analysis was high outside this range. For each ratio's linear regression equation a subsequent equation was determined that was a 95% cutline, ie a line above only which 5% of ratios from  $\beta$ -VLDL negative patients would lie. By requiring multiple ratios to be positive the specificity for excluding  $\beta$ -VLDL negative samples on chemical criteria can be increased without compromising sensitivity. Using a single criterium the specificity is 95% by definition.

The following cutlines are suggested:

Ratio 1: Positive if  $> 0.33 -TG \text{ ( in mg/dl)}/10000$

Ratio 2: Positive if  $> 0.24$

Ratio 3: Positive if  $> 0.38 -TG \text{ ( in mg/dl ) }/10000$

Adjusting the cutlines for triglycerides takes into consideration, that as triglycerides increase chylomicrons can appear, as well as that the composition of  $\beta$ -VLDL shifts more towards that of triglyceride rich pre- $\beta$  VLDL. This adjustment increased sensitivity and specificity of the analysis, remembering that the gold standard was the presence of  $\beta$ -VLDL. In this study there was also much better concordance between high chemical ratios and the presence of  $\beta$ -VLDL than in the study reported by Fredrickson(14). In Fredrickson's study 27 of 59 subjects with  $\beta$ -VLDL had a ratio

( corresponding to Ratio 1 ) of less than 0.30. In this study 152/183 patient with  $\beta$ -VLDL had a triglyceride adjusted ratio that was positive. Explanations provided for this discrepancy is the use of paper electrophoresis vs agarose gel and different criteria for assigning  $\beta$ -VLDL positivity.

Regarding other electrophoretic techniques: Heparin/magnesium chloride precipitation was positive in 87% of patients that had  $\beta$ -VLDL and were positive on their chemical ratios. Patients without  $\beta$ -VLDL and with negative ratios had 18% false positives, most of which occurred when triglycerides were more than 5.6 mmol/l. Polyacrylamide gel electrophoresis performed poorly in this study, with only 60% of otherwise positive patients being diagnosed by this technique. In addition 26% of negative samples were considered false positive by this technique. The reason why this technique performed so poorly compared to the diagnostic accuracy claimed by Masket (61) is not immediately obvious. Again agarose gel electrophoresis was used instead of paper electrophoresis and patients had lower triglyceride levels on average in the current study. The polyacrylamide gel technique performed better in the current study at higher triglyceride levels and the inclusion of more patients with low triglyceride levels may have influenced the results.

In summary regarding compositional analysis of VLDL the following can be said. Despite differences in techniques and research methodology ( using  $\beta$ -VLDL as a gold standard or describing the frequency distribution of a ratio and isolating an abnormal population ) several authors define ratios within a similar range that differentiate a subgroup of patients with abnormal VLDL composition from other patients. The frequency distribution of these ratios is clearly not Gaussian, allowing one to select a population that lies outside the normal range of distribution. As with many biological variables, there is however a range of distributions with no clear absolute differentiation between the normal and abnormal. Therefore sensitivity and specificity will never be 100% for any of these ratios. Ratios are an attempt at chemical detection of the abnormal  $\beta$ -VLDL and have the advantage that no subjective interpretation of electrophoresis results is required. Ratio calculation is

entirely dependant on good technical practice and standardization in performing ultracentrifugation to recover VLDL. Incomplete recovery of VLDL supernatant, as may occur when triglycerides are markedly elevated and stick to the tube, will obviously invalidate the ratio calculated that compares  $C_{VLDL}$  ( directly measured) with plasma triglyceride. The ratio of cholesterol to plasma triglyceride within VLDL does not depend on complete recovery of VLDL. When measuring cholesterol and triglycerides of very low concentrations in the VLDL most standard assays are at the limit of performance and coefficients of variation increase. In addition ratios represent a single snapshot of a very dynamic metabolic process-they are therefore not constant in any given patient and will reflect dietary and drug influences. This is reflected by the experience in our clinic where some patients with known E2/E2 status have had negative ratios on occasions, only to be positive when tested at some other stage. A few patients also have had multiple ultracentrifugations that are negative with one positive result in between. These ratios are therefore not absolute diagnostic criteria but should be used together with clinical and genetic information to make a diagnosis of dysbetalipoproteinaemia. It is clear that with extreme hypertriglyceridaemia the ratios are unlikely to be diagnostically positive, while if normotriglyceridaemic patients are analysed the chances of false positive ratios are increased.

Apart from the use of electrophoresis and analysis of VLDL other diagnostic techniques have been described. Further development of diagnostic techniques has always been driven by the goal to diagnose dysbetalipoproteinaemia without the use of ultracentrifugation. In 1993 März(63) explored the use of the **Apolipoprotein E to Apolipoprotein B ratio** as a diagnostic marker for dysbetalipoproteinaemia. ApoE concentrations are increased in patients with dysbetalipoproteinaemia ( except in the rare instances of ApoE deficiency ) while their ApoB levels will be normal or low. In this study 40 patients with well-defined dysbetalipoproteinaemia ( all patients had the E2/E2 genotype,  $\beta$ -VLDL and a ratio of Cholesterol in VLDL to plasma triglycerides of more than 0.30 ) were compared with a control group matched for lipid values. The control group all were negative for the E2/E2 genotype as well as

having a ratio of less than 0.30. ApoB and ApoE were determined by kinetic nephelometry and the ratio calculated. Patients with dysbetalipoproteinaemia had higher ratios than control patients. Choosing a cutline of 0.09 sensitivity was 95% with a specificity of 88%. This is therefore a reasonable screening test for patients with mixed hyperlipidaemia. Assuming a frequency of 1:100 of dysbetalipoproteinaemia amongst patients with hyperlipidaemia the negative predictive value of the test approaches 100%. Technical problems relate to standardization of apolipoprotein measurements. As values measured will differ somewhat by technique this ratio is only applicable if the same techniques and calibration plasmas are used. Each laboratory therefore needs to establish an internal reference range for this ratio.

In 1999 Wang(64) published a paper proposing the use of the ratio between **remnant-like particle cholesterol** and plasma triglyceride as a diagnostic test for dysbetalipoproteinaemia. In previous work (79) this group had described the development of immuno-affinity gels loaded with monoclonal antibodies to ApoB100 and ApoA-I bound to sepharose beads. These monoclonal antibodies were raised in mice immunised with human apolipoproteins. The microepitopes of an apolipoprotein that are accessible to antibody binding can vary according to the size and composition of the lipoprotein of which it forms a part. Some monoclonal antibodies can therefore select certain lipoprotein subclasses based on their binding affinity to exposed microepitopes. The authors showed that the specific ApoA-I antibody they used quantitatively bound lipoproteins containing ApoA-I while the ApoB100 antibody they used bound most lipoproteins containing ApoB100. Lipoproteins enriched in ApoE were not well bound, presumably as different microepitopes on the ApoB100 were exposed. The unbound fraction of lipoproteins is eluted and then analysed. This unbound fraction of lipoproteins was enriched in ApoE and cholesterol-esters. It resembled VLDL-and chylomicron remnants by compositional analysis. The unbound lipoproteins were subsequently designated as remnant-like-particles. These remnant-like-particles are heterogenous in size and generally are larger in patients with hyperlipidaemia.(80).

Assaying the cholesterol in this fraction proved to be the easiest and most reliable method of quantifying its size. Evaluating this assay(64) directly measured VLDL cholesterol was shown to be highly correlated with remnant-like particle cholesterol. Twenty-three subjects with dysbetalipoproteinaemia ( defined by the presence of  $\beta$ -VLDL ) were matched by plasma lipids with control patients selected from patients who had Type IIb and Type IV hyperlipidaemia. A ratio of 0.23 ( when cholesterol and triglycerides are expressed in molar terms ) was found to be 95% sensitive with a specificity of 94%. The principle is therefore similar to ultracentrifugation of plasma and determination of VLDL chemical composition, except that differential binding to antibodies is used to isolate a population of lipoproteins that has characteristics similar to remnants found in the supernatant after ultracentrifugation. Cost is obviously high due to the use of monoclonal antibodies, but if the kit is obtained the procedure is said not to be technically demanding.

In 1999 März together with others (62) described the use of a precipitation based method in screening for dysbetalipoproteinaemia. Serum is treated with dextran sulfate /magnesium chloride which results in the precipitation of LDL and VLDL. Cholesterol and triglyceride are measured in the supernatant and LDL cholesterol + LDL triglyceride ( =LDL-C<sub>DS</sub> and LDL-TG<sub>DS</sub> ) are calculated as the difference between total plasma values and values measured in the supernatant. LDL-C<sub>DS</sub> shows good correlation with LDL-C measured after ultracentrifugation of plasma and isolation of LDL. This however only applies to patients who do not have dysbetalipoproteinaemia. In patients with dysbetalipoproteinaemia LDL-C<sub>DS</sub> is markedly higher than that measured by ultracentrifugation reflecting the precipitation of  $\beta$ -VLDL. Triglyceride content calculated in the precipitate was also markedly high. In analysing 1317 sera, including 29 patients with dysbetalipoproteinaemia ( defined by E2/E2 genotype and mixed hyperlipidaemia ) the authors suggest that in patients with cholesterols of more than 6.45 mmol/l and triglycerides of more than 2.82 mmol/l precipitated levels of LDL-C<sub>DS</sub> and LDL-TG<sub>DS</sub> of more than 4.52 and 1.07 be considered suspicious for dysbetalipoproteinaemia. This technique is a sensitive screening method but not specific enough to make a confident diagnosis

and specimens found positive on screening require further testing.

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## 5.2 Apolipoprotein E and Genotyping in the Diagnosis of Dysbetalipoproteinaemia

In the 1970's while more refined chemical and electrophoretic techniques were explored and developed for the demonstration of  $\beta$ -VLDL ( or remnant ) accumulation, further work was in progress in an attempt to understand the genetic nature of this disease. Apolipoproteins were intensively studied and Utermann was the first to report the specific deficiency of the E3 isoform of ApoE in the VLDL of patients with dysbetalipoproteinaemia.(81) Subsequently multiple modifications were made in the method used for isoelectric focussing of the apolipoproteins in VLDL(82-84), including methods to remove the sialic acid residues ( on threonine 194 ) on the minor isoforms of ApoE. In 1982 a uniform nomenclature for the ApoE isoforms was agreed upon. (44)Sequencing of the gene for ApoE showed that the three major isoforms differed from each other by single base pair substitutions at either residue 112 or 158.

Population studies established the frequency distribution of the major alleles and the strong association between the E2/E2 form and the occurrence of dysbetalipoproteinaemia. It also became clear that hyperlipidaemia was not the inevitable consequence of E2/E2 status and that most subjects who are homozygous for ApoE2 in fact were hypolipidaemic. Patients with typical dysbetalipoproteinaemia and non E2/E2 status were also found, leading to the description of multiple other ApoE mutations, usually causing dysbetalipoproteinaemia in an autosomal dominant fashion. ( See Table 4.2)

Hixson and Vernier(85) described a rapid method for genotyping ApoE based on PCR amplification of DNA and subsequent digestion of DNA by restriction enzyme and separation of restriction fragments by length in polyacrylamide gels in 1990. A 244 basepair region, encompassing both sites of mutation for the major genotypes, is amplified by PCR with two oligonucleotide primers and the product digested with *Hha I*. The resulting fragments after separation by electrophoresis allow

classification of the patient's genotype. ApoE with the 145 ( R→C ) mutation is classified as ApoE2 with isoelectric focussing while it is ApoE3 by genotyping(86) according to Hixson and Vernier.

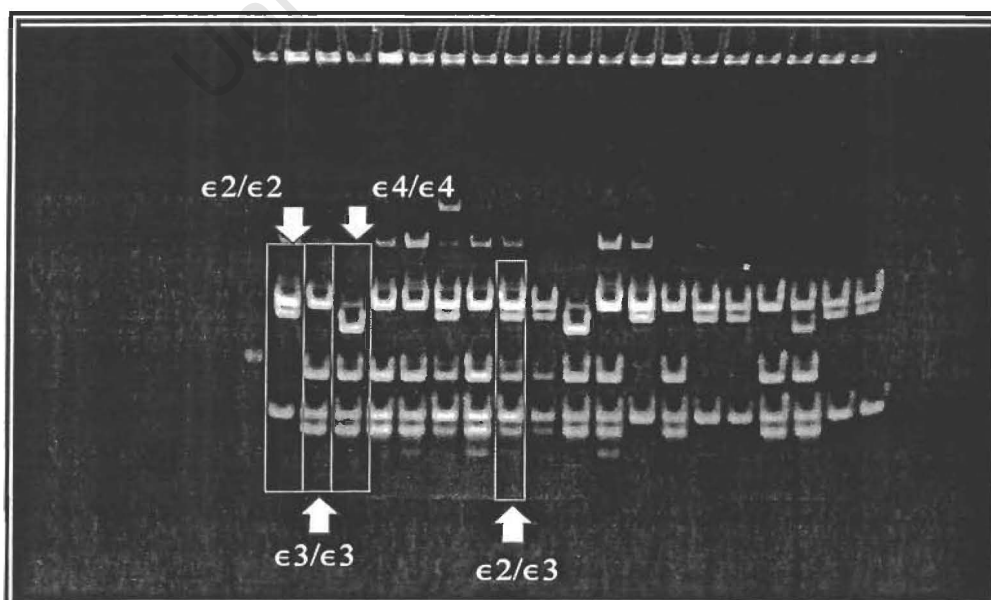
Genotyping used in the right setting can therefore certainly be confirmatory of the suspicion of dysbetalipoproteinaemia, remaining aware of the fact that dominant ApoE mutations are not detected by the routine methods of genotyping. The degree to which one can rely simply on determination of E-isoform for diagnostic purposes will therefore be directly dependant on the local prevalence of other ApoE-mutations. Certainly the absence of E2/E2 cannot be taken as an absolute indication that dysbetalipoproteinaemia is not present.

The following picture illustrates the genotyping of ApoE according to the method of Hixson and Vernier (85)

The first three highlighted lanes illustrate the ApoE2/E2, ApoE3/E3 and Apo E4/4 homozygous states. The next highlighted lane shows a E2/E3 heterozygote.

### **Fig 5.6**

ApoE genotyping according to Hixson and Vernier



## Chapter 6

### **Non -Denaturing Gradient Gel Electrophoresis (GGE )in the Diagnosis of Dysbetalipoproteinaemia**

#### 6.1 Introducing Gradient Gel Electrophoresis

Previously multiple electrophoretic techniques were reviewed for the diagnosis of dysbetalipoproteinaemia. Nearly all these methods separate lipoproteins according to their charge. Using the technique of gradient gel electrophoresis ( GGE ) lipoproteins are separated according to size and not charge. This technique is therefore conceptually similar to the separation of lipoproteins on agarose columns as described by Shepherd (73;74) The advantage is that with GGE it is not necessary to isolate lipoproteins by ultracentrifugation as they can be selectively stained with a lipid stain. In addition the gels are much smaller than columns and technically much less demanding, not requiring the collection and analysis of multiple eluent fractions. It is also possible to run multiple specimens simultaneously on one gel, allowing for direct comparisons between multiple samples.

To make acrylamide gels, acrylamide monomer is mixed with chemicals which generate free radicals and a linking agent is added. The acrylamide polymerizes in these conditions and forms a three-dimensional mesh. The length of the polymers is dependant on the concentration of acrylamide used. The amount of linking agent added will determine the extent of cross-linkage that occurs. The usual linking agent used is N', N'-methylene bisacrylamide. . In a gradient gel, in which the concentration of acrylamide rises progressively, the size of pores will therefore decrease with an increase in acrylamide content. Particles will therefore be able to migrate to a certain point in the gel, dependant on their own size and the running conditions. Small particles migrate the furthest in the gel.

Regarding lipoprotein work gradient gel electrophoresis has been used mainly as

an alternative method to ultracentrifugation to study subspecies of HDL and LDL. Only small volumes of serum are needed and the cost of equipment is much less. Many more specimens can be handled this way than with density gradient ultracentrifugation. (87-90) Both methods show good correlation with each other, although the physical characteristics used to separate particles are different. Density gradient ultracentrifugation separates particles according to buoyant density while GGE separates according to size. These two physical properties are however closely correlated (90) and therefore give similar results.

## 6.2 Description of Technique in the UCT/GSH Lipid Laboratory(91)

The method currently in use in the UCT/GSH Lipid Laboratory was devised in 1990. Polyacrylamide gels are freshly prepared in the laboratory with a polyacrylamide gradient from 2-8 %. The stacking gel has a 3% polyacrylamide concentration. The minigel so created is about 6 cm long. The gel is sandwiched between two glass plates during electrophoresis. Fifteen lanes can be run per gel. . Specimens are usually run in lanes 1-6 and 9-12. The middle lanes seven and eight are used to run control specimens to permit standardization between gels. In lane seven a specimen with a large LDL species ( termed A- see text further down for the nomenclature of species in this system) is run while lane eight carries a small dense LDL species (70) as a marker. To stain the lipoproteins, 100 µl of plasma is mixed with 50 µl of Sudan Black lipid stain and left to incubate for an hour. This mixture is subsequently spun ( 20 min at 10000G ) and the supernatant taken off and mixed with an equal volume of saturated sucrose solution. The denser sample makes for better loading. Twelve µl of this final mixture is loaded in each well and the gels are subsequently run for 18-24 hours at 130V at a temperature of 4° Celsius. Gels are prefocussed at 20V for 30 min before running.

After the gels have been run, they are removed from the minigel apparatus. As the samples have been prestained with Sudan Black, the gels do not need to be removed from the glass sandwich for further handling. Permanent records are

obtained in the following ways:

- Gels are photocopied (on a background of white paper ) and a copy of every gel is kept. On the photocopy the samples in each lane are identified, together with the report given
- Slides are taken of the gel and stored
- From July 1998 onwards all gels have been scanned and recorded using the GEL DOC system from Bio Rad. In this system gels are scanned using a video camera and the image is stored as an electronic file for subsequent analysis. The GEL DOC software allows the user to change the viewing properties of the gels, select lanes and display densitometric profiles of the lanes. It is also possible to produce good quality printed images of the gel. Data can also be exported to be analysed by other software packages.
- All gel reports are captured in a Corel Paradox database, identifying the gel and the lane in which a particular patient's specimen can be found

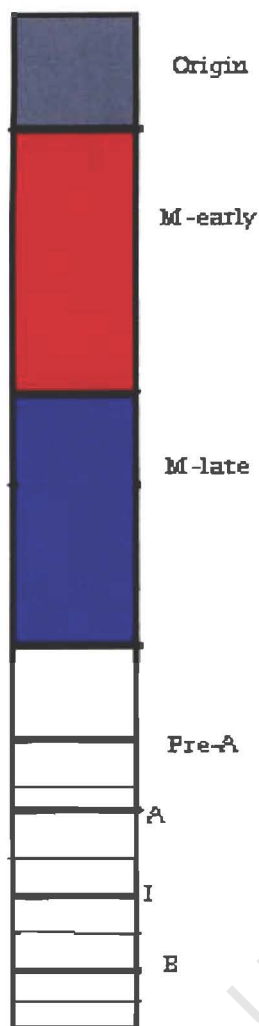
### 6.3 Reporting of gels

All gels have been reported on by a single observer. ( D Marais ). The total number of lanes run on this system stood at about 17800 in March 2001. The gels are all read blindly without knowledge of the patient's identity or clinical data, including the lipid values at time of electrophoresis. After the gels have been reported on, the patients may be identified and patients with interesting patterns selected for further studies.

As the lipoproteins are separated according to size, with size being inversely proportional to migration distance the largest lipoproteins are found at the origin. Large particles of chylomicron size do not migrate and remain at the origin. Such staining is referred to as O. Particles migrating further than the origin but not into the " LDL area" stain in the midzone of the gel and are termed M. The M region is quite large, making up about two thirds of the gel. Therefore lipoproteins migrating to the beginning of the M zone are termed M-early ( M-e) while those migrating

further are termed M-late ( M-I ). Particles that migrate to the lower third of the gel are termed to be in the " LDL" area. The largest particle is assigned the letter A while the smallest particle is termed B. Faintly staining bands are indicated by lowercase letters. The assignment of lowercase or upper-case letters in the reporting is subjective by nature, but as all reports have been give by a single observer at least the problem of inter-observer variability is eliminated. I is found in an intermediate position between A and B and further subdivisions are made- termed Aearly (Ae), A/I and I/B. Particles that migrate further than the B marker are termed B-post. Unusually an extraordinary large LDL may be seen above A, and is termed pre-A. It represents Lipoprotein (a) (Lp(a)). This can be demonstrated by adding a reducing agent to the specimen, breaking the disulfide bond in Lp(a). The pre-A band is then no longer visible on the gel.

This particular gel system has a maximal acrylamide concentration of 8% and is therefore not " designed" for evaluation of HDL species which require gels with smaller pore sizes. In the laboratory a separate system of gels is run for HDL, here a gradient of 4-18% acrylamide is utilised. With the " LDL gel " HDL is not visible at all. A schematic drawing illustrates the way gels are reported.



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## 6.4 Retardation Factors

To quantitatively describe the migration of lipoprotein species and to allow for slight differences between gels, it is useful to relate the distance travelled to a standard, deriving a numerical expression. This can be done by describing the retardation factor ( $R_f$ ) of any given species. To calibrate the system it is useful to assign the  $R_f$  value of 1.0 to the B species on the gel, as this is the species that will generally migrate the furthest. The migration of all other species can then be described as a fraction—the distance travelled by the species in question divided by the distance travelled by the B marker.

The minigel system has been standardised against ultracentrifugally isolated plasma fractions—this standardisation indicates how far lipoproteins of a certain density will migrate in the gel.(91)

**Table 6.1**

Lipoprotein migration according to Retardation Factor

Ultracentrifugal fraction	Retardation factor
VLDL 1 ( Sf 60-400)	0.2-0.45
VLDL 2 ( Sf 20-60)	0.45-0.7
IDL ( Sf 12-20)	0.7-0.85
LDL (Sf 0-12)	0.85-1.0

## 6.5 Rationale for evaluating GGE in the diagnosis of Dysbetalipoproteinaemia

Diagnosing dysbetalipoproteinaemia is still difficult and the ultracentrifugal tests or

genotyping needed are not generally available. These tests can only evaluate a limited number of specimens simultaneously and are generally technically fairly demanding and time-consuming.

It is therefore very desirable to have a test available that will screen patients with mixed hyperlipidaemia with a higher degree of accuracy than is currently possible with standard agarose gel electrophoresis. Patients who have a pattern compatible with dysbetalipoproteinaemia could then either directly proceed to genotyping ( which is more widely available than ultracentrifugation- requiring only facilities for PCR ) or have ultracentrifugation done. Using GGE it is possible to screen large numbers of samples, making it a suitable technique for use in population surveys etc.

During the course of examining GGE for LDL species, it was noted that there may be a specific pattern in the M1 fraction of the gel, with absent or variable intensity of LDL staining at the position of LDL B. This called for a closer examination to evaluate the utility of the GGE as a diagnostic tool for dysbetalipoproteinaemia in the context of a mixed hyperlipidaemia.

## 6.6 The Pattern of Dysbetalipoproteinaemia

In patients with overt hyperlipidaemia secondary to dysbetalipoproteinaemia the excess cholesterol and triglyceride is found mainly in remnant particles. These remnant particles have abnormal chemical composition when compared to normal pre- $\beta$  VLDL as well as differing in size.

**Table 6.2**

Particle Diameters of Various Lipoproteins

Lipoprotein	Mean Particle Diameter in nm
Intestinal $\beta$ -VLDL(=remnant particles)	82
Hepatic $\beta$ -VLDL	38
Hepatic pre- $\beta$ VLDL	44

Source(1)

Patients with dysbetalipoproteinaemia also generally have low levels of LDL and this is a useful pointer towards the diagnosis.

In patients with dysbetalipoproteinaemia one would therefore expect to find little or no LDL staining with GGE, with most of the stain being found in the region of the gel in which particles of the size corresponding to small VLDL and IDL are found.

In a preliminary review (92)of the GGE experience at the UCT Lipid Clinic 27 patients with dysbetalipoproteinaemia ( on chemical criteria of VLDL composition ) were compared with 22 subjects with similar lipid values but negative chemical ratios. The M only pattern was found in 48% of these patients and in no other patients and is therefore highly specific for dysbetalipoproteinaemia. A M pattern with a faint LDL band was seen in 18% of dysbetalipoproteinaemic patients but only in 9% of

controls.

To further evaluate the utility of GGE in the diagnosis of dysbetalipoproteinaemia all gel-reports issued for patients with known dysbetalipoproteinaemia were reviewed and compared with a control group of other patients with mixed hyperlipidaemias. In addition a quantitative technique was developed, analysing the area under the curve on the densitometric profile of the gel.

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## Chapter 7

### Methods

#### 7.1 Analysis of GGE Reports

By linkage of databases all GGE ever done on patients with **unequivocal** dysbetalipoproteinaemia ( defined here as patients with a genotype known to be associated with dysbetalipoproteinaemia as well as positive ratios (  $C_{VLDL} / TG_{VLDL} \geq 0.42$  or  $C_{VLDL} / TG_{Plasma} \geq 0.30$  ) on analysis of VLDL composition on at least one occasion ) were found. Currently there are results of about 700 ultracentrifugal analyses on 519 patients with mixed hyperlipidaemia. The corresponding GGE reports were found and analysed in a descriptive fashion. GGE done at time of presentation in untreated patients were evaluated in a sub-analysis. The GGE reports were compared to those obtained from a control group of patients with mixed hyperlipidaemia but negative ultracentrifugation. The criteria for selection were:

- Ultracentrifugation done and negative on every occasion when performed
- TC >7.0 mmol/l and TG between 3 and 10 mmol/l

These patients all were initially suspected of possibly being dysbetalipoproteinaemic, leading to ultracentrifugation being performed. This group therefore does represent the patients who need differentiation between dysbetalipoproteinaemia and other dyslipidaemias.

#### 7.2 Patient selection for quantitative analysis of gel-calculation of area under the curve:

For inclusion patients had to fulfill the following criteria

- $C_{VLDL} / TG_{VLDL} \geq 0.42$  or  $C_{VLDL} / TG_{Plasma} \geq 0.30$  by mass

- GGE performed **at time of ultracentrifugation on the same specimen**
- GGE performed after July 1998 and therefore captured on the GEL DOC system
- Control patient found in database with closely matched lipid values but non-diagnostic ratios on ultracentrifugation and simultaneous GGE available for analysis.
- GGE on patient and control technically satisfactory

In this selection patients with all genotypes are included, they are subsequently analysed separately according to genotype status in a sub-analysis.

### 7.3 Analysis of gels

The scanned image of the gel was located on the hard drive of the GEL DOC computer and subsequently compared to the photocopy stored, to ensure that the gel had been labelled and identified correctly. The lane carrying the sample of interest was identified both from the results database and the hardcopy gel reports.

In the GEL DOC system the lane of interest was manually selected on the screen by overlaying the lane with the lane selection tool. The top of the lane was positioned at the junction of stacking and separation gel ( easily visible on the scans ) while the lane was extended to run the length of the gel to the position of the B marker plus about ten % extra distance added on-giving a  $R_f$  of about 1.1 at the end of the lane. A profile of the lane can then be displayed automatically by the GEL DOC system. In this profile the optical density is plotted on the Y-Axis with distance migrated( =D ) plotted on the X -axis. The GEL DOC system measures the distance migrated from the top of the scanned image, irrespective whether this is the actual top of the gel or not. The distance measured in cm will therefore be dependant on the positioning of the gel in the scanner and how far the edge of the gel is from the scanned area. The lane will therefore not commence at 0 cm with the separation gel, but at a distance, noted as S=Start ( in cm ).

The profile of the gel was exported from GEL DOC to a Microsoft Excel spreadsheet. When the profile is thus exported, Excel generates a graph of the profile and two columns detailing optical density and migration distance. In Excel these columns were selected and copied into a new spreadsheet. This second spreadsheet is then saved onto a floppy disk as a .txt file. This floppy is then used to import the data into a second PC running GraphPad Prism. This two stepped approach was necessitated by the fact that the GEL DOC system runs on a different operating system from the other computers at the university and is therefore not linked to the university network. To make the data available for use with other computers it is therefore necessary to manually export it from the GEL DOC computer.

Once the data has been imported into Prism the following manipulations are carried out

- Subtract S from all migration distances ( D ) and make a new results table of D-S. This adjusts the graph so that the start of the profile is at value zero cm.
- The graph is subsequently standardised so that the B marker will lie at 1.0

#### 7.4 Standardising the Graph

As the gels are not mass produced and electrophoretic conditions may vary slightly no absolute value for B marker migration can be derived. As each gel has two control lanes, these can be utilised to calculate the  $R_f$  within each gel.

While analysing the gels, several gels were found in which the B marker had not migrated appropriately. This results from prolonged storage of control plasma ( even in the frozen state ) in which the small dense lipoproteins tend to spontaneously acquire additional lipid and enlarge in size. The marker used for B therefore becomes unsuitable after a few weeks and has to be replaced. Unfortunately there were still

several gels which did not carry a B migrating marker and could therefore not be standardised using this marker. The A marker is however very stable and does not undergo significant size change with time and was therefore found to have appropriately migrated on every gel. To utilise the A marker its position relative to the B marker was determined by examining a sample of 15 gels that carried both markers unambiguously. Migration distance of A relative to B was calculated and found to be 0.88 ( SD 0.014 ).

In every gel analysed the migration distance of A was therefore measured and noted as AM(=A-marker)

To standardise the graph the results table containing migration distances corrected for S was divided by a standardisation factor.

Standardisation factor =  $(A - M - S) * 1/0.88$

The results were graphed again. The resulting graph had a X-axis ranging from zero to about 1.1 to 1.2, depending on how much of the terminal part of the lane had been included in the overlay. AM is positioned at 0.88 with B at 1.0. The optical density ( OD ) values remain unchanged during these initial manipulations.

#### 7.5 Baseline Correction:

It is important to subtract the baseline from the graph as calculations of the area under the curve (AUC) otherwise become invalid. This is readily appreciated if considering a graph that is completely flat ( slope=0) from the origin to midgel. With a baseline OD of more than zero an area will be calculated under this section that may make up a significant portion of total calculated area under the curve. This area however does not correspond to the staining of any lipoprotein but simply represents the optical density of the gel itself. Baseline correction was performed by subtracting the optical density measured on unstained gel from all the optical density values.

A final graph is thus obtained which can now be analysed for AUC.

#### 7.6 Calculating the area under the curve:

Prism can calculate the area under the curve for any given graph. The area under the curve is calculated using the trapezoid rule. The software is also able to find peaks in a graph according to user-defined criteria. The user can either define a peak as having a specified minimum height or percentage rise above baseline. In addition the minimum width of a peak can be defined as well. Prism does however not allow the user to specify points directly between which the area under the curve needs to be calculated. As our aim was to calculate the area under the curve of each segment of the gel as previously standardised with the ultracentrifugal fractions, Prism was not ideally suited to this task. The problem was overcome by using the facility that Prism offers to “prune rows”. This function allows user-defined restrictions on the X-axis values allowing segments of the graph to be analysed individually. With this function datasets were generated covering the following areas.

Table 7.2

#### Labels for Retardation Factor Ranges

Retardation factor Range	Term applied
0.0 to 1.1	Total area under curve
0.0 to 0.2	Area I
0.2 to 0.45	Area II
0.45 to 0.7	Area III
0.7 to 0.85	Area IV
0.85 to 1	Area V
1.0 to 1.1	Area VI

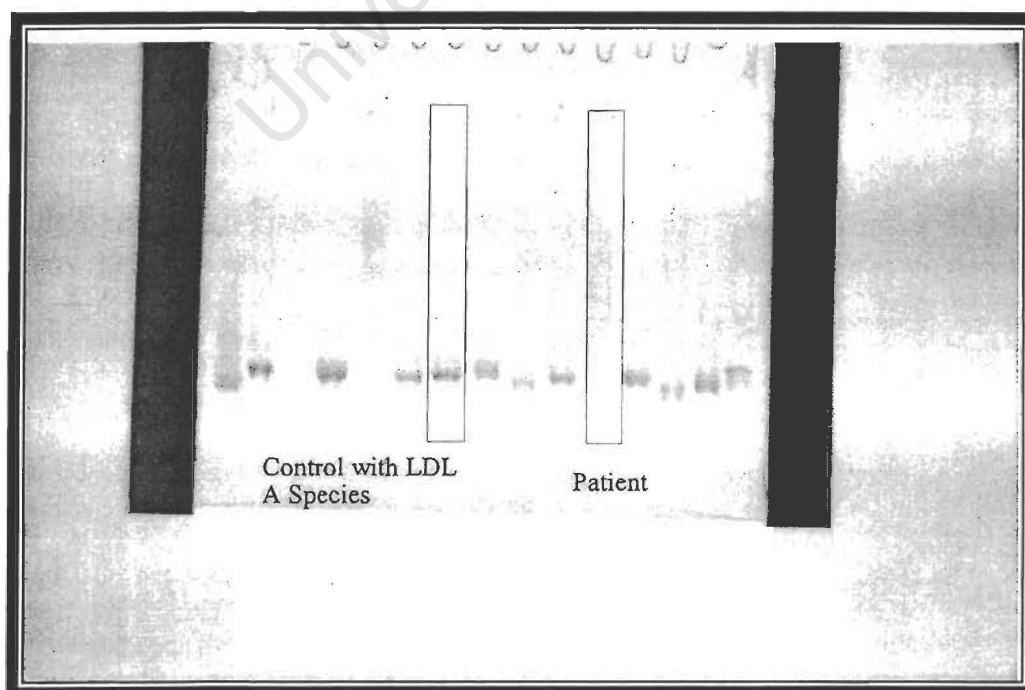
The area under the curve was calculated by Prism for each of the above datasets. In addition the total area calculated by Prism was compared to the total area obtained as the sum of all the areas from I to VI to validate that the subdivisions of the dataset had been correct. Each area was then expressed as a percentage of the total area. The decision to express the areas as a relative percentage was taken as absolute areas on the gels are difficult to compare due to differences in staining intensities of the gels. This may well relate to the freshness of the Sudan Black and the acrylamide, which both do influence the final appearance of the gel.

All the patient and control scans were analysed in this way and the results collated in a Quattro Pro spreadsheet. The results were subsequently statistically analysed using the statistical functions of the Quattro Pro software package as well as InStat, a statistics programme developed by GraphPad

### 7.7 Examples

The following is an illustration of the procedure described above.

**Fig 7.1**



The gel is that of a patient with dysbetalipoproteinaemia that displays the typical pattern of M only with no LDL visible

Lipid Values at time of running gel

Total Cholesterol 5.6 mmol/l

Triglyceride 2.4 mmol/l

$$C_{VLDL}/TG_{VLDL} = 0.46$$

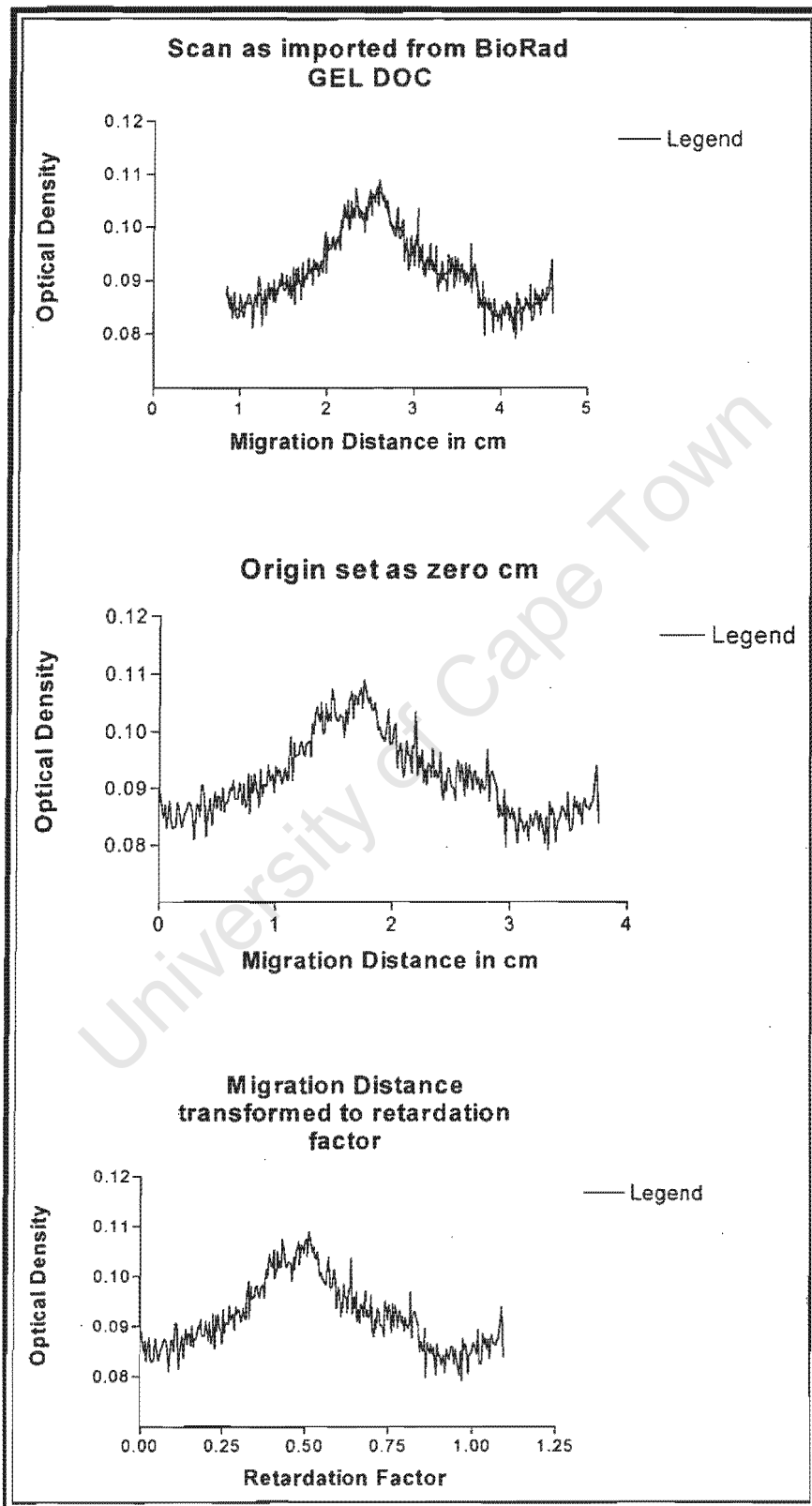
$$C_{VLDL}/TG_{Plasma} = 0.34$$

Note: This gel is somewhat unusual in that both markers in lane seven and eight have nearly identical migration patterns. Lane seven is highlighted in addition to the patient. It is possible that the same marker was loaded in both lanes as there is a faint Pre-A visible in both lanes. In lane eight the LDL band is however not as sharply defined as in lane seven. The marker in lane eight would have been expected to migrate to about the position of the LDL band in lane five.

The following page illustrates the graphs that are obtained from the densitometric profile and their subsequent processing.

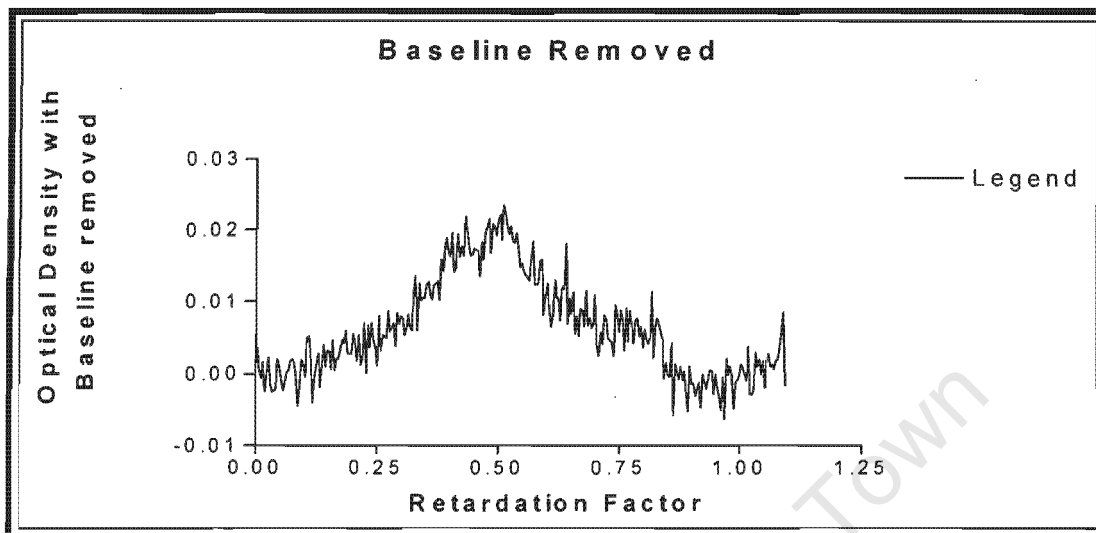
### **Fig 7.3**

#### **Processing of Densitometric Profile**



**Fig 7.4**

Final Densitometric Profile



The last scan is the scan that is utilised for calculating the area under the curve.

The results for this patient are given as an example.

**Table 7.2**

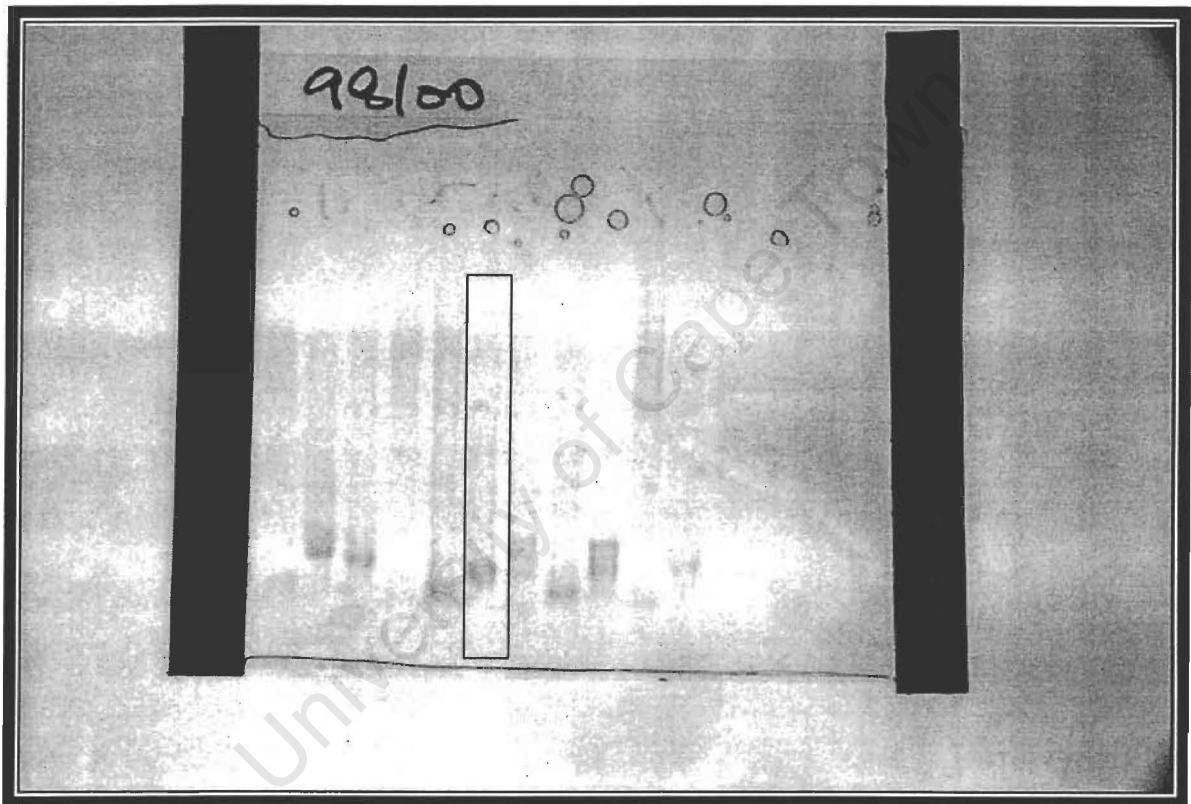
Area Calculation

Area designation	Absolute Area	Percentage of total ( % )
I	0.0003976	5.25
II	0.002437	32.19
III	0.003457	45.67
IV	0.0008560	11.30
V	0.0002416	3.18
VI	0.0001800	2.38
Ratio Area IV/V	3.553	

The following scan served as a control for this patient

**Fig 7.5**

Control Hyperlipidaemia

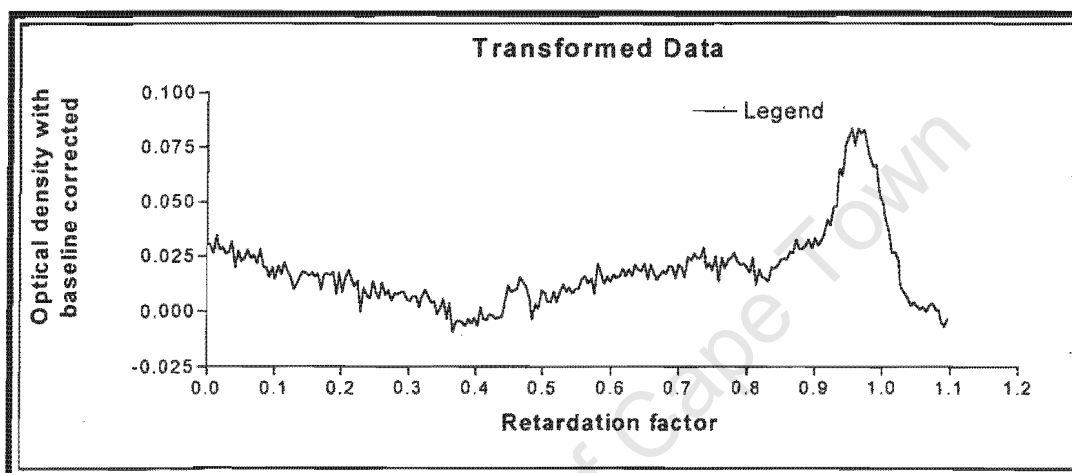


Note this gel is sub-optimal in quality, containing air-bubbles, but the lane of interest does remain interpretable

This is the final edited tracing of the scan

**Fig 7.6**

Control Hyperlipidaemia Densitometric Profile



Lipid values are

TC 5.6 mmol/l

TG 2.2 mmol/l

$C_{VLDL}/TG_{VLDL}=0.28$

$C_{VLDL}/TG_{Plasma}=0.17$

The areas calculate as follows

**Table 7.4**

Area Calculation for Control Hyperlipidaemia

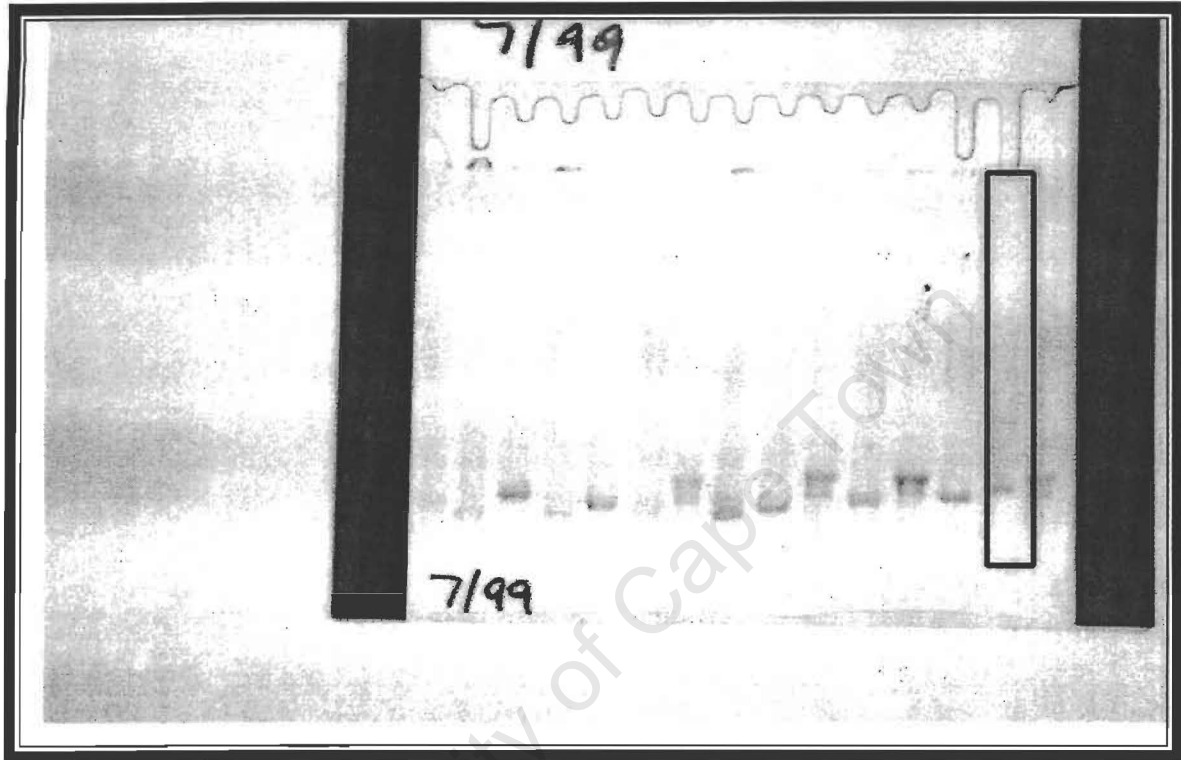
Area	Percentage
I	20.04
II	7.59
III	16.22
IV	14.68
V	36.03
VI	5.44
Ratio Area IV/V	0.407

Visual inspection of the tracings shows that the control scan has a strong and dominant LDL band, while this is absent in the patient with dysbetalipoproteinaemia. Calculation of the area under the curve confirms this impression.

The patient chosen as illustration here exhibits the pattern most strongly associated with dysbetalipoproteinaemia, VLDL/IDL staining only with no visible LDL staining. In these cases visual inspection of the gel will often strongly suggest the diagnosis. Difficulties arise when there is faint staining in the LDL area as well as VLDL/IDL staining as this pattern is also found in other mixed hyperlipidaemias. The following example shows the graphs of a patient with dysbetalipoproteinaemia and the control patient. The graphs illustrate the utility of the area under the curve analysis in distinguishing between the two patterns.

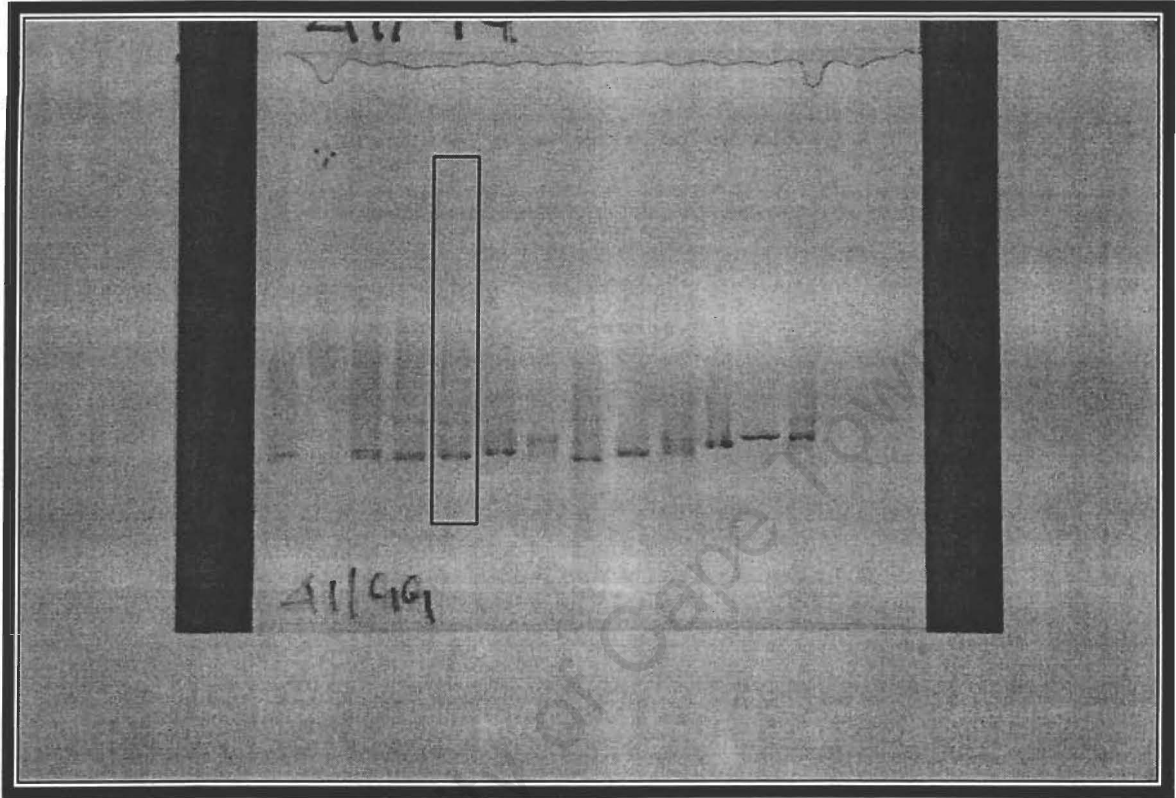
**Fig 7.7**

Dysbetalipoproteinaemic Patient



**Fig 7.8**

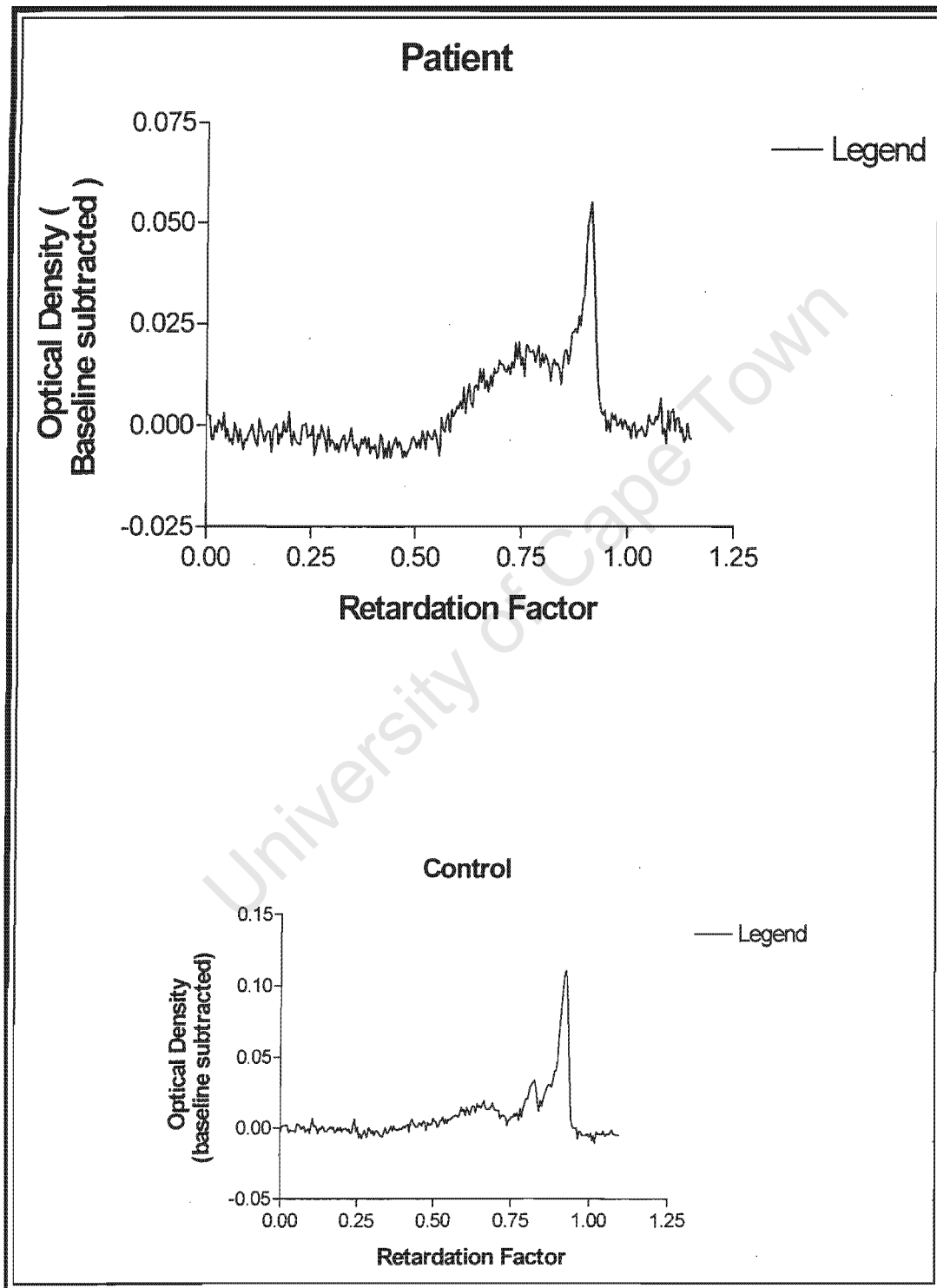
Control Hyperlipidaemia



The graphs generated from the two scans are illustrated below

**Fig 7.9**

Densitometric Profiles of Patient and Control Hyperlipidaemia



Note that the control graph shows a minor peak before the major LDL peak, this minor peak represents Lp ( a), or pre-A using the GGE terminology.

The relevant lipid values and area values are given in the following table.

**Table 7.5**

Data for Patient and Control Hyperlipidaemia

	Patient	Control
Total Cholesterol	6.4	6.3
Triglycerides	3.6	3.4
$C_{VLDL}/TG_{VLDL}$	0.43( 5 of 6 ultracentrifugations positive)	0.24 ( 3 ultracentrifugations performed- all negative)
$C_{VLDL}/TG_{Plasma}$	0.23	0.18
Genotype	E2/E2	Not known
Area I	5.52	3.00
Area II	13.13	5.68
Area III	18.56	20.67
Area IV	29.23	19.92
Area V	31.52	46.63
Area VI	2.03	4.08
Ratio Area IV/V	0.9273477	0.4271928

### 7.8 Statistical Analysis

All statistical analyses were performed using commercially available software packages

- Graph Pad InStat
- Graph Pad Prism
- Corel Quattro Pro

Variables are expressed as mean  $\pm$  standard deviation. Analysis of variance is by unpaired *t*-test for continuous variables. Categorical variables were analysed with the chi-square test. A p-value of less than 0.05 was taken as being statistically significant.

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## Chapter 8

### Results

#### 8.1 Description of GGE Reports

There were 68 patients with analysable GGE that were genotype positive ( E2/E2 or E145 mutation ) and also had at least one positive ultracentrifugation for dysbetalipoproteinaemia. Some of the genotype positive patients who presented more than 15-20 years ago have not had GGE performed. In total there were 222 GGE runs on genotype positive dysbetalipoproteinaemic patients. Of the 222 GGE done, 218 had been reported on. The remaining four could not be reported on because of failed loading of serum onto gel or other technical problems. For analysis the reports were grouped into four categories.

- Reports indicating presence only of VLDL or IDL species and no staining in the LDL region ( M, Mearly, Mlate, Monly, O M)
- Reports indicating staining in the VLDL and IDL region as well as the presence of a faint band in the LDL region, denoted by a lowercase letter ( M b, M a, M i .....)
- Reports indicating staining in the VLDL and IDL region as well as a clearly discernible band in the LDL region, indicated by upper-case letters ( M B, M A, M I ...)
- Reports indicating staining only in the LDL area ( B, A, I .....)

**Table 8.1**

#### Reports of Dysbetalipoproteinaemic Patients

Report	Number	Percentage
VLDL/IDL only	105	48%
VLDL/IDL+faint LDL	60	28%
VLDL/IDL+distinct LDL	36	16%
LDL only	17	8%

The control hyperlipidaemic group contained 145 electrophoresis runs that could be analysed.

**Table 8.2**

Reports for Control Hyperlipidaemia

Report	Number	Percentage
VLDL/IDL only	4	3%
VLDL/IDL + faint LDL	7	5%
VLDL/IDL +distinct LDL	104	72%
LDL only	30	21%

As can be seen the reports are clearly different amongst these two groups with the control patients having much more LDL staining than patients with dysbetalipoproteinaemia. The p-value using the chi-square test is less than 0.0001. The VLDL/IDL only pattern that is common in dysbetalipoproteinaemia was found twice in two control patients who are both clinically still strongly suspected of having dysbetalipoproteinaemia ( based on their excellent response to therapy )but have not had diagnostic ultracentrifugations. Their genotypes are currently being determined.

GGE's that only show VLDL/IDL staining are therefore highly specific ( 96% ) for a diagnosis of dysbetalipoproteinaemia with the sensitivity being 48%. Combining the categories of VLDL/IDL staining only and VLDL/IDL staining with faint LDL the sensitivity improves to 76%. The finding of VLDL/IDL staining and faint staining in the LDL area was found in 28% of patients and 5% of controls. On its own it is therefore not very sensitive ( 28% ) but has a reasonable specificity of 89%.

8.2 Untreated Dysbetalipoproteinaemic Patients

Thirty-nine genotype and ultracentrifugation positive patients were found that had

GGE performed at their initial presentation and in addition had not been given any lipid modifying therapy prior to being seen. The untreated state was verified by review of patient records. The GGE reports were distributed as follows:

**Table 8.3**

Reports for Untreated Dysbetalipoproteinaemic Patients

Report	Number	Percentage
VLDL/IDL only	28	72%
VLDL/IDL + faint LDL	8	20%
VLDL/IDL +distinct LDL	3	8%
LDL only	0	0%

The distribution of reports is significantly different (  $p=0.0037$  by chi-square test ) when this group of untreated patients is compared to the remaining GGE done on patients with dysbetalipoproteinaemia in the treated state. The untreated patients presented with a mean cholesterol of 12.18 mmol/l ( SD 5.4 with a range of 6.7-34.0) and mean triglycerides of 6.78 mmol/l ( SD 5.207 and range 2.4-25.6 ). In spite of the marked hypercholesterolaemia very little staining was seen in the LDL area, with most of the particles in the VLDL/IDL size range. This reflects the accumulation of remnant particles which are not taken up and catabolized appropriately. There is poor conversion of VLDL-remnants to LDL and low levels of LDL result. The effects of treatment were studied by identifying all patients in whom an initial GGE off therapy was available and who had subsequently had at least one further GGE performed while they were on therapy and had improved lipid values ( as a surrogate marker of compliance and therapeutic efficacy ).

Thirteen patients fulfilled these criteria. In these patients the cholesterol decreased by a mean of 6.25 mmol/l with triglycerides showing a mean drop of 5.18 mmol/l. The distribution of the initial reports was as follows.

## Table 8.4

Reports for Dysbetalipoproteinaemic Patients in the Untreated State where Treatment Effect was Studied

Report	Number	Percentage
VLDL/IDL only	8	62%
VLDL/IDL + faint LDL	2	16%
VLDL/IDL +distinct LDL	3	22%
LDL only	0	0%

This group is not significantly different ( $p=0.3196$  by chi-square test) from the group comprising other untreated patients. With treatment the following changes occurred

- Of the eight patients with only VLDL/IDL sized particles visible initially, four subsequently had staining ( usually faint ) in the LDL area on the gel. The other four patients did not develop a visible LDL species. The size of the VLDL/IDL species visible did become smaller in all patients as their lipid values improved, this is reflected by a change from M-early to mainly M-late staining.
- The two patients with faint LDL species remained unchanged
- Patients with a distinct LDL species never lost their LDL species, in one patient it was thought to be less distinct and labelled as faint once she was receiving therapy

### 8.3 Analysis of Area under the Curve for Dysbetalipoproteinaemic Patients

Sixteen patients were found with suitable gels and controls for analysis. The gels reflect a cross-section of both treated and untreated patients.

### 8.3.1 Characterisation of Patients at time of Gel Electrophoresis and Ultracentrifugation

Female : 6

Male:10

**Table 8.5**

Lipid Values of Dysbetalipoproteinaemic Patients at Time of Ultracentrifugation

	Triglycerides mmol/l	Total Cholesterol mmol/l
Mean	3.812	7.14
Median	2.95	6.6
Range	2.2-7.9	4.3-11.3
Standard Deviation	1.95	1.755

### 8.3.2 Characterization of VLDL chemical composition (mass ratios)

The ratio of cholesterol in VLDL to triglyceride in VLDL ranged between 0.42 and 0.82 with a mean of 0.5088 and a standard deviation of 0.1106. The mean ratio of VLDL cholesterol to plasma triglycerides was 0.3069 with a range between 0.14 and 0.49 and a standard deviation of 0.09673. The first ratio was positive in all patients while both were positive in eight patients.

### 8.3.3 Genotype of patients

Seven patients had the E2/E2 genotype while there were two patients with the E145 mutation. In one patient the genotype is not known as yet and six patients have

dysbetalipoproteinaemia by chemical criteria with the ApoE mutation being unknown. Comparing the VLDL composition of patients with known mutations to that of other patients both ratios have a higher mean in the patients with known mutations, but this does not quite reach statistical significance. ( Mean gene+ 0.51; mean gene- 0.44  $p=0.1005$  for the ratio within VLDL. Mean gene + 0.34 and mean gene - 0.25 and  $p=0.063$  for the ratio of  $C_{VLDL}/TG_{Plasma}$  )

#### 8.4 Analysis of Area under the Curve for Control Patients

##### 8.4.1 Characterisation of Control Patients

Males: 10

Females: 6

The mean difference between the total cholesterol of the patients and controls was 0.03 mmol/l ranging from -0.5 to 1.3 mmol/l. The triglycerides were matched with a mean difference of 0.0125 mmol/l but a wider range of -4.1 to 1.9 mmol/l. The extremes of range given are the only two patients where a match within 0.5 mmol/l could not be found.

##### 8.4.2 Characterisation of VLDL chemical composition ( mass ratios )

As expected the chemical composition of VLDL differs significantly (  $p < 0.001$  ) between the patients and the control group. Results are illustrated in a table.

#### **Table 8.6**

##### VLDL Chemical Composition

	Patients	Control HL
$C_{VLDL}/TG_{VLDL}$ Mean	0.5088	0.2243
$C_{VLDL}/TG_{VLDL}$ Median	0.4650	0.2050
$C_{VLDL}/TG_{VLDL}$ Standard Deviation	0.1106	0.05785
$C_{VLDL}/TG_{VLDL}$ Range	0.42-0.82	0.14 -0.35
$C_{VLDL}/TG_{Plasma}$ Mean	0.3069	0.16437
$C_{VLDL}/TG_{Plasma}$ Median	0.3150	0.1650
$C_{VLDL}/TG_{Plasma}$ Standard Deviation	0.09673	0.04676
$C_{VLDL}/TG_{Plasma}$ Range	0.14 to 0.49	0.07-0.25

The genotypes are not available for the control group, as patients that do not have chemical ratios diagnostic of dysbetalipoproteinaemia are in general not genotyped.

### 8.5 Results of Area Analysis

Each area on the gel was compared with the corresponding area of the control gel to look for differences in the relative proportion of total area under the curve occupied. The most striking difference was found when comparing Area V ( " LDL Area " ). The mean percentages here were 19.045 for patients with dysbetalipoproteinaemia and 40.448 for controls with a p-value of less than 0.0001. Regarding Area III ( "VLDL 2 " ) there was a difference in means ( 28.64 vs 16.91) that is significant with a p-value of 0.004. In the other areas no significant differences could be found. Patients with dysbetalipoproteinaemia therefore have more staining in the VLDL2 area and less in the LDL area than matched controls.

Although the percentage area under the curve is significantly different for some

portions of the gel, the overlap is too great for the areas alone to serve as a diagnostic test. However no patient with dysbetalipoproteinaemia had a LDL area of more than 45% while in no control the VLDL2 area was greater than 25%.

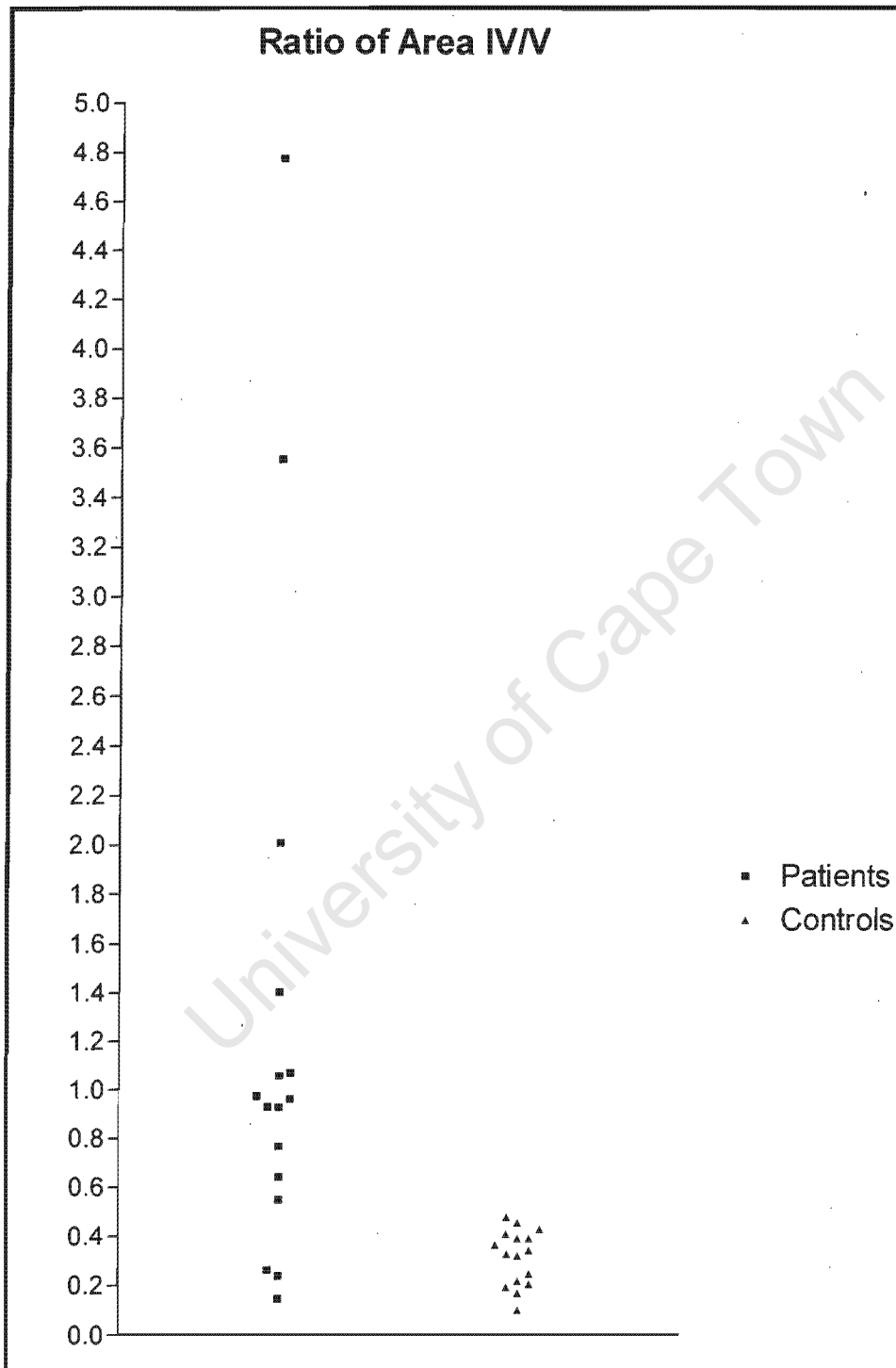
The best discrimination between patients and controls was found when a ratio of Area IV/Area V was derived

The distribution of this ratio is illustrated in the following graph.

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**Fig 8.1**

Distribution of Ratio Area IV/V for Patients and Control Hyperlipidaemia.



The following table reflects the actual numeric results

**Table 8.7**

Numeric Results for Fig 8.1

Patients	Controls
4.774319	0.3248312
2.005769	0.3194566
0.6396369	0.2434964
0.2364341	0.191301
0.9717314	0.3899083
0.7657658	0.2142523
3.553459	0.4074382
0.9273477	0.4271928
1.05527	0.1656714
0.9597781	0.3436682
1.066939	0.4547141
0.1458286	0.4767299
1.399439	0.3639207
0.9259259	0.390625
0.2608847	0.1001296
0.545737	0.2027538

**Table 8.8**

Statistical Data for Table 8.7

	Patients	Controls
Mean	1.265	0.3135
Median	0.9436	0.3342
Range	0.1458- 4.774	0.1001-0.4767
Standard Deviation	1.241	0.1135

P-value=0.0006 after logarithmic transformation of data and analysis by student *t*-test.

If a cut-off is selected at a ratio of 0.5 the following sensitivity and specificity result.

**Table 8.9**

Sensitivity and Specificity at Cut-off Ratio of 0.5

	Spin +	Spin-	Sum
Area Ratio +	13	0	13
Area Ratio -	3	16	19
Sum	16	16	

Sensitivity = 0.8125 ( 95% Confidence Interval 0.54-0.95 )

Specificity = 1 ( 95% Confidence Interval 0.79 -1 )

The three patients with positive spin ratios but area ratios of less than 0.5 were reviewed separately.

**Table 8.10**

Patients with Positive Ultracentrifugation but Area Ratio less than 0.5

	Patient 1	Patient 2	Patient 3
Sex	Male	Male	Male
Lipids at time of GGE	TC=6.4 TG=5.0	TC=6.7 TG=2.8	TC=7.4 TG=3.6
VLDL composition ratios at time of GGE	$C_{VLDL}/TG_{VLDL}=0.42$ $C_{VLDL}/TG_{Plasma}=0.18$	$C_{VLDL}/TG_{VLDL}=0.42$ $C_{VLDL}/TG_{Plasma}=0.28$	$C_{VLDL}/TG_{VLDL}=0.47$ $C_{VLDL}/TG_{Plasma}=0.34$
Number of ultracentrifugations ever performed	2	2	1
Mean ratios	$C_{VLDL}/TG_{VLDL}=0.355$ $C_{VLDL}/TG_{Plasma}=0.18$	$C_{VLDL}/TG_{VLDL}=0.365$ $C_{VLDL}/TG_{Plasma}=0.255$	$C_{VLDL}/TG_{VLDL}=0.47$ $C_{VLDL}/TG_{Plasma}=0.34$
Number of times positive on either ratio	1	1	1
Genotype	ApoE145 mutation present	Not known	E2/E3 ApoE145 mutation not present

Both the total cholesterol (  $p=0.94$  ) and the triglycerides (  $p=0.38$  ) are not significantly different amongst dysbetalipoproteinaemic patients who have a positive area ratio and those that have a negative ratio. The chemical composition within VLDL (  $p=0.15$  ) is also not significantly different as is the case for the ratio of cholesterol in VLDL to plasma triglycerides (  $p=0.54$  ).

Patient 1 clearly has to be classified as dysbetalipoproteinaemic with a positive ultracentrifugation and the presence of a mutation known to cause dysbetalipoproteinaemia. Patient 2 only has one positive ultracentrifugation and no confirmatory genotype is available and he may therefore falsely been classified as having dysbetalipoproteinaemia. Patient 3 has only had one ultracentrifugal study, but both ratios were positive making the chances of an incorrect classification less likely.

### 8.6 Analysis of Area under Curve by Genotype

A sub-analysis of the area ratios was undertaken with patients divided according to the presence or absence of a genotype known to be associated with dysbetalipoproteinaemia.

Patients with a positive genotype had significantly higher chemical composition ratios within VLDL ( Mean 0.56 vs 0.44  $p=0.038$  ) but the difference between the ratio of cholesterol in VLDL and plasma triglycerides did not quite reach statistical significance. ( Mean 0.34 vs 0.25  $p=0.06$ ).

There was no difference in the lipid values amongst the two groups, with a p-value for total cholesterol of 0.26 and triglycerides of 0.07.

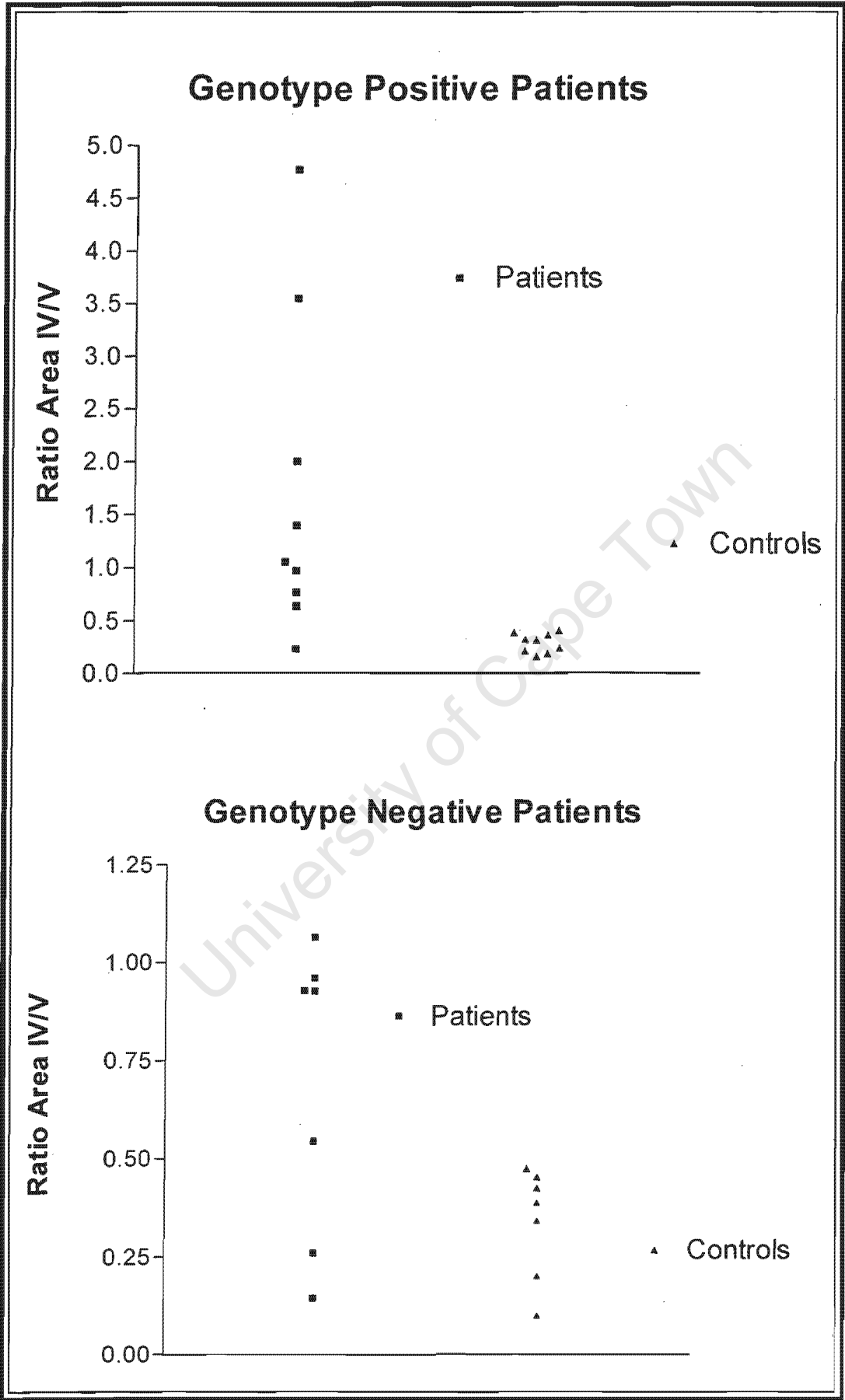
The sensitivity of GGE area analysis using an area ratio of 0.5 as a diagnostic criterion is 89% with a specificity of 100% in patients with a positive genotype. For the genotype negative patients the sensitivity is 72% with a specificity of 100%.

Genotype positive patients do not have significantly different area ratios (  $p=0.11$  ) from those in whom no dysbetalipoproteinaemic genotype has been identified. The area ratios in the two control groups were also not significantly different. (  $p=0.25$  )

A graphical illustration of the results divided by genotype is provided.

## **Fig 8.2**

Ratio of Area IV/V for Genotype Positive and Genotype Negative Patients



## Chapter 9

### **Discussion**

The diagnosis of dysbetalipoproteinaemia still presents considerable challenges to the physician today. In routine clinical practice patients with a mixed hyperlipidaemia will often not be further investigated, and a specific diagnosis is subsequently often not arrived at. Only patients that present with palmar crease xanthoma, that are recognised as such by the physician, can be confidently diagnosed on clinical grounds alone. A common differential diagnosis that is entertained in the context of a mixed hyperlipidaemia is that of FCH which is not believed to give rise to xanthoma.(2)The presence of a cutaneous tuberoeruptive xanthoma, though suggestive of dysbetalipoproteinaemia, is not as specific. The presence of a tendon xanthoma is compatible with dysbetalipoproteinaemia but is uncommon and could indicate FH and another defect raising the triglycerides. Not making a specific diagnosis will usually not result in immediate harm to the patient, as the response to dietary therapy and either HMG-CoA reductase inhibitors or fibrates is usually good(1). In the public health sector in South-Africa treatment with a fibrate is much more cost -effective than therapy with HMG-CoA reductase inhibitors and should therefore be the initial choice for most patients. ( Cost to the public sector ( Western Cape Province) of Bezafibrate controlled release preparation 400 mg/day for one month is +- R 30 with Atorvastatin 10 mg/day for one month being R70 ) In the private sector the price of fibrates is generally much higher ( Bezafibrate 400 mg/d +- R 400 ) so that the fibrates may lose their cost advantage. Certainly patients with marked hypertriglyceridaemia should commence therapy with a fibrate as the initial agent, as the hypertriglyceridaemia can theoretically be exacerbated by the use of HMG-CoA reductase inhibitors. A trial of each drug class as well as the combination may yield the best information for planning long term treatment.

Not making a specific diagnosis does deprive the patient of the opportunity for genetic counselling and family screening. In addition diagnosing

dysbetalipoproteinaemia should always lead to a careful search for precipitating factors, leading in some cases to the diagnosis of other unsuspected disorders such as hypothyroidism or clinically asymptomatic diabetes. Patients with dysbetalipoproteinaemia in general have an excellent response to therapy(1;3;8) and if this does not occur in a patient with an established diagnosis of the disorder, the physician will be prompted to revisit issues such as compliance and secondary factors. Making a definite diagnosis, apart from being scientifically and clinically satisfying for the physician, provides the patient with an explanation for their disorder as well as the knowledge that effective control of the hyperlipidaemia is possible.

Of all the diagnostic techniques described electrophoresis is the one that is most readily available and which can be performed with the least expense. Using electrophoretic techniques it is possible to process many samples while with ultracentrifugation the amount of specimens processed is limited by the capacity of the rotor and the duration of the spin. Agarose gel electrophoresis is unfortunately not sufficiently sensitive (40-60 % ) to be used on its own diagnostically. Although the finding of a broad beta band was initially said to be specific for the diagnosis of dysbetalipoproteinaemia (4)subsequent reports(1) dispute this finding. No exact figures are available for the specificity of this finding, but local experience is that there are certainly patients with a broad beta band on electrophoresis that on repeated attempts cannot be shown to have the characteristic cholesterol enriched remnants or a genotype compatible with the diagnosis of dysbetalipoproteinaemia. Demonstration of beta-VLDL by electrophoresis requires ultracentrifugation and is therefore again not routinely practicable GGE is an attractive alternative electrophoretic technique, as it has greater sensitivity and specificity. Lipoprotein staining patterns in the "LDL " or ApoB GGE system in use at the UCT Lipid Clinic reflect the proportional size distribution of ApoB containing lipoproteins.

Dysbetalipoproteinaemic patients presenting with an uncontrolled hyperlipidaemia usually have only lipoproteins of VLDL/IDL size present with little or no staining in the LDL size region. If hypertriglyceridaemia is marked chylomicrons may be present and the bulk of the lipoproteins is relatively large and triglyceride rich. As control improves with diet and drug therapy the lipoproteins generally tend to become smaller and less

triglyceride rich -staining mainly in the IDL size range. LDL may also become visible, as remnants decrease and are better metabolised, resulting in the formation of LDL.

The finding of only VLDL/IDL staining only is highly specific ( 96% ) for the diagnosis of dysbetalipoproteinaemia. Approximately half ( 48% ) of patients have this finding, sensitivity is improved to 76% when combining patients with VLDL/IDL only with those that have VLDL/IDL and a faint LDL band. The specificity of this finding in differentiating patients with dysbetalipoproteinaemia from those with other forms of mixed hyperlipidaemia is 92%.

Using a quantitative approach to the analysis of GGE sensitivity and specificity are improved even further. ( Sensitivity: 81%, specificity: 100% ) In addition a quantitative approach eliminates the subjective element involved in reporting a prominent or faint LDL band on the gel.

Potential problems with this approach are that Lp ( a ) migrates in the region of IDL particle size ( Rf 0.7-0.85 ) which has been termed Area IV. If there is excessive staining in this area due to high levels of Lp ( a ) the ratio of area IV/V may be falsely high, suggesting the diagnosis of dysbetalipoproteinaemia. Of the patients with dysbetalipoproteinaemia only one had visible Lp(a) on the gel whole amongst the patients serving as controls there were three with staining in the pre-A region. The area analysis of these patients did however not differ significantly from other patients. Lipoprotein (a) stains as a narrow peak of generally low optical density and therefore does not seem to affect the calculated area under the curve greatly. Lp ( a ) is generally identifiable as a narrow band on the gel in contradistinction to the more diffuse size distribution seen with lipoproteins of IDL size. Lp ( a ) values were also not significantly different between the two groups, a finding similar to a previously published report.(93)If there is concern that a high Lp ( a ) level may influence the analysis, the serum sample can be treated with a reducing agent to break the disulfide -bond between ApoB100 and apoprotein ( a ) and then electrophoresed again.

Patients with Type I and Type V hyperlipidaemia will also have elevated triglyceride

and cholesterol values with low levels of LDL. The hypertriglyceridaemia is however a much more marked in these patients than the hypercholesterolaemia and on GGE they have prominent staining at the origin reflecting the presence of chylomicrons that do not migrate in the gel. On inspection the pattern will therefore clearly be different with staining mainly at the origin and very little elsewhere. Patients with Type IV hyperlipidaemia generally have readily detectable LDL in addition to VLDL sized particles.

Patients genetically predisposed to the development of dysbetalipoproteinaemia generally do not express the phenotype in childhood or adolescence. If no remnant lipoproteins have accumulated, diagnosis by GGE will not be possible. The transition from a normal lipoprotein phenotype to dysbetalipoproteinaemia has not been sufficiently explored and we have no GGE data available. The same caution applies to patients with dysbetalipoproteinaemia that have very well controlled lipid values in that the characteristic pattern may again not be identifiable.

The following strategy is suggested for the diagnosis of dysbetalipoproteinaemia at the UCT/GSH Lipid Clinic. All patients with mixed hyperlipidaemia should have especially careful clinical evaluation to detect the presence of palmar crease and other xanthoma. GGE is routinely performed on all patients and is first inspected visually. The finding of a M-only pattern can be taken as highly suggestive of dysbetalipoproteinaemia and the patient should be genotyped directly. The finding of ApoE2/E2 or the ApoE145 mutation in this setting can be taken as sufficient confirmatory proof to firmly establish the diagnosis. Patients with M-staining as well as LDL staining should have their graphs quantitatively analysed by the method described previously. Area ratios of more than 0.5 should again prompt genotyping. Using this approach some patients with dysbetalipoproteinaemia may be missed, but repeating the GGE later on may well be more diagnostic. Positive results here can again be accepted as sufficient confirmatory proof. Patients who fulfill the above criteria, but who do not have a positive genotype require ultracentrifugation to demonstrate whether they have cholesterol enriched VLDL or not. It is important to remember that dysbetalipoproteinaemia is a very dynamic disease, the relative proportion of large triglyceride rich VLDL or small dense cholesterol enriched

remnants being very dependant on dietary fat intake, control of diabetes etc ...Ultracentrifugation therefore is generally not diagnostic in the poorly controlled patient with marked hypertriglyceridaemia. Using the above approach the number of ultracentrifugal studies required can be reduced quite markedly as it is reserved for patients in whom the diagnosis cannot be confirmed by genotyping, which is generally more readily available and performed more rapidly.

If the suspicion of dysbetalipoproteinaemia remains high, GGE and ultracentrifugation should be repeated on multiple occasions. Genotyping here will also be useful as a positive genotype will help to establish the diagnosis. A negative genotype will not rule out the disorder since not all known mutations are routinely screened for.

GGE is therefore a cost-effective screening method for dysbetalipoproteinaemia that is effective over a wide range of lipid values ( Range evaluated in this study: Cholesterol 4.3-11.3 and TG 2.2 -7.9 ). Of special relevance is the fact that some patients with **dysbetanormolipoproteinaemia** may be detected using this method. It can therefore be used to screen large number of samples in population studies aimed at establishing local prevalence of this disorder. In addition some patients can be identified before they become hyperlipidaemic and subsequently prospectively studied to identify factors that result in hyperlipidaemia becoming manifest. It should also be possible to study the atherogenic properties of remnant particles prospectively by following these patients with techniques such as measurement of carotid intima-media thickness and determine at what levels and how rapidly atherosclerosis sets in.

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## ApoE Nomenclature

### ApoE alleles

- $\epsilon 2$  112 Cys 158 Cys
- $\epsilon 3$  112 Cys 158 Arg
- $\epsilon 4$  112 Cys 158 Arg

### Phenotypes by Isoelectric focussing

- The major asialo apoE isoproteins found in plasma by two dimensional gel electrophoresis are designated apoE4, apoE3 and apoE2
- ApoE4 is the most basic isoprotein with apoE2 being the most acidic isoprotein
- The minor plasma isoproteins which are eliminated by treatment with neuraminidase are designated as apoE<sub>s</sub>
- The sialo isoform of apoE3 will therefore be designated as apoE3<sub>s</sub>
- Sialo isoforms can in addition be sublabelled as for instance apoE3<sub>s1</sub>, apoE3<sub>s2</sub> from basic to acidic

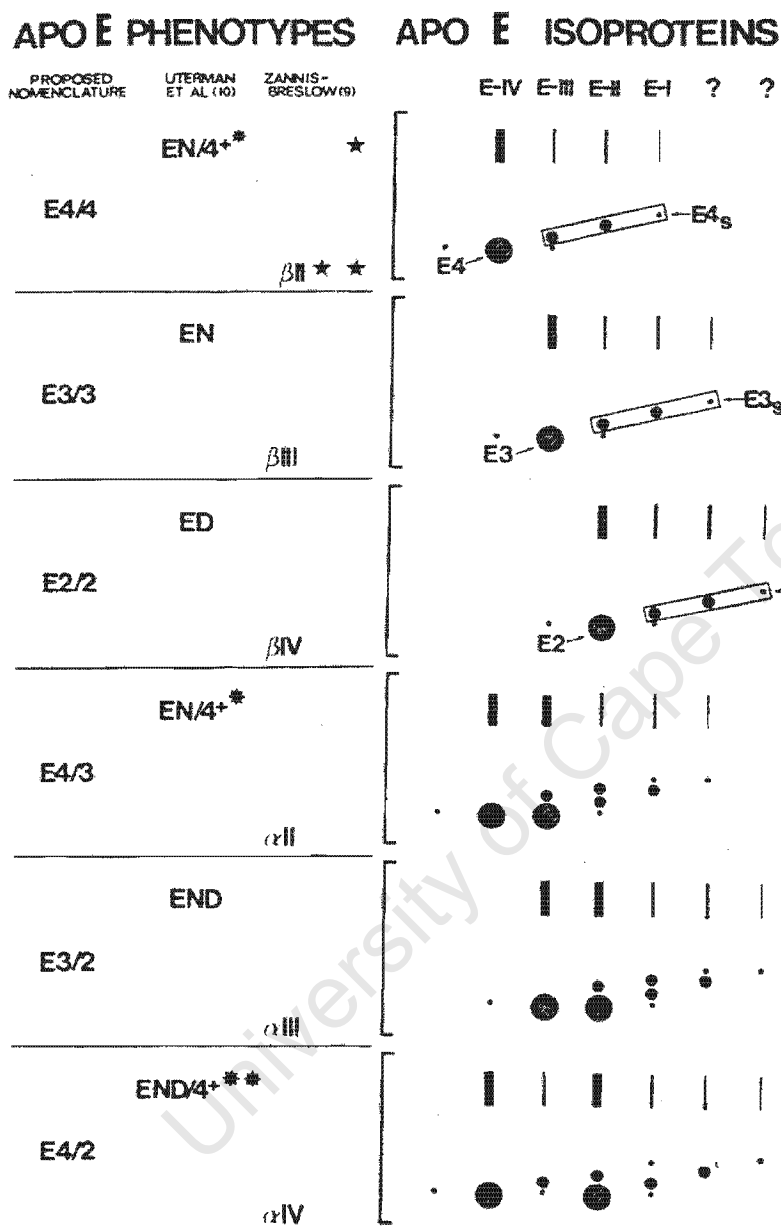
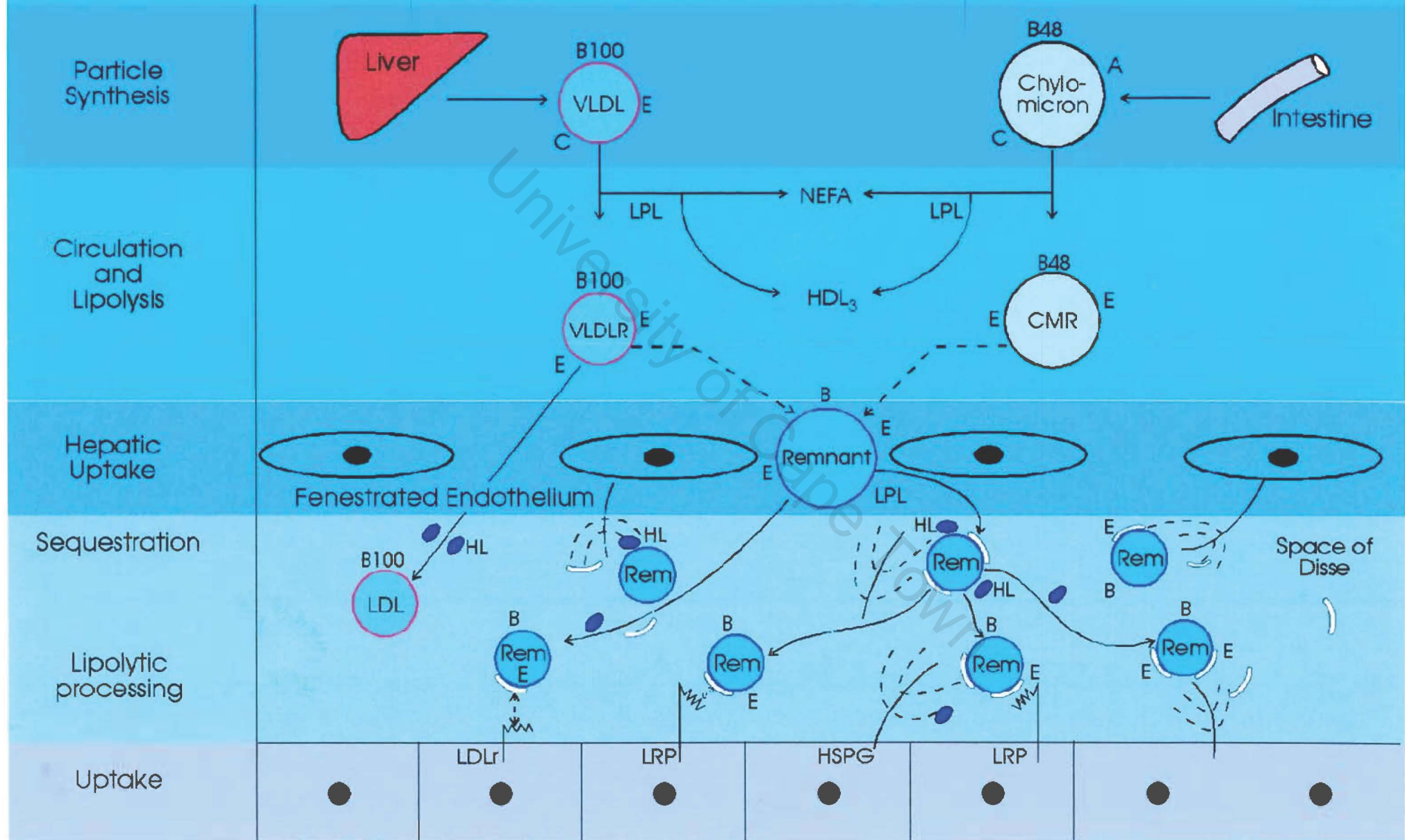


Fig. 3. Schematic presentation and proposed nomenclature of apoE isoproteins and phenotypes seen by one-dimensional isoelectric focusing and two-dimensional gel electrophoresis. \*, Phenotypes E4/4 and E4/3 were not differentiated in the original work of Utermann, et al. (10). Both were collectively designated EN/4+ \*\*Phenotype E4/2 ( $\alpha$ IV) ought to correspond to Utermann's hypothetical apoE phenotype ED/4+ (10). Due to interference of the sialo apoE isoprotein E4<sub>s1</sub> (13), the phenotype observed was not originally recognized as such, but was designated as END/4+ (10). ★, Phenotypes and isoproteins observed by one-dimensional isoelectric focusing. ★★. Phenotypes and isoproteins observed by two-dimensional gel electrophoresis.

# REMNANT METABOLISM



# Apo E Genotyping by PCR and Hha I digest

