MUTATION ANALYSIS AT THE LIPOPROTEIN LIPASE GENE LOCUS IN TWO SOUTH AFRICAN KINDREDS

Mohammed Fahri Hassan

1996

A thesis submitted to the Faculty of Medicine, University of Cape Town, Cape Town, in the fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Familial lipoprotein lipase (LPL) deficiency is a rare disorder of lipid metabolism associated with massive chylomicronaemia. Patients often present early in life with abdominal pain, pancreatitis, hepatosplenomegaly, eruptive xanthomata and zero to near zero levels of LPL activity in post-heparin plasma. The genetic heterogeneity underlying this disease is well-characterised and over 40 mutations have been described at the LPL gene loci. In this report three mutations are described at the LPL locus in two unrelated probands, namely, JJ (Kindred I) and LB (Kindred II).

JJ presented early in childhood with signs and symptoms suggestive of LPL-deficiency. These were abdominal pain, hepatosplenomegaly and a markedly reduced LPL activity (38% of normal) in post-heparin plasma. DNA studies showed JJ to be a compound heterozygote for two point mutations in the LPL gene, these being, the I194T and C418Y substitutions, which occur in exons 5 and 9, respectively. Several mutation detection systems were set up as part of the characterisation and screening workup for these mutations; these were, allele-specific oligonucleotide (ASO) hybridisation, "ARMS" PCR, PCR-SSCP, RT-PCR and DNA sequence analysis. In an earlier separate study, *in vitro* transfection results showed that the I194T mutant was catalytically inactive. Our findings of zero LPL activity in JJ's post-heparin plasma, implies that the C418Y mutation is also likely to produce an inactive protein product. The differences in LPL activity observed during the pre- and post-pubertal stages, if not artefactual, may be due to differential processing of LPL during human development with loss of activity post puberty.

LB was first diagnosed with pancreatitis during the third trimester of her pregnancy. Although her child, BB, was successfully delivered by caesarian section, LB died of haemorrhagic pancreatitis with the marked hyperlipidaemia being suggestive of an underlying deficiency in LPL activity. Genomic DNA from her parents was first subjected to mutation analysis, since only slide specimens of post-mortem material were available from LB. Maternal DNA revealed a G→A transition at nucleotide position 1516 which results in the substitution of lysine for glutamic acid at codon 421 in exon 9 (E421K), while paternal DNA show a single polymorphism at codon 108 in exon 3 of the LPL gene. Analysis of archival DNA obtained from histopathological slides of spleen tissue from LB also showed the E421K mutation. This mutation was also detected in her offspring, BB indicating maternal inheritance in three generations. While, this mutation may produce a catalytically defective product, the evidence is insufficient to propose a role for LPL deficiency as the primary cause of death in this patient, hence the search for a second mutation in the LPL gene of LB is imperative to establish this association.

.DECLARATION

I, Mohammed Fahri Hassan, hereby declare that the work on which this thesis is based is my original work (accept where acknowlegements indicate otherwise) and that neither the whole work nor any part of it has been , is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate Signature Removed

M.F. Hassan.

25 July 1995

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Professor H.E. Henderson for his guidance, criticisms and discussions throughout this work. I also wish to express my gratitude to those who offered advice and encouragement, namely, Dr T. Egan, Dr D. Ross and Mr D.P. Naidoo. I am also deeply indebted to Ms F. Leisegang for her expert assistance with the archival DNA extractions and single-standed conformational polymorphism analysis. To my parents for their support and especially my sister, Ms T. Hassan, for her assistance in the typing of this thesis. To a special person, Ms S. Ryklieff, for her encouragement and belief in me.

This study was undertaken in the Department of Chemical Pathology and the core facilities of the Institute of Child Health, University of Cape Town situated at the Red Cross War Memorial Children's Hospital.

To my sister, Ms T. Hassan

CONTENTS

	Page
ABSTRACT	ii
DECLARATION	iii
ACKNOWLEDGEMENTS	iv
DEDICATION	v
LIST OF TABLES	xi
LIST OF FIGURES	xii
ABBREVIATIONS AND SYMBOLS	xv
CHAPTER 1 INTRODUCTION	1
1.1 The Role of LPL in Lipoprotein Metabolism	1
1.1.1 Triglyceride Transport	1
1.1.2 The role of LPL as a Ligand in the Uptake of Lipoproteins	3
1.2 Molecular Genetics	5
1.2.1 Gene Structure	5
1.2.2 Gene Variants	8
1.3 Structural and Functional Properties of LPL	9
1.3.1 Structural Properties	12
1.3.1.1 Molecular Size	12
1.3.1.2 Monomer and Dimer Forms	12
1.3.2 Functional Domains	17
1.3.2.1 Active Site	17
1.3.2.2 "Lid" or Loop Domain	18
1.3.2.3 ApoC-II Binding Site	21
1.3.2.4 Heparin Binding Domain	22
1.3.2.5 Lipid Binding Site	23
1.3.3 Functional Properties	24
1.3.3.1 Interfacial Activation, active site mechanism and physical properties	24

1.3.3.2 Substrate Specificity 25
1.3.3.3 Interaction with ApoC-II 26
1.3.3.4 Interaction with Heparin 27
1.3.3.5 Effect of ApoC-III 27
1.3.3.6 Effect of Fatty Acids 28
1.4 Type I Hyperlipoproteinemia 30
1.4.1 Familial LPL Deficiency 30
1.4.1.1 Age of Onset 30
1.4.1.2 Clinical and Biochemical Profile 31
1.4.2 Apolipoprotein C-II Deficiency 32
1.4.2.1 Age of Onset 33
1.4.2.2 Clinical Profile 33
1.4.2.3 Genetics 33
1.4.3 Site-specific and Transient Expression LPL Variants 35
1.4.4 Circulating LPL Inhibitors 35
1.5 Aims of this Work 36
1.6 Layout of this Work 36

CHAPTER 2 KINDRED I ANALYSIS 38

2.1 Case History 39
2.2 Clinical and Biochemical Data 40
2.3 Results (Mutation Analysis) 43
2.3.1 The I194T Mutation 43
2.3.1.1 Haplotype Analysis 43
2.3.1.2 Sequence Analysis 44
2.3.1.3 Detection 46
2.3.1.3.1 Allele-Specific Oligonucleotide (ASO) Hybridisation 46
2.3.1.3.2 "ARMS" PCR 47
2.3.1.3.3 PCR-SSCP Analysis 48
2.4 Mutagenesis and Transfection Studies 50
2.5 The Second Mutation 51
2.5.1 RT-PCR Analysis 51
2.5.1.1 Monocyte-derived Macrophage Preparation 52

2.5.1.2 cDNA Synthesis 53
2.5.1.3 PCR Amplification of cDNA fragments 53
2.5.1.4 Cloning of cDNA fragments 53
2.5.1.5 PCR Screening 54
2.5.1.6 I194T "ARMS" PCR Screening 55
2.5.1.7 Sequencing of the Alternate Allele 56
2.5.2 Detection of the C418Y mutation 58
2.5.2.1 C418Y ARMS PCR 58
2.5.2.2 PCR Restriction Analysis 58
2.5.2.3 PCR-SSCP Analysis 60
2.6 Discussion 62

CHAPTER 3 KINDRED II ANALYSIS 66

3.1 INTRODUCTION 66
3.1.1 Pregnancy and Hyperlipidemia 66
3.1.2 Pregnancy, Hyperlipidemia and Pancreatitis 66
3.1.3 Kindred II 67
3.2 Case History and Laboratory Findings 68
3.3 Results (Mutation Analysis) 69
3.3.1 RT-PCR Analysis 69
3.3.1.1 Total RNA Assessment 69
3.3.1.2 cDNA Synthesis 69
3.3.1.3 PCR Amplification of LPLcDNA Fragments 69
3.3.1.4 Cloning of Fragments 69
3.3.1.5 PCR Screening 70
3.3.1.6 Sequence Analysis of Positive Colonies for DR 70
3.4 Genomic DNA Analysis of Kindred II 71
3.4.1 DNA Extraction of LB 71
3.4.2 PCR Amplification of LB 72
3.4.3 PCR-SSCP of Kindred II 74
3.5 Discussion 77

CHAPTER 4 CONCLUSIONS AND FUTURE STUDIES 80

4.1 General Conclusions 80
4.2 Future Studies 81

CHAPTER 5 MATERIALS AND GENERAL METHODS 82

5.1 Materials, Reagents and Buffer Solutions 82
5.2 General Methods 86
5.2.1 LPL Activity Assay 86
5.2.1.1 Materials and Reagents 86
5.2.1.2 Assay Conditions 91
5.3 DNA Isolation 92
5.4 End Labelling of Allele-Specific Oligonucleotides 92
5.4.1 Materials and Reagents 92
5.4.2 Procedure 93
5.5 ASO Hybridisation 94
5.5.1 Materials and Reagents 94
5.5.2 Sample Preparation 94
5.5.3 Procedure 94
5.6 Southern Blot Hybridisation 95
5.6.1 Sample Analysis 95
5.6.2 Protocol 96
5.7 Large-Scale Preparation of Plasmid DNA 97
5.7.1 Materials and Reagents 97
5.7.2 Preparation of LPL DNA Probe 97
5.8 SSCP Analysis 100
5.8.1 Materials and Reagents 100
5.8.2 Protocol 100
5.9 PCR Amplification of Histopathological Slide Extract DNA 102
5.10 PCR Restriction Analysis of the C418Y Mutation 102
5.10.1 Enzymatic Amplification 102
5.10.2 PvII Restriction Analysis 103

5.11 Ficoll-Histopaque Gradient Separation 103
5.12 Mononuclear Cell Culture 104
5.12.1 Preparation of Autologous Serum 104
5.12.2 Preparation of Growth Media 104
5.12.3 Cell Culture 104
5.12.4 Harvesting of Cells 105
5.13 Total RNA Isolation 105
5.13.1 Materials and Reagents 105
5.13.2 Protocol 105
5.14 Electrophoretic Analysis of RNA 106
5.14.1 Materials and Reagents 106
5.14.2 Sample Preparation 107
5.14.3 Preparation of 1% agarose/1.85% formaldehyde gels 107
5.15 cDNA Synthesis 107
5.15.1 Materials and Reagents 107
5.15.2 Protocol 108
5.16 PCR Amplification of LPLcDNA Products 108
5.16.1 PCR Protocol 108
5.16.2 Product Analysis 109
5.16.3 Purification of PCR Products 109
5.17 Cloning of LPLcDNA Fragments 110
5.17.1 Materials and Reagents 110
5.17.2 Ligation Reaction 110
5.17.3 Transformation 110
5.17.4 Preparation of Agar Plates and Colony Selection 110
5.17.5 PCR Screening of Colonies 110
5.18 Colony Selection and Plasmid Purification 112
5.18.1 Protocol 1 112
5.18.2 Protocol 2 112
5.19 DNA Sequence Analysis 113

APPENDIX 1 PRIMER DESIGN 114-118

REFERENCES 119-144

LIST OF TABLES

Table		Page
1.	Tissue distribution of LPL	2
2.	Location of potential cis-acting regulatory motifs in the promoter region of the human LPL gene	5
3.	Amino acid sequence comparisons corresponding to the β-ϵSer-α structural motif	11
4.	Clinical Manifestations associated with the Chylomicronaemia Syndrome and Type I Hyperlipoproteinaemia	31
5.	Conditions associated with hypertriglyceridaemia	32
6.	Gene variants reported at the ApoC-II gene locus up to 1993	34
7.	Lipid profiles of two kindreds with LPL deficiency due to a I194T mutation	41
8.	RFLP Haplotype Frequencies at the LPL gene locus for 33 normolipidaemic South African Afrikaners.	44
9.	Transformation efficiencies of cloned LPL gene fragments from patient, JJ (Kindred I)	54
10.	Transformation efficiencies of cloned LPL gene fragments from subjects, DR and ER (Kindred II)	69

LIST OF FIGURES

Figure		Page
1.	Role of LPL in Lipoprotein Metabolism.	1
2.	LPL-mediated lipoprotein uptake via LDLR and LRP.	4
3.	The cDNA sequence of Lipoprotein Lipase.	6
4.	LPL gene variations and structure.	7,8
5.	Central homology of the regions of human lipoprotein (hLPL), hepatic (hHL) and pancreatic (hPL) lipases.	10
6.	Schematic diagram of the proposed LPL dimer conformations.	13
7.	Ribbon diagram showing the α/β type structure of the human pancreatic lipase molecule. Location of LPL specific sites.	14
8.	Ribbon diagram showing the α/β type structure of the human pancreatic lipase molecule. Location of LPL specific mutations.	15
9.	The location of disulfide bridges and conserved cysteine residues in the N- and C-terminal domains of human lipoprotein (hLPL), hepatic (hHL) and pancreatic lipase (hPL).	16
10.	Stereodiagram of the superimposed Asp - His - Ser catalytic triads of human pancreatic lipase and trypsin.	17
11.	Sequence alignment and topology of the lid domain of human lipoprotein lipase.	20,21

12.	The catalytic charge-relay reaction mechanism of the serine esterase/ protease and lipase enzymes.	25
13.	ApoC-II peptide sequence and structure.	26
14.	Family Pedigree of Kindred I.	39
15.	Nucleotide sequence of the sense strand from exon 5 of patient, JJ.	45
16.	Autoradiograph of ASO hybridization blots of LPL exon 5 from a control kindred and kindred I.	47
17.	SSCP analysis of PCR-amplified LPL exon 5 products from kindred I.	49
18.	Schematic diagram of the the protocol followed in the RT-PCR of the LPL coding sequence.	51
19.	Total RNA isolates from patient and control monocyte and culltured monocyte-macrophage cell preparations.	52
20.	Colony screening by ARMS PCR for the verification of insert DNA.	55
21.	Nucleotide sequence of the sense strand of exon 9 of the LPL gene of index patient, JJ (Kindred I) and a normal control subject.	57
22.	PCR Restriction Analysis of LPL exon 9 for kindred I.	59
23.	SSCP analysis of LPL exon 9 PCR products from kindred I subjects.	61
24.	Family Pedigree of Kindred II.	68
25.	Archival DNA extracts from tissue embedded in paraffin-wax and mounted on histopathological slides.	72

26.	PCR amplification of exon 9 from slide DNA of index patient, LB and genomic DNA of three kindred II subjects.	73
27.	SSCP analysis of LPL exon 9 amplicons from kindred II subjects.	75
28.	Nucleotide sequence of the sense strand of exon 9 of the LPL gene for the kindred II subject, ER and a normal control subject.	76

ABBREVIATIONS AND SYMBOLS

ATP	Adenosine Triphosphate
ASO	Allele-specific Oligonucleotide
ARMS	Amplification Refractory Mutation System
Ab	Antibody
apoC-II	Apolipoprotein C-II
apoC-III	Apolipoprotein C-III
BSA	Bovine Serum Albumin
bLPL	Bovine Lipoprotein Lipase
BamHI	Restriction enzyme from Bacillus amyloliquefaciens H
C-terminal	Carboxy-terminal
CD	Circular Dichroism
cLPL	Chymotryptic fragment of LPL
CRL	Candida rugosa lipase
DNA	Deoxyribonucleic acid
DNAses	Deoxyribonucleic acid nucleases
DMSO	Dimethyl Sulfoxide
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
DMEM	Dulbecco's modified eagle medium
ddH_2O	Distilled Deionized Water
dNTP	Deoxynucleotide triphosphate
dATP	Deoxyadenosine triphosphate
dGTP	Deoxyguanosine triphosphate
dCTP	Deoxycytosine triphosphate
dTTP	Deoxythymidine triphosphate
EtOH	Ethanol
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
FFA	Free fatty acid
GPL	Guinea-pig Phospholipase
GCL	Geotrichum Candidum Lipase
Gly	Glycine

Glu	Glutamic acid
hPL	Human Pancreatic Lipase
hHL	Human Hepatic Lipase
HindIII	Restriction enzyme from Haemophilus influenzae R_b
HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein cholesterol
HDL_2	High Density Lipoprotein isoform
HSPG	Heparan Sulfated Proteoglycan
IDL	Intermediate Density Lipoprotein
IPTG	Isopropyl-β-D-thiogalactopyranoside
Ile	Isoleucine
LPL	Lipoprotein Lipase
LPLmRNA	Lipoprotein Lipase messenger Ribonucleic acid
LPLcDNA	Lipoprotein Lipase complementary Deoxyribonucleic acid
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LRP	Low Density Lipoprotein receptor-related protein/α_2-macroglobulin Receptor
Leu	Leucine
MW	Molecular Weight
M-MLuV RT	Murine Molony Leukemia Virus Reverse Transcriptase
N-terminal	Amino terminal
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
poly A	Polyadenylate
pOH	Post-heparin plasma
pEH	Pre-heparin plasma
PBS	Phophate buffered saline
Pro	Proline
PGL	Pseudomonas glumae lipase
PAGE	Polyacrylamide Gel Electrophoresis
PvuII	Restriction enzyme from Proteus vulgaris
RNA	Ribonucleic acid

RNAses	Ribonucleic acid nucleases
RML	Rhizomucor Miehei lipase
RNasin	Ribonuclease inhibitor
RT-PCR	Reverse Transcription PCR
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single-stranded Conformational Polymorphism
SDS	Sodium Dodecyl Sulfate
ss-DNA	Single-stranded DNA
SacI	Restriction enzyme from Streptomyces achromogenes
TG	Triglyceride
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
TLC	Thin-layer Chromatography
3D	Three dimensional
UV	Ultraviolet
VLDL	Very Low Density Lipoprotein
W/W^v	Mast cell-deficient mice
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

α	alpha
β	beta
d	dalton
ϵ	epsilon
kd	kilodalton
M	Molar or moles/liter
mM	millimolar or millimoles/liter
μM	micromolar or micromoles/liter
ml	milliliter
μl	microliter
K_d	Dissociation constant
V_{max}	Rate constant of enzyme reaction
rpm	revolutions per minute
Cl^-	Chloride anion
F^-	Fluoride anion
I^-	Iodide anion

NO^-_3	Nitrate anion
OH	Hydroxyl group
CN^-	Cyanide anion

CHAPTER 1

INTRODUCTION

1.1 The Role of LPL in Lipoprotein Metabolism.

1.1.1 Triglyceride Transport.

Lipoprotein Lipase (LPL) plays a pivotal role in lipid metabolism and triglyceride transport through the hydrolysis of triglyceride present in circulating chylomicrons and very low density lipoproteins (VLDL)(Fig. 1). LPL is synthesised predominantly in the parenchymal cell and is found in a variety of organ tissues with muscle, adipose tissue, Kupffer cells, and monocyte-derived macrophages being the major sites of synthesis (Table 1). The enzyme is transported from it's site of synthesis via an unknown mechanism to the luminal surface of the capillary endothelium where it is bound through ionic interactions to heparan sulfated proteoglycan (HSPG) moieties. This is the primary site of hydrolysis of the triglyceride core of circulating chylomicrons and very low density lipoproteins (VLDL).

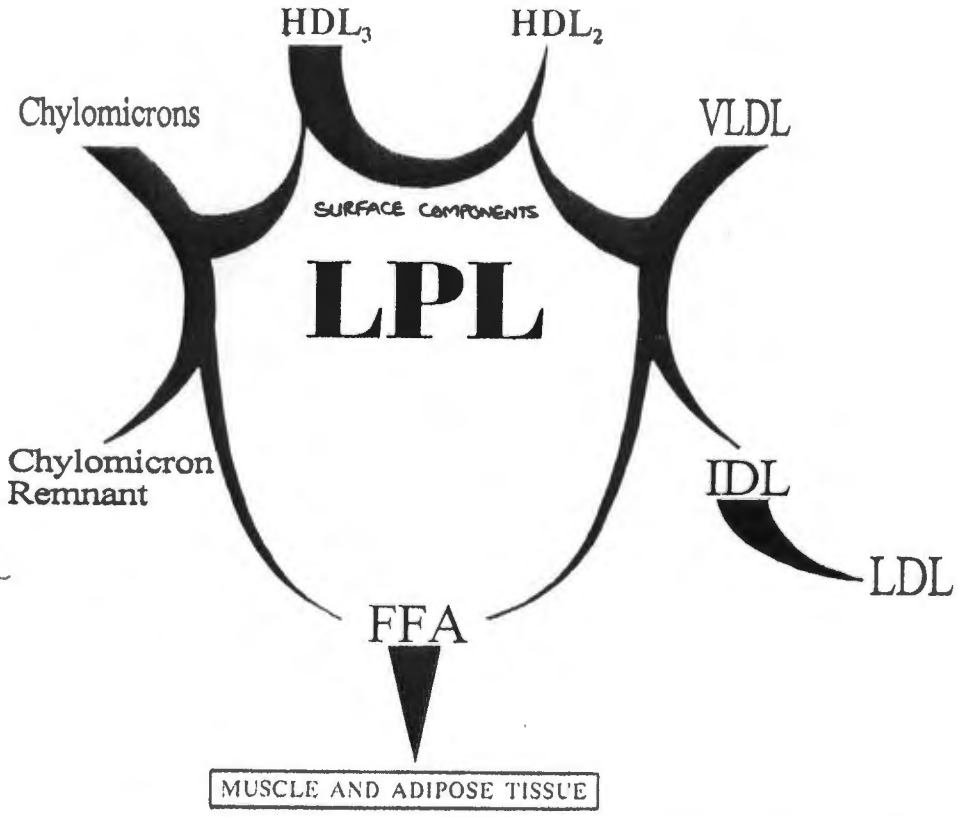


Fig. 1: **Role of LPL in Lipoprotein Metabolism. VLDL - very low density lipoproteins, IDL - intermediate density lipoproteins, LDL - low density lipoproteins, FFA - free fatty acids.**

Table 1 : Tissue distribution of LPL.

ORGAN	CELL TYPE	FUNCTION	REFERENCES
Aorta	(a)	(b)	Semenkovich *et.al.*, 1989
Brain	Purkinje cells of the cerebellum, substantia nigra	Glycolipid synthesis	Vilaro *et. al.*, 1990 Goldberg *et. al.*, 1989 Eckel and Robbins, 1984
Heart	Mesenchymal cells, cardiac myocytes	FFA provides oxidative energy	Rodrigrues *et. al.*, 1992, Liu and Olivecrona, 1991 Stein *et. al.*, 1991 Pederson *et. al.*, 1983
Intestine	(a)	(b)	La Rosa *et. al.*, 1972
Kidney	Renal medulla	(b)	Camps *et. al.*, 1990 Cryer, 1987
Lungs	Macrophages	Synthesis of pulmonary surfactant	Camps *et. al.*, 1991
Lactating mammary gland	Mammary adipocyte	Milk synthesis	Jensen *et. al.*, 1991 Camps *et. al.*, 1990 Rebuffé-Scrive *et. al.*, 1986 Ramirez, 1983
Neonatal liver	Hepatocyte	Organ development, catabolism of LPL	Vilaro *et. al.*, 1988
Ovaries	(a)	(b)	Camps *et. al.*, 1990
Skeletal muscle	(a)	(b)	Borenzstayn, 1987
Spleen	Macrophages	(b)	Camps *et. al.*, 1991
Uterus	Parametrial adipose tissue	Contraction and prostaglandin synthesis	Gray and Greenwood, 1983

The cell type indicates the proposed site of synthesis of LPL within these specific organ tissues.

(a) - undetermined, (b) - unknown

Chylomicrons are assembled in the endoplasmic recticulum of enterocytes in the intestine after a fat-containing meal (Brunzell, 1989; Glueck, 1977). These particles are then transported via the thoracic duct into the circulation where they acquire apoC-II, the physiological activator of LPL. LPL is principally responsible for the conversion of chylomicrons into remnant particles which are rapidly removed from the circulation. This action of LPL results in the clearance of all chylomicrons by 9 hours after a fatty meal. The process of chylomicron conversion to remnant particles involves a depletion of triglyceride and a loss of cholesterol, phospholipid and surface proteins. Most of these surface components are either transferred to or converted into high density lipoproteins (HDL)(Lewis, 1988). The liberated fatty acids are either used as fuel by the various tissues or are taken up by adipose tissue for storage.

VLDL are synthesised mainly in the sinusoidal cells of the liver from where they are secreted into the space of the Disse. These lipoproteins serve as a means of transport for liver derived triglyceride which originates from remnant uptake, lipogenesis and reesterification of fatty acids. VLDL are metabolised to the remnant particles, namely, intermediate density (IDL) and low density (LDL) lipoproteins. IDL are avidly taken up via the hepatic apoB/E receptor, with 50% being removed in this manner. The rest are converted to LDL by further remodelling of the surfaces and core lipids, probably under the action of hepatic lipase. LDL are catabolised in various peripheral tissues with the liver being their major site of uptake and degradation.

1.1.2 The Role of LPL as a Ligand in the Uptake of Lipoproteins.

In addition to it's role in the hydrolysis of triglyceride, LPL also appears to mediate the uptake of lipoproteins. The evidence for this comes mainly from *in vitro* studies which suggest that this process occurs via two independent receptor-mediated endocytotic pathways, involving the low density lipoprotein receptor (LDLR, also known as the apoB,E receptor) and the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP)(Fig. 2).

LPL has been shown to facilitate uptake of LDL and VLDL via the LDL receptor pathway in normal fibroblasts (Gianturco *et. al.*, 1983) and HepG2 cells (Mulder *et. al.*, 1993). This process occurs as a direct result of LPL-mediated bridging between the lipoprotein and HSPG's. Transfer of VLDL and LDL from the complex to the LDL receptor is followed by rapid internalisation and degradation. Likewise, Choi *et. al.* (1990) demonstrated that 25-35% of chylomicron remnant uptake also occurs via the LDL receptor. The remainder appears to be taken up by a 600kd LRP receptor which has been shown to bind to LPL-lipoprotein complexes with high affinity (Beisegel *et. al.* 1991). LPL appears to act as a ligand in enhancing the binding of lipoproteins to LRP. Chappell *et. al.* (1992, 1993, 1994) demonstrated that this process was mediated by the specific binding of the C-terminal domain of LPL to LRP and was independent of LPL activity. In addition, they showed that apoE and HSPG's were essential requirements for the binding of these large triglyceride-rich lipoproteins to LRP. A third mechanism of LPL-mediated uptake is the endocytosis of the entire LPL-HSPG-lipoprotein complex, a process which occurs at a much slower rate. Likewise, chylomicron remnants have also been shown to be catabolised via similar pathways (Mahley and Hussain, 1991; Brown *et. al.*, 1991; Nagata *et. al.*, 1988).

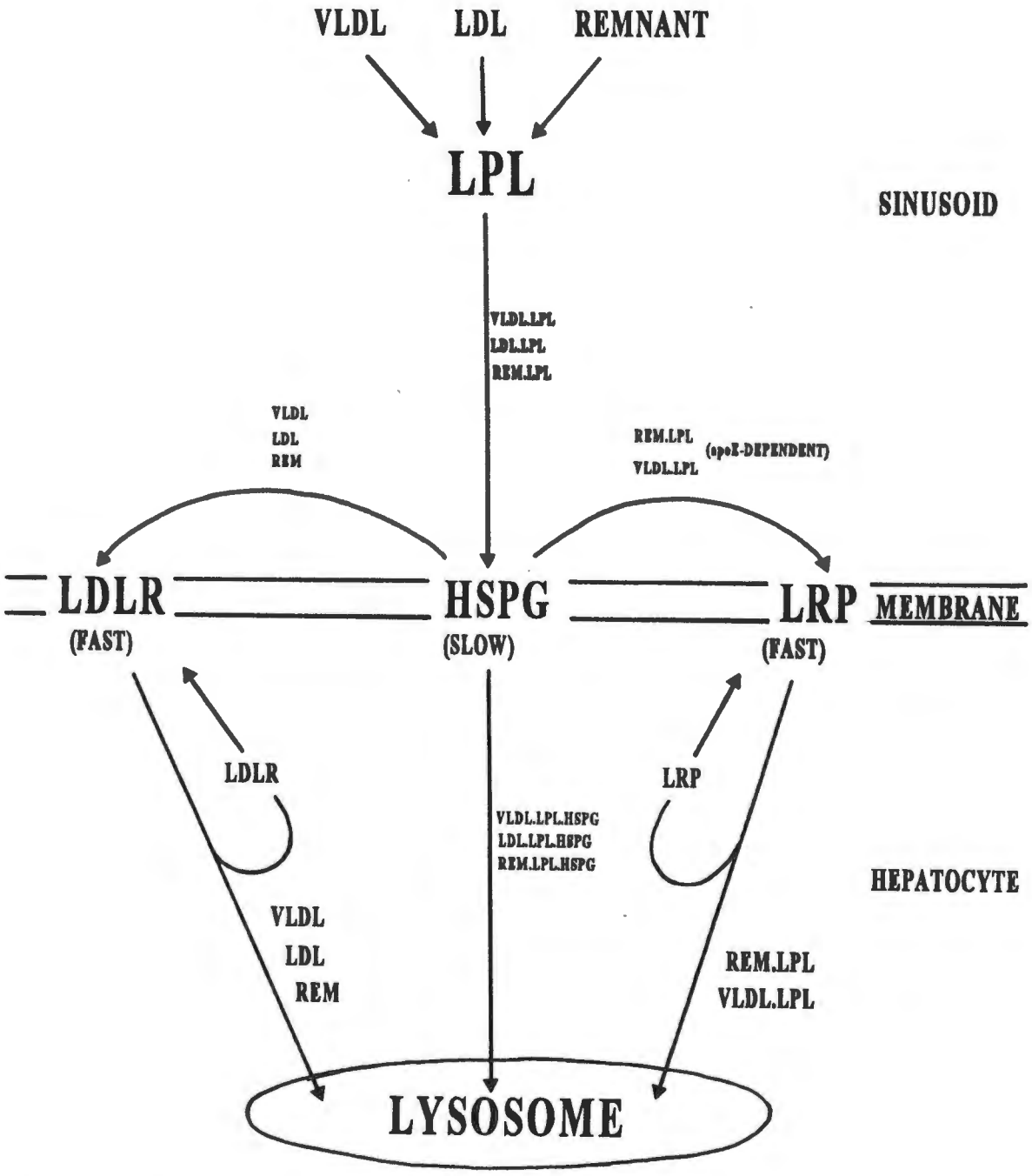


Fig 2: LPL-mediated lipoprotein uptake via LDLR and LRP.

VLDL - very low density lipoprotein, LDL - low density lipoprotein, REM - lipoprotein remnant particles, LDLR - low density lipoprotein receptor, LRP - low density lipoprotein receptor-related protein, HSPG - heparan sulfated proteoglycan.

1.2 Molecular Genetics.

1.2.1 Gene Structure.

The human LPLcDNA was cloned and sequenced in 1987 (Wion *et. al.*)(Fig. 3). The gene was subsequently localised to chromosome 8 (p22 region) (Sparkes *et. al.*, 1987) and determined to be 30kb in length, with the coding sequence comprising 10 exons (Deeb *et. al.*, 1989; Oka *et. al.*, 1990)(Fig. 4). Exon 1 comprises 188 bp of the 5'-untranslated sequence, and the coding sequence of the signal peptide and the first two amino acid residues of the mature protein (Kirchgessner *et. al.*, 1987). Several cis-acting regulatory motifs have been identified in the 5'-untranslated region of the LPL gene (Table 2). Exons 2 to 9 code for the rest of the protein sequence while exon 10 codes for the last base of the termination codon and the entire 1948 bp 3' untranslated region which contains two poly-adenylation signals (Wion *et. al.*, 1987)(Fig. 3). These signals generate two mRNA species, approximately 3350 and 3750 nucleotides in length, and of roughly equal frequencies.

Table 2: Location of potential cis-acting regulatory motifs in the promoter region of the human LPL gene.

REGULATORY MOTIFS	LOCATION (bp)	REFERENCES
CAAT	-64, -506	Deeb *et. al.*, 1989 Dorn *et. al.*, 1987
TATA	-26	Hua *et. al.*, 1991
tsp (ATG)	+189	Hua *et. al.*, 1991
last nt of exon1	+276	Hua *et. al.*, 1991
Oct 1 (5'-ATTTGCAT-3')	-581, -186, -46	Currie *et. al.*, 1992 Singh *et. al.*, 1986
GRE (5'-AGATCA-3')	-1534, -1272, -777, -645	Scheidereit *et. al.*, 1984
TRE (5'-GGAGGAGG-3')	+22	Umek *et. al.*, 1991
FSE 1 FSE 2 (5'-GAGAGGAG-3')	-362 -630	Scheidereit *et. al.*, 1984
CRE (5'-ACGTCA-3')	-306	Rupp *et. al.*, 1990
C/EBP	-68, -509	Umek *et. al.*, 1991

CAAT - potential binding sites for the nuclear factor, NF-1; Oct 1 - octamer transcription factor recognition sequences; GRE - glucocorticoid response element; TRE - thyroid response element; FSE 1/FSE 2 - fat specific elements; CRE - cAMP response element; C/EBP - liver and adipocyte differentiation transcriptional factor or CCAAT/enhancer binding protein; tsp - transcriptional start site.

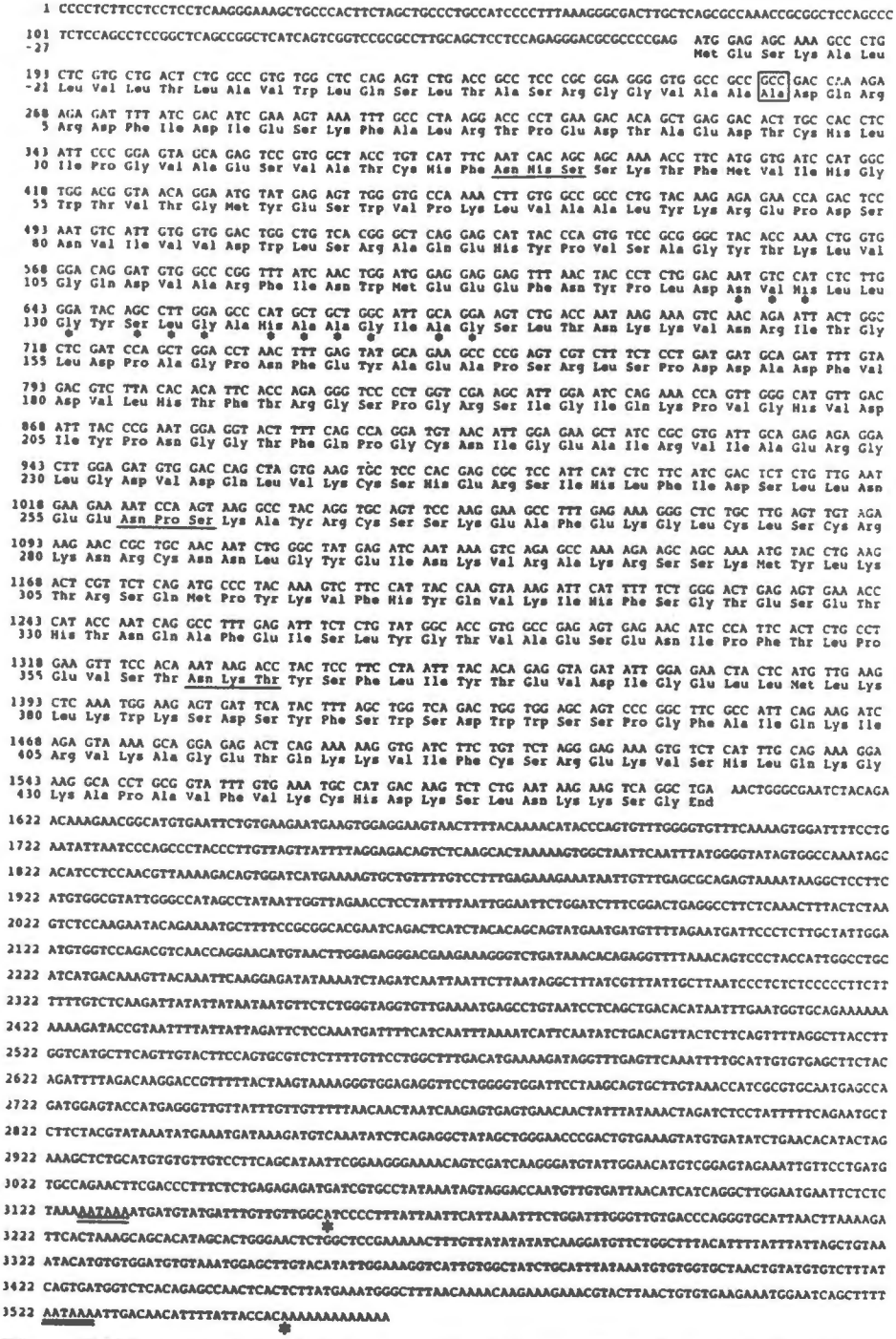


Fig. 3: **The cDNA sequence of Lipoprotein Lipase (Reprinted from Wion *et. al.*, 1987). The complete predicted amino acid sequence of LPL is shown below the cDNA sequence. Negative amino acid numbers refer to the predicted signal peptide sequence. Also included is 174 nucleotides of the 5'-untranslated region and the entire 1948 nucleotides of the 3'- untranslated region. The first amino acid of the mature protein is boxed. Amino acids predicted as part of the interfacial recognition site in porcine pancreatic lipase are indicated below with an asterisk. The three potential N-glycosylation sites are underlined. The two polyadenylation signals (AATAAA) at positions 3126 and 3522 are double underlined and precede the two alternative sites of polyadenylation at positions 3155 and 3550 indicated by asterisks.**

Human Lipoprotein Lipase - Gene Variations

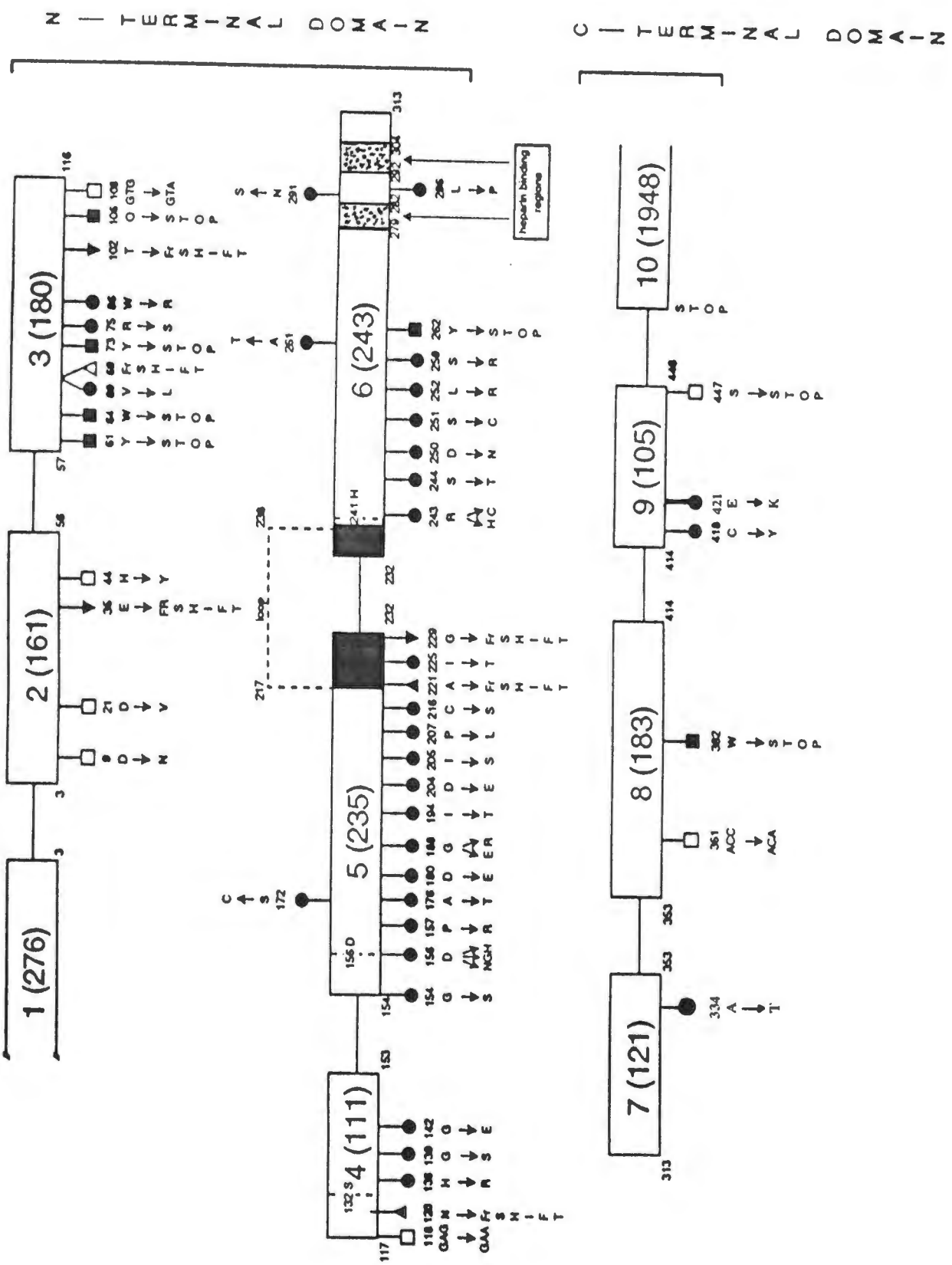


Fig. 4: **LPL gene variations and structure.**

All missense (●), frameshift (▲) and nonsense (□■) mutations described in literature (up to 1994) including the two novel missense mutations described in this manuscript are shown (Reprinted from Hayden and Henderson, 1995). Exon numbers (1 - 10) and sizes [eg: (276)] in base pairs are indicated. Exon-intron boundaries are indicated. The catalytic triad residues (eg: 132 S) are shown. The disulfide bridge (217-------238) at the base of the loop domain (▇) are indicated. The proposed heparin binding regions (▨) are shown. The N-terminal and C-terminal domains refer to the distinct structural domains of the monomeric subunit of LPL as deduced from the homologous 3D molecular model of human pancreatic lipase (Winkler *et. al.*, 1990). The amino acids are indicated by the single letter code with gene variations indicated.

1.2.2 Gene Variants.

A large number of patients with familial LPL deficiency have now been examined for coding sequence variations at the LPL locus (Bruin *et. al*, 1994; Lalouel *et. al.*, 1992). These sequence variants include, insertional, deletional and point mutations (Lalouel *et. al.*, 1992; Devlin *et. al.*, 1990; Langlois *et. al.*, 1989). The majority of mutations described to date are missense mutations which cluster in the central exons 4, 5 and 6 (Fig. 4). Fig 4 is a schematic diagram of the LPL gene showing the location of some the mutations described in this section. Also included in the diagram are the two novel exon 9 mutations which are the subject of this dissertation.

The population frequency of primary LPL deficiency has been estimated at 1 in 10^6 worldwide. This gives a carrier frequency of 1 in 500 in the general population (Nikkilä, 1978). Higher frequencies have been reported and these occur within the French Canadian population, in areas surrounding Quebec City, Canada, where founder genes have increased the carrier rate to 1:40 (Gagne *et. al.*, 1989). Genealogies constructed from several restriction fragment length polymorphisms (RFLP haplotypes) indicated that these founder mutations were introduced into Quebec by French settlers who had migrated mainly from Brittany, Normandy in the 17th century.

They settled mainly in and around Quebec City where they remained socially and linguistically isolated, with little expansion of their gene pool. Two founder mutations, the Pro207↪Leu (P207L) (Ma *et. al.*, 1991) and Gly188↪Glu (G188E) (Monsalve *et. al.*, 1990) predominate in this French Canadian population. For instance, the P207L mutation occurs in 54 (73%) out of 74 mutant alleles in 37 patients with LPL deficiency, while the G188E mutation accounted for 15 (21.6%) of the LPL deficient alleles. The G188E mutation is not only found in the French Canadians but has been reported in persons of different ancestries, including people of Polish, English, German, Dutch, Austrian (Miesenbock *et. al.*, 1993) and East Indian (Henderson *et. al.*, 1992) descent. In the South African population this mutation was detected in 9 probands from 4 families of Indian extraction. The higher than expected frequency of LPL deficiency in this population group can be attributed to a few carriers who arrived in South Africa in the early part of this century. Haplotype analysis has shown that the G188E mutation associates predominantly with a single haplotype in all the population groups, suggesting an ancient origin possibly pre-dating the spread of Caucasoid populations. Another missense mutation which occurs at codon 194 in exon 5 of the LPL gene (Ile194↪Thr or I194T mutation) was reported in 5 unrelated kindreds of different ancestries (Dichek *et. al.*, 1991; Henderson *et. al.*, 1991). It has been reported in four South African (S.A.) patients, one of Dutch, one of French and one of mixed ancestry with the fourth patient being of unknown ancestry. This mutation was demonstrated to be associated with two divergent RFLP haplotypes in the S.A patients and most likely is indicative of recurrent mutations occurring on different chromosomal backgrounds, giving a multicentric origin for this mutation.

1.3 Structural and Functional Properties of LPL

Technological advances in molecular biology have greatly facilitated the characterisation of pro- and eucaryotic lipases and their corresponding gene sequences. The complete structure and organisation of LPL genes from a variety of species is now known, for example, human (Deeb *et. al.*, 1989; Wion *et. al.*, 1987; Kirchgessner *et. al.*, 1989; Oka *et. al.*, 1990), guinea pig (Enerback and Bjursell, 1989), rat (Brault *et. al.*, 1992; Wicker-Planquart and Puigserver, 1992), cow (Senda *et. al.*, 1987), mouse (Kirchgessner *et. al.*, 1987) and chicken (Cooper *et. al.*, 1992). Sequence comparisons of all known lipases reveal the existence of several lipase gene families. Human pancreatic lipase (hPL)(Lowe *et. al.*, 1989), human hepatic lipase (hHL)(Martin *et. al.*, 1988; Ameis *et. al.*, 1990) and human LPL (as shown) show a high degree of homology, indicative of a common ancestral gene, and

represent one of these families (Kirchgessner *et. al.*, 1989). A striking feature in this family is the extensive homology observed for a region located between residues 120 and 260 (Fig. 5)(Kirchgessner *et. al.*, 1989). This region contains the catalytic triad residues, Ser, Asp and His (Wang *et. al.*, 1992) and the "nucleophilic elbow" motif, G-X-S-X-G, reminiscent of the serine protease family of enzymes (Derewenda and Derewenda, 1991)(Table 3).

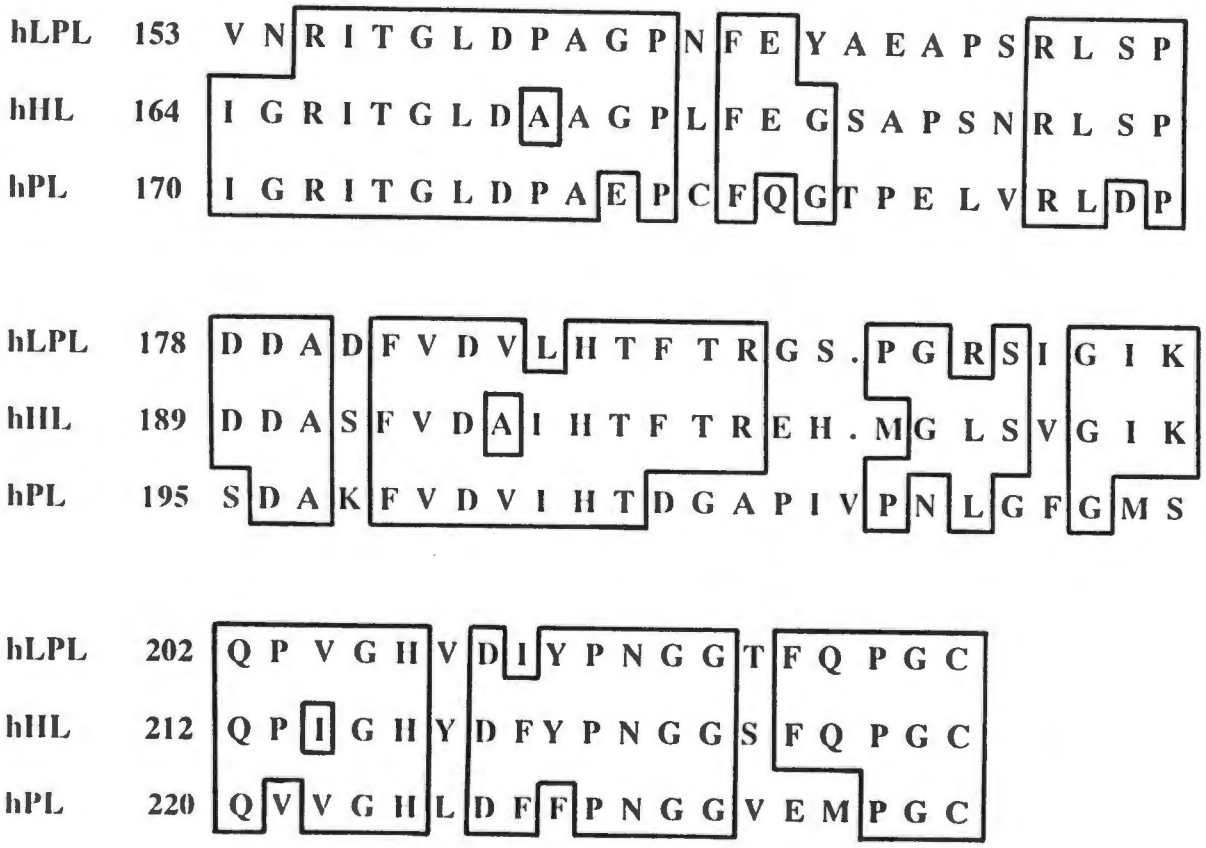


Fig 5 : Central homology of the regions of human lipoprotein (hLPL), hepatic (hHL) and pancreatic (hPL) lipases. Portions of the protein sequences of hLPL, hHL and hPL are shown with the numbers indicating the amino acid number in thesecretedprotein. Maximal homology was obtained by the introduction of gaps (indicated by dots). Regions of homology are boxed.

Mammalian Lipases and Related Proteins												
hLPL	V	H	L	L	G	Y	S	L	G	A	H	A	A
bLPL	V	H	L	L	G	Y	S	L	G	A	H	A	A
hHL	V	H	L	I	G	Y	S	L	G	A	H	V	S
hPL	V	H	V	I	G	H	S	L	G	A	H	A	A
hGL	L	H	Y	V	G	H	S	Q	G	T	T	I	G
hBAL	I	T	L	F	G	E	S	A	G	G	A	S	V
hLCAT	V	F	L	I	G	H	S	L	G	C	L	H	L
rLL	I	H	Y	V	G	H	S	Q	G	T	T	I	G
Fungal Lipases													
RML	V	A	V	T	G	H	S	L	G	G	A	T	A
GCL	V	M	I	F	G	E	S	A	G	A	M	S	V
CCL	V	T	L	F	G	E	S	A	G	S			
PCL	L	V	V	V	G	H	S	L	G	A	A	V	A
Bacterial Lipases													
P. fragi	V	N	L	I	G	H	S	Q	G	A	L	T	A
P. cepacia	V	N	L	V	G	H	S	Q	G	Q			
S. aureus	V	H	L	V	G	H	S	M	G	G	Q	T	I
Other													
F. sol. cutinase	L	I	A	G	G	Y	S	Q	G	A	A	L	A
A. Hydrophila	V	H	F	L	G	H	S	L	G	A	S	R	V
h. prolyl oligopeptidase	L	T	I	N	G	G	S	N	G	L	L	V	A
Comparison to serine esterases													
T. cal. AChE	V	T	L	F	G	E	S	A	G	A	A	S	V

Table 3: **Amino acid sequence comparisons corresponding to the β-ϵSer-α structural motif. The active site Ser and conserved residues making up the "nucleophillic elbow", GXSXG, are boxed. Adjacent residues involved in the helix-β-sheet packing of this motif are double-underlined. The sequences compared are human lipoprotein lipase (hLPL)(Wion *et. al.*, 1987), bovine lipoprotein lipase (bLPL)(Senda *et. al.*, 1987), human hepatic lipase (hHL)(Datta *et. al.*, 1988), human pancreatic lipase (hPL)(Lowe *et. al.*, 1989), human gastric lipase (hGL)(Bodmer *et. al.*, 1987), human bile-salt activated lipase (hBAL)(Wong and Hartsuck, 1993), human lecithin-cholesterol acyltransferase (hLCAT)(McLean *et. al.*, 1986), rat lingual lipase (rLL)(Docherty *et. al.*, 1985), Rhizomucor Miehei lipase (RML)(Boel *et. al.*, 1988), Geotrichum candidum lipase (GCL)(Shimada *et. al.*, 1990), Cadida rugosa lipase (CRL)(Longhi *et. al.*, 1992), Penicillium camembertii lipase (PCL)(Isobe and Nokihara, 1993), Pseudomonas fragi lipase (Wohlfarth and Winkler, 1991), Pseudomonas cepacia lipase (Cygler *et. al.*, 1992), Staphylococcus aureus lipase (Lee *et. al.*, 1986), Fusarium solani cutinase (Soliday *et. al.*, 1984), Aeromonas Hydrophila extracellular lipase (Anguila *et. al.*, 1993), human prolyl oligopeptidase (Polgár, 1992), Torpedo Californica acetylcholinesterase (T. cal. AChE)(Sussman *et. al.*, 1991).**

Several approaches have been adopted to study structure-function relationships of lipases. While much data has been obtained from earlier protein and enzyme kinetic studies, the recent X-ray crystallographic data from the human pancreatic lipase (hPL)(Winkler *et. al.*, 1990) and fungal lipases, Rhizmucor Miehei (RML)(Brady *et. al.*, 1990), Geotrichum candidum (GCL)(Shrag *et. al.*, 1991), Candida rugosa (CRL)(Grochulski *et. al.*, 1993) and the bacterial lipase, Pseudomonas glumae (PGL)(Noble *et. al.*, 1993) have provided structural models for lipase function. The PL model has been used as a general blueprint for LPL and HL function, even though each of these enzymes possess distinctive properties, which alludes to local domain-specific differences.

1.3.1 Structural Properties.

1.3.1.1 Molecular Size

Molecular weight (MW) determination utilising radiation inactivation (Olivecrona *et. al.*, 1982, 1985) and gel filtration (Osborne *et. al.*, 1985) techniques, revealed apparent molecular weights for the non-covalent LPL homodimer of between 83 000d and 110 000d. While monomeric LPL was shown to have apparent MW's ranging from 34 000d to 96 900d (Quinn *et. al.*, 1982), determination of the human LPLcDNA sequence (Wion *et. al.*, 1987)(Fig. 3) revealed a mature protein of 448 amino acids preceded by a 27 amino acid signal peptide with a calculated molecular weight of 50 394d. With an estimated carbohydrate content of 8%, the predicted apparent MW may be around 60 000d. A similar apparent MW (60 000d) was obtained upon SDS-PAGE of human milk LPL (Zechner, 1990).

1.3.1.2 Monomer and Dimer Forms.

Sedimentation equilibrium dialysis and gel filtration performed on bLPL shows that lipase activity is essentially associated with the protein dimer species (Osborne *et. al*, 1985). Mild treatment of intact bLPL with guanidinium chloride, leads to an irreversible loss of activity, due to dimer to monomer dissociation, as evidenced by the loss in secondary structure based on circular dichroic (CD) measurements (Osborne *et. al*, 1985). These findings provide evidence that the intact LPL dimer is an absolute requirement for full catalytic activity. Earlier studies have shown that heparin stabilises the LPL dimer against denaturation presumably by spanning the dimeric unit

following binding to specific binding sites on each individual subunit (Jackson *et. al.*, 1980; Clarke *et. al.*, 1983). While this may represent an attractive vehicle for subunit stabilisation, direct protein-protein interactions cannot be discounted. Hydrophobicity has been established as the major factor in stabilising protein-protein associations (Chothia and Janin, 1975). Wong *et. al.* (1991) proposed two possible models for lipase dimer formation, (i) a head-to-head or (ii) a head-to-tail association (Fig. 6). In both structures a two-fold axis of symmetry exists with the head-to-head conformation having the two N-terminal catalytic domains juxtaposed and the two C-terminal domains next to each other. The head-to-tail conformation would bring the C-terminal domain in close proximity to the N-terminal active site.

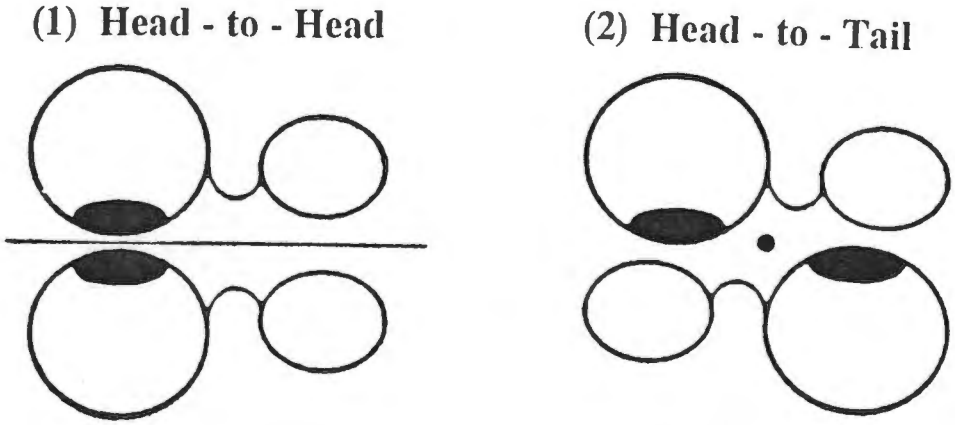


Fig. 6: **Schematic diagram of the proposed LPL dimer conformations (reprinted from Wong *et. al.*, 1990). Monomeric LPL is represented with a larger N-terminal domain linked via a spacer arm to the smaller C-terminal domain. The catalytic sites (●) are shown.**

Biochemical studies of monomeric LPL following proteolytic digestion had earlier suggested the existence of distinct functional domains (Bengtsson and Olivecrona, 1980; Bengtsson-Olivecrona *et. al.*, 1986). This was confirmed by the construction of a molecular model of the homologous human pancreatic lipase (PL)(Winkler *et. al.*, 1990) which clearly shows a two domain structure for the members of this lipase gene family (Fig. 7 and 8). The N-terminal domain is a globular α/β type structure whereas the C-terminal domain is of the β-sandwich type. The α/β-type scaffold of the N-terminal domain appears to be a conserved topological feature of all lipases although their overall structures are different. The location of cysteine residues involved in the formation of five disulfide bridges in LPL (Yang *et. al.*, 1989), appear to have been conserved in HL and PL indicating potentially similar tertiary conformation (Wang *et. al.*, 1992; Persson *et. al.*, 1989)(Fig. 9). The larger N-terminal domain of LPL (res 1-312) contains the catalytic triad and the highly conserved consensus sequence, G-X-S-X-G (Derewenda and Derewenda, 1991).

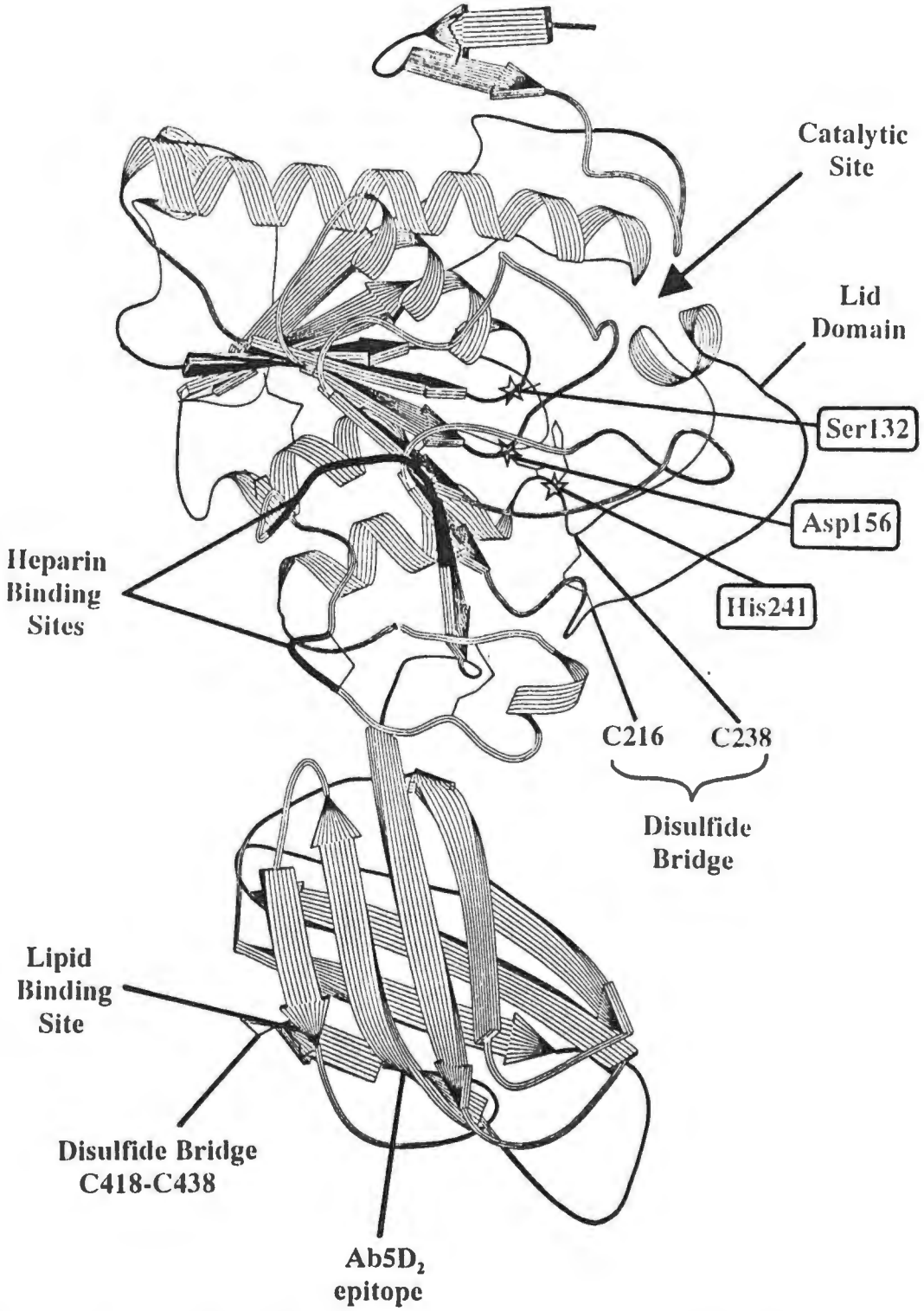


Fig 7: **Ribbon diagram showing the α/β type structure of the human pancreatic lipase molecule. The proposed location of specific functional sites and residues of the homologous LPL molecule are superimposed. The active site residues (☆) forming the catalytic site are shown. The residues involved in the formation of the disulfide bridges at the base of the lid domain and at the C-terminus are shown. The heparin binding sites and lipid binding motifs are shown.**

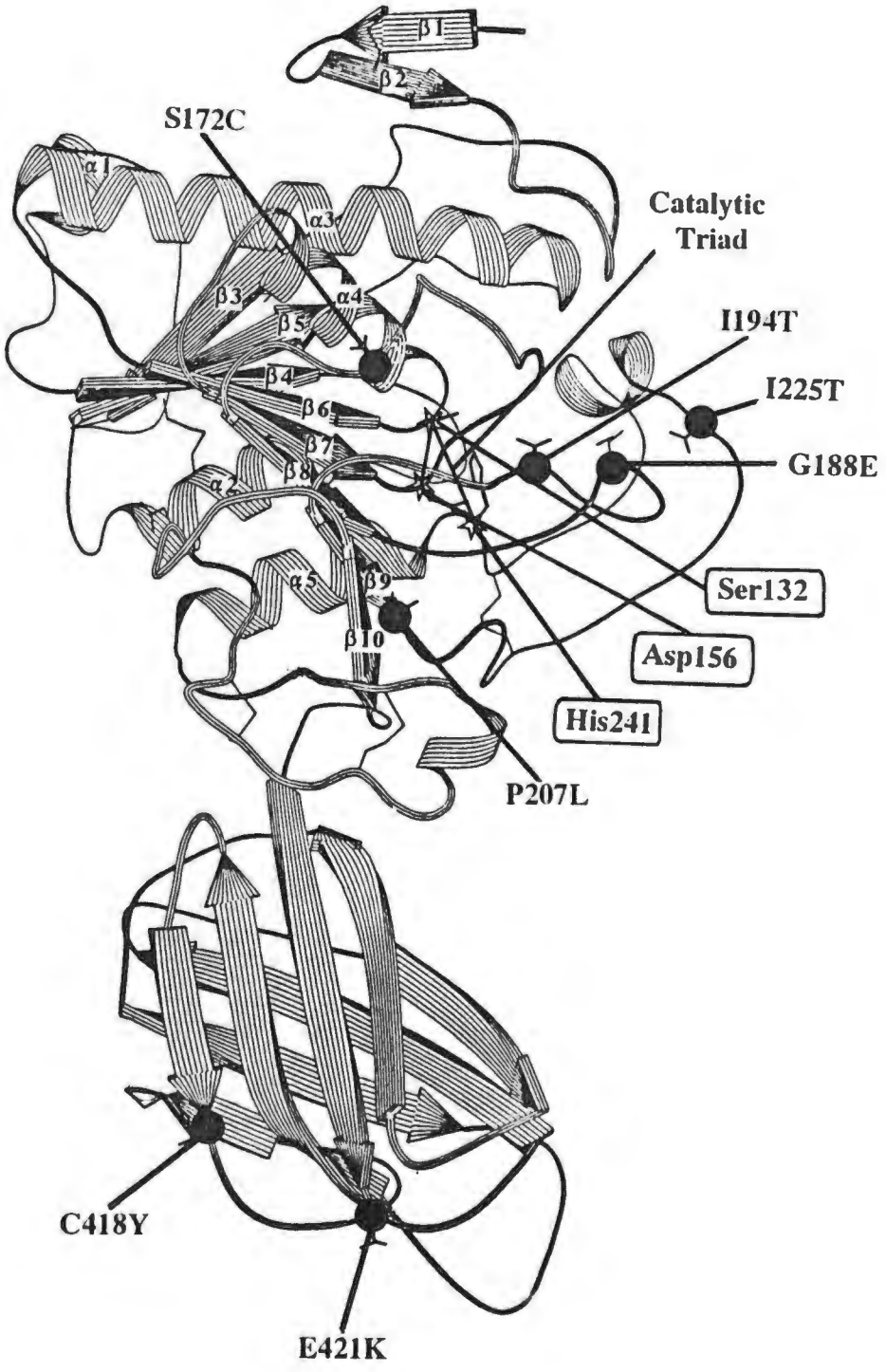


Fig 8: **Ribbon diagram showing the α/β type structure of the human pancreatic lipase molecule. Location of LPL mutations discussed in the manuscript. The mutations are indicated by (●). The active site residues (☆) involved in the formation of the catalytic triad are shown. The α-helix and β-sheet structures of the N-terminal domain are numbered to facillitate the discussion.**

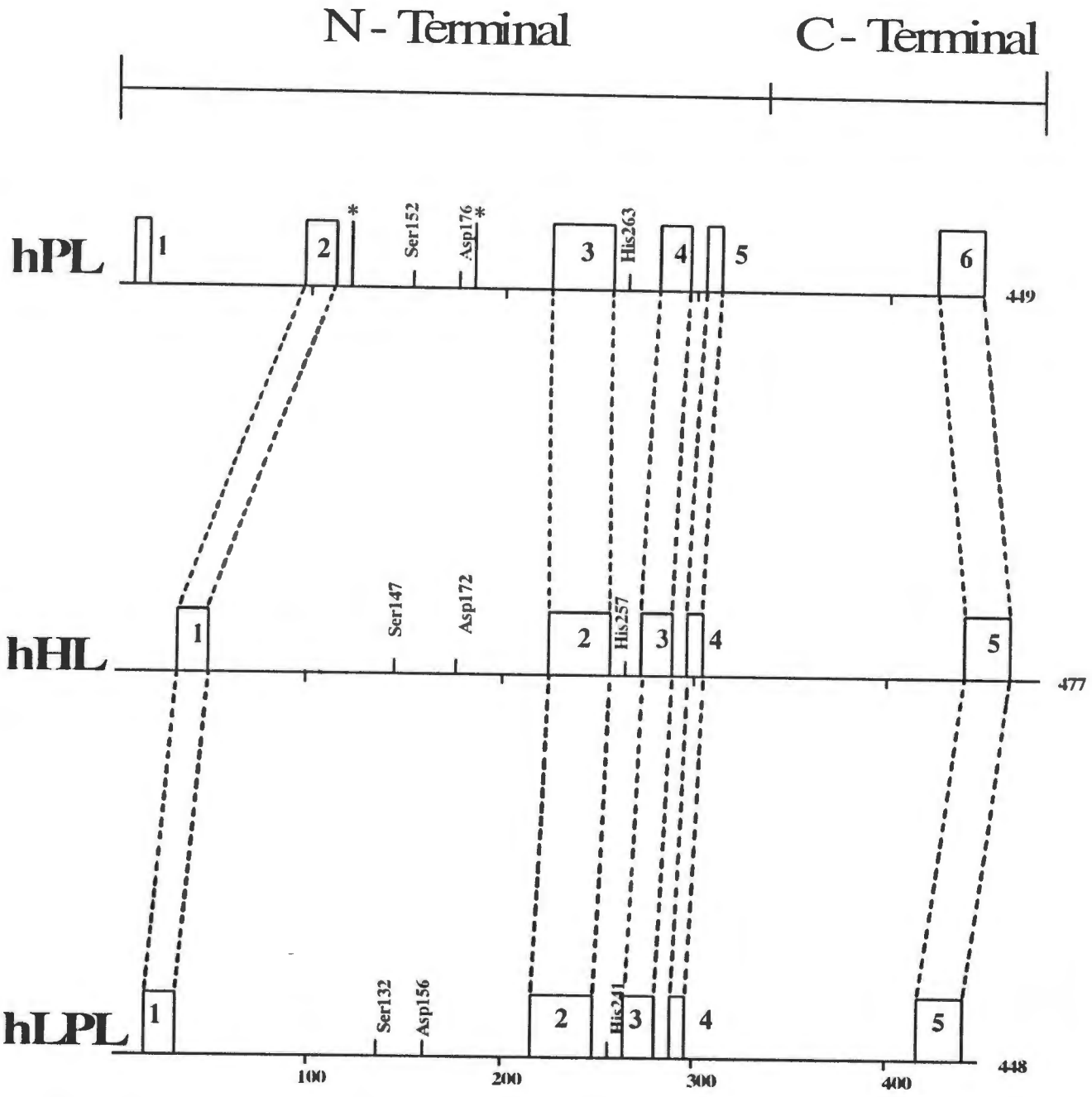


Fig. 9: **The location of disulfide bridges and conserved cysteine residues in the N- and C- terminal domains of human lipoprotein (hLPL), hepatic (hHL) and pancreatic lipase (hPL). Disulfide bridges are numbered 1 to 6 and free cysteine residues are marked (*). Also shown is the size of the individual proteins and the position of the catalytic triad residues, Ser, Asp and His.**

1.3.2 Functional Domains.

1.3.2.1 Active Site.

The catalytic triad, Ser...His...Asp, of the serine protease and esterase family of enzymes (Rosenberry, 1975) is identical to that found in most lipases (Fig. 10), except for the *Geotrichum candidum* lipase, where a Glu replaces an Asp in the triad. The putative catalytic Ser forms part of the conserved consensus sequence, G-X-S-X-G (Table 3) and several lines of evidence show this residue to be the active site serine in LPL.

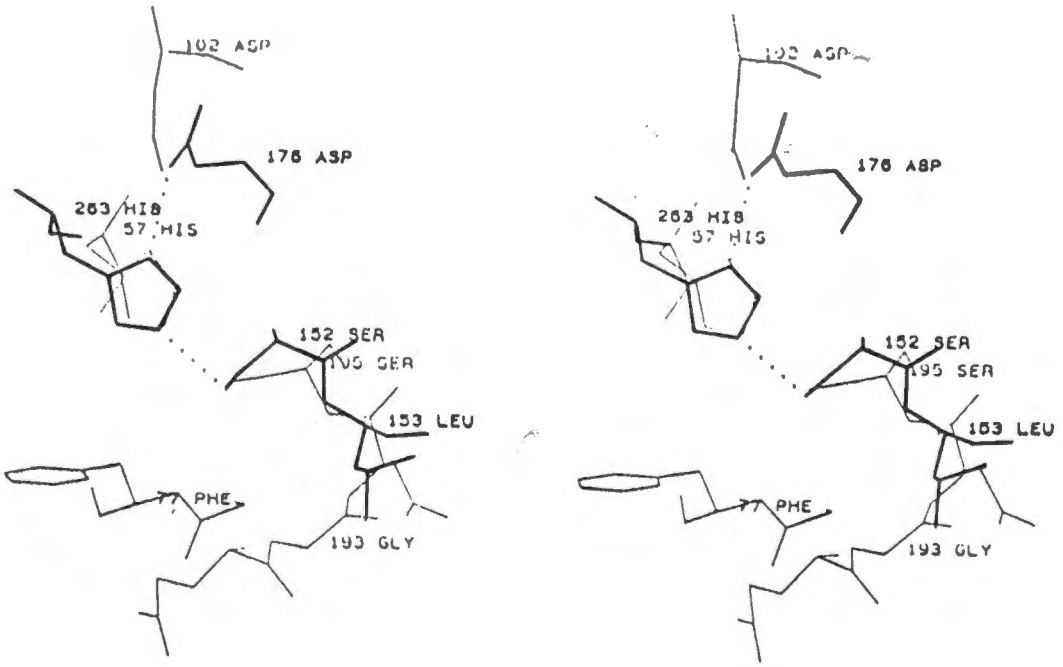


Fig. 10: Stereodiagram of the superimposed Asp-Ser-His catalytic triads of human pancreatic lipase (thick lines) and trypsin (Winkler, 1990).

The catalytic triad in hPL (Ser152, His263, Asp176)(Winkler *et. al.*, 1990) and RML (Ser144, His257, Asp203)(Brady *et. al.*, 1990) was demonstrated to be situated in a cleft, buried under a surface loop, called the "lid", rendering it inaccessible to the aqueous environment. Sequence alignment and structure homology predictions suggest that Ser132, Asp156 and His241 would be the functionally equivalent counterparts in LPL (Fig. 7). Several investigators have shown that

substitutions at all three active site residues lead to the expression of catalytically inactive LPL mutant products (Faustinella *et. al.*, 1991; Toshiro *et. al.*, 1992; Emmerich *et. al.*, 1992). The mutant LPL protein mass levels and heparin binding properties were similar to native LPL. These findings indicate that these substitutions disrupted only the hydrolytic activity of LPL, without affecting any of the other functional domains. Substitution of the homologous Ser147 in hepatic lipase (Davis *et. al.*, 1990) caused a similar lack of activity against triolein emulsions and tributyrin substrates.

Based on structural predictions for RML, the catalytic Ser144 was demonstrated to be part of a β-ϵSer-β conformation, unique to lipases (Derewenda and Derewenda, 1991). In identical fashion, Ser132 of LPL is located at the C-terminal end of β-strand 6 which is connected it to α-helix 4 (Derewenda and Cambillau, 1991)(Fig. 8). In this position Ser132 is under considerable strain forming part of a classical β-hairpin type II' turn or "nucleophilic elbow (Sibanda *et. al.*, 1989) where it assumes the rare ϵ conformation. The other triad residues, His241 and Asp156 are both located in flexible loops which vary markedly in size and structure between species. His241 is located at the end of helix 6 adjacent to a conserved disulfide bridge which forms the base of a loop structure called the "flap" or "lid" domain. Asp156 is located in the loop which connects strands, β7 to β8. This loop which is made up of eleven residues, partially obscures the catalytic Ser.

Interestingly, an active site geometry similar to lipases has been described in Dienelactone Hydrolase (DLH) from Pseudomonas sp. B13 (Pathak and Ollis, 1990). This enzyme which is also of the α/β type has an active site Cys123 in place of a Ser, which also assumes the identical β-ϵCys-β conformation. Evolutionary relationships based on codon interconversions confirmed a Cys residue as the precursor to all active site Ser residues (Brenner, 1988).

1.3.2.2 " Lid " or Loop Domain.

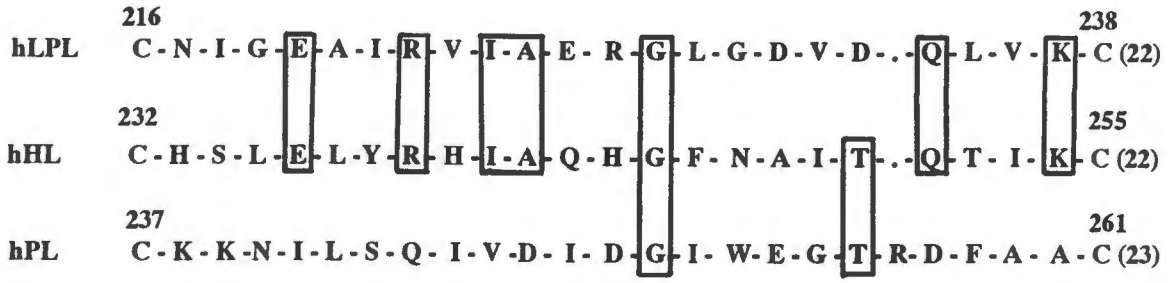
All the lipases crystallised to date have yielded molecular models showing an active site buried beneath surface loops which renders it inaccessible to solvent. In both RML (Cygler *et. al.*, 1992; Dodson and Lawson, 1992) and hPL (vanTilbergh *et. al.*, 1993)(Fig. 8), this "lid" or loop domain is formed by a short coil which blocks the active site cleft and is linked to the main body by two short flanking α-helices. In hPL, however the lid is completed by disulfide-bridging at its base.

At the lipid-water interface this structure undergoes a conformational change which involves the unwinding of the one-turn α-helix causing the lid to role back exposing the active site to substrate (van Tilbergh *et. al.*, 1993). This lid movement in turn causes a main chain movement of an adjacent active site loop resulting in the formation of the oxy-anion hole. This overall movement exposes the hydrophobic faces of the amphipathic lid helices which creates an extended hydrophobic surface with the non-polar surface of the cofactor, colipase, facillitating interaction with the lipid substrate.

Secondary structural predictions have also suggested a helix-turn-helix motif for the 22 amino acid LPL lid domain (res 217-238)(Dugi *et. al.*, 1992; Faustinella *et. al.*, 1992). This motif is disulfide-bridged (res Cys216-Cys239) at its base and contains helix A (8 amino acids in length), followed by a 3 amino acid β-turn or coil and a second helix B consisting of 7 amino acids (Fig. 11). Both helices are highly amphipathic in nature with hydrophobic moments 0.64 and 0.68, respectively, which are amongst the highest reported in the literature (Eisenberg *et. al.*, 1990). The projected α-helical character of the LPL lid domain is characteristic of proteins involved in lipid binding. Both helices in the LPL lid domain are of the A-helix type (high hydrophobic moments) which are characterised by the strong hydrophobicity of the non-polar faces and the charge distribution, with negatively charged residues in the centre and positively charged residues at the border between the hydrophobic and polar faces (Segrest *et. al.*, 1990).

Structural features of the lid domain such as amphilicity, length, periodicity and charge distribution are important for catalytic function. They may also be major determinants of substrate specificity, although this is yet to be explored. The secondary structural regions of the loop in hLPL, hHL and hPL are likely to be different as sequence conservation for this lid domain is poor (Fig. 11) and loops cannot be interchanged between enzymes. For example, replacement of the LPL lid with the HL lid generates a LPL/HL variant with 60% activity when compared to the normal LPL protein, while the LPL/PL loop mutant was totally inactive (Faustinella *et. al.*, 1992; Dugi *et. al.*, 1992). Alteration of the charge profile in the lid region of LPL (Henderson *et. al.*, 1994; Dugi *et. al.*, 1992) by the substitution of positively-charged residues with negatively-charged residues resulted in a markedly reduced or complete loss of activity against emulsified triolein, but activity against tributyrin was unaffected. This demonstrated that amphilicity and secondary structure in the lid domain was essential for activity (Dugi *et. al.*, 1992). A naturally-occurring point mutation, namely, the I225T, which occurs within the LPL lid domain caused a marked decrease in activity towards long-chain

A


216
238
hLPL C-N-I-G-E-A-I-R-V-I-A-E-R-G-L-G-D-V-D-Q-L-V-K-C (22)
232
255
hHL C-H-S-L-E-L-Y-R-H-I-A-Q-H-G-F-N-A-I-T-Q-T-I-K-C (22)
237
261
hPL C-K-K-N-I-L-S-Q-I-V-D-I-D-G-I-W-E-G-T-R-D-F-A-A-C (23)

B

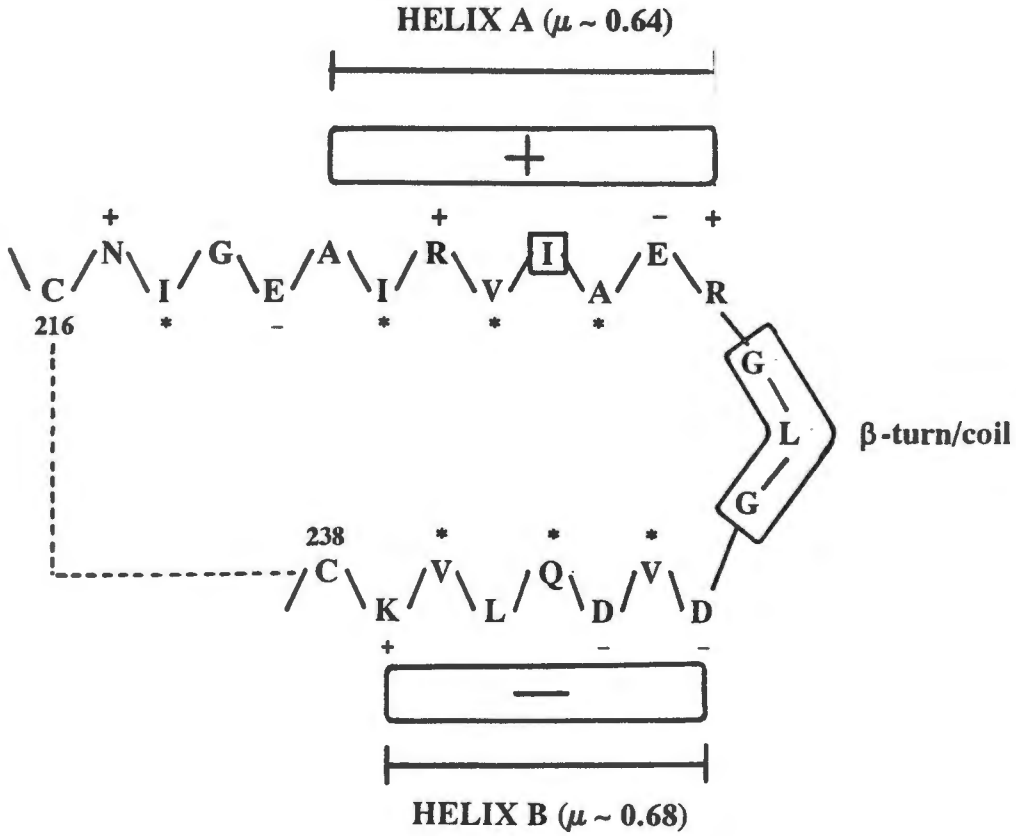

HELIX A (μ ~ 0.64)
C N I G E A I R V I A E R
216
G
L
G
β-turn/coil
238
C K V L Q D V D
HELIX B (μ ~ 0.68)

Fig. 11: **Sequence alignment and topology of the lid domain of human lipoprotein lipase.**
A. The sequence and size of the lid domains of human lipoprotein (hLPL), hepatic (hHL) and pancreatic (hPL) lipase are shown by the single amino acid notation. Sequence homology is indicated by boxed residues. The cysteine residues involved in the formation of the disulfide bridge forming the base of the lid domain are numbered. The size of the individual lid domains are shown in brackets.
B. The derived structure for the lid domain of human lipoprotein lipase is shown. This consists of helix A (res A221 - R228), helix B (res D232 - K237) and a β-turn (res G229 - G231). Helix A contains an overall positve charge and helix B a negative charge as indicated. Hydrophobic residues are indicated by an *. A naturally-occurring mutation at position 225 (I225T) is boxed. The disulfide bridge at the base of the lid domain is shown (C216-----C238).

---

lipid substrates (Fig. 8 and 11)(Henderson *et. al.*, 1993). The introduction of a hydroxyl (-OH) group from Thr in a highly hydrophobic region (flanked by Val and Ala residues) possibly disrupts interaction with lipid substrates or the main body of the enzyme.

1.3.2.3 ApoC-II Binding Site.

While the evidence for the specific sites of interaction between apoC-II and LPL remains obscure, several studies suggest an N-terminal domain location. Characterisation of chimeric lipases, consisting of an N-terminal LPL domain and a rat (Davis *et. al.*, 1991) or human (Dichek *et. al.*, 1992) HL C-terminal domain, demonstrated lipase activity only in the presence of apoC-II. Since HL is known to have no cofactor requirement for apoC-II, the interaction with the chimeric lipases are likely confined to the N-terminal domain. This is supported by the lack of an apoC-II enhancing effect on the activity of the reverse HL/LPL chimera. More recently, Bruin *et. al.* (1994) constructed

site-specific mutants of LPL to locate the exact apoC-II binding site. Their approach was based on the hypothesis that the two C-terminal glutamic acids of apoC-II mediate binding via ionic interaction with opposing positively-charged residues on LPL. Mutant constructs for the five dibasic sequences identified in LPL, revealed one mutant (K147A-K148A) with reduced LPL activity (35% of normal) in the presence of apoC-II, following expression in COS cells. Taken together, these findings are suggestive of an N-terminal domain location for the site of interaction of apoC-II. Lookene and Bengtsson-Olivecrona (1993) demonstrated effective binding of apoC-II to an N-terminal chymotryptic fragment of LPL (cLPL).

1.3.2.4 Heparin Binding Domain.

Several heparin binding proteins, such as, the apolipoproteins, apoB-100 and apoE (Yang *et. al.*, 1986; Innerarity *et. al.*, 1983) have been shown to have regions rich in basic (B) amino acids, such as, lysine and arginine, conforming to consensus sequences, -X-B-B-X-B-X and -X-B-B-B-X-X-B-X. Similar regions have been identified in LPL between residues 279-282 and 291-304 having the sequence CRKNRC and KVRAKRSSKMYLK, respectively (Enerback *et. al.*, 1987; Yang *et. al.*, 1989). The other heparin-binding lipase, hHL, has 8 Lys or Arg residues (Martin *et. al.*, 1988) while hPL has only 3 basic amino acid residues in the corresponding segments (Hata *et. al.*, 1993). This is consistent with the differences in their heparin affinity, LPL > HL, with PL, devoid of any heparin binding affinity.

Mutagenesis studies involving the substitution of charged residues in these regions support the contention that they form part of the heparin binding domain in hLPL (Hata *et. al.*, 1993) and avian LPL (Berryman and Bensadoun, 1993). In the human PL model the residues homologous to the heparin binding domain in hLPL are located in a solvent exposed β-turn structure, positioned close to the junction of the N-and C-terminal domains, on the face, opposite the active site (Fig. 7). This places the heparin binding domain in an ideal position for interaction with the endothelial-bound HSPG's. While this may be so, the contribution to heparin binding from the other regions mentioned cannot be discounted. A further arginine-rich region in LPL, residues 146-151, was postulated to be involved in interaction with heparin, but abolition of charge by mutagenesis did not affect binding affinity (Hata *et. al.*, 1993).

A heparin binding site localised to the C-terminal domain was proposed by several investigators. For the chimera construct, LPL/HL (Dichek *et. al.*, 1993; Wong *et. al.*, 1991) heparin affinity was shown to be associated with the C-terminal domain. The hLPL/hHL chimera was eluted at a salt concentration of 0.9M from Heparin-Sepharose columns which represents an intermediate affinity for heparin between that for hLPL (1.1M) and hHL (0.75M)(Dichek *et. al.*, 1993). This differs from the elution profile observed for a human LPL/rat HL chimera, which eluted at 0.75M NaCl similar to native human HL (Davis *et. al.*, 1992). This may in part be explained by the sequence differences in the C-terminal domain between the human HL (residues 330-476) and rat HL (330-472) consisting of 22 as opposed to 16 basic amino acids residues, respectively. Thus the greater basic residue content of the human HL domain may impart a higher affinity for heparin.

1.3.2.5 Lipid Binding Site.

An ideal lipid binding motif exists in the C-terminal domain and comprises residues proximal and distal to the Cys418-Cys438 disulfide bridge. The proximal six residues, APAVFV, are brought into close proximity with a three residue hydrophobic segment, juxtapositioned next to Cys418, following disulfide bridge formation (Fig. 7). Strong support for the localisation of a lipid binding domain in the region was provided by the expression in COS cells of C-terminal truncated LPL mutants (Kozaki *et. al.*, 1993). Interestingly in this study, LPL mutants, 435→stop and 436→stop exhibited respective lipase activities of 22 and 88% compared to native LPL, when using triacylglycerol emulsions as substrates. Notably, only four of the nine corresponding residues are conserved in HL with no such conservation observed in PL consistent with their variable substrate specificities.

In addition, a mutant construct of a naturally occurring mutation, Trp^{382}→stop, produced a catalytically inactive protein establishing the importance of this hydrophobic domain in the expression of full activity towards emulsified lipid substrates. Further evidence for the involvement of the C-terminal domain in lipid binding comes from the localisation of the epitope for the LPL monoclonal antibody, $Ab5D_2$, to residue 400 (Lui *et. al.*, 1992)(Fig. 7). This antibody inhibits activity to long-chain triacylglycerols but does not affect activity towards tributyrin, implying a role for the C-terminal domain in lipid binding.

1.3.3 Function Properties.

1.3.3.1 Interfacial activation, active site mechanism and physical properties

Schønheyder and Volqvartz (1945) were the first to describe the phenomena of "Interfacial Activation" for pancreatic lipase (PL). Chapus *et. al.* (1976) confirmed this mechanism of action for PL by demonstrating an increase in enzyme activity in the presence of substrates with hydrophobic interfaces e.g. inert silicon beads. The recent publication of the crystal structures of a fungal lipase-inhibitor complex (Brzozowski *et. al.*, 1991) and the hPL-procolipase-phosphatidylcholine/bile salt complex (van Tilbergh *et. al.*, 1993) provided a model for "interfacial kinetics". Since the long-chain triacylglycerols embedded in large lipoproteins form emulsions in aqueous medium it would suggest that lipases work at the two dimensional (lipid-water) interface of an emulsion (Potts *et. al.*, 1991; Chapus *et. al.*, 1976; McLean *et. al.*, 1986; Shirai *et. al.*, 1982, 1984; Quinn *et. al.*, 1982). Subsequently, this phenomena was also confirmed for LPL following monomolecular lipid film studies (Macritchie, 1978; Vainio *et. al.*, 1983; Burdette *et. al.*, 1986).

A classical acyl-enzyme pathway based on kinetic studies was demonstrated for the lipase reaction (Chapus *et. al.*, 1976)(Fig. 12). This catalytic charge-relay reaction mechanism has been well documented, as the preferred mechanism in serine proteases (Warshel *et. al.*, 1989) and esterases (Rosenberry *et. al.*, 1975; Blow, 1976). The role of the Ser ··· Asp ··· His catalytic triad is now well established as the constellation of residues responsible for catalysis in the lipase family of proteins. The triglyceride substrate, upon binding to the interfacial binding site appears to migrate down a hydrophobic channel to the catalytic site by facilitated diffusion (Scot *et. al.*, 1990). An ester bond between the carboxyl group of the fatty acid and serine hydroxyl of the enzyme characterises the acyl-enzyme intermediate that forms. Since the deacylation step is base-catalysed in both the lipase (Rojas *et. al.*, 1989) and serine esterase (Rosenberry *et. al.*, 1975) reaction, the histidine imidazole is implicated as the base catalyst in these reactions. The aspartic acid residue in turn stabilises the transition state complex by neutralising the positively-charged imidazole ring that forms during the reaction (Brady *et. al.*, 1990)(Fig. 12).

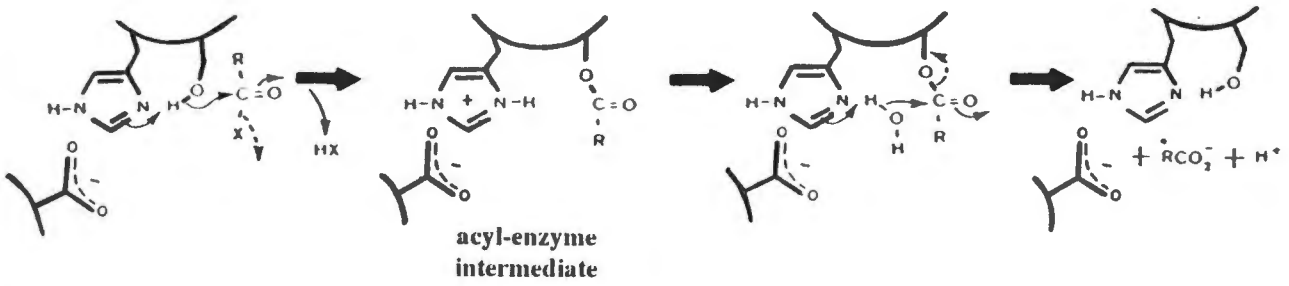


Fig. 12: The catalytic charge-relay reaction mechanism of the serine esterase/ protease and lipase enzymes. The acyl-enzyme intermediate is shown.

LPL manifests maximal activity at an alkaline pH of 8.8 to 9.0 (Ehnholm *et. al.*, 1975) and is inhibited by salt solutions of high ionic strength (Fielding and Fielding, 1976). This salt-mediated inhibition follows the Hofmeister series ($I^- > SCN^- > NO^{3-} > Cl^- > F^-$) and is fully reversible. Fielding and Fielding also showed that salt inhibition at 1M NaCl could be reversed by lowering the ionic strength. LPL from a variety of sources, including adipose tissue and post-heparin plasma from the rat (Parkin *et. al.*, 1982) and bovine milk (Kinnunen *et. al.*, 1976) exhibit similar properties. Op Den Kamp *et. al.* (1974) demonstrated stability of the active LPL over a wide temperature range (17°C to 32°C) with maximal activity achieved at 37°C (Quinn *et. al.*, 1982).

1.3.3.2 Substrate Specificity.

While long-chain triacylglycerides are the preferred substrates, LPL has also been shown to hydrolyse medium and short chain triglycerides, diglycerides and monoglycerides; long- and short-chain phosphatidylcholines and water-soluble p-nitrophenylesters (Cryer, 1985; Speake, 1985). While substrate specificity is low, positional specificity at the ester bonds in acylglycerols is strict, as the sn-1 and sn-3 positions are preferentially hydrolyzed, generating sn-2 monoglycerides and free fatty acids. It has been shown that LPL is more effective in the catalysis of triglyceride-rich substrates containing the n-3 and n-6 class of fatty acids (Murphy *et. al.*, 1993).

1.3.3.3 Interaction with ApoC-II.

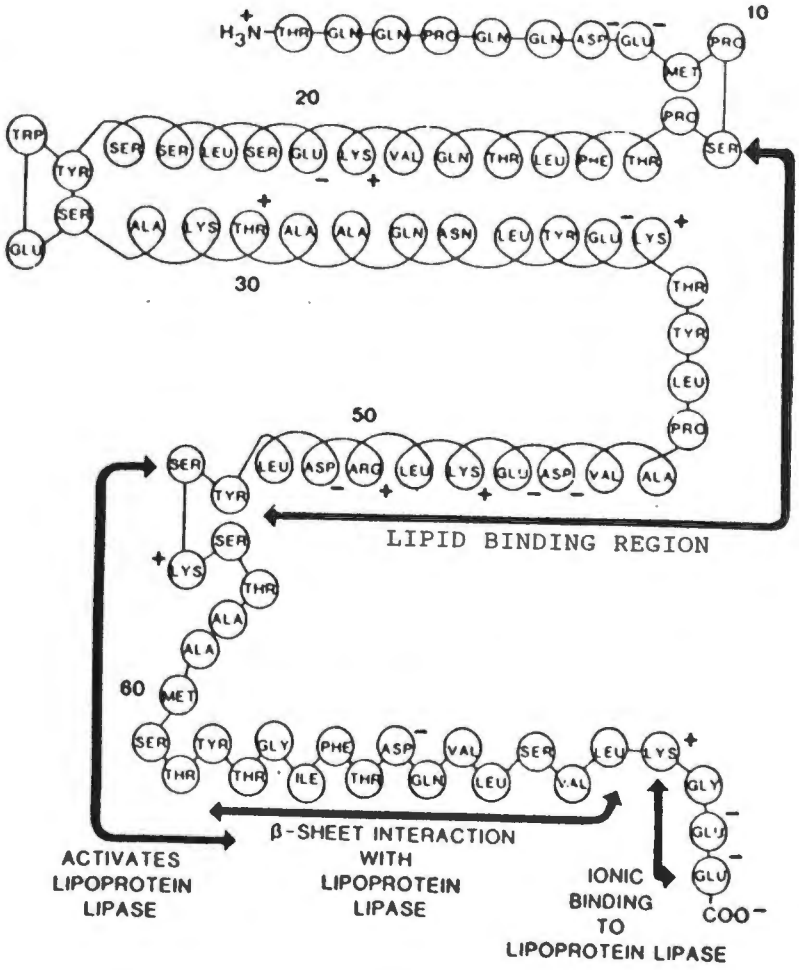


Fig. 13: ApoC-II peptide sequence and structure. (Reprinted from Smith and Pownall, 1984). Specific active domains are indicated.

The exact mechanism for apoC-II activation of LPL is still obscure, but a unifying hypothesis suggests that apoC-II orientates LPL at the interface such that it's active site is readily accessible to substrate (Olivecrona *et. al.*, 1987). Direct protein-protein interaction between the LPL and apoC-II was demonstrated by monitoring intrinsic flourescence upon interaction of dansylated apoC-II with LPL (Clarke and Holbrook, 1985). They were able to show a 1:1 stoichiometry for this interaction, with each subunit of the LPL homodimer binding a single apoC-II molecule with a dissociation constant of 0.2μM (Olivecrona and Bengtsson-Olivecrona, 1987). This asssociation appears to

become tighter in the presence of lipid as evidenced by a 40 fold decrease in K_d (0.05μM)(McLean *et. al.*, 1986). Varying the apoC-II concentration had no effect on the apparent K_m but the apparent V_{max} was increased 20 fold for the LPL hydrolysis of emulsified triacylglycerol substrate (Posner *et. al.*, 1983). The LPL-apoC-II complex formation is therefore not the rate-determining step. Furthermore, it has been shown that the apoC-II protein contains distinct regions involved in the activation of LPL (Fig. 13). A LPL binding domain spans residues 61 to 79, with the N-terminal residues 1-49 representing a lipid binding domain (Cetapano *et. al.*, 1979). The C-terminal apoC-II tetrapeptide, KGEE appears to be involved in direct binding to LPL (Cheng *et. al.*, 1990; Musliner *et. al.*, 1977) and is therefore thought to represent an ionic binding site involved in electrostatic association with LPL.

1.3.3.4 Interaction with Heparin.

More than 50 years ago it was noted that intravenous heparin administration enhanced lipolysis by releasing a "clearing factor", later classified as "Lipoprotein Lipase", into the blood circulation of dogs (Hahn, 1943; Korn, 1955). This functional property of heparin was also demonstrated in humans (Ehnholm *et. al.*, 1975 and 1985). Heparin appears to have a dual role, in that it was reported to stimulate the secretion of active LPL from guinea-pig adipocytes in a constitutive manner without affecting it's cellular activity (Semb and Olivecrona, 1987; Ashby *et. al.*, 1978). It is also known to stabilise the active LPL dimer against degradation (Cupp *et. al.*, 1987). In support of these proposed roles, mast-cell deficient W/W^v mice (mast cells are heparin producing cells) were found to have markedly reduced levels of LPL and HL in post-heparin plasma, which correlated with their negligible heparin levels (Hatanaka *et. al.*, 1986). Also, Vannier *et. al.* (1989) demonstrated a quantitative increase in LPL secretion from adipocytes in the presence of heparin While Cupp *et. al* (1991) showed that incubation of cultured adipocytes with heparin resulted in reduced degradation of LPL. Heparin may therefore play an integral role in the regulation of LPL secretion *in vivo.*

1.3.3.5 Effect of ApoC-III.

The role of apoC-III on LPL function is still unclear but the available evidence suggests an inhibitory role (McConathy *et. al.*, 1992). Brown and Baginsky (1972) for instance, showed that the

hydrolysis of emulsified triglyceride by apoC-II-stimulated bovine LPL was inhibited by the addition of excess apoC-III. Earlier, Carlson and Ballantyne (1976) had demonstrated an inverse relationship between VLDL-triglyceride levels and the apoC-II/apoC-III_1 ratio, suggesting that alterations in the apoC-II and apoC-III, content in VLDL may cause decreases in LPL activity. In support of these findings, increased apoC-III_2 levels in association with variant triglyceride-rich lipoproteins have been described in some patients with severe hypertriglyceridemia (Holdsworth *et. al.*, 1982) and chronic renal failure (Staprans *et. al.* 1979). This variant lipoprotein was demonstrated to be a poor substrate for bovine LPL (Holdsworth *et. al.*, 1982) which is attributed to elevated levels of the apoC-III_2 isoform of this lipoprotein, since normalisation of the apoC-III_2/apoC-III_1 ratio following neuraminidase treatment, resulted in a normal lipoprotein species and interaction with LPL. In order to investigate the physiological role of apoC-III, a transgenic mouse model, containing an excess of human apoC-III gene copies, was shown to overexpress the apoC-III product leading to severe hypertriglyceridemia (Ito *et. al.*, 1990). VLDL was the predominant lipoprotein species which accumulated in the plasma of these hypertriglyceridemia transgenic mice. Less secure evidence for a regulatory role for apoC-III comes from association studies where a Sac I restriction fragment length polymorphism (RFLP) in the 3' untranslated region of the apoC-III gene was shown to segregate with severe hypertriglyceridemia in English Caucasians (Tas, 1989; Aalto-Selätä *et. al.*, 1987) and the Arab population in Kuwait (Tas, 1989). Subjects with the Sac I allele also had decreased HDL consistent with a depressed LPL activity in these individuals. These findings do imply a possible regulatory role for apoC-III.

1.3.3.6 Effect of Fatty Acids

LPL hydrolysis of the triglyceride core of chylomicrons and VLDL liberates free fatty acids which are sequestered mainly by albumin. In the absence of albumin, the fatty acids become rate-limiting due to their accumulation at the lipid-water interface. They may therefore act as competitive inhibitors with the enzyme forming complexes with the fatty acids, suggesting that a feedback inhibition mechanism may prevail (Peterson *et. al.*, 1990). Saxena *et. al.* (1989) were able to demonstrate that the release of endothelial cell-bound LPL by its physiological substrates, chylomicrons and VLDL, was as a direct result of the increase in fatty acid production. Furthermore, incubation of cultured endothelial cells with fatty acids caused a dramatic release of LPL into the medium, due to dissociation of the LPL from it's binding sites, in accord with it's LPL inhibitory role

(Karpe *et. al.*, 1992; Saxena *et. al.*, 1990). This process occurred in a dose-dependent and specific manner, since fatty acids were unable to release antithrombin-III, another protein known to bind to endothelial cell-surface glycosaminoglycans (Shimada and Ozama, 1987; Stern *et. al.*, 1985). Furthermore, free fatty acids also appear to destabilise the binding of LPL to heparin-sepharose columns (Olivecrona and Bengtsson-Olivecrona, 1985).

1.4 Type I Hyperlipoproteinemia.

This rare disorder of lipid metabolism manifesting as the chylomicronemia syndrome is characterised by the presence in plasma of large triglyceride-rich chylomicrons, while in the fasted state. While the clinical phenotype presents a preliminary diagnosis for this condition, it is not sufficient to distinguish the heterogeneity underlying Type I hyperlipoproteinemia (Brunzell *et. al.*, 1980) which may be subdivided into four distinct inborn errors of lipid metabolism namely:

1. Familial Lipoprotein Lipase Deficiency
2. Apolipoprotein C-II Deficiency
3. Transient expression and/or site-specific LPL deficiency
4. Circulating Inhibitors of Lipoprotein Lipase

1.4.1 Familial LPL Deficiency.

The classical work by Havel and Gordon in 1960, established the role of LPL in the aetiology of Type I hyperlipoproteinemia. They reported on three siblings with fasting hyperchylomicronemia who demonstrated an inability to remove post-prandial fat from the circulation. The introduction of a fat-free diet failed to reduce the circulating triglyceride levels to within the normal range. Their finding of a decreased LPL activity in patient plasma led to the conclusion that a genetic deficiency of LPL was responsible for the defective lipolysis in these subjects. This condition is characterised by a marked reduction or complete absence of LPL activity in post-heparin plasma and has been shown to be due to mutations in the LPL gene. The genetic heterogeneity underlying this disease is well documented, with over 40 mutations being reported in literature to date (Fig. 4).

1.4.1.1 Age of Onset.

Clinical symptoms usually manifest in childhood, often in the first year of life (Sadan *et. al.*, 1977) but late diagnosis has been reported, with the oldest subject being a 75 year old man (Hoeg *et. al.*, 1983). This variable age of onset, again highlights the heterogeneity underlying this disorder.

1.4.1.2 Clinical and Biochemical Profile.

A full clinical examination often reveals a history of recurrent abdominal pain, probably due to pancreatitis as the major complaint in association with one or two of the other clinical symptoms (Table 4)(Brown *et. al.*, 1977). Serum amylase levels are often raised during the episodes of abdominal pain and pancreatitis. In early infancy, the disease is usually characterised by colicky pain, failure to thrive and eruptive xanthoma (Brunzell and Biermann, 1982). These patients present with creamy, milky plasma in the fasted state indicative of chylomicrons in the circulation. Chylomicronemia is usually indicative of triglyceride levels in excess of 15-20 mmol/l (normal < 2 mmol/l); fasting levels in excess of 80 mmol/l have been reported in some patients. LPL activity is markedly reduced or completely absent in post-heparin plasma. Hepatic lipase activity levels are usually found to be normal. Parents often present with reduced post-heparin plasma LPL activity levels in keeping with the familial nature of the condition. VLDL may be normal to moderately elevated, but LDL and HDL levels may be markedly depressed. A low cholesterol/triglyceride ratio is often recorded, with a ratio of less than 0.2 being diagnostic.

Table 4: Clinical Manifestations associated with the Chylomicronemia Syndrome and Type I Hyperlipoproteinemia.

Clinical Manifestations
Abdominal pain/Pancreatitis
Eruptive Xanthomata
Hepatosplenomegaly
Lipemia Retinalis
Objective Dyspnoea
Recent Memory Loss(Dementia)

Obligate heterozygotes usually present with reduced post-heparin plasma LPL activities but sometimes may overlap with levels in normals (Kondo *et. al.*, 1985; Barbirak *et. al.*, 1989). Fasting triglyceride levels often are in the upper normal range, while VLDL-cholesterol levels can be raised in the presence of low LDL and HDL_2-cholesterol levels (Wilson *et. al.*, 1990). Carriers have been shown to exhibit an abnormal lipoprotein pattern and pronounced lipemia in the post-prandial state. HDL_2 were found to be enriched in triglyceride, VLDL and IDL were enriched in cholesterol esters, IDL levels were elevated and LDL was reduced in size (Miesenbock *et. al.*, 1993). These findings are consistent with a defect in LPL function. Secondary hyperlipidemia (Table 5) may often complicate diagnoses with the underlying primary LPL deficiency only detected following treatment of the former condition.

Table 5: Conditions associated with hypertriglyceridemia.

Secondary Hypertriglyceridemias	
Common	**Rare**
Diabetes Mellitus **Hypothyroidism** **Renal Failure (Uremia)** **Nephrotic Syndrome** **Alcoholism** **Obesity**	**Cushing's Syndrome** **Acromegaly** **Systemic Lupus Erythematosus** **Dysglobulinemias** **Lipodystrophy** **Storage Diseases (Gaucher etc.)** **Pregnancy** **Post-transplantation**

1.4.2 Apolipoprotein C-II Deficiency.

Apolipoprotein C-II (apoC-II) deficiency is a rare autosomal recessive disorder characterised by the absence of the physiological activator of LPL, apoC-II, in the circulation. Molecular defects at this gene locus result in a clinical phenotype indistinguishable from familial LPL deficiency. It was originally described by Breckenridge *et. al.* (1978) in a 59 year old man with diabetes mellitus showing typical symptoms of LPL deficiency (Brunzell, 1989). A blood transfusion and substitution therapy with normal HDL caused a dramatic increase post-heparin plasma LPL activity, demonstrating that a deficiency in apoC-II was the primary defect in this patient. Yamamura *et. al.*

(1979) established apoC-II deficiency as the basis for the Type I phenotype in two siblings. Consanguinity was demonstrated in both studies with all parents showing apoC-II levels at 50 % of normal, confirming a familial nature for this disease.

1.4.2.1 Age of Onset.

It usually has a later age of onset than classical LPL deficiency, ranging from 12 to 60 years of age with symptoms often traced back to childhood (Brunzell, 1989).

1.4.2.2 Clinical Profile.

A diagnosis is often confirmed when the absence of apoC-II can be demonstrated on immunoblot or radioimmunoassay. Intravenous infusion of normal plasma usually results in a marked reduction in chylomicron, VLDL and triglyceride levels. Also the recovery of normal post-heparin plasma LPL activity in the presence of an exogenous source of apoC-II again confirms the diagnosis. Symptoms are often less severe than that observed in familial LPL deficiency. Heterozygotes usually have 50% plasma apoC-II levels compared to normal individuals.

1.4.2.3 Genetics.

ApoC-II deficiency occurs at a frequency of less than 1 in 10^6 in the general population. The apoC-II gene was mapped to chromosome 9pter-q13 (Jean-Pierre *et. al.*, 1984)(Hayden *et. al.*, 1986). The availability of the gene sequence greatly facilitated the characterization of this disorder in more than 11 kindreds to date (Brunzell, 1989). Evidence of a worldwide ethnic distribution including individuals of Canadian (Breckenridge, 1978; Huff *et. al.*, 1990; Connelly *et. al.*, 1987), Japanese (Yamamura *et. al.*, 1979), English (Miller *et. al.*, 1981), American (Kashyap *et. al.*, 1980; Sprecher *et. al.*, 1988; Menzel *et. al.*, 1986), Peurto Rican (Saku *et. al.*, 1984), Turkish (Fojo *et. al.*, 1988), French (Parrot *et. al.*, 1992) and Italian (Hayden *et. al.*, 1986)(Sprecher *et. al.*, 1988)(Fojo *et. al.*, 1989)(Baggio *et. al.*, 1986) origin has been described. Consanguinity is a common feature in this disorder. To date fifteen different mutations with several sequence variants as well as base deletions, insertions and splice-junction defects have been described at this locus, underlying the genetic heterogeneity in this disorder (Table 6).

Table 6:Gene variants reported at the ApoC-II gene locus upto 1993.

Designation (Exon)	Amino Acid Substitution/ Mutation	Reference
$ApoC\text{-}II_{St.\ Michael}$ (exon 4)	$Gln^{70}\rightarrow Pro$ / base insertion causes frameshift with extension to Pro^{96}.	Connelly *et. al.*, 1987
$ApoC\text{-}II_{Nijmegen}$ (exon 3)	$Val^{18}\rightarrow stop$ / G deletion causes frameshift.	Fojo *et. al.*, 1988
$ApoC\text{-}II_{Hamburg}$ (exon 2)	G→C substitution causes a donor splice defect in intron 2.	Fojo *et. al.*, 1988 Beil *et. al.*, 1992
$ApoC\text{-}II_{Toronto}$ (exon 4)	$Leu^{75}\rightarrow stop$ / T deletion causes a frameshift.	Cox *et. al.*, 1988
$ApoC\text{-}II_{Padova}$ (exon 3)	$Tyr^{37}\rightarrow stop$ / C→A transversion.	Fojo *et. al.*, 1989
$ApoC\text{-}II_{Paris1}$ (exon 1)	$Met^{-22}\rightarrow Val$ / A→G transition.	Fojo *et. al.*, 1989
$ApoC\text{-}II_{Bari}$ (exon 3)	$Tyr^{37}\rightarrow stop$ / C→G transversion.	Crecchio *et. al.*, 1990
---------- (exon 4)	$Phe^{67}\rightarrow Ser$ / T→C transition.	Fojo *et. al.*, 1990
---------- (exon 2)	$Lys^{19}\rightarrow Thr$ / A→C transversion.	Hegele *et. al.*, 1991
$ApoC\text{-}II_{Japan}$ (5'-untranslated)	-82bp / C deletion and -28bp / T→G transversion upstream of initiation codon.	Xiong *et. al.*, 1991 Fojo and Brewer, 1992
(exon 3)	$Gln^{2}\rightarrow stop$ / C deletion.	
(polyadenylation site/exon 4)	AT deletion.	
$ApoC\text{-}II_{Venezuela}$ (exon 3)	$Gln^{2}\rightarrow stop$ / C deletion.	Xiong *et. al.*, 1991 Fojo and Brewer, 1992
(exon 3)	$Ser^{21}\rightarrow Pro$ / T→C transition.	
(exon 4)	$Gln^{70}\rightarrow stop$ / C→T transition.	
(exon 4)	$Glu^{79}\rightarrow Val$ / A→T transversion.	
(3'-untranslated)	C→T transition.	
$ApoC\text{-}II_{Paris2}$ (exon 1)	$Arg^{-19}\rightarrow stop$ / C→T transition.	Reina *et. al.*, 1992 Parrott *et. al.*, 1992
$ApoC\text{-}II_{Wakayama}$ (exon 3)	$Trp^{26}\rightarrow Arg$ / T→C transition.	Inadera *et. al.*, 1993
$ApoC\text{-}II_{San\ Francisco}$ (exon 3)	$Glu^{38}\rightarrow Lys$ / G→A transition.	Pullinger *et. al.*, 1993
Variants (Polymorphisms)		
$ApoC\text{-}II_{Bethesda}$	Lower apparent MW, more basic pI.	Sprecher *et. al.*, 1988
$ApoC\text{-}II_2$ (Black Africans)	$Lys^{55}\rightarrow Gln$.	Menzel *et. al.*, 1986
$ApoC\text{-}II_V$	More acidic pI.	Huff *et. al.*, 1990

1.4.3 Site-specific and Transient Expression LPL variants.

A 21 year old patient with no detectable LPL activity in post-heparin plasma was shown to process LPL normally in the endoplasmic recticulum but translocation to the golgi complex was impaired, indicative of a transport defect. Berger (1986) demonstrated disproportionate expression of LPL activity in five patients; four patients exhibited low levels of both adipose tissue LPL (1% of normal) and muscle LPL (5 to 20% of normal) activity, while one had normal muscle LPL activity with a significantly depressed adipose tissue LPL activity. In another report, a 33 year old female of East Indian extraction had a markedly reduced post-heparin plasma LPL activity (10% of normal) and a normal AT-LPL activity. However, a peculiar finding was the return to normal post-heparin plasma LPL activity 4 hours after continuous heparin infusion (Burton and Nadler, 1977).

1.4.4 Circulating LPL Inhibitors.

Brunzell *et. al.* (1983) reported on two Caucasian subjects; a 47 year old mother and her 27 year old son, presenting with chylomicronemia and low post-heparin plasma LPL activities due to the presence of a heat-stable, non-apolipoprotein inhibitor of LPL. Autoimmune chylomicronemia due to the presence of antibodies to LPL in patient plasma has also been described (Kihara *et. al.*, 1989).

1.5 Aims of this Work.

The general aims of this study were twofold (i) to establish the molecular basis for the LPL deficiency in the index patient, JJ(Kindred I) and (ii) to verify if LPL deficiency was the underlying condition that led to the death of the index patient, LB (Kindred II), by searching for gene mutations at the LPL gene locus.

The specific aims were:

i) To search for mutations at the LPL gene loci, through the application of:
   a) Direct DNA sequencing of individual LPL exons.
   b) RT-PCR technology.

ii) To set up and apply mutation detection systems for screening of gene mutations, such as:
   a) Allele-Specific Oligonucleotide Hybridisation.
   b) "Amplification Refractory Mutation System" PCR.
   c) PCR Restriction analysis.
   d) PCR-SSCP analysis.

1.6 The Layout of this work.

The current knowledge on LPL structure-function relationships, molecular genetics, metabolic function and its role in the aetiology of Type I hyperlipoproteinaemia is reviewed in Chapter 1. The case report of index patient, JJ (Kindred I) is given in Chapter 2 which contains:

i) a short Introduction
ii) a Case History section, which describes clinical histories including findings from an earlier workup on this patient.
iii) a Clinical and Biochemical Data section which describes the clinical and laboratory investigations performed on patient JJ and his available family members.
iv) a Results section.
v) a Discussion section, in which a detailed summary of the findings is presented and specific conclusions are proposed.

Chapter 3 contains the case report of index patient, LB (Kindred II) which comprises a:

i) brief review (Introduction) of the role of LPL in pregnancy and some case reports published in the literature to highlight the association of pregnancy with hyperlipidaemia.

ii) Case History and Laboratory Findings section presenting the patient clinical profile.

iii) Results section

iv) Discussion section, which summarises the major findings and conclusions.

General Conclusions and Future Studies are discussed in Chapter 4. A detailed outline of the methodologies employed in these studies is described in Chapter 5. Appendix 1 describes the Primer Design and oligonucleotides utilised in the respective studies. A full list of references is given at the end.

CHAPTER 2

KINDRED I ANALYSIS

This section of the thesis describes the genetic workup of patient, JJ, who presents with familial LPL deficiency. He is shown to be a compound heterozygote for two missense mutations located in exons 5 and 9 of the LPL gene. These point mutations result in amino acid substitutions of Ile194→Thr (I194T) and Cys418→Tyr (C418Y), respectively. Earlier transfection studies in COS cells established that the I194T substitution produces a catalytically defective protein. The absence of LPL activity in post-heparin plasma of JJ, is rather consistent with a primary LPL deficient status, in contrast to the earlier suggestion of a variable phenotype.

Part of this workup was published as a journal article:

Henderson H.E., Ma Y., Hassan M.F., Monsalve M.V., Marais A.D., Winkler F., Gubernator K., Peterson J., Brunzell J.D. and Hayden M.R. (1991). **Amino Acid Substitution (Ile^{194}→Thr) in Exon 5 of the Lipoprotein Lipase Gene Causes Lipoprotein Lipase Deficiency in Three Unrelated Probands: Support for a Multicentric Origin.** J. Clin. Invest., 87; 2005 - 2011.

2.1 Case History.

The index patient, JJ, is a 26 year old South African male of mixed ancestry, being derived from the indigenous Khoisan peoples with admixture from early Dutch, German and French settlers (Kindred I, Fig. 14)(Berger *et. al.*, 1987). JJ was first investigated at the age of 4, upon presentation of hepatosplenomegaly and complaints of pain in the right subcostal region. Although his triglyceride level was only moderately raised at 4.67mmol/l (normal, 0.3-2.3), lipoprotein electrophoresis showed a chylomicron and an elevated pre-beta band. He was diagnosed as having Type I hyperlipoproteinemia and placed on a strict low-fat diet. Although he remained symptomless on this regimen, fluctuating VLDL levels (normal to moderately elevated) led to an unstable lipoprotein pattern with a Type V or even a Type IV phenotype being recorded at times. The patient's father, WJ, had triglyceride levels in the upper normal range, from 1.91 to 2.41 mmol/l; his mother and his only sibling, WilJ, were normolipidemic. A complete family study revealed occasional hypercholesterolemia in paternal relatives. Consanguinity was ruled out in this kindred.

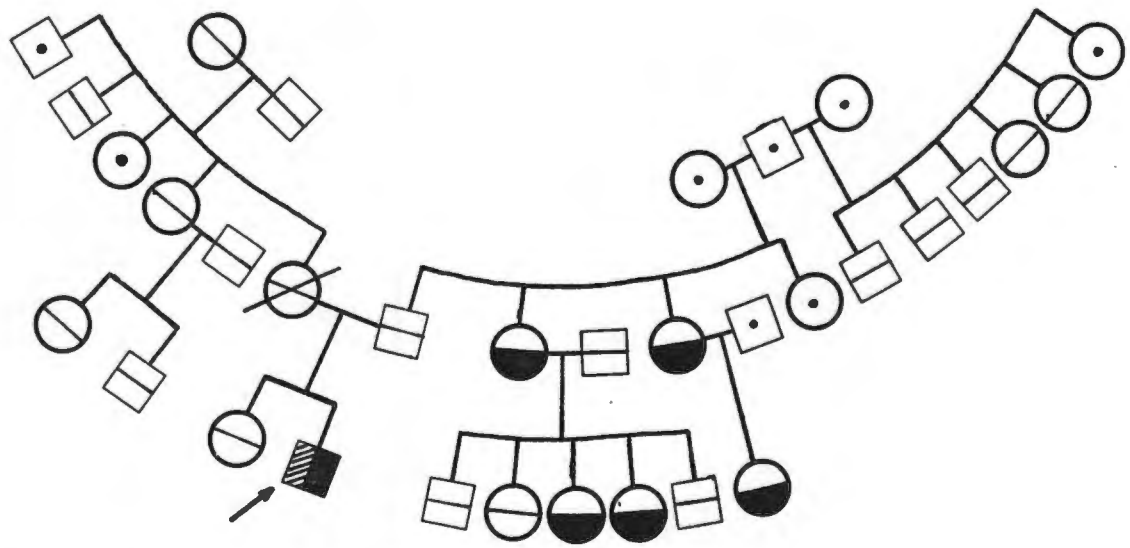


Fig. 14: Family Pedigree of Kindred I.
(Reprinted from Berger, 1987)
- not investigated, - normolipideamic, - proband,
- hypercholesteroleamic.

Patient JJ was hospitalised at the age of 11 years, essentially to determine his response to dietary fat reduction and to monitor his post-heparin lipolytic activity. The diet instituted had a high carbohydrate content (65% of total calories) with a moderate fat intake (25% of total calories). A significant decrease in plasma triglyceride levels was achieved, representing a reduction in chylomicron and VLDL fractions. Reducing the fat intake further to 5% of total calories caused a further drop in triglyceride levels with a concommitant disappearance of chylomicrons after 24 hours. Replacing this regimen with a normal western diet (fat content equivalent to 40% of total calories), caused a rapid rise in plasma triglyceride levels (75 mmol/l) after two days which was characterised by the presentation of severe chylomicronemia with near normal VLDL levels, typical of a Type I phenotype. Despite this acute response to a fat bolus, JJ has remained relatively symptom-free. Post-heparin plasma lipolytic activity measured before this dietary trial, by the protamine sulfate method (Berger *et. al.*, 1982) revealed significant LPL activity, of the order of 38% of normal, while hepatic lipase (HL) activity was within normal range. However, later assays of his post-heparin plasma LPL activity using a different procedure revealed a complete absence of activity (Table 7).

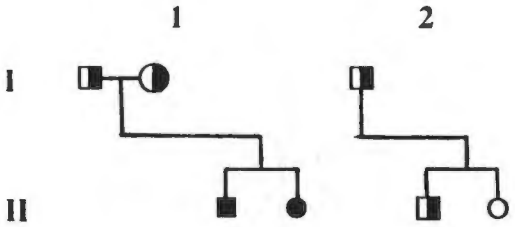
While, JJ, has not maintained a strict low-fat diet, he has remained symptom-free except for occasional bouts of acute pancreatitis. The working diagnosis at this stage was Type I hyperlipoproteinemia with a variant form of LPL deficiency (Berger *et. al.*, 1987).

2.2 Clinical and Biochemical Data.

Following the cloning of the LPLcDNA and the publication of the gene sequence (Wion *et. al.*, 1987) it was decided to reexamine JJ and identify the underlying gene mutations. Lipid profiles were again determined for JJ and all available family members which are given in Table7.

Grossly lipemic fasting plasma was recorded for JJ with a triglyceride level of 43 mmol/l (normal, 0.3-2.3). Lipoprotein electrophoresis showed the presence of a large chylomicron peak (46% of total) and a broad pre-βeta fraction (47% of total)(Table 7), rather indicative of a Type V phenotype (Brunzell, 1989), corroborating the earlier findings of a variable lipoprotein phenotype for this patient. A markedly elevated cholesterol level of 15mmol/l (normal < 5.2) with a cholesterol/triglyceride ratio of 0.3 (Type I <0.2)(Beaumont *et. al.*, 1970) is in agreement with a Type V diagnosis, but this may not be definitive, since overlap with the Type I phenotype has been documented (Nikkilä, 1978).

A. LIPID PROFILE



Plasma Triglyceride*	2.0	1.4	41.8	17.8	8.1	26.6	1.2
Total Cholesterol*	5.2	6.8	7.4	5.7	8.8	12.4	5.4
LDL_c*	3.5	4.6	·	·	7.2	·	4.0
HDL_c*	0.8	1.6	0.3	0.4	1.1	0.4	1.0
ApoA-I**	106	134	·	·	114	520	113
ApoB**	90	96	·	·	201	250	92
LPL Activity #	130	60	0	·	110	0	92
HL Activity #	244	168	412	·	124	237	178
LPL Mass T = 0' # #	-15	244	27	·	-18	-7	-5
LPL Mass T = 10'# #	435	621	354	·	426	286	75
Incremental Mass# #	435	377	327	·	426	286	75

B. LIPID ELECTROPHORESIS

FRACTIONS	%
ChMi (1)	46.4
Preβeta (2)	47.6
Alph (3)	6.0

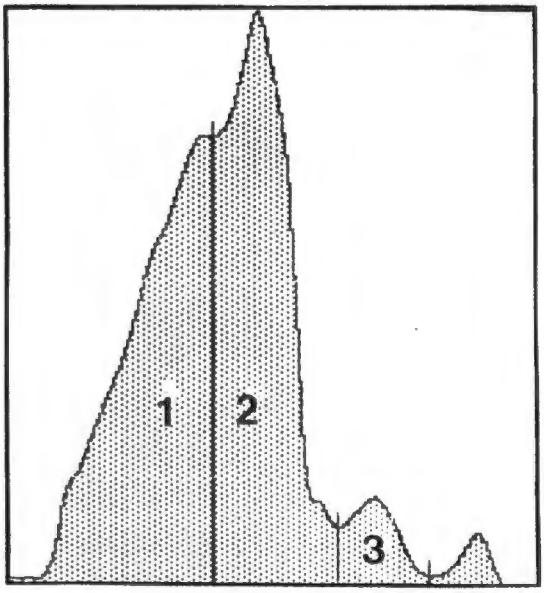


Table 7: A. Lipid profiles of two kindreds with LPL deficiency due to a 1194T mutation. Control Kindred (1), Kindred I (2). Fasting plasma lipid levels, apolipoprotein A-I and B concentrations, LPL and HL activities are shown. Concentrations are given as : * - mmol/l, **- μg/ml, # - nmol FFA/min/ml, # # - ng/ml.

B. Lipid electrophoresis on index patient, JJ(↗), are shown. ChMi - chylomicron, Preβeta - βVLDL, Alph - HDL.

■ ● – homozygous, ◨ ◑ – heterozygous, □ ○ – normolipideamic.

Other findings were a low HDL-cholesterol (HDL-C) level (0.4mmol/l; normal, 0.8-2). Both ApoA-I (52mg/dl; normal, 100-170) and apoB levels (25mg/dl; normal, 60-120) were also depressed (Crepaldi *et. al.*, 1979; Brunzell. 1989; Nikkilä, 1978) consistent with inefficient conversion of

circulating triglyceride-rich lipoproteins into remnant particles. ApoC-II deficiency was ruled out, since apoC-II activator activity in JJ plasma was normal. Post-heparin plasma LPL activity on this occasion was found to be zero (Table 7). However a different assay was used which incorporated the use of a LPL-specific monoclonal antibody, $Ab5D_2$ (Methods). Repeat post-heparin plasma samples were assayed on several occasions with zero activities consistently being recorded.

Further evidence in support of a familial defect was derived from post-heparin plasma results of JJ's father, WJ where a 50% reduction in LPL activity was found (110 nmol FFA/min/ml; normal ~ 214 - 244). His lipid profile was also consistent with a heterozygous state for LPL deficiency in association with a normal LPL immunoreactive mass (Table 7) and moderate lipemia. He presented with a plasma triglyceride level of 8.1 mmol/l, a low HDL-C level (1.1mmol/l; normal, 0.8-2) and a raised apoB level (201mg/dl; normal, 60-120). These are fairly common features in carriers over the age of 40 (Wilson *et. al.*, 1990)(Miesenbock *et. al.*, 1993; Fager *et. al.*, 1990). Also note that the low incremental mass recorded for JJ's sibling is not unusual for normals. The patient's mother was unavailable for study.

2.3. Results (Mutation Analysis).

The author must acknowledge that while he was not involved in the sequence analysis which led to the identification of the I194T mutation in patient JJ, he was responsible for carrying out and setting up most of the other methodologies used, such as, DNA isolation, plasmid preparation, probe labelling, southern blot hybridisation, haplotype analysis, DNA sequencing, PCR Restriction analysis, "ARMS" PCR, PCR-SSCP analysis, LPL activity assays.

2.3.1 The I194T Mutation.

2.3.1.1 Haplotype Analysis.

An early indication of genetic heterogeneity in monogenic disorders is often obtained through haplotype analysis of mutant alleles as different mutations tend to manifest different haplotypes. This has been investigated in LPL deficiencies by constructing haplotypes at the LPL gene from three restriction fragment length polymorphisms (RFLP), namely, HindIII, PvuII and BamHI (Heizmann *et. al.*, 1987 and 1991; Fisher *et. al.*, 1987; Chamberlain *et. al.*, 1989; Oka *et. al.*, 1989). These haplotypes were determined for nine South African Type I patients, comprising four of Indian extraction, three from the Afrikaner community, one of Malay origin and one of mixed ancestry (JJ) (Henderson *et. al.*, 1990, 1991 and 1992).

The use of three RFLP's gives a theoretical possibility of 8 [$(2)^3$] different haplotypes. An assessment of the haplotype distribution in the Afrikaner community was determined by analysing DNA from 33 normolipidemic subjects. The haplotypes are listed in Table 8 and the distribution is similar to that reported for American Caucasians (Funke *et. al.*, 1987; Fisher *et. al.*, 1987). Haplotype 2 is the most common and accounts for 26 (39%) of 66 alleles examined in the South African control population; all the others are 12% or less. JJ (proband of kindred I) is homozygous for haplotype 1, which thus characterises the I194T mutation in this kindred. However this same mutation associates with a different haplotype, haplotype 4, in our I194T reference family. Neither of these two haplotypes are common in the Afrikaner population, each being less than 10%.

Table 8: RFLP Haplotype Frequencies at the LPL gene locus for 33 normolipidemic South African Afrikaners.

HAPLOTYPE	HindIII	BamHI	PvuII	Alleles	%
1	+	-	-	7	10
2	+	-	+	26	39
3	-	+	+	3	5
4	-	-	+	8	12
5	+	+	-	8	12
6	-	+	-	8	12
7	+	+	+	3	5
8	-	-	-	3	5
TOTAL				66	100

Haplotype nomenclature is according to Monsalve *et. al.*, 1990.

2.3.1.2 Sequence Analysis.

The author was not involved in the sequence determination of the I194T substitution which has previously been reported in Henderson *et. al.* (1991). Briefly, exons 1-10 of the LPL gene were individually amplified by PCR from genomic DNA. Single-stranded DNA (ss-DNA) was generated by assymetric PCR using one primer only and the product was subjected to direct sequencing using the dideoxy chain termination method using the alternate primer in the sequencing reaction (Methods).

Fig. 15 shows a sequence ladder from exon 5 of the LPL gene of proband, JJ (Kindred I) and a normal control. A single T→C transition at position 836 in codon 194 of the published LPLcDNA sequence (Wion *et. al.*, 1987) is clearly evident with the ATT codon of isoleucine being converted to the ACT of threonine. To verify that this transition was not a PCR error both the sense and anti-sense strands of exon 5 were sequenced from several independent PCR amplications.

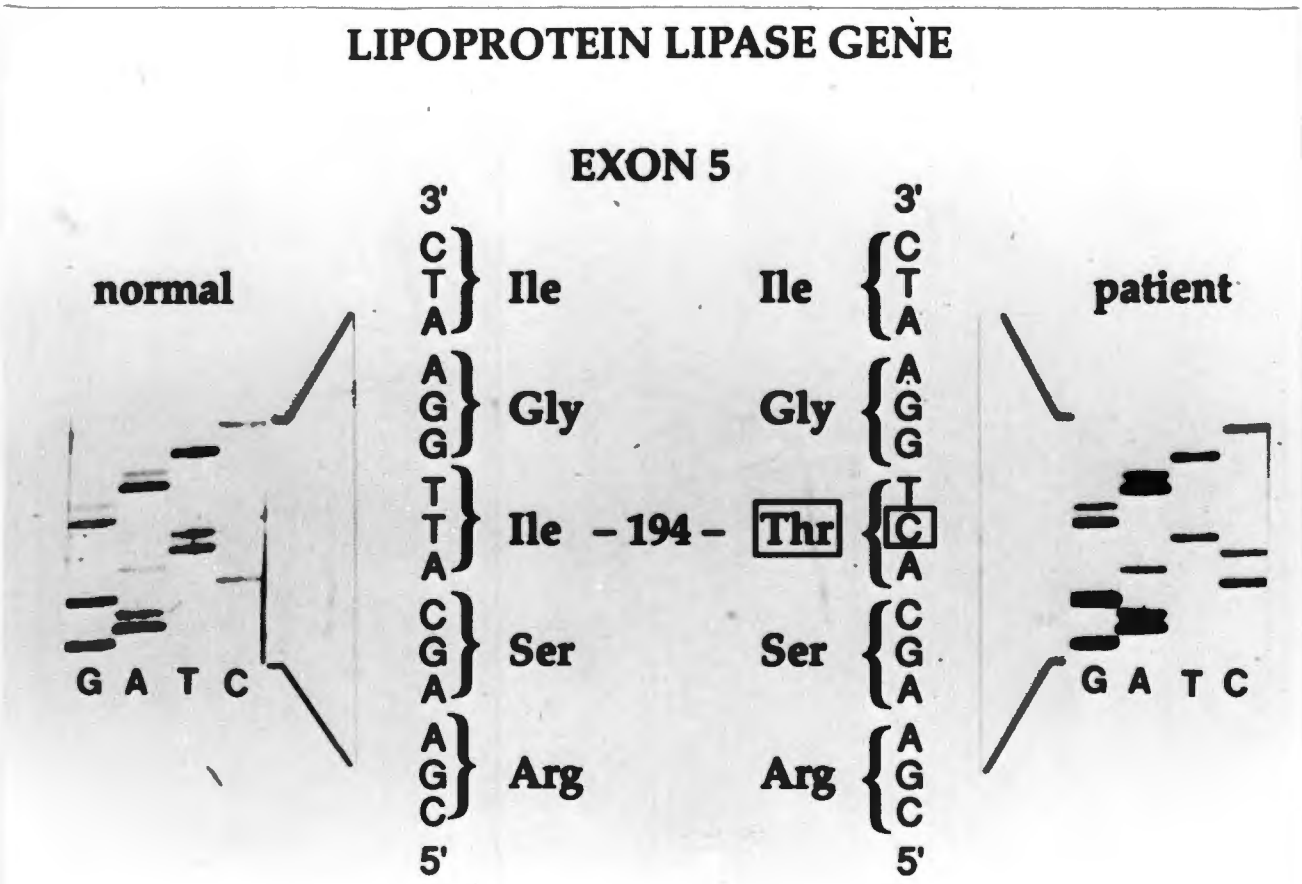


Fig. 15: Nucleotide sequence of the sense strand from exon 5 of patient, JJ, showing the T→C transition at codon 194, resulting in the threonine to isoleucine substitution. Sequence analysis was performed using the Sequenase Version 2.0 Kit (USB Biochemicals, Cpy, Ltd). Codon positions are taken from the published LPLcDNA sequence (Wion et. al., 1987).

Heterozygosity for this mutation was confirmed in JJ, since a consistent finding was the presence of both the T and C bands at position 836 in the sequence ladder. Inspection of the rest of exon 5 revealed no additional base substitutions. Sequence analysis of the other exons was similarly determined and no evidence of a second mutation was found. The 1194T mutation neither created nor destroyed a restriction site, which necessitated an alternative technique to restriction analysis for detecting the presence of this mutation.

2.3.1.3 Detection.

Several methods were developed to detect the I194T mutation.

2.3.1.3.1 Allele-Specific Oligonucleotide(ASO) Hybridisation.

ASO hybridisation has proven to be a reliable test for the detection of point mutations in genomic DNA and is amenable to rapid screening of a large number of subjects (Farr *et. al.*, 1988). It is however, restricted to the detection of known mutations. The method employs two oligonucleotides usually 19-20 bases in length, whose sequence spans the point mutation and are either complemetary to the sequence of the normal or mutant allele. The specificity of the assay is based on the marked differences in melting temperature of DNA duplexes formed between the matched and mismatched oligonucleotides and the template DNA.

Approximately 20ng of PCR product was transferred to Hybond N^+ membrane filters (Amersham Corp., Arlington Heights,IL) and hybridised separately with a [γ-^{32}P]labelled ASO, complementary to the normal sequence (ASOI) or the mutant sequence (ASOII)(Primer Design), spanning the T→C transition. Following hybridisation, the membrane filters were washed and autoradiographed as descibed in (Methods).

Fig. 16 represents a schematic diagram of an autoradiograph of hybridisation blots from LPL exon 5 DNA. Exon5 DNA of JJ was found to hybridise to both the normal probe, ASOI and the mutant probe, ASOII, consistent with the assignment of a heterozygous status for the I194T mutation. Similar results was also noted for WJ, the father of JJ, which demonstrates a paternal inheritance for this mutant allele. Exon5 DNA from WilJ hybridises to ASOI only, demonstrating a normal sequence for this sibling. The possibility that the I194T substitution was a common polymorphism in the general population was discounted by the absence of this mutation in 50 normolipidemic control subjects.

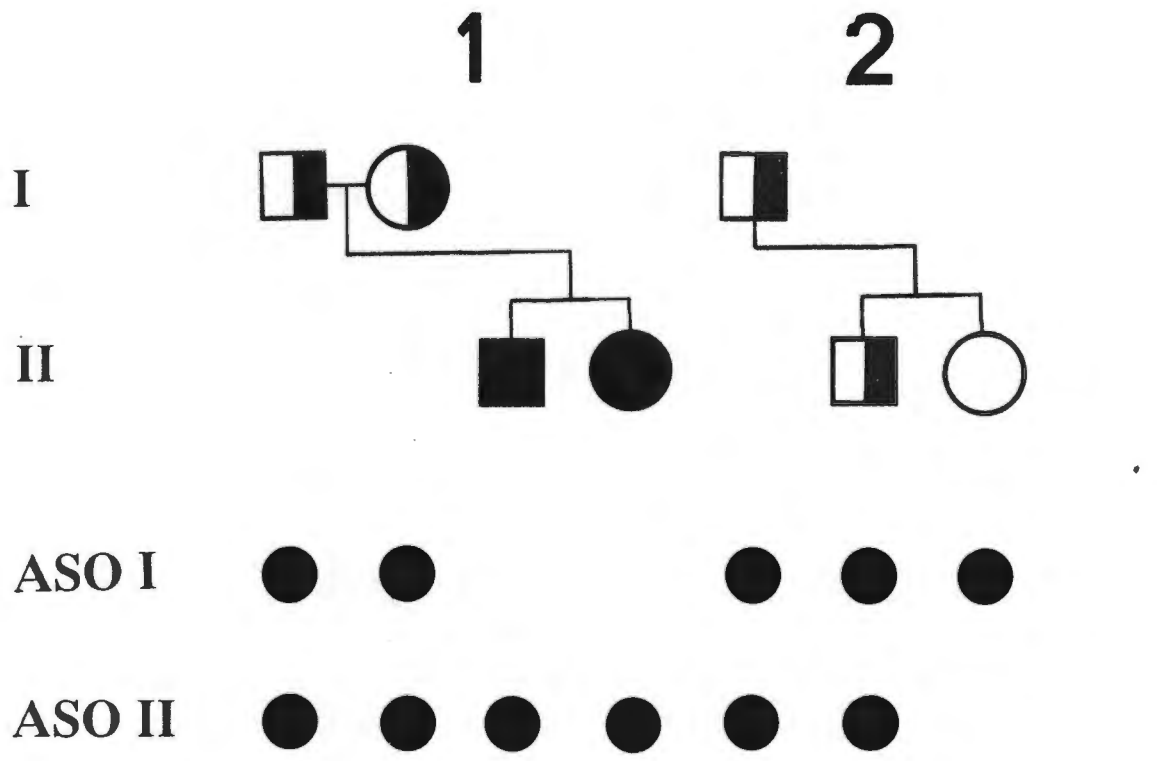


Fig. 16: Schematic diagram of an autoradiograph of exon 5 ASO hybridisation blots from a control kindred (1) and kindred I (2). Blots were probed with 32P-labelled allele-specific oligomers complimentary to the normal (ASOI) or mutant allele sequence (ASOII).

2.3.1.3.2 "ARMS" PCR.

The Amplification Refractory Mutation System (ARMS) is a simple PCR-based assay, designed to discriminate between mutant and normal alleles through the use of two sequence specific primers (Newton *et. al.*, 1989; Sarkar *et. al.*, 1990; Wenham *et. al.*, 1991; Wittwer *et. al.*, 1993). The success of the assay is dependent on the design of the PCR primers which are identical except, the nucleotide at 3'-terminal end is matched with the normal or mutant sequence. The thermostable Taq polymerase utilised in the PCR assay requires a non-refractory 3'-terminal end (matched) to facillitate amplification. Primer extension would thus only be achieved with the "normal" or "mutant" complemetary primer if the normal or mutant sequence were present. Several studies have shown

that the deliberate introduction of additional mismatches, close to the 3'-terminal end i.e. to within 2-3 bp, enhances the specificity of the procedure significantly. This modification was introduced into the primer sets for the I194T mutation.

Genomic DNA from JJ and his family members were subjected to ARMS PCR utilising two internal primers, one non-refractory (194 mutant primer, 5'-GGGTCCCCTGGTCGAAGTAC-3', the other refractory (194 wild type primer, 5'-AACTGGTTTCTGGATTCTAA-3') for the T→C transition at position 836 of the LPLcDNA sequence in combination with two flanking LPL exon 5 primers (Primer Design, Methods). In spite of extensive optimisation procedures the results obtained with these primers were unsatisfactory and often unreproducible.

2.3.1.3.3 PCR-SSCP Analysis.

The conformation of small single-stranded DNA fragments in non-denaturing environments are infleunced by its primary sequence (Orita *et. al.*, 1989; Suzuki *et. al.*, 1991; Poduslo *et. al.*, 1991; Sheffield *et. al.*, 1993). Sequence alterations thus induce conformational changes which may be detected as altered banding patterns (mobility shifts) following non-denaturing gel electrophoresis and silver staining. Other factors which may infleunce conformer formation are, the gel temperature, the acrylamide concentration, buffer ionic strength and glycerol content.

PCR-amplified LPL exon 5 DNA fragments from proband, JJ and his family members were subjected to SSCP analysis as described (Methods). The banding patterns obtained are shown in Fig. 17. Proband, JJ (lane 3), and his father, WJ (lane 1), share a pattern which is identical to that observed for another I194T heterozygote (lane 7; N/M) as well as bands B2 and B5 with a normal individual (lane 8; N/N). These findings are consistent with heterozygosity for the I194T mutation in JJ and WJ. Carrier status could be ruled out for the maternal uncle, FD (lane 4), maternal grandmother, JD (lane 5) and JJ's only sibling (lane 2; WilJ) as their banding patterns were identical to that of the normal individual (lane 8; WT).

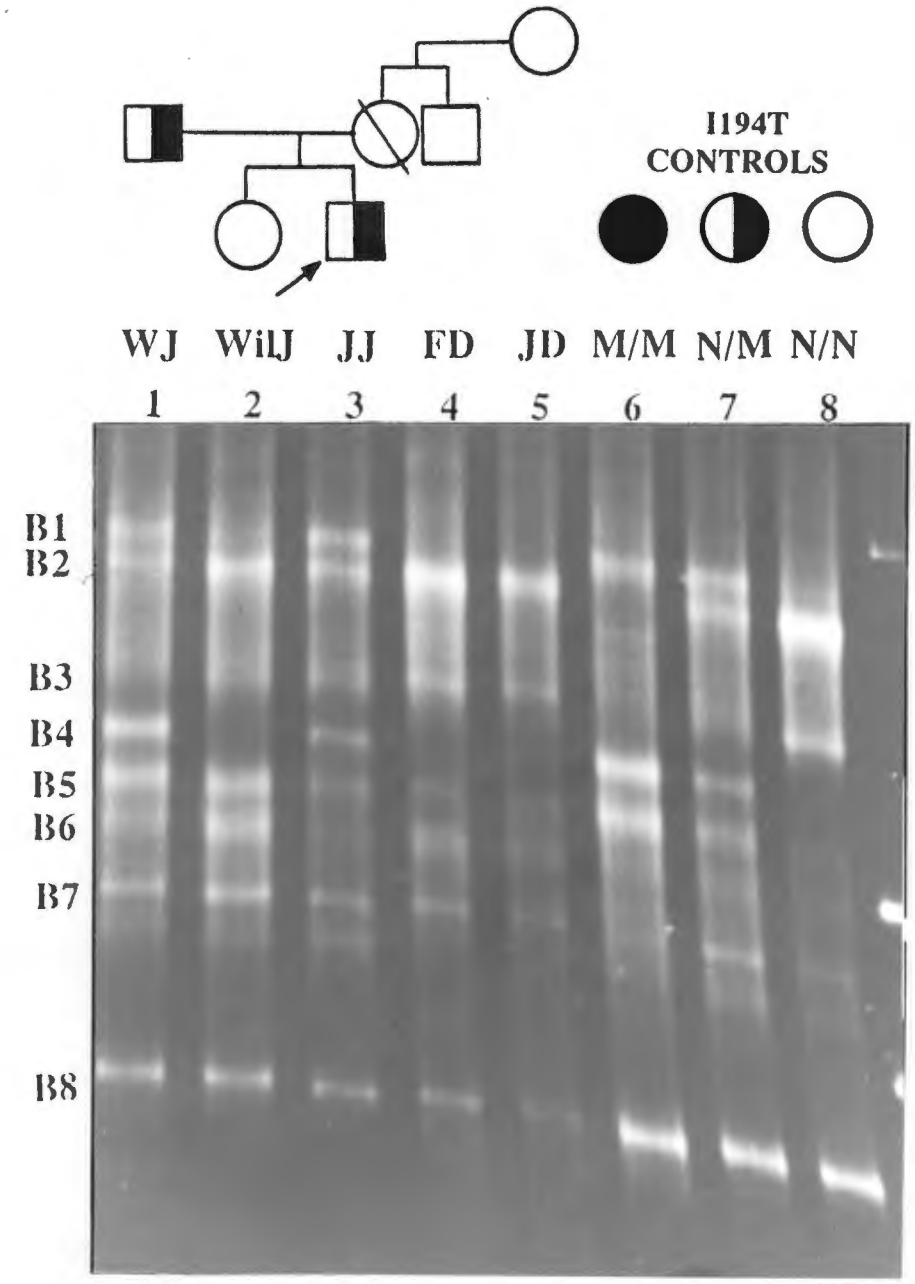


Fig. 17: **SSCP analysis of PCR-amplified LPL exon 5 products from kindred I. PCR amplification was performed using two LPL specific primers, LPL57 and LPL58 (See Primer Design, Methods). Electrophoresis was performed on 0.25×Hydrolink MDE polyacrylamide gels with approximately 5μl of PCR products loaded per lane. Gels were visualised by silver staining. Bands are labelled, B1 - B8. Lanes 1 - 5 represent patient samples.**

-homozygous (M/M), - heterozygous (N/M), deceased, -normolipidemic (N/N), - proband.

2.4 Mutagenesis and Transfection Studies.

To demonstrate the abolishment of catalytic activity by the I194T mutation, expression plasmids were constructed containing the normal LPLcDNA sequence as well as the I194T mutant sequence (Henderson *et. al.*, 1991). Transfection studies were carried out in COS-1 cells and LPL activity and protein mass measurements were assessed in the medium. These experiments clearly showed that the I194T substitution produced a catalytically inactive LPL product (activity < 1%), while mass levels were similar to that of the normal plasmid. Similar LPLmRNA levels were found in both the normal and I194T mutant transfected cell cultures which likely indicates that the mutation did not influence transcriptional rates or the stability of the mRNA species. Secondly, the mass assay used, only detected the dimer form of LPL and as similar mass levels were recorded for the mutant lipase variant, it must be secreted as a stable dimer.

Transfection studies clearly establish that the I194T allele of JJ generates a catalytically defective protein. The second mutation in this patient is therefore likely to produce a partially defective gene product which underlies the variable phenotype observed in JJ.

2.5 The Second Mutation.

Direct sequencing of PCR-amplified LPL exons had failed to reveal the second mutation in JJ, therefore it was decided to try a new approach and utilise Reverse Transcriptase-PCR (RT-PCR)(Gibbs *et. al.*, 1989; Naylor *et. al.*, 1991) on total RNA isolated from monocyte-derived macrophages which are known to synthesize LPL. The first objective was to demonstrate the expression of the alternate allele by cloning of LPLcDNA fragments and identifying clones with a normal sequence at codon 194. The second objective was to sequence cDNA from the alternate allele in search of the second mutation.

2.5.1 RT-PCR Analysis.

RT-PCR has been widely used to generate cDNA copies of mRNA from a variety of cell types; either total RNA or purified mRNA preparations have been used. Once transcibed, the cDNA preparation is amenable to any number of DNA manipulations in use today. An outline of the experimental protocol followed in the workup of JJ is given in Fig. 18.

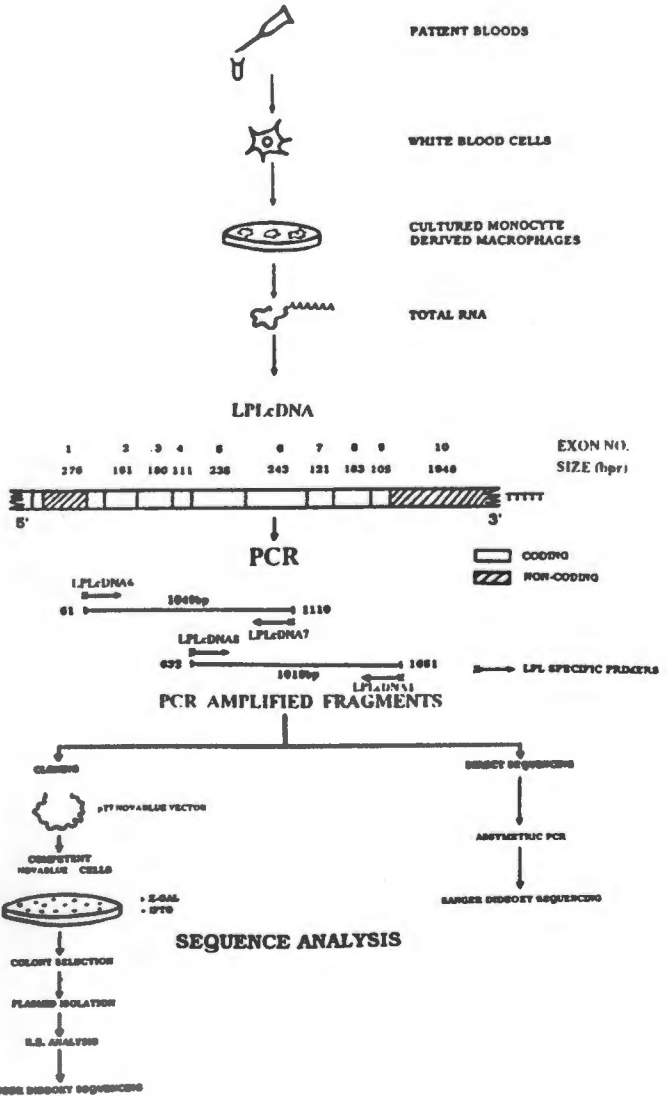


Fig. 18: Schematic diagram of the the protocol followed in the RT-PCR of the LPL coding sequence. Total RNA was isolated by the AGPC method (Chomczynski and Sacchi, 1987) and first strand synthesis was carried out using reverse transcriptase followed by PCR using the indicated primers. Amplified LPLcDNA fragments were cloned and sequenced as indicated.

2.5.1.1 Monocyte-derived Macrophage Preparations.

The monocyte-derived macrophage is one of a variety of cell types synthesizing LPL and was used as a source of LPLmRNA. Monocytes (buffy coat layer) were isolated from 50ml blood samples on Ficoll-Histopaque gradients as described (Methods). Recoveries were of the order of 20%. The isolated monocytes were either processed directly or cultured for 1-2 weeks under controlled conditions (Methods). 2-3 x 10^7 cells/dish were cultured, with approximately half (1-1.5 x 10^7 cells) being recovered as adherent, differentiated macrophages. Total RNA was isolated using the acid-guanidium-phenol-chloroform (AGPC) method as described by Chomczynski and Sacchi (1987). Following quantitation, the quality of the RNA preparations were checked by electrophoretic separation on agarose/formaldehyde gels and visualised by ethidium bromide staining (Methods)(Fig. 19). A good preparation was characterized by the appearance of clear 28S and 18S rRNA bands on a background of streaking. Intact, non-degraded RNA is required for the generation of full-length cDNA. Spectrophotometric analyses were used to assess RNA concentrations. On average, the total RNA yield obtained from monocyte-derived macrophages of a 50ml blood specimen was 100μg. Absorbance ratios (A_{260}/A_{280}) of 1.5-1.7 were generally recorded.

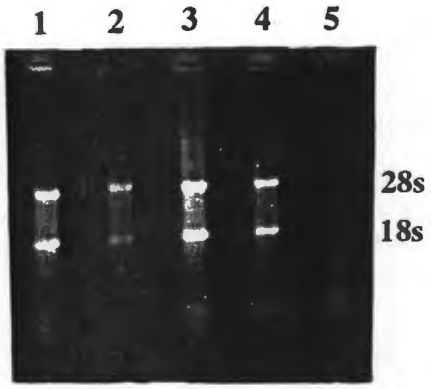


Fig. 19: **Total RNA isolates from patient and control monocyte and cultured monocyte-macrophage cell preparations. RNA samples were electrophoresed on 1% agarose/1.85% formaldehyde gels with visualisation by EtBr staining. Lane 1 - patient, JJ , monocyte cell prep., Lane 2 - patient, JJ, monocyte-macrophage cell culture prep., Lane 3 - control monocyte cell prep., Lane 4 - control monocyte-macrophage cell culture prep., Lane 5 - blank isolate. 28S and 18S rRNA bands are indicated.**

2.5.1.2 cDNA Synthesis.

Total RNA was subjected to first strand synthesis using oligo dT and random hexamers as initiation primers (Methods)(Gibbs *et. al.*, 1989). Oligo dT hybridises to the polyadenylate (poly-A) tail, common to all mRNA's and the random hexamers hybridise along the stretch of the RNA templates. RNasin, a ribonuclease inhibitor (Promega), was included in the reaction mix to preserve the integrity of the RNA template. 10 ug of total RNA yielded sufficient single-stranded cDNA product for the subsequent PCR amplication,cloning and sequence analysis.

2.5.1.3 PCR Amplification of cDNA fragments.

Two sets of PCR primers, LPLcDNA1 and LPLcDNA8, and LPLcDNA6 and LPLcDNA7, were designed to produce two overlapping fragments (Fig. 18)(See Primer Design). A 1/50th of the cDNA preparation was used for each amplification generating fragments 1049 bp (5'-fragment) and 1019 bp (3'-fragment) in length. These fragments spanned 1585 bp of sequence which included the entire coding region of the LPL gene. The amplified fragments were assumed to represent the products of both alleles with the 5'-fragment, spanning exons 2-6 and the 3'-fragment spanning exons 5-9, in addition to portions of exons 1 and 10 of the LPL gene (Fig. 18). Each individual fragment was purified and quantitated (as described). For control purposes, the full-length LPLcDNA (pLPL35) was subjected to fragment amplification in a similar fashion.

2.5.1.4 Cloning of cDNA fragments.

Cloning of PCR-amplified cDNA fragments allowed for the selection of clones representative of both alleles. The T-tailed cloning vector, pT7BLUE T-vector (Novogen)(Methods) was used, which allows for blue-white screening of recombinants. Ligation to this vector exploits the template independent terminal transferase activity, inherent in most thermostable Taq (thermas aquaticus) polymerases, which generates a single-3' dA nucleotide overhang. Optimally, 1/10th of the ligation mix was used to transform competent cells by the heat shock process in a standard transformation reaction (Methods). A vector:insert ratio of 1:3 was found to be optimal for ligation and transformation. After overnight culture at 37°C, recombinant clones (white) were picked onto a gridded library. Transformation efficiencies are shown in Table 9.

Table 9: Transformation efficiencies of cloned LPL gene fragments from patient, JJ (Kindred I).

Colonies screened	Colony forming units per μg (cfu/μg)	% Ligation
Control plasmid	7.00×10^7	-
pT7 + control insert	2.50×10^4	-
pT7 + pLPL35(6-7)	2.20×10^4	60
pT7 + JJ1-8	1.86×10^4	58
pT7 + JJ6-7	1.30×10^4	58

Transformed colonies were screened by using LPL-specific primers, LPLcDNA1, LPLcDNA8 and LPLcDN6, LPLcDNA7 (see Primer Design)(Fig. 18). The pT7 - vector was used for cloning, pLPL35(6-7) - 5'-fragment of the full-length LPLcDNA, JJ1-8 - 3'-fragment of the LPLcDNA from JJ, JJ6-7 - 5'-fragment of the LPLcDNA from JJ.

Generally, the number of recombinant colonies obtained were similar to the number observed for the control insert. Recombinant colonies were screened for the presence of the insert DNA as described below.

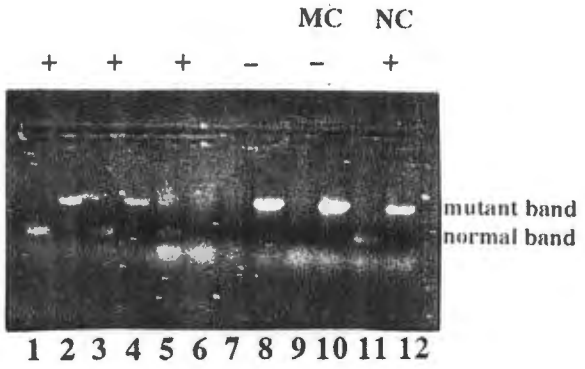
2.5.1.5 PCR Screening.

Screening of clones by direct colony PCR (Methods) was carried out using a primer set comprising a vector-specific primer, U-20 (reverse) and a LPL-specific primer (See Primer Design). Positive identification of LPL DNA insert, was denoted by the presence of a distinct band of about ± 1.1kb on agarose gel electrophoresis. Rapid PCR screening of the order of 100 white colonies, revealed a 58% fragment incorporation (Table 9) with the remainder of the recombinant clones most likely representing ligated vector.

2.5.1.6 I194T "ARMS" PCR Screening.

Clones containing insert DNA were subjected to direct colony "ARMS" PCR utilising the primer sets as described earlier (Primer Design)(Methods). Representative agarose gels of direct colony ARMS PCR for fragments JJ1-8 and JJ6-7, of patient, JJ, are shown in Fig. 20. Gel A shows six recombinant clones which contain the JJ1-8 insert and which were screened for the I194T mutation. Duplicate lanes represent a single colony screened for both the wild-type and mutant sequence. Four colonies contain the wild-type sequence (lanes 1-6) and two colonies (lanes 7-10), the mutant sequence, when compared to the "wild-type" control sample (lanes 11, 12). Gel B shows eight colonies which contain the JJ6-7 insert and which were screened for the 194 mutation. Again, duplicate lanes represent a single colony (see above). The mutant sequence is present in four colonies (lanes 5-10, 15 and 16). Wild-type and I194T mutant control samples are indicated, their sequences previously confirmed by sequence analysis.

Gel A



Gel B

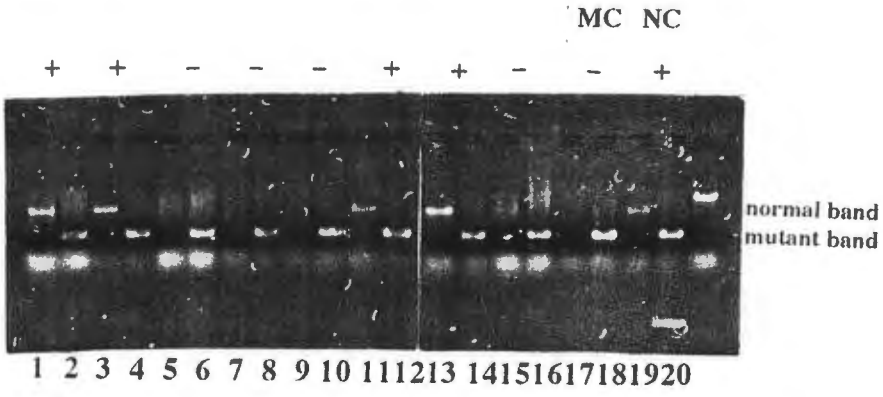


Fig. 20: Colony screening by "ARMS" PCR for the verification of insert DNA. Duplicate lanes represent single colonies screened with the normal and mutant primers (see Primer Design). GEL A : Colonies screened for the JJ1-8 insert. GEL B : Colonies screened for the JJ6-7 insert. + : the normal insert is present, - : the mutant insert is present. MC - mutant control, NC - normal control.

Unfortunately, amplification of the normal clone (NC) was achieved with both the normal (wild-type) and mutant 194 primer, whereas the mutant 194 primer amplifies up the mutant clone (MC) only, as expected. In conclusion, the results are therefore not definitive, since optimal assay conditions for the discrimination of the wild-type sequence could not be established. However the assay did allow for distinction between clones containing the I194T "mutant" and the "normal" (alternate) allele.

2.5.1.7 Sequencing of the Alternate Allele.

The colonies identified by ARMS PCR containing the LPL gene fragments (JJ1-8, JJ6-7) of the alternate allele were picked and each individual overnight culture was subjected to bulk plasmid purification (Methods). A total of four colonies (2 - JJ1-8; 2 - JJ6-7) were sequenced (Sequenase Version 2.0 kit, USB)(Methods). 6-8μg of purified plasmid DNA were generally used per sequencing reaction in combination with a cohort of sequencing primers (see Primer Design) spanning the entire LPL coding region (Methods).

Sequence analysis of the four clones revealed a single G→A transition at position 1508 of the LPL gene (Wion *et. al.*, 1987)(Fig. 21 - see next page). This transition is located in exon 9, in the second position of codon 418, resulting in a codon change from TGT to TAT, substituting tyrosine(Y) for cysteine(C). No other sequence alteration was detected in the remainder of the 1585 bp sequence of this allele. The possibility that the substitution was a PCR error was discounted by repeat PCR, cloning and sequencing which revealed the same base substitution.

These findings clearly demonstrate compound heterozygosity for proband, JJ. As discussed above, *in-vitro* transfection studies have established that the protein product derived from the I194T "mutant" allele is catalytically inactive. Similar studies are still to be carried out for the C418Y mutant cDNA, but it is expected that this mutation will only partly abolish catalytic activity which could possibly explain the hypervariable nature of the LPL deficiency in patient JJ (see Discussion).

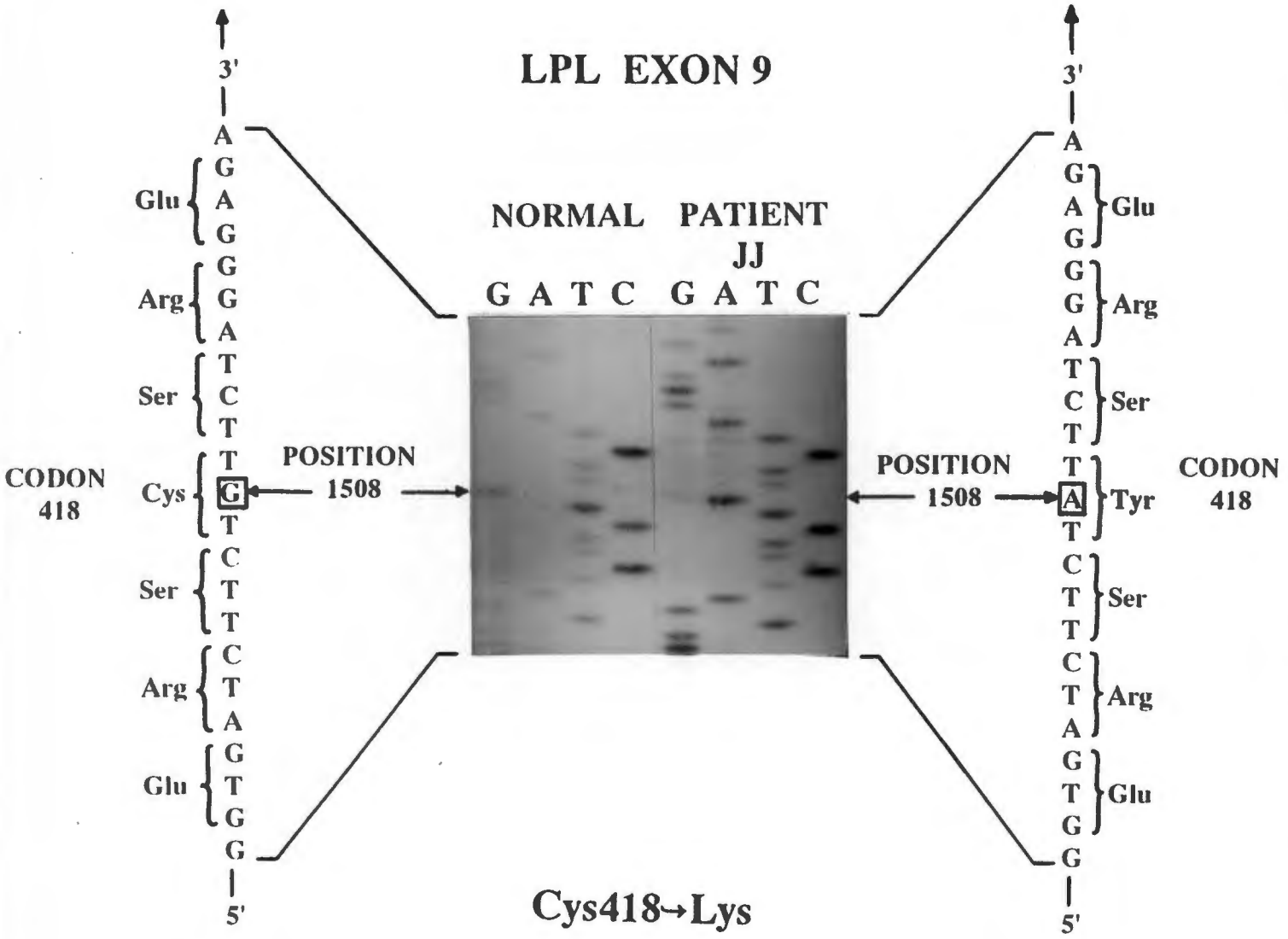


Fig. 21: Nucleotide sequence of the sense strand of exon 9 of the LPL gene of index patient, JJ (Kindred I) and a normal control subject. The G→A transition (⟶) is boxed. The amino acid substitution and nucleotide position is indicated and the codon is numbered.

2.5.2 Detection of the C418Y mutation.

Several mutation detection systems were employed to detect the C418Y mutation as the sequence alteration neither creates nor destroys a restriction site. These are presented below.

2.5.2.1 C418Y "ARMS" PCR.

Primer design was identical to that employed for the I194T "ARMS" PCR assay. Amplification was accomplished using two internal primers, one, refractory (wild type; 5'-CTTCCACAGGGTGATCTTATG-3') and one, non-refractory (mutant; 5'-CTTCCACAGGGTGATCTTATA-3') for the G→A transition in combination with two intronic external primers (see Primer Design). To improve the specificity of the assay, each of the internally, matched and mismatched primers, had an additional mismatch base introduced two nucleotides from the 3'-terminus.

Difficulty was again experienced with these ARMS primers as absolute discrimination between the normal and mutant allele could not be obtained.

2.5.2.2 PCR Restriction Analysis.

PCR restriction analysis is a simple mutation detection system, first described by Haliassos, *et. al.* (1989). The aim here was to introduce a sequence change in the amplified fragment by altering the primer sequence such that the combination of the primer sequence and the patient base transition creates a restriction palindrome.

A 21bp primer, LPL23 (forward, 5'-TATTCACATCCATTTTCTTC-3') was designed to span the exon 9 intron-exon junction with the 3' terminus adjacent to the C418Y mutation. Two deliberate mismatched bases were introduced, three and four bases upstream from the 3'- terminus (see Primer Design). Substitution of AG for TT at the 3rd and 4th base, respectively, creates a PvuII restriction enzyme site (5'- CAGCTG -3') in combination with the guanine nucleotide of the normal allele. Amplification of the mutant allele abolishes this PvuII site by altering the palindromic sequence to 5'- CAGCTC-3'. These primers in combination with a 20bp LPL intronic primer (LPL24, reverse, 5'-GTCAGCTTTAGCCCAGAATG-3') yields a 114 bp fragment.

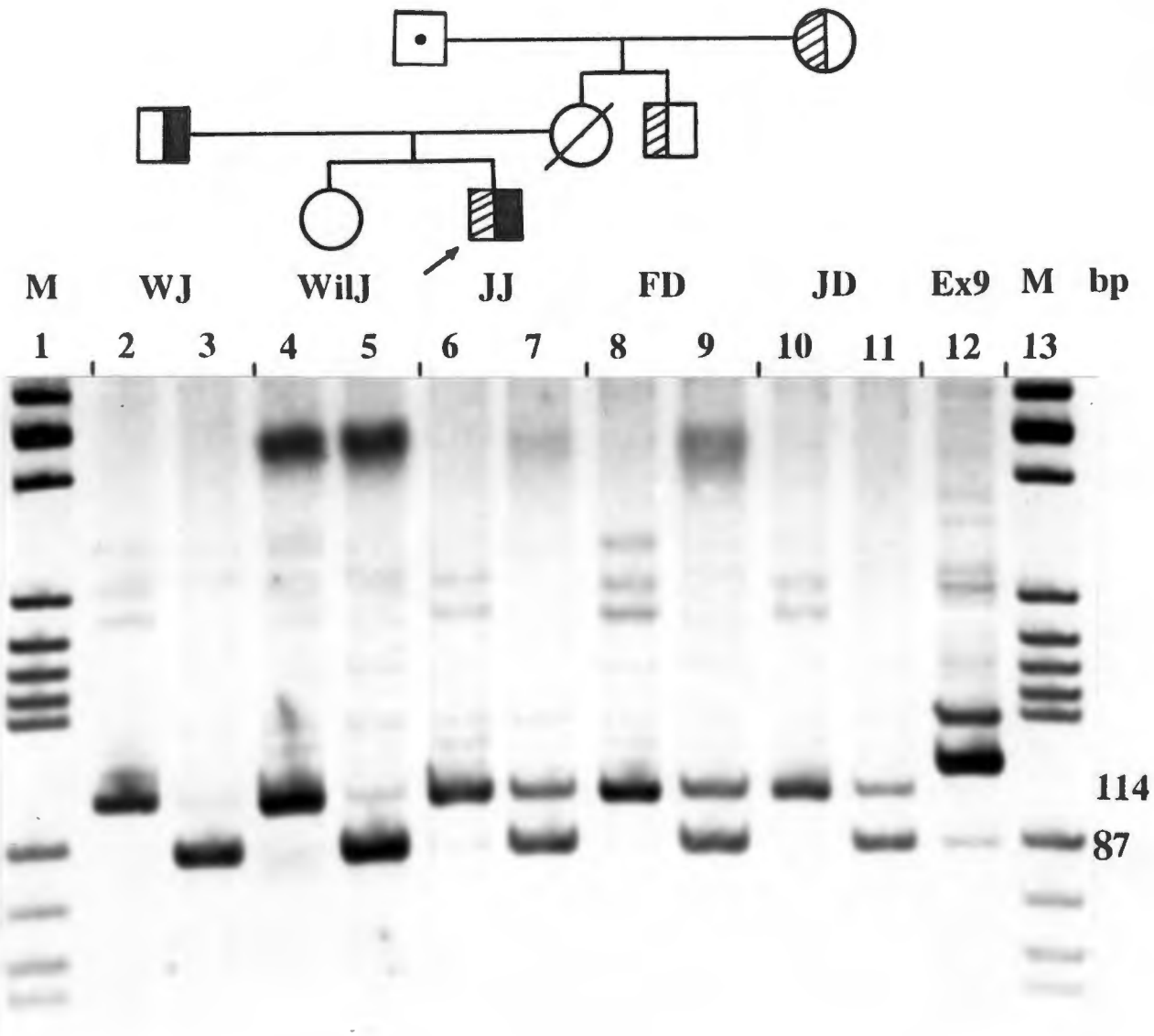


Fig. 22: PCR Mismatch Analysis of LPL exon 9 for kindred I.

Genomic DNA was subjected to PCR amplification utilizing two specific primers, a 5'-GTCAGCTTTAGCCCAGAAT-3' (reverse, 20mer) and a 5'-TTTCTTCCACAGGGTGATCAGCT-3' (forward, 23mer) designed to generate or destroy a PvuII restriction site in combination with the normal or mutant sequence, respectively. Individual samples are represented in duplicate lanes as undigested and digested samples, respectively. Fragment sizes are indicated. DNA standard size markers are shown in lanes 1 and 13. ◨ - I194T heterozygote, ◪◐ - C418Y heterozygote, ▨ - compound heterozygote, ○ - normolipidemic, ⊘ - deceased, ⊡ - not investigated, ↗ - index patient.

Genomic DNA from kindred I was amplified using these primers and subjected to digestion with PvuII. The resultant banding pattern observed on PAGE gels show clear discrimination between the "normal" and "mutant" alleles (Fig. 22). A 27 bp decrease in size of the PCR fragment following PvuII digestion is indicative of the normal allele. The presence of both the 114 and 87 bp fragments, for proband, JJ (lanes 6, 7) confirms heterozygosity for the C418Y mutation. As the proband's mother was recently deceased and therefore unavailable for study, his maternal uncle (FD) (lanes 8, 9) and maternal grandmother (lanes 10, 11) were included in the study. Both these individuals were found to be carriers indicating a maternal origin for this mutation. The banding pattern observed for father, WJ (lane 2, 3) and sibling, WilJ (lane 4, 5) of JJ indicates homozygosity for the normal allele.

The success of the PCR mismatch methodology renders it amenable to broad-based population screening procedures. It is a rapid test with no need for radioisotopic-labelling and it is not labour-intensive since it provides a direct method of analysis, without the need for blotting, probe labelling and hybridisation as required for other techniques. The test is applicable to prenatal testing, newborn screening and general carrier status detection. It is however limited to the detection of known mutations only.

2.5.2.3 PCR-SSCP Analysis of exon 9

A similar approach to that outlined for the I194T mutation was adopted in detecting the C418Y mutation. Gels were visualised as described (Methods).

Results from PCR-SSCP analysis are shown in Fig. 23 (see next page). Proband, JJ (lane 3), his maternal uncle, FD (lane 4) and maternal grandmother, JD (lane 5) share a banding pattern distinct from the other family members. As expected father, WJ (lane 1) and sibling, WilJ (lane 2), show a banding pattern identical to that of the normal control subject (lane 6). B3, is a unique conformer and is presumed to contain the C418Y sequence alteration. The individual DNA bands were excised and subjected to DNA cycle sequence analysis using a PCR-based dideoxy chain termination protocol [fmol™ DNA Sequencing System (Promega)](Methods). The results confirmed the G→A transition at position 1508, as the only base change in exon 9 of the LPL gene sequence. Similar sequencing of the "normal" band, B4, confirmed the presence of the normal guanine base, at nucleotide position 1508. This technique clearly confirmed the carrier status for proband, JJ, and his maternal relatives, for the C418Y mutation.

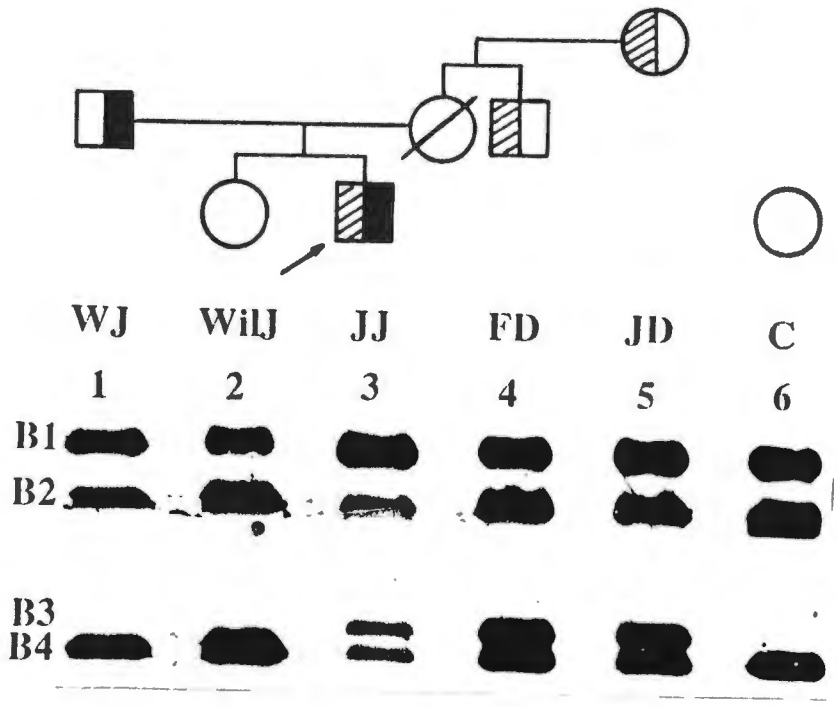


Fig. 23: SSCP analysis of LPL exon 9 PCR products from kindred I subjects. PCR was performed using two primers, LPL23 and 24 (Primer Design). Electrophoresis was performed on a 0.55×Hydrolink MDE polyacylamide gels with visualisation by silver staining. DNA bands are numbered B1 - B4. Lanes 1 - 5 are patient samples and Lane 6 is a control DNA sample.

- heterozygote, - compound heterozygote, - normolipidemic, - deceased, - index patient.

2.6 Discussion.

The evidence presented in this report confirms LPL deficiency as the primary cause for the hyperchylomicronaemia syndrome in the index patient, JJ. In the initial report, Berger *et. al.* (1987) proposed an atypical and milder phenotype for this patient, but this was based on the residual LPL activity level (38% of normal) measured in JJ's post-heparin plasma when assayed by the protamine sulfate method. In our study, however, when utilising a more specific LPL assay, we consistently demonstrated a complete absense of LPL activity in his plasma. He also presented with a history of phenotypic sequelae (hepatosplenomegaly and abdominal pain) since the age of 4. Laboratory investigations revealed the following characteristic features, namely (i) a 4°C stored plasma test showed a typical dense chylomicron-rich band over a slightly turbid infranatant (ii) markedly elevated plasma triglyceride levels and (iii) a significantly depressed HDL-cholesterol level. Also, a fat tolerance test was consistent with a Type I phenotype. Despite these findings, JJ has been relatively symptom-free since puberty, except for occasional bouts of pancreatitis.

The detection of two gene mutations at the LPL gene locus and the establishment of compound heterozygosity in JJ is consistent with the above findings. Only one of these mutations has however been shown by *in vitro* studies to yield a catalytically defective enzyme; the other is likely to be partially defective and may explain the variable levels of LPL activity recorded in post-heparin plasma in the prepubertal stage as opposed to during puberty. The first of these mutations comprises a T→C transition at position 836 in codon 194 of exon 5, causing a substitution of a Thr for an Ile. The second mutation was detected through the application of RT-PCR methodology on monocyte-derived macrophage mRNA which revealed a G→A transition at position 1508 giving rise to substitution of a Tyr for a Cys, at codon 418. Confirmation of compound heterozygosity for JJ was obtained by using various mutation detection systems.

The majority of missense mutations causing LPL deficiency occur in exons 4, 5 and 6 of the LPL gene (Peterson *et. al.*, 1992; Hayden *et. al.*, 1995). These exons, in particular exon 5, are located in an evolutionary conserved section showing strong homology to the other members of the LPL/HL/PL gene family. They code for residues making up the structural core of the N-terminal domain and active site residues. As reported earlier, the I194T mutation is located in exon 5. Structural predictions for LPL and the effect the I194T mutation may have on the overall LPL

structure can be inferred from the molecular model of the homologous hPL molecule (Fig. 9)(van Tilbergh *et. al.*, 1992; Derewenda and Cambillau, 1991). Residue 215 in PL, which corresponds to isoleucine 194 in LPL, is located in the loop linking β-strands 8 and 9 (Fig. 9), and packs against the fragment containing the His residue of the catalytic triad (Fig. 9)(Derewenda and Cambillau, 1991). This residue also makes Van der Waal's contact with Tyr^{114}, Ala^{171} and Pro^{180}, all three of which are conserved in LPL. The conservation of hydrophobicity around Ile^{194} in LPL is supported by the finding of a Val in the homologous position in hHL. This implies an important structural role for these amino acids. The introduction of a hydrophillic residue, such as, Thr, is likely therefore to disrupt the local hydrophobic enviroment essential for the maintenance of the active site motifs. Only those motifs required to process long chain acyl esters are affected as the mutant has been shown to have normal activity towards soluble esters. The exact mechanism of this inactivation is unknown but it is possible that construction of the hydrophobic channel as well as repositioning of the active site lid is abnormal. The I194T mutant protein has also been shown to form a stable dimer and has normal heparin binding properties as it manifests similar elution kinetics to that of normal LPL on heparin-sepharose chromatography. This implies that the heparin binding site of the mutant enzyme is intact and is separate from the catalytic site.

The second mutation in patient, JJ, the C418Y mutation is located in the C-terminal domain of the LPL molecule, in a region which appears to play a role in lipid binding and has been implicated in heparin binding (Fig. 8). The substitution of a Cys in this position destroys the only disulphide bridge in this domain. While the functional effects of the loss of this cysteine have not been established, other workers have shown by *in-vitro* transfection studies on LPL deletion mutants that alterations in the C-terminal domain are usually associated with partial catalytic deficiencies. The M437→stop mutant, for example, which has lost the C-terminal decapeptide including the Cys^{438} residue involved in the Cys^{418}-Cys^{438} disulfide bridge, shows a 41% decrease in LPL activity (Bruin *et. al.*, 1994). Presumably this mutation disrupts the local structure, and hence interferes with lipid binding or dimer formation. In support of this proposal, several workers have demonstrated an absolute requirement for an intact LPL C-terminal domain for maximal activity against long-chain triacylglycerol emulsions (Wong *et. al.*, 1991; Davis *et. al.*, 1992). Furthermore, strong support for the presence of a secondary lipid binding site localised to the C-terminal domain, proximal to the disulfide bridge, Cys^{418}-Cys^{438} have been derived from expression studies in COS-1 cells of C-terminal truncated mutants (Kozaki *et. al.*, 1993). Residue C^{438} is juxtaposed to six highly conserved

hydrophobic residues, APAVFV, while residue C^{418} has three such conserved residues in close proximity. Disulfide-bridge formation bring these two regions into close proximity, which could form a large, hydrophobic lipid binding domain. Disruption of this crucial disulfide bridge would clearly prevent the formation of this potential lipid binding domain and thereby affect funtional activity. The truncated LPL mutants, 435→stop and 436→stop, expressed lipase activities of 22% and 88%, respectively, compared to native LPL in keeping with the other data and demonstrating the importance of Val^{436} in enhancing the lipid substrate association with LPL. These data suggest that the C418Y mutant may exhibit partial activity. Evidence for the presence of a heparin binding region in the LPL C-terminal domain has been presented (Fig. 8)(Bengtsson-Olivecrona *et. al.,* 1986; Wong *et. al.,* 1991; Davis *et. al.,* 1992). The presence of this mutation may therefore lead to the disruption of this region, thus altering the heparin binding properties and stability of the mutant protein. The only other mutation described in exon 9, a Ser447→stop (Kobayashi *et. al.* 1992; Hata *et. al.*, 1990), produces a truncated LPL molecule, two residues shorter than the native protein. The removal of this carboxy-terminal dipeptide (Ser - Gly) has no apparent effect on the catalytic activity of LPL. In fact, one study proposed an enhancement in LPL activity (Hata *et. al.*, 1990).

The initial proposal of an atypical and milder phenotype for the index patient, JJ (Berger *et. al.*, 1987) which was based on residual LPL activity measured in post-heparin plasma, muscle and adipose tissue biopsies, needs to be reassessed in the light the of our findings of zero activity in post-heparin plasma. We were unfortunately, unable to confirm the initial report of residual LPL activity as measured by the protamine sulfate assay. This difference is likely to be explained by differential processing and/or alterations in post-translational modifications which may occur at various stages of human development with a loss of LPL activity post puberty. The LPL dimer/protein mass levels are within the normal range in patient JJ and therefore it is likely that both the I194T and C418Y mutant proteins are expressed normally and form stable dimers. *In vitro* expression studies have previously shown the I194T/I194T dimer to be inactive towards triolein emulsions thereby implying that both the C418Y/C418Y dimer and the heterodimer species may also be inactive *in vitro.*

Our laboratory reported earlier the association of haplotype 4 with the mutant I194T allele in two unrelated kindreds of Dutch and French origin, including the control kindred in this study. While,

proband JJ is of mixed ancestry, the finding of a different haplotype (haplotype I) associating with the 194 allele in kindred I was unexpected. Haplotype 1 and 4 differ at the HindIII and PvuII restriction sites. Generally, single haplotypes segregate with single mutations in genetic disorders which is compatible with singular ancestral events (DiLella *et. al.*, 1987; Wainscoat *et. al.*, 1986). The association therefore of the mutation at codon 194 with two divergent RFLP haplotypes, more than likely indicates an identical ancestral mutation occurring separately on two different chromosomal backgrounds rather than these haplotype changes occurring on a common ancestral gene. These haplotype associations suggest a multicentric origin for the I194T mutation in the South African population.

CHAPTER 3.

3.1 INTRODUCTION.

3.1.1 Pregnancy and Hyperlipidaemia.

Mild hyperlipidaemia has long been recognised as a common feature in the third trimester of pregnancy with triglyceride levels increasing between two and four fold; increases in cholesterol and phospholipid being less common (Warth *et. al.,* 1975). Earlier work on rats (Scow *et. al,* 1964*),* revealed that hyperlipidaemia was associated with pregnancy at late gestation in both the fed and fasted states. This hyperlipidaemia was aggravated by dietary fat, since pregnant animals fed on a fat-free diet had significantly reduced lipid levels compared to rats fed on a normal diet.

The changes in lipid metabolism during the pregnancy-lactation-involution period results from the down regulation of adipose tissue LPL activity and the significant increase in VLDL production in response to higher estrogen levels. As early as 1970, Hamosh *et. al.*, described reciprocal changes in the adipose tissue and mammmary gland LPL activity during gestation in the rat. They also showed a marked increase in LPL activity (2 fold) in the parametrial adipose tissue during the second trimester which decreased significantly at parturition and remained low throughout lactation. In contrast, in the mammary glands of the rat, LPL activity increased very slowly throughout the first and second trimester with a dramatic rise being observed during the third trimester. This was followed by a transient decrease in LPL activity at parturition with levels increasing and remaining high throughout the lactation period. These tissue-specific reciprocal changes in LPL activity serve to divert plasma triglyceride from storage in adipose tissue to the mammary gland for milk-fat synthesis (Scow and Chernick, 1987).

3.1.2 Pregnancy, Hyperlipidaemia and Pancreatitis.

Several reviews and case reports have described the association of pancreatitis with pregnancy-induced hyperlipidaemia, a condition which is estimated to have a mortality rate of 21% for both mother and child. In the majority of these patients the underlying defect was gallstone formation and blockage of the bile duct (Montgomery and Miller, 1970). Hyperlipidaemia is rarely encountered

as a cause of pancreatitis but several patients have now been described with hypertriglyceridaemia-induced pancreatitis and most of these patients have been shown to have a clearance defect as a result of LPL deficiency. Gleuck *et. al.* (1980) described three patients whom developed pancreatitis during their pregnancies. One of these patients had a known Type V hyperlipoproteinemia phenotype, and the increase in triglycerides during pregnancy was controlled by the introduction of a fat-free diet leading to an uncomplicated pregnancy and the delivery of a healthy infant. Watts *et. al.* (1992) reported two cases; a clinical profile of both patients, revealed a history of recurrent bouts of abdominal pain, raised triglycerides and chylomicronaemia. The diagnosis of LPL deficiency in these patients provided a suitable explanation for their clinical presentation. One of these patients miscarried her foetus as a result of a bout of acute pancreatitus. The second patient, after being placed on a strict lipid lowering diet, had an uneventful antenatal course despite hypertriglyceridaemia being recorded. Following the birth of a healthy infant, the patient's triglyceride levels decreased rapidly. Ma *et. al.* (1993, 1994) reported five patients, four of which were diagnosed with partial LPL deficiency after developing pancreatitis during pregnancy. In three of these patients, the apoE2 isoform (associated with defective receptor binding) was elevated, which implies that remnant uptake was also impaired. The occurance of this mild hyperlipidaemia in these patients, is thus exacerbated during the third trimester of pregnancy. Gestational pancreatitis thus carries a significant risk of death for both mother and foetus and hence the early detection of any underlying lipid disorder is essential for the proper management of pregnant individuals.

3.1.3 Kindred II.

The marked hyperlipidaemia recorded in the index patient, LB, during her acute attack of pancreatitis was suggestive of a primary defect in LPL activity, hence we set out to search for the gene defect/s at the LPL loci. Due to the non-availability of suitable sample preparations from the deceased proband, LB, analysis was initially confined to the study of her parents, father, DR and mother, ER. RT-PCR, cDNA cloning, PCR amplification, PCR-SSCP and DNA sequencing were the techniques employed. During the course of our investigation, histological slide preparations of a spleen biopsy from the index patient, LB, and a genomic DNA specimen from her only female offspring, BB, became available.

3.2 Case History and Laboratory Findings.

Patient, LB, a 24 year-old woman presented in 1987 with lower back pain and pyrexia during the 36th week of her pregnancy. She was admitted to a nursing home for observation on the assumption of a urinary tract infection. It's important to note that she had no previous clinical history and her pregnancy had thus far been uneventful. Shortly after admission, she suffered spontaneous rapture of membranes and a foul smelling liquor was noted. Signs of foetal distress necessitated an emergency caesar and a healthy female infant was delivered. Inspection of the peritoneal cavity during surgery revealed a thin milky fluid which was thought to be pus but the source of the infection could not be determined.

Her condition deteriorated and she was admitted to the surgical ICU where an ultrasonar graft showed a diffusely enlarged pancreas, abdominal distention leading to a diagnosis of acute pancreatitis. In spite of aggressive therapy her condition deteriorated further and she developed all nine of Ransin's Criteria of fulminant acute pancreatitis. She progressed into total organ failure and died, five days after admission. Post mortem examination confirmed the diagnosis of fulminant haemorrhagic pancreatitis. Blood samples taken a day after admission revealed a serum amylase level of 3290 mmol/l and marked lipemia with a cholesterol of 21 mmol/l (n = 3 - 7.3) and a triglyceride level of 87 mmol/l (n = 3 - 2.3).

The patient's, father, DR and mother, ER, 63 and 62 years old, respectively, were normolipidemic with no notable history of disease or illness. Following their informed consent, bloods were obtained for later genetic analysis. The mother, ER, who gave birth to two offspring, the proband, LB, and a male child had experienced no complications during either pregnancy. A family pedigree is shown in Fig. 24.

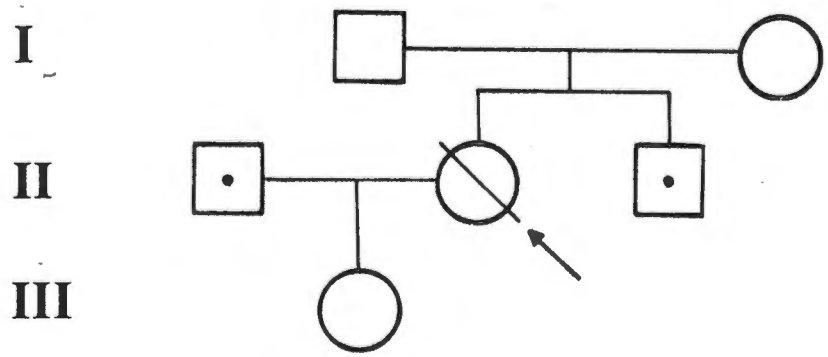


Fig. 24: Family Pedigree of Kindred II.
deceased proband, not investigated.

3.3 Results (Mutation Analysis).

3.3.1 RT-PCR Analysis.

3.3.1.1 Total RNA Assessment: RNA was isolated from monocyte-macrophages from both parents, as described previously (Methods).

3.3.1.2 cDNA Synthesis: cDNA was synthesised from total RNA preparations as described previosly (Methods).

3.3.1.3 PCR Amplification of LPLcDNA Fragments: The primer design and PCR conditions employed, were as described for kindred I (Chapter I).

3.3.1.4 Cloning of Fragments.

The PT7Blue T-vector (Novagen) was used as a vehicle for cloning the 5'- and 3'- of amplified fragments from LPLcDNA of subjects, DR and ER (Methods). Ligation was carried out at a vector:insert ratio of 1:3. Approximately 1/10th of the ligation mix was used to transform "Novablue" competent cells. Transformation efficiencies are shown in Table 10. Based on the blue/white colony selection process, several recombinant colonies (white phenotype) were identified and libraries of recombinants were prepared.

Table 10: Transformation efficiencies of cloned LPL gene fragments from subjects, DR and ER.

Colonies screened	Colony forming units per μg	% Ligation
Control plasmid	7.0×10^4	-
pT7 + control insert	2.5×10^4	-
pT7 + pLPL35(6-7)	2.2×10^4	60
pT7 + DR1-8	0.2×10^4	25
pT7 + DR6-7	1.3×10^4	20
pT7 + ER1-8	0.4×10^4	0
pT7 + ER6-7	0.4×10^4	0

Transformed colonies were screened by PCR using LPL-specific primers, LPLcDNA1, LPLcDNA8 and LPLcDNA6, LPLcDNA7(See Primer Design)(Fig. 18). The pT7 - vector was used for cloning, pLPL35 - 5'-fragment of the full length LPLcDNA; DR1-8 and ER1-8 - 3'- fragment of the LPLcDNA from subjects, DR and ER; DR6-7 and ER6-7 - 5'-fragment of the LPLcDNA of subject, DR and ER.

3.3.1.5 PCR Screening.

Direct colony PCR screening was carried out as described before (Kindred I)(Methods). The presence of a DNA fragment approximately 1.1 kb in length, following gel electrophoresis, indicates a clone positive for the insert DNA. Only 20-25% of colonies (white phenotype) representing the 5'- and 3'- LPLcDNA fragments of subject, DR, contained insert DNA of the expected size. The greater proportion of the remainder of the clones contained DNA inserts of 200 to 600bp in length. However, enough clones containing the correct insert size were obtained for analysis of the entire LPL coding sequence of subject, DR. As for the mother, ER, despite screening over 30 recombinant clones containing the 5'-fragment, no 1.1kb DNA fragment was observed; instead a 200bp fragment was consistently amplified. A similar pattern was observed when screening recombinant clones containing the ER 3'-fragment.

3.3.1.6 Sequence Analysis of Positve Colonies for DR.

Insert DNA, from colonies containing DR1-8 and DR6-7 LPLcDNA fragments were prepared for DNA sequence analysis as follows: Insert DNA from colonies positive for the 1.1kb fragment were subjected to PCR amplification, followed by gel electrophoresis on 0.7% agarose gels and the DNA fragments gel purified as described (Methods). The concentration of each DNA fragment was carefully assessed and subjected to direct PCR-based dideoxy chain termination DNA sequence analysis (Promega)(Methods). 50-100 ng of insert DNA was generally used per sequencing reaction. Due to the constraints imposed by the sequencing conditions only 300 bases could be sequenced per sequencing reaction. This prompted the use of a repertoire of LPL-specific sequencing primers (see Primer Design), spanning the entire LPLcDNA sequence. Successful sequence was only obtained for the region spanning exons 2 to 9. Further sequence analysis of several clones including the 5'-untranslated region are in progress to verify the above results.

3.4 Genomic DNA Analysis of Kindred II.

The availability of genomic DNA from both parents allowed for an alternate approach to detecting mutations in the LPL gene in this kindred. The problems encountered during the investigation of the LPL gene locus for ER using RT-PCR methodology could be circumvented by amplifying and sequencing individual exons. Postmortem histological slide preparations from the index patient, LB was available for analysis. A protocol for the extraction of archival DNA from slide preparations, was developed, as was a PCR protocol for amplifying exonic DNA from these archival specimens. Genomic DNA was also obtained from the patient's daughter, BB.

3.4.1 DNA Extraction of archival samples from LB.

Histological tissue slide preparations obtained from the postmortem performed on LB, were the only samples available since all the wax blocks were destroyed by a fire in the mortuary. The DNA extraction protocol for Heamatoxylin/Eosin stained slides and wax blocks was developed by our laboratory staff as described (Methods).

Patient spleen tissue DNA extracts were assessed for their degree of degradation by electrophoresis on a 1% EtBr stained agarose gel. Fig. 25 represents DNA extracts from histopathological slides (lanes 7,8) compared to controls from paraffin wax blocks (lanes 6,7). Each extract represents material from a single slide preparation or wax block section. All postmortem tissues yielded highly degraded DNA consisting predominantly of low molecular weight DNA (200-300 bpr). Several reports, have suggested that the sample extraction process, the tissue scraping and/or vortexing steps (Provan *et. al.*, 1992) as well as the fixation process (Rogers *et. al.*, 1990) may be the cause of mechanical shearing of high molecular weight DNA. Despite the problems encountered, the quality and yield of the DNA template recovered by this extraction protocol was good enough for PCR amplification of smaller LPL exons.

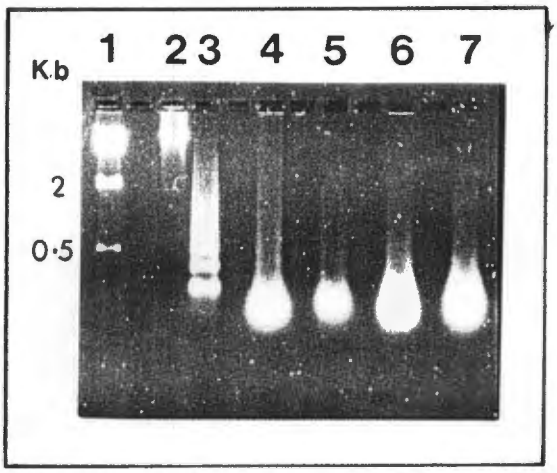


Fig. 25: **Archival DNA extracts from tissue embedded in paraffin-wax and mounted on histopathological slides. Electrophresis was performed on 1% agarose gels and stained with ethidium bromide. Lane 1 - DNA molecular weight marker, Lane 2 - Undegraded genomic DNA, Lane 3 - Degraded genomic DNA, Lanes 4,6 - DNA from wax blocks, Lanes 5,7 - DNA from histopathological slides.**

3.4.2 PCR Amplification of LB Slide DNA.

Several groups have been successful in amplifying archival DNA, extracted from either paraffin-wax blocks or histology slides (An and Fleming, 1991; Provan *et. al.*, 1992; Neubauer *et. al.*, 1992; Volkenandt *et. al.*, 1992; Gall *et. al.*, 1993; Chen and Clejan, 1993). PCR amplification of archival DNA is usually successful with target sequences of 300 bp or less (Neubauer *et. al.*, 1992). A problem sometimes encountered is the presence of PCR inhibitors in extracts (An and Fleming, 1991). Also, formalin fixation appears to induce crosslinking of DNA thereby adversely affecting PCR amplification (Neubauer *et. al.*, 1992). Mercury-based fixatives, such as B-5 and fixatives containing picric acid, such as Bouiins, are to be avoided as they extensively degrade DNA, limiting PCR analysis (Herbert *et. al.*, 1989).

The following protocol was used in this study: (i) A series dilution of the extracted DNA (1/5 to 1/100) was performed to dilute out any inhibitors. This proved to be an important step in determining the optimal concentration for successful amplification. (ii) The Taq polymerase enzyme was added under denaturing conditions (94°C), called a "hot start". (iii) The PCR cycle number was increased to 40, generating a stronger signal. Following amplification, DNA products were applied to 1% agarose gels and visualized by ethidium bromide staining.

Fig. 26 shows amplification products of exon 9 from kindred II. Primers, specific for exon 9, namely, LPL 23 (reverse) and LPL 24 (forward) (Primer Design) were used, giving a DNA fragment of 159 bp. Lanes 1 to 5 represent a dilution series of amplified extract DNA from the proband, LB, clearly indicating a positive signal at a sample dilution of 1/25 (lane 3)(Methods). DR, ER and BB amplified DNA products (lanes 6, 7, 8) are shown. These amplification products were subjected to SSCP analysis.

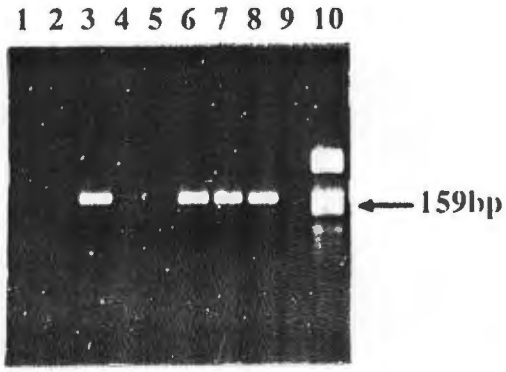


Fig. 26: PCR amplification of exon 9 from slide DNA of index patient, LB and genomic DNA of three kindred II subjects. Electrophoresis was performed on 1% agarose gels and stained with EtBr. Lanes 1 to 4 - PCR products from 1/100, 1/50, 1/25, 1/5 dilutions of LB slide DNA. Lane 5 - a PCR blank, Lane 6 - exon 9 PCR product from the father of LB, Lane 7 - exon 9 PCR product from mother of LB, Lane 8 - exon 9 PCR product from the daughter of LB, Lane 9 - PCR blank, Lane 10 - DNA molecular weight marker.

Despite the successful amplification, the sensitivity and reliability of the assay remains a problem, since PCR product was not always obtained. On a positive note, the results corroborate earlier reports, suggesting improved amplification following dilution of extract DNA since inhibitor concentrations are presumably diluted out (Volkenandt, *et. al.*, 1992). Also, the "hot start" utilised is very effective in reducing primer loss through primer-dimer formation.

3.4.3 PCR-SSCP of Kindred II.

The principles and strategy of this technique were outlined earlier.

PCR-SSCP analysis was adopted in order to search for sequence alterations in the LPL gene as this methodology provides an efficient means for the detection of point mutations. This was effectively demonstrated for proband, JJ (Kindred I) in the previous chapter. Following electrophoresis of DNA fragments, SSCP gels were visualised by silver staining, as described in Methods. After excision of the abnormal bands from the stained gel, sequence variations were determined directly by PCR cycle sequencing from eluted DNA.

Fig. 27 shows a SSCP gel of LPL exon 9 DNA for kindred II (see next page). Initially, SSCP analysis of exonic DNA was performed for DR and ER only. Band shift anomalies were only detected for exon 9 of ER (lane 2) while DR (lane 1) has a banding pattern identical to the normal LPL exon 9 control (lane 5). An interesting observation for ER, is the absence of a single "normal" band, B8 as well as the presence of variant bands B1, B3, B5, B6, B7 and B9 which suggested that she may be homozygous for a single sequence alteration or a compound heterozygote for two distinct base variations in exon 9. Individual bands were sequenced directly by a cycle sequencing protocol as described [fmol DNA sequencing system (Promega)] (Methods). Successful sequence was only obtained for the variant band, B9 of ER, which showed a single G→A transition at position 1516 in the first positon of codon 421 in exon 9. This gave rise to a substitution of lysine (K)(AAG) for glutamic acid (E)(GAG) and was designated E421K (Fig. 28). Further investigation is necessary to determine the sequence the other ER SSCP bands.

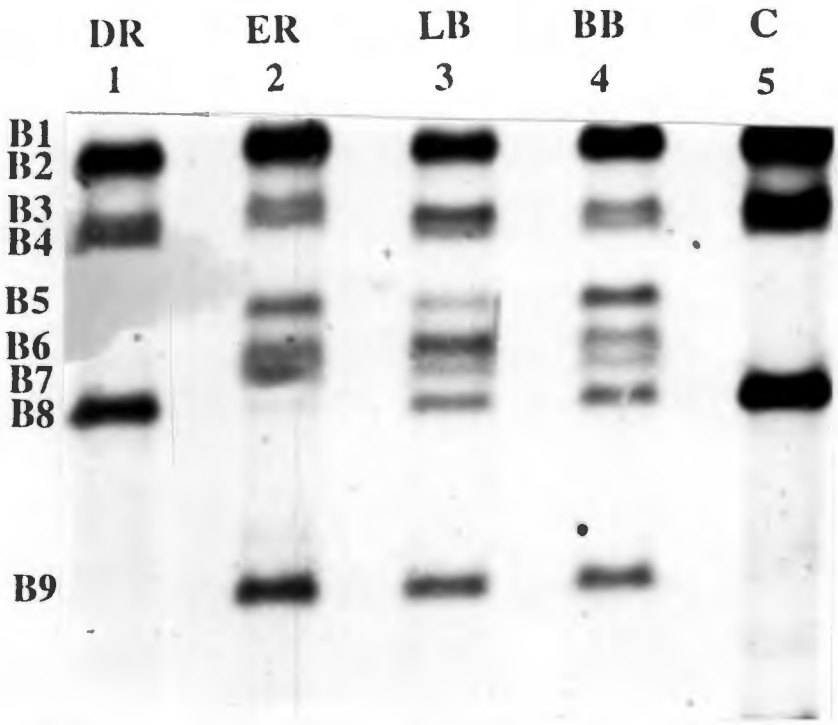


Fig. 27: **SSCP analysis of LPL exon 9 amplicons from kindred II subjects. PCR amplification was performed using two primers, LPL23 and LPL24 (See Primer Design). Electrophoresis was performed on 0.55×Hydrolink MDE polyacrylamide gels with visualisation by silver staining. DNA bands are numbered B1 - B9. Lanes 1 - 4 are kindred II samples. Lane 5 is a normal control specimen. The normal control DNA gives three bands, B2, B4 and B8. Bands B1 and B2 cannot be distinguished in lane 2 due to overexposure to bring up band, B7.**

SSCP analysis of exon 9 was also carried out for amplification products from the index patient (LB)(lane 3) and her only offspring (BB)(lane 4). The presence of the variant band, B9 in both subjects suggest that they had inherited the same maternal band. Sequence analysis of this band confirmed the inheritance of the E421K allele for both LB and BB. Therefore, the cluster of bands, B5, B6 and B7, probably represent "conformers" of the E421K allele, since they were observed in

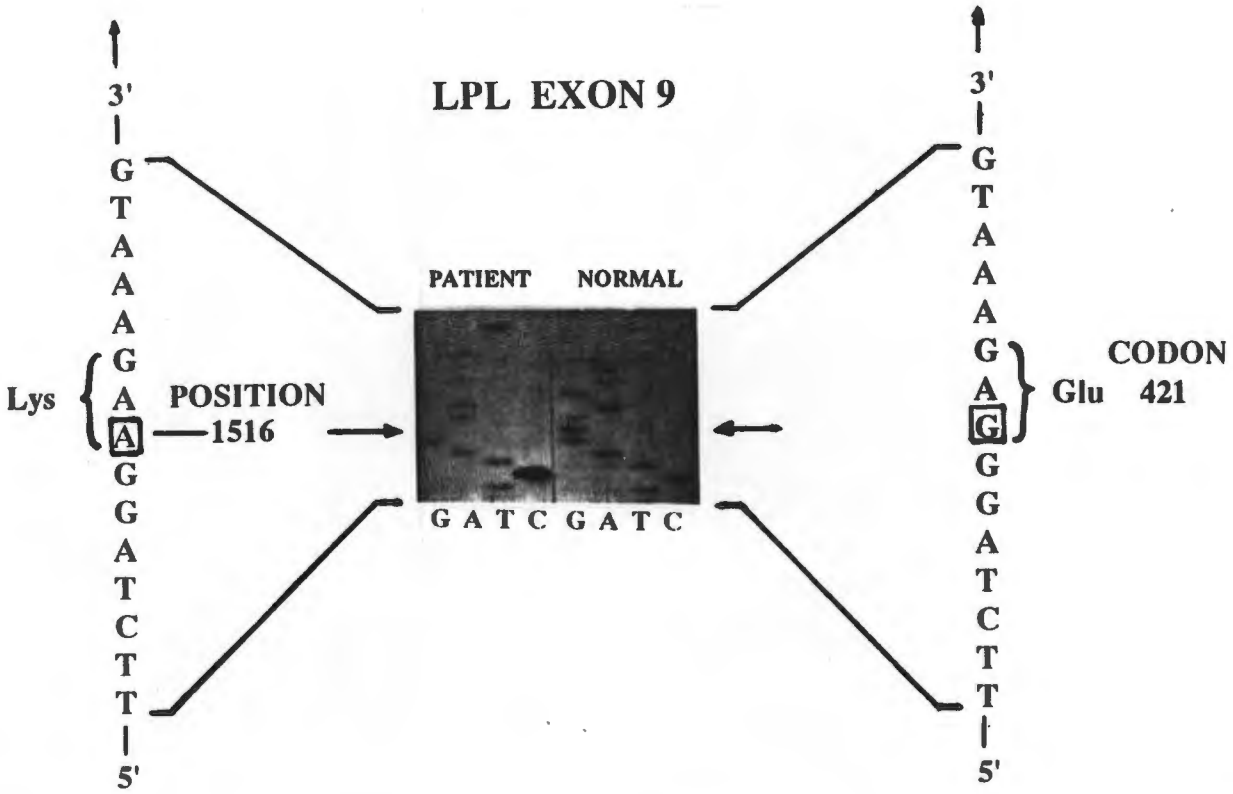


Fig. 28: **Nucleotide sequence of the sense strand of exon 9 of the LPL gene for ER, the mother of LB and a control subject. The G→A base transition (⟶) is boxed. The amino acid subsitution is indicated and the codon is numbered.**

---

the three subjects ER, LB and BB but not in DR. Bands B2, B4 and B8 present in DR, and normal controls also occur in LB and BB suggesting that they also received a copy of the paternal allele which is most probably of normal exon 9 sequence. These band shifts were confirmed through several independent PCR-SSCP analyses. In a search for the paternal mutation, PCR-SSCP analysis of exons 2-9 from DR, spanning the entire coding region, including the exon-intron boundaries of the LPL gene locus, revealed no sequence variations, except for a G→A transition in the third position of codon 108 in exon 3 (data not shown). DR proved to be heterozygous for this rare polymorphism (data not shown) and DNA analysis is in progress to establish whether LB has inherited this allele or not.

3.5 Discussion.

Familial LPL deficiency is known to exacerbate the hyperlipidaemia commonly associated with individuals in the third trimester of their antenatal course (Gleuck *et. al.*, 1980; Watts *et. al.*, 1992). The index patient, LB, despite having no history or clinical sequelae alluding to an underlying hyperlipidaemic condition, presented upon admission with acute pancreatitis. This condition progressed to fulminant haemorrhagic pancreatitis which eventually led to her death. The gross hypertriglyceridaemia (TG, 87mmol/l) was considered the most likely cause in the absence of other precipitating factors such as, diabetes or alcohol abuse, which rendered her a strong candidate for a defect in LPL activity.

A search for mutations in the LPL coding sequence in the parents of LB led to the discovery of a novel G→A transition at position 1516 in exon 9 at codon 421 leading to a substitution of lysine for glutamic acid. This mutant allele was shown to be inherited by LB and was passed on to her offspring, BB. While completing the investigation on ER, a second mutation, a C→G transversion (a known polymorphism) at position 1595 in exon 9 of the alternate allele, was identified by our research staff. This sequence change introduces a premature termination codon at Ser447 and gives rise to a truncated protein, lacking the C-terminal Ser-Gly dipeptide. Compound heterozygosity for the E421K mutation and the Ser447→stop polymorphism clearly explains the abberant banding observed upon PCR-SSCP analysis in the mother of our patient. The fact that she has no history of hyperlipidemia and that both her pregnancies were uneventful would suggest that the expression of these two mutant LPL gene products does not manifest as a clearance defect. A search for the second mutation in LB by examining DNA from her father has thus far been unsuccessful. Her father was however shown to be heterozygous for a silent mutation in exon 3 at codon 108. This polymorphism can be detected by restriction analysis with Sau961. A possible defect in the LPL exon 1 and the 5'-regulatory region of DR cannot be ruled out. Further analysis is also necessary to determine expression levels for both alleles. Complete sequencing of all the LPL exons of LB is now necessary as not all sequence alterations can be determined from SSCP band shifts. At present time we have shown that our patient, LB, is heterozygous for the E421K mutation, the "mutant" allele being of maternal origin, while the paternal allele has a "normal" sequence at this codon position.

LPL deficiency manifests a baseline carrier frequency of 1:500. While homozygotes present with marked hyperlipidaemia, establishing an autosomal recessive mode of inheritance, several reports have recently alluded to a possible role for the heterozygous LPL-deficient state in the genesis of hypertriglyceridaemia. This has been facillitated by the ability to unequivocally identify carrier status by DNA screening techniques. Previous attempts to identify carriers had to rely on measurement of LPL activity levels in post-heparin plasma and adipose tissue. Obligate carriers have been reported with a 50% reduction in LPL activities in post-heparin plasma (Kondo *et. al.*, 1985; Miesenbock *et. al.*, 1993) and adipose tissue (Wilson *et. al.*, 1990) compared to normal controls. On this basis mild and variable hyperlipaemia have earlier been shown to segregate with the carrier state (Babirak *et. al.*, 1989) and was characterised by slightly elevated plasma triglyceride and VLDL-cholesterol levels with a reduced HDL_2 component (Wilson *et. al.*, 1990; Miesenbock *et. al.*, 1993; Patsch *et. al.*, 1983 and 1987). In the post-prandial state, the carrier status manifests itself as reduced clearance of dietary triglyceride from plasma called "the syndrome of impaired TG tolerance". Earlier, Wilson *et. al.* (1990) confirmed that fasting hypertriglyceridaemia in carriers were strongly age-related with individuals older than 40 years showing increased plasma triglyceride levels suggesting a later age of onset for this condition. In addition, multiple secondary factors such as obesity, hyperinsulinaemia, hypertension and lipid-raising drugs (contraceptives) are known to aggravate fasting hypertriglyceridaemia in heterozygous LPL-deficient individuals (Wilson *et. al.*, 1990). Pregnancy must now also be recognised as one of the secondary factors that interact with the heterozygous LPL-deficient state. While a defect in LPL activity has not been adequately established it is clear that our patient manifested with a lipoprotein clearance defect.

Female carriers of LPL-deficient alleles, however do not usually present with overt hypertriglyceridaemia during pregnancy. We therefore expected that our patient would manifest with two defective LPL allelic products generating a LPL-deficient state exacerbated by the pregnancy-induced lipaemia. One mutation, which may cause the production of a catalytically defective product has been found, while the other remains to be elucidated.

The identification of two mutations in this kindred located to exon 9, brings to three the number of missense mutations identified in this exon of the LPL gene. Recently, five other groups have also reported the identical C→G transversion at position 1595 (Ser447→stop) in individuals of different nationalities. This mutation appears to have no effect on LPL function (Faustinella *et. al.*,

1991) with most of the LPL activity being retained (Ma *et. al.*, 1992). Stocks *et. al.* (1992) reported no clear relationship between the presence of the mutation (Ser447→stop), hypertriglyceridaemia and variable lipid levels as commonly associated with mutations causing LPL deficiency. This sequence variant may therefore be regarded as a common DNA polymorphism. Recently, a 30 year old East Indian woman presented for the first time at 33 weeks gestation, when she was diagnosed with pancreatitis precipitated by pregnancy (Ma *et. al.*, 1993). She was confirmed to be homozygous for a C→G transversion at nucleotide position 770 in exon 5 resulting in a serine to cysteine substitution. *In vitro* expression studies of this mutant LPLcDNA, produced a partially active product with approx. 6% LPL activity compared to the wild type. The concluding remarks in this case may be reported as, hyperlipidaemic pancreatitis as a result of partial LPL deficiency compounded by pregnancy. This case report appears to be identical to our index patient, LB, except our patient is heterozygous for the E421K mutation in exon 9 of the LPL gene. LPL-deficient carriers do not usually present with hyperlipidaemia in pregnancy. Hence, there is presumed to be a undetected second mutation or defective allele in our patient not usually associated with a defect in lipoprotein clearance. However, the E421K mutation may produce a partially active LPL protein product, which in combination with a second partially defective protein and a secondary factor such as pregnancy, may manifest as LPL deficiency. Another possibility, is the association of the "impaired triglyceride tolerance" syndrome with the LPL-deficient heterozygote in the post-prandial state, which in combination with all these genetic and enviromental triggers may have been the underlying cause for our index patient's condition, leading to the catastrophic events in 1987.

CHAPTER 4

CONCLUSIONS AND FUTURE STUDIES

The specific conclusions have been discussed at the end of each chapter. General conclusions relevant to the topic are presented in this chapter and a short summary on future studies is given at the end.

4.1 General Conclusions.

The findings in the two kindred studies corroborates the well described phenotypic and genetic heterogeneity that underlies Type I hyperlipoproteinemia. In kindred I, proband, JJ, has a milder phenotype and was shown to be a compound heterozygote for two mutations. Transfection studies showed the I194T mutant to be catalytically inactive. In the absence of transfection studies, the preliminary evidence suggests that the C418Y mutant product is partially active. In the kindred II study (Chapter 3), the relatively benign clinical history and uneventful pregnancies of the mother, ER, implies that the E421K mutant product, more than likely is partially active. This mutation was also inherited by the kindred II proband and while it may have contributed to her condition, it is unlikely to have been the major determinant of the hyperlipidaemia which led to her death. This is in conformity with the findings of other workers, who propose that gene mutations located in the C-terminal domain of LPL generate protein products that are partially active. It becomes evident that the number of mutations at this gene locus is underestimated since many of these sequence variants may only be detected in association with a secondary hyperlipidaemic state. It is therefore necessary to screen individuals with moderate hypertriglyceridemia in the presence of environmental stress factors commonly associated with hyperlipidaemia.

In our hands RT-PCR has proved a reliable and powerful tool in the analysis of the coding sequence of genes and is now routinely applied to mutation analysis in a variety of genetic disorders presenting at this hospital. We have also applied several mutation detection techniques, which allow for broad-based screening programs for any known polymorphism or mutation prevalent in the general population.

4.2 Future Studies.

In-vitro expression studies on the C418Y and E421K mutants and elucidation of the biochemical properties of the mutant proteins will clarify their functional properties and demonstrate any defect in intracellular processing. Furthermore, transfection experiments that allow for the expression of specific LPL dimers would facillitate the study of biochemical properties of the I194T/C418Y heterodimer.

The I194T and C418Y ARMS PCR system requires optimisation to facilitate screening for these mutations in the general population.

CHAPTER 5

MATERIALS AND GENERAL METHODS

This chapter contains a comprehensive list of all materials used in this work as well as the methods used.

5.1 Materials, Reagents and Buffer Solutions

All the water used in this work was deionized and doubly distilled in glass using a Quickfit 3 DWS Double Distillation Water Still. The abbreviation, ddH_2O will be used wherever water is used in procedures. The sources of all other reagents and solvents are shown in brackets.

1) 1M TRIS. HCl

121.1g of Tris base (Boehringer Mannheim (BM) Pty, Cat. No.: 708976) was dissolved in 800ml of ddH_2O. The pH was adjusted to the desired level by adding concentrated HCl. The final volume was adjusted to 1 liter with ddH_2O and the solution was autoclaved.

2) 0.5M EDTA (pH 8.0)

186.1g of Disodium ethylene diamine tetraacetic acid.dihydrate ($EDTA.2H_2O$)(BDH Ltd., Cat. No.: 10093 5V) was dissolved in 800ml of ddH_2O and the pH was adjusted to 8.0 with a 10N NaOH (Merck, Cat. No.: 6498.0500) solution. This solution was autoclaved.

3) 10×TE (Tris.EDTA)

100mM TRIS.HCl (pH 7.4 - 8.0)

10mM EDTA (pH 8.0)

4) 1M Dithiothreitol (DTT)

3.09g of DTT (Cleland's Reagent)(Sigma, Cpy; Cat. No.: D5545) was dissolved in 20ml of 0.01M sodium acetate (pH 5.2). The solution was filter sterilised and stored at -20°C.

5) 20×SSC

175.3g of NaCl (Saarchem (Pty) Ltd., Cat. No.: 582 25 00) and 88.2g of trisodium citrate (Saarchem (Pty) Ltd., Cat. No.: 582 250) was dissolved in 800ml of ddH_2O. The pH of the solution was adjusted to 7.0 with 10N NaOH and made up to 1 liter with ddH_2O and autoclaved.

6) 20×SSPE

174g of NaCl, 27.6g of $NaH_2PO_4.H_2O$ (Merck, Cat. No.: 6346.0500) and 7.4g of EDTA was dissolved in 800ml of ddH_2O. The pH was adjusted to 7.4 with a 10N NaOH solution. The volume was adjusted to 1 liter and sterilised by autoclaving.

7) 50×Denhardt's Solution

5g of Ficoll (Merck, Cat. No.: 498.0250), 5g of Polyvinylpyrrolidone (Sigma Cpy., Cat. No.: PVP-40) and 5g of Bovine serum albumin (Fraction V) (Sigma Cpy., Cat. No.: A 7906) was dissolved in 500ml of ddH_2O. The solution was filter sterilised and stored at -20°C.

8) 10% SDS

100g of Sodium Dodecyl Sulfate (SDS)(Merck, Cat. No.: 13760) was dissolved in 900ml of ddH_2O. The solution was heated to 60°C to facilitate dissolution and the pH was adjusted to 7.2 with concentrated HCl. The volume was adjusted to 1 liter and the solution was stored at room temperature.

9) Luria-Bertani Medium (LB Broth)

10g of Bactotryptone (Oxoid, Unipath Ltd, Cat. No.: CM129), 5g of Bacto-yeast extract (Biolab Diagnostics (Pty) Ltd, Cat. No.: BX6) and 10g of NaCl was dissolved in 1 liter of ddH_2O. The pH was adjusted to 7.5 with 10N NaOH and the solution was autoclaved.

10) LB Agar

15g of Bacto-agar (Oxoid Ltd, Cat. No.: L13) was added to 1 liter of LB broth and autoclaved. The solution was cooled to 55°C before adding the antibiotics. Approximately 30 to 35 ml of LB agar was poured per 85mm petri dish. The top surface of the agar plate was flamed with a bunsen burner to sterilise and remove any surface bubbles. The agar was allowed to set and the plates were stored at 4°C until use.

11) 5×TBE

54g of TRIS.HCl (pH 7.4), 27.5g boric acid (Saarchem (Pty) Ltd, Cat. No.: 140 5200) and 20ml 0.5M EDTA (pH8.0) were dissolved in ddH_2O and made up to 1 liter .

12) Taq DNA Polymerase (for PCR) (Concentration: 5 units/μl; Promega, Cat. No.: M1861)

Taq DNA Polymerase Buffer (Promega, Cat. No.: M190A)

100mMM TRIS.HCl (pH 9.0), 500mM KCl, 1% Triton X-100.

Magnesium Chloride (25mM; Promega, Cat. No.: A351)

Note: The above PCR reagents were used in all subsequent PCR reactions.

13) Cell Lysis Buffer

109.5g of Sucrose (0.32M; BDH Ltd, Cat. No.: 10274), 5mM $MgCl_2$, 10ml Triton X-100 (1%), and 10mM TRIS.HCl (pH 7.6) were mixed and made up to 1 liter with ddH_2O, autoclaved and stored at 4°C.

14) Saline-EDTA, pH 8.0

Fifty milliliters of a 100mM EDTA stock solution was mixed with 3.75ml of a 4M NaCl stock solution and made up to 200ml with ddH_2O. The solution was autoclaved and stored at room temperature.

15) Proteinase K

The Proteinase K (Sigma Cpy, Cat. No.: P-0390) solution was prepared as a 10mg/ml stock with sterile ddH_2O. 1ml aliquotes were snap frozen and stored at -70°C.

16) Sodium-perchlorate

70.23g of $NaClO_4.H_2O$ (5M; Merck, Cat. No.: 6564.0100) was dissolved in 100ml of ddH_2O. The solution was autoclaved and stored at room temperature.

17) Phenol/Chloroform (T.E. saturated) Mixture

Redistilled Phenol (Merck, Cat. No.: 7632.0100) was saturated with a 1×T.E. solution. An equal volume of this solution was mixed with an equal volume of Chloroform (BDH Ltd, Cat. No.: 10077).

18) Chloroform/Octanol Solution (24:1)

480ml of Chloroform was mixed with 20ml of Octanol (Merck, Cat. No.: 991.1000) and stored in a dark bottle at room temperature.

19) Ethidium Bromide (10mg/ml)

1g of Ethidium Bromide (EtBr; Sigma Cpy, Cat. No.: E 8751) was dissolved in 100ml of ddH_2O.

20) Terrific Broth

To 900ml of ddH_2O was added 12g of Bactotryptone, 24g of Bacto-yeast extract and 4ml of glycerol. This solution was mixed and autoclaved for 20mins at 15 lbs. A solution containing 2.31g of KH_2PO_4 and 12.54g of K_2HPO_4 was prepared separately in 90ml of ddH_2O, made up to 100ml with ddH_2O and autoclaved. Both solutions were allowed to cool to 60°C before combining the two solutions, which results in a final concentration of 0.17M for KH_2PO_4 and 0.72M for K_2HPO_4.

5.2 General Methods

5.2.1 LPL Activity Assay

5.2.1.1 Materials and Reagents

1) 0.233M TRIS.HCl Buffer, pH 8.5

29.289g of TRIS was dissolved in 1 liter of ddH_2O and the pH adjusted to 8.5 with 10N NaOH.

2) L-α-Phosphatidylcholine (Type 1x-E) from egg (lecithin), 50mg/ml:

This phospholipid is supplied as a thick gel by Sigma Chemical Co. (Cat. No. P8640). The reagent was prepared as a 50mg/ml stock solution in chloroform and stored at 20°C.

3) Salt Correction Solution (0.78M NaCl in TRIS buffer)

4.558g of NaCl was diluted in 100ml of 0.223M TRIS buffer, pH 8.5 and stored at 4°C until use.

4) Krebs-Ringer Phosphate (KRP) buffer:

NaCl (45 g/l)	-	100.0ml
KCl (57.4 g/l)	-	5.0ml
$CaCl_2.2H_2O$ (8.14 g/l)	-	1.5ml
$MgSO_4.7H_2O$ (191 g/l)	-	1.0ml
$NaH_2PO_4.H_2O$ (13.8 g/l)	-	15.0ml
$Na_2HPO_4.H_2O$ (14.196 g/l)	-	85.0ml

432ml ddH_20 was added to the above mixture and the pH was adjusted to 7.4 with HCl. The solution was stored at 4°C.

4) Heparin (1mg/ml in TRIS or KRP buffer)

Sodium heparin was obtained from Sigma Chemical Co. (Cat. No.: H3125) and dissolved in TRIS or KRP buffer at a final concentration of 1mg/ml and stored at -20°C in 2ml aliquotes.

5) Belfrage Extraction mixture (methanol:chloroform:heptane = 1.41:1.25:1)

141 ml methanol

125 ml chloroform

100 ml heptane

The solvent was prepared in a dark bottle and stored at 4°C until use.

6) 0.05M Carbonate-Borate buffer, pH 10.5

6.91g of Potassium Carbonate (Merck, Cat. No.: 4928.0500) and 3.092g of boric acid was dissolved in ddH_2O and the pH was adjusted to 10.5 with 5N potassium hydroxide. The solution was made up to 1 liter and stored at 4°C.

7) Bovine serum albumin (BSA)

a) Delipidation of BSA (Removal free fatty acids).

Activated charcoal (5g; Merck, Cat. No.: 2186.0250) was layered on Whatman (No.1) filter paper in a buchner funnel and washed 3 to 4 times with ddH_2O. The fines floating on top of the suspension was syphoned off using a venturi pump. A BSA solution (10g BSA in 100ml ddH_2O) was prepared and mixed with the washed charcoal and while stirring, the pH was adjusted to 3 by the slow addition of 0.2M HCl. The mixture was placed in an ice-bath and stirred for 1 hour. The charcoal was removed by centrifugation at 19500 rpm for 30 minutes. The supernatant was collected and the pH was adjusted to 7 with 0.2N NaOH. The delipidated BSA solution was dialysed against ddH_2O for several hours or overnight. The dialysate was freeze-dried overnight and the dried, delipidated, BSA powder was stored in a brown bottle at 4°C until use.

b) The BSA, Fraction V (lyophilized and fatty acid free) obtained from Sigma (Cat. No. A7030) was used directly.

8) Preparation of the Non-radioactive and Radioactive Triolein substrate (200 mg/ml).

a) Reagents

i) The Glycerol trioleote [C18:1, (cis)-9] stock material was obtained from the Sigma Chemical Cpy. (Cat. No. T-7140) as a 1g quantity. A stock solution of 0.5mg/5μl were aliquoted and stored at -20°C until use.

ii) Glycerol tri [1-^{14}C] oleate (conc: 50μCi/0.5ml)(Spec.Act. 54.3mCi/mmol)(Amersham, Cat. No.: CFA 258). 5μl and 10μl were assayed.

b) Protocol

Non-radioactive triolein (0.1mg/μl) and radiolabelled triolein (Stock, 0.5-1μCi) were subjected to thin-layer chromatography (TLC), utilising precoated, TLC (KIESEL) Silica gel 60 plates (20x20; thickness 0.25mm)(Merck Cpy, Cat. No.: 5721.0001) and chromatographed for approximately 40 minutes in a Panglas TLC chromatank (Shandon, Ltd) containing 100ml of a petroleum ether: diethyl ether: glacial acetic acid (90:10:1) buffer solution. Following chromatography, the plates were air-dried for approximately 30 minutes and allowed to develop in the presence of iodine crystals. The stained spots were marked and allowed to destain by evaporation. The spots were scraped, pooled and the triolein extracted as follows:

i) Triglycerides were liberated from silica gel by shaking vigorously in diethyl ether for 10 minutes.

ii) The solution was centrifuged for 10 minutes at 3000 rpm to pellet the gel pieces.

iii) The supernatant was transferred to a clean tube and the gel pellet extracted twice with 5ml of diethyl ether.

iv) The supernatants were pooled and evaporated to dryness under N_2 gas.

v) The dried and purified samples were reconstituted in benzene.

vi) The concentrations and recoveries of purified triolein were assessed as described below. The concentration of non-radioactive triolein was determined on a Technicon Random Access (RA) 1000 SYSTEM. The radioactive triolein concentration was assessed on a Beckman Scintillation Counter with 0.1μCi being used per assay.

9) Preparation of non-radioactive and radioactive oleic acid (cis-9-Octadecenoic Acid) solution.

a) Reagents

i) Radioactive [1-^{14}C]oleic acid (Spec. Act. 54.9mCi/mmol; Amersham Ltd; Cat. No.:CFA 243) was prepared as 50μCi aliquotes in toluene and evaporated to dryness under N_2.

ii) Non-radioactive oleic acid (Sigma, Cat. No.: O 3879) was prepared in hexane as a 50μM solution (14.2mg/100ml).

iii) The working stock solution was prepared as follows: 50μCi of radioactive oleic acid was diluted in 100 ml of a 50μM solution of non-radioactive oleic acid. This solution was stored at 20°C until use.

10) Pre-heparin plasma (pEH)

Approximately 20ml of blood was obtained from each subject and collected into heparinised tubes. The cell debris were removed by centrifugation at 3000 rpm at 4°C; the plasma was collected and 1ml aliquotes were snap frozen and stored at -70°C.

11) Post-heparin plasma (pOH) sample preparation

After an overnight fast, subjects were administered a heparin bolus (50-100 units per kg of body weight) by intravenous injection. Infusion was usually carried out over a period of 1 minute. This was followed by an intravenous saline wash (5ml). Following a 10 minute incubation period, bloods were drawn and collected into EDTA- or heparin-containing tubes (prevent clotting). The tubes were inverted several times and immediately placed on ice. Plasma was separated by centrifugation at 4°C and 1ml aliquotes were immediately snap frozen and stored at -70°C until use.

12) Pooled Serum

The bloods obtained were transferred to non-heparinised tubes and allowed to clot. After centrifugation the serum was collected, pooled and heat-inactivated at 56°C. 1ml aliquotes were snap frozen and stored at -70°C.

13) Bovine Skim Milk standard preparation

Fresh unpasteurised bovine milk was obtained from the dairy distribution centre and the cream removed by centrifugation at 4°C. Approximately 14.7g of solid trisodium citrate dihydrate (0.1M) was mixed with 500ml of the skim milk supernatant and stirred for 30 minutes at 4°C. This solution was then dialysed against a 5mM sodium phosphate buffered-saline (0.15M) solution (pH7.4) for 18 hours with buffer changes at 6 hourly intervals. After dialysis, glycerol was added to 30% (V/V) and 1ml skim milk aliquotes were snap frozen and stored at -70°C.

14) Preparation of the Triglyceride Substrate Emulsion

L-α-phosphatidylcholine (6mg/120μl), triolein (50mg/250μl) and radioactive triolein (1μCi/100μl) were aliquoted into a scintillation vial (2.2x7cm/flat-bottomed) and the contents were evaporated under a stream of nitrogen. To this mixture was added 4.95 ml of 0.233M Tris buffer (pH 8.5), 1ml of Salt Correction solution and 549.9mg of delipidated BSA was layered on top and allowed to dissolve. The mixture was cooled on ice and sonicated at 60 Watts for 3 minutes and 20 seconds (10 repeated cycles of 10 sec bursts interrupted by 10sec pauses) using a Sonifier B12 sonicator (Brandon Sonic Power, Cpy). Each 6ml aliquote is enough for 10 assay incubations.

15) Preparation of samples

a) Post-heparin plasma (pOH)

i) Just before analysis, aliquotes of pOH were rapidly thawed in cold water and 0.05ml of pOH was mixed with 0.2ml of pooled serum, 0.05ml heparin (1mg/ml) and 0.2 KRP or TRIS buffer. 0.2ml of this preparation was assayed for lipase activity.

ii) Antibody inhibition: Antibody $5D_2$ (A monoclonal antibody raised against bovine LPL in mice)(1mg/ml) dilutions were prepared in KRP or TRIS buffer. The diluted pOH (1:10) was mixed in a 1:1 ratio with the diluted antibody preparations and incubated at 4°C for 2hrs prior to assay. A 0.2ml aliquote was assayed for lipase activity.

b) Cell Cultures

0.2ml of culture medium was assayed without further dilution.

c) Skim milk standard

An aliquote of skim milk was rapidly thawed in cold water and diluted 1:10 or 1:20 in KRP or TRIS buffer. 0.2ml of the diluted sample was assayed for lipase activity.

5.2.1.2 Assay Conditions

The complete assay mixture (pH 8.2 at 37°C and ionic strength 0.16) contained 0.178M TRIS-HCI buffer (ionic strength 0.05), 0.11M NaCl, 55mg/ml of albumin, 0.01 mg/ml of heparin, 5% of serum (V/V), 5mg/ml of triolein and 0.05μCi/mg of C^{14}-triolein emulsified with 0.6mg/ml of lecithin. 0.6ml of this mixture was aliquoted per assay tube. All tubes were kept in an ice-bath until the enzyme substrates were added. Blank assay incubations contains 0.6ml emulsified assay mixture, 0.2ml TRIS buffer and 0.2ml heparin in KRP solution. To each tube containing 0.6 ml emulsified assay mixture, 0.2 ml of TRIS buffer was added. The enzyme solution (0.2ml)(i.e. pEH, pOH or skim milk standard) was added just before incubation is commenced.

Specific radioactive oleic acid recovery: A known amount of the oleic acid stock mixture (0.2ml) was aliquoted into test tubes in triplicate and evaporated under a stream of N_2 gas. 0.6ml assay mixture, 0.2ml TRIS and 0.2ml pEH solution was added to these test tubes and incubated as above. Also 0.2ml oleic acid stock mixture was aliquoted into two scintillation vials; scintillation fluid (4ml) was added and the radioactivity (cpm) assessed.

Specific radioactive triolein solution: 100μl of the assay mixture was added in triplicate to scintillation vials, dissolved in 4ml scintillation fluid and the radioactivity was counted.

Incubation: After addition of the enzyme solution, the test tubes were covered, mixed and incubated with shaking at 37°C for 60 minutes.

Incubations were terminated by the extraction of free fatty acids as follows:

(i) 3.25ml of a methanol:chloroform:heptane solution was added to 13x100mm test tubes.

(ii) A 0.2ml volume of the incubation mixture was transferred to the above tubes and vortexed.

(iii) This was followed by the addition of 1.05ml of carbonate-borate buffer solution.

(iv) The solution was again vortexed and centrifuged at 3000 rpm for 20 mins at room temperature to facillitate phase separation.

(v) The upper phase (2ml) was pipetted into scintillation vials and 5ml scintillation fluid was added and each tube was counted for 10 mins in a scintillation counter.

5.3 DNA Isolation

Anticoagulated blood (20ml) was collected in 50ml sterile evergreen tubes (heparinised or 1.5mg/ml EDTA). The blood was mixed thoroughly and centifuged at 4°C (2700rpm/15min). The plasma supernatant was aspirated off, ensuring that the packed cell layer is not disturbed. Ice-cold lysis buffer was added to cell layer up to the 50ml mark and mixed by inverting 10 times and stood on ice for 30 min. This mixture was centrifuged at 4°C (2700rpm/15min) and the supernatant aspirated off up to the 10 ml mark. About 40ml of ice-cold lysis buffer was added and the mixture inverted several times. The nuclei was pelleted by centrifugation at 2700rpm for 15min at 4°C. The supernatant was carefully poured off, leaving behind the gelatinous white cell/nuclei pellet. The tube was inverted to allow for drainage of the excess supernatant. To the pellet was added 8ml of Saline-EDTA and 0.8ml of 10% SDS and the mixture gently vortexed. One hundred microliters of Proteinase K solution (10mg/ml) was added to the mixture and incubated at 37°C for approximately 8 hours to overnight. To the mixture was added 0.5ml of 5M Na-perchlorate and 8ml phenol/chloroform (TE-saturated), which was gently stirred at 4°C for approximately 60 min, until the solution was homogenous. The solution was transferred to a sterile corex tube and centrifuged for 15min (10°C/8000rpm). The aqueous phase was carefully removed and transferred to a 50ml evergreen tube containing 10ml chloroform/octanol (24:1). This mixture was vigorously shaken for 30 min, transferred to a sterile corex tube and centrifuged for 15min (10°C/8000rpm). The aqueous phase was transferred to a sterile 50ml evegreen tube and 2 volumes of ice-cold absolute ethanol was added and mixed to precipitate the DNA. The DNA was recovered with a flamed-sealed glass pipette, dissolved in 1ml ddH_2O and placed on a roller-mixer overnight at 4°C. A 1/100 dilution of the DNA sample was prepared and spectrophotometric analysis was performed at wavelengths of 260 and 280nm. Two micrograms of purified DNA were electrophoresed on 0.7% agarose gels to assess the quality of the DNA preparation. The DNA was aliquoted, snap frozen and stored at -70°C until use.

5.4 End Labelling of Allele-Specific Oligonucleotides

5.4.1 Materials and Reagents

1) 10 x Kinase Buffer

121.1g of TRIS (1M)(BM Ltd, Cat. No.: 708 967) was dissolved in 800 ml of ddH_2O and the

pH was adjusted to 8 with HCl. 15.43g of DTT (0.1M) and 20.33g of magnesium chloride-6-hydrate (0.1M) ($MgCl_2.6H_2O$; BDH Ltd, Cat. No.: 10149) was dissolved in the above solution, which was made up to 1 liter with ddH_2O. 1ml aliquotes were stored at -20°C.

2) T4 Polynucleotide Kinase (BM Ltd, Cat. No.: 709 557)

5.4.2 Procedure

5.4.2.1 Sample Preparation

The following labelling mix was prepared: To a sterile, screw-capped 1.5ml eppendorf tube was added 20pmol of oligonucleotide, 2μl of 10×kinase buffer and 5μl of γ^{32}P-ATP (10μCi/μl)(Amersham, Cat. No.: PB 10168) and the solution was made up to 18μl with ddH_2O. The solution was vortexed and briefly centrifuged in a microfuge to collect the sample. T4 polynucleotide kinase (10U/μl) was added and the solution incubated for 45 min at 37°C. This was followed by a 10 min incubation at 65°C to inactivate the enzyme.

To 19μl of end-labelling mix was added 1μl loading dye (0.25% bromophenol blue; 30% glycerol in 1xTE) and the mixture made up to 100 μl with ddH_2O.

5.4.2.2 Purification (Spun Column Chromatography)

A 1ml G-25 Sephadex spun column (Pharmacia Fine Chemicals: Cat. No.: 6139) was prepared as follows: The bottom of a 1ml syringe or pipette tip was plugged with a small amount of sterile, siliconised glass wool. The column was filled with the G-25 sephadex gel (equilibrated with TE, pH8.0). The column was placed inside a glass or polypropylene centrifuge tube and centrifuged at 1000-1200 rpm for 2 mins to pack the gel and to remove excess fluid. Sephadex gel was added until the packed volume was approximately 0.9ml. The reproducibility of the packed column was monitored by loading approximately 100μl 1xTE and the column was centrifuged as before. One hundred microliters of sample was loaded and centrifuged as before. The eluant was recentrifuged (to ensure that the excess dNTP's were completely removed) and transferred to an eppendorf tube. An aliquote (1μl) of the eluant was assayed to determine the recovery which is usually between 30-40% of the total label used in the labelling reaction.

5.5 ASO Hybridisation

5.5.1 Materials and Reagents

1) Denaturation Solution: (0.4N NaOH/25mM EDTA):
   16g of NaOH was dissolved in 800ml of ddH_2O. To this solution was added 40ml 0.5M EDTA and the volume made up to 1 liter with ddH_2O.
2) Hyperfilm MP (Amersham, Cat. No.: RPN 7)

5.5.2 Sample Preparation

1/200th of the PCR-amplified DNA product was adjusted to 0.4N NaOH/25mM EDTA in a 200μl volume.

5.5.3 Procedure

5.5.3.1 Blotting

The wells of the Bio-Dot Spotting Apparatus (Bio-Rad) were overlayed with 3MM Whatman filter paper which had been soaked in ddH_20. Hybond N^+ filter paper (Amersham Ltd, Cat. No.: RPN 1210B) was soaked in ddH_2O and placed on top of the filter paper. The apparatus was clamped tight and a vacuum applied. Approximately 400μl 0.4N NaOH/25mM EDTA was loaded into each well and the flow rate monitored. DNA samples (200μl) were loaded into the wells and the DNA allowed to blot under vacuum. The sample blotting process was followed by an additional wash with 200μl of denaturation solution. This was followed by a 10×SSE wash and the blotted filter was baked at 80°C for 2 hours.

5.5.3.2 Hybridisation

Individual filters were pre-hybridised in a solution containing 5ml 5×SSPE, 5×Denhardt's, 0.5% SDS, and 100μg/ml salmon sperm DNA (from salmon testes; Sigma Cpy, Cat. No.: D1626) for 2-4 hours at 55°C with shaking. Hybridisation chambers were preheated at 55°C. ASO probes were used at specific activities of 1 and $2x10^6$ $cpm.pmol^{-1}$ and boiled for 2-3 minutes just before use. The boiled probe was added to 5ml hybridisation buffer (pre-hybridisation buffer containing 0.01M EDTA)

(probe solution). The filters were placed in appropriately labelled chambers and overlayed with the probe solution. The chambers were clamped tight and incubated overnight at 55°C with vigorous shaking. The filters were removed and washed twice at room temperature for 30mins in 2×SSPE, 0.1% SDS. This was followed by a high stringency wash at 55°C for 10 mins. The filters were autoradiographed overnight at -80°C using a single intensifying screen.

5.6 Southern Blot Analysis

5.6.1 Sample Analysis

5.6.1.1 Restriction Enzyme Digests

Usually 5μg of sample DNA were mixed with 10× enzyme buffer (A, B. L, M OR H) and 40 units of restriction enzyme in a total volume of 20μl. Samples were briefly mixed, centrifuged and incubated overnight at 37°C.

5.6.1.2 Agarose Gel Electrophoresis

1) Ordinary Electrophoresis

For the purpose of RFLP analysis, gels were generally caste in 200ml 1×TBE buffer. Gels usually contained 0.7 to 1.5% agarose (BM Ltd, Cat. No.: 1388 983); the powdered agarose were mixed with the buffer solution and the mixture was melted in a microwave oven for 5-6 mins. The solution was allowed to cool and the gel was caste and allowed to set at room temp. A few drops 20-50μl of ethidium bromide (10mg/ml, EtBr) may be added to the molten gel just before casting, to aid in visualization of the separated DNA fragments. Restriction enzyme digests were prepared as described, loaded into the wells and subjected to electrophoresis at room temperature overnight at a constant voltage of 55V. The gels may now be visualized under UV light.

2) Pulsed-Field Gel Electrophoresis

In order to separate large DNA fragments, 0.7% agarose gels were prepared as described. The restriction digests was mixed with loading dye and subjected to electrophoresis under a reverse pulse field (field-inversion) utilising a programmable switching/pulsing device (forward pulse setting ~ 150 milliseconds; reverse pulse setting ~ 50ms)(Hoeffer Instruments). The overall applied voltage for the 48 hour run was a constant voltage of 100V. Since the power dissipation may cause the buffer

temperature to rise, gels were generally run at 15°C. The gel was stained with EtBr for visualisation after the electrophoretic run.

5.6.2 Protocol

5.6.2.1 Multiprime Labelling of Probe (Random-primer)(Amersham RPN. 160 Y/Z)

DNA was diluted in ddH_20 and denatured at 95-100°C for 2 min and immediately placed on ice. 10μl of buffer solution (containing dATP, dGTP and dTTP in TRIS.HCl, pH7.8, $MgCl_2$ and β-mercaptoethanol), 5μl of random primer solution (containing random hexanucleotides in BSA solution) and 5μl ^{32}P-dCTP (Spec. Act.: 15 - 30 Ci/mmol)(Amersham; Cat. No.: TRK 625) were mixed with the above DNA solution. Two units of DNA polymerase were mixed with the reaction mixture and the total volume was made up to 50μl. The solution was mixed briefly, centrifuged and incubated at 37°C for 60mins or overnight at room temperature. The radiolabelled DNA was purified and quantitated as described (Spun Column chromatography).

5.6.2.2 Blotting Procedure

After electrophoresis of the DNA fragments on agarose gels, the DNA was denatured *in situ* and transferred from the gel to a solid membrane support (nitrocellulose paper or nylon filters) as follows: The gel was placed in a tray, overlayed with denaturation solution (0.5M NaOH/1.5M NaCl) and incubated at room temperature for 1 to 2 hours with gentle shaking. The denaturation solution was poured off and replaced with a renaturation solution (0.5M TRIS in 20×SSC) for a few mins. The excess gel was removed by trimming the edges with a clean blade and the gel was placed on a wick of 3MM Whatman filter paper that had been placed on a glass support over a tray; the wick extends over the glass support and into the 20×SSC buffer contained in the tray. The exposed areas surrounding the gel were covered in saran wrap to prevent any evaporation. A piece of membrane filter of appropriate size was cut, presoaked in 20×SSC buffer and placed on top of the gel matrix. Three to five pieces of 3MM Whatman filter paper were cut a few millimeters smaller than the gel, soaked in 20×SSC buffer and placed on top of the membrane. In turn, several layers of ordinary tissue paper were placed on top of the filter paper. A heavy weight placed on top of this pyramid completes the blotting sandwich. After the overnight DNA transfer, the blotted membrane filters were cut into appropriate sizes, placed between two sheets of 3MM Whatman filter paper and baked dry at 80°C for 2 hours.

5.6.2.3 Pre-hybridisation, Hybridisation, Post-hybridisation

The filters were placed in a container or sealed bag, overlayed with 100ml of prehybridisation solution (3×SSC buffer) and incubated with shaking at 65°C for 30 mins. [Note: Filters are soaked individually into buffer solution to allow homogeneous wetting.]

The filters were transferred to a preheated container containing 3×SSC, 10×Denhardt's solution and shaken for 30 mins at 65°C. In the meanwhile, 100ml of hybridisation solution (3×SSC/10×Denhardt's/0.1% SDS) was preheated at 65°C and boiled salmon-sperm DNA (1μg/ml) was added. The filters were transferred to the above solution and incubated at 65°C for 60min. The filters were transferred to the hybridisation chambers such that the solution just cover the filters. The radiolabelled probe was denatured by heating at 95°C for 3 mins, added to the hybridisation solution and incubation was carried out overnight at 65°C with shaking.

For the post-hybridisation step, the filters were transferred to a 3×SSC, 0.1% SDS solution and incubated for 10 mins at 65°C . The filters were transferred to a 1×SSC, 0.1% SDS solution and the incubation was continued for 45min at 65°C. The filters were then transferred to a 0.2×SSC, 0.1% SDS solution and incubated for a further 45min at 65°C. The excess probe was removed by washing the filters with 2×SSC and transferred to a plastic bag. The bag was sealed and the filters were set up for autoradiography as before.

5.7 Large Scale Isolation of Plasmid DNA

5.7.1 Materials and Reagents

Sample: pLPL35 - contains full-length LPL gene copy cloned into the single ECORI site of the pUC19 plasmid vector. Stored in slope agar cultures.

5.7.2 Preparation of LPL DNA Probe

5.7.2.1 Isolation of Plasmid DNA

A stock culture was plated out on agar plates and incubated at 37°C overnight. Single colonies were picked and two 1 liter LB broth cultures were inoculated and incubated at 37°C with gentle shaking. Ampicillin (final concentration ~ 25μg/ml) was added to each culture and cell growth was monitored

by measuring the turbidity (OD_{600}). At OD_{600} = 0.5, chloramphenicol (final conc ~ 170μglml) was added and incubation was continued overnight at 37°C with gentle shaking. The cells were pelleted by centrifugation at 8000 rpm (4°C/10min) and the supernatant discarded. Ten milliliters of solution I (25mM TRIS.HCl, pH 8.0; 50mM glucose; 10mM EDTA) was added to each 500ml cell pellet. The suspension was thoroughly mixed, followed by the addition of lysozyme (final conc.~ 5mg/ml) and incubation at room tempemperature for 5 min. Twenty milliters of solution II (0.2N NaOH/1% SDS) was added to each suspension and mixed until the solution became gelatinous and stood on ice for 10 min. Fifteen milliters of 5M potassium acetate was added to the above suspensions, mixed and stood on ice for 10 mins. The residue was pelleted by centrifugation at 10000 rpm for 10 mins at 4°C. The supernatant was transferred to a fresh centrifuge tube and centrifuged at 20000 rpm for 20 mins at 4°C. To each supernatant was added 0.6 volumes of isopropanol, which was thoroughly mixed and incubated at room temperature for 15 min. This solution was centrifuged at 10000 rpm for 30 min at room temperature. The supernatant was discarded and the white precipitate was washed with 70% ethanol by centrifugation for 10 mins at room temperature. The pellet was dried and resuspended in 8ml 1×TE (pH8.0).

5.7.2.2 Purification by Eqiulibrium-Cesium Chloride-Ethidium Bromide Centrifugation

One gram of solid CsCl was added per ml of plasmid DNA. The mixture was allowed to dissolve, and 0.8ml of EtBr (10mg/ml) was added for every 10ml of the CsCl/DNA solution. The final density of the solution was 1.55g/ml and the concentration of EtBr was approximately 600μg/ml. The solution was centrifuged at 45000 rpm for 36 hours at 20°C (Beckman T-50 rotor, Beckman Ultracentrifuge). The rotor was carefully removed so as to ensure that the separated layers, consisting of linear DNA, nicked circular DNA (upper band) and the closed circular plasmid DNA (lower band), was not disturbed. A hypodermic needle was inserted just below the meniscus of the lower band and the closed circular plasmid DNA was collected. This solution was transferred to an eppendorf tube and an equal volume of isopropanol/CsCl solution (1:1) was added (EtBr dissolves in isopropanol). This solution was centrifuged at 3000 rpm for 3 min at room temperature to separate the isopropanol layer. This step was repeated 3 times or until the EtBr hue had disappeared. The aqueous phase was dialysed against several changes of 1×TE to remove the CsCl.

5.7.2.3 Precipitation of Plasmid DNA

The dialysate was transferred to a 25ml siliconised, polycarbonate, centrifuge tube. A 1/20th volume of 4M NaCl was added to the dialysate which was mixed with 2 volumes of ice-cold ethanol. The mixture was vortexed thoroughly, stoppered and incubated at -20°C for 2-3 hours. The mixture was then centrifuged at 7000rpm for 30 mins at 10°C. The supernatant was discarded and the white DNA pellet was reconstituted in approximately 0.5 to 1.0 ml ddH_2O. Again, a 1/20th volume of 4M NaCl and 2 volumes of ice-cold ethanol was added to the DNA suspension. This mixture was incubated overnight at -20°C and centrifuged at 12000 rpm for 15min. The supernatant was discarded and the pellet washed with 70% ethanol. The pellet was dried and reconstituted in 0.5ml ddH_2O. The concentration was assessed by spectrophotometric analysis at wavelengths of 260 and 280nm. Purity was assessed by electrophoresis on 0.7 to 1% EtBr-stained agarose gels.

5.7.2.4 Recovery and Electroelution of insert DNA (probe)

Of the order of 100μg of plasmid DNA was digested overnight at 37°C with 100 units of EcoRI enzyme (10U/μl)(BM, Cat. No.: 703 737)(buffer H). Aliquotes of DNA restriction digests were electrophoresed on 0.7 to 1% agarose gels at 80V for 30 to 60mins. The EtBr stained gel was visualised on a transilluminator box under UV light and blotted dry. Using a sterile scalpel blade, a trough (1-2mm wide) was cut in front of the band of interest. The gel was again set up for electrophoresis, ensuring that the electrophoresis buffer (1xTBE) was just below the gel surface. The trough was filled with 1xTBE buffer and electrophoresis was continued and the progress of the DNA band of interest was monitored by visualisation under UV light. As the DNA band moved into the trough, the solution was collected and transferred to an eppendorf tube. The trough was refilled and the band collection continued until all of the DNA was recovered. The aliquotes were pooled and the DNA precipitated by adding 0.5 volumes of ice-cold 7.5M NH_4Ac and 2.5 volumes of ice-cold ethanol. This mixture was incubated for 1 to 2 hours at -70°C and centrifuged at 12000rpm for 10-15mins at room temperature. The supernatant was discarded and the pellet washed with 70% ethanol. The pellet was air-dried and reconstituted in 1xTE buffer. Quantitation of DNA was accomplished by visual comparison against a known standard, BioMarker™ Low (Bio Ventures, Inc, Lot. No.: 021594) under equivalent electrophoretic conditions as described above. Insert DNA were prepared as 30ng/μl aliquotes and stored at -70°C.

5.8 SSCP Analysis

5.8.1 Materials and Reagents

1) Stock plate glue solution: Dilute 50μl of γ-methacryloxypropyl trimethoxysilane (Sigma Cpy, Cat. No.: M6514) in 100ml absolute ethanol.
2) Mutation Detection Enhancement gel (MDE™)(2 × Concentrate)(Hydrolink, AT Biochem Inc., Cat. No. 500-MDP).
3) Sequencing Apparatus (BRL, Life Technologies Inc., Model S2; Cat. Series 1105)

5.8.2 Protocol

5.8.2.1 Preparation of Plates

The surfaces of both glass plates were thoroughly washed with 70% ethanol. Three to five milliters of siliconising agent (dimethyl dichlorosilane solution; BDH, Cat No: L186291 426) were spread over the long plate with a tissue and allowed to dry. Three milliters of the diluted plate glue containing 90μl 10% acetic acid was applied to the short plate and rubbed vigorously with a tissue, covering the entire surface of the plate. The short plate was allowed to dry for a few mins, bathed in 100% ethanol and re-rubbed to remove the excess glue.

5.8.2.2 Preparation of the SSCP gel

A SSCP gel was prepared in a total volume of 60ml as follows:

	VOL	0.55×	0.5×	0.35×	0.25×
MDE (2×)	ml	16.5	15	10.5	7.5
GLYCEROL	ml	6	6	6	6
5xTBE	ml	6	6	6	6
10% AMPS	μl	0.24	0.24	0.24	0.24
TEMED	μl	24	24	24	24
ddH_2O	ml	31.36	32.86	37.36	40.36

The gel was poured and allowed to polymerise for 60mins.

Note: A well-forming comb was used and clamped tightly in place until polymerisation was complete.

5.8.2.3 Sample preparation and analysis

To each 100-200ng DNA sample was added 1μl of loading dye (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were heated at 80°C for 2-3mins and loaded directly into the wells. Electrophoresis was performed at 5 to 10V overnight at room temperature or at 4°C.

5.8.2.4 Detection and Visualisation

Silver staining was the method of choice:

The plates were disassembled and the gel-bound plate was immersed in ddH_2O and gently shaken for 60min, with several changes (~5 times) of water. The water was discarded, replaced with the staining solution (ammoniacal solution; 0.1% $AgNO_3$, 0.1% NaOH, 0.4%(v/v) NH_3) and the gel was soaked for 20 min without shaking. This solution was decanted and the gel was washed for 30sec with ddH_2O. The gel was now immersed in the developing solution (0.005% citric acid, 0.02% formaldehyde) and allowed to develop for approximately 5min or until the bands appeared. The reaction was stopped by washing with ddH_2O.

5.8.2.5 Elution and PCR of DNA from SSCP Gels

The bands of interest were excised by cutting with a sharp scalpel blade. The gel slice was transferred to a 1.5ml sterile, eppendorf tube containing 50μl 1×TE buffer. The DNA was eluted by incubating with shaking for 1-2 hours at 37°C. The suspension was centrifuged at 12000g for 10 min to pellet the gel debris. The supernatant was collected and stored at -20°C until use.

PCR-amplification was carried out as follows: 1/10th of the eluted DNA (template) was heated at 95°C for 5min in the presence of the two primer species. The mixture was immediately placed on ice to allow annealing to take place. The master reation mix contained; 10mM TRIS.HCl, pH 9, 50mM KCl, 0.1% Triton X-100 (V/V); 1.5mM $MgCl_2$, 0.2mM dNTP stock solution (contains 4 dNTP's). The annealing and master mixes were combined and the total volume was made up to 24μl. The mixture was overlayed with mineral oil and heat denatured at 95°C. The Taq polymerase was added at 1 unit/reaction tube at 94°C, then the temperature cycling process was started; 94°C at 1 min, 50°C at 1 min, 72°C at 1 min. This program was repeated for 30 cycles, then the PCR samples was heat denatured at 94°C, a further 1 unit of Taq polymerase was added at 94°C and the above cycling program was repeated for a further 10 to 15 cycles. A final 5 min-extension step at 72°C completed the amplification procedure. PCR amplified products were visualised on 1% EtBr-stained agarose gels.

5.9 PCR Amplification of Histopathological Slide Extract DNA

A sample mix was prepared as follows: A dilution series of slide extract DNA were prepared such that the final concentrations were 1/100, 1/50, 1/25, 1/10 and neat. Primers, LPL23 (forward) and LPL24 (reverse)(see Primer Design) were mixed with the DNA sample at a concentration of 30pmol. The mixture was heated at 95°C for 5 min and then cooled on ice.

A master reaction mix was prepared as follows: 5μl of 10×concentrated Taq polymerase buffer, 1.5mM $MgCl_2$ and 0.2mM dNTP solution (contains 4 dNTP's) were mixed. The sample mix and master reaction mix were combined and the volume was made up to 49μl with ddH_2O. The mixture was overlayed with mineral oil and the sample heated to 94°C. Two units of Taq polymerase were added and the temperature cycling program was started: 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, for 30 cycles. The sample again heat denatured at 94°C and again 2 units of Taq polymerase were added and the above temperature cycling program was continued for a further 10 cycles. Aliqoutes of products were analyzed on 1% agarose gels and visualised by EtBr staining under UV light.

5.10 PCR Restriction Analysis of the C418Y Mutation

5.10.1 Enzymatic Amplification

The sample mix was prepared as follows: To 0.5μg of sample DNA was added 30pmol of each primer, LPL24 (reverse) and 418MIS (forward)(see Primer Design). This solution was heat denatured at 94°C for 5 mins and snap cooled on ice.

A master reaction mix was prepared as follows: 5μl of 10×concentrated Taq polymerase buffer, 1.5mM $MgCl_2$, 0.2mM dNTP's solution (contains 4 dNTP's) and 2 units of Taq polymerase were mixed. The sample mix and master mix were combined and made up to 50μl with ddH_2O. The mixture was overlayed with mineral oil and the temperature cycling program was commenced: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, for 30 cycles. Amplification products were analysed on 1% EtBr-stained agarose gels and visualised under UV light.

5.10.2 PvuII Restriction Analysis

Reagents: Restriction Buffer M (BM, Cat. No.: 1417 983)

TRIS.HCl, 20mM; NaCl, 100mM; EDTA, 1mM; β-mercaptoethanol, 10mM; Triton X-100, 0.01% (V/V); glycerol, 50% (V/V); pH 7.7.

To 1/25th of the PCR product (~ 100ng) was added 1μl of 10×concentrated buffer M and 5 units of PvuII Restriction enzyme (BM, Cat. No.: 642 690) in a total volume of 10μl. The solution was vortexed, briefly centrifuged and incubated for two hours at 37°C. The restriction digests were analysed on 10% polyacrylamide gels and detection was accomplished by a silver staining protocol as described.

5.11 Ficoll-Histopaque Gradient Separation

Twenty milliters of fresh whole blood were collected in EDTA tubes to prevent coagulation. The bloods were immediately centrifuged at 4°C for 20min at 1500 rpm. The mononuclear cell layer (buffy coat) was recovered by aspirating with a clean pasteur pipette and transferred to a clean tube. The cell suspension was diluted in an equal volume of Dullbecco's phosphate buffered saline solution (Highveld Biologicals, Pty, Ltd, Cat. No.: 1686-2) containing 1mg/ml EDTA. Two milliters of Ficoll-Histopaque R-1077 (Sigma, Cpy., Cat. No.: 1077-1) solution were pipetted into a clean centrifuge tube. The cell/PBS/EDTA suspension was carefully layered on top of the Ficoll gradient solution and centrifuged at 2500 rpm for 10min at room temperature. The mononuclear cells which bands as an opaque layer of cells at the interphase were recovered and mixed with an equal volume of PBS/EDTA solution. The Ficoll gradient separation was repeated until all traces of red blood cells were removed. The erythrocyte-free monoclear band was aspirated off, transferred to a clean centrifuge tube and pelleted at 2500 rpm for 10min at room temperature. The supernatant was discarded and the pelleted cells are now ready for processing. Aliquotes of cells were taken for the assessment of cell number.

Note: Lipemic plasma were treated as above, except where the buffy coat layer was not visible, approximately 1ml of plasma was left above the cell pellet following aspiration of the plasma, such that the cell layer is not disturbed.

5.12 Mononuclear Cell Culture

5.12.1 Preparation of Autologous serum

5.12.1.1 Lipemic serum:

Fifty milliters of whole blood from the subject was collected into a 50ml evergreen tube and allowed to clot. The clotted serum was centrifuged at 20000 rpm for 60min at 4°C using polycarbonate centrifuge tubes. The lipids band as a fat cake on top of a clear infranatant. The bottom of the tube was punctured with a hypodermic needle and the serum infranatant was collected and transferred to a clean tube. This serum was heat inactivated at 56°C for 30min and filter sterilised.

5.12.1.2 Non-lipemic serum:

A 50ml blood specimen was collected as a clotted specimen and centrifuged at 3000 rpm for 15-30 min. The serum component was aspirated off, transferred to a fresh tube, heat inactivated and filter sterilised as above.

5.12.2 Preparation of Growth Media

To 90ml of Dulbecco's modified eagle medium (DMEM; Highveld Biological Ltd., Cat. No.: 1299-3) or 90ml RPMI1640 medium (+L- glutamine)(Highveld Biological Ltd., Cat. No.: L07) was added 10ml of autologous serum (as prepared above), 50μl penicillin [100U/ml; Labethica (Ltd)] and 50μl streptomycin [166.7mg/ml; Novo Nordisk (Ltd)]. This mixture may be stored at 4°C until use, but should be warmed to room temperature before use.

5.12.3 Cell Culture

The freshly isolated mononuclear cell pellet was added to 10ml growth media and thoroughly mixed. The cells were plated out at 2-3x10^7 cells per dish (35mm petri dish, Falcon Ltd). An equal volume of fresh growth medium was added to the culture dish and incubated at 37°C (5% CO_2/95% humidity) for 30 min. This allowed for cells to adhere to the dish. Half of the culture medium was aspirated off and replaced with an equal volume of fresh growth medium. This would ensure that enough growth factors remain behind. The cell density was checked under a microscope. The cells were cultured for 8-10 days with growth media supplementation carried out every 2 days until harvesting.

5.12.4 Harvesting of Cells

The growth medium was aspirated off and the monolayers washed three times (2ml) with ice-cold PBS. Approximately 200μl of ice-cold PBS were added to the monolayer of cells and placed on ice for a few mins to allow for diffusion of the solution. The cell sheet was scraped off using a rubber policeman. The cell suspension was collected with a wide-mouthed pipette and transferred to a clean, sterile eppendorf tube. A further 200μl of ice cold PBS were added to the plate and the procedure repeated until no cells were visible under the microscope. The cells were pelleted at 1500 rpm for 5min at 4°C. The supernatant was aspirated off; the cells were snap frozen and stored at -70°C.

OR

The growth medium was aspirated off and the cell monolayer washed three times (2ml) with ice cold PBS. The excess PBS was aspirated off and the plates were stored at -70°C until use.

5.13 Total RNA Isolation

(Ref: Chomczynski and Sacchi; 1987)

5.13.1 Materials and Reagents

All solutions, where possible were prepared in 0.1% DEPC-treated H_2O. Glassware and spatulas were baked overnight at 180°C . These treatments are designed to destroy any RNAases that may be present.

5.13.2 Protocol

Frozen cells were allowed to thaw on ice and 1ml solution D (4M guanidium thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% sarcosyl, 0.1M β-mercaptoethanol) was added to the tissue culture dish. Using a rubber policeman the monolayer cells were scraped, dislodged and transfered to a fresh tube containing 4ml of solution D or freshly isolated mononuclear cells were mixed directly with 5ml of solution D. These suspensions were homogenised on ice in a glass-teflon homogeniser (approximately 10 strokes). The homogenate was poured into a polypropylene tube. Five milliliters of phenol (TRIS-EDTA saturated), 1ml of chloroform: isoamyl alcohol (49:1) and 1ml 2M sodium acetate, pH 4.5 were added sequentially to the homogenate which was vigorously agitated for 60secs and incubated on ice for 15min. This suspension was centrifuged at 7000 rpm for 15min at 4°C. The upper aqueous phase was transfered to a fresh polypropylene tube and re-extracted as above. Again

the suspension was centrifuged as before and the aqueous phase mixed with an equal volume of isopropanol and placed for 1 hour or overnight at -20°C. The mixture was centrifuged at 7000 rpm for 15mins at 4°C, to pellet the RNA. The excess supernatant was drained off and the pellet was reconstituted in 0.3ml solution D (without β-mercaptoethanol) and transferred to an eppendorf tube. An equal volume of isopropanol was added and the mixture was placed at -20°C for 1 hour. The mixture was centrifuged at 10000rpm for 10min at 4°C; the pellet was washed with 70% ethanol and briefly dried under vacuum. The dried RNA pellet was dissolved in 20μl DEPC-treated H_2O. Aliquotes may now be subjected to spectrophotometric (OD_{260} and 280) and electrophoretic analysis to assess RNA concentration, purity and integrity.

5.14 Electrophoretic Analysis of RNA

5.14.1 Materials and Reagents

1) 10× MOPS/EDTA, pH 7.0

41.86g 3-(N-Morpholino) Propanesulfonic Acid (0.2M)(MOPS; Sigma Cpy, Cat. No.: M-8899), 40mls 2M sodium acetate (50mM)(NaAc; Merck, Cat. No.: 6264.0500) and 3.72g EDTA (10mM) were dissolved in 1 liter of DEPC-treated H_2O and the pH was adjusted to 7.0 with approximately 30ml 10N NaOH. The solution was autoclaved at 15lbs/sq. inch for 30min.

2) Deionized Formamide

One hundred milliters of formamide solution (Merck, Cat. No.: 9684.1000) was mixed and stirred with 10g of DOWEX TMD-8 mixed bed resin (Sigma Cpy, Cat. No.: M 8157) at room temperature for 60min. The solution was filtered twice through 3MM Whatman No.1 filter paper and stored as 1ml aliquotes under nitrogen at -70°C.

3) Electrophoresis Sample Buffer

The sample buffer was prepared as follows: 0.75ml of deionized formamide, 0.15ml 10×MOPS/EDTA buffer, 0.24ml formaldehyde, 0.1% DEPC-ddH_2O, 0.1ml glycerol and 0.08ml 10% (W/V) bromophenol blue was mixed and stored in 1ml aliquotes at -20°C.

4) Electrophoresis buffer

1×MOPS/EDTA buffer.

5.14.2 Sample Preparation

Five micrograms of the total RNA preparation were added to 25μl electrophoresis sample buffer and the solution was made up to 30μl with DEPC-treated ddH_2O. The samples were heated at 65°C for 15min; they were briefly centrifuged and 2μl of a 1mg/ml EtBr solution were added. The samples were thoroughly mixed and immediately loaded onto the gel .

5.14.3 Preparation of 1% agarose/1.85% formaldehyde gel

Two grams of agarose (Bio-rad Laboratories, Cat. No.: 162-0100)) were weighed and transferred to a RNAse-free flask. Twenty milliters 10×MOPS/EDTA buffer were added and the mixture was made up to 200ml with 0.1% DEPC-treated ddH_2O. The agarose solution was melted and allowed to cool to approximately 50°C before adding 10ml of 37% formaldehyde (This is carried out in a fume-hood). The gel was poured and allowed to set for 60min at room temperature.

Samples were electrophoresed at 25V for 18 hours at room temperature. The gel was visualised under UV.

5.15 cDNA Synthesis

5.15.1 Material and Methods

1) 5M dNTP stock

A 5M dNTP stock solution was prepared as follows:

dNTP	Source/Cat. No.	mg/ml	mM
dATP	BM/103 977	14.73	20.24
dGTP	BM/104 094	13.78	21.14
dTTP	BM/104 272	14.26	20.50
dCTP	BM/104 035	12.78	20.34

Each dNTP solution was adjusted to pH 7. 250μl of each dNTP solution was mixed to give a final working stock of 5mM for each dNTP.

2) 5×RT buffer

The following solution was prepared: 250mM TRIS buffer, pH 8.2; 35mM $MgCl_2$; 200mM KCl; 50mM DTT; 0.5mg/ml bovine serum albumin (BSA stock, 20mg/ml; BM, Cat. No.: 652 237) was prepared in DEPC-treated ddH_2O. 1ml quantities were aliquoted and stored at -20'C.

5.15.2 Protocol

First strand cDNA synthesis was accomplished by preparing a sample mixture containing 10μg of total RNA, 0.5μg oligo dT (BM, Cat. No: 814270), 0.5μg random hexamer primers (BM; Cat. No: 1034731) and 1μl dimethyl sulfoxide (DMSO)(Burdick and Jackson Inc; Cat No. 081) in a total volume of 10μl with DEPC-treated ddH_2O. The mixture was vortexed, heated at 65°C for 3 min and immediately placed on ice, to allow for primer annealling. A reaction mixture was prepared by adding the following sequentially: 2×RT buffer; 5mM dNTP stock (final conc. ~ 0.5mM), 40 units rRNasin (RNase Inhibitor)(Promega Corp, Cat. No: N251/1-4), 1/20th DMSO, in a total volume of 40μl with DEPC-treated ddH_2O. The reaction and sample mixtures were combined and thoroughly mixed. Two hundred units of the Murine-Molony Leukemia Virus Reverse Transcriptase (M-MLUV RT)(Promega Corp; Cat. No.: M530/1,2) was added, followed by incubation at 37°C for 60 min to facilitate linear amplification. To improve the transcription efficiency, the transcribed product was denatured at 95°C for 3 mins, followed by the addition of a further 200 units M-MLUV RT enzyme and 40 units of RNasin; the mixture was thoroughly mixed and again incubated at 37°C for a further 60mins. This procedure was repeated three times and the samples stored at -20°C until use.

5.16 PCR Amplification of LPLcDNA Products

5.16.1 PCR Protocol

The polymerase chain reaction was carried out as follows: To 1/25th cDNA product was added 30pmol of each LPL primer (LPL fragment 1-8; forward primer, LPLcDNA8; reverse primer, LPLcDNA1) or (LPL fragment 6-7; forward primer, LPLcDNA6; reverse primer, LPLcDNA7). This mixture was heat denatured at 95°C for 2-3min and snap cooled on ice to allow for primer-template annealing. A master mix was prepared containing 5μl of a 10 × concentrated Taq polymerase buffer (100mM TRIS buffer, pH 8.5, 500mM KCl, 0.1% gelatin)(Promega; Cat No: PL M190A); 2mM $MgCl_2$; 0.2mM dNTP stock (containing 4 dNTP's) and 2 units of Taq Polymerase. The total volume was adjusted to 50μl with ddH_2O. The samples were overlayed with mineral oil. This was followed

by the temperature cycling process performed on a Hybaid thermal cycler with the following program: 1 min at 94°C, 1 min at 56°C (LPL fragment 1-8) or 1 min at 60°C (LPL fragment 6-7) and 2 min at 72°C, over 30 cycles. The final cycle was followed by a 5 min extension step at 72°C.

5.16.2 Product Analysis

Aliquotes of PCR products were analysed directly by electrophoresis on 1% agarose or 10% polyacrylamide gels and visualised by EtBr staining and photographed under UV light.

5.16.3 Purification of PCR products

The Qiaex DNA Gel Extraction Kit (Qiagen; Cat. No.: QXGE 4111) was used for the purification of the amplified PCR products as follows :

Following agarose gel electrophoresis, a clean scalpel blade was used to excise the gel slice containing the DNA fragment of interest. The gel slice was transferred to an eppendorf tube and 0.3ml of the QX1 buffer was added per 100mg of gel. The solubilisation process was allowed to go to completion, followed by the addition of 10μl of QIAEX beads. The suspension was thoroughly mixed by inverting the tube several times and incubated for 10min at 50°C with intermittant mixing at 2min intervals. The sample was centrifuged for 30sec at 12000g in a bench top microcentrifuge. The supernatant was discarded and the pellet washed twice in 0.5ml QX2 buffer. This was followed by a second wash in 2 × 0.5ml QX3 buffer. After each wash the supernatant was discarded and the pellet retained. After the final wash the pellet was dried at room temperature. The DNA was generally eluted from the beads by reconstituting in 20μl of 1xTE buffer. After incubating the beads for several minutes the suspension was centrifuged at 12000g for 30sec; the supernatant was recovered and stored at -20°C until use. To ensure that no beads were carried over, a second reconstitution and centrifugation step was carried out. The yield of DNA was assessed by electrophoresis on agarose gels against a known standard (Biomarker™ Low, Bioventures Inc. Lot No: 021594).

5.17 Cloning of LPLcDNA Fragments

5.17.1 Materials and Reagents

The pT7BLUE T-Vector Kit (Novagen Inc.; Cat. No.: 69836-1) was used for the cloning of the PCR- amplified LPL fragments.

5.17.2 Ligation Reaction

A standard ligation reaction was carried out as follows : A 50ng or 0.3pmol of pT7BLUE T-Vector aliquote was mixed with 100ng of PCR-amplified product (0.2pmol) in a total volume of 10μl. This mixture was combined with the reaction mix which contained 10×Ligase buffer (200mM TRIS.HCl pH 7.6, 50mM $MgCl_2$); 0.5μl 100mM DTT and 0.5μl 10mM ATP. Two to three Weiss Units of T4 DNA ligase was added to the above mixture. The total volume was made up to 10μl with ddH_2O and the ligation reaction was allowed to proceed at 16°C overnight.

5.17.3 Transformation

The required number of Novablue competent cells was thawed on ice to ensure that the cells were evenly suspended. Twenty microliters of cells were pipetted into pre-chilled microfuge tubes on ice. One microliter of the ligation mix was added directly to the cells and gently stirred with the pipette tip. This mixture was incubated on ice for 30 mins. The tubes were heated at 42°C for 40 secs without shaking and immediately placed on ice for 2 mins. Eighty microliters of room temperature SOC medium was then added to each tube and vigorously shaken at 200-250 rpm for 60 mins at 37°C.

5.17.4 Preparation of Agar Plates and Colony Plating

LB agar plates containing 75μg/ml ampicillin and 15μg/ml tetracycline were prepared. Approximately 100μl of X-GAL (20mg/ml in dimethylformamide) and 40μl of IPTG (100mM) were spread over the plate surface and allowed to soak in for 2 hours at room temperature. Approximately 100μl of each transformation mix was plated out and the excess fluid allowed to evaporate, followed by overnight incubation of the plates at 37°C.

5.17.5 PCR Screening of Colonies

PCR amplification was the method of choice for screening large numbers of colonies for the presence of the insert DNA.

5.17.5.1 Bulk PCR Screening

Individual colonies were picked with a sterile pipette tip or loop. The bacteria was then transfered either onto a master plate (grid library) and then into an eppendorf tube containing 50μl ddH_2O. The cells were vortexed and the tube was placed in a boiling water bath for 5 mins to lyse the cells and to denature DNAses. The tube was centrifuged at 12000g for 1 min to pellet the cell debris. 1/50th of the cell lysate (supernatant) was subjected to PCR amplification under the following reaction conditions. The cell lysate was mixed with 30pmol of each primer and the mixture heat denatured at 95°C for 5 min. The tube was immediately placed on ice to allow primer-template annealling to take place. A master reaction mix was prepared containing 1×TRIS.HCl, pH 8.2; 2mM $MgCl_2$, 0.2mM dNTP stock (containing each of the four dNTP's) and 1 unit of Taq DNA polymerase. This mixture was added to the annealling mix and made up to 25μl with ddH_2O. The samples were overlayed with mineral oil and subjected to 30 cycles of : 94°C at 1 min, 56°C at 1 min; 72°C at 1 min. The final cycle was followed by a 5 min-extension step at 72°C. Products were analysed on 1% EtBr stained agarose gels and visualised and photographed under UV light.

5.17.5.2 1194T ARMS PCR Screening

The following sample mix was prepared : One tenth of the colony extract or 0.5μg of genomic DNA was mixed with 15pmol of each primer (LPLcDNA1 - forward primer; 194MUT - reverse primer) or (LPLcDNA8 - reverse primer; 194WT - forward primer)(Primer Design). This mixture was heat denatured at 95°C for 5 min and snap cooled on ice to allow for primer-template annealling.

A master reaction mix was prepared as follows : Mix 2.5μl 10×concentrated Taq polymerase buffer, 2mM $MgCl_2$, 0.2mM dNTP stock (contains 4 dNTP's) and 1 unit of Taq polymerase. The sample mix and reaction mix was combined and the total volume was adjusted to 25μl with ddH_2O. The solution was overlayed with a few drops of mineral oil. The temperature cycling program was performed in a Hybaid Thermal Cycler for 30 cycles of : 1 min at 94°C, 1 min at 62°C, 1 min at 72°C. The final cycle was followed by a 5 min extension step at 72°C. Aliquotes of PCR products were analysed directly by electrophoresis on 1% agarose gels and visualised by EtBr staining under UV light.

5.18 Colony Selection and Plasmid Purification

5.18.1 Protocol 1

Colonies of interest were picked and inoculated into 25ml terrific broth media (containing 75μg/ml ampicillin, 15μg/ml tetracycline). These cultures were incubated at 37°C for two days with shaking at 250rpm. The cells were pelleted by centrifugation at 3000 rpm for 10 to 15mins. The cell pellet was resuspended in 2.5ml cell lysis buffer (25mM TRIS.HCl, pH 8.0; 10mM EDTA, pH 8.0; 50mM glucose; containing 5mg/ml lysozyme), vortexed and incubated at room temperature for 5mins. Five milliters of 0.2N NaOH/1% SDS was added, inverted 2 to 3 times and placed on ice for 5mins. 3.75ml of 7.5M NH_4Ac, pH 7.6 was added to the above mixture which was inverted several times (a white flocculent debris observed) and then placed on ice for 5mins. The suspension was centrifuged at 8000 rpm for 15min at 4°C. The white pellet was washed with 70% EtOH, then air dried and resuspended in 1ml 1×TE buffer. RNase was added to a final concentration of 100μg/ml and the solution was incubated at 37°C for 30 to 60mins. The DNA was precipitated by adding 0.5 volumes of 7.5M NH_4Ac, pH 7.6 and 2.5 volumes of 100% EtOH to this solution followed by incubation at -70°C for 30mins. The DNA was pelleted by centrifugation at 12000g for 10 to 15mins. The pellet was washed with 70% EtOH, air dried and resuspended in 100μl 1×TE buffer. The concentration and purity of the plasmid DNA was assessed by spectrophotometric analysis and electrophoresis on 1% EtBr stained agarose gels.

5.18.2 Protocol 2

5.18.2.1 Materials and Reagents

1) Magic Minipreps™DNA Purification System (Promega Pty, Cat. No.: A7100).
2) All solutions were as indicated in the protocols.
3) Column Wash Solution : Dilute 1:1 in 50ml absolute ethanol.

5.18.2.2 Plasmid Minipreps

One to three ml of cells were pelleted by centrifugation. The cell pellet was resuspended in 0.2ml of Cell Resuspension Solution. This suspension was transferred to a microcentrifuge tube and 0.2ml of Cell Lysis Solution was added. The suspension was mixed by inverting the tube several times until the suspension cleared. 0.2ml of the Neutralization Solution was added and the suspension was centrifuged at 14000g for 5 min. The supernatant was transferred to a fresh eppendorf tube and 1ml of Magic Miniprep DNA purification resin was added and mixed by inverting the tube. A 3ml

disposable syringe barrel was attached to the luer-lok extension of each mini-column. The Resin/supernatant suspension was poured into the syringe barrel. The syringe plunger was inserted and the slurry was gently pushed through the mini-column. The column was washed with 2ml of Column Wash Solution. The column was transferred to an eppendorf tube and centrifuged for 20 secs to dry the resin. The column was transferred to a fresh eppendorf tube and 50μl of preheated ddH_2O or TE buffer (65-70°C) was applied to the column. The plasmid DNA was eluted by centrifugation of the column for 20 secs. The eluant was collected and store at -20°C. The yield and quality of the DNA preparations were assessed as before.

5.19 DNA Sequence Analysis

1) The Sequenase(R) Version 2.0 DNA Sequencing kit (United States Biochemical and Amersham, Cat. No.: 70770) was generally used. The protocols followed were as per instructions (see the accompanying step-by-step protocol booklet).

2) The PCR-based Sequencing Kits.
   a) Sequenase™ PCR + Product Sequencing Kit (USB and Amersham, Product No: 70170). See Step-by-Step protocols for instructions.
   b) fmol™ DNA Sequencing System (Promega; Cat No: Q4100). Instructions as per protocol booklet.

APPENDIX 1

PRIMER DESIGN

The oligonucleotides (primers) described below have been utilised at various stages in the experimental procedures. R - reverse primer, F - forward primer. The numbers in brackets denotes the primer length (number of bases).

A1) Primers for the amplification and sequencing of individual LPL exons.

a) Exon2:	LPL8	(F)	5'-CTCATATCCAATTTTTCCTT-3'	(20mer)
	LPL9	(R)	5'-CTCTTCCCCAAAGAGCCTCC-3'	(20mer)
b) Exon3:	LPL10	(F)	5'-AAGCTTGTGTCATCATCATCTTC-3'	(23mer)
	LPL11	(R)	5'-ATAAGTCTCCCTTCTCCCAGT-3'	(20mer)
c) Exon4:	LPL12	(F)	5'-GCAGAACTGTAAGCACCTTC-3'	(20mer)
	LPL13	(R)	5'-GACAGTCTTTTCACCTCTTA-3'	(20mer)
d) Exon5:	LPL57	(F)	5'-TACGGATCCCATGCGAATGTCATACGAATGG-3'	(31mer
	LPL58	(R)	5'-AGTGAATTCGAAGCTACTGAGTAGGACATTGGG-3'	(33mer)
e) Exon6:	LPL16	(F)	5'-TCTTGGTGTCTCTTTTTTACC-3'	(21mer)
	LPL17	(R)	5'-TTATTTACAACAGTCTCCAGC-3'	(21mer)
f) Exon7:	LPL19	(F)	5'-ACAGGATCCATGTTCGAATTTCC-3'	(23mer)
	LPL20	(R)	5'-GATGACCGCCCCCAGAGCTAC-3'	(21mer)
g) Exon8:	LPL21	(F)	5'-CCAAATTTATTGCTTTTTTGT-3'	(21mer)
	LPL22	(R)	5'-AAGGAAGAAAAATACATTTAATT-3'	(23mer)

h) Exon9: LPL23 (F) 5'-TATTCACATCCATTTTCTTC-3' (21mer)
LPL24 (R) 5'-GTCAGCTTTAGCCCAGAATG-3' (20mer)

A2) Primers used for amplification and sequencing of the LPLcDNA.

a) $LPLSP_2$ 5'-AGAGAACCAGACTCCAA-3' (17mer)
b) $LPLSP_3$ 5'-AGAGATTTTATCGACATCG-3' (19mer)
c) LPLcDNA3 5'-TGCAGATCTCAGATGCCCTACAAAGTCT-3' (28mer)
d) LPLcDNA4 5'-GATCACCTTTTTCTGAGTTCTCTCCT-3' (25mer)
e) LPL62 5'-CATTTACCCGAATGGAG-3' (17mer)
f) LPL65 5'-GTGGGACAGGATGTGGC-3' (17mer)
g) LPL66 5'-CATTGCAGGAAGTCTGA-3' (17mer)
h) PCR screening and sequencing primers for LPLcDNA (see Fig 18)
i) Fragment LPLcDNA1-8
LPLcDNA1: 5'-GCCGTACACTTAAGACACTTCTT-3' (23mer)
LPLcDNA8: 5'-CTAGGATCCATCTCTTGGGATACAGC-3' (27mer)
ii) Fragment LPLcDNA6-7
LPLcDNA6: 5'-TCAGTCGACTTGCTCAGCGCCAAAC-3' (25mer)
LPLcDNA7: 5'-TTGCTGCAGCGGTTCTTTCTACAA-3' (24mer)
i) Vector-specific primer for PCR screening and sequencing
U-20 5'-GGTTTTCCCAGTCACGACGT-3' (20mer)

A3) Allele-Specific Oligonuleotide (ASO) Primers for the detection of the 194 mutation.
LPL59 (ASO I): 5'-TCGAAGCA<u>T</u>TGGAATCC-3' (17mer)
LPL60 (ASOII): 5'-TCGAAGCA<u>C</u>TGGAATCC-3' (17mer)
The T→C nucleotide change is double underlined.

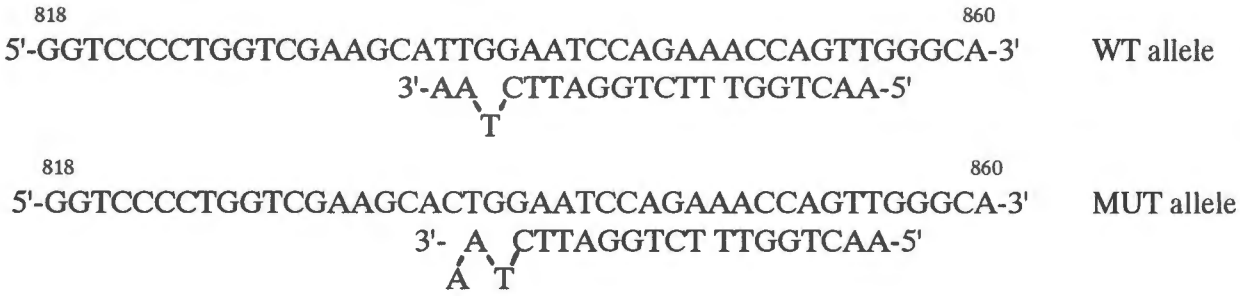
A4) "ARMS" PCR Primers for the detection of the I194T mutation.

194 WT Primer: 5'-AACTGGTTTCTGGATTCTAA-3' (20mer)

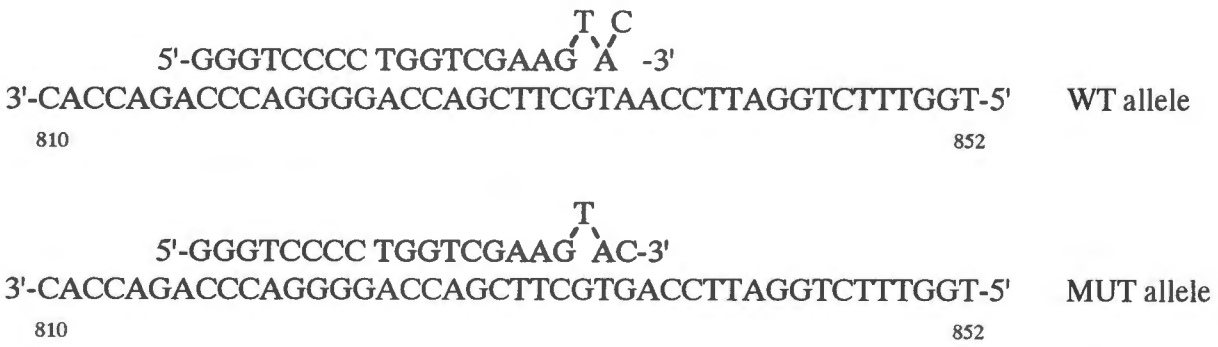
194 MUT Primer: 5'-GGGTCCCCTGGTCGAAGTAC-3' (20mer)

The nucleotide complementary to the normal (WT) or 194 mutant (MUT) sequence is indicated in bold. The deliberate base incorporated two nucleotides upstream from the 3'-terminal end of the primer is double underlined. Primers, 194 WT and 194 MUT were used in combination with primers, LPL57 and LPL58, respectively (see above). Nucleotide positions according to Wion *et. al.* (1987). See Fig 20 for the detection of the 194 mutant allele.

a) 194 Wild-Type Primer



b) 194 Mutant Primer



A5) Primers for the detection of the C418Y mutation

a) "ARMS" PCR Analysis

i) 418 WT Primer : 5'-cttccacagGGTGATCTT**A**TG-3' (21mer)

ii) 418 MUT Primer : 5'-cttccacagGGTGATCTT**A**TA-3' (21mer)

iii) The nucleotide base complementary to the normal (WT) and 418 mutant (MUT) sequence is indicated in bold. The deliberate mismatch introduced is double underlined. These primers span the intron8-exon9 boundary, with the intronic sequence shown in small letters. These primers were used in combination with the reverse primer, LPL24 (see above). Nucleotide positions according to Wion *et. al.* (1987).

iv) 418 Wild-Type Primer

```
                          A
5'-c ttccacagGGTGATCTT   TG-3'
3'-gtaggtaaaagaaggtgtcCCACTAGAAGACAAGATCCCTCTTTCACAGAGTA-5'  WT allele
                1497                                    1530

                          A  G
5'-cttccacagGGTGATCTT    T  -3'
3'-gtaggtaaaagaaggtgtcCCACTAGAAGATAAGATCCCTCTTTCACAGAGTA-5' MUTallele
                1497                                    1530
```

v) 418 Mutant Primer

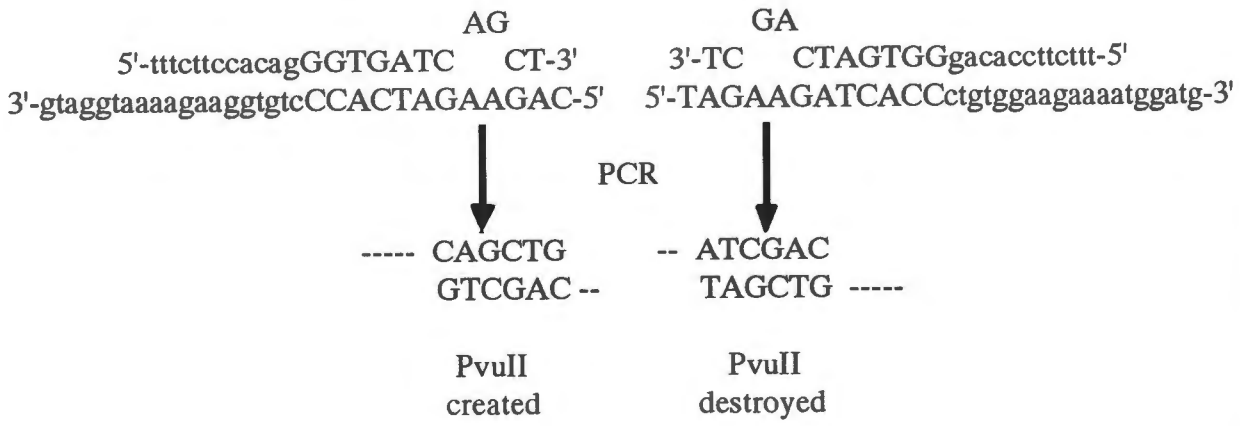
```
                          A  A
5'-cttccacagGGTGATCTT    T  -3'
3'-gtaggtaaaagaaggtgtcCCACTAGAAGACAAGATCCCTCTTTCACGAGTA-5'  WT  allele
                1497                                    1530

                          A
5'-cttccacagGGTGATCTT    TA-3'
3'-gtaggtaaaagaaggtgtcCCACTAGAAGATAAGATCCCTCTTTCACGAGTA-5' MUT allele
                1497                                    1530
```

b) PCR Restriction Analysis

i) 418 MIS Primer : 5'-tttcttccacagGGTGATC**AGCT**-3' (23mer)

The deliberate nucleotide bases introduced are double underlined. The nucleotide bases shown in bold completes the PvuII restriction site (-CAGCTG-) in the presence of the normal allele. The primer spans the intron8-exon9 boundary, with the intronic sequence shown in small letters. This primer is used in combination with LPL24 (see above). The sequence patterns shown below demonstrates how the PvuII restriction site is created or destroyed in the presence of the wild-type or mutant allele, respectively.

ii) Wild-Type Allele Mutant Allele



REFERENCES

1. Aalto-Setälä K., Fisher E.A., Chen X., Chajek-Shaul T., Hayek T., Zechner R., Walsh A., Ramakrishnan R., Ginsberg H.N. and Breslow J.L. (1992). **Mechanism of hypertriglyceridemia in human apolipoprotein (Apo) C-III transgenic mice: Diminished very low density lipoprotein fractional catabolic rate associated with increased apo C-III and reduced apo E on the particles.** J. Clin. Invest., 90; 1889 - 1900.

2. Ameis D., Stahnke G., Kobayashi J., McLean J., Lee G., Büscher M., Schotz M.C., Will H. (1990). **Isolation and characterisation of the human hepatic lipase gene.** J. Biol. Chem., 265 (12); 6552-6555.

3. Ameis D., Kobayashi J., Davis R.C., Ben-Zeev O., Malloy M.J., Kane J.P., Lee G., Wong H., Havel R.J. and Schotz M.C. (1991). **Familial chylomicronemia (Type I hyperlipoproteinemia) due to a single missense mutation in the lipoprotein lipase gene.** J. Clin. Invest., 87; 1165 - 1170.

4. An S.F. and Fleming K.A. (1991). **Removal of inhibitor(s) of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues.** J. Clin. Pathol., 44; 924 - 927.

5. Anguita J., Rodríguez Aparicio L.B. and Naharro G. (1993). **Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate.** Applied and Enviromental Microbiology, 59 (8); 2411 - 2417.

6. Aoyama, S., Yoshida, N. and Inouye, S. (1988). **Cloning, sequencing and expression of the lipase gene from Pseudomonas fragi IFO-12049 in E. Coli.** FEBS *Lett.*, 242; 36-40.

7. Ashby P., Bennett D.P., Spencer I.M. and Robinson D.S. (1978). **Post-translational regulation of lipoprotein lipase activity in adipose tissue.** Biochem J., 176; 865 - 872.

8. Auwerx J.H., Babirak S.P., Hokanson J.E., Stahnke G., Will H., Deeb S.S., Brunzell J.D. (1990). **Coexistence of abnormalities of hepatic lipase and lipoprotein lipase in a large family.** American J. Human Genetics. 46; 470-477.

9. Bagby G.J. and Pekala P.H. (1987). **Chapter 10: Lipoprotein lipase in trauma and sepsis;** pg. 245 - 277: *In*, Lipoprotein Lipase, J. Borensztayn (eds), Evener Publishers Inc., Chicago.

10. Baggio G., Manzato E., Gabelli C., Fellin R., Martini S., Baldo Enzi G., Veriato F., Baiocchi M.R., Sprecher D.L., Kashyap M.L., Brewer H.B. and Crepaldi G. (1986). **Apolipoprotein C-II Deficiency Syndrome: Clinical features, lipoprotein characterisation, lipase activity and correction of hypertriglyceridemia after apolipoprotein C-II administration in two affected patients.** J. Clin. Invest., 77; 520 - 527.

11. Barbirak S.P., Iveruis P-H., Fujimoto W.Y. and Brunzell J.D. (1989). **Detection and characterisation of the heterozygote state for lipoprotein lipase deficiency.**

Arteriosclerosis, 9; 326 - 334.

12. Beaumont J.L., Carlson L.A., Cooper G.R., Fejfar Z., Fredrickson D.S. and Strasser T. (1970). **Classification of hyperlipidaemias and hyperlipoproteinaemias.** Bulletin of W.H.O., 43; 891-908.

13. Beg O.U., Meng M.S., Skarlatos S.I., Previato L, Brunzell J.D., Brewer H.B. Jr., Fojo S.S. (1990). **Lipoprotein lipase$_{Bethesda}$: A single amino acid substitution (Ala-176→Thr) leads to abnormal heparin binding and loss of enzymic activity.** Proc. Natl. Acad. Sci. USA., 87; 3474 - 3478.

14. Beil F.U., Fojo S.S., Brewer H.B. Jr., Greten H. and Beisiegel U. (1992). **Apolipoprotein C-II deficiency syndrome due to apoC-II$_{Hamburg}$: Clinical and biochemical features and *Hph*I restriction enzyme polymorphism.** Eur. J. Clin. Invest., 22; 88 - 95.

15. Beisiegel U., Weber W. and Bengtsson-Olivecrona G. (1991). **Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein.** Proc. Natl. Acad. Sci. USA., 88; 8342 - 8346.

16. Bengtsson G. and Olivecrona T. (1980). **Interaction of lipoprotein lipase with native and modified heparin-like polysaccharides.** Biochem J., 189; 625 - 633.

17. Bengtsson G. and Olivecrona T. (1980). **Lipoprotein lipase: Some effects of activator proteins.** Eur J. Biochem., 106; 549 - 555.

18. Bengtsson G. and Olivecrona T. (1980). **Lipoprotein Lipase: Mechanism for product inhibition.** Eur. J. Biochem., 106; 557 - 562.

19. Bengtsson-Olivecrona G, Olivecrona T. and Sornvall H. (1986). **Lipoprotein lipases from cow, guinea pig and man: Structural characterisation and identification of protease-sensitive internal regions.** Eur. J. Biochem., 161; 281 - 288.

20. Bengtsson-Olivecrona G., Olivecrona T. and Jörnvall H. (1986). **Lipoprotein lipase from cow, guinea-pig and man: Structural characterisation and identification of protease-sensitive internal regions.** FEBS *Lett.*, 161; 281 - 288.

21. Berger G.M.B. (1986). **Clearance defects in primary chylomicronemia: A study of tissue lipoprotein lipase activities.** Metabolism, 35 (11); 1054-1061.

22. Berger G.M.B. (1987). **Single case reports: An incomplete form of familial lipoprotein lipase deficiency presenting with type I hyperlipoproteinemia.** Am. J. Clin. Pathol., 88(3),.

23. Berryman D. E. and Bensadoun A. (1993). **Site-directed mutagenesis of a putative heparin binding domain of avian lipoprotein lipase.** J. Biol. Chem., 268 (5); 3272 -3276.

24. Bijvoet S.M., Bakker H.D., Bruin T., Kastelein J.J.P., Hayden M.R. and Fruchart J.C. (1993). **Compound heterozygosity for an Asp^{250}→Asn and Ser^{251}→Cys substitution in**

the lipoprotein lipase gene.; pg. 15: *In*, Human Lipoprotein Lipase; Molecular Genetics & Structure Function Analysis. T. Bruin (eds), Thesis Publishers, Amsterdam.

25. Bijvoet S.M., Bruin T., Tuzgöl S., Bakker H.D. and Kastelein J.J.P. (1994). **Homozygosity for a mutation in the lipoprotein lipase gene ($Gly^{139}\rightarrow Ser$) causes chylomicronemia in a boy of Spanish descent.**; pg. 117 - 127: *In*, Human Lipoprotein Lipase; Molecular Genetics & Structure Function Analysis. T. Bruin (eds), Thesis Publishers, Amsterdam.

26. Blow D.M. (1976). **Structure and mechanism of chymotrypsin.** Accounts Chem. Res., 9; 145 - 152.

27. Bodmer M.W., Angal S., Yarranton G.T., Hams T.J.R., Lyons A., King D.J., Pierone G, Riviere C., Verger R. and Lower P.A. (1987). **Molecular cloning of a human gastric lipase and expression of the enzyme in yeast.** Biochim. Biophys. Acta., 909; 237 - 244.

28. Boel E., Huge-Jensen B., Christensen M., Thim L. and Fiil N.P. (1988). ***Rhizomucor miehei* triglyceride lipase is synthesized as a precursor.** Lipids, 23 (7); 710 - 706.

29. Borensztajn J. (1987). **Chapter 5: Heart and skeletal muscle lipoprotein lipase**, pg. 133 - 148; *In*, Lipoprotein Lipase, J. Borensztajn eds., Evener Publishers Inc., Chicago.

30. Brault D., Noé L., Etienne J., Hamelin J., Raisonnier A., Souli A., Chuat J-C., Dugail I., Quinard-Boulangé A., Lavau M. and Galibert F. (1992). **Sequence of rat lipoprotein lipase-encoding cDNA.** Gene, 121; 237 - 246.

31. Brady L., Brzozowski A.M., Derewenda Z.S., Dodson E., Dodson G., Tolley S., Turkenburg S.P., Christiansen L., Huge-Jensen B., Norskov L, Thin L. and Menge U. (1990). **A serine protease triad forms the catalytic centre of a triacylglycerol lipase.** Nature, 343; 767 - 770.

32. Breckenridge W.C., Little J.A., Steiner G., Chow A. and Poast M. (1978). **Hypertriglyceridemia associated with deficiency of apolipoprotein C-II.** New. Engl. J. Med., 298 (23); 1265 - 1273.

33. Brenner S. (1988). **The molecular evolution of genes and proteins: a tale of two serines.** Nature, 334; 528 - 530.

34. Brown W.V. and Baginsky M.L. (1972). **Inhibition of lipoprotein lipase by an apoprotein of human very low density lipoprotein.** Biochem. Biophys. Res. Commun., 46 (2); 375 - 382.

35. Brown W.V., Baginsky M.L. and Ehnholm C. (1977). **Chapter 6: Primary type I and V hyperlipoproteinemia;** pg. 93-112: *In*, Hyperlipidemia: Diagnosis and Therapy, Basil M. Rifkind and R.L. Levy (eds), Grune and Stratton Inc., New York.

36. Brown M.S., Herz J., Kowal R.C. and Goldstein J.L. (1991). **The low-density lipoprotein receptor-related protein: double agent or decoy?.** Current Opinion in Lipidology, 2; 65 - 72.

37. Bruin T., Kastelein J.P.P., van Diermen D.E., Ma Y., Henderson H.E., Stuyt P.M.J., Stalenhoef A.F.H., Sturk A., Brunzell J.D. and Hayden M.R. (1992). **A missense mutation Pro157Arg in lipoprotein lipase ($LPL_{Nijmegen}$) resulting in the loss of catalytic activity.** Eur. J. Biochem., 208; 267 - 272.

38. Bruin T., Tuzgöl S., van Diermen D.E., Hoogerbrugge-van der Linden N., Brunzell J.D., Hayden M.R. and Kastelein J.J.P. (1993). **Recurrent pancreatitis and chylomicronemia in an extended Dutch kindred is caused by a Gly154↪Ser substitution in lipoprotein lipase.** J. Lipid Res., 34; 2109 - 2119.

39. Bruin T., Tuzgöl S., Mulder W.J., van den Ende A.E., Jansen H., Hayden M.R. and Kastelein J.J.P. (1994). **Chapter 6: A compound heterozygote for lipoprotein lipase deficiency, Val^{69}↪Leu and Gly^{188} ↪Glu; correlation between *in-vitro* LPL-activity and clinical expression.**; pgs. 101 - 115: *In,* Human Lipoprotein Lipase; Molecular Genetics & Structure Function Analysis. T. Bruin (eds), Thesis Publishers, Amsterdam.

40. Brunzell J.D., Chait A., Nikkilä E.A, Ehnholm C., Huttunen D.K. and Steiner G. (1980). **Heterogeneity of primary lipoprotein lipase deficiency.** Metabolism, 29(7); 624.

41. Brunzell J.D. and Biermann E.L. (1982). **Chylomicronemia Syndrome: Interaction of genetic and acquired hypertriglyceridemia. Symposum on lipid disorders.** Medical Clinics of North America, 66 (2): 455-468.

42. Brunzell S.D., Miller N.E., Alaupovic P., St. Hilaire J., Wang C.S, Sarson D.L., Bloom S.R. and Lewis B. (1983). **Familial chylomicronemia due to a circulating inhibitor of lipoprotein lipase activity.** J. Lipid Res., 24; 12 - 19.

43. Brunzell J.D. (1989). **Chapter 45: Familial lipoprotein lipase deficiency and other causes of the chylomicronemia syndrome**; Part 7: Lipoprotein and Lipid Metabolism Disorders; pg. 1165: *In,* The Metabolic Basis of Inherited Disease, C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle (eds), Sixth Edition, vol. 1, Magraw-Hill Inc., USA.

44. Brzozowski A.M., Derewenda U., Derewenda Z-S., Dodson G.G., Lawson D.M. Turkenburg J.P., Bjorkling F., Huge-Jensen B., Packer S.A. and Thon L. (1991). **A model for interfacial activation in lipases from the structure of a fungal lipase inhibitor complex.** Nature, 351; 491 - 494.

45. Burdette R.A. and Quinn D.M. (1986). **Interfacial reaction dynamics and acyl-enzyme mechanism for lipoprotein lipase-catalysed hydrolysis of lipid ρ-nitrophenyl esters.** J. Biol. Chem., 261 (26); 12016 - 12021.

46. Byron M.A. and Hughes G.R.V. (1988). **Systemic Lupus Erythromatosis**; pg. 16.20-16.28; *In,* Oxford Textbook of Medicine, 2nd edition; vol. 2, D.J. Weatherall, J.G.G. Ledingham and D.A. Warrell (eds); Oxford Medical Publications, Britain.

47. Baginsky M.L. and Brown W.V.: **A new method for the measurement of Lipoprotein Lipase in post-heparin plasma using sodium dodecyl sulfate for the inactivation of hepatic triglyceride lipase.** J.Lipid Res., 20; 548-, 1979.

48. Camps L., Reina M., Llobera M., Vilaro S. and Olivecrona T. (1990). **Lipoprotein Lipase: cellular origin and functional distribution.** Am J. Physiol., 258; C673 - C681.

49. Camps L., Reina M., Llobrea M., Bengtsson-Olivecrona G., Olivecrona T. and Vilaro S. (1991). **Lipoprotein lipase in lungs, spleen and liver: synthesis and distribution.** J. Lipid Res., 32; 1877 - 1888.

50. Carlson L.A. and Ballantyne D. (1976). **Changing relative proportions of apolipoproteins C-II and C-III of very low density lipoproteins in hypertriglyceridaemia.** Atherosclerosis, 23; 563 - 568.

51. Catapano A.L., Kinnunen P.K.J., Breckenridge W.J., Gotto A.M. Jr., Jackson R.L., Little J.A., Smith L.C. and Sparrow J.T. (1979). **Lypolysis of apoC-II deficient very low density lipoproteins: Enhancement of lipoproetin lipase action by synthetic fragments of apoC-II.** Biochem. Biophys. Res. Commun., 89 (3); 951 - 957.

52. Chait A., Iverius P-H. and Brunzell J.D. (1982). **Lipoprotein lipase secretion by human monocyte-derived macrophages.** J. Clin. Invest., 69; 490 - 493.

53. Chamberlain J.C., Thorn J.A., Oka K., Galton D.J. and Stocks J. (1989). **DNA polymorphisms at the lipoprotein lipase gene: associations in normal and hypertriglyceridaemic subjects.** Atherosclerosis, 79; 85 - 91.

54. Chappell D.A., Fry G.L., Waknitz M.A., Iverius P-H., Williams S.E. and Strickland D.K. (1992). **The low density lipoprotein receptor-related protein/α_2-macroglobulin receptor binds and mediates catabolism of bovine milk lipoprotein lipase.** J. Biol. Chem., 267(36); 25764 - 25767.

55. Chappell D.A., Fry G.L., Waknitz M.A., Muhonen L.E., Pladet M. W., Iverius P-H. and Strickland D.K. (1993). **Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor *in vitro*.** J. Biol. Chem., 268(19); 14168 - 14175.

56. Chappell D.A., Inoue I., Fry G.L., Pladet M.W., Bowen S.L., Iverius P-H., Lalouel J-M. and Strickland D.K. (1994). **Cellular catabolism of normal very low density lipoproteins via the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor is induced by the C-terminal domain of lipoprotein lipase.** J. Biol. Chem., 269 (27); 18001 - 18006.

57. Chapus C., Sémériva M., Bovier-Lapierre C. and Desneulle P. (1976). **Mechanism of pancreatic lipase action. 1. Interfacial activation of pancreatic lipase.** Biochemistry, 15 (23); 4980 - 4987.

58. Chen B-F. and Clejan S. (1993). **Rapid preparation of tissue DNA from paraffin-embedded blocks and analysis by polymerase chain reaction.** J. Histochem. Cytochem., 41 (5); 765 - 768.

59. Cheng Q., Blackett P., Jackson K.W., McConathy W.J. and Wang C-S. (1990). ***C*-terminal**

domain of apolipoprotein CII as both activator and competitive inhibitor of lipoprotein lipase. Biochem. J., 269; 403 - 407.

60. Choi S.Y., Fong L.G., Kirvin M.J. and Cooper A.D. (1990). **Effect of anti-LDL receptor (R) antibody on clearance of LDL and chylomicron remnants (CR) in mice [abstract].** Arteriosclerosis. 10; 766 -.

61. Chomczynski P. and Sacchi N. (1987). **Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction.** Anal. Biochem., 162; 156 - 159.

62. Chothia C. and Janin J. (1975). **Principles of protein-protein recognition.** Nature, 256; 705 - 708.

63. Clarke A.R. and Holbrook J.J. (1985). **The mechanism of activation of lipoprotein lipase by apoliporotein C-II. The formation of a protein-protein complex in free solution and at a triacylglycerol/water interface.** Biochim. Biophys. Acta., 827; 358 - 368.

64. Clarke A.R., Luscombe M. and Holbrook J.J. (1983). **The effect of the chain length of heparin on its interaction with lipoprotein lipase.** Biochim. Biophys. Acta., 747; 130 - 137.

65. Connelly P.W., McGuire G.F. and Little J.A. (1987). **Apolipoprotein C-II$_{St. Michael}$: Familial apolipoprotein C-II deficiency associated with premature vascular disease.** J. Clin. Invest., 80; 1597-1606.

66. Connelly P.W., MacGuire G.F., Hofmann T. and Little J.A. (1987). **Structure of apolipoprotein C-II$_{Toronto}$, a non-functional human apolipoprotein.** Proc. Natl. Acad. Sci. USA, 84; 270 - 273.

67. Cooper D.A., Lu S-C., Viswarnath R., Freiman R.N., Bensadoun A. (1992). **The structure and complete nucleotide sequence of the avian lipoprotein lipase gene.** Biochim. Biophys. Acta., 1129; 166 - 171.

68. Craik C.S., Rocznaik S., Largman C. and Rutter W.J. (1987). **The catalytic role of the active site aspartic acid in serine proteases.** Science, 237; 909 - 914.

69. Crepaldi G., Fellin R., Baggio G., Augustin J. and Greten H. (1979). **Lipoprotein and apoprotein, adipose tissue lipoprotein and hepatic lipase levels in patients with familial hyperchylomicronemia and their immediate family members.**; pg. 250 -254: In, Atherosclerosis V: Proceedings of the Fifth International Symposium, A.M. Gotto, L.C. Smith and B. Allen (eds), Springer-Verlag, New York.

70. Cryer A. (1985). **Lipoprotein lipase: molecular interactions of the enzyme.** Biochem. Soc.Trans., 13; 27 - 28.

71. Currie R.A. and Eckel R.H. (1992). **Characterisation of a high affinity octamer transcription factor binding site in the human lipoprotein lipase promoter.** Arch. Biochem. Biophys., 298 (2); 630 - 639.

72. Cupp M., Bensadoun A. and Melford K. (1987). **Heparin decreases the degradation rate of lipoprotein lipase in adipocytes.** J. Biol. Chem., 262 (13); 6383 - 6388.

73. Cygler M., Schrag J.D. and Ergan F. (1992). **Chapter 2: Advances in structural understanding of lipases.** Biotech. Genet. Eng. Rev., 10; 143 - 184.

74. Datta S., LuoC-C., Li W-H., Van Tuinen P., Ledbetter D.H., Brown M.A., Chen S-H., Liu S-W. and Chan L. (1988). **Human Hepatic Lipase: Cloned cDNA sequence, restriction fragment length polymorphisms, chromosomal localization, and evolutionary relationships with lipoprotein lipase and hepatic lipase.** J. Biol. Chem., 263 (3); 1107 - 1110.

75. Davis R.C., Stalinke G., Wong H., Doolittle M.H., Ameis D., Will H. and Schotz M.C. (1990). **Hepatic lipase: Site-directed mutagenesis of a serine residue important for catalytic activity.** J. Biol. Chem., 265 (11); 6291 - 6295.

76. Davis R.C., Diep A., Hunziksi W., Klisak T., Monandas T., Schole M.C., Sparkes R.S. and Lusis A.J. (1991). **Assignment of human pancreatic lipase gene (PNCIP) to chromosome 10q24-q26.** Genomics, 11; 1164 - 1166.

77. Davidson P.J., Stalenhoef A.F.H. and Humphries S.E. (1987). **Apolipoprotein C-II (apo C-II) gene expression defect in an individual with familial apo C-II deficiency.** Biochem. Biophys. Res. Common., 148 (1), 320-328.

78. Deeb S.S. and Peng R. (1989). **Structure of the human lipoprotein lipase gene.** Biochemistry, 28 (10); 4131 - 4135.

79. Derewenda Z.S. and Derewenda U. (1991). **Relationships among serine hydrolases: evidence for a common structural motif in triacylglyceride lipases and esterases.** Biochem. Cell. Biol., 69; 842 - 851.

80. Derewenda Z.S. and Cambillau C. (1991). **Effects of gene mutations in lipoprotein and hepatic lipases as interpreted by a molecular model of the pancreatic triglyceride lipase.** J. Biol. Chem., 266 (34); 23112 - 23119.

81. Derewenda U., Brzozowski A.M., Lawson D.M. and Derewenda Z.S. (1992). **Catalysis at the interface: The anatomy of a conformational change in a triglyceride lipase.** Biochemistry, 31; 1532 - 1541.

82. Devlin R.H., Deeb S., Brunzell J. and Hayden M.R. (1990). **Partial gene duplication involving exon-alu interchange results in lipoprotein lipase deficiency.** Am. J. Hum. Genet., 46; 112 - 119.

83. Dichek H.L., Fojo S.S., Beg O.U., Skarlatos S.I., Brunzell J.D., Cutler G.B. Jr. and Brewer H.B. Jr. (1991). **Identification of two separate allelic mutations in the lipoprotein lipase gene of a patient with the familial hyperchylomicronemia syndrome.** J. Biol. Chem., 266 (1); 473 - 477.

84. Dichek H.L., Parrott C., Ronan R., Brunzell J.D., Brewer H.B. Jr. and Santamarina-Fojo S. (1993). **Functional characterisation of a chimeric lipase genetically engineered from human lipoprotein lipase and human hepatic lipase.** J. Lipid Res., 34; 1393 - 1401.

85. DiLella A.G., Marvit J., Brayton K. and Woo S.L.C. (1987). **An amino-acid substitution involved in phenylketonuria is in linkage diseqiulibruim with DNA haplotype 2.** Nature, 327; 333 - 336.

86. Docherty A.J.P., Bodmer M.W., Angal S., Verger R., Riviere C., Lowe P.A., Lyons A., Emtage J.S. and Harris T.J.R. (1985). **Molecular cloning and nucleotide sequence of the rat ligual lipase cDNA.** Nucl. Acid Res., 13 (6); 1891 - 1902.

87. Dodson G.G. and Lawson D.M. (1992). **Structural and evolutionary relationships in lipase mechanism and activation.** Faraday Discuss, 93; 95 - 105.

88. Dorn A., Bollekens J., Staub A., Benoist C. and Mathis D. (1987). **A multiplicity of CCAAT box-binding proteins.** Cell, 50; 863 - 872.

89. Dugi K.A., Dichek H.L., Talley G.D., Brewer H.B. Jr. and Snatamarima-Fojo S. (1992). **Human lipoprotein lipase: The loop covering the catalytic site is essential for the interaction with lipid substrates.** J. Biol. Chem., 267 (35); 25086 - 25091.

90. Eckel R.H. and Robbins R.J. (1984). **Lipoprotein Lipase is produced, regulated and functional in rat brain.** Proc. Natl. Acad. Sci. USA. 81; 7604 - 7.

91. Ehnholm C., Shaw W., Greten H. and Brown W.V. (1975). **Purification from human plasma of a heparin-released lipase with activity against triglyceride and phospholipids.** J. Biol. Chem., 250 (17); 6756 - 6751.

92. Eisenberg D. and Wesson M. (1990). **The most highly amphiphilic α-helices include two amino acid segments in human immunodeficiency virus glycoprotein 41.** Biopolymers, 29; 171 - 177.

93. Emi M., Hata A., Robertson M., Iveruis P-H., Hegele R., Lalouel J-M. (1990). **Lipoprotein lipase deficiency resulting from a nonsense mutation in exon 3 of the lipoprotein lipase gene.** Am. J. Hum. Genet. 47; 107 - 111.

94. Emi M., Wilson D.E., Iveruis P-H., Wu L., Hata A., Hegele R., Williams R.R., Lalouel J-M. (1990). **Missense mutation (Gly→Glu^{188}) of human lipoprotein lipase imparting functional deficiency.** J. Biol Chem. 265 (10); 5910 - 5916.

95. Emmerich J., Beg O.U., Peterson J., Previato L., Brunzell J.D., Brewer H.B. Jr. and Santamaria-Fojo S. (1992). **Human lipoprotein lipase: Analysis of the catalytic triad by site directed mutagenesis of Ser-132, Asp-156, His-241.** J. Biol. Chem., 267 (6); 4161 - 4165.

96. Enerbäck S. and Bjursell G. (1989). **Genomic organization of the region encoding guinea pig lipoprotein lipase; evidence for exon fusion and unconventional splicing.** Gene, 84;

391 - 397.

97. Farr C.J., Saiki R.K., Erlich H.A., McCormick F. and Marshall C.J. (1988). **Analysis of *RAS* gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes.** Proc. Natl. Acad. Sci. USA, 85; 1629 - 1633.

98. Faustinella F., Smith L.C., Semenkovich C.F. and Chan L. (1991). **Structural and functional roles of highly conserved serines in human lipoprotein lipase.** J. Biol. Chem., 266 (15); 9481 - 9485.

99. Faustinella F., Chang A., Van Biervliet J.P., Rosseneu M., Vinaimont N., Smith L.C., Chen S-H. and Chan L. (1991). **Catalytic triad residue mutation ($Asp^{156}\rightarrow Gly$) causing familial lipoprotein lipase deficiency : Co-inheritance with a nonsense mutation ($Ser^{447}\rightarrow Ter$) in a Turkish family.** J. Biol. Chem., 266 (22); 14418 - 14424.

100. Faustinella F., Smith L.C. and Chan L. (1992). **Functional topology of a surface loop shielding the catalytic center in lipoprotein lipase.** Biochemistry, 31; 7219 - 7223.

101. Fielding C.J. and Fielding P.E. (1976). **Mechanism of salt-mediated inhibition of lipoprotein lipase.** J. Lipid Res. 17; 249 - 255.

102. Fisher K.L., Fitzgerald G.A. and Lawn R. (1987). **Two polymorphisms in the lipoprotein lipase (LPL) gene.** Nucl. Acid Res., 15; 7567.

103. Fojo S.S., Low S.W. and Brewer H.B. Jr. (1984). **Human apolipoprotein C-II: Complete nucleic acid sequence of pre-apolipoprotein C-II.** Proc. Natl. Acad. Sci. USA, 81; 6354 - 6357.

104. Fojo S.S., Stalenhoef A.F.H., Marr K., Gregg R.E., Ross R.S., Brewer H.B.Jr. (1988). **A deletion mutation in the apoC-II gene ($ApoC\text{-}II_{Nijmegen}$) of a patient with a deficiency of apolipoprotein C-II.** J. Biol. Chem., 263 (34); 17913-17916.

105. Fojo S.S., Beisiegel U., Beil U., Higuchi K., Bojanoski M., Gregg R.E., Greten H. and Brewer H.B. Jr. (1988). **Donor splice site mutation in the apolipoprotein (Apo) C-II gene ($ApoC\text{-}II_{Hamburg}$) of a patient with Apo C-II deficiency.** J. Clin. Invest., 82; 1489 - 1494.

106. Fojo S.S., deGennes J.L., Chapman J. *et. al.* (1989). **An initiation codon mutation in the apoC-II gene ($apoC\text{-}II_{Paris}$) of a patient with deficiency of apolipoprotein C-II.** J. Biol. Chem., 264; 20839-20842.

107. Fojo S.S., Lohse P., Parrott C., Baggio G., Gabelli C., Thomas F., Hoffan J. and Brewer H.B. Jr. (1989). **A nonsense mutation in the apolipoprotein $C\text{-}II_{Padova}$ gene in a patient with apolipoprotein C-II deficiency.** J. Clin. Invest., 84; 1215-1219.

108. Fojo S.S., Lohse P., Lohse P., Alsayed N., Rebourcet R. and Brewer H.B. Jr. (1990). **Apo C-II and Apo E Deficiency. A unique kindred with point mutations in two separate apolipoprotein genes.** Arteriosclerosis, 10 (5); 772a

109. Fojo S.S. and Brewer H.B. (1992). **Hypertriglyceridaemia due to genetic defects in lipoprotein lipase and apolipoprotein C-II.** J. of Internal Medicine, 231: 669-677.

110. Funke H., Wiebusch H., Paulweber B. and Assmann G. (1990). **Identification of the molecular defect in a patient with type I hyperlipidemia.** Arteriosclerosis. 10 (5); 830a.

111. Gagne C., Brun L.D., Julien P., Moorjani S. and Lupien P.J. (1989). **Primary lipoprotein lipase activity deficiency: clinical investigation of a French Canadian population.** Can. Med. Assoc. J. 140; 405 - 411.

112. Gall K., Pavičić D., Pavelić J., Audy-Jurković S. and Pavelić K. (1993). **PCR amplification of DNA from stained cytological smears.** J. Clin. Pathol., 46; 378 - 379.

113. Gianturco S.H., Gotto A.M.Jr., Hwang S-L. C., Karlin J.B., Lin A.H.Y., Prasad S.C. and Bradley W.A. (1983). **Apolipoprotein E mediates uptake of S_f 100-400 hypertriglyceridemic very low density lipoproteins by the low density lipoprotein receptor pathway in normal human fibroblasts.** J. Biol. Chem., 258(7); 4526-4533.

114. Gibbs R.A., Nguyen P-N., McBride L.J., Koepf S.M. and Caskey C.T. (1989). **Identification of mutations leading to the Lesch-Nyhan syndrome by automated durect DNA sequencing of *in vitro* amplified cDNA.** Proc Natl. Acad. Sci. USA, 86; 1919 - 1923.

115. Glueck C.J. (1977). **Chapter 2: Classification of diagnosis of hyperlipoproteinemia**; pg. 17 - 39: *In*, Hyperlipidemia: Diagnosis and Therapy, Basil M. Rifkind and Robert I. Levy (eds), Grune and Stratton Inc., New York.

116. Glueck C.J., Christopher C., Mishkel M.A., Tsang R.C. and Mellies M.J. (1980). **Pancreatitis, familial hypertriglyceridemia, and pregnancy.** Am. J. Obstet. Gynecol., 136; 755 - 761.

117. Goldberg I.J., Soprano D.R., Wyatt M.L., Vanni T.M., Kirchgessner T.G. and Schotz M.C. (1989) **Localization of lipoprotein lipase mRNA in selected rat tissues.** J. Lipid Res., 30; 1569 - 1577.

118. Gotoda T., Murase T., Ishibashi S., Shimano H., Harada K. and Yamada N. (1990). **Splicing, nonsense, and missense mutations in familial lipoprotein lipase deficiency.** Arteriosclerosis, 10 (5); 833a.

119. Gotoda T., Yamada N., Murase T., Inaba T., Ishibashi S., Shimano H., Koga S., Yazaki Y., Furuichi Y. and Takaku F. (1991). **Occurrence of multiple abberantly spliced mRNA's upon a donor splice site mutation that causes familial lipoprotein lipase deficiency.** J. Biol. Chem., 266 (36); 24757 - 25762.

120. Gotoda T., Yamada N., Kawamura M., Kozaki K., Mori N., Ishibashi S., Shimano H., Takuka F., Yazaki Y., Furuichi Y. and Murase T. (1991). **Heterogeneous mutations in the human lipoprotein lipase gene in patients with familial lipoprotein lipase deficiency.** J. Clin. Invest., 88; 1856 - 1864.

121. Gotoda T., Yamada N., Murase T., Miyake S., Murakami R., Kawamura M., Kozaki K., Mori N., Shimano H., Shimada M. and Yazaki Y. (1992). **A newly identified null allelic mutation in the human lipoprotein lipase (LPL) gene of a compound heterozygote with familial LPL deficiency.** Biochim. Biophys. Acta, 1138; 353 - 356.

122. Grochulski P., Li Y., Shrag J.D., Bouthillier F., Smith P., Harrison D., Rubin B. and Cygler M. (1993). **Insights into interfacial activation from an open structure of *Candidum rugosa* lipase.** J. Biol. Chem., 268 (17); 12843 - 12847.

123. Hahn P.F. (1943). **Abolishments of alimentary lipemia following injection of heparin.** Science, 98; 19 - 20.

124. Haliassos A., Chomel J.C., Tesson L., Baudis M., Kruh J., Kaplan J.C. and Kitzis A. (1989). **Modification of enzymatically amplified DNA for the detection of point mutations.** Nucl. Acid Res., 17 (9); 3606.

125. Hamosh M., Clary T.R., Chernick S.s. and Scow R.O. (1970). **Lipoprotein lipase activity of adipose and mammary tissue and plasma triglyceride in pregnant and lactating rats.** Biochim. Biophys. Acta, 210; 473 - 482.

126. Hata A., Emi M., Luc G., Basdevant A., Gambert P., Iverius P-H. and Lalouel J-M. (1990). **Compound heterozygote for lipoprotein lipase deficiency: Ser→Thr^{244} and transition in 3' splice site of intron 2 (AG→AA) in the lipoprotein lipase gene.** Am. J. Hum. Genet., 47; 721 - 726.

127. Hata A., Ridinger D.N., Sutherland S.D., Emi M., Kwong L.K., Shuhua J., Lubbers A., Guy-Grand B., Basdevant A., Iveruis P-H., Wilson D.E. and Lalouel J-M. (1992). **Missense mutations in exon 5 of the human lipoprotein lipase gene: Inactivation correlates with loss of dimerization.** J. Biol. Chem., 267 (28); 20132 - 20139.

128. Hata A., Ridinger D.N., Sutherland S., Emi M., Shuhua Z., Myers R.L., Ren K., Cheng T., Inoue I., Wilson D.E., Iveruis P-H. and Lalouel J-M. (1993). **Binding of lipoprotein lipase to heparin: Identification of five critical residues in two distinct segments of the amino-terminal domain.** J. Biol. Chem., 268 (12); 8447 - 8457.

129. Hatanaka K., Tanishita H., Ishibashi-Ucda H. and Yamamoto A. (1986). **Hyperlipidemia in mast cell-deficient W/W^{v} mice.** Biochem. Biophys. Acta., 878; 440 - 445.

130. Havel R.J. and Gordon R.S. (1960). **Idiopathic Hyperlipidemia: Metabolic studies in an affected family.** J. Clin. Invest., 39; 1797 - 1790.

131. Hayden M.R., Vergani C., Humphries S.E., Kirby L., Shukin R. and McLeod R. (1986). **The genetics and molecular biology of apolipoprotein C-II**: *In*, Lipoprotein Deficiency Syndromes, A. Angel and J. Frolich (eds), Plenum Press New York.

132. Hayden M.R. and Henderson H.E. (1995). **The molecular biology and genetics of human lipoprotein lipase**: *In*, Lipoproteins in Health and Disease, J. Betteridge, R. Illingworth and J. Shepard (eds), Edward Arnold Publishers (in press).

133. Hegele R.A., Connelly P.W., Maguire G.F., Huff M.W., Leiter L., Wolfe B.M., Evans A.J. and Little J.A. (1991). **An apolipoprotein C-II mutation, C-II$_{Lys19\rightarrow Thr}$ identified in patients with hyperlipidemia.** Disease Markers, 9; 73 - 90.

134. Heizmann C., Ladias J.A., Antonarakis S.F., Kirchgessner T., Schotz M.C. and Lusis A.J. (1987). **RFLP for the human lipoprotein lipase (LPL) gene: *Hin*dIII.** Nucl. Acid Res., 15; 6763.

135. Heizmann C., Kirchgessner T., Kwiterovich P.O., Ladias J.A., Derby C., Antonarakis S.F. and Lusis A.J. (1991). **DNA polymorphism haplotypes of the human lipoprotein lipase gene: possible association with high density lipoprotein levels.** Hum. Genet., 86; 578 - 584.

136. Henderson H.E., Devlin R., Peterson J., Brunzell J.D. and Hayden M.R. (1990). **Frameshift mutation in exon 3 of the lipoprotein lipase gene causes a premature stop codon and lipoprotein lipase deficiency.** Mol. Biol. Med., 7; 511 - 517.

137. Henderson H.E., Ma Y., Hassan M.F., Monsalve M.V., Marais A.D., Winkler F., Gubernator K., Peterson J., Brunzell J.D. and Hayden M.R. (1991). **Amino acid substitution (Ile$^{194}\rightarrow$Thr) in exon 5 of the lipoprotein lipase gene causes lipoprotein lipase deficiency in three unrelated probands.** J. Clin. Invest., 87; 2005 - 2011.

138. Henderson H.E., Hassan M.F., Berger G.M.B., Hayden M.R (1992). **The lipoprotein lipase Gly$^{188}\rightarrow$Glu mutation in South Africans of Indian descent: evidence suggesting common origins and an increased frequency.** J. Med. Genet., 29; 119-122.

139. Henderson H.E., Ma Y., Liu M-S., Clarke-Lewis I., Maeder D.L., Kastelein J.J.P., Brunzell J.D. and Hayden M.R. (1993). **Structure-function relationships of lipoprotein lipase: Mutation analysis and mutagenesis of the loop region.** J. Lipid Res., 34; 1593 - 1602.

140. Herbert D.J., Nishiyama R.H., Bagwell C.B., Munson M.E., Hitchcock S.A. and Lovett E.J. (1989). **Effects of several commonly used fixatives on DNA and total nuclear protein analysis by flow cytometry.** Am. J. Clin. Pathol., 91; 535 - 541.

141. Hjorth A., Carriére F., Cudrey C., Wöldike H., Boel E., Lawson D.M., Ferrato F., Cambillau C., Dodson G.G., Thim L. and Verger R. (1993). **A structural domain (the lid) found in pancreatic lipases is absent in the guinea pig (phospho) lipase.** Biochemistry, 32; 4702 - 4707.

142. Hoeg J.M., Osborne J.C., Gregg R.E. and Brewer H.B. (1983). **Initial diagnosis of lipoprotein lipase deficiency in a 75 year old man.** The American J. of Medicine, 75; 889-892.

143. Holdsworth G., Stocks J., Dodson P. and Galton D.J. (1982). **An abnormal triglyceride-rich lipoprotein containing excess sialylated apolipoprotein C-III.** J. Clin. Invest., 69; 932 - 939.

144. Hua X., Enerback S., Hudson J., Youkhana K. and Gimble J.M. (1991). **Cloning and characterisation of the promoter of the murine lipoprotein lipase-encoding gene: Structural and functional analysis.** Gene, 107; 247 - 258.

145. Huff M.W., Evans A.J., Wolfe B.M., Connelly P.W., Maguire G.F. and Strong W.L.P. (1990). **Identification and metabolic characteristics of an apolipoprotein C-II variant isolated from a hypertriglyceridemic subject.** J. Lipid. Res., 31; 375 - 396.

146. Inadera H., Hibino A., Kobayashi J., Kanzaki T., Shirai K., Yukawa S., Saito Y. and Yoshida S. (1993). **A missense mutation ($Trp^{26}\rightarrow Arg$) in exon 3 of the apolipoprotein C-II gene in a patient with apolipoprotein C-II deficiency (Apo $C\text{-}II_{Wakayama}$).** Biochem. Biophys. Res. Commun., 193 (3); 1174 - 1183.

147. Ishimura-Oka K., Fuastinella F., Kihara S., Smith L.C., Oka K. and Chan L. (1992). **A missense mutation ($Trp^{86}\rightarrow Arg$) in exon 3 of the lipoprotein lipase gene: A cause of familial chylomicronemia.** Am. J. Hum. Genet., 50; 1275 - 1280.

148. Isobe K. and Nokihara K. (1993). **Primary structure determination of mono- and diacylglycerol lipase from *Penicillium camembertii*.** FEBS *Lett.*, 320; 101 - 106.

149. Ito Y., Azrolan N., O'Connell A., Walsh A. and Breslow J.L. (1990). **Hypertriglyceridemia as a result of human apo C-III gene expression in transgenic mice.** Science, 249; 790 - 793.

150. Jackson R.L., PattusF. and de Haas G. (1980). **Mechanism of action of milk lipoprotein lipase of substrate interfaces: Effects of apolipoproteins.** Biochemistry, 19; 373 - 378.

151. Jean-Pierre M., Weil D., Hors-Cayla M.C., Williamson R., Junien C. and Humphries S.F. (1984). **Gene for apolipoprotein C-II is on chromosome 19.** Somatic Cell Mol. Genet., 10; 645 - 649.

152. Jensen D.R., Bessesen D.H., Etienne J., Eckel R.H. and Neville M.C. (1991). **Distribution and source of lipoprotein lipase in mouse mammary gland.** J.Lipid.Res., 32: 773 - 742.

153. Jorgensen J., Skor K.W. and Diderichsen B. (1991). **Cloning, sequence and expression of a lipase from Pseudomonas cepacia: lipase production in heterologous host require two Pseudomonas genes.** J. Bacteriol., 173; 559 - 567.

154. Karpe F., Olivecrona T., Walldius G. and Hamsten A. (1992). **Lipoprotein lipase in plasma after an oral fat load: relation to free fatty acids.** J. LIpid Res., 33; 975 - 984.

155. Kihara S., Matsuzawa Y. Kubo M., Kozaki S., Funahashi T., Yamashita S., Sho N. and Tarui S. (1989). **Autoimmune Hyperchylomicronemia.** N. Engl. J. Med., 320 (9); 1255 - 1259.

156. Kinnunen P.K.J., Huttunen J.K. and Ehnholm C. (1976). **Properties of purified bovine milk lipoprotein lipase.** Biochim. Biophys. Acta., 450; 342 - 351.

157. Kirchgessner T.G., Svenson K.L., Lusis A.J., Schotz M.C. (1987). **The sequence of cDNA encoding Lipoprotein Lipase: A member of a lipase gene family.** J. Biol. Chem. 262 (18); 8463 - 8466.

158. Kirchgessner T.G., Chuat J-C., Heinzmann C., Etienne J., Guilliot S., Svenson K., Ameis D., Pilon C., D'Auriol L., Andalibi A., Schotz M.C., Galibert F. and Lusis A.T. (1989). **Organisation of the human lipoprotein lipase gene and evolution of the lipase gene family.** Proc. Natl. Acad. Sci. USA, 86; 9647 - 9651.

159. Kobayashi J., Shirai K., Saito Y. and Yoshida S. (1989). **Lipoprotein lipase with a defect in lipid interface recognition in a case with type I hyperlipidemia.** Eur. J. Clin. Invest., 19; 424 - 432.

160. Kobayashi J., Nishida T., Ameis D., Stahnke G., Schotz M.C., Hashimoto H., Fukamachi T., Shirai K., Saito Y., Yoshida S. (1992). **A heterozygous mutation (the codon for Ser^{447}→a stop codon) in lipoprotein lipase contributes to a defect in lipid interface recognition in a case with type I hyperlipidemia.** Biochem. Biophys. Res. Commun., 182 (1); 70 - 77.

161. Kobayashi J., Sasaki N., Tashiro J., Inadera H., Saito Y. and Yoshida S. (1993). **A missense mutation (Ala^{334}→Thr) in exon 7 of the lipoprotein lipase gene in a case with type I hyperlipidemia.** Biochem. Biophys. Res. Commun., 191 (3); 1046 - 1054.

162. Komaromy M.C. and Schotz M.C. (1987). **Cloning of rat hepatic lipase cDNA: Evidence for a lipase gene family.** Proc. Natl. Acad. Sci. USA, 84; 1526 - 1530.

163. Kondo Y., Kurobane I., Omura K., Sano R., Abe R., Chida N. and Tada K. (1985). **Post-heparin plasma lipoprotein lipase activity in heterozygotes of familial lipoprotein lipase deficiency.** Tohoku J. Exp. Med., 145; 1 - 6.

164. Korn E.D. (1955). **Clearing factor, a heparin-activated lipoprotein lipase: Isolation and characterization of the enzyme from normal rat heart.** J. Biol. Chem., 215, 1 - 14.

165. Kozaki K., Gotoda T., Kawamura M., Shimano H., Yazaki Y., Ouchi Y., Orimo H. and Yamada N. (1993). **Mutational analysis of human lipoprotein lipase by carboxy-terminal truncation.** J. Lipid Res., 34; 1765 - 1772.

166. Lalouel J-M., Wilson D.E. and Iveruis P-H. (1992). **Lipoprotein lipase and hepatic triglyceride lipase: molecular and genetic aspects.** Current Opinion in Lipidology, 3; 86 - 95.

167. Langlois S., Deeb S.S., Brunzell J.D., Kastelein J.J.P. and Hayden M.R. (1989). **A major insertion accounts for a significant proportion of mutations underlying human lipoprotein lipase deficiency.** Proc. Natl. Acad. Sci. USA. 86; 948 - 952.

168. LaRosa J.C., Levy R.I., Windmueller H.G. and Fredrickson D.S. (1972). **Comparison of the triglyceride lipase of liver, adipose tissue, and postheparin plasma.** J. Lipid Res., 13; 356 - 363.

169. Lee C.Y. and Iandolo J.J. (1986). **Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a genome into the lipase structural gene.** J. Bacteriol., 166; 385 - 391.

170. Lewis B. (1988). **Disorders of Lipid Transport**; pg. 9.108 - 9.123; *In,* Section 9: Metabolic Disorders of the Oxford Textbook of Medicine, Second edition, Vol. 1; D.J. Weatherall, J.G.G. Ledingham and D.A. Warrell (eds): Oxford University Press, New York.

171. Liu M-S., Ma Y., Hayden M.R. and Brunzell J.D. (1992). **Mapping of the epitope on lipoprotein lipase recognized by a monoclonal antibody (5D2) which inhibits lipase activity.** Biochim. Biophys. Acta., 1128; 113 - 115.

172. Lohse P., Beg O.K., Brunzell J.D., Fojo S.S. and Brewer H.B. Jr. (1990). **Familial chylomicronemia: Identification of a unique patient homozygote for two separate mutations in the LPL gene.** Arteriosclerosis and Thrombosis, 11; 1415a-.

173. Lookene A. and Bengtsson-Olivecrona G. (1993). **Chymotryptic cleavage of lipoprotein lipase: Identification of cleavage sites and functional studies of the truncated molecule.** Eur. J. Biochem., 213; 185 - 194.

174. Lowe M.E., Rosenblum J.L. and Strauss A.W. (1989). **Cloning and characterization of human Pancreatic lipase cDNA.** J. Biol. Chem., 264 (33); 20042-20048.

175. Lui G. and Olivecrona T. (1991). **Pulse chase study on lipoprotein lipase in perfused guinea pig heart.** Am. J. Physiol. (Heart Circ. Physiol 30); H2044, H2080.

176. Ma Y., Henderson H.E., Ven Murthy V., Roederer G., Monsalve M.V., Clarke L.A., Norman T., Julien P., Gagne C., Lambert M., Davignon J., Lupien P.J., Brunzell J. and Hayden M.R. (1991). **A mutation in the human lipoprotein lipase gene as the most common cause of familial chylomicronemia in French Canadians.** N. Engl. J. Med., 324; 1761 - 1766.

177. Ma Y., Bruin T., Tuzgol S., Wilson B.I., Roederer G., Liu M-S., Davignon J., Kastelein J.J.P., Brunzell J.D. and Hayden M.R. (1992). **Two naturally occurring mutations at the first and second bases of codon aspartic acid 156 in the proposed catalytic triad of human lipoprotein lipase: *In vivo* evidence that aspartic acid 156 is essential for catalysis.** J. Biol. Chem., 267 (3); 1918 - 1923.

178. Ma Y., Wilson B.I., Bijvoet S., Henderson H.E., Cramb E., Roederer G., Ven Murthy M.R., Julien P., Bakker H.D., Kastelein J.J.P., Brunzell J.D. and Hayden M.R. (1992). **A missense mutation ($Asp^{250} \rightarrow Asn$) in exon 6 of the human lipoprotein lipase gene causes chylomicronemia in patients of different ancestries.** Genomics, 13; 649 - 653.

179. Ma Y., Liu M-S., Zhang H., Forsythe I.J., Brunzell J.D. and Hayden M.R. (1993). **A 4 basepair deletion in exon 4 of the human lipoprotein lipase gene results in type I hyperlipoproteinemia.** Human Molecular Genetics. 2 (7); 1049 - 1050.

180. Ma Y., Liu M-S., Ginzinger D., Frohlich J., Brunzell J.D. and Hayden M.R. (1993). **Gene-**

Enviroment interaction in the conversion of a mild-to-severe phenotype in a patient homozyguos for a $Ser^{172}\rightarrow Cys$ mutation in the lipoprotein lipase gene. J. Clin. Invest., 91; 1953 - 1958.

181. Ma Y., Ooi T.C., Liu M-S., Zhang H., McPherson R., Edwards A.L., Forsythe I.J., Brunzell J.D. and Hayden M.R. (1994). **High frequency of mutations in the human lipoprotein lipase gene in preganancy-induced chylomicronemia: possible association with apolipoprotein E2 isoform.** J. Lipid Res., 35; 1066 - 1075.

182. Macritchie F. (1978). **Proteins at interfaces.** Adv. Protein Chem., 32; 283 - 326.

183. Mahley R.W. and Hussain M.M. (1991). **Chylomicron and chylomicron remnant catabolism.** Current Opinion in Lipodology, 2; 170 - 176.

184. Martin G.A., Busch S.J., Meredith G.D., Cardin A.D., Blankenship D.T., Mao S.J.T., Rechlin A.E., Woods C.W., Racke M.M., Schafer M.P., Fitzgerald M.C., Burke D.M., Flanagen M.A. and Jackson R.L. (1988). **Isolation and cDNA sequence of human post-heparin plasma hepatic triglyceride lipase.** J. Biol. Chem., 263 (22); 10907 - 10914.

185. McConathy W.S. , Gesquiere J.C., Bass H., Tartar A., Fruchart J.C. and Wang C-S. (1992). **Inhibition of lipoprotein lipase activity by synthetic peptides of apolipoprotein C-III.** J. Lipid Res., 33; 995 - 1003.

186. McLean L.R., Larsen W.J. and Jackson R.L. (1986). **Interaction of lipoprotein lipase with phospholipid vesicles: Effect on protein and lipid structure.** Biochemistry, 25; 873 - 878.

187. McLean L.R., Demel R.A., Socorro L., Shinomiya M. and Jackson R.L. (1986). **Chapter 44: Mechanism of action of lipoprotein lipase.** Methods of Enzymology, 129; 738 - 763.

188. McLean J., Fielding C., Drayna D., Dieplinger H., Baer B., Kohr W., Henzel W. and Lawn R. (1986). **Cloning and expression of human lecithin-cholesterol acyltransferase cDNA.** Proc. Natl. Acad. Sci. USA, 83; 2335 - 2339.

189. Menzel H-J., Kane J.P., Malloy M.J., Havel R.J. (1986). **A variant primary structure of apolipoprotein C-II in individuals of African descent.** J. Clin. Invest., 77; 596 - 601.

190. Miesenbock G., Hölzl B., Föger B., Brandstätter E., Paulweber B., Sandhofer F. and Patsch J.R. (1993). **Heterozygous lipoprotein lipase deficiency due to a missense mutation as the cause of impaired triglyceride tolerance with multiple lipoprotein abnormalities.** J. Clin. Invest., 91; 448 - 455.

191. Miller N.E., Rao S.N., Alaupovic P., Noble N., Stack J., Brunzell J.D. and Lewis B. (1981). **Familial apolipoprotein CII deficiency: plasma lipoproteins and apolipoproteins in heterozygous and homozygous subjects and the effects of plasma infusion.** European J. Clin. Invest., 11; 69 - 75.

192. Monsalve M.V., Henderson H.E., Roederer G., Julien P., Deeb S., Kasletein J.J.P., Pentz L., Devlin R., Bruin T., Murthy M.R.V., Cagne C., Davignon J., Lupien P.J., Brunzell J.D. and Hayden M.R. (1990). **A missense mutation at codon 188 of the human lipoprotein lipase gene is a frequent cause of lipoprotein lipase deficiency in persons of different ancestries.** J. Clin. Invest., 86; 728 - 734.

193. Montgomery W.H. and Miller F.C. (1970). **Pancreatitis and Pregnancy.** Obstetrics and Gynecology, 35 (4); 658 - 664.

194. Mulder M., Lombard P., Jansen H., van Berkel T.J.C., Frants R.R. and Havekes L.M. (1993). **Low density lipoprotein receptor internalizes low density and very low density lipoproteins that are bound to heparan sulfate proteoglycans via lipoprotein lipase.** J. Biol. Chem., 268 (13); 9369 - 9375.

195. Murphy M.C., Zampelas A., Puddicombe S.M., Furlonger N.P., Morgan L.M. and Williams C.M. (1993). **Adipose tissue site specificity of lipoprotein lipase mRNA expression in rats fed diets containing different fatty acid compositions.** Biochem. Soc. Trans., 21; 145S.

196. Musliner T.A., Church E.C., Herbert P.N., Kingston M.S. and Shulman R.S. (1977). **Lipoprotein lipase cofactor activity of a carboxyl-terminal peptide of apolipoprotein C-II.** Proc. Natl. Acad. Sci. USA., 74 (12); 5358 - 5362.

197. Nagata Y., Chen J. and Cooper A.D. (1988). **Role of low density lipoprotein receptor-dependent and -independent sites in binding and uptake of chylomicron remnants in rat liver.** J. Biol. Chem., 263; 15151 - 15158.

198. Naylor J.A., Green P.M., Montandon A.J., Rizza C.R. and Giannelli F. (1991). **Detection of three novel mutations in two haemophilia A patients by rapid screening of whole essential region of factor VIII gene.** The Lancet, 337; 635 - 639.

199. Neubauer A., Neubauer B., He M., Effert P., Iglehart D., Frye R.A. and Liu E. (1992). **Analysis of gene amplification in archival tissue by differential chain reaction.** Oncogene, 7; 1019 - 1025.

200. Newton C.R., Graham A., Heptinstall L.E., Powell S.J., Summers C., Kalsheker N., Smith J.C. and Markham A.F. (1989). **Analysis of any point mutation in DNA: The amplification refractory mutation system (ARMS).** Nucl. Acid Res., 17 (7); 2503 - 2516.

201. Noble M.E.M., Cleasby A., Johnson L.N., Egmond M.R. and Frenken L.G.J. (1993). **The crystal structure of triacylglycerol lipase from Pseudomonas glumae reveals a partially redundant catalytic aspartate.** FEBS *Lett.*, 331 (1,2); 123 - 128.

202. Nikkilä E.A. (1978). **Chapter 30: Familial Lipoprotein Lipase Deficiency and related disorders of chylomicron metabolism**; pg. 622: *In*, The metabolic basis of inherited disease, 4th edition, J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson (eds), McGraw-Hill Book Cpy, USA.

203. O'Brien K.D., Gordon D., Deeb S., Ferguson M. and Chait A. (1992). **Lipoprotein lipase is synthesised by macrophage derived from cells in human coronary artherosclerotic plaques.** J. Clin. Invest., 89; 1344 - 1550.

204. Oka K., Tkalcevic T., Stocks J., Galton D.J. and Brown W.V. (1989). **Nucleotide sequence of PvuII polymorphic site at the human lipoprotein lipase gene locus.** Nucl. Acid Res., 17 (16); 6752.

205. Oka K., Tkalcevic G.T., Nakano T., Tucker H., Ishimura-Oka K. and Brown W.V. (1990). **Structure and polymorphic map of human lipoprotein lipase gene.** Biochim. Biophys. Acta., 1049; 21 - 26.

206. Olivecrona T., Bengtsson G. and Osborne J.C. Jr. (1982). **Molecular properties of lipoprotein lipase: Effects of limited trypsin digestion on molecular weight and secondary structure.** Eur. J. Biochem., 124; 629 - 633.

207. Olivecrona T., Bengtsson-Olivecrona G., Osborne J.C. Jr. and Kempner E.S. (1985). **Molecular size of bovine lipoprotein lipase as determined by radiation inactivation.** J. Biol. Chem., 260 (11); 6888 - 6891.

208. Olivecrona T. and Bengtsson-Olivecrona G. (1987). **Lipoprotein lipase from milk. The model enzyme in lipoprotein lipase research**, pg. 15-58; *In,* Lipoprotein Lipase. J. Borensztajn (eds): Evener Publishers Inc., Chicago.

209. Op Den Kamp J.A.K., De Gier J. and Van Deenen L.L.M. (1974). **Hydrolysis of phsphatidylcholine liposomes by pancreatic phospholipase A_2 at the transition temperature.** Biochim. Biophys. Acta., 345; 253 - 256.

210. Orita M., Suzuki Y., Sekiya T. and Hayashi K. (1989). **Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction.** Genomics, 5; 874 - 879.

211. Osborne J.C., Bengtsson-Olivecrona G., Lee N.S., Olivecrona T. (1985). **Studies on inactivation of lipoprotein lipase: Role of the dimer to monomer dissociation.** Biochemistry, 24; 5606 - 5611.

212. Paduslo S.E., Dean M., Kolch U. and O'Brien S.J. (1991). **Detecting high-resolution polymorphisms in human coding loci combining PCR and single-strand conformation polymorphism (SSCP) analysis.** Am. J. Hum. Genet., 49; 106 - 111.

213. Parkin S.M., Speake B.K. and Robinson D.S. (1982). **Purification and characterization of rat adipose tissue lipoprotein lipase.** Biochem. J., 207; 485 - 495.

214. Parrott C.L., Alsayed N., Rebourcet R. and Santamarina-Fojo S. (1992). **ApoC-II_{Paris2} : a premature termination mutation in the signal peptide of apoC-II resulting inthe familial chylomicronemia syndrome.** J. Lipid Res., 33; 361- 367.

215. Patsch J.R., Prasad S., Gotto A.M. Jr. and Patsch W. (1987). **High Density Lipoprotein$_2$: Relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase.** J. Clin. Invest., 80; 341 - 347.

216. Pathak D. and Ollis D. (1990). **Refined structure of Dienelactone Hydrolase at 1.8 Å.** J. Mol. Biol., 214; 497 - 525.

217. Pedersen M.E., Cohen M. and Schotz M.C. (1983). **Immunocytochemical localization of the functional fraction of lipoprotein Lipase in the perfused heart.** J.Lipid.Res., 24; 512 - 520.

218. Persson B., Bengtsson-Olivecrona G., Enerback S., Olivecrona T., Tornvall H. (1989). **Structural features of lipoprotein lipase: Lipase family relationships, binding interactions, non-equivalence of lipase co-factors, vitellogenin similarities and functional subdivision of lipoprotein lipase.** Eur. J. Biochem., 179; 39 - 45.

219. Peterson J., Bihain B.E., Bengtsson-Olivecrona G., Deckelbaum R.J., Carpentier Y.A. and Olivecrona T. (1990). **Fatty acid control of lipoprotein lipase: A link between energy metabolism and lipid transport.** Proc. Natl. Acad. Sci. USA., 87; 809 - 913.

220. Phillipson B.E., Bolhrock D.W., Connor W.E., Harris W.S. and Illingworth D.R. (1985). **Reduction of plasma lipids, lipoproteins and apoproteins by dietary fish oils in patients with hypertriglyceridemia.** New. Engl. J. Med., 312; 1210 - 1216.

221. Polgár L. (1992). **Structural relationship between lipases and peptidases of the prolyl oligopeptidase family.** FEBS *Lett.*, 311; 281 - 284.

222. Posner I., Wang C-S. and McConathy W.J. (1983). **Kinetics of bovine milk lipoprotein lipase and the mechanism of enzyme activation by apolipoprotein C-II.** Biochemistry, 22; 4041 - 4047.

223. Provan A.B., Hodges E., Smith A.G. and Smith J.L. (1992). **Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA.** J. Clin. Pathol., 45; 763 - 765.

224. Pullinger C.R., Zysow B.R., Hennessy L.K., Frost P.H., Malloy M.J. and Kane J.P. (1993). **Molecular cloning and characteristics of a new apolipoprotein C-II mutant identified in three unrelated individuals with hypercholesterolemia and hypertriglyceridemia.** Human Molecular Genetics., 2 (1); 69 - 74.

225. Quinn D., Shirai K., Jackson R.L. (1982). **Lipoprotein Lipase: Mechanism of action and role in lipid metabolism.** Progress in Lipid Research, 22; 35 - 78.

226. Rebuffé-Scrive M., Eldh J., Hafström L-O. and Björntorp P. (1986). **Metabolism of mammary, abdominal, and femoral adipocytes in women before and after menopause.** Metabolism, 35 (9); 792 - 797.

227. Reina M., Brunzell J.D. and Deeb S.S. (1992). **Molecular basis of familial chylomicronemia: mutations in the lipoprotein lipase and apolipoprotein C-II genes.** J. Lipid Res., 33; 1823 - 1832.

228. Rodrigues B., Spooner M. and Severson D.L. (1992). **Free fatty acids do not release LPL from isolated cardiac myocytes or perfused hearts.** Am. J. Physiol. 262; E216 - E223.

229. Rogers B.B., Alpert L.C., Hine E.A.S. and Buffone G.J. (1990). **Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction.** Am. J. Pathol., 136; 541 - 548.

230. Rojas C., Wang H-H., Lively C.R., Gustafson W.G., Schulz L.O. and McFarland J.T. (1989). **Spectral observation of an acyl-intermediate of lipoprotein lipase.** Biochemistry, 28 (10); 4476 - 4481.

231. Rosenberry T.L. (1975). **Acetylcholinesterase.:** *In* Advances in Enzymology, 43; pg 103 - 218, A. Meister (eds), Interscience, John Wiley and Sons publications, N.Y.

232. Rupp E., Mayer H and Wingender E. (1990). **The promoter of the human parathyroid hormone gene contains a functional cyclic AMP-response element.** Nucl. Acid Res., 18 (19); 5677 - 5683.

233. Sadan N., Drucker M.M., Arber I., Joseph D., Franco S. and Shapiro M.S. (1977). **Type I hyperlipoproteinemia in an 8-day-old infant.** J. of Pediatrics, 90 (5); 775 - 777.

234. Sarkar G., Cassady J., Bottema C.D.K. and Sommer S.S. (1990). **Characterization of polymerase chain reaction amplification of specific alleles.** Anal. Biochem., 186; 64 - 68.

235. Saxena U., Witte L.C. and Goldberg I.J. (1989). **Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids.** J. Biol. Chem., 264 (8); 4349 - 4355.

236. Saxena U., Klein M.G. and Goldberg I.J. (1990). **Identification and characterisation of the endothelial cell surface lipoprotein lipase receptor.** J. Biol. Chem., 266 (26); 17516 - 17521.

237. Saxena U., Klein M.G. and Goldberg I.J. (1990). **Metabolism of endothelial-cell-bound lipoprotein lipase: Evidence for heparan sulfate proteoglycan-mediated internalisation and recycling.** J. Biol. Chem., 265 (22); 12880 - 12886.

238. Saxena U. and Goldberg I.J. (1990). **Interaction of lipoprotein lipase with glycosaminoglycans and apoC-II: Effects of free-fatty acids.** Biochim. Biophys. Acta., 1043; 161-168.

239. Scheidereit C. and Beato M. (1984). **Contacts between hormone receptor and DNA double helix within a glucocorticoid regulatory element of mouse mammary tumor virus.** Proc. Natl. Acad. Sci. USA, 81; 3029 - 3033.

240. Schønheyder F. and Volqvartz K. (1945). **On the affinity of pig pancreas lipase for tricaproin in heterogeneous solutions.** Acta. Physoil. Scand., 9; 57 - 67.

241. Schrag J.D., Li Y., Wu S. and Cygler M. (1991). **Ser-His-Glu forms the catalytic site of the lipase from Geotrichum candidum.** Nature, 351; 761 - 764.

242. Scow R.O., Chernick S.S. and Brindley M.S. (1964). **Hyperlipidemia and ketosis in the pregnant rat.** Am. J. Physiol., 206; 796 - 804.

243. Scow R.O. and Chernick S.S. (1987). **Chapter 6: Role of lipoprotein lipase during lactation**; pg. 149 - 185: *In*, Lipoprotein Lipase, Jayme Borensztajn (eds), Evener Publishers, Inc., Chicago.

244. Scott D.L., White S.P., Otwnowski Z., Yuan W., Gelb M.H. and Sigler P.B. (1990). **Interfacial catalysis: The mechanism of phospholipase A_2.** Science, 250; 1541 - 1546.

245. Segrest J.P., jones M.K., De Loof H., Brouillette C.G., Venkatachalapathi Y.V. and Anantharamaiah G.M. (1992). **The amphipathic helix in the exchangeble apolipoproteins: a review of secondary structure and function.** J. Lipid Res., 33; 141 - 166.

246. Semb H. and Olivecrona T. (1987). **Mechanisms for turnover of lipoprotein lipase in guinea pig adipocytes.** Biochim. Biophys. Acta., 921; 104 - 115.

247. Semenkovich C.F., Chen S-H., Wims M., Luo C-C., Li W-H. and Chan L. (1989). **Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation and evolution.** J. Lipid. Res. 30; 423 - 431.

248. Senda M., Oka K., Brown W.V., Qasba P.K. and Furuichi Y. (1987). **Molecular cloning and sequence of a cDNA coding for bovine lipoprotein lipase.** Proc. Natl. Acad. Sci. USA, 84; 4369 - 4373.

249. Sheffield V.C., Beck J.S., Kwitek A.E., Sandstrom D.W. and Stone E.M. (1993). **The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions.** Genomics, 16; 325 - 332.

250. Shimada Y., Sugihara A., Iizumi T. and Tominaga Y. (1990). **cDNA Cloning and characterisation of *Geotrichum candidum* Lipase II.** J. Biochem., 107; 703 - 707.

251. Shirai K., Matsuoka N., Saito Y. and Yoshida S. (1984). **Post-heparin plasma hepatic triacylglycerol lipase-catalyzed hydrolysis of tributyrin: Effect of lipid interface.** Biochim. Biophys Acta., 795; 1 - 8.

252. Shrag J.D., Li Y., Wu S and Cygler M. (1991). **Multiple crystal forms of lipases from *Geotrichum candidum*.** J. Mol. Biol., 220; 541 - 543.

253. Sibanda B.L., Blundell T.L. and Thornton J.M. (1989). **Conformation of β-hairpins in protein structures: A systematic classification with applications to modelling by homology, electron density fitting and protein engineering.** J. Mol. Biol., 206; 759 - 777.

254. Singh H., Sen R., Baltimore D. and Sharp P.A. (1986). **A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes.** Nature, 319; 154 - 158.

255. Soliday C.L., Flurkey W.H., Okita T.W. and Kolattukudy P.W. (1984). **Cloning and structure determination of cDNA for cutinase, an enzyme involved in fungal penetration of plants.** Proc. Natl. Acad. Sci. USA, 81; 3939 - 3943.

256. Sparkes R.S., Zollman S., Klisak I., Kirchgessner J.G., Komaromy M.C., Mohandas T., Schotz M.C. and Lusis A.J. (1987). **Human genes involved in lipolysis of plasma lipoproteins: Mapping of loci for lipoprotein lipase to 8p22 and hepatic lipase to 15q21.** Genomics, 1; 138 - 144.

257. Sprecher D.L., Taom L., Gregg R.F., Fojo S.S., Wilson D.M., Kashyap M.L. and Brewer H.B. Jr. (1988). **Identification of an apoC-II variant (apoC-$II_{Bethesda}$) in a kindred with apoC-II deficiency and type I hyperlipoproteinemia.** J. Lipid. Res., 29; 273 - 278.

258. Sprecher D.L., Kobayashi J., Rymaszewski M., Goldberg I.J., Harris B.V., Bellet P.S., Ameis D., Yunker R.L., Black D.M., Stein E.A., Schotz M.C. and Wiginton D.A. (1992). **$Trp^{64}\rightarrow$nonsense mutation in the lipoprotein lipase gene.** J. Lipid Res. 33; 859 - 866.

259. Staprans I., Anderson C.D., Lurz F.W. and Felts J.M. (1980). **Separation of a lipoprotein lipase cofactor from the α_1-acid glycoprotein fraction from the urine of nephrotic patients.** Biochim. Biophys. Acta., 617; 514 - 523.

260. Stalenhoef A.F.H., Caspane A.F., Demacker P.N.M., Stouten J.T.J., Lutterman J.A, van't Laor A. (1981). **Combined deficiency of apolipoprotein C-II and lipoprotein lipase in familial hyperchylomicronemia.** Metabolism, 30 (9); 919 - 926.

261. Stein O., Stein Y., Schwartz S.P., Reshef A., Chajek-Shaul T., Ben-Naim M., Friedman G. and Leitersdorf E. (1991). **Expression of lipoprotein lipase mRNA in rat heart is localized mainly to mesenchymal cells as studied by *in situ* hybridization.** Arteriosclerosis and Thrombosis. 11; 857 - 863.

262. Stern D., Nawroth P., Marcum J., Handley D., Kisiel W., Rosenberg R. and Stern K. (1985). **Interaction of antithrombin III with bovine aortic segments: Role of heparin in binding and enhanced anticoagulant activity.** J. Clin. Invest. 75; 272 - 279.

263. Stocks T., Thorn J.A. and Galton D.J. (1992). **Lipoprotein lipase genotypes for a common premature termination codon mutation detected by PCR-mediated site-directed mutagenesis and restriction digestion.** J. Lipid. Res., 33; 856 - 857.

264. Sussman J.L., Harel M., Frolow F., Oefner C., Goldman A., Toker L. and Silman I. (1991). **Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein.** Science, 253; 872 - 879.

265. Suzuki Y., Sekiya T. and Hayashi K. (1991). **Allele-specific polymerase chain reaction:**

A method for amplification and sequence determination of a single component among a mixture of sequence variants. Anal. Biochem., 192; 82 - 84.

266. Takagi A., Ikeda Y., Tsutsumi Z., Shoji T. and Yamamoto A. (1992). **Molecular studies on primary lipoprotein lipase (LPL) deficiency: One base deletion (G^{916}) in exon 5 of the LPL gene causes no detectable LPL protein due to the absence of LPLmRNA transcript.** J. Clin. Invest., 89; 581 - 591.

267. Tas S. (1989). **Strong association of a single nucleotide substitution in the 3'-untranslated region of the apoC-II gene with common hypertriglyceridemia in Arabs.** Clin. Chem., 35/2; 256 - 259.

268. Tashiro J., Kobayashi J., Shirai K., Saito Y., Fukamachi I., Hashimoto H., Nishida T., Shibui T., Morimoto Y. and Yoshida S. (1992). **Effects of substitutions of glycine and asparagine for serine^{132} on activity and binding of human lipoprotein lipase to very low density lipoproteins.** FEBS *Lett.*, 298 (1); 36 - 38.

269. Taskinen M-R. (1987). **Lipoprotein lipase in hypertriglyceridemias**; pg. 201-228: *In*, Lipoprotein Lipase, J. Borensztajn (eds), Evener Publishers Inc., Chicago.

270. Tsujita T., Nakagawa A., Shirai K., Saito Y. and Okuda H. (1984). **Methyl Butyrate-hydrolyzing activity of hepatic triglyceride lipase from rat post-heparin plasma.** J. Biol. Chem., 259 (18); 11215 - 11220.

271. Umek R.M., Friedman A.D. and McKnightS.L. (1991). **CCAAT-Enhancer binding protein: A component of a differentiation switch.** Science, 251; 288 - 292.

272. Vainio P., Virtanen J.A., Kinnunen P.K.J., Voyta J.C., Smith L.C., Gotto A.M. Jr., Sparrow J.T., Pattus F. and Verger R. (1983). **Action of lipoprotein lipase on phospholipid monolayers. Activation by apolipoprotein C-II.** Biochemistry, 22; 2270 - 2275.

273. Vainio P., Virtanen J.A., Kinnunen P.K.J., Gotto A.M. Jr., Sparrow J.T., Pattus F., Bougis P. and Verger R. (1983). **Action of lipoprotein lipase on mixed triacylglycerol/phophatidylcholine monolayers: Activation by apolipoprotein C-II.** J. Biol. Chem., 258 (9); 5477 - 5482.

274. Vannier C. and Ailhaud G. (1989). **Biosynthesis of lipoprotein lipase in cultured mouse adipocytes. II. Processing, subunit assembly and intracellular transport.** J. Biol. Chem., 264 (5); 13206 - 13216.

275. van Tilbeurgh H., Sarda L., Verger R. and Cambillau C. (1992). **Structure of the pancreatic lipase-procolipase complex.** Nature, 359; 159 - 162.

276. van Tilbeurgh H., Egloff M-P., Martinez C., Rugani N., Verger R. and Cambillau C. (1993). **Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography.** Nature, 362; 814 - 820.

277. Vilaró S., Llobera M., Bengtsson-Olivecrona G., Olivecrona T. (1988). **Lipoprotein lipase uptake by the liver: localization, turnover and metabolic role.** Am. J. Physiol., 254; G711 - G722.

278. Vilaró S., Llobera M., Bengtsson-Olivecrona G., Olivecrona T. (1988). **Synthesis of lipoprotein lipase in the liver of newborn rats and localization of the enzyme by immunoflourescence.** Biochem. J., 249; 549 - 556.

279. Vilaró S., Camps L., Reina M., Perez-Clausell J., Llobera M. and Olivecrona J. (1990). **Localization of lipoprotein lipase to discrete areas of the guinea pig brain.** Brain Res., 405; 249 - 253.

280. Volkenandt M., Koch O., Fanin R., Banerjee D., Seger A., Vogel J., Bierhoff E., Heidl G., Neyses L. and Bertino J.R. (1992). **Sequence analysis of polymerase chain reaction amplified t(14;18) chromosomal breakpoints in formalin, paraffin wax embedded follicular lymphoma.** J. Clin. Pathol., 45; 210 - 212.

281. Voyta J.C., Vainio P., Kinnunen P.K.S., Gotto A.M Jr., Sparrow S.T. and Smith L.C. (1983). **Interaction of synthetic N-5-dimethylaminonaphthalene-1-sulfonyl apolipoprotein C-II peptides with lipoprotein lipase.** J. Biol. Chem., 258 (5); 2934 - 2939.

282. Wainscoat J.S., Hill A.V.S., Boyce A.L., Flint J., Hernandez M., Thein S.L., Old J.M., Lynch J.R., Falusi A.G., Weatherall D.J. and Clegg J.B. (1986). **Evolutionary relationships of human populations from an analysis of nuclear DNA polymorphisms.** Nature, 319; 491 - 493.

283. Warth M.R., Arky R.A. and Knopp R.H. (1975). **Lipid metabolism in pregnancy. III. Altered lipid composition in intermediate, very low, low, and high-density lipoprotein fractions.** J. Clin. Endocrinol. Metab., 41; 649 - 655.

284. Wang C-S., Hartsuck J., McConathy W.J. (1992). **REVIEW: Structure and functional properties of lipoprotein lipase.** Biochem. Biophys. Acta. 1123; 1 - 17.

285. Warden C.H., Davis R.C., Yoon M-Y., Yui D.Y., Svenson K., Xia Y-r., Diep A., He K-Y. and Lusis A.J. (1993). **Chromosomal localization of lipolytic enzymes in the mouse: pancreatic lipase, colipase, hormone-sensitive lipase, hepatic lipase and carboxyl ester lipase.** J. Lipid Res., 34; 1451 - 1455.

286. Watts G.F., Morton K., Jackson P. and Lewis B. (1992). **Management of patients with severe hypertriglyceridaemia during prenancy: report of two cases with familial lipoprotein lipase deficiency.** British J. Obstet. Gynaecol., 99; 163 - 166.

287. Wenham P.R., Newton C.R. and Price W.H. (1991). **Analysis of apolipoprotein E genotypes by the amplification refractory mutation system.** Clin. Chem., 32/2; 241 - 244.

288. Wicker-Planquart C. and Puigserver A. (1992). **Primary structure of rat pancreatic lipase mRNA.** FEBS *Lett.*, 296 (1); 61 - 66.

289. Wilson D.E., Edwards C.Q. and Chan I-F. (1983). **Phenotypic heterogeneity in the extended pedigree of a proband with lipoprotein lipase deficiency.** Metabolism, 32 (12); 1107 - 1114.

290. Wilson D.E., Emi M., Iveruis P-H., Hata A., Wu L.L., Hillas E., Williams R.R. and Lalouel J-M. (1990). **Phenotype expression of heterozygous lipoprotein lipase deficiency in the extended pedigree of a proband homozygous for a missense mutation.** J. Clin. Invest., 86; 735 - 750.

291. Wilson D.E., Hata A., Kwong L.K., Lingam A., Shuhua J., Ridinger D.N., Yeager C., Kaltenborn K.C., Iveruis P-H. and Lalouel J-M. (1993). **Mutations in exon 3 of the lipoprotein lipase gene segregating in a family with hypertriglyceridemia, pancreatitis, and non-insulin-dependent diabetes.** J. Clin. Invest., 92; 203 - 211.

292. Winkler F.K., D'Arcy A., Hunziker W. (1990). **Structure of human pancreatic lipase.** Nature. 343; 771 - 774.

293. Wion K.L., Kirchgessner T.G., Lusis A.J., Schotz M.C. and Lawn R.M. (1987). **Human lipoprotein lipase complementary DNA sequence.** Science, 235; 1638 - 1641.

294. Wittwer C.T., Marshall B.C., Reed G.H. and Cherry J.L. (1993). **Rapid cycle allele-specific amplification: Studies with the Cystic Fibrosis ΔF_{508} locus.** Clin. Chem., 39/5; 804 - 809.

295. Wong H., Davis R.C., Nikazy J., Seebart K.E. and Schotz M.C. (1991). **Domain exchange: Characterization of a chimeric lipase of hepatic lipase and lipoprotein lipase.** Proc. Natl. Acad. Sci. USA, 88; 11290 - 11294.

296. Wong C-S. and Hartsuck J.A. (1993). **Bile salt-activated lipase. A multiple function lipolytic enzyme.** Biochim. Biophys. Acta., 1166; 1 - 19.

297. Xiong W., Li W-H., Posner I., Yamamura T., Yamamoto A., Gotto A.M. Jr. and Chan L. (1991). **No severe bottleneck during human evolution: Evidence from two apolipoprotein C-II deficiency alleles.** Am. J. Hum. Genet., 48; 383 - 389.

298. Yamamura T., Sudo H., Ishikawa K. and Yamamoto A. (1979). **Familial Type I hyperlipoproteinemia caused by apolipoprotein C-II deficiency.** Atherosclerosis, 34; 53 - 65.

299. Yang C-Y., Gu Z-W., Yang H-X., Rohde M.F., Gotto A.M. Jr. and Pwnall H.J. (1989). **Structure of bovine milk lipoprotein lipase.** J. Biol. Chem., 264 (28); 16822 - 16827.

300. Yost T.J. and Eckel R.H. (1992). **Regional similarities in the metabolic regulation of adipose tissue lipoprotein lipase.** Metabolism, 41 (1); 33 - 36.

301. Zechner R. (1990). **Rapid and simple isolation procedure for lipoprotein lipase from human milk.** Biochim. Biophys Acta., 1044; 20 - 25.

302. Zinder O., Hamosh M., Fleck R.C. and Scow R.O. (1974). **Effect of protactin on lipoprotein lipase in mammary gland and adipose tissue of rats.** Am. J. Physiol., 226 (3); 744 - 748.

303. Zsoldos S.J. and Heinemann H.O. (1964). **Lipolytic activity of rabbit aorta *in vitro*.** Am. J. Physiol., 206; 615 - 617.