Studies on the Structural and Metabolic Heterogeneity in Low Density Lipoprotein

Muriel Caslake, B.Sc. (Belfast)

Department of Pathological Biochemistry
Royal Infirmary University/NHS Trust
Glasgow

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow, UK.

Submitted October 1996

© Muriel Caslake 1996

ProQuest Number: 13832496

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 13832496

Published by ProQuest LLC (2019). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Thesis
10585
Copy 1

GLASGOW
UNIVERSITY
LIBRARY

Abstract

This thesis sought to investigate the relationship between structural and metabolic heterogeneity in LDL and examine the influence of plasma triglyceride on the properties of this lipoprotein.

A technique to quantify individual LDL subfractions was developed which was superior to previously published methods and was employed to characterise three LDL subfractions in normals and in subjects with coronary disease, primary hyperlipidaemia and the secondary hyperlipidaemia observed in non insulin dependent diabetes mellitus and the nephrotic syndrome. In a case controlled study, a sevenfold increased risk of myocardial infarction was found to be associated with a concentration of small dense LDL III > 100mg/100ml. Subjects with hypertriglyceridaemia, combined hyperlipidaemia and the secondary hyperlipidaemia were found to exhibit an atherogenic lipoprotein phenotype of raised plasma triglyceride, low HDL cholesterol and a preponderance of small dense LDL. The data in the entire cohort was pooled and multivariate analysis revealed that plasma triglyceride was the most important independent predictor of LDL III.

Pharmacological modulation of the LDL profile was investigated in normals and in a variety of hyperlipidaemic states using the main classes of lipid lowering drugs- resins, nicotinic acid derivatives, fibrates and statins. Those drugs which act through the lowering of plasma triglyceride reduced the concentrations of LDL III and those whose mode of action is by activation of the LDL receptor, reduced the concentrations of the larger LDL I and LDL II species. An important finding was in the unexpected beneficial effect of statins in combined hyperlipidaemia when LDL III concentrations were lower than expected for the plasma triglyceride level.

Kinetic heterogeneity of LDL was studied by undertaking radioactive turnovers in normals and hypercholesterolaemic subjects who were given triglyceride lowering therapy. A two pool model for plasma LDL was developed to explain the radioactive decay curves of plasma and urine. One pool (pool A) was rapidly removed by the LDL receptor while the other (pool B) was more slowly removed and its synthesis was positively related to plasma triglyceride. The kinetics of apo-LDL were further examined in subjects with moderate hypertriglyceridaemia. Here it was necessary to expand the model to a third plasma pool (pool C) to account for an LDL species which was rapidly catabolised by receptor-independent mechanisms. A high level of receptor -independent catabolism was associated with an increased ratio of LDL III to LDL II. Pool C was seen in subjects with plasma triglyceride >2.5mmol/l in whom there was the presence of an abnormally small, dense LDL.

On the basis of these observations and published VLDL metabolic studies, a model was postulated in which pool A LDL was produced from the delipidation of hepatic lipoproteins secreted in the S_f 0-60 density range and circulated in the form LDL I and LDL II. Pool B

LDL, on the other hand, was derived from large triglyceride-rich VLDL of S_f 60-400 and circulates in most subjects in the form of LDL II. Neutral lipid exchange and lipolysis via the agency of hepatic lipase converts a proportion of pool B LDL II to LDL III, depending on the level of plasma triglyceride and the activity of the lipase enzyme.

The data presented in this thesis are in line with current concepts of the atherogenicity of small dense LDL and provide evidence that it is an independent risk factor for coronary disease. It demonstrates that the properties of LDL are strongly influenced by its pedigree. Furthermore it shows that there are currently available drugs that are capable of altering not only the quantity of LDL but also its quality and offer hope in the continuing problem of tackling coronary heart disease.

Contents

Page

Abstract......II
Table of Contents......IV
List of Tables......IX
List of Figures......XII
Acknowledgements......XIV
Author's Declaration......XV
Dedication......XVI

Chapter 1 Introduction

1.1 Coronary Heart Disease and Plasma Lipids......1
1.2 Lipoprotein Structure and Function......2
1.2.1 Chylomicrons
1.2.2. Very Low Density Lipoprotein
1.2.3 Intermediate Density Lipoprotein
1.2.4 Low Density Lipoprotein
1.2.5 High Density Lipoprotein
1.2.6 Lipoprotein (a)
1.3 Lipoprotein Metabolism......9
1.3.1 Intestinal Lipoprotein Secretion
1.3.2 Intravascular Lipolysis of Chylomicrons
1.3.3 Metabolism of Chylomicron Remnants
1.3.4 Lipoprotein Assembly in the Liver
1.3.5 Metabolism of Apo B Containing Lipoproteins
1.3.6 High Density Lipoprotein and Reverse Cholesterol Transport
1.4 Receptors in Lipoprotein Metabolism......15
1.4.1 The LDL Receptor and its Ligands
1.4.2 LDL Receptor Related Protein Receptor (LRP)
1.4.3 VLDL Receptor
1.4.4 Scavenger Receptors
1.5 Structural Heterogeneity in Low Density Lipoprotein......18
1.5.1 Historical Concept of Heterogeneity
1.5.2 Classification of LDL Subfractions
1.5.3 LDL Heterogeneity in Relation to Coronary Heart Disease
1.5.4 Determinants of LDL Subfraction Distribution
1.5.5 Properties of LDL Subfractions and Relationship to Coronary Heart Disease
1.5.6 LDL Receptor Binding and LDL Subfractions
1.6 Metabolic Heterogeneity of Low Density Lipoprotein......28
1.7 Aims and Objectives......32

Chapter 2 Materials and Methods

2.1 Materials......33
2.2 Lipid Analyses......33
2.2.1 Cholesterol
2.2.2 Free Cholesterol
2.2.3 Triglyceride
2.2.4 Phospholipid
2.3 Protein and Apolipoprotein Assays......34
2.3.1 Protein
2.3.2 Apolipoprotein AI
2.3.3 Apolipoprotein AII
2.3.4 Apolipoprotein B
2.3.5 Lipoprotein (a)
2.3.6 Apolipoprotein E Phenotyping
2.4 Lipoproteins......36
2.4.1 β Quantification
2.4.2 Isolation of $VLDL_1(S_f60\text{-}400)$*, VLDL2*$(S_f20\text{-}60)$*, IDL*$(S_f12\text{-}20)$*, LDL*$(S_f0\text{-}12)$
2.4.4 Preparation of LDL by Zonal Ultracentrifugation
2.4.5 Isolation of VLDL, IDL, LDL and HDL by Sequential Flotation Ultracentrifugation
2.4.6 Compositional Analysis of Lipoproteins
2.4.7 HDL Subfractions by Analytical Ultracentrifugation
2.4.8 LDL Subfractions by Gradient Gel Electrophoresis
2.4.9 LDL Subfraction Analysis by Density Gradient Ultracentrifugation
2.5 Electron microscopy......41
2.6 LDL Turnover Protocols......42
2.6.1 LDL Preparation
2.6.2 Labelling of LDL
2.6.3 Modification with 1,2-cyclohehanedione
2.6.4 Sterilisation and Calculation of Injection Dose
2.6.5 Subject Preparation
2.6.6 Injection
2.6.7 Sampling Protocol
2.6.8 Data Collection and Handling
2.6.9 Calculation of Apo-LDL Pool Size
2.6.10 Kinetic Analysis
2.7 Ethical Implications......44
2.8 Statistical Analysis......44

Chapter 3 Epidemiology of Low Density Lipoprotein Subfractions

3.1 Introduction......45

3.2 Methods......46
3.2.1 Gradient gel electrophoresis
3.2.2 Zonal Ultracentrifugation
3.2.3 Density Gradient Ultracentrifugation
3.2.4 Development of final LDL Subfractionation Method
3.3 Results......47
3.3.1 Comparison of Methods
3.3.2 Characterisatics of final Density Gradient Ultracentrifugation Method
3.3.3 LDL Subfraction Profiles in Normal and Hyperlipidaemc Subjects
3.3.4.Regulation of LDL Concentration, Composition and Subfractions
3.4 Discussion......67

Chapter 4 Modification of LDL Subfractions by Hypolipidaemic Agents

4.1 Introduction......71
4.2 Method......72
4.2.1 Protocol
4.2.2 Subjects
4.3 Results......73
4.3.1 Bile-acid Sequestrants
4.3.2 HMG-CoA Reductase Inhibitors
4.3.3 Nicotinic Acid Derivatives
4.3.4 Fibrates
4.3.5 Combination Therapies
4.3.6 Influence of Triglyceride on LDL III
4.4 Discussion......92

Chapter 5 Metabolic Heterogeneity of Low Density Lipoprotein in Normals

5.1 Introduction......97
5.2 Methods......98
5.2.1 Protocol
5.2.2 Subjects
5.2.3 Kinetic Analysis
5.3 Results......103
5.3.1 Lipids, Lipoproteins and Apolipoproteins
5.3.2 Receptor-mediated and Receptor-independent Apo LDL Metabolism
5.3.3 Two compartmental Model of Apo LDL Metabolism
5.7 Discussion......112

Chapter 6 Fibrates and LDL Metabolism in Moderate Hypercholesterolaemia

6.1 Introduction........120
6.2 Protocol........121
6.3 Recruitment and Conduct of the Study........121
6.4 Subjects........121
6.5 Fenofibrate........122
6.5 1 Lipids and Lipoproteins and Apoproteins
6.5.2 Receptor-mediated and Receptor-independent Metabolism of LDL
6.5.3 Two Compartment Model of Apo B Metabolism
6.6 Ciprofibrate........129
6.6.1 Lipids, Lipoproteins and Apolipoptoteins
6.6.2 Receptor-mediated and Receptor-independent Metabolism of LDL
6.6.3 Two Compartment Model of Apo B Metabolism
6.7 Discussion........137

Chapter 7 Ciprofibrate and LDL Metabolism in Moderate Hypertriglyceridaemia

7.1 Introduction........142
7.2 Method........143
7.2.1 Protocol
7.2.2 Recruitment and Conduct of the Study.
7.2.3 Kinetic Analysis
7.2.4 Subjects
7.3 Results........147
7.3.1 Lipids, Lipoproteins, Apolipoproteins and HDL Subfractions
7.3.2 ApoB Lipoprotein Subfractions
7.3.3 Receptor-mediated and Receptor-independent Apo LDL Metabolism
7.3.4 Three compartmental Model of Apo LDL Metabolism
7.4 Discussion........156

Chapter 8 Impact of Heterogeneity in LDL Structure and Metabolism

8.1 LDL Heterogeneity and Apo-B Metabolism........158
8.2 Relating LDL Structure to Metabolism........161
8.3 Correction of an Atherogenic Lipoprotein Phenotype by Drug Therapy........162
8.4 Directions for Future Research........162

References......164

Appendix 1......193

Glossary......197

List of Tables

Page

1.1. Apolipoproteins of Human Plasma....4
1.2 Physical Properties of the Plasma Lipoprotein Classes....4
1.3 Composition of Normal Plasma Lipoproteins....5
1.4 Classification of LDL Subfractions....20
2.1 Centrifugation Conditions for VLDL-LDL Isolation....37
3.1 Particle Size Distribution of LDL....48
3.2 Size and Density of LDL Subfractions....51
3.3 Apoprotein Content of LDL Subfractions....52
3.4 Lipoprotein Composition of LDL Subfractions....53
3.5 Lipids, Lipoproteins and Apoproteins in Normal Subjects....55
3.6 LDL Subfractions in Normal Subjects....56
3.7 Lipids, Lipoproteins and Apoproteins in Subjects with Coronary Disease....57
3.8 LDL Subfractions in Subjects with Coronary Disease....58
3.9 Relative Coronary Risk associated with LDL III > 100mg/100ml, Triglyceride > 1.5 mmol/l and Cholesterol:HDL cholesterol ratio > 6.0....58
3.10 Lipids, Lipoproteins and Apoproteins in Subjects with Primary Moderate Hyperlipidaemia....60
3.11 LDL Subfractions in Subjects with Primary Moderate Hyperlipidaemia....61
3.12 Lipids, Lipoproteins and Apoproteins in Subjects with Familial Hypercholesterolaemia....62
3.13 LDL Subfractions in Subjects with Familial Hypercholesterolaemia....62
3.14 Lipids, Lipoproteins and Apoproteins in Subjects with Secondary Hyperlipidaemia....63
3.15 LDL Subfractions in Subjects with Secondary Hyperlipidaemia....64
3.16 Comparison of Low and High Triglycerides....65
3.17 Correlations with LDL Subfractions....66
3.18 Determinants of LDL Subfraction Concentration....66
4.1 Effect of Cholestyramine on Lipids in Normal Subjects....73
4.2 Effect of Cholestyramine on LDL Subfractions in Normal Subjects....74
4.3 Effect of Colestipol on Lipids in CABG Subjects....74
4.4 Effect of Colestipol on LDL Subfractions in CABG Subjects....75
4.5 Effect of Simvastatin on Lipids in Moderate Hypercholesterolaemia....76
4.6 Effect of Simvastatin on Lipoprotein Subfractions in Moderate Hypercholesterolaemia....77
4.7 Effect of Simvastatin on Lipids in Combined Hyperlipidaemia....77
4.8 Effect of Simvastatin on LDL Subfractions and Apoproteins in Combined Hyperlipidaemia....78
4.9 Effect of Atorvastatin on Lipids in Combined Hyperlipidaemia....79

4.10 Effect of Atorvavastatin on LDL Subfractions and Apoproteins in Combined Hyperlipidaemia......79
4.11 Effect of Acipimox on Lipids in Normal Subjects......80
4.12 Effect of Acipimox on LDL Subfractions in Normal Subjects......81
4.13 Effect of Acipimox on Lipids in Moderate Hypercholesterolaemia......81
4.14 Effect of Acipimox on Lipoprotein Subfractions in Moderate Hypercholesterolaemia......82
4.15 Effect of Acipimox on Lipids in Combined Hyperlipidaemia......83
4.16 Effect of Acipimox on Lipoprotein Subfractions in Combined Hyperlipidaemia....83
4.17 Effect of Fenofibrate on Lipids and Apoproteins in Moderate Hypercholesterolaemia......84
4.18 Effect of Fenofibrate on Lipoprotein Subfractions in Moderate Hypercholesterolaemia......85
4.19 Effect of Fenofibrate on Lipids, Lipoproteins and Apoproteins in Non Insulin Dependent Diabetes Mellitus......85
4.20 Effect of Fenofibrate on Lipoprotein Subfractions in Non Insulin Dependent Diabetes Mellitus......86
4.21 Effect of Ciprofibrate on Lipids, Lipoproteins and Apoproteins in Moderate Hypercholesterolaemia......87
4.22 Effect of Ciprofibrate on Lipoprotein Subfractions in Moderate Hypercholesterolaemia......87
4.23 Effect of Ciprofibrate on Lipids, Lipoproteins and Apoproteins in Moderate Hypertriglyceridaemia......88
4.24 Effect of Ciprofibrate on Lipoprotein Subfractions in Moderate Hypertriglyceridaemia......89
4.25 Effect of Acipimox and Cholestyramine in Combination on Lipids in Moderate Hypercholesterolaemia......89
4.26 Effect of Acipimox and Cholestyramine in Combination on Lipoprotein Subfractions in Moderate Hupercholesterolaemia......90
4.27 Effect of Colestipol and Simvastatin in Combination on Lipids in Moderate Hypercholesterolaemia......91
4.28 Effect of Colestipol and Simvastatin in Combination on LDL Subfractions in Moderate Hypercholesterolaemia......91
5.1 Summary of Subjects Characteristics......99
5.2 Characteristics Summarised by Quintile......104
5.3 Plasma Lipid and Lipoproteins by Quintile......105
5.4 Apo-LDL and HDL Subfraction Concentrations by Quintile......105
5.5 Compostion of LDL by Quintile......106
5.6 Matthews Kinetic Parameters by Quintile......107
5.7 Relationships between Plasma Lipids, Lipoproteins, Body Weight and Apo-LDL Kinetic Parameters......108
5 .8 Effect of ApoE Phenotype on LDL Kinetic Parameters......108
5.9 Kinetic Parameters of Pool A Apo-LDL by Quintile......110
5.10 Kinetic Parameters of Pool B Apo-LDL by Quintile......110

5.11 Relationships between Plasma Lipids, Lipoproteins, Body weight and Kinetic Parameters of Pools A and B Apo-LDL....111
6.1 Fenofibrate StudyPatient Characteristics....121
6.2 Ciprofibrate Study Patient Characteristics....122
6.3 Lipids and Lipoproteins of Subjects on Fenofibrate Therapy....123
6.4 Apoproteins and HDL Subfractions of Subjects on Fenofibrate Therapy....124
6.5 LDL Composition in Subjects on Fenofibrate Therapy....124
6.6 LDL Subfractions on Fenofibrate Therapy....125
6.7 Matthews Kinetic Parameters of Apo-LDL on Fenofibrate Therapy....126
6.8 Kinetics of Pools A and B Apo-LDL on Fenofibrate Therapy....129
6.9 Lipids and Lipoproteins of Subjects on Ciprofibrate Therapy....130
6.10 Apoproteins and HDL Subfractions of Subjects on Ciprofibrate Therapy....131
6.11 LDL Composition in Subjects on Ciprofibrate Therapy....132
6.12 LDL Subfractions in Subjects on Ciprofibrate Therapy....133
6.13 Matthews Kinetic Parameters of Apo-LDL on Ciprofibrate Therapy....134
6.14 Kinetic Parameters of Pools A and B apo-LDL on Ciprofibrate Therapy....137
7.1 ABC Model Catabolic Rates....145
7.2 Characteristics of the Subjects....146
7.3 Lipid and Lipoprotein Levels....147
7.4 Apoprotein and HDL Subfraction Concentrations....148
7.5 Masses of Lipoprotein Subfractions (S_f 0-400)....149
7.6 Composition of Lipoprotein Subfractions (S_f 0-400)....150
7.7 LDL Subfraction Concentrations....151
7.8 Matthews Kinetic Parameters of Apo-LDL....153
7.9 Distribution of apo-LDL Mass in Pools A, B and C....155
7.10 Synthesis into Pools A, B and C....156

Appendix to Chapter5

1 Individual Lipid and Lipoprotein Levels in Normal Subjects....114
2 Individual Apo-LDL and HDL Subfraction Concentrations in Normal Subjects..115
3 Lipoprotein Composition of Apo-LDL in Normal Subjects....116
4 Kinetic Parameters by Matthews Analysis in Normal Subjects....117
5 Kinetic Parameters of Mathematical Pool A in Normal Subjects....118
6 Kinetic Parameters of Mathematical Pool B in Normal Subjects....119

List of Figures

Page

1.1 Structure of Very Low Density Lipoprotein....3
1.2 Exogeneous Lipid Metabolism....6
1.3 Role of Enzymes and Receptors in Endogeneous Lipid Metabolism....13
1.4 LDL Subclass Patterns A and B obtained by Gradient Gel Electrophoresis....19
1.5 Foster's A, B, C and D Models of LDL Kinetics....29
2.1 LDL Subfraction Profiles....41
3.1 LDL Subfraction Profiles by Gradient Gel Electrophoresis....48
3.2 Density Gradient formed in swing-out Centrifuge Tube....49
3.3 Relationship between Particle Size obtained by Gradient Gel Electrophoresis and Density Gradient Ultracentrifugation....50
3.4 Gradient Gel of Purified Subfractions....51
3.5 Electron Micrographs of Purified LDL I, LDL II and LDL III....52
3.6 Effect of Storage and Prolonged Centrifugation on LDL Subfraction Profile....54
3.7 Frequency Distribution Plots of LDL Subfractions....59
3.8,b Relationship of Plasma Triglyceride with LDL I, LDL II and LDL III....65
3.9 LDL Subfraction Profiles....68
3.10 Proposed Model for Formation of LDL III....70
4.1 Plasma Triglyceride and LDL III on Statin and Fibrate Therapy....92
4.2 Pharmacological Regulation of LDL Subfractions....93
5.1 Relationship between LDL Cholesterol and Plasma Triglyceride....97
5.2 Matthews Analysis of Plasma Radioactivity Curve....100
5.3 Two pool Model of LDL Kinetics....101
5.4 Urine to Plasma Radioactivity Ratios....102
5.5 Computer Simulations of Urine to Plasma Radioactivity Ratios....103
5.6 Plasma and Urine Radioactivity Decay Curves of Native and Cyclohexanedione-modified LDL....109
6.1 Plasma and Urine Radioactive Decay Curves at Baseline and on Fenofibrate....127
6.2 Urine and Plasma Radioactivity Ratios on Fenofibrate....128
6.3 Urine to Plasma Radioactivity Ratios for Native LDL at Baseline and on Ciprofibrate Therapy....135
6.4 Plasma and Urine Radioactivity Decay Curves at Baseline and on Ciprofibrate...136
6.5 Mean LDL Kinetic Parameters in Normal Subjects, Subjects off and on Fenofibrate and Ciprofibrate....139
6.6 Relationship Between Plasma Triglyceride and Pool B Synthesis....140
7.1 Urine to Plasma Radioactivity Ratios at Baseline and on Ciprofibrate (II)....144
7.2 Plasma Decay Curves at Baseline (Cipro II)....145
7.3 ABC Kinetic Pool of LDL Kinetics....146

7.4 LDL Subfractions at Baseline and on Ciprofibrate (II)..152
7.5 Receptor-independent LDL Catabolism and Ratio of LDL III to LDL II.............154
8.1 Plasma Triglyceride and Fractional Catabolic Rate of LDL..................................159
8.2 Association of $VLDL_1$ and $VLDL_2$ with A and B LDL Kinetic PLasma Pools.....160
8.3 Hepatic Lipoprtein Metabolism...160
8.4 Linking Structural and Metabolic Heterogeneity in LDL......................................161

Acknowledgements

The nature of the work presented in this thesis meant the co-operation of numerous colleagues in patient care and recruitment, technical and moral support. I wish to thank them all for making this thesis an example of true scientific collaboration. In particular I acknowledge:

Professor Jim Shepherd for providing excellent facilities and giving me this opportunity.

Professor Chris Packard, my supervisor, a model example of a good researcher in his intellect and how things should be done. I wish to thank him for his encouragement and patience, for teaching me how to acquire the skills I lacked, for his belief in me and even for making me carry out those oral presentations 'for my own good'.

Dorothy Bedford for being a constant source of encouragement and always being there.

Michael McConnell and Philip Stewart for their assistance in computing and patience with a learner.

Lipid Research staff (*Jacqui Louden, Liz Murray, Linda McCusker, Julie MacKenzie, Grace Stewart, Jim Thompson and May Watkins*) for assistance with lipoprotein compositions and apo E phenotyping.

Lipid Research staff, past and present, for discussions and moral support at conferences, *(Vian Anber, Marina Cuchel, Lorne Forster, Dilys Freeman, John Millar, Chee-eng Tan and Tim Watson).*

Bruce Griffin who worked with me on the early LDL structural studies.

Nancy Thompson for always being so helpful and keeping intrusions to the minimum during my appointments with Chris.

Dairena Gaffney and Jackie Reid for identifying FDB patients.

Anne Bell and staff in Lipid Routine Section, Institute of Biochemistry for carrying out βquant analyses.

Clinical staff listed below involved in patient care and recruitment. In particular I wish to acknowledge *Professor Ross Lorimer and Dr Denis O'Reilly* for giving permission for recruitment to take place at the Lipid Clinic, Glasgow Royal Infirmary University/NHS Trust, *Allan Gaw* who was involved in many of the early studies and *John Hinnie* for his persistence in recruiting the hypertriglyceridaemic subjects. *(John Betteridge, Chris Deighan, Moira Devine, Beth Farish, Mike Feher, Irene Hendry, Grace Lindsay, Margaret Sinnott, Sue McCarthy, John Series, Graeme Tait, Margaret Towland, Brigitte Yip, David Walsh)*

Patients and volunteers for giving so freely of their time and blood without any obvious benefit to themselves.

Gifts of drugs and educational grants from *Bristol Myers Squibb, Farmitilia, Fournier, Merck, Sharpe and Dohme, Sanofi-Winthrop and Parke-Davis.*

British Heart Foundation for funding.

Author's Declaration

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

Muriel Caslake, October 1996

Dedication

I dedicate this work to my family, especially my mother and father who encouraged me to have an enquiring mind, and to my husband Chuck and children Robert and Sarah-Jane for their love and constant support.

Chapter 1 Introduction

1.1 Coronary Heart Disease and Plasma Lipids

Coronary heart disease (CHD) continues to be a major cause of death in most industrialised populations. However, evidence that the burden of CHD can be reduced is seen in countries such as the USA and Finland where appropriate preventative strategies have been successfully implemented. Epidemiological studies have identified a number of risk factors for first events and recurrent CHD. Modifiable factors include hypertension, smoking, lack of exercise, obesity and abnormal lipid levels, while other risk factors are age, sex, family history of CHD and diabetes mellitus. Men are affected by CHD at an earlier age than women but after the menopause women lose the protective effect of oestrogen and the incidence rises. Among these risk factors abnormal blood lipid profiles have a central position. The largest prospective study was the Multiple Risk Factor Intervention Trial (MRFIT) which provided evidence that blood pressure, smoking, dyslipidaemia and glucose intolerance explained the majority of CHD cases (Stamler *et al*, 1986). This study demonstrated that the relationship between CHD death and serum cholesterol was continuous, graded and independent of smoking and hypertension (Martin *et al*, 1986). In countries where habitual fat intake is low, mortality from CHD is low and total life expectancy exceeds that in many Western countries. However a positive correlation between CHD and cholesterol exists even in countries such as China where average cholesterol levels are low, ranging from 2.4 to 4.2 mmol/l (Chen *et al*, 1991). Law et al (1994) recently highlighted the previous underestimation of the link between cholesterol and CHD risk by re-analysing ten prospective and three international studies based on single cholesterol measurements and correcting for regression dilution and surrogate dilution effects. He estimated that a 10 % decrease in cholesterol (0.6 mmol/l) would be associated with a reduction in incidence of CHD events in the cohort studies of 54% at age 40 years, 39% at age 50 and 27% at 60.

Epidemiological studies carried out over the last three decades have consistently shown an association between elevated levels of plasma triglyceride and an increased risk of CHD. The interpretation of this relationship has been controversial as some studies found that following adjustment for other risk factors, in particular HDL cholesterol, triglyceride failed to be an independent predictor of disease. Others such as the Caerphilly and Framingham studies (Bainton *et al*, 1992 Castelli *et al*, 1986) found that triglyceride persisted as a risk factor even when other potential confounding factors were taken into account. Austin in 1991 comprehensively re-evaluated the epidemiological data and suggested that the strength of the association between triglyceride and CHD has been significantly underestimated because of the biological variation of this plasma lipid and shortcomings in the multivariate analysis approach. In a recent meta-analysis she confirmed an independent triglyceride/CHD link (Hokannson & Austin, 1993a). Data from the Helsinki Heart Study (Manninen *et al*, 1992) and the Prospective Cardiovascular Munster study (PROCAM) (Assmann & Schulte, 1992) highlight the particularly high CHD risk associated with moderately raised triglycerides, low HDL cholesterol and an

LDL:HDL cholesterol ratio greater than 5. Furthermore in the Helsinki Heart Study most benefit from therapy with gemfibrozil was observed in this high risk group.
Further support for the benefits of cholesterol-lowering have come from the angiographically - monitored trials which have demonstrated arrested progression and even regression of coronary lesions but the most definitive evidence has been obtained recently in trials using 3-hydroxy 3 methylglutaryl coenzyme A (HMGCo A) reductase inhibitors. The Scandinavian simvastatin survival study (4S) provided proof of the effects of cholesterol lowering not only on CHD but also on overall mortality of high risk individuals (Scandinavian Simvastatin Survival Study Group, 1994). Over 5.4 years, simvastatin produced mean changes in total cholesterol, LDL cholesterol and HDL cholesterol of -25%, -35% and +8% respectively. The treated group had significantly less total mortality (8% vs 12%) and fewer coronary events (19% vs 28%). The West of Scotland Coronary Prevention Study (WOSCOPS) showed that treatment with pravastatin led to a 22% reduction in overall mortality and a 30% reduction in fatal and non-fatal myocardial events in 6,595 middle aged men with no previous coronary disease but raised plasma and LDL cholesterol (Shepherd *et al*, 1995).
In a placebo controlled trial Coronary Atherosclerosis Intervention Trial (BCAIT), bezafibrate has recently been shown to induce atheroma regression in 42 men who had survived a first coronary below the age of 45 (Ericsson *et al*, 1996). The authors compared their results with two recent regression trials involving statin therapy and noted similar effects on lesion regression despite different effects on plasma lipids. The statin studies (Regression Growth Evaluation Statin Study (REGRESS) and Multicentre Anti-Atheroma Study (MAAS)) showed reductions of 20-23% in total cholesterol, 30% in LDL cholesterol and 7-18% in plasma triglyceride whereas in BCAIT the major lipid reduction was a 31% drop in serum triglyceride with little change in LDL.
Although there are minor variations among the various national and international guidelines for management of dyslipidaemia, there is now considerable agreement on the need for risk stratification and the treatment of lipid risk factors in the context of individual global CHD risk. LDL cholesterol remains the major target for therapy but the importance of HDL cholesterol and triglyceride is acknowledged. Clearer guidelines on the management of hypertriglyceridaemia will become possible when further intervention data becomes available.

1.2 Lipoprotein Structure and Function

It is estimated that as much as 80% of the carbon and hydrogen in energy substrates used by the human body passes through lipid intermediaries at some point before being oxidised to terminal products. Also there is constant bi-directional movement of the lipid constituents of membranes between cells and blood. Transport of lipid in plasma is chiefly accomplished by a system of lipid:protein complexes (lipoproteins) which generally take the form of spherical microemulsion particles, comprised of a core region containing the hydrophobic cholesteryl esters and triglycerides in variable proportions surrounded by a mixed monolayer of phospholipids and unesterified cholesterol. The charged head groups of the phospholipid and the free hydroxyl group of cholesterol associate with the water in the surrounding aqueous medium, while the hydrophobic fatty acid chains of the fatty acids

and the sterol ring structure are in contact with each other and with the hydrophobic core lipids. Fig. 1.1 shows the structure of a typical lipoprotein particle.

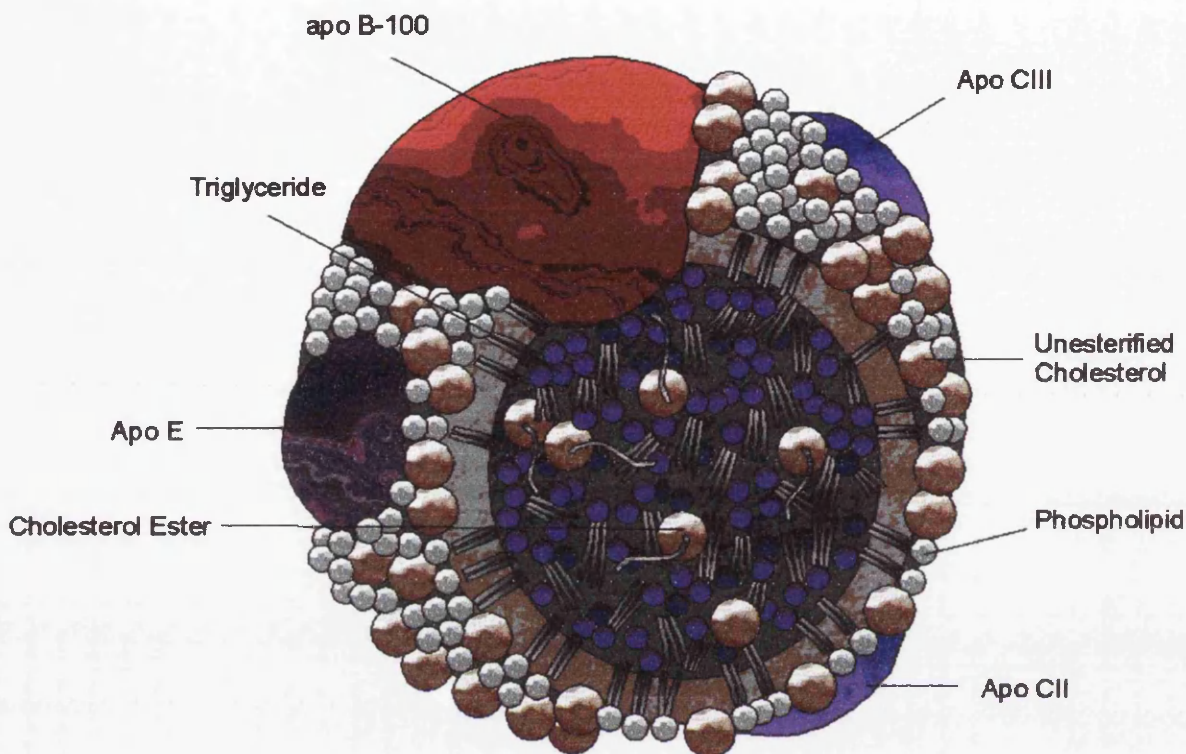

Fig. 1.1 Structure of Very Low Density Lipoprotein
The major lipid component of the nonpolar core is triglyceride and cholesteryl ester while the surface is composed of unesterified cholesterol, phospholipid and apoproteins B, E, C I, C II and CIII.

A number of specific proteins (apolipoproteins) interact with the lipid microemulsion, associated primarily with the surface monolayers (table 1.1). A group of seven proteins with a relatively small molecular weight (AI, AII, AIV, CI, CII, CIII, E) are members of a gene family characterised by tandem repeats of codons (Luo *et al*, 1986). These proteins have several sequences that form amphipathic helices and are readily exchangeable among the lipoprotein species. In contrast, the B apolipoprotein group is comprised of two very large proteins (apo B100, apo B48) which are the products of a single gene. They form very few amphipathic helices and contain a large amount of β sheet structure. At certain points on the phospholipid monolayer they displace surface lipids and interact directly with the core lipids. Throughout their lifetime in plasma the B apoproteins remain with the lipoprotein particles on which they were secreted and so are a useful marker for

investigation of the metabolism of those particular lipoproteins. A group of relatively hydrophobic circulating proteins are associated with the lipoprotein system. They include apo D, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein which function in lipid exchange mechanisms.

Table 1.1. Apolipoproteins of Human Plasma

Apoprotein	*Average concentration in plasma (mg/dl)*	*Aminoacid molecular weight*
Apo AI	130	29,016
Apo AII	40	8,700
Apo AIV	40	44,465
Apo B100	85	512,723
Apo B48	variable	240,800
Apo CI	6	6,630
Apo CII	3	8,900
Apo CIII	12	8,800
Apo D	10	19,000
Apo E	5	34,145

Generally, there are three roles for plasma lipoproteins in lipid transport , namely ; transport of triglyceride to body tissues (the triglyceride-rich lipoproteins),delivery of cholesterol to the tissues (via low density lipoprotein) and retrieval of lipids from the peripheral tissues (via high density lipoprotein).

Table 1.2 Physical Properties of the Plasma Lipoprotein Classes

Class	*Density (g/ml)*	*Sf value*	*Diameter (nm)*	*Molecular weight*	*Electrophoretic Mobility*
Chylomicron	<0.93	>400	75-1,200	$50\text{-}1{,}000 \times 10^6$	origin
VLDL	0.93-1.006	20-400	30-80	$10\text{-}80 \times 10^6$	pre-β
IDL	1.006-1.019	12-20	25-35	$5\text{-}10 \times 10^6$	slow preβ
LDL	1.019-1.063	0-12	18-25	$2\text{-}3 \times 10^6$	β
HDL	1.063-1.21	-	5-12	$65\text{-}386 \times 10^3$	α

The separation of the individual plasma lipoproteins was first carried out in the 1950's (Gofman *et al*, 1950; Turner *et al*, 1951) employing the technique of preparative ultracentrifugation. The most widely used nomenclature defines five main classes of lipoproteins (table 1.2) based on their hydrated density in g/ml (Havel *et al*, 1955). Other classifications include electrophoretic mobility, apolipoprotein composition or, as described by Lindgren et al (1972), by the rate of flotation through a salt solution expressed in Svedberg units (S_f).

1.2.1 Chylomicrons

Chylomicrons of enteric origin are the largest triglyceride-rich lipoproteins that carry exogenous triglycerides into the plasma. Circulation of chylomicrons serves to distribute dietary triglyceride to adipose tissue and to muscle. The chylomicron is characterised by its high triglyceride content and its specific apo B variant (tables 1.2 and 1.3). The intestine produces an apo B which has the same amino acid complement as the N terminal 48% of full length apo B, and is therefore called apo B48 (Kane *et al*, 1980). Apolipoproteins AI, AII, AIV, CI, CII, CIII and E are also found on chylomicrons (Green *et al*, 1979; Wu *et al*, 1979, Krause *et al*, 1981). The chylomicrons secreted by the intestine are transported via the thoracic duct and enter the blood stream in the left subclavianvein. Once the chylomicron has reached the blood stream several compositional changes take place. Apo AI is transferred to HDL (Tall *et al*, 1979, Schaefer *et al*, 1982) and apolipoproteins circulating in the plasma bound to HDL (apos CI, CII, CIII and apo E) are rapidly transferred to the chylomicron (Havel *et al*, 1973). Of these, apo CII has a critical action as an activator of lipoprotein lipase (LPL) (La Rosa *et al*,1970). Hydrolysis of core triglyceride is mediated by endothelium bound LPL, liberating free fatty acids that are taken up locally. During the delipidation of the core of the chylomicron, redundant surface materials (phospholipids and apoproteins) are transferred back to HDL (Redgrave *et al*, 1979, Tall *et al*, 1979). As a result the chylomicrons decrease in size and the core becomes relatively enriched in cholesteryl esters to form remnants. The delipidated chylomicron remnant contains apo B48, surface lipids, residual triglycerides, and accumulated cholesteryl esters and under normal conditions is taken up rapidly by the specific receptors of the liver recognising apo E (Hoeg *et al*, 1985). Fig.1.2 illustrates exogenous lipid metabolism.

Table 1.3 Composition of Normal Plasma Lipoproteins

Class	*Free Cholesterol* ⇐	*Cholesteryl Esters* Percentage	*Phospholipids* Lipoprotein	*Triglycerides* Mass	*Protein* ⇒	*Apolipoprotein content*
Chylo-microns	2	3	7	86	2	AI, AIV, B48, C, E
VLDL	7	12	18	55	8	B100, C, E
IDL	9	29	19	23	19	B100, C,E
LDL	8	42	22	6	22	B100,C,E
HDL	4	14	34	4	45	AI,AII,C,D,E

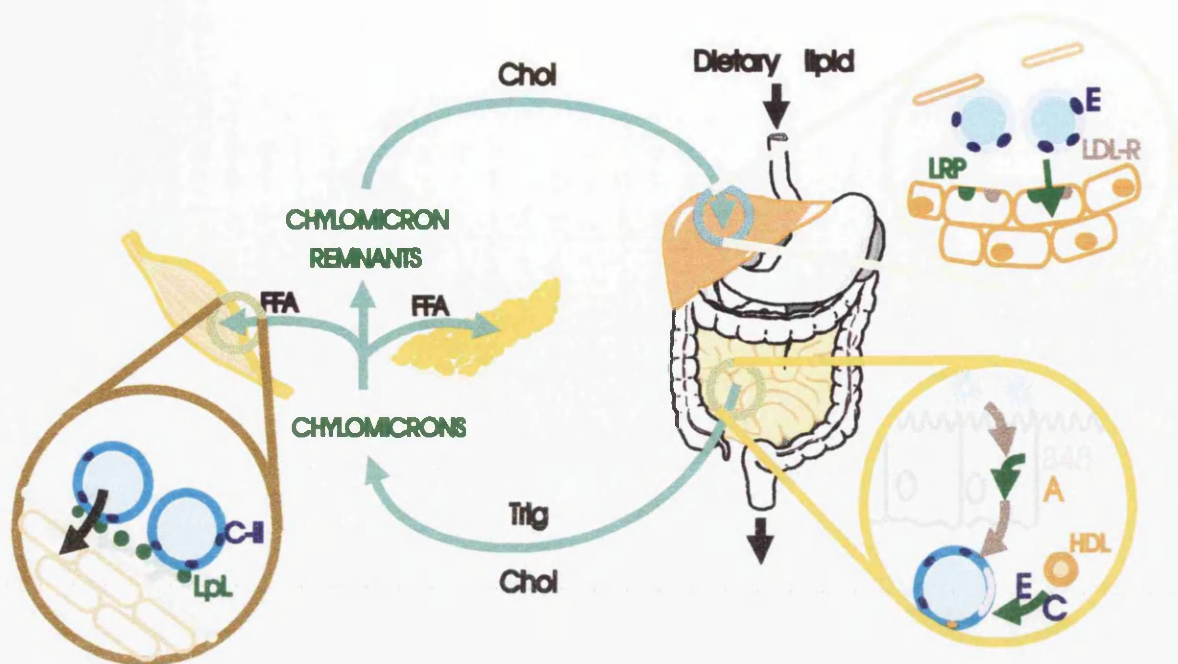

Fig.1.2 Exogenous Lipid Metabolism
(adaptation of an illustration by Professor Packard)
Chol cholesterol, FFA free fatty acids, Trig triglyceride, HDL high density lipoprotein, LDL-R low density lipoprtein receptor, LpL lipoprotein lipase, LRP low density lipoprotein receptor related protein receptor, apolipoproteins A, B48, C, CII, E.

1.2.2. Very Low Density Lipoprotein (VLDL)

VLDL synthesised in the liver, is the endogenous triglyceride transporter in plasma. The triglyceride in VLDL originates either from hepatic *de novo* synthesis of fatty acids, from plasma free fatty acids taken up into the liver or from residual triglycerides in chylomicron remnants. The apo B released in VLDL is the complete apo B100 polypeptide. Like chylomicrons, newly secreted VLDL acquire apo Cs and apo E from circulating lipoprotein, mainly HDL (table 1.3). The triglyceride core is rapidly hydrolysed by the action of LPL, i.e. there is a common saturable triglyceride removal mechanism for both chylomicrons and VLDL (Brunzell *et al*, 1973). VLDL is not homogeneous, and can be divided into at least two components of differing size, density and metabolic properties (Patsch *et al*, 1978, Kuchinskiene *et al*, 1982, Packard *et al*, 1984). $VLDL_1$ (S_f 60-400) is larger and triglyceride-rich, whereas $VLDL_2$ (S_f 20-60) is smaller and comparatively rich in cholesteryl ester (Packard *et al*, 1984). Small VLDL is derived in part by delipidation of large VLDL but may also be secreted by the liver (Demant *et al*, 1995). Parallel processing pathways operate within the delipidation cascade (Packard *et al*, 1984) so that large $VLDL_1$ are inefficiently converted to a class of LDL (S_f 0-12) that is cleared slowly from the plasma while newly synthesised $VLDL_2$ is rapidly and quantitatively delipidated to an LDL species which is catabolised rapidly (Packard *et al*, 1984).

1.2.3 Intermediate Density Lipoprotein (IDL)

IDL is the lipoprotein with hydrated density 1.019-1.063 g/ml and a flotation S_f 12-20 and was initially regarded as a single class of lipoproteins generated from VLDL by lipolysis. Generally in the quantification of lipoproteins, IDL cholesterol is included with LDL. Musliner *et al* (1986) have shown the existence of two populations IDL_1 and IDL_2 by gradient gel electrophoresis of ultracentrifugal fractions from normal subjects. IDL_1, the larger subfraction, was richer in triglycerides and was often observed to be the same size as small VLDL particles. On the other hand, IDL_2 was relatively cholesterol enriched. The same group (Musliner *et al*, 1987) showed that small VLDL gives rise to a lipoprotein with the size and density of intermediate LDL.

1.2.4 Low Density Lipoprotein (LDL)

LDL is the major cholesterol carrier in plasma and its function is to deliver cholesterol to the peripheral tissues principally for the maintenance of cell membranes. In certain specialised cells the cholesterol extracted from LDL has other roles. In the adrenal gland and the ovary it is converted into, respectively, the steroid hormones cortisol and oestradiol, and in the liver it is transformed to make bile acids which have a digestive function in the intestine. LDL contains tocopherols and the lipoprotein acts as a transport mechanism for this minor lipid (Kayden *et al*, 1993). Each LDL particle is spherical with a diameter of 22-28nm, and is isolated at hydrated density 1.019-1.063 g/ml or flotation rate S_f 0-12. It contains one copy of apo B100 which is the ligand for the LDL receptor (Goldstein & Brown, 1984) by which means the particle enters the cell. The apo B is disposed in a circumferential distribution around the lipid core (Phillips *et al*,1989, Chatterton *et al*,1991, Schumaker *et al*,1994). LDL was once considered structurally and metabolically homogeneous but has now been shown not only to exhibit inter-individual heterogeneity but it also exists in the plasma of all individuals as a group of discrete but overlapping populations of particles (Hammond *et al*,1977, Fisher *et al*,1983, Krauss *et al*,1980, Shen *et al*, 1981, Krauss & Burke, 1981, Chapman *et al*,1988).

1.2.5 High Density Lipoprotein (HDL)

Plasma HDL lies in the density range 1.063-1.21 g/ml and is comprised of small, dense, spherical lipid-protein complexes consisting of approximately equal proportions of lipid and protein (Eisenberg *et al*, 1984). The major lipids are phosphatidylcholine, cholesterol, cholesteryl ester and triglyceride. The two main structural apoproteins are AI (MW 28,000) and AII (MW 17,000). In addition HDL contains small amounts of apo CI, CII, CIII, E, and AIV (table 2.3) and trace quantities of CETP, phospholipid transfer protein and lecithin: cholesterol acyl transferase (LCAT), which play an important part in HDL and lipoprotein metabolism. HDL also contains several components of unknown function including apo D, apo J (clusterin), apo SAA (an acute phase reactant) and PI-glycan-specific phospholipase D (Tall & Breslow, 1996). HDL newly formed by secretion or a by-product of lipolysis (nascent HDL) has a distinctive discoidal structure (Tall et al, 1990). The particle lacks neutral lipids and is an excellent substrate for the LCAT enzyme whose action rapidly leads to the conversion to a spherical structure. The major subclassification of HDL is the division into HDL_2 (d 1.063-1.125 g/ml) and HDL_3 (d 1.125-1.210 g/ml) in

the preparative ultracentrifuge (Eisenberg *et al*, 1984). HDL_2 contains 60% lipid and 40% protein, while HDL_3 consists of about 45% lipid and 55% protein. The mean diameter of HDL_2 is 10-12nm and that of HDL_3 is 8-9nm. Further subdivisions can be achieved by gradient gel electrophoresis (Blanche *et al*, 1981) which yields HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c}, in decreasing order of size.
An important distinctive subclassification of HDL is based on immunochemical differences between HDL particles. The primary classification is based on differences in content of apo AI and AII (Cheung *et al*, 1982, 1984, 1991, Duverger *et al*, 1993). HDL consists of particles containing apo AI (LpAI), apo AI and AII (LpAI/AII) and apo AII (LpAII). There is increasing evidence that Lp(AI) and Lp(AI/AII) have distinct metabolic properties as well as possible differences in atherogenesis. The overall metabolic function of HDL is related to the reverse cholesterol transport which is the centripetal movement of cholesterol from the periphery back to the liver. HDL is believed to remove cholesterol from cells, either by a process of simple diffusion or by apoprotein, particulary AI, interaction with the cell surface. Cholesterol in HDL is esterified and transferred to other lipoprotiens for subsequent clearance by the liver. Alternatively HDL cholesterol and cholesteryl ester are directly removed by the liver (Tall & Breslow, 1996).

1.2.6 Lipoprotein (a) (Lp(a))
Since its discovery in 1963 by Berg and his colleagues at Oslo University, Lp(a) has become recognised by most investigators as an independent risk factor for cardiovascular disease. This observation has been seen in a number of retrospective (Armstrong *et al*, 1986, Durrington *et al*, 1988, Genest *et al*, 1991,1992) and prospective studies including the Lipid Research Clinics Primary Prevention Trial (Schaefer *et al*, 1994) and the Gottingen Risk Incidence and Prevention Study (Cremer *et al*, 1994). However two recent prospective studies - the Helsinki Heart Study (Jauhiainen *et al*, 1991) and Raker *et al* (1993) failed to show a significant association.
Lp(a) has the structure of LDL bound to a specific glycoprotein, apolipoprotein (a) via a disulphide bridge and non polar interactions. Apo(a) varies in mass between 300 - 800 kDa. As a consequence of the marked size polymorphism of apo(a), the Lp(a) particle exhibits an important variability in size and weight (Fless *et al*, 1984) and impaired binding to a number of cell and extracellular surfaces (Maartnan-Moe *et al*, 1981; Armstrong *et al*, 1985, Harpel *et al*, 1989, Salonen *et al*, 1989). Through sequencing cDNA, McLean *et al* (1987) showed that apo(a) has a striking similarity to plasminogen. Both proteins have identical secretion signal peptides and protease domains but plasminogen contains a zymogen region and three kringles that have no close counterpart in apo(a). The 4th and 5th kringles have been conserved with Lp(a) having up to forty exact copies of kringle 4. All components of the Lp(a) particle are synthesised in the liver. The normal function of the particle is not known, however the homology with plasminogen has lead to the speculation that Lp(a) might serve as the link between thrombosis and atherosclerosis and this has been the focus of recent research. This topic has recently been reviewed by Lawn & Scanu (1996).

1.3 Lipoprotein Metabolism

1.3.1 Intestinal Lipoprotein Secretion

The most abundant lipid in the diet is triglyceride which under normal conditions is absorbed virtually completely following hydrolysis by pancreatic lipase. Unesterified cholesterol is present in foods of animal origin and is liberated from ingested cholesteryl esters by a cholesteryl esterase (fig. 1.2). Enterocytes selectively absorb unesterified cholesterol while excluding phytosterols. This process appears to involve one or more transmembrane proteins (Thurnhofer *et al*, 1991) while the intracellular transport of free fatty acids derived from ingested triglycerides is probably facilitated by one or more fatty acid binding proteins (Xu *et al*, 1992, Schaeffer *et al*, 1994). After esterification with CoA, the fatty acyl groups directly trans acylate the β-monoglycerides to triglycerides. Some free cholesterol is esterified, by acyl CoA:cholesterol acyl transferase (ACAT) to yield esters that appear in chylomicrons.

The form of apo B (apo B48) that is found in chylomicrons is completely homologous with the N terminal 48% of apo B100 and results from tissue-specific editing process that introduces a stop codon (Driscoll *et al*, 1990). The process of lipidation of apo B in the intestine is inferred from a more detailed knowledge of the formation and secretion of VLDL in hepatocytes. However caution should be exercised in this extrapolation since in chylomicron retention disease there is an inability to secrete chylomicrons while VLDL synthesis and secretion proceed normally (Roy *et al*, 1987), suggesting that there is a difference in the two processes.

Scission of a 27 amino acid signal peptide probably occurs cotranslationally and partial lipidaton of nascent apoB48 chain proceeds as the protein is translocated across the membrane of the endoplasmic reticulum, forming a small spherical precursor with partially lipidated apo B. In a second step, the partially lipidated apo B particle fuses with a large triglyceride droplet (Christensen *et al*, 1983). The nascent particles are then transported to the Golgi apparatus where a further complement of phospholipid may be added and newly synthesised apos AI, AII, and AIV are associated with the particle.

Secretion of chylomicrons into extracellular space occurs when membranes of the trans-cisternae of the Golgi fuse with the basolateral membrane. The chylomicrons then pass into the intestinal lacteals, transit the thoracic duct and spill into the blood via the subclavian vein. The rates of synthesis of the C and E apoproteins are very low in the intestine (Imaizumi *et al*, 1978a, 1978b) and so it is likely that limited amounts are present in chylomicrons.

The capillaries of the intestine are fenestrated in such a way that macromolecules in the size range of HDL can pass into lymph spaces and this allows extensive exchange of phospholipid from chylomicrons to HDL and, in the reverse direction, acquisition of apo Cs and E by chylomicrons. Apoproteins AI, AII and AIV dissociate very rapidly on exposure to lymph and plasma and enter the HDL pool.

The principal mechanism by which the intestine accommodates large fluxes of triglyceride transport is by increasing the particle volumes of the chylomicrons secreted. Synthesis of apo B48 appears to be increased only minimally or not at all with fat feeding in the rat (Imaizumi *et al*,1978b) whereas triglyceride transport can increase twenty-fold.

1.3.2 Intravascular Lipolysis of Chylomicrons

Lipolysis of lipoprotein-borne triglyceride takes place at the vascular endothelium. Electron microscopy has shown that chylomicrons and VLDL marginate on the capillary walls in adipose tissue and in skeletal and cardiac muscle. There is a complex interaction with lipoprotein lipase (LPL) (Olivecrona *et al*, 1993) with LPL binding both to the lipoprotein and heparin sulphate of endothelial proteoglycans (Eisenberg *et al*, 1992, Williams *et al*, 1992a) (fig.1.2). The active form of LPL is a homodimer and it is activated by binding to the C terminal of apo CII. LPL is synthesised and secreted in adipose tissue, striated muscle and mammary gland and moves across the endothelium to the luminal surface of each of these tissues. LPL is regulated by poorly defined metabolic and endocrine signals. Fasting reduces the activity in adipose tissue and increases activity in cardiac and skeletal muscle (Arner *et al*, 1991; Enerback *et al*, 1988). This effect spares free fatty acids for use in skeletal and cardiac muscle when energy substrates are not abundant. In insulin deficiency there are low levels of LPL activity in adipose tissue (Frayn *et al*, 1993).

Intravascular hydrolysis of triglyceride liberates free fatty acids and glycerol. Free fatty acids are taken up in the tissue where hydrolysis/ occurs and glycerol released into plasma is removed by tissues in which there is glycerokinase activity, principally the liver and intestine. Albumin is a transient ligand for the fatty acids which go to cell membranes where a lipid binding protein facilitates uptake (Schaeffer *et al*, 1994).

Depletion of the core lipids leads to the production of progressively smaller spherical particles until approximately 70% of the core triglyceride is removed. The surface monolayer of phospholipid and free cholesterol sheds amphipathic lipids to HDL apace with the decrease in particle diameter. This process is dependent on phospholipid transfer protein (PLTP) (Day *et al*, 1994; Tollefson *et al*, 1988). PLTP is synthesised in a number of tissues including placenta, pancreas, lung, kidney, liver and brain. Deficiency of PLTP leads to retention of phospholipids in chylomicrons and VLDL remnants (Malloy et al, 1994) and in homozygous PLTP deficiency there is a virtual absence of HDL.

As triglyceride hydrolysis proceeds, apolipoprotein Cs and a portion of apo E dissociate from the particles and associate with HDL. Small (50-80 nm in diameter), triglyceride-depleted lipoprotein products of lipolysis that contain B48 and E are termed chylomicron remnants. The process of lipolysis from secretion to the remnant stage takes about 15 minutes in normals (Stalenhoef *et al*, 1984)

1.3.3 Metabolism of Chylomicron Remnants

Chylomicron remnants are removed with great efficiency via endocytosis into the hepatocytes (Shafi *et al*, 1994) (fig. 1.2). The process is still poorly understood but is mediated by a receptor or receptors for which apo E is the ligand. Though chylomicron remnants bind the LDL receptor with high affinity, the absence of significant retention of chylomicron remnants in homozygous LDL receptor-defective animals and humans points to the existence of an additional receptor. The LDL-receptor-like protein (LRP) may be the responsible receptor (lipoprotein receptors are discussed in detail in section 1.4).

Kinetic studies of endocytosis of chylomicron remnants by the liver reveals a delay after binding, suggesting that modification of the particles is required before endocytosis proceeds. Both the triglyceride and cholesterol of chylomicron remnants are used in the

secretion of VLDL by the liver. Tocopherols also enter the hepatocyte in endocytosed chylomicron remnants and are resecreted in VLDL (Kayden *et al*, 1993).

1.3.4 Lipoprotein Assembly in the Liver

Fatty acids employed in triglyceride synthesis come from several sources. Free fatty acids released by the activity of intracellular lipase (hormone-sensitive lipase, HSL) in adipose tissue are transported in plasma bound to albumin. About one third of plasma free fatty acid is extracted by the liver independent of the triglyceride level. Hence in the situation in which lipolysis in the adipocytes is increased, there will be an increase influx of free fatty acids to hepatocytes. The most potent down regulator of HSL is insulin. Plasma fatty acids undergo two competing fates following uptake by the liver, either oxidation or deposition in the storage pool as triacylglycerol, awaiting incorporation into VLDL (Gibbons *et al*, 1995). Biosynthesis of fatty acids proceeds using carbon sources of glucose, amino acids and ethanol. Fatty acid synthesis is enhanced in the fed state and suppressed during fasting. Cholesterol in liver may be present as the result of *de novo* synthesis or due to retrieval from the peripheral tissues mediated through HDL. All the body's cells have the capacity to synthesise cholesterol but the bulk is made in the liver. The cholesterol biosynthetic pathway is initiated at acetate with multiple steps to mevalonic acid through to lathosterol and then cholesterol. The pathway is finely regulated by the negative feedback of cholesterol exerted on the rate limiting enzyme HMG Co A reductase. The liver cell has the unique ability to eliminate cholesterol into the bile either unchanged or following oxidation to bile acids (Dietschy & Wilson, 1970). This is regulated by cholesterol 7 α hydroxylase which is the rate limiting enzyme of bile acid secretion. The rate of hepatic cholesterol synthesis correlates with the rate of hepatic VLDL-apo B secretion in stable isotope turnover studies in normolipaemic subjects (Watts *et al*, 1995) and subjects with heterozygous familial hypercholesterolaemia (Cummings *et al*, 1995).

Apo B100 is the sole B protein produced in the liver and each nascent VLDL particle contains a single copy of B100 (Kane *et al*, 1994). The protein is approximately 549 kDa including carbohydrate linked at up to 16 sites and contains about 40 lipophilic sequences distributed evenly through the molecule which allow it to associate with the moieties of VLDL, IDL and LDL with such high affinity that it remains with a single lipoprotein particle from secretion to ultimate endocytosis. These sequences include several amphipathic helices and a large number of sequences yielding amphipathic β structure. Apo B100 message appears to be constituitively expressed in Hep G2 cells (Pullinger *et al*, 1989), insulin inhibits the synthesis and secretion of apo B from rat hepatocytes (Patsch *et al*, 1983, Sparks *et al*, 1986) and oestrogens increase the synthesis of apo B in Hep G2 cells (Tam *et al*, 1986). Apo B100 may be partially lipidated cotranslationally during transmembrane transfer into the endoplasmic reticulum by a 'pause transfer' mechanism. The lipids at this stage of lipidation probably come from the inner leaflet of the endoplasmic membrane. A substantial fraction of newly synthesised apo B is degraded without being secreted, suggesting that lipidation may induce conformational changes to allow it to proceed to the second stage of lipidation (Thrift *et al*, 1992). Hamilton (1994) was one of the first to postulate that lipidation of apo B is a two step process. The first phase of which appears, at least in Hep G2 cells, to produce spherical particles that are

approximately 22nm in diameter. These cells appear to lack the ability to carry out the next stage and consequently secrete LDL-like nascent particles (Tao *et al*, 1991, Spring *et al* 1992). The second phase seems to involve the coalescence of these small spherical particles with triglyceride-rich particles produced in the endoplasmic reticulum. Microsomal triglyceride transport protein (MTP), a heterodimeric protein that includes a protein disulphide isomerase subunit (Werrerau *et al*, 1992, Hamilton *et al*, 1993) appears to be involved in the first step and might also be involved in the formation of the triglyceride-rich droplets. The strongest evidence of MTP involvement comes from studies of several cases of recessive abetalipoproteinaemia in which no apo B containing lipoproteins can be secreted from liver or intestine (Werrerau *et al*, 1992, Hamilton *et al*, 1993). In these subjects functional MTP activity is absent.

This two step approach to lipoprotein assembly suggests that the liver is capable of secreting particles with varying amounts of triglyceride and also apo B100 in different conformational states and of variable particle size. However there is still uncertainty about the precise mechanisms of these events: is there an insulin-regulated step, is cholesteryl ester synthesis an obligatory requirement for the earliest stages of lipoprotein secretion and in its absence does apo B fail to assemble and be degraded, does apo B undergo incomplete translocation in endoplasmic reticulum and what is the pathway and regulation for mobilisation of triacylglycerol from intracellular storage and incorporation into VLDL? These topics have been reviewed recently by Pease & Leiper (1996).

1.3.5 Metabolism of Apo B Containing Lipoproteins

Fully lipidated VLDL is transported to the Golgi vesicles where glycosylation of the apoproteins proceeds before VLDL is transported to the plasma membrane and released into the space of Disse. Nascent VLDL isolated from the Golgi apparatus contains newly synthesised E and Cs. As soon as they emerge from the hepatocyte, exchanges take place with HDL: phospholipid to HDL mediated by phospholipid transfer protein (PLTP) (Tollefson *et al*, 1988; Malloy *et al*, 1994) and esterified cholesterol from HDL to VLDL mediated by CETP. Apoproteins C and E also associate with HDL and disassociate from VLDL. VLDL lipolysis takes place by LPL bound to capillary endothelium of skeletal and cardiac muscle and adipose tissue (fig. 1.3). Particles competing with chylomicrons have a higher affinity for the enzyme than VLDL. The presence of apoCII and CIII play opposing roles in the regulation of the rate hydrolysis of core triglycerides as apo CII is a cofactor (Eckel *et al*, 1989) whereas apo CIII has been shown to inhibit LPL activity (Wang *et al*, 1985). In a study in subjects with apo CIII deficiency, Ginsberg *et al* (1986) observed a sevenfold increase in VLDL lipolysis.

In 1978 Berman *et al* showed that in studies with radioiodinated VLDL that apo B is transferred through a delipidation cascade to IDL and LDL. This concept was further elaborated to include the existence of VLDL-IDL-LDL delipidation parallel processing pathways as proposed by Fisher *et al* (1982). Fig.1.3 shows the enzymes, receptors and apoproteins involved in the progressive hydrolysis of core triglycerides and subsequent delipidation from VLDL to IDL to LDL.

$VLDL_1$ has two metabolic fates: first it is delipidated to $VLDL_2$ and second, a substantial amount is removed directly from the circulation by an unknown process possibly involving receptor activity (discussed in section 1.4). Both appear to involve the action of LPL. In a

study of lipoprotein lipase deficiency, Demant *et al* (1991) observed a delay in the conversion of $VLDL_1$ to $VLDL_2$ as well as a 90% reduction in direct clearance of $VLDL_1$ apo B. Remnants derived from $VLDL_1$ delipidation contribute to the apo B found in IDL and LDL but the conversion to these species is partial and inefficient (Demant *et al*, 1988, Packard *et al*, 1995). $VLDL_2$ conversion to IDL and LDL does not depend on LPL as these steps were normal in LPL-deficient patients (Demant *et al*, 1991). In contrast, in hepatic lipase deficiency, $VLDL_1$ to $VLDL_2$ and $VLDL_2$ to IDL conversion is normal whereas there is a block on IDL to LDL conversion.

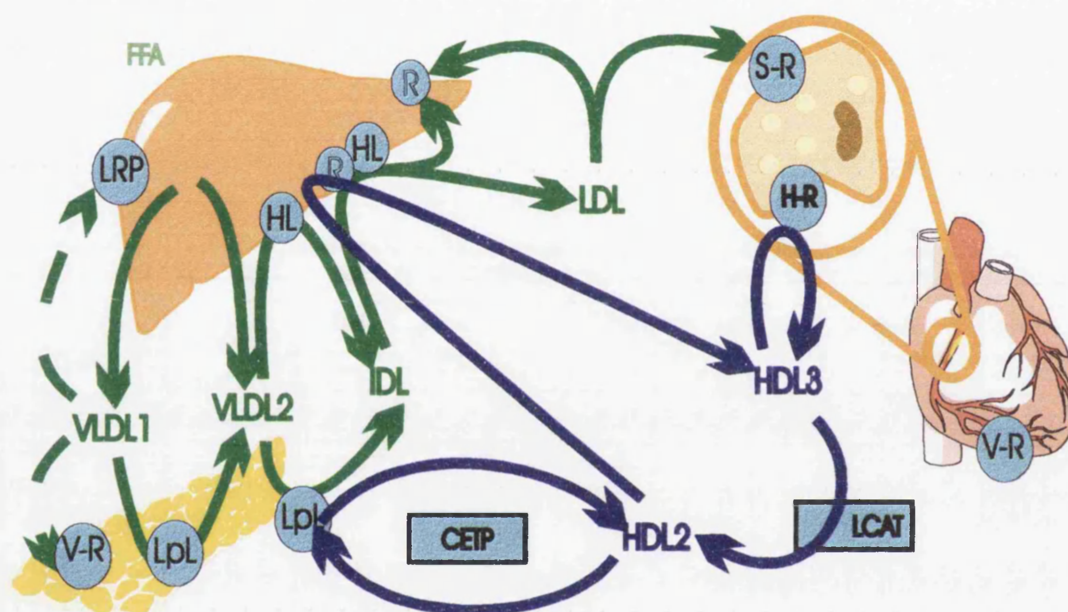

Fig.1.3 Role of Enzymes and Receptors in Endogenous Lipoprotein Metabolism
(adapted from illustrations by Professor Packard).
VLDL very low density lipoprotein, IDL intermediate density lipoprotein, LDL low density lipoprotein, HDL high density lipoprotein, FFA free fatty acid R LDLreceptor, V-R VLDLreceptor, H-R HDL receptor, LRP LDL recptor related protein receptor (remnant receptor), S-R scavenger receptor, LpL lipoptotein lipase, HL hepatic lipase, CETP cholesterol ester transfer protein, LCAT lecithin cholesterol acyl transferase.

The surface components of the apo B containing lipoproteins change constantly, affecting the ability to interact with the various enzymes and receptors. During the course of lipolysis, the C apolipoproteins are lost from the particle and so lipolysis by LPL becomes less efficient. Both apo B and E can potentially mediate cellular uptake and degradation by the LDL receptor. However it appears that these apoproteins are not always in a conformation appropriate for receptor binding. In normal subjects neither apo B or E on

$VLDL_1$ are able to act as a ligand on the receptor but as lipolysis continues, their conformation alters towards a receptor-active state. In addition apo E acquired by exchange onto VLDL enhances the particle's affinity for receptors. Thus it is possible to envisage a scheme where VLDL is removed by LDL receptor-related protein (LRP) or VLDL receptor via apo E and as lipolysis progresses the surface of the particles alters facilitating removal by the classical LDL receptor (see section 1.4).
The circulating half life of LDL is approximately 2.5 days in normal humans. LDL is the major source of cholesterol for the maintenance of cell membranes and also delivers tocopherol to the cells. Endocytosis of LDL via the LDL receptor for which apo B100 is the ligand accounts for about half of the uptake of LDL. Mutations in the LDL receptor in familial hypercholesterolaemia (FH) and in apo B100 in familial defective B lipoproteinaemia (FDB) lead to accumulation of LDL in plasma. LDL can also be taken up in all cells by non receptor mediated processes. Macrophages and transformed smooth muscle cells can endocytose chemically or physically modified LDL via a pair of structurally related scavenger receptors in a process that can lead to foam cell formation and atherosclerosis.

1.3.6 High Density Lipoprotein and Reverse Cholesterol Transport

HDL is the most malleable of lipoprotein species since all of its components undergo rapid interparticle exchange. It is composed of a number of subspecies - pre beta HDL, HDL_2 and HDL_3, whose interconversions are governed by the exchange and lipolysis mechanisms among the apo B containing lipoproteins as well as by the action of the enzyme lecithin:cholesterol acyl transferase (LCAT). The role of HDL is that of a cellular cholesterol acceptor which removes excess sterol from the cell surfaces and traps it in the form of cholesteryl ester and a gradient of reverse cholesterol transport is maintained, mediated by cholesterol ester transfer protein (CETP), whereby HDL cholesteryl ester is transferred to apo B containing particles, which are subject to rapid uptake by the liver (Tall *et al*, 1990) (fig. 1.3). Pre-beta HDL is thought to play an important role in the initiation of this centripetal transport pathway (von Eckardstein *et al*, 1994). Pre-beta HDL is a small sized HDL (about 60kDa), contains only AI and constitutes about 5% of normal HDL(Ishida *et al*, 1987, Kunitake *et al*, 1992). When plasma is exposed to cholesterol-rich cells in culture much of the sterol lost from the cell membrane is found initially in this fraction (Castro & Fielding, 1988). Apo AI is a cofactor for activity of LCAT and the action of the enzyme triggers a change in the disposition of cholesterol within the particle. Transfer of a fatty acid from lecithin to the hydroxyl residue on cholesterol leads to the generation of lysolecithin, which dissociates readily from the lipoprotein into the aqueous environment and cholesteryl ester. Esterification of the sterol increases its hydrophobicity and causes it to partition into the nonpolar interior of the particle. The surface site vacated by the sterol is then available to accept additional cholesterol and the HDL increases in size (von Eckardstein *et al*, 1994). Acquisition of cholesterol proceeds until the nascent disc is fully transformed into a pseudomicellar spherical structure. CETP exchanges the cholesteryl ester in the core of HDL for triglyceride acquired from chylomicrons or VLDL. The triglyceride-enriched HDL_2 then becomes more susceptible to intravascular lipolysis by hepatic lipase which renders it smaller and denser until it becomes HDL_3 (Patsch *et al*, 1988). Thus the size of circulating HDL depends on the balance between the opposing

forces of cholesterol reesterification, which increases particle size and lipolytic digestion, which decreases particle size. HDL_3 is postulated to be converted back to HDL_2 by the acquisition of phospholipid and free cholesterol shed from lipolysed chylomicrons and VLDL and the subsequent action of LCAT.
Recent studies have shown that mechanisms exist for regeneration of pre-beta HDL from mature HDL particles. James & Pometta (1994) have shown that when HDL is incubated with physiological concentrations of VLDL and LDL in the presence of CETP there is dissociation of up to one third of apo AI with restructuring of the remaining HDL. This apo AI is lipid free and its interaction with a cell surface or with redundant surface phospholipids, released during lipolysis of triglyceride-rich lipoproteins, is thought to lead to the formation of small pre-beta HDL (Hana & Yokoyama,1992, von Eckardstein *et al*, 1994, Liang *et al*, 1994).
The validity of this exchange/lipolysis cycle in regulating HDL is demonstrated by examining the characteristics of HDL in a number of deficiency states. LCAT deficiency is a recessive condition resulting from structural mutations in the LCAT gene and the manifestations are dependent on whether the deficiency is complete (classical LCAT deficiency) or partial (fish eye disease). Patients develop corneal opacities, anaemia and renal failure and are susceptible to premature coronary artery disease. The HDL levels are markedly reduced and apo AI levels are 25-30% of normal. There is a high proportion of pre-beta HDL and disc shaped larger particles are seen under electron microscopy (Torsvik *et al*, 1970, Soutar *et al*, 1982). Four different types of CETP genetic deficiency have been described in Japanese subjects. They have high levels of cholesterol-rich HDL_2 and apo AI (Koizumi *et al*, 1985) and there is a gene dosage effect resulting in decreased levels of cholesteryl ester to triglyceride ratio in VLDL and IDL. In HL deficiency (Demant *et al*, 1988) HDL_2 is the major if not only HDL species and is enriched in triglyceride. Despite the increase HDL levels, some HL-deficient subjects have premature CHD. In abetalipoproteinaemia, where there are no triglyceride-rich lipoproteins to act as donors, HDL exists as mainly cholesteryl ester rich HDL_2 (Deckelbaum *et al*, 1982). Studies in transgenic animals have shown that possession of apo AI, CIII and CETP genes is sufficient to cause expression of a high plasma triglyceride/low HDL phenotype in mice (Hayek *et al*, 1991). A number of genetic studies have shown that polymorphisms at the CETP (Kondo *et al*, 1994; Freeman *et al*, 1994) and AI/CIII/AIV and HL loci (Cohen *et al*, 1994) can markedly influence HDL cholesterol levels.

1.4 Receptors in Lipoprotein Metabolism

The picture of lipoprotein receptors drawn by Brown & Goldstein in 1986 has become more complicated with the appearance of several new receptors, particularly receptors of triglyceride-rich lipoproteins. The catabolism of these triglyceride-rich lipoproteins may be determined by the tissue distribution of the functional receptor proteins. The LDL receptor, or the apo B/E receptor is mainly responsible for LDL catabolism mediated by LDL apo B100 and has been observed in all mammalian cells tested except erythrocytes (Brown & Goldstein, 1986). The LDL receptor-related protein (LRP) seems to bind mainly to chylomicron remnants through apo E and lipases and as the main organ of this catabolism, the liver expresses high levels of LRP. The VLDL receptor is most abundant in muscle and adipose tissue and may be important in the delivery of endogenous

triglycerides carried in VLDL or IDL from the liver to places of fatty acid metabolism. The ligand binding to these three families of receptors is inhibited by the receptor-associated protein (RAP) (Mokuno *et al*, 1994) which may therefore play an important role in the regulation of triglyceride-rich lipoprotein catabolism.

1.4.1 The LDL Receptor and its Ligands

Receptor-mediated removal of LDL and lipoprotein remnants occurs mostly via classical LDL receptors. The LDL receptor is a single transmembrane glycoprotein with molecular weight 164,000 daltons and its coding gene is localised on chromosome 19 (Schneider *et al*,1982, Francke *et al*, 1984). The ligand binding domain is found in the 300 amino acid N-terminal segment, which consists of seven 40 amino acid repeat units, each of which is enriched in aspartate and glutamate residues arranged in a configuration that facilitates interaction with complementary arginine and lysine residues found on apo B and apo E (Mahley & Innerarity, 1983). The receptor therefore recognises and interacts with lipoprotein particles containing these proteins but with different affinities since apo E binds ten times more effectively than apo B, thus apo E containing lipoproteins (VLDL and IDL) can be cleared very efficiently by this pathway. Cellular cholesterol content is the major regulator of the synthesis of LDL receptors and so the number of LDL receptors synthesised decreases when the cholesterol content of the cell increases, and vice versa (Brown & Goldstein, 1986). The most important clinical implication of the LDL receptor relates to familial hypercholesterolaemia, an autosomal codominant genetic disorder in which the LDL receptor activity is decreased either because of a reduced number of LDL receptors, or the formation of structurally altered LDL receptors. This leads to an elevation of serum cholesterol via accumulation of LDL particles in the circulation and is accompanied by the development of premature atherosclerosis. Recent observations that similar effects may result from mutations in its primary ligand, apo B100. The apo B arginine-to glutamine change at codon 3500 has become established as a cause of failure of binding of LDL to its receptor and consequent hypercholesterolaemia (Myant *et al*, 1993).

1.4.2 LDL Receptor Related Protein Receptor (LRP)

LRP has been described as an apo E binding protein (Beisiegel *et al*, 1989) and its potential role as a remnant receptor has been elucidated in several laboratories using β-VLDL (Nykjaer *et al*, 1993) and human chylomicrons as ligands (Beisiegel *et al*, 1991). LRP interacts directly with apo E (Beisiegel *et al*, 1989), LPL (Nykajaer *et al*, 1993) and HL (Nykajaer *et al*, 1994). Like apo E, both lipases are heparin-binding proteins that can bind to cell-surface proteoglycans with high affinity. Using heparanase-treated cells, Eisenberg *et al* (1992) demonstrated that the binding of LPL to heparan sulphate on the cell surface facilitated the transfer of lipoproteins to cellular receptors. Beisiegel (1995) has proposed a model in which first triglyceride-rich lipoprotein remnants containing apo E in a sufficient density bind to cell surface proteoglycans. Then they are presented to membrane receptors (probably LRP) which mediates internalisation of the remnants.

In 1990, soon after the LRP sequence was published, Strickland *et al* and Kristensen *et al* identified it as the receptor for activated alpha$_2$-macroglobulin. Other protease and protease inhibitor complexes are recognised by LRP as well as a group of human rhinoviruses

(Hofer *et al*, 1994) and tissue factor pathway inhibitor (Warshawssky *et al*, 1994). Studies on the distribution of LRP in human tissues have revealed its presence in various cell types, including hepatocytes, fibroblasts, neurons and monocyte-derived macrophages (Moestrup *et al*, 1992). In 1994 Bu *et al* confirmed the abundance of LRP in mammalian central neurons and on the basis of earlier studies on the presence of apo E in the brain they have proposed an important role for LRP in apo E metabolism in the brain. This is particularly interesting in light of the recently discovered connection between apo E and Alzheimer's disease.
The induction of LRP during the differentiation of monocytes and macrophages (Watanabe *et al*, 1994) has opened up discussion on the role of this receptor in the development of atherosclerotic plaques. The possible involvement of LRP in atherogenesis is supported by studies from Louma *et al* (1994) and Lupu *et al* (1994) who demonstrated that LRP was expressed not only in macrophages from early and advanced lesions but also in smooth muscle cells.

1.4.3 VLDL Receptor

In 1994, Shimano *et al* described a VLDL receptor in rabbits which is an LDL receptor-like protein that binds and internalises apo E-containing lipoproteins. In humans , the VLDL receptor gene has been characterised, sharing 96% homology with the rabbit (Sakai *et al*, 1994) and has been assigned to chromosome 9 (Oka *et al*, 1994). VLDL receptor messenger RNA is most abundant in skeletal muscle, heart, kidney and brain (Oka *et al*, 1994). In the rat, Jokinen *et al* (1994) have found no effect on fasting and refeeding but VLDL receptors were reduced by 80% in the skeletal muscle of hyperthyroid rats.

1.4.4 Scavenger Receptors

Scavenger receptors recognise chemically and biologically altered lipoproteins. Unlike LDL receptors, the expression of scavenger receptors is not dependent on cellular cholesterol content, which leads to the cholesterol accumulation in the macrophages (Goldstein *et al*, 1979). Lipid-loaded macrophages can be seen in the earliest lesions of atherosclerosis (Ross, 1986). Modified LDL particles extracted from the aorta (Hoff & Morton, 1987) differ from the normal lipoprotein by being more electronegative, like the artificially produced acetyl-LDL, which is avidly assimilated and deposited in macrophages (Goldstein *et al*, 1979). These cells exhibit on their membranes proteins that facilitate the rapid uptake of acetyl LDL to the extent of generating foam cells similar to those found in the atherosclerotic lesion. Because a wide variety of negatively charged compounds can compete with acetyl-LDL for these receptors, they have become known as scavenger receptors (Brown *et al*, 1980). Steinberg *et al* (1989) have found that charge modified lipoproteins, in the form of oxidised LDL, compete with acetyl-LDL and cause foam cell formation. They concluded that modified lipoproteins may be the in vivo ligand for these scavenger receptors. Support for the hypothesis that oxidised LDL is important in the development of atheromatous plaques comes from several lines of evidence. Firstly, antibodies to oxidised LDL have shown its presence in atheromatous lesions and human fatty streaks(Haberland *et al*, 1988,Yla-Herttuala *et al*, 1989, 1991). Secondly, LDL isolated from atherosclerotic plaques is electronegative (Hoff & Morton, 1987, Yla-Herttuala *et al*, 1989) and produces foam cell transformation in vitro. Thirdly, when the

drug probucol was administered to Watanabe rabbits, Carew *et al* (1987) observed that LDL uptake into atherosclerotic lesions fell by 65% compared to a group given lipid lowering drugs. This they attributed to the ability of probucol to scavenge free radicals and limit the rate of oxidation of LDL. The hypothesis concerning the pathophysiological importance of modified LDL as an atherogenic particle has been summarised by Steinberg *et al* (1989).

1.5 Structural Heterogeneity in Low Density Lipoprotein

Low density lipoprotein was once considered to be structurally and metabolically homogeneous but it is now known to comprise a heterogeneous spectrum of particles which differ in density, size and chemical composition. An increasing body of evidence indicates that within this spectrum there are discrete physiochemical forms of LDL and that plasma concentrations and biochemical properties of these LDL subclasses may have physiological importance.

1.5.1 Historical Concept of Heterogeneity

In 1951, Lindgren, Elliot & Gofman were the first to show that it was possible to fractionate LDL into three subclasses by preparative ultracentrifugation in a fixed angle rotor. In 1969, Lingren *et al* showed that like the other lipoproteins, LDL (S_f 0-12) exhibited a range of flotation rates when subjected to centrifugation in a solvent of appropriate density. In particular they observed that the peak flotation rates were different in normal males and females. Early studies by Lee & Alaupovic (1970) used sequential ultracentrifugation and immunological techniques to demonstrate the presence of a series of LDL subfractions differing in composition and measurement of flotation rates were used by Hammond & Fisher (1971) to characterise a discrete series of LDL in subjects with hyperprebetalipoproteinaemia. The best characterised structural alteration was found in individuals with hypertriglyceridaemia (Fisher *et al*, 1983). Since then a variety of high resolution techniques such as density gradient ultracentrifugation and gradient gel electrophoresis have been employed to identify and characterise subfractions of particles along the LDL density spectrum. In 1982, Lee & Downs used density gradient ultracentrifugation to demonstrate compositional changes between LDL in normal males and females and in the same year Krauss & Burke identified multiple subclasses in normals using both density gradient ultracentrifugation and gradient gel electrophoresis. Of the many density gradient ultracentrifugation procedures in the literature, some isolate LDL first (Shen *et al*, 1981, Marzetta *et al*, 1986, Chapman *et al*, 1988) while others isolate the subfractions in a single spin directly from plasma (Lee & Alaupovic, 1986, Swinkles *et al*, 1987, de Graaf *et al*, 1991).

The most commonly used technique of examining LDL heterogeneity is non denaturing gel electrophoresis, which determines particle size. Application of this technique to the analysis of LDL or plasma has confirmed the existence of multiple discrete subpopulations of LDL (Krauss & Burke, 1982, Nichols *et al* 1986, Campos *et al*, 1992a,1992b, Williams *et al*, 1992b). The technique is suitable for large-scale clinical applications and has been used to examine the participants in the Framingham Offspring Study (McNamara *et al*, 1987). Use of either lipid or protein staining makes it suitable for plasma or isolated LDL and no changes in pattern have been observed with freezing plasma at -80°C. Calibration

can be a problem due to the lack of standards at 20nm but generally latex particles at 30 nm and high molecular weight markers have been used (Krauss & Burke, 1982) and in a few instances calibrated LDL standards (McNamara *et al*, 1987). Although LDL particle diameters calculated by this method correlate well with other methods of size measurement, there is a systematic bias towards larger molecular size with gel electrophoresis (Krauss & Blanche, 1992).

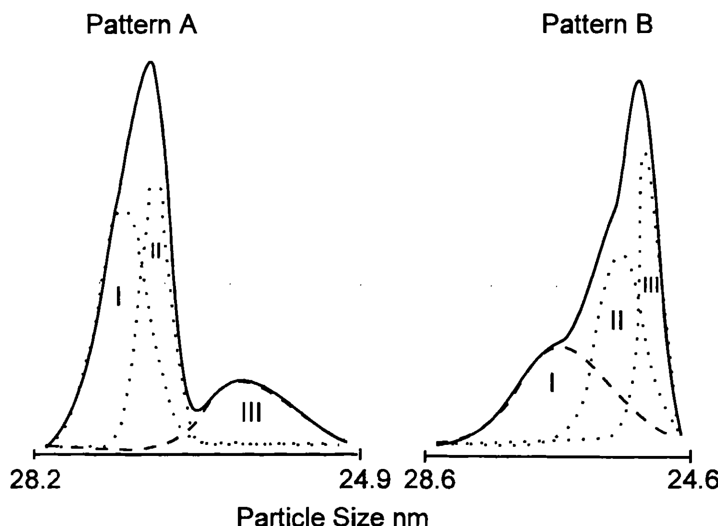

Fig.1.4 LDL Subclass Patterns A and B determined by gradient gel electrophoresis

(Adapted from Austin & Krauss, 1986)

Solid bands are obtained from densitometric tracing of gradient gel bands. The dotted lines are determined by mathematical modelling.

Austin & Krauss (1986) described a mathematical modelling technique which was used to facilitate the identification of LDL subclasses by separating the gradient gel electrophoresis curves into a series of Gaussian curves (fig. 1.4). They identified two distinct subclasses A and B in which A has a major peak with larger diameter >25.5 nm and B has, as the major component, particles of a smaller diameter. This classification is widely used and subjects are described as having LDL phenotype A or phenotype B, but has been criticised as not all gel scans fitted into these two categories. In a recent publication from the same laboratory (Krauss & Dreon, 1995) an intermediate subclass has been identified with a mean particle diameter of 25.6-26.3 nm.

1.5.2 Classification of LDL Subfractions

Application of these techniques has revealed the existence of many subfractions of LDL in the density range 1.019-1.063 g/ml and care should be taken when reading the literature as there is no universal classification system and use of the terms 'large', 'buoyant', 'small', 'dense' refer to different species depending on the method of isolation. Most investigators have attempted to remedy this and rationalise the number of species identified. For example, Chapman *et al* (1988) in most recent publications from the same laboratory (Guerin *et al*, 1996) refer to five subfractions instead of the original fifteen. Table 1.4 shows classifications used by various laboratories.

Table 1.4 Classification of LDL Subfractions

Krauss				Chapman			Swinkles		de Graaf	
class	*density g/ml*	*peak size nm*	*Sf*	*class*	*size*	*density g/ml*	*class*	*density g/ml*	*class*	*density g/ml*
I	1.022-1.032	26.5-28.5	7.5-11	1	light	1.019-1.023	1a	1.025-1.028	1	1.030-1.033
				2		1.023-1.029				
IIa	1.032-	26.0-26.5	5.5-8	3	inter-mediate	1.029-1.039	1b	1.030-1.033	2	1.033-1.040
IIb	1.038	25.5-26.0	5-6				2	1.036-1.041		
IIIa	1.038-	24.7-25.5	3-5	4	dense	1.039-1.050			3	1.040-1.045
IIIb	1.050	24.2-24.7	0-4						4	1.045-1.049
IVa	1.050-	23.2-24.2		5		1.050-1.063			5	1.049-1.054
IVb	1.063	22.0-23.2								

1.5.3 LDL Heterogeneity in Relation to Coronary Heart Disease

Several studies have shown the association between small dense LDL and coronary disease. In 1985 Crouse *et al* observed the presence of a low molecular weight LDL isolated by

sequential ultracentrifugation in 46 men with CAD and observed its association with serum triglyceride. Austin *et al* (1988a) determined the LDL particle size distribution (pattern A or B) in 109 subjects who had previous nonfatal myocardial infarction and in 121 healthy controls. There was a preponderance of pattern B associated with a three-fold risk of MI independent of gender, age or body weight, but dependent on HDL cholesterol and plasma triglyceride. Campos *et al* (1992b) analysed LDL particle size in 275 men with CAD and 822 controls. CAD subjects had a decreased particle size which was dependent on serum triglyceride and HDL cholesterol and so was not an independent predictor of risk. In a study population of 100 women and 98 men undergoing elective diagnostic coronary angiography (Coresh *et al*, 1993), small LDL particles were associated with coronary disease independent of age, gender, smoking, diabetes, LDL cholesterol and HDL cholesterol but dependent on serum triglyceride levels. Tornvall *et al* (1991) measured LDL subfractions by density gradient ultracentrifugation in 36 post -MI patients and 14 normolipidaemic controls. Dense LDL was more prevalent in CAD patients and the triglyceride content of both dense and light LDL was related most strongly to the progression of coronary atherosclerosis. The same group (Tornvall *et al*, 1993) showed that the number of apo B-containing particles and the concentration of LDL triglyceride were related to the degree of coronary atherosclerosis and distinct stenosis measured by angiography in 64 young (<45 years) infarct patients. Very dense, triglyceride-rich particles and small cholesteryl ester -rich VLDL particles were also related to the severity of the disease.

Strong evidence for the increased atherogenicity of small dense LDL came from the Stanford Coronary Risk Intervention Program trial (SCRIP), a multifactorial risk reduction trial performed in 300 patients with angiographically verified coronary disease (Haskell *et al*, 1994). Most of the patients received a hypolipidaemic drug in addition to the consumption of an American Heart Association Step 2 diet. Patients with pattern A and B had similar disease at baseline and displayed comparable reductions in LDL concentration , but only in pattern B was progression of atherosclerosis reduced as measured by coronary angiography (Krauss *et al*, 1992). This outcome was also associated with a simultaneous decrease in serum triglyceride.

1.5.4 Determinants of LDL Subfraction Distribution

Influences of Other Lipoproteins

Individuals with small dense LDL usually have increased levels of plasma triglyceride, triglyceride-rich lipoproteins and low concentrations of HDL cholesterol (Austin *et al*, 1990a). There is abounding evidence that plasma triglyceride has a significant effect on the properties of LDL subfractions (Deckelbaum *et al*, 1984, Swinkels *et al*, 1989, Campos *et al*, 1992a, 1992b, McNamara *et al*, 1992, Coresh *et al*, 1993, Stewart *et al*, 1993, Watson *et al*, 1994, Hokanson *et al*, 1995, Tan *et al*, 1995a). Nikkila *et al* (1994) showed that postprandial increment in serum triglyceride is enhanced in subjects with small dense LDL. Associations between LDL size and HDL cholesterol have been reported (McNamara *et al*, 1987, Swinkels *et al*, 1989, Campos *et al*, 1992a, 1992b, McNamara *et al*, 1992, Coresh *et al*, 1993, Haffner *et al*, 1993, Katzel *et al*, 1994, Nikkila *et al*, 1994). However as plasma triglyceride levels, HDL and LDL size are closely linked it is difficult to distinguish one from the other. In most studies LDL cholesterol has not been related to LDL size.

The mechanism by which plasma triglyceride affects LDL subclass distribution and consequently particle size has been the focus of many investigations. CETP, HL and LPL are the key factors responsible for the remodelling of circulating lipoproteins. Deckelbaum *et al* (1984) first suggested that neutral lipid transfer would be the mechanism determining LDL subfraction distribution. Lagrost *et al* (1994) have shown that CETP and HL are both important in this process. As the concentration of VLDL triglyceride increases, CETP-mediated transfer of cholesteryl ester and triglyceride between LDL and VLDL is enhanced and so LDL becomes enriched in triglyceride. This triglyceride-rich LDL is a good substrate for HL which hydrolyses triglyceride in LDL so forming smaller and denser LDL particles. Differences between males and females observed by Tan *et al* (1995a) suggest that HL concentrations in the male range as well as plasma triglyceride > 1.3 mmol/l are necessary to generate small LDL above 100mg/100ml.

Role of CETP

Plasma CETP is a glycoprotein and its main function is to transfer esterified cholesterol from HDL to VLDL/LDL in exchange for triglyceride (Tall *et al*, 1990). The activity of plasma CETP is regulated by several factors. Under physiological conditions the amount of acceptor lipoprotein has been shown to be the major determinant of plasma CETP activity (Mann *et al*, 1991). In CETP deficient patients LDL consists of two populations of particles, one representing normal LDL in size, and another small dense LDL which is depleted in cholesteryl ester (Sakai *et al*, 1991). This natural experiment indicates the important part that CETP plays in regulating LDL size. *In vitro* studies have shown that CETP incubation with LDL and HDL results in an increase in particle size (Gambert *et al*, 1990, Lagrost *et al*, 1993).

Role of Hepatic Lipase and Lipoprotein Lipase

The main function of HL is to hydrolyse triglycerides in denser lipoproteins, such as IDL, LDL and HDL, and has a crucial role in the conversion of IDL to LDL as shown by Demant *et al* (1988) in enzyme deficient subjects. In HL deficiency LDL particles are large and triglyceride-rich (Auwerx *et al*, 1989). HL activity is lowered by oestrogens, hypothyroidism, and endurance training (Appelbaum et al, 1977, Kuusi et al, 1982a, Tikkanen et al, 1982a) and is increased with alcohol consumption, anabolic steroids, progestins, fibrates, smoking and thyroid hormones (Ehnholm *et al*, 1975, Tikkanen *et al*, 1981, 1982b, Taskinen *et al*, 1982, Chen *et al*, 1989, Packard *et al*, 1993, Moriguichi *et al*, 1990).

Several studies have revealed the importance of HL as a determinant of LDL subfraction distribution (Auwerx *et al*, 1989, Zambon *et al*, 1994, Jansen *et al*, 1994, Lagrost *et al*, 1994, Watson *et al*, 1994, Tan *et al*, 1995). An inverse correlation between HL activity and LDL particle size being observed (Zambon *et al*, 1994, Jansen *et al* 1994, Watson *et al*, 1994) and Tan *et al* (1995a) suggested that HL concentrations >15 (μmol fatty acid/ml/h) are required to generate large amounts of small dense LDL.

LPL is the rate limiting enzyme for the conversion of $VLDL_1$ into VLDL2. It does not have any direct action on LDL subfraction distribution but because it acts on its precursors it has an indirect impact. In LPL deficiency there is hypertriglyceridaemia and LDL

particle size distribution changes towards smaller and denser particles if HL activity is normal.

Role of Apo E

In population studies serum cholesterol has been shown to progressively increase from homozygous E2/E2 phenotype to E4/E4 phenotype (Davignon *et al*, 1988), while one study (Wilson *et al*, 1994) has demonstrated the positive association between apo E4 allele and plasma triglyceride. Only a few studies have examined the relationship between apo E phenotype and LDL size with contradictory findings. No correlation was observed in normal healthy males (Zhao *et al*, 1993) and in subjects with CAD (Nikkila *et al*, 1994). In the Framingham study cohort (Schaefer *et al*, 1994) apo E4phenotype was observed to be associated with small LDL and Nikkila *et al* (1996) have shown an association of E4/4 with small LDL in a survey of 40 and 70 year old men and women. Zhao *et al* (1993) have shown that LDL particle size is negatively correlated with plasma apo E content irrespective of apo E phenotype. In this study triglyceride and apo E concentration were found to be the independent risk factors most closely associated with LDL size.

Gender

Premenopausal women usually have larger and less dense LDL particles than males of the same age, which is considered to be due to lower activity of HL in women (McNamara *et al*, 1987, Swinkels *et al*, 1989, Williams *et al*, 1992, Watson *et al*, 1994, Tan *et al*, 1995a, Nikkila *et al*, 1996). Oestrogens decrease the activity of HL and postmenopausally, when ovaries no longer synthesise oestrogen, LDL subclass distribution is suggested to shift towards dense LDL (Tikkanen *et al*, 1982, Schaefer *et al*, 1983, McNamara *et al*, 1987, Campos *et al*, 1988) Austin *et al*,(1988) proposed a dominant role of inheritance for LDL pattern B and reported a reduced inheritance in women. Cross sectional studies have failed to find any increase of small dense LDL in postmenopausal women (Campos *et al*, 1992a, 1993).

Body Weight

Campos *et al* (1991) found that small particles were associated with higher body mass index (BMI) and total body fat in men and women, with smaller size being associated with abdominal obesity in women. Serum triglyceride was elevated and HDL cholesterol was decreased in subjects with high waist-to-hip ratio. In contrast, no association was observed between LDL particle size in a cohort of Japanese men (Suehiro *et al*, 1995). Patients with small dense LDL had an increased waist to hip ratio as well as elevated concentrations of triglyceride and fasting serum insulin.

Weight loss is usually associated with an increase in particle size. Williams *et al* (1990) studied the effects of weight reduction by calorie restriction and by calorie expenditure on LDL subclasses in moderately overweight men and observed comparable increases in LDL particle diameter, decreases in masses of VLDL and small dense LDL. Men with pattern B were more resistant to effects of weight reduction.

Exercise

Cross sectional studies have shown that subjects who train regularly have a larger average LDL particle size mainly due to a reduced amount of small dense LDL (Williams *et al*, 1986, Lamon-Fava *et al*, 1989a, Berg *et al*, 1992). One longitudinal study (Williams *et al*, 1989) has shown that during exercise-induced loss of body mass there is a decrease in small LDL concentrations and an increase in LDL peak flotation rate. After an endurance triathlon, 7 out of 40 subjects had increased particle size related to an acute decrease in serum triglyceride (Lamon-Fava *et al*, 1989b). Baumstark *et al* (1993) investigated the effects of acute exercise on LDL subclasses and found an acute decrease in serum triglyceride and in the triglyceride content of all subfractions. In those subjects with greatest reductions in triglyceride, there was a decrease in concentration of small dense LDL. Houmard *et al* (1994) observed an increase in molecular weight, particle size and total lipid content of LDL particles associated with a reduction in fat, plasma triglyceride and fasting plasma glucose in subjects who had undergone endurance exercise training.

Diet

A diet rich in carbohydrates is associated with increased levels of small, dense LDL (Campos *et al* 1991). The effect is mediated by serum triglyceride since excess intake of carbohydrates increases the influx of free fatty acids to the liver and increases the production of VLDL (Abbott *et al*, 1990). A diet rich in saturated fat on the other hand increased buoyant LDL in relation to an increase in LPL activity (Campos *et al*, 1995a). The decrease observed in small LDL was not associated with the changes in HL or LPL activities.

Dreon *et al* (1995) studied the effects of a reduced fat diet on LDL subclass density in association with the response to apo E phenotype in 105 healthy men. Subjects with E4 allele showed the largest response which was a reduction due to a shift from buoyant LDL to small dense LDL. Krauss *et al* (1996) examined the responses in these subjects in relation to the distribution of LDL profile *ie* whether the subject exhibited pattern A or pattern B . On the low fat diet almost half of pattern A subjects switched to pattern B *ie* there was a shift in particle size from larger to smaller. On the other hand, subjects with B pattern showed greatest increases in large VLDL, greater decreases in HDL_3 and greater reductions in LDL cholesterol, apo B, LDL II and LDL III concentrations. Suzukwa *et al* (1995) observed that fish oil supplementation increased LDL particle size with a decrease in serum triglyceride and an increase in LDL cholesterol, whereas corn oil supplementation had no effect on these parameters.

Genetic Influences

The earliest report of genetic influences on LDL heterogeneity by Fisher *et al*, (1975) indicated that the molecular weight of monodisperse LDL appeared to be inherited in 5 families. Subsequently two large family studies, one in primarily healthy families and one in families with familial combined hyperlipidaemia have shown that the inheritance of LDL subclass phenotype B is consistent with the presence of a single major gene defect, based on complex segregation analysis (Austin *et al*, 1988b, Austin *et al*, 1990b). Phenotype B was found to be closely associated with increased levels of triglyceride, apo B, mass of VLDL, mass of IDL and decreased HDL, HDL_2 and apo AI. Austin *et al* (1990a) named

this the atherogenic lipoprotein phenotype (ALP) a proposed genetic marker for CHD. The first study (Austin *et al*, 1988b) was based on 61 nuclear families in 29 kindreds obtained by community sample in San Francisco and the model providing the best fit to the family data was a single major locus model with a dominant mode of inheritance for the major gene. The allele frequency for the proposed phenotype B allele was estimated to be 0.25 and the model included reduced penetrance in young males and premenpoausal females. When the same study population was analysed using the LDL peak diameter as a continuous variable *ie* adjusted for age and gender (Austin *et al*, 1993a,b) the mode of inheritance could not be distinguished. In the second study (Austin *et al*, 1990b), 7 large multigenerational kindreds with well-characterised familial combined hyperlipidaemia were used, including 250 individual family members. LDL subclass phenotype B was inherited in these families consistent with the presence of a single genetic locus and an additional multifactorial inheritance component. Similar to the results in the healthy families, the mode of inheritance for the major locus was either dominant or additive, and the allele frequency for phenotype B was estimated to be 0.3. Reduced penetrance was again found in males under 20 years and females under 50. Although segregation analysis is susceptible to ascertainment bias, the uniformity of these results in these two studies with different sampling schemes is striking. Complex segregation analysis performed in healthy Dutch families (de Graaf *et al*, 1992) have further strengthened the view of the inheritance of LDL distribution, the results suggesting a single gene effect with multifactorial inheritance, and additionally the penetrance of small dense LDL being dependent on age, gender, hormone use and menopausal status in women. These studies provide evidence for a single major gene effect on the inheritance of LDL subclass phenotypes.
Twins studies provide a different approach to detecting genetic influences and LDL subclasses were analysed in 348 women from the Kaiser Permanente Women Twins study (Austin *et al*, 1987). The heritability of LDL peak particle diameter was 0.54 based on the classical heritibality estimate and the within pair estimate was 0.48. The NHLBI (Lamon-Fava *et al*, 1991) study conducted in 250 male twin pairs in their seventies found a heritability estimate of 0.52 for unadjusted LDL size and 0.39 when adjusted for BMI, alcohol consumption, smoking and physical activity.
Because of the strong evidence for a major gene effect based on the studies described, a candidate gene approach (Lusus 1988) has been used in attempting to map the chromosomal location of the proposed locus controlling LDL subclass phenotypes. The first candidate examined was the apo B locus on chromosome 2, but linkage analysis showed strong evidence against linkage (La Belle *et al*, 1991, Austin, 1991). Nishna *et al* (1992) found a significant linkage of LDL pattern B to the LDL receptor gene locus on chromosome 19, and moderate LOD scores between small LDL and the insulin receptor located on the short arm of chromosome 19. Rotter *et al* (1994) observed a significant linkage of LDL particle size with the LDL receptor gene among 260 members of 24 kindreds in which the proband had coronary disease. In the same study the haplotypes at the apolipoprotein AI-CIII-AIV gene complex on chromosome 11, manganese superoxide dismutase locus on chromosome 6 and the CETP gene on chromosome 16 were found to be linked to LDL particle diameter. The AI-CIII-A IV cluster was found to be the main locus for familial combined hyperlipidaemia in seven families, characterised as having a preponderance of small LDL (Wojciechoeski *et al*, 1991).

1.5.5 Properties of LDL Subfractions and Relationship to Coronary Heart Disease

Oxidation of LDL has been implicated in the formation of the fatty streak in the arterial intima (Steinberg *et al*, 1989) Oxidised LDL has diminished affinity for the classical LDL receptor and is more readily bound to the scavenger receptor in the macrophages thus loading them with cholesterol and initiating the formation of the foam cell. Several studies have shown that small dense LDL particles are more susceptible to oxidative modification in both normal individuals (de Graaf *et al*, 1991, Tribble *et al*, 1992) and in hypertriglyceridaemia (de Graaf *et al*, 1993, Chait *et al*, 1993) and combined hyperlipidaemia (Dejager *et al*, 1993). The prevention of oxidation of LDL using antioxidants is widely discussed at present as a promising antiatherogenic therapy (Witzum *et al*, 1993). α-Tocopherol or vitamin E is on a molar base the major antioxidant in LDL (Esterbaur *et al*, 1992) and a decreased content in small dense LDL is one possible explanation for its increased tendency to oxidative modification. However, Dejager *et al* (1993) found that the oxidative susceptibility of LDL species was independent of vitamin E and could not be accounted for by enrichments in polyunsaturated fatty acids. Tribble *et al* (1993) observed greater susceptibility to oxidation of dense LDL when compared with buoyant LDL and related this to a reduced free cholesterol content and a 50% reduction in α-tocopherol content. In a later study Tribble *et al* (1995) observed an enhanced susceptibility to oxidation of the surface monolayer compared to the outer core in small dense LDL which may lead to the enhanced susceptibility of this species by external agents. This has physiological relevance and therapeutic implications depending on the oxidising conditions *in vivo*. If external oxidising agents are the predominant mediators of LDL oxidation *in vivo*, then the surface properties are important and the surface may then be the most appropriate site for intervention. Barbosa *et al* (1995) observed that heavy LDL was more susceptible to oxidation in patients with combined hyperlipidaemia compared to controls, hypercholesterolaemics and hypertriglyceridaemics. They also found that the sialic acid content was increased in heavy compared to light LDL in these subjects and neutral sugar content was reduced. This is in contrast to La Belle *et al* (1990) who observed reduced content of neutral carbohydrates and sialic acid in small dense LDL. Sialic acid was observed to play a protective role against oxidation of heavy LDL in hypercholesterolemia and hypertriglyceridaemia but not in combined hyperlipidaemia where the sialic acid content was highest, the LDL was most susceptible to oxidation and desialition increased the resistance to oxidation

Orekhov *et al* (1989) demonstrated that the LDL from patients with CHD had a two to three-fold lower content of sialic acid and one and a half to two-fold lower content of neutral sugars (glucosamine, galactosamine, galactose, glucose) when compared to that of healthy subjects. Furthermore, a negative correlation was found between the capacity of LDL to stimulate intracellular lipid content *in vitro* and its sialic acid content. The same group (Tertov *et al*, 1993) isolated a sialic acid poor subfraction in LDL and observed it caused an increased accumulation of cellular cholesterol when isolated from CHD patients when compared to controls. This LDL was found to be smaller and more dense than the sialic acid rich LDL fraction. This is in agreement with the La Belle & Krauss (1990) who found a lower sugar and sialic acid content in LDL isolated from subjects with a

predominant B LDL phenotype. Filipovic *et al* (1979, 1988) have shown that desialiation of LDL increases binding and uptake by fibroblasts. Taken together these findings suggest that desialiation may increase the atherogenicity of LDL.
LDL with low sialic acid content has shown an increased affinity to artery wall by forming insoluble complexes with negatively charged lipoprotein -complexing proteoglycan found in the artery wall (Camejo *et al*, 1976, Camejo *et al*, 1985, Avila *et al*, 1978). The atherosclerotic lesion is characterised by lipoprotein deposition and increased intimal proteoglycans. Proteoglycans are highly charged electronegative glycoproteins consisting of glycosaminoglycan chains covalently attached to a protein core through *O* or *N*-glycosidic linkages. These macromolecules influence the viscoelasticity and permeability of arterial wall intima. There is general agreement that arterial wall proteoglycans play a key role in the development of atherosclerosis (Camejo *et al*, 1993). LDL isolated from post MI patients has been shown to have greater binding reactivity to arterial proteoglycans than normal subjects (Linden *et al*, 1989). Anber *et al* (1996) have shown that proteoglycan -LDL complex formation was significantly higher in subjects with ALP defined as LDL III concentration > 100mg/100ml. A recent study from Camejo's laboratory (Wiklund *et al*, 1996) observed reductions in proteoglycan-LDL complex formation on treatment with gemfibrozil and/or pravastatin, the effect being greater on gemfibrozil. This fits in well with their earlier observations that purified large LDL particles have a lower affinity to proteoglycan than small dense LDL (Hurt-Camejo *et al*, 1990, Linden *et al*, 1989). Parks *et al* (1991) have suggested that proteoglycan binding may be related to E:B ratio in LDL. Reduced binding of large LDL to proteoglycans on fish oil feeding was related to decreased E content in this subfraction and may be due to conformational alterations on the particle surface.
Weisser *et al* (1993) examined the effects of purified LDL subclasses on Ca^{2+} content in vascular smooth muscle cells cultured from rat aorta and found that dense LDL caused a more pronounced increase in Ca^{2+} than the lighter LDL fraction. As Ca^{2+} is an important second messenger system involved in many processes leading to atherosclerosis (Ross & Glomset, 1976) this is another possible atherogenic mechanism of small dense LDL.
Initiation of blood coagulation via the intrinsic pathway occurs when tissue factor (TF) is exposed to plasma and the resulting factor VIIa-TF complex then proteolytically activates factors IX and X (Osterund *et al*, 1977). Physiological regulation of this pathway is mediated by a specific factor Xa-dependent inhibitor of the factor VIIa-TF complex known as tissue factor pathway inhibitor (TFPI) (Broze *et al*, 1987). Lesnik *et al* (1993) determined the distribution of TFPI among lipoprotein particles and found that it was closely related to antithrombolytic activity which was primarily due to a preferential interaction with small dense LDL, HDL_3, VLDL and Lp(a). Small dense LDL provided the most favourable surface structure for efficient TFPI binding and expression of its anticoagulation activity.
Demuth *et al* (1996) have isolated a minor electronegative LDL with a higher sialic acid, apo CIII and apo E content. It has a higher proportion of dense particles than electropositive LDL and displayed enhanced cytotoxic effects on cultured human umbilical vein endothelial cells.

1.5.6 LDL Receptor Binding and LDL Subfractions

Differences in the receptor-binding affinity of the various LDL subfractions have been reported, but the earliest results were inconsistent. This may be partially explained by the differences in the LDL subfractions isolated. In 1985, Witzum *et al* reported that large LDL is cleared more rapidly by the LDL receptor and in the same year Kleinman *et al* observed defective binding in vitro of the small LDL from hypertriglyceridaemic subjects. On the other hand, Knight *et al* (1986) found that light and heavy LDL subfractions from a normolipidaemic subject bound with similar affinity to the LDL receptors of both cultured human fibroblasts and monocyte-derived macrophages and Swinkels *et al* (1987) found no differences to receptor binding or degradation of LDL1 (d 1.023-1.034 g/ml) or LDL2 (d 1.036-1.041 g/ml) to LDL receptors on fibroblasts or Hep G2 cells. Since then there has been a general consensus of opinion that large LDL is more rapidly removed by the LDL receptor. Jaakhola *et al* (1989), Nigon *et al* (1991) found that both light (d 1.031-1.037 g/ml, d 1.0244-1.0297 g/ml respectively) and heavy (d 1.041-1.047 g/ml, d 1.0358-1.0435 g/ml respectively) LDL subfractions had reduced affinity when compared with medium density LDL (d 1.037-1.041 g/ml, d 1.0297-1.0358 g/ml). These observations were confirmed in a recent report from the laboratory of Krauss. Campos *et al* (1996) examined receptor binding affinities of LDL (d 1.020-1.050 g/ml) isolated from subjects with predominantly large LDL (pattern A), predominantly small LDL (pattern B) and intermediate LDL pattern. They found a 16% reduction in binding affinity in pattern A and a 14% reduction in pattern B subjects when compared with the intermediate pattern. In the same report LDL I (d 1.026-1.032 g/ml), LDL II (d 1.032-1.038 g/ml) and LDL III (d 1.038-1.050 g/ml) were isolated from individuals with pattern A and pattern B and binding affinities to LDL receptors measured. LDL II had similar binding affinities while affinity of LDL III was lower in both groups. Investigators using specific monoclonal antibodies to block LDL receptor binding domains on apo E and apo B have attributed these different binding affinities among the LDL species to conformational changes (Chen *et al*, 1994, Galeano *et al*, 1994, Campos *et al*, 1996).

1.6 Metabolic Heterogeneity of Low Density Lipoprotein

The earliest estimations of catabolism of iodinated proteins were carried out by calculating the urine:plasma ratios which gave an estimate of the daily catabolic potential of the protein injection of radioiodinated LDL (Berson & Yalow, 1954). There followed a series of studies on the kinetics of LDL in which it was assumed that LDL was a homogeneous particle (Signurdsson *et al*, 1976, Packard *et al*, 1976, Bilheimer *et al*, 1979, Shepherd *et al*, 1980). At least two routes for LDL apo B catabolism were identified (Goldstein & Brown, 1972) one involving initial binding to the lipoprotein high affinity cell membrane receptors and the other occurring independently of this receptor mechanism. Comparison of the clearance rates of native and chemically modified by cyclohexanedione LDL permitted an assessment of the relative importance of the receptor and non-receptor pathways in vivo (Mahley *et al*, 1980). The fractional catabolic rate via the receptor pathway was calculated as the difference between the fractional catabolic rates of native LDL and cyclohexanedione modified LDL (Shepherd *et al*, 1979). Glycosylated LDL in which the lysine residues are blocked has also been used to measure receptor and receptor-independent LDL kinetics (Kesaniemi *et al*, 1983).

It became more evident that the assumption of LDL homogeneity was invalid both from the measurement of urine to plasma ratios of radioactivity and from growing evidence that LDL was structurally heterogeneous (Krauss & Burke, 1982). A number of studies took this into account when analysing the kinetics of LDL (Goebel *et al*, 1976, Malmendier *et al*, 1978, Wastney *et al*, 1982, Fisher *et al*, 1983), especially in hyperlipidaemic subjects (Fisher *et al*, 1980, Chait *et al*, 1986, Vega *et al*, 1986).

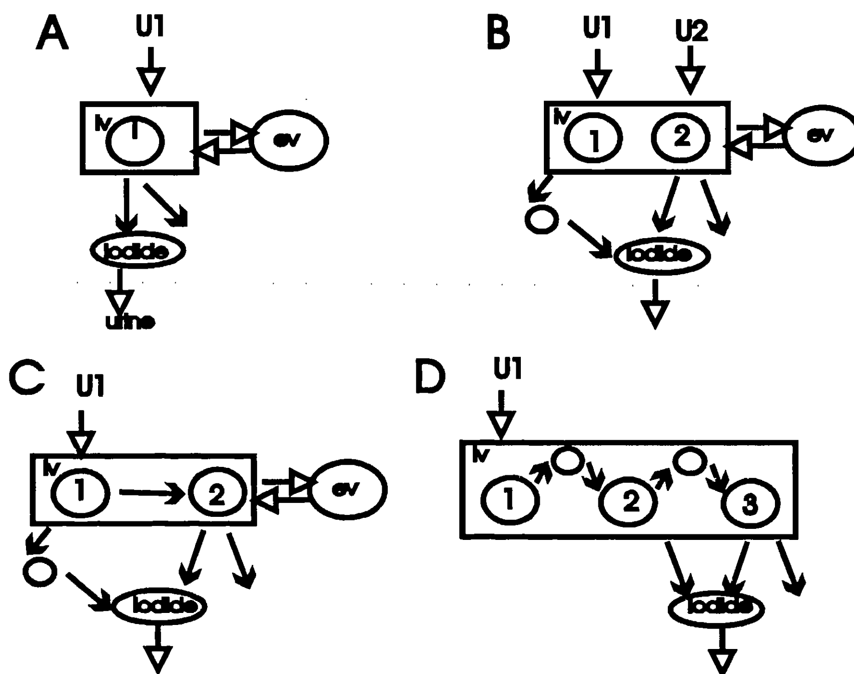

Fig.1.5 Foster's A, B, C and D Models of LDL Kinetics
iv intravascular, ev extravasclar, U input. The plasma compartments are in the rectangles. Model A has 1 plasma compartment, model B has 2 plasma compartments and 2 sited of production, model C has 2 plasma compartments and 1 site of input, model C is characterised by 3 plasma compartments and 1 site of synthesis.

In 1986 Foster *et al* examined the effect of kinetic heterogeneity in the analysis of LDL apo B turnover and developed and tested four integrated kinetic models (fig.1.5) which considered the heterogeneity of plasma LDL apo B and were consistent with the plasma and urine radioactivity data. The models were applied to studies in familial hypercholesterolaemia and familial combined hyperlipidaemia by Chait *et al* (1986). Model A assumed that LDL apo B was kinetically homogeneous and that degradation occurred only from the plasma compartment. To include urine data a compartment for whole body iodide was included which turned over at the rate of 2.5 pools/day. This model did not fit the data from all the individuals. Model B was characterised by two LDL apo B

compartments, one of which was allowed extravascular exchange, and a body iodide compartment. This model was an extension of model A to allow for two heterogeneous pools of apo B LDL and was not improved by allowing both plasma compartments to exchange with an extravascular pool. This was compatible with all the data. Model C was a modification of model B and was similar to that proposed by Phair *et al* (1975) and used by Fisher *et al* (1980) to analyse LDL turnover in hypertriglyceridaemia. It included a pathway where one plasma compartment was the sole precursor for a second plasma compartment and the rates of removal from the two plasma compartments were identical. Not all individuals fitted this model as some required model B in which the rates of removal from the two plasma compartments were different. Model D tested the possibility that LDL apo B which returns from the extravascular compartment has different kinetics and was a radical departure from the traditional LDL apo B models. It required three plasma compartments and two extravascular pools but was compatible with the data.
Different model configurations were devised to accommodate the complexity of LDL kinetics in different patient groups. Malmendier *et al* (1989a) developed a model for receptor and non receptor routes, each particle population having its own independent subsystem with their own production and catabolic rates. In a companion paper, Malmendier *et al* (1989b) the model was applied to describe the LDL kinetics in normocholesterolaemic volunteers at baseline and on treatment with simvastatin.
The most powerful tool that has been used in this area of lipoprotein metabolism is the Simulation, Analysis And Modeling (SAAM) computer program generated by Berman & Weiss (1974). This package was originally devised to operate in a batch mode but is now available in an interactive version, CONSAM (Boston *et al*., 1982) and a windows based version (SAAM II). This form of the program has a conversational potential for interrogation, display, modification of the model and the solutions and for setting up and executing various modelling tasks. These modelling programs, given an observed data set and initial estimates, will alter the parameters in an iterative manner until they reach a minimum sum of squares for the residual differences between observed and calculated data. Multicompartmental modelling can be used for parameter estimation in complex metabolic pathways and is limited only by the quality and quantity of information contained in the data set. It also allows quantitative hypotheses to be generated and tested.
There have been very few studies designed to examine the kinetics of individual LDL subfractions and to date investigators have concluded that the kinetics of LDL is not simply a function of density. Teng *et al* (1986) examined the kinetics of 'heavy' LDL (mean density 1.048 g/ml) and 'light' LDL (mean density 1.0405 g/ml) in normal volunteers and subjects with familial hypercholesterolaemia and hyperapobetalipoproteinaemia. In hyperapobetalipoproteinaemia there was an increased synthesis of light LDL with a normal conversion to heavy, whereas in FH there was a decreased conversion of light to heavy and independent synthesis of heavy LDL. In 1986, Vega & Grundy examined two subfractions in normals and subjects with moderate and severe hypertriglyceridaemia. The densities of light and heavy LDL were not the same in all subjects but they found that more dense LDL had a higher catabolic rate than normal and in moderate hypertriglyceridaemia a similar picture was present. Thomson *et al* (1987) carried out turnovers of light and heavy LDL in FH and hyperapobetalipoproteinaemia and concluded that light LDL was a precursor of heavy LDL. In hyperbetalipoproteinaemia there was overproduction of both subfractions

whereas in FH the kinetics of the light fraction was normal and heavy LDL was independently synthesised.

The origin of LDL subclasses is likely to be remodelling of VLDL through neutral lipid exchange or as a result of delipidation. A seminal paper by Packard *et al* (1995) describes and defines the multicompartmental model that has been used in Glasgow in a number of dual radioactive label VLDL turnover studies. The model is complex but has been used to explain human apo B metabolism in all circumstances of primary and secondary hyper or dyslipoproteinaemia in which it has been employed, including moderate hypercholesterolaemia, FH, lipoprotein and hepatic lipase deficiencies, non insulin dependent diabetes mellitus, hypothyroidism, nephrotic dyslipidaemia and apo E2 and E4 homozygosity. Following the administration of the radiolabelled tracer, transfer and catabolic rates can be determined by multicompartmental modelling analysis of the appearance and disappearance curves. Apo B derived from smaller VLDL particles ($VLDL_2$) appears rapidly in LDL fraction and is cleared rapidly in a bi-exponential curve. On the other hand LDL apo B derived from large triglyceride-rich $VLDL_1$ was produced at a slower rate and the catabolism was slower and monoexponential. The interpretation for these differences in metabolism of large and small VLDL particles is that $VLDL_1$ gives rise to an LDL population whose affinity for receptors is relatively low and remains in the circulation for a longer time and $VLDL_2$ contains not only $VLDL_1$-derived lipoproteins but also those synthesised *de novo* by the liver. It is the latter fraction that is processed rapidly by the receptor pathway. When hepatic triglyceride levels are high (due to *de novo* synthesis or delivery of dietary lipid) large triglyceride-rich particles are formed which delipidate to smaller VLDL and slowly metabolised LDL. Conversely, when the liver has a reduced triglyceride load, smaller VLDL are made which give rise to rapidly metabolised LDL.

1.7 Aims and Objectives

The aim in setting out on the work reported in this thesis was to investigate the structural and metabolic heterogeneity in LDL, to ascertain how important it might be in determining the properties of the lipoprotein and its involvement in the atherogenic process. The ultimate goal was to provide a quantitative description of the important subfractions within LDL, their sources and their route of catabolism.

In pursuit of this aim a number of objectives were set:
1) The development of a method to allow the quantitation in plasma of LDL subfractions in both normal and hyperlipidaemic subjects.

2) Investigation of the perturbation of the LDL subfraction profile induced by lipid lowering drugs in order to determine both how the drugs affected the subfraction distribution and, because the mechanism of action in most cases was known, to reveal further features of regulation of the profile.

3) Development of a method for quantifying the metabolic heterogeneity within LDL based on traditional LDL turnover methods to which multicompartmental modelling could be added as a technique for defining the behaviour of potential subspecies.

4) To understand the regulation of metabolic heterogeneity within the lipoprotein in hyperlipidaemic subjects in the basal state and during treatment with the fibrate class of lipid lowering drugs.

5) To relate the results of structural and kinetic studies in a physiological model which describes the heterogeneity of LDL.

Chapter 2 Materials and Methods

2.1 Materials

All reagents used were of analytical grade and the names and addresses of all suppliers are shown in appendix 1.

2.2 Lipid Analyses

Lipid analyses were carried out using enzymatic colorimetric assays. The final step was common to all i.e. the production of hydrogen peroxide which in the presence of the enzyme peroxidase, phenol and 4-aminophenazone produces the chromophore 4-(p-benzoquinone-mono-imino)-phenazone. Absorbance of this product was measured at 505nm on a Hitachi 704 or 717 autoanalyser or the Encore chemistry centrifugal analyser from Baker Instruments or on a Beckman DU70 spectrophotometer or a Dynatech MR 5000 Microtitre Plate Reader. Measurements were determined on potassium EDTA plasma (final concentration 1 mg/ml) or lipoprotein fractions. The salt concentration did not affect the analyses.

2.2.1 Cholesterol

Cholesterol was determined using a Boehringer kit (catalogue number 704121) in which the cholesterol esters in the sample were converted to cholesterol by cholesterol esterase. The enzyme cholesterol oxidase then oxidised all the cholesterol to form 4-cholestenone and hydrogen peroxide, the quantity of which was measured as described above. Cholesterol assays were standardised through the Center for Disease Control Cholesterol Reference Method Network. Coefficient of variation was 1.6%.

2.2.2 Free Cholesterol

Free cholesterol was measured using Boehringer kit number 310328. The cholesterol esterase which was present in the cholesterol assay was omitted and so only free cholesterol present in the sample was measured by oxidation with cholesterol oxidase. Quality control was monitored with Precinorm and Precipath materials and coefficient of variation was 4.3%.

2.2.3 Triglyceride

Triglyceride was determined using Boehringer kit number 704113. The first enzyme was lipase which converted the triglyceride into glycerol. Then, in the presence of ATP, glycerol kinase mediated the formation of glycerol-3-phosphate which was oxidised by the third enzyme, glycerol phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide, the quantity of which was analysed as above. Triglyceride assays were standardised through the Center for Disease Control Triglyceride Reference Method Network. Coefficient of variation was 2.1%.

2.2.4 Phospholipid

Phospholipid was measured in lipoprotein fractions using an enzymatic colorimetric kit from Boehringer (catalogue number 691844). Phospholipase D converted the phospholipids to choline and phosphatidic acid and the second enzyme choline oxidase mediated the formation of betaine and hydrogen peroxide in the presence of oxygen. Quality control was monitored by using Precinorm and Precipath materials and coefficient of variation was 4.8%.

2.3 Protein and Apolipoprotein Assays

2.3.1 Protein

Protein measurements in the lipoprotein fractions and apolipoprotein B isolates were carried out by a modification of the method of Lowry *et al* (1951).

i) Reagents

a) Stock Reagents:

A 2% (w/v) Na_2CO_3 in 0.1N NaOH
B 2% (w/v) Na_2CO_3 in deionised H_2O
C 2% (w/v) NaK tartrate in deionised H_2O
D 1% (w/v) $CuSO_4$ in deionised H_2O
Folin - Ciocalteu Reagent (BDH)

b) Working Reagent:

Biuret reagent - To 100ml A (for lipoprotein fractions) or B (for apolipoprotein B), 1ml each of solutions C and D were added. If the samples were turbid (eg VLDL1) sodium dodecyl sulphate was added to A at a concentration of 1 mg/ml (Curry *et al* 1978).

ii) Standards:

a) Stock Standard

Human serum albumin (fraction V, Sigma) at a concentration of 1mg per ml was stored at -70 °C. A fresh aliquot was thawed immediately prior to use.

b) Working Standards

A working standard curve in the range 0-500µg/ml was prepared in duplicate by pipetting 0-50µl stock standard. When analysing apoB, 100µl of 2N NaOH was also added. The final volume of the standard curve was made up to 400µl with deionised H_2O.

iii) Samples

a) Lipoprotein fractions

Appropriate aliquots of each fraction were taken and the final volume was adjusted to 400µl with deionised H_2O. Typical volumes taken were: 200µl VLDL1, 100µl VLDL2, 50µl IDL, 25µl LDL (S_f 0-12) and 10µl LDL (d 1.019-1.063 g/ml).

iv) Quality Control

Aliquots of bovine serum albumin (fraction V, Sigma) at 15 µg/100µl and at 30 µg/100µl were stored at -70°C. Fresh aliquots were used in each assay, the final volume of 400µl being reached by the addition of 100µl 2N NaOH and 200 µl deionised H_2O or 300 µl deionised H_2O, depending on which kind of sample was being analysed.

v) Procedure

a) Two mls of working biuret reagent was added to 400µl standard, control or sample, mixed and allowed to stand at room temperature for 10 minutes.
b) 200µl working Folin's reagent (diluted 1:1 with deionised H_2O) was added and mixed immediately.
c) Absorbances were read at 750nm on Beckman DU70 spectrophotometer after 30 minutes and within 2hours and a straight line standard curve was obtained.
d) Unknown values were read from a standard curve drawn on graph paper and latterly these unknown values were calculated by linear regession.

vi) Precision of the Assay

The within batch coefficient of variation was 1.9% and between batch was 2.4%.

2.3.2 Apolipoprotein AI

Apolipoprotein AI in plasma was quantified by immunonephelometry using a kit (catalogue number 67265) from Orion Diagnostics. The extent of immunoprecipitation was measured at 292nm on the Encore clinical chemistry centrifugal analyser (Baker Instruments). In conditions of antibody excess, the amount of precipitant is proportional to the apoprotein concentration. The sensitivity of the assay was 0.3 g/l and the coefficient of variation 5%. The quality control was monitored using lyophilised material from Immuno and Behring.

2.3.3 Apolipoprotein AII

Apolipoprotein AII in plasma was quantified by immunonephelometry using a kit (catalogue number 67356) from Orion Diagnostics. Immunoprecipitation was measured at 292nm on the Encore clinical chemistry centrifugal analyser (Baker Instruments). The sensitivity of the assay was 0.3 g/l with a coefficient of variation of 5%. Quality control was monitored using lyophilised material from Immuno and Behring.

2.3.4 Apolipoprotein B

Apolipoprotein B in plasma was quantified by immunonephelometry using a kit (catalogue number 67249) from Orion Diagnostics. Immunoprecipitation was again measured at 292nm on the Encore clinical chemistry centrifugal analyser. The sensitivity of the assay was 0.3 g/l and coefficient of variation 5%. Quality control was monitored using lyophilised materials from Immuno and Behring.

2.3.5 Lipoprotein (a)

Lipoprotein (a) (Lp(a)) in plasma and lipoprotein fractions was measured using the Innotest (Innogenetics) enzyme linked immunosorbent assay (ELISA). The wells of the polystyrene microplate were coated with a mouse monoclonal anti-Lp(a). A standard curve from 0 - 100mg/dl or diluted sample bound to this solid-phase antibody and unbound substances were removed by washing. The second antibody, a sheep polyclonal anti-apo B labelled with the enzyme horseradish peroxidase, was added and bound to the retained Lp(a) due to the apo B moiety. Incubation with substrate produced a blue colour which turned yellow when the reaction was stopped with sulphuric acid. The amount of colour produced was

proportional to the amount of Lp(a) originally present in the sample or standard. Samples were stored at -70°C and thawed immediately prior to assay.
The assay was carried out in duplicate and read at absorbance 450nm using a Dynatech MR 5000 Microtitre Plate Reader. The sensitivity of the assay was 2mg/dl in diluted samples and concentrations as low as 0.001 mg/dl could be detected in undiluted samples. The between batch precision of the assay was 12% and quality control monitored using in-house frozen plasma and lyophilised material from Innogenetics.

2.3.6 Apolipoprotein E Phenotyping

Apo E phenotypes were determined by isoelectric focussing followed by Western blotting using an adaptation of the method of Havekes *et al* (1987).

i) Delipidation

Ten μl of plasma was first incubated for 30 minutes at 37°C with 28.5 mU neuraminidase (EC 3.2.1.18 from Clostridium Perfinges Type X, Sigma) in 0.02M acetate buffer, pH5.1, then delipidated overnight at 4°C with ethanol:ether (3:1,v/v), followed by an overnight incubation in ether. The resultant precipitate was dissolved in 200μl 0.1M Tris,6M urea,1%(w/v) SDS, pH 10.0 to which 10μl β-mercaptoethanol was added.

ii) Isoelectric focussing

Thirty μl of sample was applied to a vertical slab gel of 5% polyacrylamide in 8M urea containing 1%(v/v) ampholines pH 4-6 (Serva). Electrophoresis was carried in a Hoefer SE 600 tank (Pharmacia) at 250 volts, 12mA per gel at room temperature overnight with water cooling. The upper buffer was 0.08% NaOH (w/v), pH 10.1 and lower buffer 0.1M sodium phosphate, pH 2.3

iii) Transfer to Nitrocellulose

Transfer from the acrylamide gel to nitrocellulose was performed as described by Towbin *et al* (1979). This was carried out at room temperature with water cooling at 100 volts for 3 hours in 0.2M glycine, 0.02M Tris, 20%.(v/v) methanol, pH 9.0 in a Biorad transblot cell.

iv) Immunostaining

a) The nitrocellulose strip was first soaked in wash buffer (0.01M Tris, 0.05% (v/v) Tween 20, 0.15M NaCl, pH 7.4) containing 5% (w/v) milk powder (Marvel) as a blocking agent and then the strip was washed for 20 minutes with 3 changes of buffer. Incubation with the first antibody (mouse monoclonal anti-apoE) was overnight at room temperature with slow regular shaking. The strip was washed for 20 minutes and incubated with the second antibody (goat anti-mouse horse radish peroxidase conjugate) for 2 hours at room temperature. After washing a blue colour was developed by the addition of 4-chloro-napthol and peroxide in 0.15M saline. Apo E isoforms were identified by reading against a known apo E2/E2 phenotype.

2.4 Lipoproteins

2.4.1 β Quantification

Plasma VLDL cholesterol, LDL cholesterol and HDL cholesterol were analysed according to the Lipid Research Clinics Program Manual of Laboratory Operations (1975). VLDL (d 1.006 g/ml) was isolated by ultracentrifugation at 35,000rpm (110,000g) in a Beckman Ti

50.2 rotor at 4° C for 18 hours. The apo B containing lipoproteins in the infranatant were precipitated by the addition of an equal volume of sodium heparin ($5x10^5$units)/Mn Cl_2 (0.092M) (Warnick & Albers,1978), followed by centrifugation at 10,000 rpm at 4°C for 30 min. This left HDL in solution and cholesterol measurements were carried out on total plasma, VLDL and HDL. LDL cholesterol was obtained by subtracting the HDL level from the cholesterol content in the d >1.006 g/ml infranatant.

2.4.2 Isolation of $VLDL_1$(S_f60-400), VLDL2(S_f20-60), IDL(S_f12-20) ,LDL(S_f0-12)

$VLDL_1$, $VLDL_2$, IDL and LDL were isolated from plasma by a modification of the cumulative ultracentrifugation density gradient technique described by Lindgren *et al* (1972). Density solutions were prepared from stock solutions at d 1.006g/ml and d 1.182 g/ml of NaBr in 0.195M NaCl, 0.001% Na_2EDTA. The densities were measured to 3 decimal places in a Paar Scientific density meter (model DMA 35).

The density of 2ml of plasma was adjusted to d 1.118 g/ml by the addition of 0.341 g NaCl and this was carefully layered over a cushion of 0.5 ml d 1.182 g/ml solution in an ultraclear Beckman SW 40 ultracentrifuge tube which had been coated with polyvinyl alcohol (Holmquist,1982). This allowed the solutions to gravity feed down the side of the tubes smoothly without disturbing the formation of the gradient. A discontinuous gradient was formed by overlayering with d 1.0988 g/ml (1ml), d 1.0860 g/ml (1ml), d 1.0790 g/ml (2ml), d 1.0722 g/ml (2ml), d 1.0641 g/ml (2ml), d 1.0588 g/ml (2ml). Centrifugation was carried out in a Beckman SW 40 rotor at 23°C in a Beckman L8 ultracentrifuge for the times and speeds given in table 2.1. Rotors were decelerated without the brake. Fractions were removed from the top of the tube using a finely drawn glass Pasteur pipette.

Table 2.1 Centrifugation Conditions for VLDL-LDL Isolation

Fraction	*Speed (rpm)*	*Time*	*Volume (ml)*
$VLDL_1$	39,000	1h 38min	1.0 *
$VLDL_2$	18,500	15h 41min	0.5
IDL	39,000	2h 35min	0.5
LDL	30,000	21h 10min	1.0

**1 ml removed and overlayered with 1 ml d 1.0588 g/ml density solution*

2.4.3 Preparation of LDL(S_f0-12)

LDL (S_f 0-12) for the LDL turnover protocol described in chapter 2.6 was isolated using solutions and materials which were sterile and equipment that was autoclaved before use. In addition, solutions were filtered through Amicon 0.22mμ filters prior to use. LDL was prepared by cumulative density ultracentrifugation as described in section 2.4.2 with a few minor alterations. 13 mls of fasting K_2EDTA plasma was adjusted to d 1.118 g/ml by the

addition of 2.2165g NaCl and overlayered with NaBr solutions d 1.0988-1.0588 g/ml as described above. $VLDL_1$, $VLDL_2$ and IDL were centrifuged to the top of the tube at 40,000 rpm for 7h 22min and 1 ml removed. After a further spin at 23°C and 34,000 rpm for 16 h 29 min, LDL was harvested in 1.0 ml from each tube and pooled to yield 6 ml LDL solution.

2.4.4 Preparation of LDL by Zonal Ultracentrifugation

LDL for the turnovers described in chapters 5 and 6 was isolated from plasma (within 3 h of venepuncture) by the rate-zonal ultracentrifugation technique described by Patsch *et al* (1974). All solutions and materials were prepared under sterile conditions and equipment was autoclaved before use. In addition the solutions were filtered through Amicon 0.22mμ filters. The density solutions d 1.0-1.3 g/ml were prepared in 0.1M Tris-0.01%(w/v) Na_2EDTA, pH 7.6 by the addition of NaBr and densities checked using a hydrometer at 20 °C.

A linear gradient of d 1.0-1.3 g/ml was formed in a Beckman Ti 14 zonal rotor spinning at 3,500 rpm (capacity 665ml) using the Beckman rotating seal assembly, a gradient mixer (1130 LKB Ultragrad) and a peristaltic pump (LKB). The density of 50 ml fasting K_2 EDTA plasma was raised to 1.3 g/ml by the addition of 15 g NaBr and carefully loaded onto the outside of the gradient. This was followed by a cushion of 25 mls of d 1.3 g/ml to ensure that all the plasma was transferred into the rotor and was not adhering to the wall of the rotor. The loading head assembly was removed and the rotor was capped. After centrifugation at 45,000 rpm for 140 minutes at 10°C, the rotor was unloaded at 3,500 rpm by pumping density 1.3 g/ml solution through the peripheral line of the loading head. The eluate was monitored at OD 280nm by a Type 6 Optical Unit connected to a Model U-A Absorbance Monitor (Isco Instrument Specialities Co.) and collected into 14 ml fractions. The one or two fractions containing the peak of LDL were pooled.

The development of a zonal ultracentrifugation method for the separation of individual LDL subfractions was carried out in a Beckman Ti 15 zonal rotor which had a capacity of 1665mls. Procedures for loading and unloading the gradients were similar to those described above. Variations of a step wise gradient were tried as well as different ultracentrifugation times as will be discussed in chapter 3.

2.4.5 Isolation of VLDL, IDL and LDL by Sequential Flotation Ultracentrifugation

VLDL (d 1.006 g/ml), IDL (d 1.006-1.019 g/ml), LDL (d 1.019-1.063 g/ml) and HDL (d 1.063-1.21 g/ml) were isolated according to the method of Havel *et al* (1955). The density solutions were prepared from stock solutions at d 1.006 g/ml, d 1.182 g/ml and d 1.478 g/ml of NaBr in 0.195M NaCl, 0.001% Na_2EDTA. Densities were measured to 3 decimal places in a Paar Scientific density meter.

4 ml of plasma was overlayered with 2 ml density1.006 solution and centrifuged in a Beckman Ti 50.3 rotor at 39,000 rpm or a Beckman Ti 50.2 rotor at 35,000 rpm for 18 hours at 15°C. The top 2ml containing VLDL was aspirated using a finely drawn glass Pasteur pipette. The remaining 4 ml was adjusted to d 1.019 g/ml by the addition of 0.32 ml d 1.182 g/ml solution and overlayered with 1.68 ml d 1.019 solution. After an overnight centrifugation (18 h, 15°C), IDL was removed in the top 2 ml. The infranatant (4ml) was

adjusted to d 1.063 g/ml by the addition of 1.47 ml d 1.182 g/ml solution and overlayered with solution at d 1.063 g/ml. After an overnight centrifugation (18 h, 15°C), LDL was harvested in the top 2ml.

2.4.6 Compositional Analysis of Lipoproteins

The major components contributing to the total mass of lipoprotein in mg/dl in each fraction are triglyceride, free cholesterol, cholesteryl ester, phospholipid and protein. The analyses of these have been described in sections 2.3 and 2.4.1. However, the masses of triglyceride and cholesterol need to be converted to mg/dl by multiplying the molar concentrations by 88.6 and 38.7 respectively. The mass of cholesteryl esters was calculated as the difference between total cholesterol and free cholesterol which was multiplied by 1.68 to correct for the mass of the esters. The contribution of each component to the total lipoprotein mass was then calculated by dividing their respective masses by their combined masses and was expressed as percentages.

2.4.7 HDL Subfractions by Analytical Ultracentrifugation

The masses of protein in HDL_2 and HDL_3 were measured by analytical ultracentrifugation in a Beckman model L8-70 equipped with an ultraviolet scanning attachment using a Beckman ANF rotor (Shepherd *et al*, 1984). 2 ml of plasma were adjusted to d 1.21 g/ml by the addition of 4 ml d 1.31 g/ml solution and total lipoproteins were prepared by centrifugation for 48 h at 39,000rpm and 4°C in a Beckman 50.3 rotor in a Beckman L7 ultracentrifuge. One ml was harvested from the top of the tube and the sample was diluted with d 1.20 g/ml so that during the analytical centrifugation run the total absorbance at 280 nm did not exceed 1.0. A microcomputer was used to control the centrifuge and it triggered the scanner after a defined centrifugal force had been applied to the sample. During centrifugation, flotation of the lipoprotein was monitored by changes in the absorbance recorded in the analytical cell. Under these conditions HDL was buoyant and so absorbance at the periphery of the cell decreased progressively and centripetally as the lipoproteins floated in the gravitational field. The rate at which they moved was a function of their density. The absorbance change in the flotation interval ($F_{1.2}$) 0-3.5 was due to HDL_3 and $F_{1.2}$ of 3.5-9 was due to HDL_2. (F1.2 is the negative sedimentation coefficient in Svedbergs of the lipoprotein at d 1.20 g/ml and 26°C). The masses of the HDL subfractions were expressed in mg/dl plasma.

2.4.8 LDL Subfractions by Gradient Gel Electrophoresis

The particle size distribution of LDL was examined by non-denaturing gradient gel electrophoresis as described by Nichols *et al* (1986). Electrophoresis was performed using commercially available polyacrylamide slab gels containing a linear gradient of 2-16% acrylamide (PAA 2/16, Pharmacia). The buffer system used within the electrophoersis apparatus (GE-4, Pharmacia) was 0.01M Tris, 0.08M boric acid, 2.5mM EDTA, pH 8.3. The gels were pre-equilibrated for 20 minutes at 70 volts. Samples of LDL (d 1.019-1.063 g/ml) were mixed in a 2:1 ratio with tracking dye made up of 4 g sucrose and 1mg bromophenol blue in 10 ml of electrophoresis buffer. Approximately 5-10 µl of sample containing 5-10µg of protein was applied to the top of the gel and electrophoresed at 20

volts for 20 minutes, 70 volts for 30 minutes and finally 120 volts for 24 hours. A high molecular weight marker (HMW, Pharmacia), containing thyroglobin (radius 8.5 nm), apoferritin (6.1 nm), lactate dehydrogenase (4.08 nm) and bovine serum albumin (3.55 nm), and a suspension of latex beads (diameter 38 nm, Dow Chemicals) were used to estimate the relative migration (Rf) rates of each band. Gels were fixed in 10% (w/v) sulphosalicyclic acid for 30 minutes, stained with 0.1% (w/v) Coomassie Brilliant Blue 250 (Bio-Rad Laboratories) in methanol:acetic acid:water (4:1:5, v/v/v) for 1 hour and destained in 7.5% (v/v) acetic acid. The gels were scanned by computer assisted video densitometry in a Bio-Rad Model 620 Video Densitometer (Bio-Rad Laboratories). LDL size was calculated with reference to the Rf values of the high molecular weight markers.

2.4.9 LDL Subfraction Analysis by Density Gradient Ultracentrifugation

A discontinuous salt gradient was devised which permitted isoltation of LDL subfractions directly from plasma within 24 hours (Griffin *et al*, 1990). The density solutions were prepared from stock solutions at d 1.006 g/ml and d 1.182 g/ml of NaBr in 0.195M NaCl, 0.001% (w/v) Na_2EDTA. The densities were measured to 3 decimal places in a Paar Scientific digital densitometer.

The density of 3 ml plasma was adjusted to 1.09 g/ml by the addition of 0.38 g KBr and this was carefully layered over a cushion of 0.5 ml d 1.182 solution in an ultraclear Beckman SW 40 ultracentrifuge tube which had been coated with polyvinyl aclohol (Holmquist *et al*,1982). This allowed the solutions to gravity feed down the side of the tubes smoothly without disturbing the formation of the gradient. A discontinuous gradient was formed by overlayering with d 1.060 g/ml (1ml), d 1.056 g/ml (1ml), d 1.045 g/ml (1ml), d 1.034 g/ml (2ml), d 1.024 g/ml (2ml), d 1.019 g/ml (1ml). Centifugation was carried out in a Beckman SW40 rotor at 23°C using a Beckman L8 ultracentrifuge. The rotor was accelerated to 170rpm over 4 minutes, centrifuged at 40,000rpm for 24 hours and decelerated without using the brake. The gradient containing the separated LDL subfractions was displaced upwards by a dense, hydrophobic material (Maxidens, 1.9g/ml, Nyegaard Ltd) which was introduced using a constant infusion pump (Sage Instruments, Orion Research Inc, USA) at a flow rate of 0.69 ml/min. The eluate was continuously monitored at 280nm and, if required, 0.3 ml fractions were collected using a Gilson fraction collector. At first the apparatus used for piercing and holding the ultracentrifuge tube in place was made from perspex by the Medical Physics Department at GRI and was a similar design to that described by Groot *et al* (1982). This was replaced by the density fractionation system from Beckman. Originally, the protein content of the eluate was monitored by passing through a UV detector (MSE, Fisons) and recorded with a Chessel chart recorder. This caused difficulties in interpretation of the LDL profile and was replaced by a Beckman DU70 spectrophotmeter integrated with the Data Leader programme (Beckman). The procedure generated LDL subfraction profiles (fig. 2.1) from which it was possible to resolve three subfractions that corresponded in size and density to those described by Krauss *et al* (1981), namely LDL I (d 1.025-1.034 g/ml), LDL II (d 1.034 - 1.044 g/ml) and LDL III (d 1.044-1.060 g/ml). The percentage areas under the curve for each individual subfraction was then quantified (Data Graphics, Beckman) and corrected for differential absorption characteristics of total lipoproteins using previously determined extinction coefficients. For LDL I this was 2.63, for LDL II it was 2.94 and for

LDL III it was 1.96. This was recalculated as a percentage of total LDL (d 1.019-1.063 g/ml) mass and was expressed in concentrations in milligrams of lipoprotein per 100ml of plasma. Within rotor coefficient of variation for LDL I-III concentrations was 5.4% and between rotors 6.5% for replicate analysis of the same plasma sample.

Fig.2.1 LDL Subfraction Profiles
Typical LDL subfraction profiles in two subjects showing 3 fractions, LDL I, LDL II and LDL III.

2.5 Electron microscopy

The sizes of LDL (d 1.019 - 1.063 g/ml) and LDL I, LDL II and LDL III subfractions isolated from normal volunteers was estimated by negative contrast electron microscopy. The fractions, at a concentration of 0.25-0.5 mg/ml of protein, were exhaustively dialysed against a volatile buffer consisting of 0.125 M ammonium acetate, 2.6 mM ammonium carbonate, 0.26 mM tetra sodium EDTA, pH 7.4 and then mixed with the negative stain phosphotungstate at pH 7.0 (Forte *et al*, 1986) immediately prior to examination and photography on Formvar-coated grids in a transmission electron microscope at a magnification of x29,000 (courtesy of Mr Jim Aitken, Department of Virology, Glasgow

University). The diameters of 100 randomly selected lipoprotein particles were determined from the images.

2.6 LDL Turnover Protocols

2.6.1 LDL Preparation

After a 14 h fast 50 ml K_2 EDTA plasma was obtained from each subject. For studies in normals (Chapter 5) and subjects with moderate hypercholesterolaemia (Chapter 6) LDL was prepared by rate zonal ultracentrifugation as described in section 2.4.4. For the turnovers in subjects with moderate hypertriglyceridaemia (Chapter 7) LDL (S_f 0-12) was prepared by cumulative ultracentrifugation as described in section 2.4.3.

LDL prepared by zonal ultracentrifugation was concentrated to approximately 1 mg/ml and dialysed against 0.15M NaCl, 0.01% Na_2EDTA (w/v), pH 8.1 using N_2 pressure filtration through an Amicon XM100 A cellulose membrane.

The LDL prepared by cumulative ultracentrifugation was passed through a 1x10cm column of Sephadex G-25 (PD 10 column, Pharmacia) using 0.15M NaCl, 0.01% Na_2EDTA (w/v), pH 8.1 as the eluting buffer. This removed the high salt content. All solutions were prepared using sterile water and filtered through 0.22mµ Amicon filters and all consumables and equipment which would come into contact with the preparation were sterilised by autoclaving.

An approximate protein concentration was calculated by measuring the absorbance of the LDL at 280nm and assuming a specific extinction coefficient of 1.0.

2.6.2 Labelling of LDL

Radiolabelling of LDL with either reductant-free Na ^{125}I or Na ^{131}I (Amersham) was carried out by the iodine monochloride method of McFarlane as modified by Shepherd *et al* (1975). This labelled the tyrosine residues in LDL protein. All solutions had been prepared using sterile water and filtered through 0.22mµ Amicon filters and all consumables and equipment which would come into contact with the preparation had been sterilised by autoclaving.

2 mls of LDL were added to 500µl 1.0 M glycine, pH10 followed by 1mCi carrier free Na ^{125}I or Na ^{131}I. An appropriate volume of ICl (stock solution- 25nmoles per µl in 1.0M NaCl) was added to give a molar ratio of ICl : protein of 2.5:1 and the solution mixed immediately. Bound and free iodine were separated by elution from a 1x10cm column of Sephadex G-25 (PD10 column, Pharmacia) with 0.15M NaCl, 0.001% (w/v) EDTA,pH 8.1.

2.6.3 Modification with 1,2-cyclohexanedione

^{131}I-apo LDL was treated with 1,2-cyclohexanedione to block the arginine residues on the apoprotein moiety (Shepherd *et al*, 1979). 2 mls of ^{131}I-apo LDL in 0.15M NaCl, 0.001% (w/v) EDTA,pH 8.1 was incubated at 35°C for 2 hours with 4 mls of freshly prepared 0.15M 1,2-cyclohexanedione in 0.2M borate buffer, pH 8.1. The modified LDL was separated from unbound reagents by passing the mixture over a 1x10cm column of Sephadex G-25 (PD10 column, Pharmacia) with 0.15M NaCl, 0.001% (w/v) EDTA,pH 8.1 as eluant.

2.6.4 Sterilisation and Calculation of Injection Dose

Labelled tracers were sterilised immediately prior to injection by filtration through a 0.45μm filter (Acrodisc, Gelman) which had been primed with the subject's unlabelled LDL. The radioactivity concentration in μCi /ml was determined after sterilisation by counting a 10μl aliquot and comparing with ^{125}I and ^{131}I simulated standards. Radioactivity was measured in a twin channel Packard gamma spectrometer.

2.6.5 Subject Preparation

Thyroidal uptake of radioiodide was blocked by the oral administration of potassium iodate (170 mg twice daily). This was commenced three days prior to injection and was continued for 28 days. The turnover studies were conducted on an out-patient basis and in order to ensure steady state conditions the subjects were encouraged to adhere strictly to their regular diet and lifestyle. Subjects received detailed instructions on the collection of 24 h urine specimens and were introduced to the nurse who would be visiting them each day to collect the samples. All subjects gave written informed consent before participation.

2.6.6 Injection

Aliquots of 25μCi of ^{125} I-native LDL and 25μCi ^{131}I-CHD modified LDL followed by a small bolus of sterile 0.15M NaCl were injected in rapid sequence into the subject's antecubital vein. Ten minutes later a 10 ml sample was taken from the peripheral vein of the opposite arm.

2.6.7 Sampling Protocol

The patient was visited at home by a community nurse every morning for the next 14 days and 20 ml of fasting blood was collected into K_2 EDTA as anticoagulant at a final concentration of 1mg/ml. Continuous 24 hour urine samples were also collected daily for the duration of the study and creatinine clearance rates were calculated.

2.6.8 Data Collection and Handling

At the end of the study, 2 ml aliquots of plasma and urine were counted for 10 minutes in a twin Packard gamma spectrometer. Plasma and urine radioactivity decay curves were constructed for the 14 days. The urine : plasma radioactivity ratios for both native and CHD modified apo LDL were calculated from the urinary output of ^{131}I or ^{131}I in a 24 hour period and the plasma radioactivity at the beginning of that period. Since there is approximately a half day delay between the clearance of radioactivity from the plasma and its appearance in urine (Boston, 1982, Foster 1986), the latter value represents the relevant mean plasma radioactivity for the 24-h urine collection.

2.6.9 Calculation of Apo-LDL Pool Size

LDL d 1.019-1.063 g/ml was prepared from 2ml fasting plasma on four occasions by sequential ultracentrifugation as described an section 2.4.5 and the composition of the protein, free cholesterol, cholesteryl ester, triglyceride and phospholipid analysed. A pool size for apo-LDL was derived from the product of plasma volume (calculated by isotope

dilution or 4% of body weight if the subject had a normal BMI) and the mean protein concentration.

2.6.10 Kinetic Analysis

Plasma radioactivity data was analysed by the procedure of Matthews (1957) as adopted by Shepherd *et al* (1979) to determine fractional catabolic rates (FCR) for total, receptor-dependent and receptor-independent apo-LDL catabolism. The rate of elimination of ^{125}I-labelled native apo-LDL represents total catabolism of this fraction, while that of the ^{131}I-CHD modified tracer is used as a measure of receptor-independent activity. The difference between these two FCR's (native and CHD) therefore provides an index of the activity of the receptor pathway. The synthetic rate for apo-LDL was calculated as the product of the apo-LDL circulating mass and the FCR. The fractional catabolic rate was expressed as pools per day and the synthetic rate as mg per kg of body weight.

In order to fit the plasma and urine radioactive decays these were modelled simultaneously using version 30 of the Simulation, Analysis And Modeling (SAAM) computer program generated by Berman & Weiss (1974). This was distributed by the SAAM Institute, University of Washington, Seattle, USA with detailed instructions in the CONSAM Users Manual (1990). Further details of kinetic analyses are given in the appropriate chapters.

2.7 Ethical Implications

All subjects gave informed written consent to the studies which met with the requirements of the Ethics Committee of each host institution ie Glasgow Royal Infirmary; Victoria Infirmary, Glasgow; Stobhill Hospital, Glasgow; Hairmyres Hospital, East Kilbride; Chelsea and Westminster Hospital, London and University College Hospital, London. All radioactive injections were carried out under the ARSAC licence of Professor James Shepherd and Dr Denis O'Reilly.

2.8 Statistical Analysis

Statistical analysis was carried out using the PC version of Minitab version 10 (Minitab Inc, PA). All variables were tested for the presence of a normal distribution by drawing normality plots and in instances where they were not normally distributed, appropriate transformations were performed. Differences in mean values between groups were compared by Student's unpaired t-tests using transformed data where appropriate. Differences between lipid, lipoprotein, compositional data and metabolic parameters at different time points in the same individuals were analysed by paired students t-test. Associations between variables were tested by calculating the Pearson correlation coefficient and the significance of association between pairs was determined by linear regression. Multivariate analysis was conducted using the analysis of variance General Linear Model (GLM) in Minitab which generated the coefficient of determination (r^2) related to the independent contribution of variables.

Chapter 3 Epidemiology of Low Density Lipoprotein Subfractions

3.1 Introduction

Early studies with the analytical ultracentrifuge showed that LDL exhibited a range of flotation rates at the standard density of 1.063 g/ml (Lindgren *et al*, 1969). Later it was shown by Hammond & Fisher (1971) and Fisher *et al* (1983) that multiple peaks or polydisperse LDL patterns were present in subjects with hypertriglyceridaemia or diabetes mellitus. Subsequent studies have led to the recognition that the LDL flotation profile in most subjects represents overlapping distributions of several components and is appropriately described and analysed as a paucidisperse system.

A number of high resolution methods employing electrophoresis and ultracentrifugation have been developed to identify and characterise subfractions along the LDL density spectrum. Non denaturing gradient gel electrophoresis as described by Nichols *et al* (1986), using commercially available 2-16% polyacrylamide gels, is commonly employed to measure the mean particle diameter of isolated LDL. A variety of ultracentrifugation methods have also been used. These have involved sequential separations at various densities (Hammond & Fisher, 1971), rate zonal ultracentrifugation (Patsch, 1986) and density gradient ultarcentrifugation (Krauss & Burke, 1982; Fisher *et al*, 1983, Lee & Downs, 1982, Marzetta *et al*, 1986, Swinkels *et al*, 1987, Chapman *et al*, 1988).

Analysis of LDL subclass distributions by both particle size and density has revealed marked variability among individual subjects, a large portion of which is related to clinical and metabolic variables, including gender, age, adiposity, diet, hormonal status and drug use (Krauss 1991, McNamara *et al*, 1987). Many of these factors result in coordinate changes among LDL subclasses, notably a reciprocal variation in levels of LDL I and LDL III (Krauss *et al*, 1988). These important effects on LDL subclass patterns may be amplified in disease states such as hypertriglyceridaemia (Hammond & Fisher, 1971; Nichols *et al*, 1986) and diabetes mellitus (Fisher 1983; Barakat *et al*, 1990).

The clinically most important variation in the LDL profile is a predominance of small dense LDL. It is an important feature in two metabolic disorders in which there is a clustering of risk factors - the insulin resistance syndrome (IRS) and the atherogenic lipoprotein phenotype (ALP). IRS, first proposed by Gerry Reaven in the 1988 Banting Lecture, is a plurimetabolic disorder linked to increased risk of CHD. It is associated with hypertriglyceridaemia, depressed HDL, central obesity, hypertension, impaired glucose metabolism and to this list Reaven *et al* added in 1993, increased proportions of small dense LDL. In 1990b, Austin *et al* identified in the general population a clustering of moderate hypertriglyceridaemia, low HDL and a predominance of small dense LDL which she termed the ALP and linked to increased risk of myocardial infarction (Austin *et al*, 1988).

The purpose of the present investigation was to develop a quantitative method of isolating LDL subfractions, to compare it with already published methods and to examine in detail the LDL subfraction distribution in normals, and subjects with coronary artery disease,

hyperlipidaemia, familial hypercholesterolaemia, non insulin dependent diabetes mellitus and the nephrotic syndrome.

3.2 Methods

The techniques of gradient gel electrophoresis, zonal ultracentrifugation and density gradient ultracentrifugation were explored in an attempt to develop a method to subfractionate LDL into discrete species.

3.2.1 Gradient gel electrophoresis

Non denaturing gradient gel electrophoresis on 2-16% Pharmacia gels as originally desribed by Nichols *et al* (1986) and in detail in 2.4.8 was employed to examine LDL (d 1.019-1.063 g/ml) in an initial survey of a mixed popultion of 143 subjects. Based on these findings of a useful but limited resolution, attempts were made using home made gels to improve the separation of LDL fractions by narrowing the gradient and by using linear and concave gradients from 2.5-5.4% and 2.5-7.8% polyacrylamide.

3.2.2 Zonal Ultracentrifugation

Zonal ultracentrifugation as described in 2.4.4 in a Ti14 rotor (capacity 665ml, path length 5.3 cm) and a Ti 15 rotor (capacity 1800ml, path length 7.3 cm) was carried out as a preparative method to isolate different LDL species. The following gradients were formed with run times varying from 4 to 24 h and speeds from 30 to 40,000 rpm: linear and concave gradients d1.006-1.21 g/ml and d 1.006-1.3 g/ml, and a stepwise gradient 1.006:1.063:1.21 in the ratio 5.2:4.0:2.5, mimicking the density gradient ultracentrifugation of Marzetta *et al* (1986).

3.2.3 Density Gradient Ultracentrifugation

Initially, three of the current published density gradient ultracentrifugation methods were evaluated. In the first (Krauss *et al*, 1981), 2 ml LDL (d 1.006-1.063 g/ml) adjusted to 1.040 g/ml was layered between 2.5 ml d 1.054 g/ml solution and 2.5 ml d 1.028 g/ml solution and centrifuged for 40h at 40,000 rpm in a Beckman SW40 rotor. In the second (Swinkels *et al*, 1987), 3.4 mls of serum stained with coomassie blue at pH 4.5-5 was layered under 2.5 ml d 1.065 g/ml, 2.5 ml d 1.020 g/ml and 2.5 ml d 1.006 g/ml and centrifuged for 19.5 h at 40,000 rpm in a swinging bucket rotor. In the third (Marzetta *et al*, 1986), 4 ml LDL at d 1.006-1.063 g/ml was layered between 2.5 ml d 1.21 g/ml and 5.2 ml d 1.006 and centrifuged for 24h at 40,000rpm.

3.2.4 Development of final LDL Subfractionation Method

With the knowledge of density intervals for each individual subfraction obtained from the above methods, various stepwise gradients in the density range 1.019-1.063 g/ml were devised and run initially with pre-isolated LDL (d 1.019-1.063 g/ml) to optimise conditions. However, as this LDL took 48h to prepare and may have suffered deterioration, it was decided in the final method, to minimise any opportunity for damage from prolonged centrifugations, to apply fresh plasma to the gradient. Various times (between 18h and 48h) and temperatures were tried to optimise conditions. The final

method chosen is described in detail in chapter 2.4.9 and consisted of a stepwise gradient - 3ml plasma at d 1.109g/ml, 1ml d 1.060g/ml, 1ml d 1.056 g/ml, 1ml d 1.045 g/ml, 2ml d 1.034g/ml, 2ml d 1.024g/ml,1ml d 1.019g/ml with ultracentrifugation carried out at 40,000 rpm and 23°C for 24 hours.

The density of the gradient formed in the centrifuge tube was measured using a Paar Scientific densitometer and the mean particle size of isolated LDL was determined both by gradient gel electrophoresis on 2-16% polyacrylamide gradient gels as described in 2.4.8 and that of purified LDL subfractions by electron microscopy as described in 2.5. The lipoprotein content of each subfraction was analysed as in 2.4.6. The content of apoproteins B, E and CIII was analysed using radioimmunoassay at Professor Fruchart's laboratory in Lille, France. The stability of the LDL subfraction profile using this methodology was determined by comparing the subfraction distribution in fresh plasma (analysis beginning within 24 h of venepuncture) with that of stored plasma (48h, 4°C) and frozen plasma (2-3 weeks, -70°C). Recovery and reproducibility measurements were also carried out.

Aspects of this work were performed jointly with Dr Bruce Griffin, in particular the automation of the method using the Beckman fractionation system and data leader program and a few of the early structural studies.

Using this methodology LDL subfractions were quantified in a number of normals and subjects with coronary artery disease, primary moderate hyperlipidaemia, familial hypercholesterolaemia, non insulin dependent diabetes mellitus and nephrotic syndrome. During these studies all subjects maintained their normal habits of diet and exercise. Normolipaemic subjects were recruited from staff at Glasgow Royal Infirmary. None were taking medication known to affect lipid metabolism and none were obese. The remaining subjects were recruited from Lipid Clinics at Glasgow Royal Infirmary University/NHS Trust; Victoria Infirmary, Glasgow and Hairmyres Hospital, East Kilbride; the Menopause Clinic at Stobhill Hospital, Glasgow; the database of Department of Cardiac Surgery, Glasgow Royal Infirmary; the Renal Unit, Glasgow Royal Infirmary; the Diabetic Clinic at Chelsea and Westminster Hospital, London and the FH register at the University College Hospital, London. Where appropriate, all subjects were screened for haematological, hepatic, endocrine or renal disorders by routine clinical laboratory testing. Except in the familial hypercholesterolaemia study, none of the subjects had clinical evidence of familial hypercholesterolaemia (FH) and none were taking any medication known to affect lipid metabolism

3.3 Results

3.3.1 Comparison of Methods

The four main types of subfraction profile observed in the gradient gel electrophoresis survey are shown in fig. 3.1. In all subjects 2 or 3 main fractions were evident. The profiles were classed according to mean particle diameter as pattern A or pattern B to correspond with the nomenclature of Krauss *et al* (1982). Table 3.1 shows that in profile A1 LDL I was the main species and had the largest particle size, A2 profile had LDL II as the major species while in B3 and B4 there were increasing proportions of small LDL

(LDL III). Most of the 143 LDL profiles examined were classified as pattern A, with 52% of the population exhibiting an A2 profile and LDL II being the major LDL species.

Table 3.1 Particle size distribution of LDL

Group	Diameter (nm)*	Characteristic	%Distribution
A1	>26.0	LDL I>II + III	21
A2	25.5-26.0	LDLII>I + III	52
B3	24.7-25.5	LDLIII = I + II	8
B4	<24.7	LDL III>I + II	18

** determined by comparison with high molecular weight standards and latex beads.*

The concentrations of LDL cholesterol and total plasma cholesterol were unrelated to LDL particle size. However there were significant associations between concentrations of plasma triglyceride and LDL particle size ($r= -0.60$, $p<0.0001$), HDL cholesterol and LDL particle size ($r=0.44$, $p<0.005$) and HDL cholesterol and plasma triglyceride ($r=-0.37$, $p<0.001$).

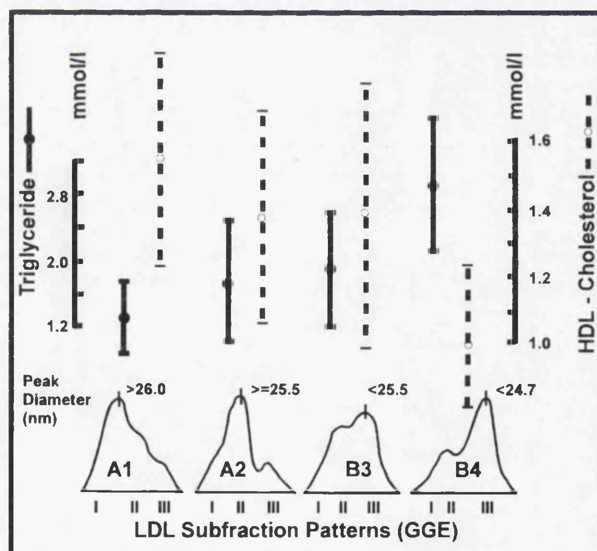

Fig.3.1 LDL Subfraction Patterns by Gradient Gel Electrophoresis
Pattern A1 peak diameter >26.0 nm. (n=30), pattern A2 peak diameter ≥25.5 nm (n=75), pattern B3 peak diameter <25.5 (n=12), pattern B4 primary peak <24.7nm (n=26). Plasma triglyceride and HDL cholesterol measurements are in mmol/l.

No apparent improvement was seen when the method was altered to give a narrow gradient on the gels. The separation between the subfractions was improved at the expense of resolution with banding becoming diffuse and irregular.
The zonal ultracentrifugation approach was encouraging and different profiles were observed in subjects with low and high plasma triglycerides and these were confirmed by gradient gel electrophoresis. However as a method of isolation and purification of the subfractions, there was insufficient resolution compared to the final method, only 1 sample could be handled at a time and too much blood was required. It was decided not to explore this technology further.
Of the 3 density gradient ultacentifugation published methods attempted, that of Marzetta *et al* looked most promising in our hands. However the modified gradient devised and described in 2.4.9 gave the best resolution and was adopted for the following studies.

3.3.2 Characteristics of final Density Gradient Ultracentrifugation Method
Isolated LDL subfractions were characterised by density, size and composition and the recovery and reproducibility of the method and the stability of the subfractions determined. Measurement of the gradient in the centrifuge tube showed it to be continuous and curvilinear (fig. 3.2) with discrete LDL bands in the range d 1.025-1.060 g/ml. The LDL bands did not reach isopycnic equilibrium under conditions of the run. Individual subfractions were harvested at d 1.025-1.034 g/ml (LDL I), d 1.034-1.044 g/ml (LDL II) and d 1.044-1.060 g/ml (LDL III).

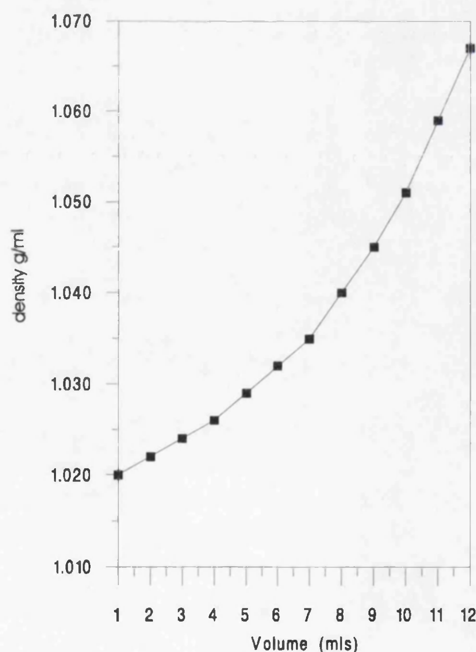

Fig.3.2 Density gradient formed in Swing-out Centrifuge Tube
Density in g/ml was measured on successive one ml fractions after an LDL gradient was centrifuged for 24h, 40,000 rpm, 23°C.

Fig. 3.3 shows the relationship between density gradient ultracentrifugation profile and gradient gel electrophoresis particle size for LDL in 2 subjects.

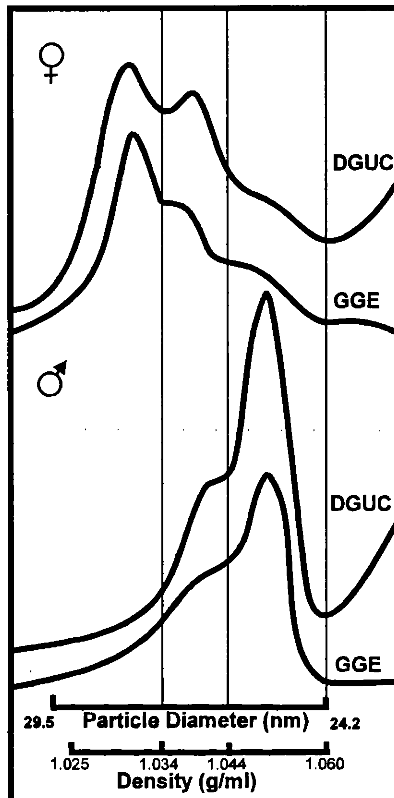

Fig.3.3 Relationship between Particle Size (GGE) and Density (DGUC) of LDL Subfractions

LDL Profiles by gradient gel electrophoresis (GGE) and density gradient ultracentrifugation (DGUC) on a female and male subject.

Fig. 3.4 shows a gradient gel of purified LDL subfractions from a normal subject. LDL I was the largest at 26.0nm, LDL II was 25.5 nm and LDL III was 24.7 nm.

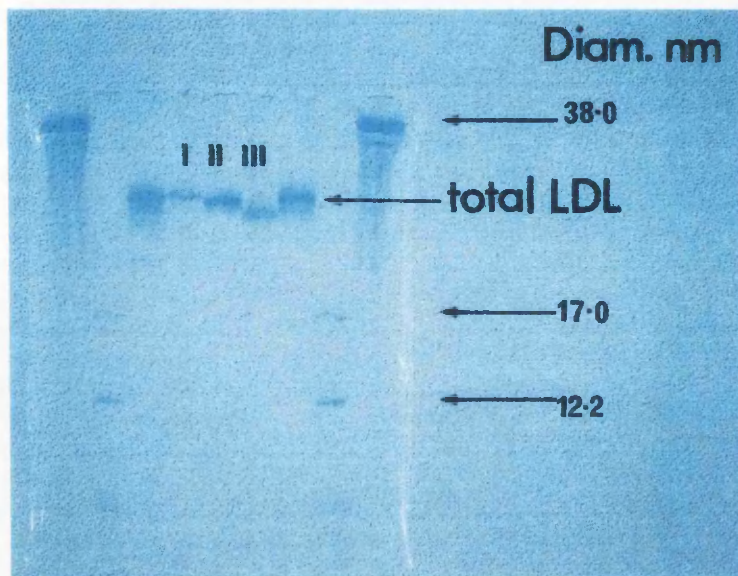

Fig.3.4 Gradient Gel of Purified LDL Subfractions
LDL I (26.0nm), LDL II (25.5nm) and LDL III (24.7nm) and LDL (d 1.019-1.063 g/ml). Standards - latex beads at 38nm, and high molecular weight standards at 17 and 12.2nm.

Fig. 3.5 is electron micrograph photographs of purified LDL subfractions in a single subject. LDL I was 23.6 nm, LDL II was 22.2 nm and LDL III was 20 nm. The size and density of LDL obtained by these 2 methods is shown in table 3.2. Those measured by gradient gel electrophoresis were larger than sizes obtained with electron microscopy.

Table 3.2 Size and Density of LDL Subfractions

Subfraction	*Density (g/ml)*	*Size by Gradient Gel Electrophoresis (nm)*	*Size by Electron Microscopy (nm)*
LDL I	1.025-1.034	26.0	23.6
LDL II	1.034-1.044	25.5	22.2
LDL III	1.044-1.060	24.7	20

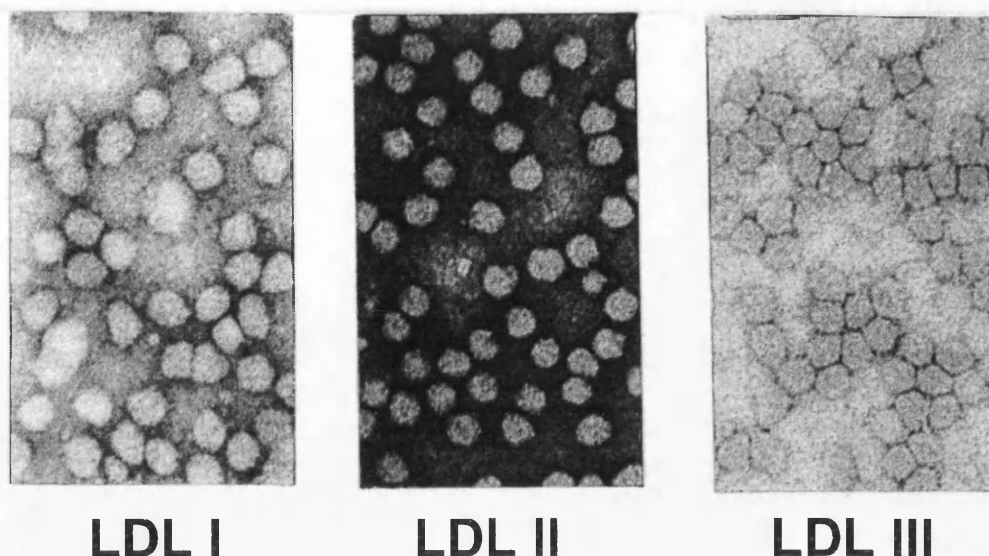

Fig. 3.5 Electron Micrograph Photographs of purified LDL I, LDL II and LDL III from a male subject

Purified LDL I, LDL II and LDL III from a subject was photographed by negative contrast electron microscopy.

Apoproteins B, E and CIII content in LDL subfractions isolated from a young male and a young female are shown in table 3.3. In both subjects the majority of apoB was in LDL II followed by LDL I and approximately 17% was in LDL III. The majority of CIII was in LDL I and the majority of E in LDL III. In both subjects the CIII to E ratio was 2.8&2.9 respectively in LDL I, 1.0 &0.6 in LDL II and 0.2 &0.6 in LDL III.

Table 3.3 Apoprotein Content of LDL Subfractions

Apoprotein *	*LDL I*	*LDL II*	*LDL III*
Apo B			
male	37 (37)	47 (47)	16.5 (16)
female	39 (36)	51 (46)	20.0 (18)
ApoCIII			
male	120 (60)	57 (28)	25 (12)
female	80 (48)	32 (19)	65 (33)
Apo E			
male	44 (21)	54 (26)	110 (53)
female	38 (16)	74 (32)	120 (52)

** apo B in mg/100ml, apo C III in μg/100ml, apo E in μg/100ml.*

Values in brackets are apoprotein content in each subfraction as % total LDL apoprotein.

Lipoprotein compositions for each subfraction isolated after one spin in a survey of 37 normal subjects is shown in table 3.4. LDL I was found to have lower percentage protein and higher percentage triglyceride content than the other subfractions whereas LDL II was enriched in cholesteryl ester. The highest protein to cholesterol ratio was found in LDL III mainly due to a lower percentage of cholesteryl ester.

Table 3.4 Lipoprotein Composition of LDL Subfractions

Subfraction	*% Protein*	*% Free Cholesterol*	*% Cholesteryl ester*	*%Phospholipid*	*%Triglyceride*
LDL I	22.0	9.1	31.1	19.1	18.7
(SEM)	(0.6)	(0.2)	(0.9)	(0.4)	(1.2)
LDL II	24.1	9.2	37.5	18.6	10.5
(SEM)	(0.5)	(0.2)	(0.8)	(0.5)	(0.6)
LDL III	24.4	7.3	27.6	17.1	12.9
(SEM)	(0.7)	(0.4)	(1.0)	(0.7)	(0.8)

Specific extinction coefficients to relate optical density at 280nm (OD_{280}) to lipoprotein concentrations were determined in 22 subjects. For LDL I, the OD_{280} for a 1 mg/ml solution of lipoprotein was 0.38, for LDL II it was 0.34 and LDL III it was 0.51. In future studies where lipoprotein mass was to be quantitated, a specific extinction coefficient was applied to the absorbance at 280 nm. To correct OD_{280} for the amount of lipoprotein in each fraction, LDL I is multiplied by 1/0.38 (=2.63), LDL II by 1/0.34 (=2.94) and LDL III by 1/0.51 (=1.96). The distribution of protein in each subfraction, as measured by OD_{280}, was calculated as % area under the curve using a computer aided package (Data Graphics, Beckman) and corrected for lipoprotein distribution using the extinction coefficients. The total lipoprotein mass in LDL (d 1.019-1.063 g/ml) was determined for each plasma sample as described in chapter 2. and the distribution of the lipoprotein was apportioned accordingly. The unit of LDL subfraction concentration is therefore mg lipoprotein /100ml plasma.

Recovery of LDL protein was 102±14% (mean ±SD). The LDL fractions were free from contamination by plasma proteins as judged by polyacrylamide gel electrophoresis and immunological techniques. When gradient fractions were assayed for Lp(a) with a wide range of plasma Lp(a) levels up to 150 mg/100 ml less than 6% of Lp(a) was found in the LDL III fraction. Lp(a) eluted from the gradient after the LDL III peak.

The analysis of 6 replicate samples within the same rotor gave a coefficient of variation of 3.4%, 2.0% and 5.4% for LDL I, LDL II and LDL III respectively. Between batch coefficient of variation was less than 6.5%, when 6 samples were analysed in 3 rotors run simultaneously.

Storage of plasma for 24 h at 4°C and at -20°C for 2-3 weeks resulted in the loss of resolution between LDL I and LDL II and was accompanied by a small increase in LDL III (fig. 3.6). A similar change was observed when LDL (d 1.019-1.063 g/ml) prepared over 48h by sequential ultracentrifugation was subjected to density gradient ultracentrifugation.

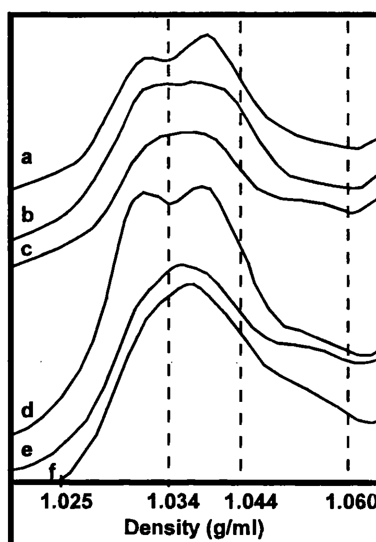

Fig.3.6 Effect of Storage and Prolonged Centrifugation upon LDL Subfraction Profile

LDL subfraction profiles prepared by the density gradient centrifugation of (a) and (d) fresh plasma, within 24h, (b) LDL (d 1.019-1.063 g/ml) derived from plasma a, (c) plasma a stored for 24h at 4°C, (e) plasma d stored for 2 weeks at -20°C, (f) plasma d stored for 3 weeks at -20°C.

3.3.3 LDL Subfraction Profiles in Normal and Hyperlipidaemc Subjects

Normal subjects

Four groups of normal subjects were studied: (i) young males, (ii) premenopausal females with the same mean age, (iii) women who had undergone hysterectomy and bilateral oophorectomy at least 2 months previously and (iv) older males. Table 3.5 shows the lipid, lipoprotein and apoprotein values and comparisons in the groups.

Young males and females had similar total cholesterol and triglyceride concentrations , but the males had significantly higher VLDL cholesterol ($p<0.01$) and lower HDL cholesterol ($p<0.01$), the difference being in HDL_2 subfraction. In the older group of men cholesterol, triglyceride, VLDL and LDL cholesterol were all significantly raised as was apoAI. When the premenopausal and post surgical menopausal women were compared, the older women had higher cholesterol and LDL cholesterol levels, but similar plasma triglyceride, VLDL

and HDL cholesterol concentrations. These older women had lower triglyceride and higher HDL cholesterol levels than men of similar age. In these groups of normals, Lp(a) levels were lower in young males.

Table 3.5 Lipids, Lipoproteins and Apoproteins in Normal Subjects

Variable	*Young males*	*Pre menopausal females*	*Post menopausal females*	*Older males*	*P*
Age	32(2)	31 (2)	42(2)	48.3(1)	b§, c†
Number	18	18	17	28	
Cholesterol	5.06(0.25)	4.73(0.16)	5.62(1.42)	6.3(0.20)	b‡, c†
Triglyceride	1.05(0.10)	0.88(0.05)	1.17(0.54)	1.75(0.26)	b*, d*
VLDL chol	0.54(0.04)	0.41(0.03)	0.44(0.24)	0.79(0.06)	a†, b‡, d†
LDL chol	3.3(0.23)	2.76(0.16)	3.54(1.00)	4.23(0.19)	b†, c*, d*
HDL chol	1.21(0.06)	1.61(0.11)	1.64(0.28)	1.33(0.06)	a†, d†
HDL2	47.6(6.0)	101(17.4)	--	--	a†
HDL3	238.5(14.4)	261(14.6)	--	--	
Apo AI	114(3.7)	130(5.3)	162(18)	143(5.8)	a*, b§, d*
Apo B	91(6.7)	72(3.8)	72(18)	--	
Lp(a)	7(0.8)	24(6.7)	--	26.5(5.6)	a*, b†

Values are mean (SEM). Cholesterol in plasma,VLDL,IDL and HDL and triglyceride values are in mmol/l , HDL subfractions and apoproteins are in mg/100ml.
P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.
a young males versus premenopausal females, **b** young males versus older males, **c** premenopausal versus postmenopausal females, **d** postmenopausal females versus older males.

The LDL subfraction profile (table 3.6) demonstrated that in normals LDL II was the major species being 50-60% of total LDL. However it was observed that in the young females (age <21 years) LDL I was the major species. The distribution of the other 2 species was significantly different between males and females, LDL I being lower and LDL III being higher in males. There was a reciprocal relationship between LDL I and LDL III with LDL II concentrations remaining relatively constant at 160-190 mg lipoprotein /100ml plasma.

Table 3.6 LDL Subfractions in Normal Subjects

Variable	*Young males*	*Pre menopausal females*	*Post menopausal females*	*Older males*	*p*
LDL mass	315(21.2)	273(14.6)	333(86)	284(35.6)	
LDL I	44(6.1)	79(10.0)	110(41)	47(7.4)	a†, d‡
LDL II	190(17.9)	162(13.4)	171(56)	162(21.7)	
LDL III	82(23.6)	32(3.5)	52(30)	76(15.8)	a*
% LDL I	14.8(2.2)	29.0(3.2)	33.0(9.8)	14.8(2.0)	a†, d‡
% LDL II	61.8(4.6)	58.9(3.1)	51.4(6.0)	57.0(4.4)	
% LDL III	23.4(5.5)	12.1(1.6)	15.6(9.5)	27.0(4.0)	a*, d*

Values are mean (SEM). LDL subfractions masses are in mg lipoprotein/100ml plasma.
P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.
a young males versus premenopausal females, **b** young males versus older males, **c** premenopausal versus surgical postmenopausal females, **d** surgical postmenopausal females versus older males.

Subjects with Coronary Artery Disease

Lipds, lipoproteins, apoprotein and lipoprotein subfractions were compared in four groups of male subjects with coronary artery disease: (i) a group identified by coronary angiography as having three-vessel disease, defined as 3 major epicardial arteries with at least 50% stenosis (denoted CAD+), (ii) a group identified by coronary angiography as having no major pericardial vessel with greater than 50% stenosis (denoted CAD-), (iii) 40 men who had sustained a documented acute myocardial infarction in the previous 6-12months (denoted PMI) and (iv) 8 men who had undergone coronary artery bypass surgery between 3 and 12 months previously (denoted CABG). Although the mean ages were similar, the CAD+ group was older and this difference was significant when compared to the older group of normal males and CAD- group. Comparisons were made between the 4 groups and the older male controls and with CAD+ and CAD -. The lipid, lipoprotein and apoprotein results are shown in table 3.7.

Table 3.7 Lipids, Lipoproteins and Apoproteins in Subjects with Coronary Disease

Variable	*CAD +*	*CAD -*	*PMI*	*CABG*	*Significance*
Age	52.8(0.81)	46.8(1.8)	50.9(1.0)	50.9(1.6)	a†, e†
Number	46	24	40	8	
Cholesterol	6.10(0.11)	5.70(0.21)	6.31(0.16)	6.57(0.17)	
Triglyceride	2.25(0.21)	1.45(0.11)	2.60(0.24)	1.87(0.14)	c*,e†
VLDL chol	0.94(0.08)	0.59(0.06)	1.21(0.11)	0.84(0.07)	b*, c†, e‡,
LDL chol	3.98(0.12)	3.77(0.16)	4.10(0.15)	4.56(0.20)	
HDL	1.18(0.05)	1.32(0.06)	0.99(0.03)	1.10(0.02)	c§, d†
Apo AI	116(5.0)	139(6.5)	122(2.6)		a‡, c†, e†
Apo B	104(5.0)	92(3.0)	123(4.2)		
Lp(a)	33.6(4.7)	15(4.6)	15(2.8)	20(11)	e†

Values are mean (SEM). Cholesterol and triglyceride units are mmol/l , HDL subfractions and apoproteins are in mg/100ml.

P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.

a CAD+ vs N older males, **b** CAD- vs N older males, **c** PMI vs N older males, **d** CABG vs N older males, **e** CAD+ vs CAD

All groups had similar total cholesterol and LDL concentrations as the group of control subjects with LDL levels at 4.0-4.5 mmol/l. PMI subjects were distinguished from normals by significantly higher triglyceride ($p<0.02$) and VLDL cholesterol ($p<0.01$), lower HDL cholesterol ($p<0.0001$) and raised LDL III ($p<0.0001$, table 3.8). The major LDL species in these subjects was LDL III being 51% of the total compared to 27% in normals. When compared to CAD-, the CAD+ subjects had significantly raised triglyceride ($p<0.01$), VLDL cholesterol ($p<0.001$) and LDL III concentrations ($p<0.001$). There were significant alterations in the distribution of the LDL species, LDL I and LDL II being in lower proportions and LDL III higher. CABG subjects were similar to control subjects with practically identical LDL subfraction distributions, the only difference being a significant decrease ($p<0.01$) in HDL cholesterol.

Table 3.8 LDL Subfractions in Subjects with Coronary Disease

Variable	*CAD +*	*CAD -*	*PMI*	*CABG*	
LDL mass	360(11.7)	368(14.4)	407(14.2)	413(32.6)	c†, d*
LDL I	54.5(5.4)	77(9.0)	44(5.0)	74(8.5)	b*
LDL II	171(12.1)	205(12.0)	187(15.1)	222(16.4)	
LDL III	135.2(12.3)	86(12.9)	176(15.6)	118(29.5)	a†, c§, e†
% LDL I	15.3(1.6)	22.1(2.2)	10.3(1.1)	18.4(2.4)	b*, e*
% LDL II	39.9(2.5)	48.7(2.4)	39.6(3.0)	55.0(4.5)	e†
% LDL III	45.2(3.7)	30.1(3.9)	51.3(3.9)	26.6(5.7)	a†, c§, e†

Values are mean (SEM). LDL subfractions masses are in mg lipoprotein/100ml plasma.
P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.
a CAD+ vs N older males, **b** CAD- vs N older males, **c** PMI vs N older males, **d** CABG vs N older males, **e** CAD+ vs CAD

Examination of the frequency distribution of LDL subfraction concentrations in the data set revealed unimodal distributions for LDL I and LDL II, but a bimodal distribution for LDL III with an inflection point at a concentration of 100mg/100ml (fig. 3.7). The relative risk of CAD associated with an LDL III in excess of 100mg/100ml was estimated by the calculation of odds ratios. This was compared with other potential predictors i.e. a plasma above the triglyceride level that appeared to be a threshold determinant of LDL subfraction distribution (1.5 mmol/l) (Tan *et al*, 1995a), and a previously published coronary risk parameter (Castelli *et al*, 1983) of cholesterol: HDL cholesterol ratio of >6.0. Table 3.9 shows the significance of these odds ratios determined by chi-square analysis with one degree of freedom.

Table 3.9 Relative Coronary Risk associated with
LDL III > 100mg/100ml,
Triglyceride > 1.5 mmol/l and
Cholesterol:HDL cholesterol ratio >6.0

	CAD+ vs CAD-		*PMI vs Controls*	
	Relative Risk	*p*	*Relative Risk*	*p*
LDL III >100 mg/100ml	4.5	<0.01	6.9	<0.001
Triglyceride >1.5 mmol/l	4.1	<0.01	6.1	<0.001
Cholesterol:HDLchol >6.0	4.8	<0.05	7.2	<0.001

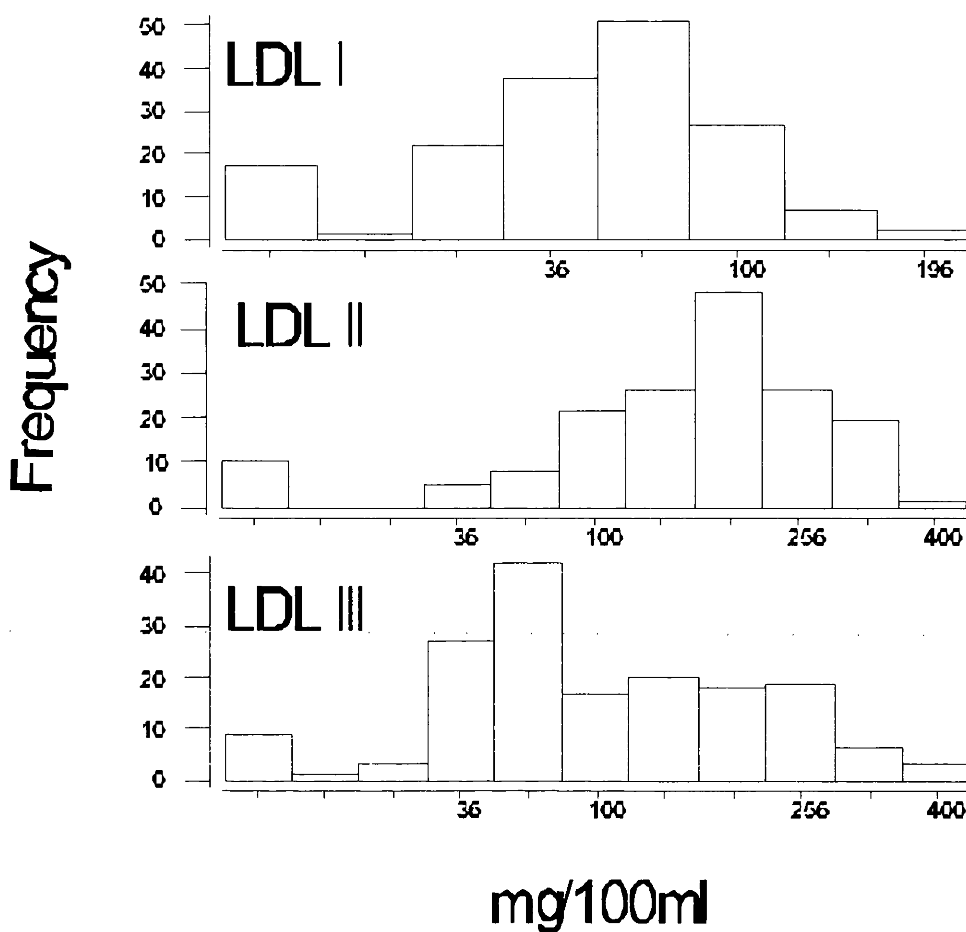

Fig. 3.7 Frequency Distribution Plots of LDL Subfractions
Frequency for LDL I and LDL II are unimodal, whereas LDL III is bimodal, showing a change at 100 mg/100ml. Data represent square-root transformed values.

A level of LDL III > 100mg/ml was associated with a 4-fold risk of CAD (CAD+vs CAD-) and 7-fold increased risk of MI (PMI vs Controls). The odds ratio calculated for a plasma triglyceride > 1.5 mmol/l was 4 for CAD and 6 for MI. A cholesterol:HDL cholesterol ratio of 6.0 gave relative risk estimates of 4.8 for CAD and 7.2 for MI. The relative risk associated with LDL III > 100mg/100ml was independent of plasma triglyceride, age, smoking and drug status whereas cholesterol:HDL cholesterol was dependent on triglyceride: odds ratio for MI being reduced to 6.4 after correction for triglyceride. LDL III measurement was therefore a reasonably sensitive and specific predictor of risk.

Primary Moderate Hyperlipidaemia

Three groups of patients with primary moderate hyperlipidaemia (moderate hypercholesterolaemia (HC), moderate hypertriglyceridaemia (HTG) and combined hyperlipidaemia (CHL)) were studied. The groups comprised males and postmenopausal females with a mean age of 50 years. Comparisons were made with the older normal males in tables 3.5 and 3.6 and between the groups. Lipids, lipoproteins and apoproteins are in table 3.10 and LDL subfractions are in table 3.11.

The HC subjects had significantly raised cholesterol ($p<0.0001$), LDL cholesterol ($p<0.0001$) and elevated concentrations of LDL I ($p<0.0001$) and LDL II ($p<0.001$), with the relative distribution of LDL subfractions being similar when compared to normals. When compared to normals, the HTG group had raised plasma triglyceride ($p<0.01$), VLDL cholesterol ($p<0.01$) and lower HDL cholesterol ($p<0.0001$). Less than 10% of total LDL was LDL I and more than 70 % was LDL III. Two of these subjects had no detectable LDL I and several exhibited the presence of an LDL species of density greater than that normally associated with LDL III. It can be termed 'LDL IV' (Krauss & Burke, 1982) but has been included for the purpose of this study in the LDL III subclass. Compared to normals, subjects with CHL had raised levels of cholesterol ($p<0.0001$), plasma triglyceride ($p<0.001$), VLDL ($p<0.001$) and LDL III ($p<0.01$). The distribution of LDL was such that there were approximately equal proportions of LDL II and LDL III (43% and 42% respectively).

Table 3.10 Lipids, Lipoproteins and Apoproteins in Subjects with Primary Moderate Hyperlipidaemia.

Variable	*Moderate Hyper-cholesterolaemia*	*Moderate Hyper-triglyceridaemia*	*Combined Hyper-lipidaemia*	*p*
Age	55.6(1.6)	53.6(2.9)	48.6(2.1)	a‡
Number	31	19	19	
Cholesterol	7.68(0.14)	6.1(0.24)	7.95(0.28)	a§, c§, d§, f§
Triglyceride	1.55(0.06)	3.2(0.32)	2.99(0.17)	b†, c‡, d‡, e§
VLDL chol	0.70(0.04)	1.46(0.15)	1.40(0.13)	b†, c‡, d‡, e§
LDL chol	5.60(0.15)	3.68(0.23)	5.44(0.20)	a§, c§, d§, f§
HDL	1.35(0.04)	0.93(0.07)	1.11(0.09)	b§, d§, e*
HDL2	70(6.5)	16(1.8)	53(12.1)	d§,f*
HDL3	250(8.5)	245(20.7)	251(19.7)	
Apo AI	126(3.5)	118(8.5)	126(5.0)	
Apo B	137(5.1)	153(6.9)	147(9.2)	
Lp(a)	42(8)	28(7.5)	61(14.9)	

Values are mean (SEM). Cholesterol and triglyceride units are mmol/l , HDL subfractions and apoproteins are in mg/100ml. P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.***a*** *moderate hypercholesterolaemia vs N males,* ***b*** *moderate hypertriglyceridaemia vs N males,* ***c*** *combined hyperlipidaemia vs N males,* ***d*** *moderate hypercholesterolaemia vs moderate hypertriglyceridaemia,* ***e*** *moderate hypercholesterolaemia vs combined hyperlipidaemia,* ***f*** *moderate hypertriglyceridaemia vs combined hyperlipidaemia.*

When the group of HC individuals was compared with the group of CHL subjects, there were similar concentrations of total cholesterol, LDL cholesterol and total LDL mass but lower plasma triglyceride ($p<0.0001$), VLDL cholesterol ($p<0.0001$) and higher HDL cholesterol ($p<0.02$) in the former group. The main difference in LDL profile was a halving of the concentration of LDL I and a doubling of LDL III in CHL. When HTG and CHL subjects were compared, the former group had lower cholesterol ($p<0.0001$), LDL cholesterol ($p<0.0001$), lower LDL I ($p<0.02$), lower LDL II ($p<0.0001$) but similar LDL III.

Table 3.11 LDL Subfractions in Subjects with Primary Moderate Hyperlipidaemia.

Variable	*Moderate Hyper-cholesterolaemia*	*Moderate Hyper-triglyceridaemia*	*Combined Hyper-lipidaemia*	*p*
LDL mass	468(20.1)	279(21.1)	392(16.4)	a§, c†, d§, e†, f‡
LDL I	129(17.5)	23(4.3)	61(10.9)	a§, d§, e†, f*
LDL II	262(14.8)	57(12.0)	171(17.9)	a‡, b‡, d§, e‡, f§
LDL III	77(13.1)	198(23.5)	160(20.3)	b‡, c†, d‡, e†
% LDL I	26.5(2.8)	8.6(4.4)	14.7(2.8)	a†, d§, e†
% LDL II	56.8(2.4)	20.7(4.4)	43.0(3.7)	b‡, d§, e†, f†
% LDL III	16.7(2.8)	70.7(5.6)	42.4(5.9)	b§, d§,e‡, f†

Values are mean (SEM). LDL subfractions masses are in mg lipoprotein/100ml plasma.
P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.
a *moderate hypercholesterolaemia vs N males,* ***b*** *moderate hypertriglyceridaemics vs N males,* ***c*** *combined hyperlipidaemia vs N males,* ***d*** *moderate hypercholesterolaemia vs moderate hypertriglyceridaemia,* ***e*** *moderate hypercholesterolaemia vs combined hyperlipidaemia,* ***f*** *moderate hypertriglyceridaemia vs combined hyperlipidaemia.*

Familial Hypercholesterolaemia

37 subjects with heterozygous familial hypercholesterolaemia (FH), defined as LDL cholesterol >4.9 mmol/l plus similar raised cholesterol in a 1st or 2nd degree relative, plus either a family history of MI below 50 of age in 1st or 2nd degree relative, or tendon xanthoma or family history of such xanthoma in 1st or 2nd degree relative, were studied. The group consisted of 19 men (mean age 51 years) and 18 females (mean age 43 years). Also examined was a group of 14 subjects with familial defective apo B100 (FDB). Five had the classic R3500Q mutation in which arginine had been replaced by glutamine, 4 were the recently discovered FDB variant R3500W (Gaffney *et al*, 1995) in which arginine was replaced by tryptophan and 5 were FDB subjects who had an arginine to cysteine change at

amino acid 3531 (Pullinger *et al,* 1995). The lipids and lipoproteins are in table 3.12 and LDL subfractions in table 3.13

Table 3.12 Lipids, Lipoproteins and Apoproteins in Subjects with Familial Hypercholesterolaemia.

Variable	FH	FDB	p
Age	46.8(2.8)		
Number	37	14	
Cholesterol	6.9(0.2)	6.7(0.39)	b†
Triglyceride	1.32(0.14)	1.75(0.35)	
VLDL chol	0.54(0.11)	0.82(0.23)	
LDL chol	5.1(0.20)	4.46(0.44)	
HDL	1.24(0.05)	1.28(0.12)	
HDL	1.24(0.05)	1.28(0.12)	

Values are mean (SEM). Cholesterol and triglyceride units are mmol/l , HDL subfractions and apoproteins are in mg/100ml. P refers to the significance of difference between groups as determined by student's unpaired t-test. p value: †<0.01. **b** Familial Hypercholesterolaemia vs moderate hypercholesterolaemia

When compared to normal males, FH subjects had higher masses of LDL I ($P<0.0001$) and LDL II ($P<0.01$) with a higher proportion of LDL I and FDB subjects had higher LDL II concentrations. FH and FDB groups were similar to HC in plasma lipid levels and LDL profile and to each other.

Table 3.13 LDL Subfractions in Subjects with Familial Hypercholesterolaemia.

Variable	FH	FDB	p
LDL mass	410(15.7)	408(22.9)	a†, c†
LDL I	110(10.0)	83(14.1)	a§
LDL II	229(12.9)	242(21.7)	a†, c*
LDL III	71(10.4)	81(24.8)	
% LDL I	26.7(2.1)	20.9(4.1)	a§
% LDL II	55.5(2.0)	61.2(5.0)	
% LDL III	17.8(2.5)	19.3(4.3)	

Values are mean (SEM). LDL subfraction masses are in mg lipoprotein/100ml plasma. P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, §<0.0001.***a*** *Familial Hypercholesterolaemia vs older normal males,* ***c*** *Familial Defective apoB100 vs older normal males*

Secondary Hyperlipidaemia

Two groups of subjects with secondary hyperlipidaemia were investigated. The first was a group of 24 subjects (14 males, 10 females) with non insulin dependent diabetes mellitus (NIDDM) who were treated with diet alone or with an oral hypoglycaemic drug. Comparisons were made with the older male population. The second group which consisted of 12 patients (10 males and 2 females) with nephrotic syndrome who had primary glomerular disease with serum creatinine <300μmol/l and urinary albumin excretion >2.5g/24h. Comparisons were made with 23 age and sex matched controls with no history of CAD. The results are shown in tables 3.14 and 3.15. Male and female NIDDM subjects had similar plasma lipid and lipoprotein levels except for plasma triglyceride (p<0.01) which was 2.69 (0.37, SEM) mmol/l in males and 4.08 (0.28, SEM) mmol/l in females.

Table 3.14 Lipids, Lipoproteins and Apoproteins in Subjects with Secondary Hyperlipidaemia

Variable	*NIDDM*	*Nephrotic Syndrome*	*Controls for Nephrotic Syndrome*	*p*
Age	62.7(1.5)	46(16)	44(14)	a§, c§
Number	24	12	23	
Cholesterol	7.32(0.21)	6.5(1.4)	5.3(1.1)	a†
Triglyceride	3.23(0.28)	3.2(2.7)	1.2(0.4)	a‡, b†
VLDL chol		1.66(1.27)	0.48(0.22)	b†
LDL chol	4.7(0.17)	3.72(1.43)	3.62(1.06)	
HDL	1.12(0.04)	1.08(0.48)	1.19(0.26)	a†

Values are mean (SEM). Cholesterol and triglyceride units are mmol/l.
P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: †<0.01, ‡<0.001, §<0.0001.
***a** NIDDM vs normals, **b** Nephrotics vs aged and sex matched controls, **c** NIDDM vs Nephrotics*

The group of NIDDM subjects were the oldest group studied and had higher cholesterol (p<0.01), higher triglyceride (p<0.001), lower HDL cholesterol (p<0.01) and similar LDL cholesterol concentrations when compared to normals. LDL III was half of the total LDL and the concentration of this subfraction was significantly higher than normal (p<0.001). Nephrotic syndrome patients had raised plasma triglyceride (p<0.01) and VLDL cholesterol (p<0.001) when compared with age and sex matched controls. In the majority LDL III was the major LDL species and the presence of LDL IV was observed in 3 subjects. When compared with controls, the total LDL mass was slightly lower (non significant), the concentration of LDL I was lower (p <0.02) and LDL III significantly higher (p<0.01). There were similarities between these 2 groups of secondary

hyperlipidaemia in levels of all lipids and lipoproteins and concentrations and distribution of the LDL subfractions.

Table 3.15 LDL Subfractions in Subjects with Secondary Hyperlipidaemia

Variable	NIDDM	Nephrotic Syndrome	Nephrotic Syndrome Controls	p
LDL mass	366(16)	285(97)	334(88)	
LDL I	38(5.2)	32(23.6)	62(26.4)	b*
LDL II	142(16.7)	121(80)	193(80.1)	
LDL III	182(21.2)	135(64.3)	75(71.3)	a‡, b‡
% LDL I	10.5(1.5)	9.3(1.8)	19.5(10.3)	
% LDL II	38.6(4.1)	40.3(6.5)	57.8(17.3)	
% LDL III	50.9(5.0)	50.4(7.4)	21.4(17.8)	a‡, b‡

Values are mean (SEM). LDL subfractions masses are in mg lipoprotein/100ml plasma.
P refers to the significance of difference between groups as determined by student's unpaired t-test. p values: *<0.02, †<0.01, ‡<0.001, §<0.0001.
***a** NIDDM vs normals, **b** Nephrotics vs age and sex matched controls*

3.3.4 Regulation of LDL Concentration, Composition and Subfractions

The data from all the subjects described above was pooled to give a cohort of 304 individuals with plasma cholesterol from 3.25-10.4 mmol/l, plasma triglyceride from 0.45-8.65 mmol/l and LDL cholesterol from 1.5 - 7.6 mmol/l. In this group LDL cholesterol rose significantly with age (r = 0.33, p <0.0001) and was significantly correlated with LDL II subfraction (r =0.51, p<0.0001). VLDL cholesterol was positively associated with the mass of LDL III (r = 0.52, p<0.0001). HDL was negatively associated with triglyceride (r = -0.41, p<0.0001) and LDL III (r = - 0.45, p<0.0001) and positively with LDL I (r = 0.40, p<0.0001). The HDL associations were with HDL_2 subfraction and not HDL_3. There were no associations between LDL subfraction concentrations and apo E phenotype or BMI. Plasma triglyceride was negatively correlated with LDL I (r = - 0.34, p<0.0001) and LDL II (r = - 0.35, p<0.0001) and positively with LDL III (r = 0.64, p<0.0001). Plasma triglyceride concentration was negatively associated with % free cholesterol in LDL (r = - 0.52, p<0.0001) and positively with % LDL triglyceride (r = 0.54, p<0.0001).

Fig. 3.8 shows the relationship of the 3 LDL subfractions with plasma triglyceride. At low levels of plasma triglyceride LDL I levels are at their highest and they fall to reach a baseline value of about 50 mg/100ml. Over the plasma triglyceride range 0.5-1.5 mmol/l, LDL II concentrations rise and then fall off above a plasma triglyceride of 1.5 mmol/l, whereas LDL III concentrations are flat and then rise sharply. This is in agreement with an observation in normal individuals in a separate paper from our laboratory (Tan *et al*, 1995a), who showed that the point of inflection where the LDL II/III changes take place

was at a plasma triglyceride concentration of 1.5 mmol/l. Table 3.16 shows the lipid, lipoprotein and LDL subfraction concentrations when the data set was divided at this level of plasma triglyceride.

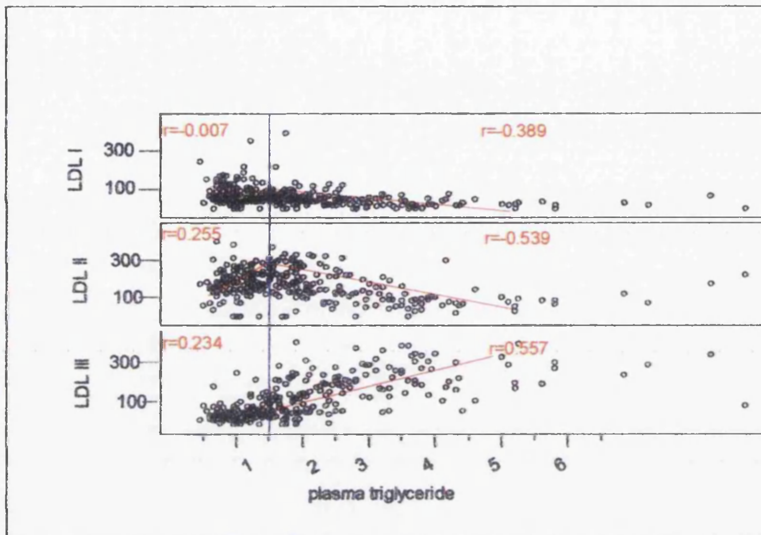

Fig.3.8 Relationship of Plasma Triglyceride with LDL I, LDL II and LDL III
At low plasma triglyceride < 1.5 mmol/l LDL II and LDL III are positively associated with triglyceride ($p<0.01$) and at triglcerude > 1.5 LDL II is negatively associated with triglyceride and LDL III positively ($p<0.0001$)

Table 3.16 Comparison of Low and High Triglycerides

Parameter	*Triglyceride < 1.5 mmol/l*	*Triglyceride>1.5 mmol/l*
Number	137	166
Triglyceride	1.05 (0.02)	2.76 (0.10)§
Cholesterol	5.86 (0.11)	6.92 (0.08)§
VLDL cholesterol	0.49 (0.02)	1.16 (0.05)§
LDL cholesterol	4.04 (0.11)	4.57 (0.08)§
HDL cholesterol	1.34 (0.03)	1.11 (0.02)§
HDL2	81 (6.0)	45 (4.2)§
HDL3	255 (7.5)	260 (9.1)
LDL I	88 (5.3)	52 (3.7)§
LDL II	207 (7.1)	171 (7.3)**
LDL III	54 (3.5)	164 (4.2)§

*Values are mean (SEM). Triglyceride, cholesterol, VLDL cholesterol and HDL cholesterol are in mmol/l. HDL and LDL subfractions are in mg/100ml. ** $p<0.0005$, § $p<0.0001$.*

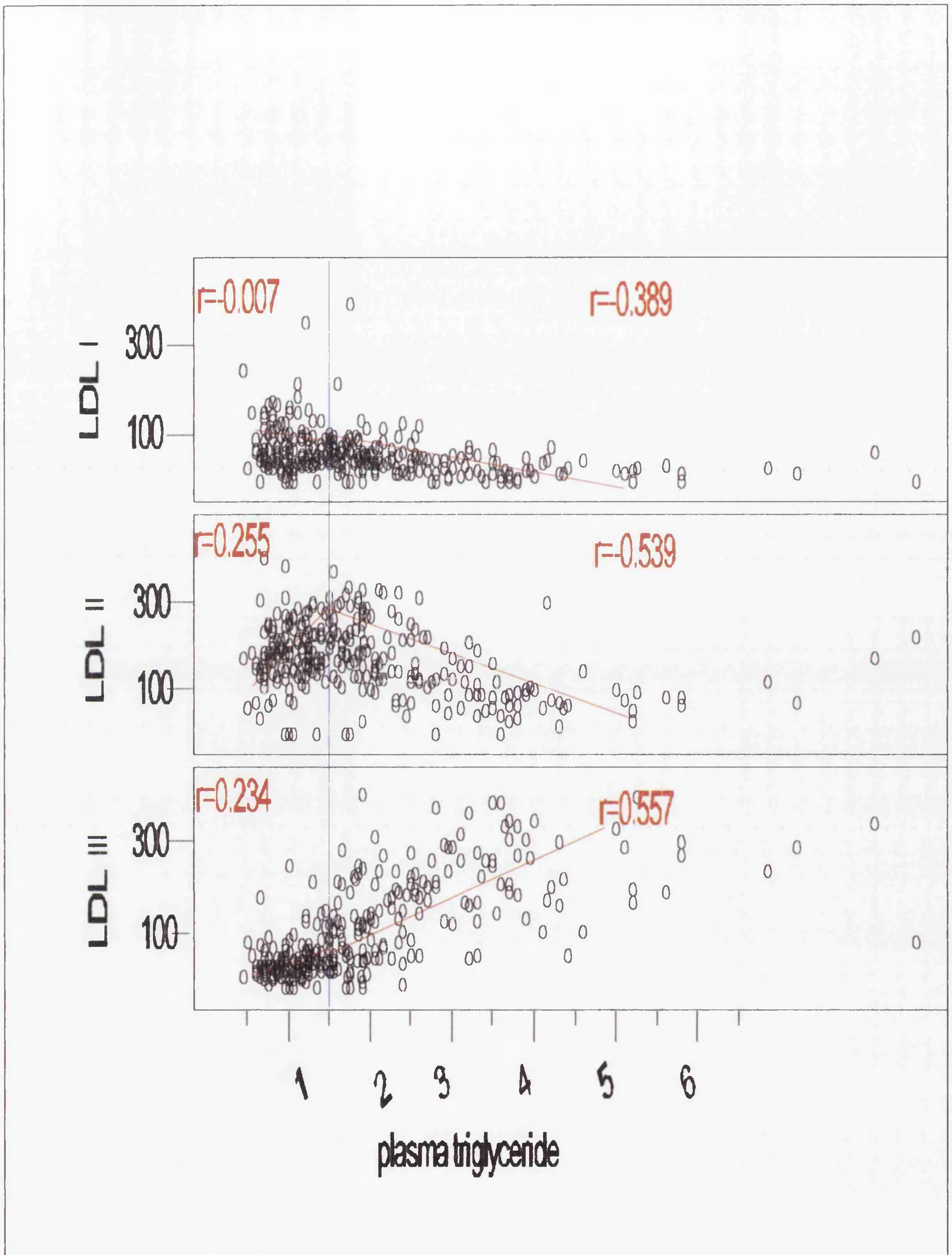

Fig.3.8b Relationship of Plasma Triglyceride with LDL I, LDL II and LDL III

At low plasma triglyceride < 1.5 mmol/l, LDL II and LDL III are positively associated with triglyceride ($p<0.01$) and at triglyceride > 1.5 mmol/l, LDL II is negatively associated and LDL III positively ($p<0.0001$)

There were highly significant differences between the groups in all parameters except for HDL_3. Plasma triglyceride > 1.5 mmol/l was associated with raised cholesterol, raised VLDL cholesterol, raised LDL cholesterol, reduced HDL cholesterol this being due to the HDL_2 fraction, lower concentrations of LDL I and LDL II and higher concentrations of small dense LDL III. Table 3.17 shows the Pearson correlations between triglyceride, cholesterol, HDL cholesterol and the LDL subfractions.

Table 3.17 Correlations with LDL Subfractions

	Triglyceride < 1.5 mmol/l			*Triglyceride > 1.5 mmol/l*		
Parameter	*LDL I*	*LDL II*	*LDL III*	*LDL I*	*LDL II*	*LDL III*
Triglyceride	- 0.007	0.255†	0.234†	- 0.389§	- 0.539§	0.557§
Cholesterol	0.550§	0.602§	- 0.001	0.320 §	0.429§	-0.116
HDL cholesterol	0.208	- 0.126	- 0.209	0.475§	0.312§	- 0.462§
HDL_2	0.348†	- 0.238	- 0.224†	0.622§	0.445§	- 0.414§

†p<0.01, §p<0.0001

Plasma triglyceride <1.5 mmol/l showed a significant positive relationship with LDL cholesterol (r = 0.34, p<0.0001) and no significant relationship when above this value (r= 0.098). Cholesterol was positively associated with LDL I and LDL II at all concentrations of plasma triglyceride. Below 1.5 mmol/l, plasma triglyceride was weakly positively correlated with LDL II and LDL III but when the concentration of the lipid was raised above this level, correlations with the LDL subfractions were very strong P(<0.0001), LDL I and LDL II being negatively and LDL III positively associated. HDL cholesterol and HDL_2 in particular, at the higher triglyceride concentration was associated positively with LDL I and LDL II and negatively with LDL III. The determinants of LDL subfraction concentration were assessed in an analysis of variance general linear model (table 3.18).

Table 3.18 Determinants of LDL Subfraction Concentration

Variable	*LDL I*	*LDL II*	*LDL III*
Age	17.8§	16.0‡‡	1.3
Sex	2.0	1.3	1.2
BMI	4.0*†	1.2	0.4
Apo E phenotype	0	0	0.8
Triglyceride	3.2	0.8	30.1*†
VLDL cholesterol	1.5	0	0
HDL_2	16.6§	1.9	0.4

values are r2. p *†<0.05, ‡‡ <0.005, §<0.0001

In the multivariate model, age and HDL_2 were significant independent predictors of LDL I, age was the only significant independent predictor of LDL II and triglyceride was the significant independent predictor of LDL III accounting for 30% of the variability of this subfraction.

3.4 Discussion

Of the published methods none met the objective of a rapid quantitative LDL subfractionation assay which could be easily used to investigate the causes of altered LDL profiles in hyperlipidaemia and coronary artery disease. Further, it was recognised early on in this project that gradient gel electrophoresis was not sufficient to generate the data necessary to understand the origin and control of heterogeneity in LDL but it was a standard to which other techniques had to be compared.

Thus the objective was to devise a density gradient ultracentifugation which generated profiles comparable to those obtained by gradient gel electrophoresis. A successful gradient was developed based on that of Marzetta *et al* (1986) and the use of fresh plasma gave improved resolution. Density gradient ultacentrifugation separation of LDL produced clear banding in the centrifuge tube and there was a high reproducibility of peaks in the eluting profile. Gradient gel electrophoresis (fig. 3.3) and electron microscopy (fig. 3.5) of the purified subfractions produced evidence for the existence of discrete LDL species. The density and size of LDL I, LDL II and LDL III were identical to those obtained by Krauss *et al* (1982) and so direct comparisons can be made with the investigations carried out on subjects identified as having LDL phenotype A or B profiles. Size by gradient gel electrophoresis was larger than that obtained by electron microscopy and Kahlon *et al* (1982) when using sedimentation equlibrium observed a systematic bias in gradient gel electrophoresis towards a larger molecular size. The application of specific coefficients of extinction for each subfraction allowed concentration distribution to be inferred from the OD280 profiles. This alleviated the necessity for the collection and analyses of individual lipoproteins in each specific fraction, and so minimised the cost and time involved.

The LDL subfraction distribution profile was characterised in normals, a number of dyslipidaemic states and CAD as summarised in fig. 3.9. In agreement with other methods of subfractionation (McNamara *et al*, 1987, Swinkels *et al*, 1989), differences were observed in normals between young males and females, particularly in LDL I and LDL III which had a reciprocal relationship, LDL I being higher in females and LDL III higher in males. This may be partly due to the sex difference in plasma triglyceride in the general population but in these subjects plasma triglyceride levels were similar and so the differences must be attributed to other factors. In a companion study (Tan *et al*, 1995a) in normals (the results of which have not been included in this thesis) we also found this pattern of subfraction distribution in young normal males and females. Gender differences were attributal, as described below, to variations in HL activity which is known to be influenced by sex hormones.

In the CAD case control study, LDL III was found to be a powerful predictor of coronary risk and was independent of age, plasma triglyceride, BMI, smoking and drug status (Griffin *et al*, 1994). This was in agreement with Austin *et al* (1988) who observed that a preponderance of pattern B LDL phenotype (small dense LDL) was associated with a

three-fold increase in the risk of MI independently of gender, age or body weight. In the present study it was clear that a concentration of small dense LDL III in excess of 100mg/100ml gives rise to a greatly increased risk of CHD independent of total circulatory LDL mass.

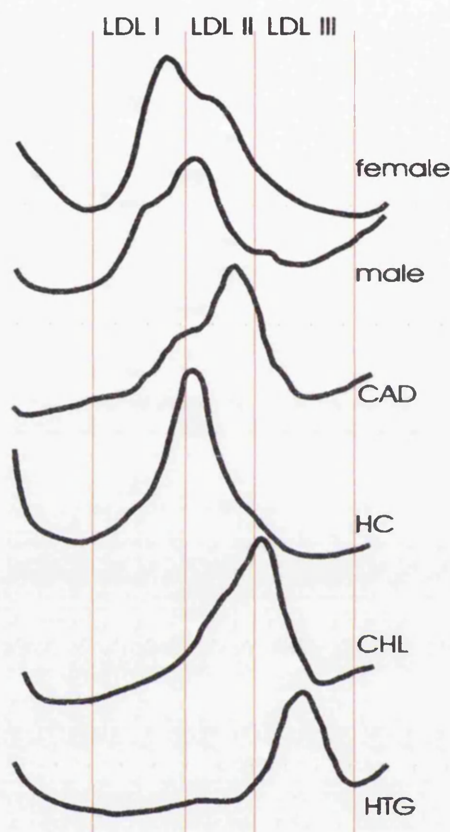

Fig 3.9 LDL Subfraction Profiles
LDL profiles by density gradient ultracentrifugation in a female, a male, subjects with coronary artery disease (CAD), hypercholesterolaemia (HC), combined hyperlipidaemia (CHL) and hypertriglyceridaemia (HTG).

LDL profile was examined in a number of dyslipidaemic states and across all there was a clear link between plasma triglyceride and LDL III as can be seen in the combined data (fig.3.8). Moderate hypercholesterolaemia and familial hypercholesterolaemia are associated with an elevation of large LDL I and LDL II and when these were accompanied by a raised triglyceride in the combined hyperlipidaemic group, there was double jeopardy with raised total LDL mass and raised LDL III. This observation goes some way to explaining why the combined hyperlipidaemic subjects are at greater risk when compared

to normotriglyceridaemics with the same LDL cholesterol level (Assmann & Schute, 1992, Frick *et al*, 1987). There is a debate at present as to whether the CHD risk is increased in hypertriglyceridaemics with normal LDL cholesterol levels. The data presented in table 3.10 indicating the presence of low HDL_2 and high concentrations of LDL III would indeed suggest that such subjects were at greater coronary risk.

Despite the different methodologies employed to determine the LDL profile, these observations in hyperlipidaemic subjects are entirely in agreement by those reported by de Graaf *et al* (1993) for FH , Innerarity *et al*, 1990 for FDB, Francheschini *et al* (1994) for FH and type IIa hyperlipidaemia, Chapman *et al* (1996) and Guerin *et al*, (1996) for combined hyperlipidaemia and Kleinman *et al* (1985) and Lahdenpera *et al*, (1995) in hypertriglyceridaemia.

The secondary hyperlipidaemic subjects studied with NIDDM and nephrotic syndrome showed similar lipid and lipoprotein profiles (tables 3.14) despite differing aetiology. The similarity in LDL profiles (table 3.15) was likely to be driven by the raised plasma triglyceride concentrations. These two groups of subjects exhibited an ALP profile and were considered at high CHD risk because of the accelerated atherosclerosis found in these conditions (Steiner *et al*, 1985, Ordonez *et al*, 1993). The findings in NIDDM patients were in agreement with numerous investigations (Barakat *et al*, 1990, Feingold *et al*, 1992, Tan *et al*,1995b, Lahdenpera *et al*, 1996) but to date there have no reports in the literature of LDL size in the nephrotic syndrome.

The primary determinant of LDL concentration was plasma triglyceride and the complex association of this plasma lipid with the individual LDL subfractions (fig. 3.8) suggested that there was a threshold for the formation of LDL III from LDL II. These findings are in agreement with the observations in normal subjects reported separately (Tan *et al*, 1995a) in which a model was proposed to account for the formation of small dense LDL (fig.3.10). It was suggested that in individuals with elevated plasma concentrations of triglyceride rich lipoproteins there was an enhanced transfer of triglyceride into LDL II via the action of CETP. LDL in turn lost cholesteryl ester in the exchange process and become triglyceride enriched so forming an ideal substrate for HL. The enzyme acts on LDL II to hydrolyse the triglyceride enriched core and generate LDL III. If HL activity is low, LDL II remains the major species despite being relatively triglyceride enriched. The HL activities from the young male and female subjects in this present study are reported in Watson *et al*, 1994 and, in agreement with those reported by Tan *et al* (1995a), HL levels in females are lower than males. Thus the data presented here are consistent with this model since at triglyceride concentrations <1.5 mmol/l there is a positive correlation between plasma triglyceride and LDL II and at plasma triglyceride concentrations >1.5 mmol/l there is a negative correlation (table 3.16). Total LDL mass is conserved across the plasma triglyceride range.

HDL, in particular HDL_2, was found to be an important predictor of LDL I mass. This may also be explained by HL which is also known to strongly influence the size and density of HDL and hence HDL_2 concentrations (Patsch *et al*, 1987) by mechanisms similar to that proposed for LDL.

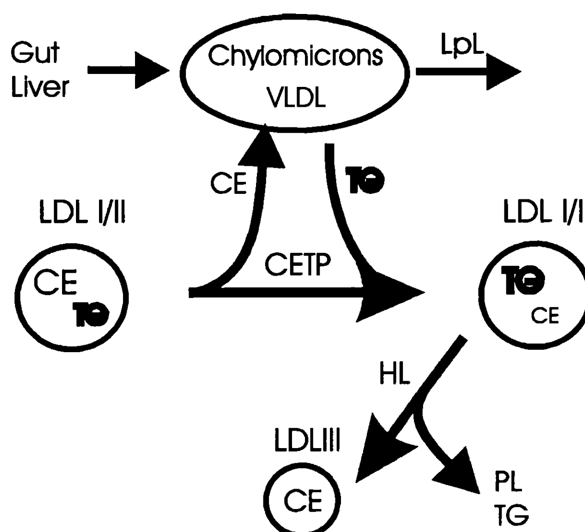

Fig.3.10 Proposed Model for Formation of LDL III

LpL lipoprotein lipase, HL hepatic lipase,CETP cholesterol ester transfer protein, CE cholesteryl ester, TG triglyceride, PL phospholipid.

CETP mediates exchange of cholesteryl ester for triglyceride, forming an LDL II particle rich in trigylceride which is an ideal substrate for HL. Hydrolysis of the trigycerude results in the formation of a small LDL III particle enriched in cholsteryl ester.

Chapter 4 Modification of LDL Subfractions by Hypolipidaemic Agents

4.1 Introduction

The pharmacological management of patients with dyslipidaemia has benefited from major advances in the last decade. Safe and effective drug therapies that have been thoroughly validated in clinical trials are available. However it is recognised in all guidelines (the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (ATPII), 1993; the European Atherosclerosis Society (EAS) , International Task Force, 1992; the British Hyperlipidaemia Association (BHA), Betteridge *et al*, 1993) that lifestyle and diet modification is essential as a first step in correcting hyperlipidaemia.
Hypolipidaemic drugs may be classified by their predominant therapeutic effect although they alter more than one lipid fraction. Cholesterol lowering agents are the bile-acid sequestrants and HMG-CoA reductase inhibitors, whereas circulating levels of triglyceride are decreased by nicotinic acid and fibric acid derivatives.

The bile-acid sequestrants have a long history of clinical use and the two currently available agents are cholestyramine and colestipol, both of which are highly charged polycationic resins which interrupt the intrahepatic circulation of bile acids. As a consequence the activity of 7-α-hydroxylase is increased, resulting in increased conversion of sterols into bile acids. This leads to a decrease in hepatic cholesterol and up regulation of the LDL receptor. In patients with an underlying metabolic propensity for hypertriglyceridaemia, triglyceride synthesis and VLDL production is promoted by resins although the mechanism of this effect remains obscure. Usually LDL cholesterol decreases by 15-30% and HDL cholesterol increases by 3-5% while triglyceride is unaffected but may be increased in a few patients.

The HMG-CoA reductase inhibitors or statins, the most recent class of hypolipidaemic agent, have had a major impact in terms of efficacy and tolerability. The currently available statins are lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin. HMG-CoA reductase is the enzyme which modulates the reduction of HMG-CoA to mevalonate. It acts as the pacemaker enzyme in the cholesterol biosynthetic pathway. Statins competitively inhibit HMG-CoA reductase and thereby decrease cholesterol synthesis. The content of cholesterol in key regulatory pools is diminished and so there is activation of the LDL receptor via the mediation of the sterol regulatory element binding protein (SREBP), a positive transcription factor for the receptor gene. Clearance of all circulating apoB/E containing lipoproteins (ie VLDL, IDL and LDL) from the circulation is increased by statins. Typical plasma changes are an LDL cholesterol decrease by 20-40%, an HDL cholesterol increase by 5-15% and triglyceride decreases of 10-20%. The drugs are the most intensively studied lipid lowering compounds in large clinical trials for example the Scandinavian Simvastatin Survival Study (4S) with simvastatin and the West of Scotland Coronary Prevention Study (WOSCOPS) with pravastatin.

Nicotinic acid is a B vitamin which acts as a coenzyme in intermediary carbohydrate metabolism and when given in large doses (2-5g/d) it has a substantial lipid lowering effect. The nicotinic acid derivative acipimox is available as a hypolipidaemic agent in the UK. Nicitonic acid suppresses free fatty acid mobilisation from peripheral adipocytes and inhibits VLDL and triglyceride production in the liver. It lowers LDL cholesterol by 10-25%, raises HDL cholesterol 15-30% and lowers triglyceride 20-50%.

The parent fibrate compound (clofibrate) was introduced in 1962 and second and third generation fibric acid derivatives gemfibrozil, bezafibrate, ciprofibrate and fenofibrate are now available. Fibrates are believed to act by stimulating (possibly through binding) peroxisome proliferator activated receptors (PPAR) in the liver. These members of the steroid hormone nuclear receptor family regulate a number of genes including apo CIII and LPL. Fibrates suppress apo CIII synthesis in the liver as a result. Plasma apoCIII concentration falls and since this protein acts to inhibit the receptor-mediated uptake of triglyceride rich lipoproteins there is increased chylomicron/VLDL uptake by the liver and, due to the further action of the drug, there is promotion of lipolysis by stimulation of lipoprotein lipase. The effect on LDL cholesterol is variable depending on the initial concentration. If it is high, LDL cholesterol is decreased by10-15% but if low, as in hypertriglyceridaemia, the LDL cholesterol is increased by therapy. Plasma triglyceride is reduced 20-40% and HDL cholesterol is increased by 10-15%.

In the previous chapter the LDL subfraction profile was examined in normal and various dyslipidaemic states. It was demonstrated that the concentration and distribution of the LDL species was not only dependent on plasma cholesterol, but that plasma triglyceride was a major determinant of the distribution as was the role of the lipolytic enzymes LPL and HL. In this chapter the perturbation of the LDL subfraction profile by hypolipidaemic drugs was observed to be predictable depending on their actions on lipoprotein and hepatic lipase, plasma triglyceride levels and LDL receptors. Drugs that lower plasma triglyceride shift the spectrum towards less dense particles and those that activate LDL receptors will reduce the larger species due to increased receptor-mediated clearance.

4.2 Method

4.2.1 Protocol

Except in the case of normal subjects, treatment with hypolipidaemic agents took place for the specified time periods after a three month standard cholesterol reducing diet. Measurements were taken before and after therapy for fasting lipids, lipoproteins, apolipoproteins and lipoprotein subfractions analyses as described in chapter 2.

4.2.2 Subjects

Normolipaemic subjects were recruited from staff at Glasgow Royal Infirmary. None were taking medication known to affect lipid metabolism and none were obese. The remaining subjects were recruited from Lipid Clinics at Glasgow Royal Infirmary University/NHS Trust; Victoria Infirmary, Glasgow; Hairmyres Hospital, East Kilbride; the Menopause Clinic at Stobhill Hospital, Glasgow; the database of Department of Cardiac Surgery, GRI

and the Diabetic Clinic at Chelsea and Westminster Hospital, London. All subjects were screened for haematological, hepatic, endocrine and renal dysfunction to exclude secondary causes of hyperlipidaemia. None of the subjects had clinical evidence of familial hypercholesterolaemia (FH) and none were taking any medication known to affect lipid metabolism. During the study, all subjects maintained their normal habits of diet and exercise.

4.3 Results

4.3.1 Bile-acid Sequestrants

Treatment with cholestyramine was investigated in a group of normal individuals and treatment with colestipol in a group of subjects who had undergone coronary artery bypass grafting (CABG).

Cholestyramine

Nine normolipaemic subjects, 4 males and 5 females aged 25-37, mean age 32 years, received cholestyramine resin at 16g/day for 4 weeks. The effect on lipids and lipoproteins is shown in table 4.1.

Table 4.1 Effect of Cholestyramine on Lipids in Normal Subjects

	Cholesterol	*Triglyceride*	*VLDL cholesterol mmol/l*	*LDL cholesterol*	*HDL cholesterol*
Baseline					
mean	4.57	0.82	0.41	2.69	1.48
(SEM)	(0.86)	(0.80)	(0.25)	(0.84)	(0.32)
Cholestyramine					
mean	3.79	0.80	0.34	1.84	1.61
(SEM)	(0.80)	(0.26)	(0.14)	(0.74)	(0.50)
p	<0.001	NS	NS	<0.001	NS

In response to cholestyramine treatment for 4weeks, 9 normal subjects showed a significant reduction in plasma cholesterol (17%, $p<0.001$) that was attributed to a decrease in LDL cholesterol (32%, $p<0.001$). Plasma triglyceride was unaffected whereas HDL showed a 9% (NS) increase.

The effect of 4 weeks treatment with cholestyramine on LDL subfractions is shown in table 4.2.

Table 4.2 Effect of Cholestyramine on LDL Subfractions in Normal Subjects

	LDL mass	*LDL I*	*LDL II mg/100ml*	*LDL III*
Baseline				
mean	253	57	162	34
(SEM)	(81)	(20)	(85)	(14)
Cholestyramine				
mean	150	32	75	43
(SEM)	(65)	(29)	(30)	(41)
p	NS	<0.05	<0.005	NS

The total lipoprotein mass of LDL was decreased as a result of a significant reduction of the large LDL I (44%, $p<0.05$) and LDL II (54%, $p<0.005$). The concentration of small, dense LDL III was increased in five subjects but showed a variable response in the remainder of the group. This resulted in an apparent enrichment in LDL III which was 28% of total LDL compared to 13% in the control phase even though the increase of 26% in the mass of LDL III was non-significant. There was no change in the lipoprotein composition of total LDL (d 1.019-1.063 g/ml).

Colestipol

Seven male subjects aged 44-64 with initial cholesterol level >6.0 mmol/l and triglyceride < 3.0 mmol/l were treated with colestipol for 10 weeks, rising to a dose of 20 g/day after 7-10 days. All had undergone coronary artery bypass grafting (CABG) between 3 and 12 months previously. The changes in lipids and lipoproteins are in table 4.3.

Table 4.3 Effect of Colestipol on Lipids in CABG Subjects

	Cholesterol	*Triglyceride*	*VLDL cholesterol mmol/l*	*LDL cholesterol*	*HDL cholesterol*
Baseline					
mean	6.58	1.84	0.82	4.59	1.09
(SEM)	(0.20)	(0.16)	(0.08)	(0.23)	(0.02)
range	6.02-7.27	1.32-2.65	0.73-1.12	3.70-5.50	1.03-1.23
Colestipol					
mean	5.62	2.01	0.89	3.57	1.17
(SEM)	(0.13)	(0.18)	(0.08)	(0.11)	(0.06)
range	5.35-6.40	1.33-2.69	0.57-1.93	3.07-4.15	0.97-1.32
p	<0.005	NS	NS	<0.005	NS

Colestipol treatment reduced cholesterol by 15% ($p<0.005$). This was due to a 22% fall in LDL cholesterol ($p<0.005$), while VLDL cholesterol and HDL cholesterol were unchanged. There was a variable response in plasma triglyceride with no change overall. The effect on LDL subfractions is shown in table 4.4.

Table 4.4 Effect of Colestipol on LDL Subfractions in CABG Subjects

	LDL mass	*LDL I*	*LDL II mg/100ml*	*LDL III*
Baseline				
mean	410	73	232	105
(SEM)	(37)	(9.8)	(15.3)	(30.9)
range	305-584	45-107	154-285	24-216
Colestipol				
mean	269	45	144	81
(SEM)	(15)	(7.4)	(13.1)	(17)
range	220-322	24-71	98-202	26-143
p	<0.01	<0.05	<0.005	NS

There was inter-individual variation in the LDL subfraction profile but the mass of LDL III both on and off colestipol was significantly associated with plasma triglyceride level ($r= 0.64$, $p<0.02$). The mass of total LDL was decreased by 34% ($p <0.01$) on drug, the most significant change being due to a 38% decrement in the major LDL II species ($p<0.005$) and a 38% reduction in LDL I ($p <0.05$). There was a variable response in LDL III resulting in an overall reduction in mass of 23%. There were no changes in Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

4.3.2 HMG-CoA Reductase Inhibitors

Simvastatin therapy was investigated in 2 groups of subjects with moderate hypercholesterolaemia and combined hyperlipidaemia and atorvastatin therapy in a group of subjects with combined hyperlipidaemia.

Simvastatin in Moderate Hypercholesterolaemia

Six subjects (4 men and 2 postmenopausal females) aged from 36-64 year were treated with simvastatin at 20mg nocte for 10 weeks, rising to this dose after 4 weeks at an initial dose of 10mg nocte. The subjects had moderate hypercholesterolaemia with LDL cholesterol >4.5 mmol/l and plasma triglyceride <2.3 mmol/l. The lipid and lipoprotein results are in

table 4.5. Treatment reduced plasma cholesterol by 29% ($p<0.001$). The decrement was due to a 40% ($p<0.0001$) fall in LDL cholesterol while VLDL and HDL cholesterol remained unchanged. Triglyceride was not significantly affected although there was a 15% reduction.

Table 4.5 Effect of Simvastatin on Lipids in Moderate Hypercholesterolaemia

	Cholesterol	*Triglyceride*	*VLDL Cholesterol mmol/l*	*LDL Cholesterol*	*HDL Cholesterol*
Baseline					
mean	7.20	1.63	0.63	5.13	1.35
(SEM)	(0.17)	(0.19)	(0.08)	(0.11)	(0.07)
range	6.5-7.6	1.0-2.25	0.35-0.85	4.75-6.0	1.1-1.6
Simvastatin					
mean	5.10	1.39	0.63	3.09	1.36
(SEM)	(0.26)	(0.16)	(0.08)	(0.23)	(0.05)
range	4.2-6.0	0.95-1.95	0.35-0.90	2.42-4.10	1.20-1.55
p	<0.001	NS	NS	<0.0001	NS

Table 4.6 documents the changes observed in the lipoprotein subfractions. There was a 37% reduction ($p <0.05$) in LDL mass. While the subfraction profile exhibited considerable variation, LDL II was the major subfraction in each subject. Quantitative analysis revealed that there was a 40% decrement in this species ($p<0.05$) while there was a 31% reduction in LDL I and 36% in LDL III. There was a tendency for LDL III to remain unchanged in those subjects in whom the mass was low and be reduced in those subjects in which the baseline mass was >100mg/100ml. HDL_2 was increased significantly ($p <0.005$) by 73%, while HDL_3 was unaffected.

Table 4.6 Effect of Simvastatin on Lipoprotein Subfractions in Moderate Hypercholesterolaemia

	LDLmass	LDL I	LDL II	LDL III mg/100ml	HDL_2	HDL_3
Baseline						
mean	382	62	231	90	40	254
(SEM)	(53)	(7.9)	(35)	(25.9)	(3.7)	(7.3)
range	283-640	30-88	163-392	17-182	28-53	236-286
Simvastatin						
mean	239	43	138	58	70	274
(SEM)	(35.9)	(9.0)	(15.0)	(15.6)	(5.5)	(17.7)
range	170-412	16-74	102-206	33-132	51-90	211-326
p	<0.05	NS	<0.05	NS	<0.005	NS

There were no changes to Lp(a). The lipoprotein composition of LDL (d 1.019-1.063 g/ml) was altered. The percentage of free cholesterol fell from 10.5 to 8.0 , a reduction of 24% ($p<0.05$) while that of triglyceride was increased by 34% ($p<0.05$).

Simvastatin Treatment in Combined Hyperlipidaemia

Five subjects, 4 male and 1 postmenopausal female with combined hyperlipidaemia (plasma cholesterol > 6.0 mmmol/l and plasma triglyceride >2.3 mmol/l) were treated with simvastatin 40mg/d for 8 weeks. The lipid and lipoprotein levels attained are summarised in table 4.7.

Table 4.7 Effect of Simvastatin on Lipids in Combined Hyperlipidaemia

	Cholesterol	Triglyceride	VLDL Cholesterol mmol/l	LDL Cholesterol	HDL Cholesterol
Baseline					
mean	6.55	2.90	1.13	4.53	0.90
(SEM)	(0.11)	(0.32)	(0.17)	(0.06)	(0.06)
range	6.4-7.0	2.25-4.10	0.75-1.70	4.35-4.70	0.70-1.05
Simvastatin					
mean	3.93	1.82	0.70	2.26	0.95
(SEM)	(0.22)	(0.21)	(0.08)	(0.13)	(0.07)
range	3.2-4.4	1.35-2.60	0.60-1.00	1.9-2.7	0.7-1.1
p	<0.001	<0.05	NS	<0.0001	NS

Simvastatin had profound reducing effects on plasma cholesterol (40%, p<0.001), plasma triglyceride (37%, p<0.05), VLDL cholesterol (38%, NS) and LDL cholesterol (50%, p<0.0001), with a 5% non-significant increase in HDL cholesterol. Apo B (table 4.8) was reduced by 33% (p<0.0005) while Apo AI was unaffected.

Table 4.8 Effect of Simvastatin on LDL Subfractions and Apoproteins in Combined Hyperlipidaemia

	LDLmass	LDL I	LDL II	LDL III mg/100ml	ApoAI	Apo B
Baseline						
mean	342	36	127	179	112	149
(SEM)	(35)	(9.1)	(23.1)	(27.7)	(9.1)	(7.6)
range	222-434	6.4-55.6	65-204	112-278	80-133	128-168
Simvastatin						
mean	189	35.9	105	49	108	83
(SEM)	(16.0)	(9.0)	(16.3)	(14.3)	(5.6)	(4.1)
range	142-240	13.5-58	67-151	17-79	87-117	70-92
p	<0.02	NS	NS	<0.01	NS	<0.0005

The LDL subfractions shown in table 4.8 were altered by therapy. At baseline, LDL III levels were high and accounted for 52% of the total LDL mass. Treatment with simvastatin decreased the levels of all 3 subfractions, the greatest fall being 73% (p<0.01) in LDL III. In all subjects concentration of this species was > 100mg/100ml at baseline and less than this value on treatment. The relevant percentages of LDL I, LDL II and LDL III on statin were 20%, 54% and 25% respectively, compared with 11%, 36% and 52% at baseline. LDL II and LDL III were negatively correlated with each other (r = -0.838, p<0.002) and LDL I and LDL III were also similarly associated (r= -0.788, p<0.01). There were no changes to Lp(a) or the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Atorvastatin in Combined Hyperlipidaemia

Four subjects, 3males and 1 postmenopausal female with combined hyperlipidaemia (plasma cholesterol > 6.0 mmmol/l and plasma triglyceride >2.3 mmol/l) were treated with atorvastatin at 40mg/day for 8 weeks. The lipid and lipoprotein levels attained are summarised in table 4.9.

Table 4.9 Effect of Atorvastatin on Lipids in Combined Hyperlipidaemia

	Cholesterol	*Triglyceride*	*VLDL Cholesterol mmol/l*	*LDL Cholesterol*	*HDL Cholesterol*
Baseline					
mean	6.84	2.84	1.18	4.71	0.95
(SEM)	(0.36)	(0.28)	(0.27)	(0.14)	(0.04)
range	6.4-7.9	2.25-3.55	0.75-1.90	4.45-5.10	0.90-1.05
Atorvastatin					
mean	3.86	1.75	0.61	2.18	1.08
(SEM)	(0.16)	(0.10)	(0.03)	(0.14)	(0.01)
range	3.45-4.15	1.55-1.95	0.55-0.70	1.8-2.5	1.05-1.10
p	<0.002	<0.05	NS	<0.0001	<0.05

Treatment with atorvastatin had dramatic effects on the lipids and lipoproteins. There were reductions in plasma cholesterol (42%, $p<0.002$), plasma triglyceride (38%, $p<0.05$), VLDL cholesterol (48%, NS) and LDL cholesterol (54%, $p<0.0001$) with a small but significant increase in HDL cholesterol (13%, $p<0.05$). Table 4.10 summarises the effect of treatment on the LDL subfraction profile and the apolipoproteins. Apo AI was unaltered by atorvastatin but apo B was significantly reduced (47%, $p<0.005$).

Table 4.10 Effect of Atorvavastatin on LDL Subfractions and Apoproteins in Combined Hyperlipidaemia

	LDLmass	*LDL I*	*LDL II*	*LDL III mg/100ml*	*ApoAI*	*Apo B*
Baseline						
mean	368	38	142	188	118	145
(SEM)	(26.1)	(11.0)	(22.2)	(31.1)	(5.6)	(8.4)
range	316-434	6.4-55.6	99-204	146-278	106-133	128-168
Atorvastatin						
mean	188	23.1	94.1	71	116	77
(SEM)	(11.0)	(5.7)	(8.0)	(11.1)	(2.1)	(2.9)
range	164-214	7.5-34.7	71-108	46-92	110-120-	71-84
p	<0.005	NS	NS	<0.05	NS	<0.005

The mass of LDL (table 4.10) was halved on atorvastatin therapy ($p<0.005$) and this was due to reductions in levels of all 3 LDL species. LDL I fell by 39% (NS), LDL II by 34% (NS) and LDL III by 62% ($p<0.05$). All subjects had LDL III levels<100mg/ml on treatment. There was a switch in the distribution of LDL II and LDL III, at baseline this was 39:51 and on atorvastatin it was 58:37. LDL I and LDL III were inversely associated ($r = -0.71$, $p<0.05$). There were no changes to Lp(a) or the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

4.3.3 Nicotinic Acid Derivatives

Acipimox treatment was investigated in normal, moderate hypercholesterolaemic and combined hyperlipidaemic groups of subjects.

Acipimox Therapy in Normals

Nine normolipaemic subjects, 5 males and 4 premenopausal females aged 25-37, mean age 31 years, received acipimox at 750mg/day for 4 weeks. The effect on lipids and lipoproteins is shown in table 4.11.

Table 4.11 Effect of Acipimox on Lipids in Normal Subjects

	Cholesterol	*Triglyceride*	*VLDL cholesterol mmol/l*	*LDL cholesterol*	*HDL cholesterol*
Baseline					
mean	5.23	1.20	0.61	3.09	1.51
(SEM)	(1.59)	(0.99)	(0.51)	(1.23)	(0.31)
Acipimox					
mean	4.59	0.77	0.36	2.68	1.55
(SEM)	(0.70)	(0.22)	(0.25)	(0.73)	(0.32)
p	NS	NS	NS	NS	NS

Treatment with acipimox for 4 weeks resulted in a 36% decrease in plasma triglyceride and slight but non-significant alterations in cholesterol, VLDL cholesterol and HDL cholesterol. The LDL subfraction data is shown in table 4.12.

Table 4.12 Effect of Acipimox on LDL Subfractions in Normal Subjects

	LDL mass	*LDL I*	*LDL II mg/100ml*	*LDL III*
Baseline				
mean	269	62	147	59
(SEM)	(88)	(29)	(64)	(14)
Acipimox				
mean	270	68	161	41
(SEM)	(58)	(16)	(51)	(14)
p	NS	NS	NS	NS

While total LDL mass was unaffected by acipimox therapy, its component subfractions showed a tendency to redistribute towards larger more buoyant species (LDL I + 10%, LDL II + 10%, LDL III - 31%). The change in LDL III correlated significantly with the fall in plasma triglyceride (r= 0.75, p<0.05). There were no changes in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Acipimox Therapy in Moderate Hypercholesterolaemia

Five subjects (3 males and 2 postmenopausal females) aged from 36-60 years were treated with acipimox at 1250 mg/day for 10 weeks. The subjects had moderate hypercholesterolaemia with LDL cholesterol >4.5 mmol/l and plasma triglyceride <2.3 mmol/l. The lipid and lipoprotein results are in table 4.13. Treatment reduced plasma cholesterol by 18% (p<0.01). This decrement was due to a significant 21% fall in LDL cholesterol (p< 0.005) with a nonsignificant fall of 26% in VLDL cholesterol and a mild 3% increase in HDL cholesterol. There was also a non-significant fall in triglyceride of 12%. The lipoprotein subfraction changes are shown in table 4.14.

Table 4.13 Effect of Acipimox on Lipids in Moderate Hypercholesterolaemia

	Cholesterol	*Triglyceride*	*VLDL cholesterol mmol/l*	*LDL cholesterol*	*HDL cholesterol*
Baseline					
mean	7.98	1.72	0.85	5.91	1.23
(SEM)	(0.19)	(0.12)	(0.12)	(0.21)	(0.09)
range	7.5-8.6	1.32-2.02	0.45-1.17	5.34-6.60	1.00-1.57
Acipimox					
mean	6.54	1.52	0.63	4.65	1.27
(SEM)	(0.29)	(0.23)	(0.12)	(0.22)	(0.10)
range	5.9-7.4	1.12-2.28	0.42-1.05	4.03-5.33	1.08-1.57
p	<0.01	NS	NS	<0.005	NS

LDL II was the major species in these subjects and there was a moderate 11% decrease with therapy. There was a redistribution of the other LDL subfractions with a 76% rise in LDL I and a 37% decrease in LDL III. None of these responses to acipimox therapy were significant. The HDL_2/HDL_3 ratio increased by 37% due to a 25% rise in HDL_2 and an 11% fall in HDL_3. Triglyceride was inversely related to LDL I ($r=-0.822$, $p< 0.005$). On treatment LDL I and LDL III were negatively associated ($r= -0.90$, $p<0.05$) as were LDL I and LDL II ($r= -0.96$, $p<0.01$).

Table 4.14 Effect of Acipimox on Lipoprotein Subfractions in Moderate Hypercholesterolaemia

	LDLmass	*LDL I*	*LDL II*	*LDL III* mg/100ml	*HDL_2*	*HDL_3*
Baseline						
mean	404	58	224	122	59	241
(SEM)	(57)	(15.8)	(49.4)	(66.4)	(13.0)	(25.6)
range	280-597	14-99	73-385	32-381	22-94	193-335
Acipimox						
mean	378	102	199	77	74	221
(SEM)	(25.3)	(27.6)	(17.2)	(33.1)	(14.5)	(14.3)
range	309-466	18-154	164-248	12-200	34-107	191-261
p	NS	NS	NS	NS	NS	NS

There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Acipimox Therapy in Combined Hyperlipidaemia

One male subject aged 49 and one postmenopausal female aged 47 were treated with acipimox at 1250 mg/day for 10 weeks. The subjects had moderate combined hyperlipidaemia with LDL cholesterol >4.5 mmol/l and plasma triglyceride >2.3 mmol/l. The lipid and lipoprotein results are in table 4.15. Treatment with acipimox in these two subjects had a favourable effect on all the lipids and lipoproteins. Cholesterol was reduced by 11% and 15% respectively in subjects Acip1 and Acip2, triglyceride by 20% and 19%, VLDL cholesterol by 34% and 20% , LDL cholesterol by 12% and 18% and HDL cholesterol was increased by 23% and 10%.

Table 4.15 Effect of Acipimox on Lipids in Combined Hyperlipidaemia

Subject	Cholesterol	Triglyceride	VLDL cholesterol mmol/l	LDL cholesterol	HDL cholesterol
Baseline					
Acip 1	7.9	2.72	1.25	5.73	0.95
Acip2	7.2	2.80	1.33	4.95	0.90
Acipimox					
Acip 1	7.0	2.17	0.83	5.02	1.17
Acip 2	6.1	2.27	1.07	4.07	1.00

LDL mass (table 4.16) was reduced in both subjects by 10% and 29%. In Acip1 there was a redistribution of LDL subfractions towards larger and lighter species. At baseline, 82% was LDL III with a complete absence of LDL I. Acipimox decreased LDL III by 84%, increased LDL II by 145% and LDL I species was present. The percentages of LDLI, LDL II and LDL III on treatment were 23, 66 and 11 respectively. At baseline LDL II was the major species in subject Acip 2. On treatment there was a 34% reduction in LDL II and a moderate 6% reduction in LDL III, resulting in a decrement of 29% in total LDL mass. There were similar alterations in HDL subfractions with a 24% rise in the ratio of $HDL_2:HDL_3$ in Acip 1 and 18% in Acip 2.

Table 4.16 Effect of Acipimox on Lipoprotein Subfractions in Combined Hyperlipidaemia

Subject	LDLmass	LDL I	LDL II	LDL III mg/100ml	HDL_2	HDL_3
Baseline						
Acip 1	430	0	79	351	31	179
Acip 2	460	64	299	97	29	213
Acipimox						
Acip 1	474	107	311	56	55	257
Acip 2	327	42	194	91	33	205

There were no changes in the concentration of Lp(a) or in lipoprotein composition of LDL (d 1.019-1.063 g/ml).

4.3.4 Fibrates

Two second generation fibrates were studied. Fenofibrate therapy was given to a group of subjects with moderate hypercholesterolaemia and a group of subjects with non-insulin dependent diabetes mellitus. Ciprofibrate was given to a group of subjects with moderate hypercholesterolaemia and another group with moderate hypertriglyceridaemia.

Fenofibrate in Moderate Hypercholesterolaemia

One male and 5 postmenopausal females aged 48 to 63 were treated with fenofibrate (100mg t.i.d.) for 8 weeks. The subjects had moderate hypercholesterolaemia with fasting LDL cholesterol >4.5 mmol/l and plasma triglyceride <2.3 mmol/l. Plasma lipid, lipoprotein and apolipoprotein levels are shown in table 4.17.

Table 4.17 Effect of Fenofibrate on Lipids and Apoproteins in Moderate Hypercholesterolaemia

	Chol	Triglyceride	VLDL Chol	LDL Chol	HDL Chol	Apo AI	Apo B
			mmol/l			mg/100ml	
Baseline							
mean	8.29	1.64	0.64	6.27	1.38	131	142
(SEM)	(0.27)	(0.14)	(0.12)	(0.32)	(0.07)	(4.1)	(6.6)
range	7.7-9.5	1.2-2.1	0.3-1.0	5.4-7.6	1.2-1.6	118-146	119-161
Fenofibrate							
mean	5.83	1.06	0.42	3.90	1.50	132	96
(SEM)	(0.31)	(0.10)	(0.05)	(0.30)	(0.08)	(7.6)	(3.8)
range	5.3-7.3	0.9-1.5	0.3-0.7	3.4-5.3	1.2-1.8	109-158	87-109
p	<0.0002	<0.01	NS	<0.0005	NS	NS	<0.0005

Treatment with fenofibrate significantly lowered plasma cholesterol by 30% (p<0.0002) and plasma triglyceride by 35% (p<0.01). The fall in VLDL cholesterol paralleled that of plasma triglyceride (34%). Fenofibrate reduced LDL cholesterol by 38% (p<0.0005) and apoB by 33% (p<0.0005, table 4.18). There was no change in the concentration of HDL cholesterol or apo AI concentration. However there was a non-significant decrease of 36% in HDL_2:HDL_3 ratio (table 4.18).

LDL II was the major subfraction (table 4.18) in most individuals. Fenofibrate therapy induced a 32% (p<0.02) fall in LDL mass, an 11% reduction in LDL I, a 44% reduction in LDL II (p<0.002) and a 44% non-significant reduction in LDL III. Where LDL III was present in small concentrations there was no change, but when it was in larger concentrations >100mg/100ml, there were dramatic reductions so that all subjects had LDL III concentrations < 50 mg/100ml.

Table 4.18 Effect of Fenofibrate on Lipoprotein Subfractions in Moderate Hypercholesterolaemia

	LDLmass	LDL I	LDL II	LDL III mg/100ml	HDL_2	HDL_3
Baseline						
mean	526	170	297	60	66	249
(SEM)	(43.2)	(50.1)	(24.0)	(19.7)	(17.9)	(18.4)
range	374-651	58-385	233-365	20-152	30-144	181-294
Fenofibrate						
mean	355	151	166	34	50	294
(SEM)	(19.5)	(19.5)	(17.5)	(5.6)	(11.8)	(20.7)
range	275-405	94-221	98-227	14-46	24-106	222-358
p	<0.02	NS	<0.002	NS	NS	NS

There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Fenofibrate in Non Insulin Dependent Diabetes Mellitus (NIDDM)
Ten patients, six men and 4 postmenopausal women, with stable NIDDM treated with either diet alone or in combination with an oral hypoglycaemic drug were treated with micronised fenofibrate for 12 weeks. The subjects were aged beteen 56 and 72 with mean BMI 27.4 (SEM 1.85). Table 4.19 shows a summary of the lipids, lipoproteins and apoproteins.

Table 4.19 Effect of Fenofibrate on Lipids, Lipoproteins and Apoproteins in Non Insulin Dependent Diabetes Mellitus

	Cholesterol	Triglyceride mmol/l	HDL chol	LDL chol	Apo AI mg/100ml	Apo B
Baseline						
mean	7.29	3.06	1.14	4.94	136	153
(SEM)	(0.22)	(0.48)	(0.07)	(0.27)	(3.1)	(7.3)
range	6.1-8.2	0.7-5.2	0.9-1.7	3.8-6.2	120-150	100-180
Fenofibrate						
mean	6.22	2.1	1.37	3.81	142	125
(SEM)	(0.28)	(0.34)	(0.06)	(0.17)	(4.1)	(7.8)
range	4.8-8.0	1.1-4.8	1.1-1.7	2.7-4.6	120-160	100-180
p	<0.01	NS	<0.05	<0.005	NS	<0.02

Treatment significantly lowered cholesterol (15%, $p<0.01$), LDL cholesterol (23%, $p<0.005$), Apo B (18%, $p<0.02$) and raised HDL cholesterol (20%, $p<0.05$). The reduction of 31% in plasma triglyceride was not significant as 2 of the 10 subjects had higher levels on fenofibrate. As was noted in NIDDM patients in chapter 3, there was a significant difference ($p=0.024$) in triglyceride between males and females. At baseline the mean (SEM) values in mmol/l were 2.23(0.50) for males and 4.30(0.52) for females, but this was lost in treatment (males 1.85(0.28)mmol/l, females 2.48(1.56)mmol/l). ApoAI was unchanged.

Table 4.20 Effect of Fenofibrate on LDL Subfractions in Non Insulin Dependent Diabetes Mellitus

	LDL mass	*LDL I*	*LDL II mg/100ml*	*LDL III*
Baseline				
mean	361	38	134	177
(SEM)	(26.1)	(7.3)	(24.5)	(28.1)
range	256-502	0-65	62-315	14-289
Fenofibrate				
mean	340	64	200	76
(SEM)	(23.1)	(6.9)	(14.6)	(22.8)
range	253-459	35-111	148-268	32-269
p	NS	<0.02	<0.05	<0.02

LDL mass of lipoproteins (table 4.20) did not alter after fenofibrate therapy, however there were significant changes in the distribution of the LDL subfractions. At baseline 53% of LDL was small and dense LDL III. On fenofibrate, the mass of this species was decreased by 60% ($p<0.02$) so that it comprised 21% of the total LDL mass. LDL I was increased by 68% ($p<0.02$) becoming 19% of total LDL and LDL II was increased by 49% ($p<0.05$) on treatment becoming the major LDL species at 60%. In these subjects, plasma triglyceride was highly correlated with the percentage of all 3 subfractions
(LDL I: $r=-0.66$, $p<0.002$; LDL II: $r=-0.731$, $p<0.0001$; LDL III: $r=0.75$, $p<0.0001$)
and the subfractions were related to each other: LDLI and LDL II: $r=0.759$, $p<0.0001$, LDL I and LDL III: $r=-0.876$, $p<0.0001$, LDL II and LDL III: $r=-0.979$, $p<0.0001$.
There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Ciprofibrate in Moderate Hypercholesterolaemia
Ciprofibrate therapy (100mg/d) was given to 9 subjects (6 men and 3 postmenopausal females), aged 37-68 for 8 weeks. In these moderate hypercholesterolaemic subjects

ciprofibrate favourably altered the lipid and lipoproteins (table 4.21). The 18% decrease in plasma cholesterol (p <0.005) was due to a 38% fall in VLDL cholesterol (p <0.005) and a 24% fall in LDL cholesterol (p <0.01), while there was a 14% non-significant rise in HDL cholesterol. There was a marked 38% fall in plasma triglyceride (p <0.0005). Apo AI was unaffected by ciprofibrate, whereas apo B concentration was decreased by 33% (p <0.005).

Table 4.21 Effect of Ciprofibrate on Lipids, Lipoproteins and Apoproteins in Moderate Hypercholesterolaemia

	Chol	*Triglyceride*	*VLDL Chol*	*LDL Chol*	*HDL Chol*	*Apo AI*	*Apo B*
			mmol/l			*mg/100ml*	
Baseline							
mean	7.46	1.38	0.71	5.32	1.39	140	130
(SEM)	(0.29)	(0.09)	(0.05)	(0.29)	(0.08)	(10.0)	(6.9)
range	6.5-9.1	1.1-2.0	0.5-1.0	4.4-6.1	1.1-1.7	88-186	110-165
Ciprofibrate							
mean	6.01	0.85	0.43	4.05	1.58	131	87
(SEM)	(0.26)	(0.07)	(0.04)	(0.32)	(0.09)	(7.8)	(6.3)
range	5.1-7.4	0.6-1.2	0.3-0.6	2.9-5.6	1.3-2.0	107-171	59-118
p	<0.005	<0.0005	<0.0005	<0.01	NS	NS	<0.0005

Analysis of LDL and HDL subfractions are shown in table 4.22. There was no change in HDL_3 with a slight decrease in HDL_2. The mass of total LDL was significantly reduced by 22% (p< 0.01). The changes in the individual subfractions were not significant, however both LDL II and LDL III fell 31%. In subjects with higher LDL III concentrations these were greatly reduced.

Table 4.22 Effect of Ciprofibrate on Lipoprotein Subfractions in Moderate Hypercholesterolaemia

	LDLmass	*LDL I*	*LDL II*	*LDL III*	*HDL_2*	*HDL_3*
				mg/100ml		
Baseline						
mean	492	163	272	56	99	259
(SEM)	(25.3)	(20.2)	(29.2)	(13.8)	(11.5)	(23.5)
range	410-618	97-252	181-451	22-141	34-158	175-392
Ciprofibrate						
mean	381	157	186	39	84	266
(SEM)	(27.7)	(13.3)	(22.8)	(6.9)	(15.8)	(25.5)
range	253-493	108-224	102-298	15-79	33-166	153-339
p	<0.01	NS	NS	NS	NS	NS

LDL I and LDL III were negatively associated (r= -0.67, p <0.05), whereas triglyceride was associated with % LDL I (r = - 0.59, p <0.02) and positively with LDL II (r = 0.58, p< 0.02). There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Ciprofibrate in Moderate Hypertriglyceridaemia

Ten men, aged 34-65, with moderate hypertriglyceridaemia were treated with ciprofibrate at 100mg/day for 10 weeks. There was a slight non-significant fall in plasma cholesterol (table 4.23) and a slight non-significant rise in LDL cholesterol (table 4.23). HDL cholesterol was also raised non-significantly (11%). The major change was a 36 % decrement in plasma triglyceride (p <0.02), which was reflected in a 44% fall in VLDL cholesterol (p <0.002). The apoproteins reflected these alterations in plasma lipoproteins with no change in apo AI and a 19% (p <0.05) fall in apo B.

Table 4.23 Effect of Ciprofibrate on Lipids, Lipoproteins and Apoproteins in Moderate Hypertriglyceridaemia

	Chol	*Triglyceride*	*VLDL Chol*	*LDL Chol*	*HDL Chol*	*Apo AI*	*Apo B*
			mmol/l			*mg/100ml*	
Baseline							
mean	6.05	3.12	1.48	3.64	0.88	115	148
(SEM)	(0.24)	(0.36)	(0.14)	(0.23)	(0.03)	(7.7)	(7.6)
range	5.2-7.1	2.0-5.2	1.0-2.1	2.6-4.5	0.8-1.1	90-166	116-180
Ciprofibrate							
mean	5.67	2.0	0.83	3.87	0.98	120	120
(SEM)	(0.30)	(0.18)	(0.09)	(0.25)	(0.06)	(5.1)	(11.0)
range	4.2-6.9	1.1-2.7	0.4-1.3	2.6-4.7	0.7-1.2	96-137	62-164
p	NS	<0.02	<0.002	NS	NS	NS	<0.05

Table 4.24 summarises the response of the lipoprotein subfractions to ciprofibrate treatment. There were small non-significant changes in HDL subfractions and no change in LDL mass. However, there were dramatic alterations to the distribution of the individual LDL subfractions. At baseline the distribution of LDLI: LDLII: LDL III was 8:20:71 and 13:46:38 on treatment. The masses of LDL I and LDL II were increased by 67% (p <0.05) and 124% (p < 0.001) respectively, whereas the mass of LDL III was decreased by 40% (p <0.05).

There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Table 4.24 Effect of Ciprofibrate on Lipoprotein Subfractions in Moderate Hypertriglyceridaemia

	LDLmass	*LDL I*	*LDL II*	*LDL III mg/100ml*	*HDL_2*	*HDL_3*
Baseline						
mean	279	23	57	198	17	248
(SEM)	(21.2)	(4.3)	(12.0)	(23.5)	(1.9)	(18.4)
range	191-356	0-42	0-112	130-354	10-26	176-374
Ciprofibrate						
mean	319	39	144	125	27	297
(SEM)	(20.9)	(5.6)	(17.1)	(24.3)	(5.9)	(16.2)
range	207-396	10-61	37-220	16-260	11-70	234-401
p	NS	<0.05	<0.001	<0.05	NS	NS

4.3.5 Combination Therapies

The combinations of acipimox and cholestyramine and colestipol and simvastatin were examined in subjects with moderate hypercholesterolaemia.

Acipimox and Cholestyramine Combination in Moderate Hypercholesterolaemia

A combination therapy of 12g/day of cholestyramine and 1250 mg/day of acipimox was given to 5 subjects with moderate hypercholesterolaemia. The subjects were 3 males and 2 postmenopausal females, aged 36-60. Table 4.25 indicates that combination therapy reduced plasma cholesterol by 25% ($p<0.001$) while plasma triglyceride fell by a non-significant 8%. There was a 19% (NS) fall in VLDL cholesterol, a 29% ($p <0.002$) fall in LDL cholesterol and a non-significant 10% increase in HDL cholesterol.

Table 4.25 Effect of Acipimox and Cholestyramine in Combination on Lipids in Moderate Hypercholesterolaemia

	Cholesterol	*Triglyceride*	*VLDL cholesterol mmol/l*	*LDL cholesterol*	*HDL cholesterol*
Baseline					
mean	7.98	1.72	0.85	5.91	1.23
(SEM)	(0.19)	(0.12)	(0.12)	(0.21)	(0.09)
range	7.5-8.6	1.3-2.0	0.45-1.17	5.34-6.60	1.00-1.57
Acipimox +	***cholestyramine***				
mean	6.02	1.58	1.68	4.17	1.36
(SEM)	(0.27)	(0.26)	(0.10)	(0.28)	(0.09)
range	5.5-7.0	1.0-2.4	0.48-1.03	3.42-4.85	1.08-1.60
p	<0.001	NS	NS	<0.002	NS

There were no significant changes in the lipoprotein subfractions (table 4.26). The individual HDL subfractions were slightly raised and LDL I decreased by 11%, and LDL II and LDL III both decreased by 27%. This resulted in a 22% non-significant decrease in LDL mass. There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL (d 1.019-1.063 g/ml).

Table 4.26 Effect of Acipimox and Cholestyramine in Combination on Lipoprotein Subfractions in Moderate Hypercholesterolaemia

	LDLmass	*LDL I*	*LDL II*	*LDL III mg/100ml*	*HDL_2*	*HDL_3*
Baseline						
mean	404	58	224	122	59	241
(SEM)	(57.1)	(15.8)	(49.4)	(66.4)	(13.0)	(25.6)
range	280-597	14-99	73-385	32-381	22-94	193-335
Acipimox + cholestryramine						
mean	314	52	161	89	66	253
(SEM)	(24.8)	(10.9)	(20.4)	(28.1)	(16.2)	(6.4)
range	261-378	18-83	124-239	26-159	23-101	236-272
p	NS	NS	NS	NS	NS	NS

Colestipol and Simvastatin Combination in Moderate Hypercholesterolaemia

Simvastatin (20mg nocte) and colestipol (20g/d) were given in combination for 10 weeks to a group of 7 male subjects with cholesterol >6.0 mmol/l and plasma triglyceride < 3.0 mmol/l. They were aged between 45 and 64 years. Plasma lipid and lipoprotein levels are displayed in table 4.27. Combined treatment reduced plasma cholesterol by 37% ($p<0.0001$), while plasma triglyceride fell by 8% (NS). This decrement was due to a 48% fall in LDL cholesterol ($p< 0.0001$) and a 19% fall in VLDL cholesterol (NS). HDL cholesterol was significantly ($p<0.05$) raised by 11%.

Table 4.27 Effect of Colestipol and Simvastatin in Combination on Lipids in Moderate Hypercholesterolaemia

	Cholesterol	*Triglyceride*	*VLDL cholesterol mmol/l*	*LDL cholesterol*	*HDL cholesterol*
Baseline					
mean	6.68	1.95	0.88	4.6	1.10
(SEM)	(0.16)	(0.14)	(0.06)	(0.22)	(0.03)
range	6.02-7.27	1.52-2.65	0.70-1.12	3.7-5.5	1.03-1.23
Colestipol + simvastatin					
mean	4.19	1.79	0.71	2.25	1.23
(SEM)	(0.15)	(0.26)	(0.11)	(0.13)	(0.05)
range	3.78-4.80	0.97-3.03	0.40-1.25	1.95-2.78	1.10-1.48
p	<0.0001	NS	NS	<0.0001	<0.05

Table 4.28 shows the effect of colestipol and simvastatin in combination on the lipoprotein subfractions. There were modest, non-significant rises in HDL subfractions with no alteration in HDL_2/HDL_3 ratio. There was a marked fall in total LDL mass (54%, p <0.001), which could be explained by profound decreases in both of the larger, more buoyant species LDL I and LDL II (72%, p<0.0005 and 59%, p<0.0005 respectively). Despite a 33% reduction in the mean value of LDL III, the overall effect was not significant. At baseline the distribution of LDL I: LDL II :LDL III was 6:53:27 and on therapy it was 11:46:42.

Table 4.28 Effect of Colestipol and Simvastatin in Combination on LDL Subfractions in Moderate Hypercholesterolaemia

	LDLmass	*LDL I*	*LDL II*	*LDL III mg/100ml*	*HDL_2*	*HDL_3*
Baseline						
mean	420	77	218	125	47	226
(SEM)	(36.8)	(9.1)	(18.5)	(33.1)	(12.6)	(9.1)
range	305-584	45-107	154-285	24-216	23-102	199-260
Colestipol + simvastatin						
mean	194	21	89	84	63	281
(SEM)	(10.7)	(5.2)	(7.6)	(16.8)	(9.3)	(24.8)
range	154-232	0-44	69-121	30-154	16-89	217-379
p	<0.001	<0.0005	<0.0005	NS	NS	NS

There were no changes in the concentration of Lp(a) or in the lipoprotein composition of LDL(d 1.019-1.063 g/ml).

4.3.6 Influence of Triglyceride on LDL III

Triglyceride showed no relationship with LDL III until > 1.5 mmol/l when there was a positive association at baseline (fig 4.1). On treatment with fibrates this association was retained (baseline r = 0.63, p<0.001, on fibrate r=0.67, p<0.001). On statins, in particular in the combined hyperlipidaemic subjects, this association between LDL III and plasma triglyceride became negative (baseline r = 0.42, p=0.12 and on statin r = -329, p=0.231).

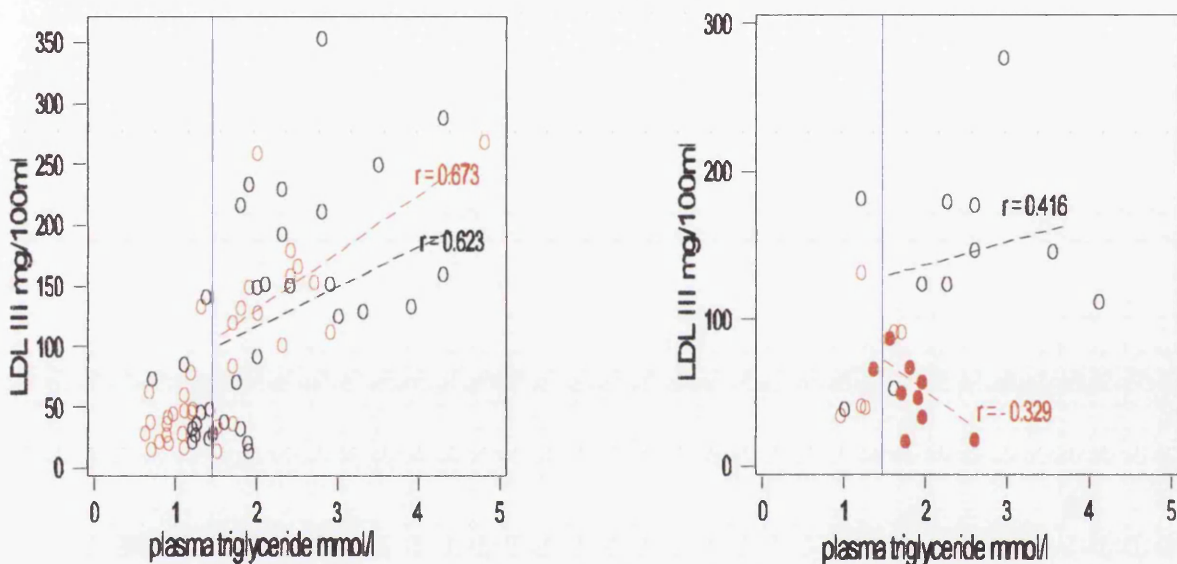

Fig.4.1 Plasma Triglyceride and LDL III on Statin and Fibrate Therapy

LDL III concentrations before (black) and after(red) therapy with fibrates (left) and statins (right). Closed cicles are combined hyperlipidaemic subjects on statin.

4.4 Discussion

Application of a quantitative procedure for LDL subfractionation to the study of the mechanisms of the action of drugs permits a closer insight into their effects compared to a simple evaluation of particle size or lipid composition of the particle. This is demonstrated repeatedly in the present studies where the influence of various drugs on the actual concentration of small dense LDL could be determined.

The resins colestipol and cholestyramine were observed to selectively decrease the mass of the larger more buoyant LDl I and LDL II species in both normal and CABG subjects.

LDL III concentration was relatively unaffected resulting in an apparent enrichment in small dense LDL III. This finding is in agreement with previous observations in miniature

pigs (Huff *et al*, 1989), guinea pigs (Witzum *et al*, 1985) and in humans (Young *et al*, 1989). When resin- and control-LDL were compared *in vitro* and *in vivo*, the control LDL had a higher affinity for the LDL receptor and a higher fractional catabolic rate. This suggests that resins by stimulating the LDL receptor induce selective clearance of an LDL species with high affinity (LDL I/II) leaving behind an LDL relatively enriched in small dense particles which have lower affinity for receptors (fig.4.2).

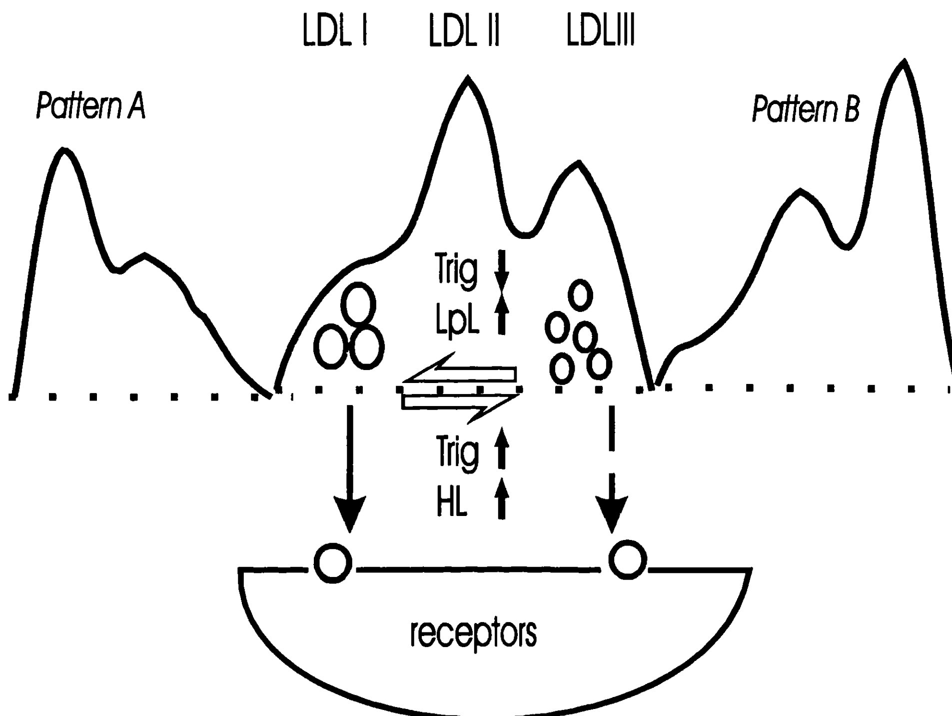

Fig. 4.2 Pharmacological Regulation of LDL Subfractions

Trig triglyceride, LpL lipoprotein lipase, HL hepatic lipase.

When hepatic lipase activity or plasma triglyceride concentrations are high,small LDL III is formed and the LDL distribution profle shifts towards a pattern B phenotype Large LDL is more activly taken up by the receptor-mediated process compared to the smaller LDL III. Drugs that lower plasma triglyceride or stimulate the activity of lipoprotein lipase (such as the fibrates) result in a reduction in small LDL III and a shift in LDL subfraction profile from pattern B to pattern A so producing a better ligand for receptors.. Drugs such as statins up regulate the LDL receptor and result in increased removal of larger LDL particles.

Therapy with HMGCoA inhibitors in moderate hypercholesterolaemia significantly reduced plasma cholesterol, LDL cholesterol and the quantities of larger LDL, with a variable effect on LDL III. Where this last subfraction was present at high concentration (> 100mg/100ml) it was reduced. These changes in LDL I and II are consistent with the observation by Witzum *et al* (1985) noted above that larger LDL is removed selectively by the LDL receptor. In combined hyperlipidaemia, both simvastatin and atorvastatin gave

approximately 40% reductions in plasma cholesterol and plasma triglyceride (tables 4.7 and 4.9). This resulted in reductions in the larger LDL subfractions but even greater reductions in LDL III. The influence of statins on the LDL profile may be due to the removal of precursors of small dense LDL from the circulation by stimulated receptors. In support of this suggestion Gaw *et al* (1995) reported that in hypercholesterolaemics, simvastatin stimulated direct removal of apo B in S_f 12-400 range resulting in a decreased LDL production rate. Furthermore stable isotope metabolic studies from this laboratory in the subjects in tables 4.7 to 4.10. have shown that there was increased clearance of $VLDL_1$ on treatment with simvastatin (Forster *et al*, 1996a) and atorvastatin (Forster *et al*, 1996b). This action reduced the amount of $VLDL_1$ remnants passing through the IDL-LDL cascade resulting in lower levels of LDL III. The effect of statins on LDL III is in clear distinction to that of the resins which tend to increase triglyceride and $VLDL_1$ levels (Packard & Shepherd, 1982).

Since plasma triglyceride is a major determinant of LDL III concentration, triglyceride lowering drugs would be predicted to have a major impact on the LDL subfraction profile. The influence of an isolated triglyceride lowering is seen in normal subjects who when treated with acipimox showed no overall change in LDL mass but a redistribution of the profile towards LDL I and LDL II. In hyperlipidaemic subjects there was a reduction in plasma cholesterol with a modest reduction in triglyceride, greater reductions being observed in those subjects with triglyceride > 2.3 mmol/l. As in normals, there was a modest decrement in LDL mass but there was a substantial redistribution of the LDL profile resulting in an increase in LDL II and a decrease in LDL III to below 100 mg/100ml. One subject in this study reduced LDL III mass from 351 to 97 mg/100ml. In one of the few studies published on the effects of nicotinic acid, Franceschini *et al* (1990) observed structural change in LDL in patients with hypertriglyceridaemia when larger more buoyant LDL appeared on treatment with an increased affinity for the LDL receptor *in vitro*. In 1992, Superko *et al* observed a change from pattern B (small LDL) by gradient gel electrophoresis to pattern A (larger LDL) in subjects treated with nicotinic acid when plasma triglyceride fell below 1.6 mmol/l. This in line with the idea of a threshold triglyceride level for B to A transition.

In all forms of dyslipidaemia investigated - moderate hypercholesterolaemia, moderate hypertriglyceridaemia and non insulin dependent diabetes - the fibrates had a profound effect on triglycerides as well as the LDL subfraction profile. The concentration of LDL III was reduced but there was a variable response in the larger subfractions which were increased in diabetes and moderate hypertriglyceridaemia and reduced in moderate hypercholesterolaemia. These observations are in agreement with studies carried out by Chapman *et al* (1996) using ciprofibrate and Guerin *et al* (1996) using micronised fenofibrate in combined hyperlipidaemia. In 1984, Homma *et al* observed reductions in small dense LDL in subjects with type II and IV hyperlipidaemia. In an elegant study in 1987, Kleinman *et al* showed that LDL in hypertriglyceridaemia was small and enriched in triglyceride and protein when compared to normal LDL and showed defective binding to LDL receptors of normal cultured fibroblasts. These structural and functional defects were reversed with bezafibrate treatment and the improvement was related to altered disposition

of apo B epitopes on LDL as measured by monoclonal antibodies, indicating a conformational change in this LDL protein.
There have been recent advances in the knowledge of the molecular mechanisms that underlie the lipid lowering action of fibrates. The fibrates activate a group of transcription factors belonging to the superfamily of nuclear hormone receptors, the peroxisome proliferator-activated receptors (PPAR) (Gottlicher *et al*, 1992). Upon activation, these receptors bind to the response elements of target genes and thus regulate their expression. Fenofibrate and bezafibrate have been observed to down regulate the apo C III gene (Staels *et al*, 1995; Haubenwallner *et al*, 1995) and upregulate the lipoprotein lipase gene (Staels *et al*, 1992; Schoonjans *et al*, 1995). These actions will enhance both the intravascular lipolysis of triglyceride-rich lipoproteins and the binding of VLDL to LDL receptors due to reduced amount of CIII relative to E on the particle. Studies in transgenic mice have shown that over-expression of apo CIII alone causes hypertriglyceridaemia by reducing clearance (Aalto-Setala *et al*, 1992), while the absence of CIII expression by gene knockout induces rapid clearance of triglyceride and very low plasma triglyceride levels (Maeda *et al*, 1994). The net effect of both these fibrate actions is the elimination of raised plasma triglyceride levels and hence reduced capacity for neutral lipid exchange. This has predictable consequences for the LDL subfraction pattern as outlined in chapter 3. However since like the statins, fibrates promote direct catabolism of triglyceride rich VLDL, then it may be that less apo B is channelled into the production of small dense LDL on such treatment also.

Combination therapy of acipimox and cholestyramine reduced plasma triglyceride and LDL cholesterol more than expected. There were reductions in all 3 subfractions. Acipimox therapy favours the formation of receptor-active LDL I and LDL II whereas cholestyramine enhances receptor expression and so these species are quickly removed. On the basis of previous discussion this is due to the formation of a receptor-active from a receptor-inactive species. The combination of colestipol and simvastatin was highly effective in reducing all 3 LDL species as well as inducing favourable changes in the lipids and lipoproteins. Metabolic studies by Gaw *et al* (1996) indicate that these reductions are due to increased IDL and LDL clearance. It is postulated that addition of a statin overcame the resin effect on LDL III.

These drug induced alterations in the LDL subfraction profile can be explained by consideration of the putative model of LDL subfraction metabolism proposed in chapter 3 and supported by the observations of Tan *et al* (1995a). Plasma triglyceride is the major controlling factor being positively associated with LDL III and negatively with LDL I. The activities of the lipolytic enzymes are important - a high level of hepatic lipase in the male range being required to lipolyse triglyceride-rich LDL II to LDL III. CETP mediated transfer of triglyceride from triglyceride-rich VLDL for cholesteryl ester in LDL forms a triglyceride-rich LDL which is a better substrate for hepatic lipase.
Reduction in triglyceride by stimulation of LPL (fibrates) or increased VLDL catabolism (fibrates, statins) reduces the extent of conversion of LDL II to LDL III and favours the retention of larger LDL particles which in turn are removed more rapidly by receptors. Fig. 4.2 shows that drugs that lower plasma triglyceride levels shift the spectrum towards less

dense particles especially if plasma triglycerides fall below 1.5 mmol/l. Activation of LDL receptors by resins or statins reduces LDL I and LDL II due to increased receptor-mediated clearance. It was interesting to note an apparent break in the link between plasma triglyceride level and LDL III in the group of combined hyperlipidaemic patients treated with simvastatin and atorvastatin in which there had been the observation that the drugs induced a rapid removal of large triglyceride-rich $VLDL_1$ (Forster *et al*, 1996a, 1996b).

Chapter 5 Metabolic Heterogeneity of Low Density Lipoprotein in Normals

5.1 Introduction

Low density lipoprotein (LDL), the major cholesterol transporter in human plasma, is generated by the stepwise delipidation of very low density lipoprotein (VLDL). In 1981 Phillips *et al* observed a strong positive link between plasma triglyceride and LDL cholesterol levels within the normal range for these parameters. This relationship persists after correction for age and weight and was confirmed in the Glasgow arm (Townhead Screening Study) of a UK epidemiological survey (Mann *et al*, 1988) carried out in the mid 1980s. Fig 5.1 demonstrates this relationship in 1843 men and 1881 women. As plasma triglyceride rose from 0.5 to 1.5 mmol/l there was a 33% increase in LDL cholesterol but when plasma triglyceride exceeded 2.0 mmo/l there was a gradual reduction in LDL levels. Above 5 mmol/l i.e. in severe hypertriglyceridaemia very low LDL cholesterol concentrations are found (Shepherd *et al*, 1985).

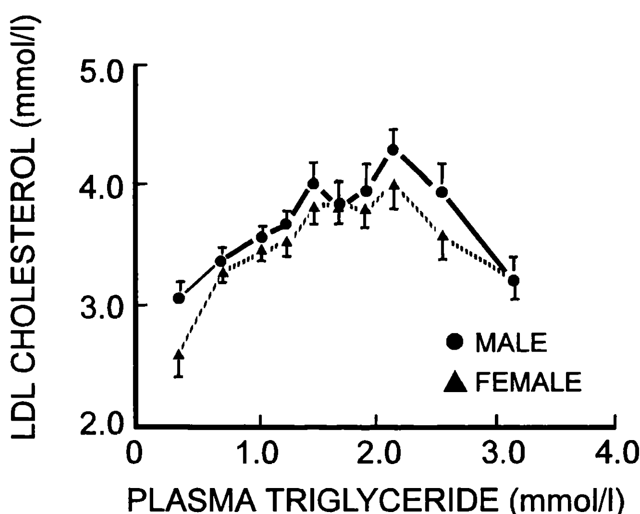

Fig. 5.1 Relationship between LDL Cholesterol and Plasma Triglyceride
The measurements were carried out in 1843 men and 1881 women. LDL cholesterol was calculated by the Friedwald equation

In order to examine more closely the link between plasma triglyceride levels and LDL metabolism, a study was designed to investigate LDL kinetics in a group of young men and women with normal lipid levels. Although early investigations of LDL metabolism in normolipaemic subjects assumed structural and metabolic homogeneity (Langer *et al*, 1972), there is evidence that LDL exists in the plasma of all individuals as a group of

discrete subfractions as has been described fully in Chapter 3. In the present study, a previously defined multicompartmental modelling procedure (Boston *et al*, 1982) was used to analyse LDL catabolism from plasma and the appearance and disappearance of its products from urine.

5.2 Methods

5.2.1 Protocol

Each subject took part in a two week study period during which they underwent measurements of lipids, lipoproteins and lipoprotein subfractions and determination of the kinetics of native and 1,2-cyclohexanedione-treated, radiolabelled LDL tracers as described in 2.6. The metabolic studies were conducted on an outpatient basis with standard dietary advice provided to all participants, based on the American Heart Association (AHA) Step 1 diet, being reinforced by interviews during the study period. The clinical aspects of the work were undertaken by Professor J Shepherd, Dr J Series and Dr B Yip.

5.2.2 Subjects

Volunteers, 21 males and 4 females, aged 19-39 years (mean 26 years) were recruited from individuals attending a local coronary screening programme. All were healthy and, on the basis of clinical laboratory investigations, were free from hepatic, renal, endocrine or haematological dysfunction. None was on medication, including oral contraceptive therapy, nor were any of the females pregnant. All subjects were within 15% of their ideal body weight and had normal lipid levels (plasma cholesterol <6.5 mmol/l and plasma triglyceride <2.3 mmol/l). The characteristics of the subjects are shown in table 5.1.

Table 5.1 Summary of Subjects Characteristics

Subject	*Sex*	*Age* y	*Weight* kg	*ApoE* phenotype
N 1	M	32	56	3/4
N 2	M	31	65	3/3
N 3	M	30	57	3/4
N 4	M	30	67	3/3
N 5	F	26	55	2/3
N 6	M	21	61	3/4
N 7	M	20	65	3/3
N 8	F	33	53	3/3
N 9	M	22	55	3/3
N 10	M	20	70	3/3
N 11	F	27	64	2/2
N 12	F	39	66	3/3
N 13	M	21	79	3/3
N 14	M	25	83	3/4
N 15	M	22	60	3/3
N 16	M	20	65	3/3
N 17	M	30	71	2/3
N 18	M	19	73	3/4
N 19	M	19	69	3/4
N 20	M	21	73	3/3
N 21	M	21	88	3/4
N 22	M	27	73	3/4
N 23	M	35	68	3/3
N 24	M	30	83	3/4
N 25	M	21	78	4/4

5.2.3 Kinetic Analysis

The kinetics of plasma apo-LDL was first analysed by a simple curve-peeling procedure and secondly plasma and urine data were analysed together by multicompartmental modelling.

Curve-peeling Kinetic Analysis

The kinetics of LDL turnover were first analysed by the standard Matthews (1957) approach used in the classic study of Langer *et al* (1972). When the daily plasma radioactivities were plotted on semi-logarithmic graph paper, the curves obtained were biexponential and indicative of a model with an extra-vascular and an intra-vascular plasma pool of apo-LDL. The curve (fig 5.2) was biexponential and was described by the equation

$$y=C_1e^{-b1t} + C_2e^{-b2t}$$

where C_1 and C_2 are the intercepts on the y-axis, and b1 and b2 are the slopes of the two exponentials. If the tracer is homogeneous and catabolised solely from the plasma compartment then a number of kinetic parameters may be determined by Matthews mathematical approach. The fractional catabolic rate (FCR) is described by the equation:

$$FCR=1/(C_1/b1+C_2/b2)$$

and the intravascular pool (IV) by the equation:

$$IV=(C_1/b1+C_2/b2)^2 / (C_1/(b1)^2 + C_2/(b2)^2)$$

Practically the plasma data was plotted to determine visually the start of the second, slow exponential. Simple regression was used to provide a line of best fit to this exponential, yielding b1 and C_1. The exponential was then extrapolated to the y-axis and these projected values were then subtracted from the plasma curve at suitable intervals (0.5, 1.0, 1.5 and 2 days) in the LDL turnover in order to calculate the first exponential. Linear regression was used to derive b2 and C_2. Absolute catabolic rates (ACR) were calculated as the product of the FCR and the mass of apo-LDL in the circulation. Plasma volume was usually calculated by isoope dilution or as 4% of body weight (both being in good agreement).

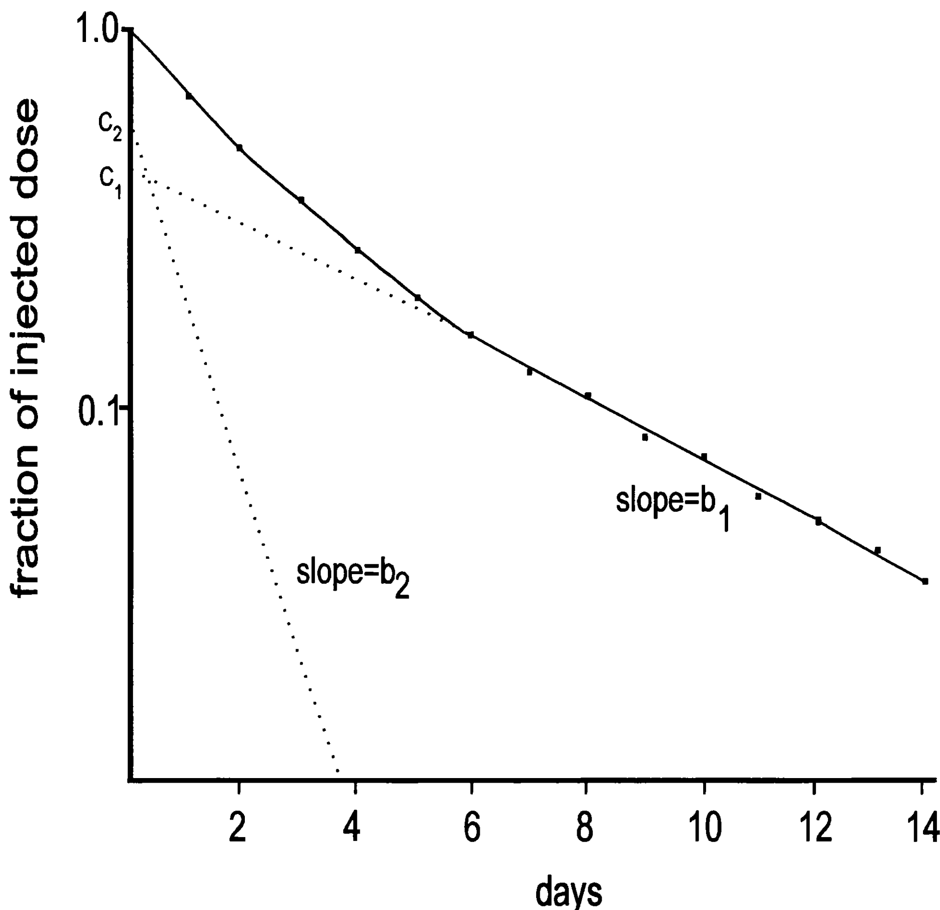

Fig.5.2 Matthews Analysis of Plasma Radioactivity Curve

The biexponential curve is resolved into two monoexponentials defined by the parameters b_1 and b_2 (slopes) and c_1 and c_2 (intercepts).

Multicompartmental Modelling
When urine and plasma radioactivities were combined together using the multicompartmental procedure in SAAM 29, two plasma apo-LDL compartments were required to provide a satisfactory fit (fig 5.3).

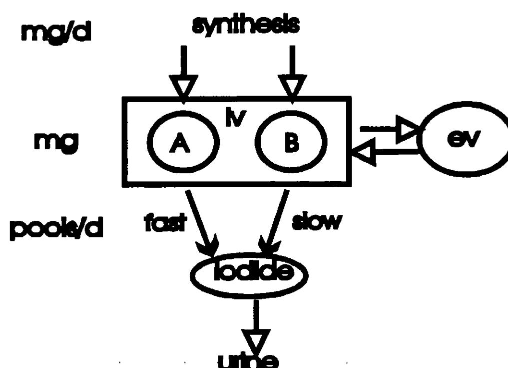

Fig.5.3 Two pool Model of LDL Kinetics
There are two plasma compartments with two points of input and pool B is allowed extravascular exchange.

One represented LDL with a rapid catabolic rate and high affinity for the receptor pathway (pool A) while the other defined a lipoprotein which was more slowly catabolised (pool B) by mechanisms involving a reduced level of receptor activity. Pool B was allowed to exchange with an extravascular pool of apo-LDL. Using this model it is possible to interpret U/P ratios obtained for the subjects in the study, seen in fig 5.4. If plasma apo-LDL was limited entirely to pool A, then there would be a constant U/P ratio of about 0.5 for quintile 1 (Q1), 0.34 for quintile 2 (Q2) and 0.29 for quintile 3 (Q3) throughout the 14 days of the turnover. On the other hand, if all the material was in pool B, there would be a constant U/P ratio of about 0.23. The fall-off in the U/P ratios from day 2 to day 12 reflects the heterogeneous mix of apo-LDL in the plasma. Individuals with lower apo-LDL mass (Q1) show a high ratio of pool A to pool B. As the more rapidly catabolised pool A species is cleared from the plasma, the U/P ratio decreases until all subjects achieve approximately the same U/P value which is determined by slowly metabolised pool B apo-LDL. The U/P ratio for cyclohexanedione modified LDL was constant at 0.2 pools/day.

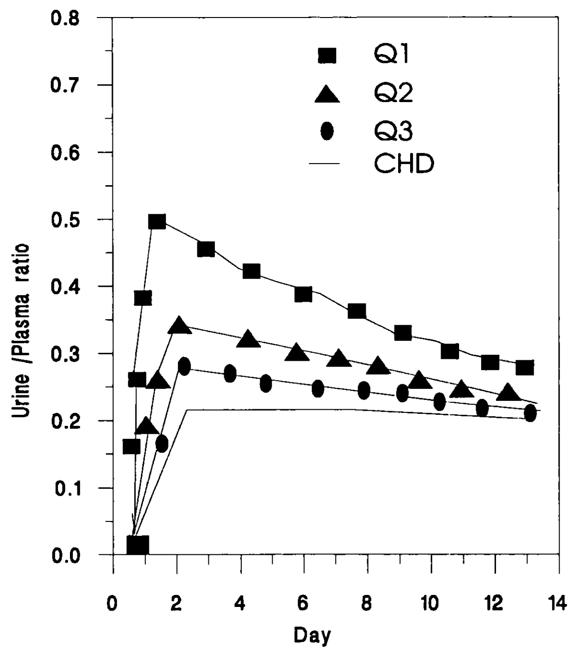

Fig. 5.4 Urine to Plasma Radioactivity Ratios
Urine to plasma radiactivity ratios in native LDL for quintiles 1,2 and 3 (Q1-3) and for cyclohexanedione modified LDL (CHD).

In this model, the overall FCR for apo-LDL can be perturbed by changing either the FCR from pool A or the distribution between the pools A and B. The influence of these parameters was examined on daily U/P ratio in a series of simulations (fig. 5.5). Changes in the elimination rate from pool A affect the shape of U/P curve in a manner that is distinct from that which occurs when the initial mass is shifted from poool A to pool B. Hence these parameters can be derived unambigously during the fitting process.

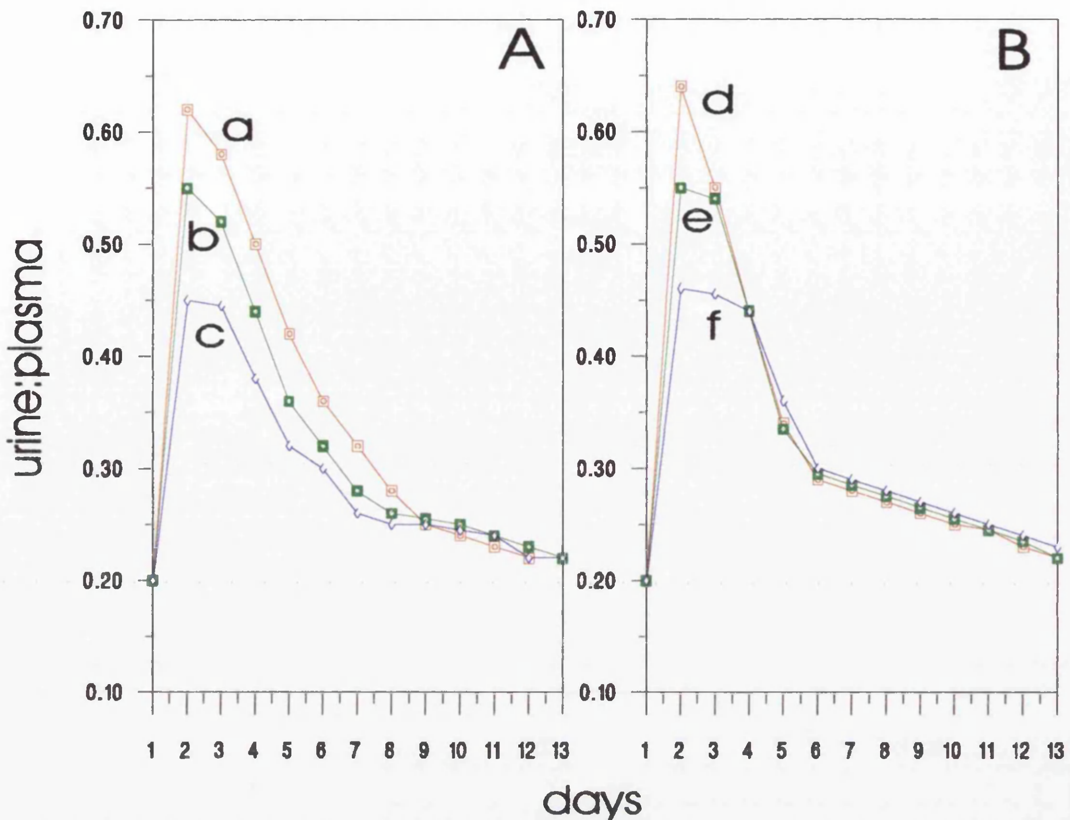

Fig. 5.5 Computer Simulations of changes in Shapes of Urine and Plasma Radioactivity Ratios

Panel A - altered mass distribution between pools A and B. a mass of pool A = mass of pool B, b mass of pool A > mass of pool B, ratio A:B = 0.6, c mass of pool A < mass of pool B, ratio A:B = 0.4. Panel B - altered elimination rates from pool A. d rate = 0.55, e rate = 0.69, f rate = 0.85.

5.3 Results

For ease of presentation, the 25 subjects were ranked according to their total circulating apo-LDL mass (table 5.2) which varied from 1.41 to 4.21 g and divided into quintiles. Apo-LDL concentration was calculated as described previously (Packard *et al*, 1976) from the cholesterol : protein ratio of LDL (d 1.019-1.063 g/ml) and the plasma LDL cholesterol concentration determined by lipoprotein quantitation. This includes a small contribution from IDL (d 1.006-1.019 g/ml). The mass of apo-LDL was the product of the concentration and plasma volume.

Table 5.2 Characteristics Summarised by Quintile

Quintile	age y	M/F	weight kg	Apo E Phenotype	Apo-LDL mass mg
I	29.8 (1.0)	4/1	60.0 (2.5)	2/3=1 3/3=2 3/4=2	1671 (75)
II	23.2 (2.5)	4/1	60.8 (3.1)	3/3=4 3/4=1	2036 (94)
III	26.8 (3.2)	3/2	70.4 (4.5)	2/2=1 3/3=3 3/4=1	2477 (35)
IV	21.8 (2.1)	5/0	70.2 (1.5)	2/3=1 3/3=2 3/4=2	2640 (36)
V	26.8 (2.7)	5/0	78.0 (3.5)	3/3=1 3/4=1 4/4=1	3371 (273)

Values are mean (SEM)

There were no significant age differences between the groups, nor did exclusion of the 4 females make any difference to the subsequent interpretation of the data. Nineteen of the subjects were aged 30 or below and only one was over 35 years. Increasing apo-LDL mass was associated with higher body weights ($r=0.69$, $p<0.001$).

5.3.1 Lipids, Lipoproteins and Apolipoproteins

The individual lipids, lipoproteins and HDL subfractions are shown in Appendix tables 1 and 2 ,with the mean (SEM) lipid and lipoprotein levels in these groups presented in tables 5.3 and 5.4.

Table 5.3 Plasma Lipid and Lipoproteins by Quintile

Quintile	*Cholesterol*	*Triglyceride*	*VLDL chol mmol/l*	*LDL chol*	*HDL chol*
I	4.46 (0.25)	0.82 (0.15)	0.38 (0.07)	2.56 (0.14)	1.54 (0.14)
II	4.56 (0.21)	1.14 (0.14)	0.36 (0.10)	2.54 (0.13)	1.66 (0.10)
III	4.70 (0.33)	1.10 (.007)	0.44 (0.02)	2.82 (0.34)	1.42 (0.12)
IV	5.34 (0.38)	1.22 (0.08)	0.62 (0.09)	3.04 (0.14)	1.58 (0.14)
V	5.90 (0.15)	1.54 (0.18)	0.64 (0.07)	3.78 (0.14)	1.50 (0.09)

Values are mean (SEM)

In this group of normolipaemic individuals, plasma triglyceride ranged between 0.5 and 1.9 mmol/l, plasma cholesterol from 3.8 to 6.6 mmol/l and LDL cholesterol from 2.0 to 4.2 mmol/l. There were significant correlations between plasma triglyceride and plasma cholesterol ($r=0.71$, $p<0.001$) and between plasma triglyceride and LDL cholesterol ($r=0.57$, $p<0.01$). Body weight was related to the concentration of triglyceride ($r=0.51$, $p<0.01$) but not to LDL cholesterol ($r=0.26$, NS). VLDL cholesterol was related to plasma triglyceride ($r=0.71$, $p<0.0001$) total cholesterol ($r=0.64$, $p<0.001$), LDL cholesterol ($r=0.49$, $p=0.013$) and LDL mass ($r=0.56$, $p<0.005$).

Table 5.4 Apo-LDL and HDL Subfraction Concentrations by Quintile

Quintile	*Apo-LDL*	*HDL_2 mg/100ml*	*HDL_3*
I	69.8 (3.8)	65 (17)	221 (30)
II	82.8 (4.3)	80 (18)	230 (13)
III	87.2 (6.2)	37 (5)	182 (14)
IV	94.2 (1.9)	81 (16)	223 (24)
V	108 (8.7)	61 (9)	206 (21)

Values are mean (SEM)

Neither HDL cholesterol nor the concentrations of HDL subfractions differed across the quintiles. Apo-LDL concentration rose 50% across the quintiles and the total circulating mass of the apoprotein in quintile V was double that in quintile I. Apo-LDL concentration and pool size correlated strongly with plasma cholesterol ($r=0.67$, $p<0.001$; $r=0.64$. $p<0.001$, respectively) and with plasma triglyceride levels ($r=0.52$, $p<0.01$; $r=0.70$, $p<0.001$ respectively).

There was no significant influence of apoE phenotype on plasma lipid and lipoprotein levels although there was a tendency for the E_4 allele to be associated with increased circulating apo-LDL mass.

The composition of LDL (Appendix table 3 and table 5.5) did not differ between the quintiles, indicating that the variation observed in LDL cholesterol concentration was associated with an altered number of lipoprotein particles in the circulation.

Table 5.5 Compostion of LDL by Quintile

Quintile	*%Protein*	*%Free cholesterol*	*%Cholesteryl ester*	*%Triglyceride*	*%Phospholipid*
I	26.2	12.2	32.3	7.6	21.6
	(1.2)	(0.8)	(1.3)	(0.8)	(1.1)
II	25.3	11.4	34.6	7.3	21.4
	(0.3)	(1.2)	(1.3)	(0.9)	(0.9)
III	24.9	9.8	34.7	8.1	22.6
	(1.1)	(0.4)	(0.6)	(0.7)	(0.8)
IV	25.6	10.5	36.2	7.2	20.6
	(0.8)	(0.5)	(0.9)	(0.9)	(0.3)
V	24.8	11.0	35.8	7.6	20.9
	(0.6)	(0.7)	(0.4)	(0.3)	(0.3)

Values are mean (SEM)

5.3.2 Receptor-mediated and Receptor-independent Apo LDL Metabolism

Apo-LDL kinetic parameters derived from by Matthews analysis for individual subjects are given in Appendix table 4 and by quintiles in table 5.6.

Table 5.6 Matthews Kinetic Parameters by Quintile

Quintile	*FCR pools/d*	*RI FCR pools/d*	*RM FCR pools/d*	*Synthetic Rate mg/kg/d*	*RI ACR mg/kg/d*	*RM ACR mg/kg/d*
I	0.45 (0.01)	0.21 (0.01)	0.24 (0.02)	12.4 (0.9)	6.0 (0.4)	7.1 (1.0)
II	0.38 (0.02)	0.22 (0.02)	0.16 (0.01)	12.7 (1.1)	7.4 (0.9)	5.4 (0.4)
III	0.36 (0.02)	0.19 (0.02)	0.17 (0.01)	12.3 (1.4)	6.5 (0.8)	5.8 (0.6)
IV	0.35 (0.02)	0.20 (0.02)	0.14 (0.03)	13.1 (0.7)	7.7 (0.8)	5.4 (1.2)
V	0.32 (0.02)	0.17 (0.02)	0.14 (0.02)	13.4 (1.0)	7.9 (1.0)	5.5 (0.8)

Values are mean (SEM). FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL; synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg; ACR is the absolute catabolic rate and is the product of either receptor independent or the receptor mediated FCR and the apo-LDL pool size divided by the body weight. Under steady state conditions synthetic rate and catabolic rate should be equal.

The rate of apo-LDL production was similar to that observed in similar studies (Packard *et al* 1976, 1983), and varied little between the quintiles. Thus the increase in mass of apo-LDL across the quintiles derived from the reduction observed in its total FCR which fell by 30%. Apo-LDL FCR was significantly associated with plasma cholesterol ($r = -0.44$, $p<0.05$), plasma triglyceride ($r = -0.54$, $p<0.01$), LDL cholesterol ($r = -0.54$, $p<0.01$) and body weight ($r = -0.53$, $p<0.01$). When catabolism was divided between the receptor-mediated and receptor-independent pathways, it was observed that the increase in circulating mass of apo-LDL was strongly related to a reduction in the receptor-mediated catabolism ($r=-0.55$, $p<0.01$) and not to any change in the FCR via receptor-independent pathways ($r=-0.30$, $p=0.143$). The relationships between these kinetic parameters using Pearson correlation are given in table 5.7.

Table 5.7 .Relationships between Plasma Lipids, Lipoproteins, Body Weight and Apo-LDL Kinetic Parameters

	Cholesterol	*Triglyceride*	*LDLchol*	*HDLchol*	*Weight*
Apo-LDL concentration	0.67‡	0.524†	0.79‡	-0.234	0.25
Apo-LDL pool	0.636‡	0.704‡	0.730‡	-0.227	0.68‡
Apo-LDL FCR	-0.436*†	-0.535†	-0.539†	0.185	-0.53†
Apo-LDL synthesis	0.131	0.017	0.255	-0.157	-0.256

*p values: *†<0.05, † <0.01, ‡ <0.001*

Categorisation of the subjects by apoE phenotype (table 5.8) indicated that in these subjects there was no impact on plasma apo-LDL concentration or kinetics.

Table 5 .8 Effect of ApoE Phenotype on LDL Kinetic Parameters

ApoE phenotype	*Apo-LDL mass mg*	*Apo-LDL Synthetic rate mg/kg/d*	*Apo-LDL FCR polls/d*
3/3	87	13.3	0.39
2/3	(3.7)	(0.6)	(0.01)
3/4	90	12.2	0.35
4/4	(7.0)	(0.6)	(0.02)
p	NS	NS	NS

Values are mean (SEM)

Examination of the urinary radioactivity excretion data (fig.5.6) and calculation of the daily urine:plasma (U/P) ratio indicated that the kinetics of apo-LDL metabolism were more complex than the Matthews analysis had first suggested. In virtually all the subjects the U/P for native LDL (fig 5.4) fell from a peak value at 2-4 days post injection to a much lower value by days 10-12. The fall-off was graded across the quintiles being steeper for those subjects in quintile I with the lowest apo-LDL mass. On the other hand, the U/P ratio observed for cyclohexanedione-modified LDL was constant over the 14 days of the study,

indicating that the increased catabolism in the early stages of the turnover was due to a receptor-mediated process. These data are incompatible with a simple one-compartment model in which LDL has a constant catabolic rate. They point to the presence of metabolic heterogeneity and so detailed kinetic analysis followed using a multicompartmental analysis approach.

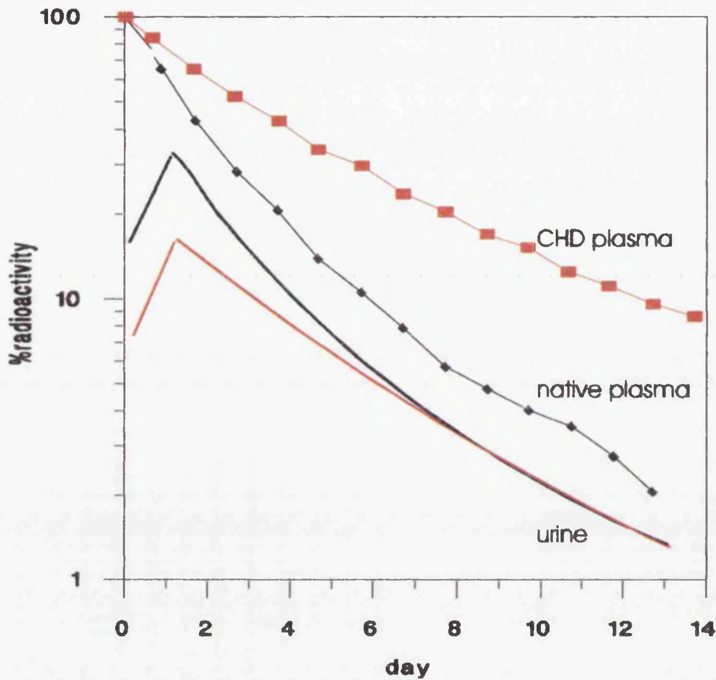

Fig 5.6 Plasma and Urine Radioactive Decay Curves of Native and Cyclohexanedione-modified LDL

Decay curves for native ^{125}I-LDL plasma (upper) and urine (lower) in black, cycloexanedione-modified ^{131}I-LDL in red.

5.3.3 Two compartmental Model of Apo LDL Metabolism

Kinetic parameters and pool sizes for apo-LDL in pools A and B in the individual subjects are given in Appendix tables 5 and 6 and by quintile in tables 5.9 and 5.10.

Table 5.9 Kinetic Parameters of Pool A Apo-LDL by Quintile

		Pool A	Apo-LDL		
Quintile	*Mass*	*Synthesis*	*FCR*	*RI FCR*	*RM FCR*
	mg	*mg/d*	*pools/d*	*pools/d*	*pools/d*
I	1058	599	0.57	0.26	0.30
	(67)	(53)	(0.03)	(0.02)	(0.04)
II	936	735	0.69	0.34	0.35
	(87)	(43)	(0.03)	(0.03)	(0.03)
III	1553	837	0.53	0.28	0.25
	(151)	(105)	(0.02)	(0.03)	(0.02)
IV	1401	680	0.49	0.23	0.26
	(154)	(94)	(0.04)	(0.02)	(0.03)
V	1491	639	0.45	0.24	0.21
	(190)	(88)	(0.07)	(0.04)	(0.05

Values are mean (SEM). FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated is the difference between native and chd modified apo-LDL

In these normal subjects, 70% (SEM 1.66) of the receptor-mediated catabolism of apo-LDL was from pool A and the amount of apo-LDL in pool A rose approximately 50% in quintiles IV and V compared to quintiles I and II. This was due to a 25% reduction in the FCR from this compartment which was significantly inversely related to apo-LDL pool A mass ($r = -0.55$, $p<0.01$). Synthesis of apo-LDL into pool A was relatively constant.

Table 5.10 Kinetic Parameters of Pool B Apo-LDL by Quintile

		Pool B	Apo-LDL		
Quintile	*Mass*	*Synthesis*	*FCR*	*RI FCR*	*RM FCR*
	mg	*mg/d*	*pools/d*	*pools/d*	*pools/d*
I	510	155	0.25	0.13	0.12
	(95)	(12)	(0.01)	(0.01)	(0.01)
II	1044	248	0.23	0.13	0.10
	(110)	(37)	(0.01)	(0.01)	(0.01)
III	924	199	0.21	0.09	0.12
	(145)	(36)	(0.01)	(0.01)	(0.004)
IV	1239	291	0.23	0.13	0.10
	(163)	(52)	(0.01)	(0.01)	(0.01)
V	1880	491	0.26	0.14	0.12
	(196)	(66)	(0.02)	(0.03)	(0.01)

Values are mean (SEM). FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL

Much greater changes were seen in the circulating mass of pool B apo-LDL. It rose more than three-fold as a result of an increase in synthesis (r = 0.95, p<0.0001). The FCR from this compartment did not change with increasing apo-LDL mass, in line with the observation that the U/P ratio fell to the same limiting value in all subjects.

Thus as total apo-LDL mass increased in these normal subjects, the ratio of pool A to pool B material fell from 2.1 in quintile I to 0.79 in quintile V. A stronger relationship was observed between the total apo-LDL mass and pool B mass (r = 0.83, p<0.0001) than between apo-LDL mass and pool A mass (r = 0.61, p<0.01). Apo-LDL mass was also related to pool A FCR (r= -0.55, p<0.01).

Pearson correlation coefficients describing the relationships between body weight, plasma lipid levels and apo-LDL kinetics in pools A and B are given in table 5.11.

Table 5.11 Relationships between Plasma Lipids, Lipoproteins, Body weight and Kinetic Parameters of Pools A and B Apo-LDL

	Pool A			*Pool B*		
	Apo-LDL mass	*Apo-LDL FCR*	*Apo-LDL synthesis*	*Apo-LDL mass*	*Apo-LDL FCR*	*Apo-LDL synthesis*
Cholesterol	0.282	-0.455*†	-0.195	0.581†	0.166	0.600†
Triglyceride	0.334	-0.336	-0.067	0.602‡	0.209	0.641‡
VLDLchol	0.267	-0.335	-0.146	0.547†	0.346	0.542†
LDLchol	0.396*†	-0.540†	-0.192	0.611‡	0.190	0.626‡
HDLchol	-0.293	0.233	0.046	-0.108	-0.200	-0.078
Weight	0.349	-0.448*†	-0.039	0.633†	0.027	0.545†

*p values: *†<0.05, †<0.01, ‡<0.001*

In univariate analysis, the FCR from pool A was negatively correlated with LDL cholesterol (r = -0.54, p<0.01), total cholesterol (r = -0.46, p<0.05) and body weight (r = -0.45, p<0.05) while the mass of apo-LDL in pool A showed a weak correlation with LDL cholesterol (r = 0.40, p<0.05). On the other hand, both mass and synthesis of apo-LDL in pool B were strongly positively correlated with cholesterol (r = 0.58, p<0.01; r = 0.60, p<0.01), triglyceride (r = 0.60, p<0.001; r = 0.64, p<0.001), VLDL cholesterol (r = 0.55, p<0.01; r = 0.54, p<0.01), LDL cholesterol (r = 0.61, p<0.001; r = 0.63, p<0.001) and body weight (r = 0.63, p<0.01; r = 0.55, p<0.01).

A General Linear Model (GLM) was employed to adjust for the effects of covariates and to identify the best combination of explanatory variables for mass of circulating apo-LDL. Synthesis of apo-LDL in pool B was the most important predictor explaining about 50% of the variation, followed by the FCR from pool A (18%) and the synthesis of pool A (16%).

5.7 Discussion

The results of this kinetic study were analysed first by the standard curve peeling approach of Matthews (1957) used previously for LDL turnovers (Langer *et al*, 1972; Packard *et al*, 1976). This showed that the rise in apo-LDL was associated with a fall in its catabolic rate. However the urine data indicated that a more complex phenomenon was occurring in these subjects. The curves that were observed reflect the earlier findings of Boston *et al* (1982), who first postulated, on the basis of a falling urine/plasma ratio over time that LDL was metabolically heterogeneous. Matthews analysis fails in this situation since its basic assumption is that the kinetics describe clearance of a homogeneous tracer by a single catabolic mechanism. In the face of tracer heterogeneity, the Matthews procedure cannot identify whether the activity of the removal mechanism is altered or the relative subcomponents of the compartment are changed.

The minimal model required to satisfy both the urine and plasma radioactivity curves necessitated the presence of two circulating pools of apo-LDL with differing kinetic characteristics. Foster *el al* (1986) described a number of two-compartment models that were all capable of accommodating urine and plasma radioactivity data. One of these (Model B in Foster *et al*, 1986) was adopted for this study because it was compatible with the LDL subsystem in a much larger model developed in the Department of Pathological Biochemistry, Glasgow Royal Infirmary (Packard *et al*, 1984; Demant *et al*, 1991, Gaw *et al* 1995) during investigations of apoB transport through the VLDL-IDL-LDL delipidation cascade. In those studies, the presence of parallel pathways of LDL transport and catabolism was demonstrated. Small VLDL (S_f 20-60) gave rise to a species of LDL that was cleared rapidly, with an FCR of 0.3-0.5 pools/day, similar to the rate seen in pool A. Large VLDL (S_f 60-400) on the other hand, when lipolysed, produced LDL that was removed more slowly with an FCR of about 0.2 pool/day, equivalent to the rate seen for pool B material. An alternative, such as model C of Foster *et al* (1986), in which pool A is converted to pool B by lipolysis would increase pool B formation as lipolysis increases. The model employed in this study has certain features that appear to have physiological significance. First,the FCR of pool A (fig.5.3) is a key variable in controlling the rate of LDL catabolism. Second, overall LDL clearance is also influenced by the distribution of mass between pools A and B. This in turn is dependent on their relative synthesis rates and possibly on the nature of the VLDL precursor made by the liver. Third, the FCR of pool B is constant. This is a reflection of the observation that parallel terminal exponentials appear after 8-10 days in both the plasma and urine radioactivity decay curves which give a U/P ratio of about 0.2 pool/day. Clearance of pool B material in our model is by both receptor-mediated and receptor-independent pathways although Malmendier *et al* (1989) restricted this to the receptor-independent pathway. This is consistent with in vitro observations of Kleinman *et al* (1987), Nigon *et al*, (1991) and Campos *et al*, (1996) that LDL from virtually all subjects has the ablilty to react with LDL receptors, but the affinity varies depending on the plasma triglyceride level and the density of the LDL subfraction.

It is concluded that in these young normolipidaemic individuals as the plasma triglyceride rose from 0.82 to 1.54 mmol/l, the mass of the apo-LDL circulating pool doubled principally due to an increase in pool B synthesis. The basis of this kinetic heterogeneity in LDL is unknown but is linked to the origin of the particle and is not due to the apoE

phenotypes of the subjects studied. It is likely that the metabolic heterogeneity observed is linked to underlying structural heterogeneity as previous chapters (3 and 4) have shown that LDL structure is strongly influenced by plasma triglyceride level. Furthermore there is increasing evidence (Chen *et al*, 1994, Galeano *et al*, 1994) that the binding affinity of LDL to the LDL receptor is reduced with increasing plasma triglyceride and density of LDL such that the LDL becomes smaller in size and is due to conformational changes in the apo B100. Further kinetic studies on the individual LDL subfractions are required to test this hypothesis.

Appendix to Chapter5
Table 1 Individual Lipid and Lipoprotein Levels in Normal Subjects

Subject	*Cholesterol*	*Triglyceride*	*VLDL chol mmol/l*	*LDL chol*	*HDL chol*
N 1	4.8	1.0	0.6	2.7	1.5
N 2	4.1	0.6	0.2	2.3	1.6
N 3	4.1	0.7	0.5	2.6	1.1
N 4	4.0	0.5	0.3	2.2	1.5
N 5	5.3	1.3	0.3	3.0	2.0
N 6	4.6	1.1	0.2	2.5	1.9
N 7	4.4	1.0	0.2	2.3	1.9
N 8	5.2	1.5	0.6	3.0	1.6
N 9	3.9	0.7	0.2	2.3	1.4
N 10	4.7	1.4	0.6	2.6	1.5
N 11	5.2	0.9	0.4	3.7	1.1
N 12	5.1	1.0	0.4	2.9	1.8
N 13	3.8	1.1	0.5	2.1	1.2
N 14	4.0	1.3	0.5	2.0	1.5
N 15	5.4	1.2	0.4	3.4	1.5
N 16	4.9	1.1	0.6	2.8	1.5
N 17	5.8	1.1	0.7	3.5	1.6
N 18	4.7	1.3	0.7	2.7	1.4
N 19	4.7	1.1	0.3	3.1	1.3
N 20	6.6	1.5	0.8	3.1	2.1
N 21	5.9	1.8	0.8	3.5	1.6
N 22	5.3	0.9	0.4	3.5	1.4
N 23	6.1	1.4	0.6	3.7	1.8
N 24	6.1	1.7	0.7	4.2	1.4
N 25	6.1	1.9	0.7	4.0	1.3

Appendix to Chapter 5
Table2 Individual Apo-LDL and HDL Subfraction Concentrations in Normal Subjects

Subject	Apo B	HDL_2 mg/100ml	HDL_3
N 1	63		
N 2	63	70	149
N 3	73	21	267
N 4	67	66	194
N 5	83	103	274
N 6	76	128	199
N 7	71	44	228
N 8	88	61	262
N 9	95	-	-
N 10	84	85	229
N 11	94	38	128
N 12	86	42	205
N 13	77	18	193
N 14	72	48	184
N 15	107	40	201
N 16	99	103	189
N 17	91	81	178
N 18	89	55	267
N 19	97	37	293
N 20	95	128	188
N 21	82	81	173
N 22	100	42	243
N 23	108	77	266
N 24	115	68	158
N 25	135	37	188

Appendix to Chapter 5
Table 3 Lipoprotein Composition of Apo-LDL in Normal Subjects

Subject	%Protein	%Free cholesterol	%Cholesteryl ester	%Triglyceride	%Phospholipid
N 1					
N 2	25.1	14.3	34.5	6.0	20.0
N 3	27.3	11.5	30.6	9.7	20.9
N 4	30.0	12.3	29.7	7.1	20.5
N 5	22.3	10.6	34.6	7.6	24.9
N 6	26.0	14.4	31.2	8.8	19.5
N 7	25.5	11.9	34.8	5.0	22.8
N 8	24.4	9.6	37.4	8.4	20.1
N 9	-	-	-	-	-
N 10	25.4	9.5	35.1	7.0	23.1
N 11	20.9	10.4	36.2	7.0	25.5
N 12	24.3	10.7	35.4	8.1	21.6
N 13	27.2	8.9	33.0	10.0	20.9
N 14	27.0	8.6	33.7	9.0	21.8
N 15	25.3	10.2	35.1	6.4	23.0
N 16	26.7	9.4	33.4	10.5	20.1
N 17	22.4	11.8	39.3	6.0	20.7
N 18	26.3	9.3	35.8	7.4	21.1
N 19	26.4	10.7	36.6	5.7	21.2
N 20	26.4	11.4	36.0	6.3	19.7
N 21	23.2	13.3	36.3	6.8	20.5
N 22	24.8	10.9	35.9	7.3	21.1
N 23	23.8	9.8	36.4	8.3	21.6
N 24	25.5	11.6	34.2	7.7	21.0
N 25	26.7	9.3	36.0	7.9	20.1

Appendix to Chapter 5
Table 4 Kinetic Parameters by Matthews Analysis in Normal Subjects

Subject	Apo-LDL mg	FCR pools/d	RI FCR pools/d	RM FCR pools/d	Synthetic Rate mg/kg/d	RI ACR mg/kg/d	RM ACR mg/kg/d
N 1	1414	0.41	0.20	0.21	10.4	5.1	5.3
N 2	1638	0.42	0.25	0.17	10.3	6.2	4.3
N 3	1664	0.47	0.21	0.26	13.7	7.6	9.5
N 4	1814	0.48	0.20	0.28	13.0	5.4	7.6
N 5	1826	0.45	0.18	0.27	14.8	5.8	9.0
N 6	1839	0.43	0.26	0.16	12.9	7.9	5.0
N 7	1846	0.34	0.20	0.14	9.6	5.6	4.0
N 8	2051	0.35	0.18	0.17	12.3	6.4	5.9
N 9	2090	0.43	0.26	0.17	16.6	10.6	6.6
N 10	2352	0.36	0.19	0.16	12.0	6.5	5.5
N 11	2406	0.31	0.16	0.14	11.5	6.2	5.4
N 12	2422	0.34	0.16	0.17	11.6	5.6	5.9
N 13	2433	0.40	0.23	0.17	12.2	6.9	5.3
N 14	2556	0.32	0.16	0.16	9.1	4.6	4.5
N 15	2568	0.41	0.22	0.19	17.3	9.2	8.1
N 16	2577	0.33	0.17	0.15	12.9	6.9	6.0
N 17	2584	0.35	0.16	0.19	12.6	5.6	7.0
N 18	2599	0.40	0.22	0.18	14.3	7.7	6.6
N 19	2677	0.28	0.26	0.02	10.9	10.2	0.6
N 20	2765	0.39	0.21	0.18	14.9	8.1	6.8
N 21	2870	0.32	0.13	0.19	10.3	6.1	4.2
N 22	2920	0.31	0.14	0.17	12.3	5.6	6.7
N 23	3024	0.37	0.22	0.15	16.0	9.6	6.4
N 24	3830	0.32	0.16	0.15	14.5	7.5	7.0
N 25	4212	0.26	0.20	0.06	14.0	10.8	3.2

FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL; synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg; ACR is the absolute catabolic rate and is the product of either receptor independent or the receptor mediated FCR and the apo-LDL pool size divided by the body weight. Under steady state conditions synthetic rate and catabolic rate should be equal.

Appendix to Chapter 5
Table 5 Kinetic Parameters of Mathematical Pool A in Normal Subjects

		Pool A	Apo-LDL		
Subject	*Mass* mg	*Synthesis* mg/d	*FCR* pools/d	*RI FCR* pools/d	*RM FCR* pools/d
N 1	795	421	0.53	0.29	0.24
N 2	1121	549	0.49	0.28	0.21
N 3	1156	630	0.55	0.25	0.30
N 4	1073	690	0.64	0.29	0.35
N 5	1143	707	0.62	0.21	0.41
N 6	1026	630	0.61	0.37	0.24
N 7	616	891	0.69	0.30	0.39
N 8	1115	725	0.65	0.26	0.39
N 9	1029	740	0.72	0.38	0.34
N 10	895	691	0.77	0.38	0.39
N 11	1152	555	0.48	0.29	0.19
N 12	1579	839	0.53	0.26	0.27
N 13	1825	1095	0.60	0.35	0.25
N 14	1278	655	0.51	0.20	0.31
N 15	1930	1041	0.54	0.30	0.24
N 16	918	519	0.57	0.20	0.37
N 17	1594	698	0.44	0.20	0.24
N 18	1719	1036	0.60	0.31	0.29
N 19	1614	598	0.37	0.21	0.16
N 20	1158	551	0.48	0.22	0.26
N 21	1183	473	0.40	0.27	0.13
N 22	1284	627	0.49	0.16	0.33
N 23	1536	956	0.62	0.36	0.26
N 24	1241	658	0.53	0.27	0.26
N 25	2211	482	0.22	0.15	0.07

FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL; synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg.

Appendix to Chapter 5
Table 6 Kinetic Parameters of Mathematical Pool B in Normal Subjects

		Pool B	Apo-LDL		
Subject	*Mass* *mg*	*Synthesis* *mg/d*	*FCR* *pools/d*	*RI FCR* *pools/d*	*RM FCR* *pools/d*
N 1	619	152	0.25	0.15	0.10
N 2	517	119	0.23	0.13	0.10
N 3	508	147	0.29	0.12	0.17
N 4	740	188	0.25	0.14	0.11
N 5	168	168	0.25	0.12	0.13
N 6	813	153	0.19	0.11	0.08
N 7	953	226	0.24	0.14	0.10
N 8	936	219	0.23	0.12	0.11
N 9	1061	265	0.25	0.17	0.08
N 10	1457	378	0.26	0.13	0.13
N 11	1254	281	0.23	0.10	0.13
N 12	843	169	0.20	0.07	0.13
N 13	608	128	0.21	0.08	0.13
N 14	1278	290	0.23	0.12	0.11
N 15	639	125	0.20	0.08	0.12
N 16	1656	438	0.26	0.16	0.10
N 17	990	204	0.21	0.09	0.12
N 18	880	216	0.25	0.12	0.13
N 19	1063	200	0.19	0.13	0.06
N 20	1607	397	0.25	0.16	0.09
N 21	1687	369	0.22	0.10	0.12
N 22	1636	391	0.24	0.12	0.12
N 23	1488	393	0.26	0.15	0.11
N 24	2589	673	0.26	0.10	0.16
N 25	2000	631	0.32	0.24	0.08

FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL; synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg.

Chapter 6 Fibrates and LDL Metabolism in Moderate Hypercholesterolaemia

6.1 Introduction

The fibrates are a family of hypolipidaemic drugs that lower plasma triglycerides by promoting the lipolysis of triglyceride-rich particles through activation of lipoprotein lipase (Wolfe *et al*, 1983) by stimulating VLDL catabolism and by the inhibition of VLDL synthesis and secretion as a result of decreased free fatty acid flux to the liver (Kissebah *et al*, 1974). In addition it has been suggested that they are able to supress hepatic cholesterol synthesis (Bernt & Gaumert, 1978) and stimulate LDL receptor activity (Stewart *et al*, 1982). The effect fibrates have on LDL varies and depends on the initial plasma triglyceride level. In severely hypertriglyceridaemic subjects, LDL particles are small and dense, and the low level of LDL in this condition is due to hypercatabolism of this species probably by receptor-independent pathways (Shepherd & Packard, 1986: Sigurdsson *et al*, 1976). Shepherd *et al* (1985) found previously that fibrate treatment supressed this high rate of clearance, and we attributed this effect to a reduction in the amount of LDL degraded by the receptor-independent pathway, whereas LDL catabolism into the receptor route was increased. The overall impact of these changes was to increase the initially low levels of plasma LDL cholesterol, alter LDL composition, and increase the average particle size. Conversely in hypercholesterolaemic, normotriglyceridaemic subjects, initially elevated LDL cholesterol levels are decreased by fibrate therapy. In a previous investigation of the mechanism of action of bezafibrate (Stewart *et al*, 1982) in type II hyperlipidaemia, the LDL lowering effect was ascribed to increased clearance via the LDL receptor pathway. At that time it was surmised that the drug operated to supress hepatic cholesterol synthesis and so activate clearance of LDL by receptors. However in the light of the present knowledge of the metabolic and structural heterogeneity of LDL, it is pertinent to ask whether it was the receptor or the ligand itself that changed during fibrate therapy. It is arguable that the drug, by changing LDL composition and size, increased the propensity of the particles for degradation by the receptor mechanism. In the present study second generation fibrates (fenofibrate and ciprofibrate) were used to treat two groups of subjects with primary hypercholesterolaemia and normotriglyceridaemia. The study was designed to test this hypothesis examining in detail the receptor-mediated and receptor-independent pathways of apo-LDL metabolism and also assessing the kinetic heterogeneity of LDL using the two-pool model of LDL metabolism proposed for normolipaemic individuals in chapter 5.

Identical protocols with the same subject inclusion criteria were used for each of the drugs, however as there was an eighteen month time difference in administration and as there may be subtle differences in the mechanism of action, the results for each drug will be described separately. FF refers to the fenofibrate study and CiproI refers to the ciprofibrate study.

6.2 Protocol

The study was carried out in three phases. The first was a three month preliminary screening period in which a standard cholesterol-reducing diet was advised and each patient was screened for cardiological, haematological, hepatic, endocrine, renal and metabolic disease by routine clinical laboratory testing. The baseline phase lasted for two weeks in which the subjects underwent measurements of lipids, lipoproteins and lipoprotein subfractions and determination of native and 1,2-cyclohexanedione-treated, radiolabelled LDL turnover. The LDL was prepared by zonal ultracentrifugation and labelled as described in chapter 2.6.1. The active treatment period followed immediately after the baseline measurement of apo-LDL metabolism. One group of patients was commenced on fenofibrate therapy at a dose of 100 mg t.i.d and the other on ciprofibrate at a dose of 100 mg/d. Patients remained on this dose for 8 weeks, the final 2 weeks of which each patient underwent a second turnover investigation with lipid, lipoprotein and lipoprotein subfraction measurements.

6.3 Recruitment and Conduct of the Study.

Recruitment took place at Lipid Clinics at Glasgow Royal Infirmary by Dr A Gaw, Victoria Infirmary, Glasgow by Dr D Wosornu and at Hairmyres Hospital, East Kilbride by Dr B Vallance. Dr Gaw was responsible for the clinical aspects of the study. The patients were visited at home each day for venepuncture by nurses namely Moira Devine and Grace Lindsay, whose contribution by encourageing the patients to adhere to the protocol was invaluable.

6.4 Subjects

All subjects showed persistent type IIa hyperlipidaemia with initial fasting plasma cholesterol > 7mol/l and triglyceride < 2.3 mmol/l. They did not have clinical evidence of familial hypercholesterolaemia (FH) ie no history of premature heart disease in first degree relatives or the presence of tendon xanthomas on clinical examination. None of the subjects was receiving drug therapy known to affect lipoprotein metabolism, including hormone replacement therapy. The women were all post-menopausal. The characteristics of the subjects are shown in tables 6.1 and 6.2.

Table 6.1 Fenofibrate Study Patient Characteristics

Subject	*Sex*	*Age*	*Weight kg*	*Apo E phenotype*
FF 1	M	63	77.5	3/3
FF 2	F	64	60.0	3/3
FF 3	F	66	68.0	3/3
FF 4	F	65	59.5	3/3
FF 5	F	53	78.5	3/3
FF 6	F	63	64.5	3/4
FF 7	F	48	58.0	2/3

Table 6.2 Ciprofibrate Study Patient Characteristics

Subject	Sex	Age	BMI kg/m^2	Apo E phenotype
Ciprol 1	M	37	24.1	3/3
Ciprol 2	M	58	24.4	3/3
Ciprol 3	F	62	25.0	4/3
Ciprol 4	F	53	27.9	4/3
Ciprol 5	F	61	23.8	3/3
Ciprol 6	M	46	26.2	3/3
Ciprol 7	M	63	25.8	3/3
Ciprol 8	M	63	23.0	3/3
Ciprol 9	M	40	27.8	3/3
Ciprol 10	M	68	23.4	3/3

6.5 Fenofibrate

Eight subjects, one man and seven post menopausal women, aged from 48 to 66 years took part in the study.

6.5.1 Lipids and Lipoproteins and Apoproteins

Plasma lipid, lipoprotein and apolipoprotein levels are shown in tables 6.3 and 6.4. All were hypercholesterolaemic due to an elevation in LDL and had normal levels of plasma triglyceride and VLDL cholesterol. Treatment with fenofibrate significantly lowered plasma cholesterol and triglyceride levels by 29% and 35% respectively, ($p<0.001$). The fall in cholesterol content of VLDL paralleled that of plasma triglycerides (36%; $p<0.01$) and there was no change in the VLDL cholesterol to plasma triglyceride ratio (0.39) before and during therapy. Fenofibrate induced a decrease in LDL cholesterol from 6.0 (0.39) to 3.86 (0.25) mmol/l; $p<0.001$), whereas plasma apoB fell by 33% ($p<0.001$). There were no changes in the concentrations of HDL cholesterol, HDL_2, HDL_3 or apo AI. Fenofibrate therapy for 8 weeks also produced no significant changes in Lp(a) levels (86(22), range 11-141 mg/100ml baseline; 91(18), range 12-140 mg/100ml on fenofibrate).

Table 6.3 Lipids and Lipoproteins of Subjects on Fenofibrate Therapy

Subject	*Cholesterol*	*Triglyceride*	*VLDL chol mmol/l*	*LDL chol*	*HDL chol*
Baseline					
FF 1	8.00	1.74	0.75	5.70	1.55
FF 2	8.20	1.30	0.35	6.45	1.38
FF 3	7.89	2.10	0.79	5.86	1.24
FF 4	6.33	1.63	0.67	4.28	1.38
FF 5	8.50	1.59	0.61	6.58	1.28
FF 6	9.50	1.20	0.30	7.60	1.62
FF 7	7.67	1.88	1.04	5.40	1.20
mean	**8.01**	**1.63**	**0.64**	**6.00**	**1.38**
(SEM)	**(0.39)**	**(0.12)**	**(0.10)**	**(0.39)**	**(0.06)**
Fenofibrate					
FF 1	6.04	0.91	0.45	3.89	1.70
FF 2	5.30	0.89	0.29	3.59	1.40
FF 3	5.54	1.19	0.45	3.71	1.38
FF 4	5.33	1.10	0.31	3.62	1.39
FF 5	5.60	0.96	0.34	3.50	1.73
FF 6	7.26	0.88	0.34	5.34	1.59
FF 7	5.26	1.51	0.66	3.39	1.21
mean	**5.71**	**1.06**	**0.41**	**3.86**	**1.49**
(SEM)	**(0.27)**	**(0.09)**	**(0.09)**	**(0.25)**	**(0.07)**
p	**<0.001**	**<0.001**	**<0.01**	**<0.001**	**NS**

Table 6.4 Apoproteins and HDL Subfractions of Subjects on Fenofibrate Therapy

Subject	Apo AI	Apo B	Lp(a) mg/100ml	HDL_2	HDL_3
Baseline					
FF 1	132	129	11	47	222
FF 2	123	142	109	46	294
FF 3	130	158	141	37	286
FF 4	134	126	34	62	256
FF 5	118	161	140	92	228
FF 6	138	143	140	144	181
FF 7	146	119	28	30	281
mean	**132**	**140**	**86**	**65**	**250**
(SEM)	**(3.5)**	**(6)**	**(22)**	**(15)**	**(15.6)**
Fenofibrate					
FF 1	150	109	12	47	358
FF 2	126	88	103	37	330
FF 3	128	101	135	24	273
FF 4	111	83	67	66	291
FF 5	158	89	130	37	322
FF 6	119	102	140	106	222
FF 7	109	87	49	46	261
mean	**129**	**94**	**91**	**52**	**294**
(SEM)	**(7.1)**	**(3.7)**	**(18)**	**(10.2)**	**(17.5)**
p	**NS**	**<0.001**	**NS**	**NS**	**NS**

Analysis of the composition of LDL (table 6.5) showed no effect of fibrate therapy, indicating that the reduction in LDL cholesterol was due to a diminished number of particles in the circulation.

Table 6.5 LDL Composition in Subjects on Fenofibrate Therapy

Subject	% Protein	% Free cholesterol	%Cholesteryl ester	%Triglyceride	%Phospholipid
Baseline					
mean	25.06	11.89	34.66	6.50	21.90
(SEM)	(0.82)	(0.47)	(1.00)	(0.47)	(0.28)
Fenofibrate					
mean	25.96	12.00	33.00	6.92	22.10
(SEM)	(0.33)	(0.56)	(0.88)	(0.34)	(0.40)
p	**NS**	**NS**	**NS**	**NS**	**NS**

There was some inter-individual variation in the LDL pattern observed with subject FF3 having 152 mg/100ml of small dense LDL III as well as the highest triglyceride level of 2.1 mmol/l. Table 6.6 shows that the decrement in mass of LDL lipoproteins (30 %, $p<0.005$) was largely due to reduction of the major LDL II species (42 %, $p<0.001$). The largest most buoyant LDL I was unaltered, while the mean level of small dense LDL III fell by 46 %. This was not significant but there was a trend in reduction of LDL III which was particularly noticeable in subjects FF 3 and FF 4 where values fell from 152 to 46mg/100ml and from 108 to 52 mg/100ml respectively.

Table 6.6 LDL Subfractions on Fenofibrate Therapy

Subject	*LDL mass*	*LDL I mg/100ml*	*LDL II*	*LDL III*
Baseline				
FF 1	546	110	365	71
FF 2	374	80	248	46
FF 3	569	58	359	152
FF 4	501	70	323	108
FF 5	594	236	320	38
FF 6	651	385	233	33
FF 7	424	150	254	20
mean	**523**	**156**	**300**	**67**
(SEM)	**(37)**	**(45)**	**(21)**	**(18)**
Fenofibrate				
FF 1	374	153	174	20
FF 2	322	94	186	42
FF 3	374	101	227	46
FF 4	417	144	221	52
FF 5	382	175	162	45
FF 6	405	221	148	36
FF 7	275	163	98	14
mean	**364**	**150**	**174**	**37**
(SEM)	**(19)**	**(17)**	**(17)**	**(19)**
p	**<0.005**	**NS**	**<0.001**	**NS**

6.5.2 Receptor-mediated and Receptor-independent Metabolism of LDL

The influence of fenofibrate therapy on apo-LDL metabolism was examined at baseline and on drug using dual-tracer, native and cyclohexanedione modified apo-LDL turnover studies as described in section 2.6. The kinetic parameters of apo-LDL metabolism derived from simple Matthews kinetic analysis are presented in table 6.7.

Table 6.7 Matthew's Kinetic Parameters of Apo-LDL on Fenofibrate Therapy

Subject	Apo-LDL mg	FCR pools/d	RI FCR pools/d	RM FCR pools/d	Synthetic Rate mg/kg/d	RI ACR mg/kg/d	RM ACR mg/kg/d
Baseline							
FF 1	4092	0.239	0.100	0.139	978	410	568
FF 2	3696	0.248	0.127	0.121	916	469	447
FF 3	3993	0.242	0.145	0.097	966	579	387
FF 4	2332	0.319	0.144	0.175	744	336	408
FF 5	2662	0.239	0.109	0.130	636	290	346
FF 6	3431	0.204	0.114	0.090	670	361	309
FF 7	2747	0.295	0.143	0.152	810	392	418
mean	**3279**	**0.255**	**0.126**	**0.128**	**817**	**420**	**412**
(SEM)	**(264)**	**(0.015)**	**(0.019)**	**(0.011)**	**(53)**	**(42)**	**(31)**
Fenofibrate							
FF 1	2790	0.444	0.140	0.304	1239	391	848
FF 2	2108	0.379	0.168	0.211	799	349	450
FF 3	2439	0.524	0.185	0.339	1278	451	827
FF 4	2142	0.508	0.180	0.328	1088	385	703
FF 5	1995	0.361	0.148	0.213	720	295	425
FF 6	3149	0.292	0.147	0.145	920	463	457
FF 7	1562	0.448	0.154	0.294	700	241	459
mean	**2312**	**0.422**	**0.160**	**0.263**	**963**	**368**	**596**
(SEM)	**(200)**	**(0.031)**	**(0.018)**	**(0.028)**	**(91)**	**(30)**	**(72)**
p	**<0.005**	**<0.0005**	**<0.001**	**<0.002**	**NS**	**NS**	**<0.05**

FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL; synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg; ACR is the absolute catabolic rate and is the product of either receptor independent or the receptor mediated FCR and the apo-LDL pool size divided by the body weight. Under steady state conditions synthetic rate and catabolic rate should be equal.

Fenofibrate therapy was associated with a 65% increase in the fractional catabolic rate of apo-LDL. This change was apparently responsible for the 30% decrement in the concentration of LDL in plasma, as fenofibrate did not significantly affect the total apo-LDL synthesis. When LDL catabolism was divided into receptor-mediated versus receptor-independent pathways, it was observed that the fractional catabolic rate of the former was increased by 105%, 0.263(0.028) pools/d on fenofibrate versus 0.128 (0.011) before drug; $p<0.002$ and the latter by 27%, 0.160 (0.018) pools/d on fenofibrate versus 0.126(0.019) pools/d before drug; $p<0.001$. Furthermore, the amount of apo-LDL degraded

by the receptor route rose by 45% ($p<0.05$), whereas that removed by the receptor-independent pathway was unchanged.

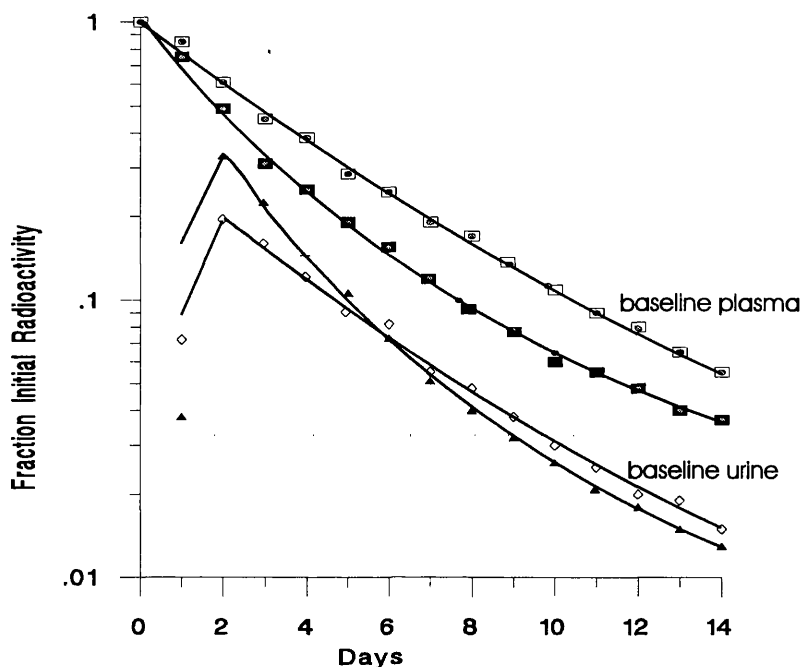

Fig 6.1 Plasma and Urine Radiactive Decay Curves at Baseline and on Fenofibrate

Plasma and urine radioactive decay curves for subject FF2 at baseline (upper curves) and on fenofibrate.

Visual examination of the plasma decay curves in conjunction with the daily urinary radioactivity excretion rates, shown for subject FF 2 in fig 6.1, revealed that fenofibrate perturbed LDL metabolism in a more complex fashion than that suggested by Matthew's analysis of the plasma data only. During the control turnover, the amount of radioactivity excreted in the urine each day was a relatively constant proportion of the radioactivity present in plasma. The calculated urine : plasma ratio (fig 6.2), a daily index of catabolic potential, fell only slightly from a peak value of 0.34 at 2-4 days after injection to 0.26 by days 10-12. On fenofibrate, however, the relation between the urine and plasma decay curves was altered. During the first 5 days of the turnover, the plasma curve showed more rapid decay than during the control phase. This change was due to a catabolic event rather than enhanced extravascular exchange because there was a concominant increase in urinary radioactivity excretion. The urine : plasma ratio ratio in this patient was initially high

(0.60), and showed a substantial fall during the turnover period to approximately the same value as the control phase. As in chapter 5, this is evidence that LDL was not behaving in a kinetically homogeneous fashion.

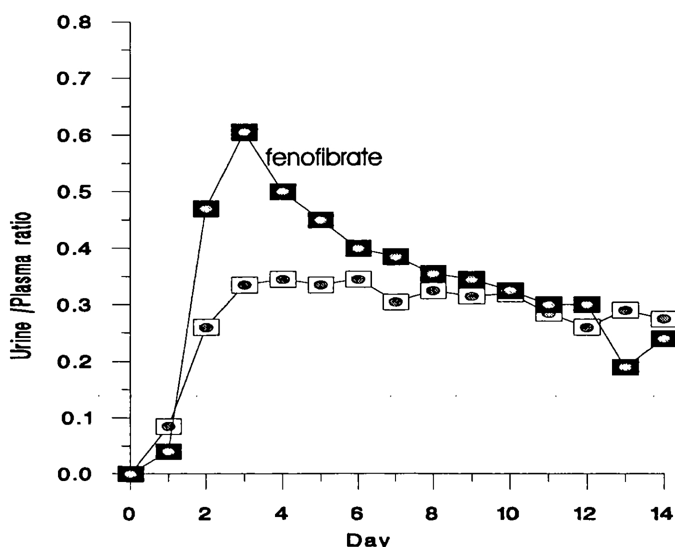

Fig. 6.2 Urine to Plasma Radioactivity Ratios at Baseline and on Fenofibrate
Ratio of radioactivity in native LDL for subject FF 2 at baseline (lower) and on fenofibrate (upper).

6.5.3 Two Compartment Model of Apo B Metabolism

The effects of fenofibrate on apo-LDL metabolism were further studied by subjecting the plasma and urine radioactivities to more detailed analysis using the multicompartmental model which divides plasma LDL into 2 compartments (A and B) that are permitted to differ in their elimination rates as is described in Chapter 5.

The kinetic behaviour of pools A and B in the hypercholesteraemic subjects at baseline and on fenofibrate therapy are shown in table 6.8. In the control situation, pool A accounted for 1,200 mg, or approximately one third, of the circulating mass of apo-LDL. Its mean synthetic rate was 400 mg/day with a fractional catabolic rate of 0.39 pools/day. The majority of apo-LDL was in pool B which had a mean mass of approximately 2,000 mg. Its fractional catabolic rate was half of that of pool A (0.2 pools/day), but the rate of production was similar (422 mg/day). Fenofibrate significantly increased the synthetic rate of pool A by 77% ($p<0.005$) and at the same time significantly decreased the synthetic rate of pool B by 39% ($p<0.02\%$). The overall result was no difference in the transport of total apo-LDL in agreement with Matthew's analysis shown in table 6.7. The fractional clearance rate of pool B was not altered, whereas that of pool A rose by 69% on fenofibrate. The net effect of these changes was that while the computed mass of apo-LDL

in pool A remained constant, that of pool B was reduced by approximately 50%. The effect on LDL overall FCR observed in Matthews analysis has been shown to be the switch from pool B to A in this model.

Table 6.8 Kinetics of Pools A and B Apo-LDL on Fenofibrate Therapy

Pool A apo-LDL				*Pool B apo-LDL*		
Subject	*Mass mg*	*FCR pools/d*	*SR mg/d*	*Mass mg*	*FCR pools/d*	*SR mg/d*
Baseline						
FF 1	2471	0.26	642	1621	0.203	329
FF 2	2064	0.29	595	1632	0.200	321
FF 3	1525	0.30	458	2468	0.210	518
FF 4	583	0.67	391	1749	0.200	353
FF 5	222	0.47	104	2440	0.220	532
FF 6	695	0.28	197	2736	0.180	502
FF 7	853	0.49	415	1894	0.210	396
mean	**1202**	**0.39**	**400**	**2077**	**0.200**	**422**
(SEM)	**(315)**	**(0.06)**	**(74)**	**(174)**	**(0.005)**	**(35)**
Fenofibrate						
FF 1	939	0.87	817	1851	0.230	426
FF 2	1441	0.47	672	667	0.190	126
FF 3	938	0.98	919	1501	0.240	360
FF 4	1254	0.69	863	890	0.250	226
FF 5	916	0.53	482	1079	0.220	238
FF 6	1646	0.39	638	1503	0.190	283
FF 7	795	0.69	550	767	0.195	150
mean	**1133**	**0.66**	**706**	**1180**	**0.220**	**258**
(SEM)	**(120)**	**(0.08)**	**(62)**	**(168)**	**(0.009)**	**(41)**
p	**NS**	**<0.05**	**<0.005**	**<0.005**	**NS**	**<0.02**

FCR-fractional catabolic rate; synthetic rate (SR) is the product of FCR in pools/d and the plasma pool of apo-LDL in mg.

6.6 Ciprofibrate

Ten subjects, 7 men and 3 postmenopausal women, aged from 37 to 69, took part in the study.

6.6.1 Lipids, Lipoproteins and Apolipoptoteins

The plasma lipids, lipoproteins and apolipoproteins are shown in tables 6.9 and 6.10.

Table 6.9 Lipids and Lipoproteins of Subjects on Ciprofibrate Therapy

Subject	*Cholesterol*	*Triglyceride*	*VLDL chol mmol/l*	*LDL chol*	*HDL chol*
Baseline					
Ciprol 1	6.5	1.25	0.64	4.78	1.06
Ciprol 2	7.2	1.10	0.60	4.72	1.72
Ciprol 3	6.8	1.20	0.65	4.41	1.68
Ciprol 4	7.4	1.24	0.51	5.54	1.30
Ciprol 5	7.3	1.40	0.70	5.10	1.55
Ciprol 6	7.5	1.42	0.75	5.50	1.26
Ciprol 7	8.6	1.36	0.81	6.60	1.15
Ciprol 8	6.7	2.00	1.02	4.47	1.17
Ciprol 9	8.9	2.35	0.88	6.06	1.93
Ciprol 10	9.1	1.45	0.69	6.80	1.58
mean	**7.6**	**1.48**	**0.73**	**5.40**	**1.44**
(SEM)	**(0.3)**	**(0.12)**	**(0.05)**	**(0.27)**	**(0.09)**
Ciprofibrate					
Ciprol 1	6.1	0.66	0.40	4.33	1.40
Ciprol 2	5.4	0.68	0.42	2.95	2.00
Ciprol 3	5.1	0.61	0.40	2.89	1.79
Ciprol 4	6.3	0.79	0.36	4.45	1.44
Ciprol 5	6.0	0.69	0.39	3.66	1.93
Ciprol 6	6.1	0.88	0.55	4.04	1.53
Ciprol 7	7.4	1.18	0.55	5.60	1.25
Ciprol 8	5.3	1.10	0.57	3.35	1.38
Ciprol 9	8.0	1.18	0.62	5.52	1.82
Ciprol 10	7.0	1.07	0.27	5.20	1.48
mean	**6.3**	**0.88**	**0.45**	**4.20**	**1.60**
(SEM)	**(0.3)**	**(0.07)**	**(0.04)**	**(0.32)**	**(0.08)**
p	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.02**

Treatment with ciprofibrate significantly affected all parameters of the lipid profile. Plasma cholesterol was reduced by 17% ($p<0.001$). This decrement was due to a 38% fall in VLDL cholesterol ($p<0.001$), and a 22% fall in LDL cholesterol ($p<0.001$). There was also a marked reduction in plasma triglyceride of 41% ($p<0.001$) and a rise in HDL cholesterol of 11% ($p<0.02$). No significant changes were observed in HDL_2, HDL_3 or Lp(a), range 1-100 mg/100ml at baseline and range 8-72 mg/100ml on ciprofibrate.

Table 6.10 Apoproteins and HDL Subfractions of Subjects on Ciprofibrate Therapy

Subject	Lp(a)	HDL_2 mg/100ml	HDL_3
Baseline			
Ciprol 1	36	34	175
Ciprol 2	7	107	392
Ciprol 3	17	158	239
Ciprol 4	51	92	229
Ciprol 5	46	132	229
Ciprol 6	14	81	219
Ciprol 7	48	102	209
Ciprol 8	1	81	290
Ciprol 9	76	134	361
Ciprol 10	100	102	348
mean	40	102	269
(SEM)	(10)	(11)	(23)
Ciprofibrate			
Ciprol 1	35	39	202
Ciprol 2	8	118	335
Ciprol 3	15	166	194
Ciprol 4	62	85	336
Ciprol 5	51	141	153
Ciprol 6	15	72	321
Ciprol 7	43	43	199
Ciprol 8	18	33	314
Ciprol 9	72	81	441
Ciprol 10	67	63	339
mean (SEM)	39	84	283
	(8)	(14)	(29)
p	NS	NS	NS

Analysis of the overall composition of LDL (table 6.11) showed that ciprofibrate therapy had no effect, suggesting that the fall in LDL cholesterol observed was due to a reduction in the number of circulating LDL particles.

Table 6.11 -LDL Composition in Subjects on Ciprofibrate Therapy

Subject	*%Protein*	*%Free cholesterol*	*%Cholesteryl ester*	*%Triglyceride*	*%Phospho-lipid*
Baseline					
mean	26.9	12.4	35.3	5.7	19.8
(SEM)	(0.6)	(0.4)	(0.7)	(0.5)	(0.5)
Ciprofibrate					
mean	25.8	11.6	36.3	6.3	19.8
(SEM)	(0.6)	(0.8)	(1.0)	(0.5)	(0.6)
p	**NS**	**NS**	**NS**	**NS**	**NS**

Results of quantative analysis of the LDL subfraction profiles are in table 6.12. Again there was some inter-individual variation in the patterns, but only one subject (Ciprol 7) had a concentration of LDL III > 100mg/100ml. On ciprofibrate therapy, the decrement (28%, $p<0.01$) in the major LDL II species was the most significant change in these subjects. LDL I, the largest fraction, was unaffected by therapy, while the mean level of small dense LDL III fell by 30%. Again this was not significant.

Table 6.12 LDL Subfractions in Subjects on Ciprofibrate Therapy

Subject	LDL mass	LDL I mg/100ml	LDL II	LDL III
Baseline				
Ciprol 1	410	147	227	36
Ciprol 2	429	141	202	86
Ciprol 3	434	230	181	22
Ciprol 4	536	241	269	27
Ciprol 5	471	252	195	24
Ciprol 6	484	107	328	49
Ciprol 7	598	116	341	141
Ciprol 8	442	97	253	92
Ciprol 9	447	181	223	43
Ciprol 10	618	139	451	28
mean	**487**	**165**	**267**	**55**
(SEM)	**(23)**	**(18)**	**(27)**	**(12)**
Ciprofibrate				
Ciprol 1	448	161	224	63
Ciprol 2	253	114	102	38
Ciprol 3	302	143	130	29
Ciprol 4	428	192	214	22
Ciprol 5	351	224	112	15
Ciprol 6	367	176	164	27
Ciprol 7	493	116	298	79
Ciprol 8	318	108	163	47
Ciprol 9	552	129	257	28
Ciprol 10	473	180	263	29
mean	**399**	**154**	**193**	**38**
(SEM)	**(30)**	**(12)**	**(22)**	**(6)**
p	**<0.02**	**NS**	**<0.01**	**NS**

6.6.2 Receptor-mediated and Receptor-independent Metabolism of LDL

The influence of ciprofibrate therapy on apo-LDL metabolism was examined at baseline and on drug using dual tracers (I^{125}-native and I^{131}-CHD modified LDL). The results from Matthews kinetic modelling are shown in table 6.13. In two turnovers, Ciprol 4 drug and Ciprol 7 baseline, the decay curves for native and CHD-modified LDL were superimposable suggesting that there was either damage to native apo-LDL before reinjection or that modification was incomplete. Examination of the decay curves and calculated kinetic parameters indicated that the former was most probable. These two subjects have been excluded from analysis of the paired data.

Table 6.13 Matthew's Kinetic Parameters of Apo-LDL on Ciprofibrate Therapy

Subject	*Apo-LDL mg/100ml*	*FCR pools/d*	*RI FCR pools/d*	*RM FCR pools/d*	*SR mg/kg/d*	*RI ACR mg/kg/d*	*RM ACR mg/kg/d*
Baseline							
CiproI 1	105	0.41	0.17	0.23	18.4	7.8	10.6
CiproI 2	108	0.30	0.14	0.16	12.8	6.0	6.7
CiproI 3	126	0.33	0.16	0.11	16.7	8.2	8.5
CiproI 4	101	0.21	0.12	0.10	8.6	4.7	3.9
CiproI 5	133	0.42	0.22	0.20	22.0	11.7	10.3
CiproI 6	141	0.35	0.18	0.17	19.8	10.3	9.5
CiproI 7	171	-	0.16	-		10.7	
CiproI 8	121	0.33	0.17	0.16	16.2	8.2	8.0
CiproI 9	110	0.23	0.11	0.12	11.9	5.7	6.1
CiproI 10	160	0.21	0.13	0.09	12.6	7.5	5.1
mean (n=8)	**126**	**0.32**	**0.16**	**0.16**	**16.3**	**8.2**	**8.1**
(SEM)	**(6)**	**(0.03)**	**(0.012)**	**(0.016)**	**(1.3)**	**(0.71)**	**(0.71)**
Ciprofibrate							
CiproI 1	128	0.40	0.19	0.21	20.2	9.6	10.7
CiproI 2	56	0.49	0.15	0.34	10.6	3.2	7.5
CiproI 3	80	0.35	0.14	0.31	11.0	4.5	6.6
CiproI 4	111	-	0.13	-		5.6	
CiproI 5	90	0.34	0.13	0.21	12.0	4.6	7.4
CiproI 6	96	0.34	0.14	0.20	13.0	5.2	7.8
CiproI 7	124	0.31	0.15	0.16	15.3	7.6	7.7
CiproI 8	88	0.46	0.19	0.27	16.4	6.8	9.5
CiproI 9	120	0.37	0.17	0.20	17.9	8.4	9.5
CiproI 10	116	0.30	0.14	0.16	13.5	6.4	7.2
mean (n=8)	**97**	**0.38**	**0.16**	**0.22**	**14.3**	**6.1**	**8.2**
(SEM)	**(8)**	**(0.02)**	**(0.01)**	**(0.02)**	**(1.2)**	**(0.76)**	**(0.51)**
p	**<0.02**	**NS**	**NS**	**<0.03**	**NS**	**NS**	**NS**

FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and chd modified apo-LDL; SR - Synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg; ACR is the absolute catabolic rate and is the product of either receptor independent or the receptor mediated FCR and the apo-LDL pool size divided by the body weight. Under steady state conditions synthetic rate and catabolic rate should be equal.

Ciprofibrate was associated with a 19% increase in the overall FCR of apo-LDL, but this was not significant. However, when LDL catabolism was divided into receptor-mediated and receptor-independent pathways, it was observed that the FCR of the former was increased by 38% ($p<0.03$), while the latter was unaltered. Since the alteration of the FCR

was accompanied by a 23% fall in apo-LDL circulating mass ($p<0.02$) here was no significant change in the absolute amount of apo-LDL cleared by either pathway.

Again examination of the plasma decay curves in conjunction with the daily urinary radioactivity excretion rates (shown for subject Ciprol 6 in fig 6.3 and for Ciprol 8 in fig 6.3) revealed that the drug was perturbing LDL metabolism in a complex fashion. During the baseline turnover, the amount of radioactivity excreted into the urine was virtually a fixed proportion of the radioactivity present. On ciprofibrate, this relationship varied among the patients. In some (eg subject Ciprol 6, fig 6.4) the radioactivity curves were virtually identical in both phases whereas in others (eg subject Ciprol 8, fig 6.4) it was substantially altered. In this subject during the first 5-6 days of the turnover, the plasma showed a more rapid decay than at baseline. The urine : plasma ratio was initially high (0.45) and showed a substantial fall over the first 5-6 days to the same value as in the control phase (0.22).

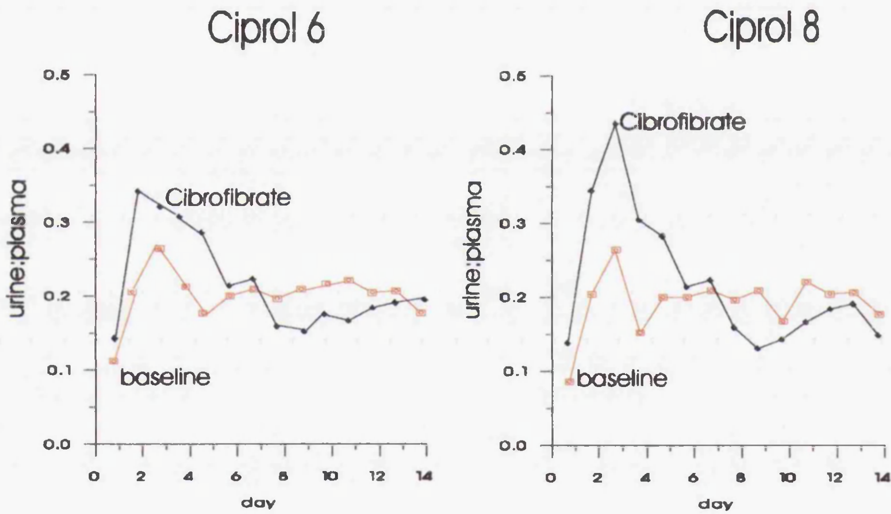

Fig.6.3 Urine to PLasma Radioactivity Ratios For Native LDL at Baseline and on Ciprofibrate Therapy

Urine to plasma ratios for ^{125}I native LDL in subjects Cipro I 6 and Cipro I 8 at baseline (red) and on ciprofibrate (black).

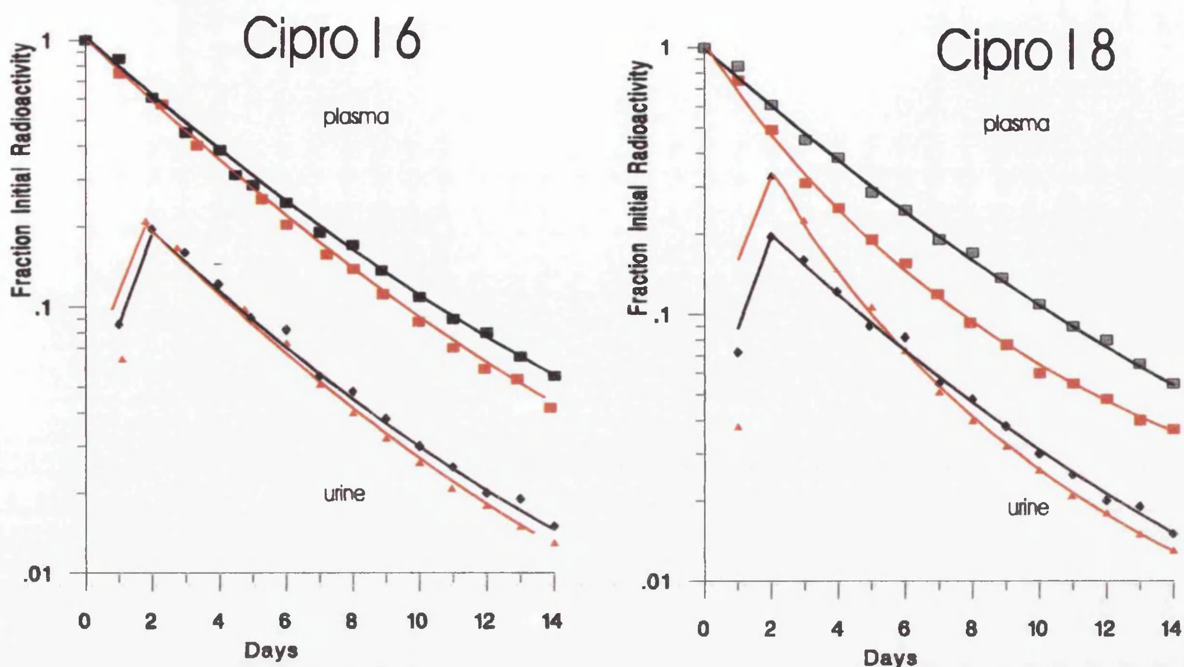

Fig.6.4 Plasma and Urine Radioactivity Decay Curves at Baseline and on Ciprofibrate
Plasma and urine radioactivity decay curves for subjects Cipro I 6 and Cipro I 8 at baseline (red) and on ciprofibrate (black).

6.6.3 Two Compartment Model of Apo B Metabolism
The effects of ciprofibrate on apo-LDL metabolism were further studied by subjecting the plasma and urine radioactivities to multicompartmental analysis using SAAM 30, during which both baseline and drug turnovers were analysed in the same SAAM deck. The results are in table 6.14. Apo-LDL in the more rapidly metabolised component (pool A) accounted for approximately half the circulating mass and had a synthesis rate of about 800 mg/day and an FCR of 0.50 pools/day. Overall the kinetic properties of this metabolic compartment were not affected by treatment with ciprofibrate. However, there were inter-individual differences in response. Those with the highest baseline plasma triglyceride (CiproI 8, CiproI 9) exhibited an increase in the synthesis rate of pool A apo-LDL on drug, while in those with the lowest starting triglyceride (CiproI 2, CiproI 3) this kinetic parameter fell. Pool B apo-LDL, on the other hand, which had a slow FCR of 0.18 pools/day was reduced in all subjects following ciprofibrate therapy. Its mean mass fell from 54% to 42% of the total, principally due to a 38% decrease in its synthesis. The net effect of these perturbances was that in subjects in whom plasma and urine curves were almost identical before and during therapy (eg CiproI 6, fig.6.4) the calculated synthesis

rates of both pools A and B fell, whereas in those in whom the curves changed (eg Ciprol 8, fig. 6.4) there was a shift in synthesis from B to A.

Table 6.14 Kinetic Parameters of Pools A and B apo-LDL on Ciprofibrate Therapy

Pool A apo-LDL				Pool B apo-LDL		
Subject	Mass mg	FCR pools/d	SR mg/d	Mass mg	FCR pools/d	SR mg/d
Baseline						
Ciprol 1	1704	0.60	1026	1752	0.22	378
Ciprol 2	1759	0.41	729	1308	0.15	201
Ciprol 3	1440	0.44	642	1035	0.17	177
Ciprol 5	1673	0.66	1100	1678	0.18	294
Ciprol 6	2698	0.46	1242	1916	0.20	379
Ciprol 8	1033	0.48	498	2267	0.26	580
Ciprol 9	881	0.62	690	2815	0.10	296
Ciprol 10	1571	0.30	460	1790	0.14	255
mean	**1567**	**0.50**	**798**	**1820**	**0.18**	**320**
(SEM)	**(550)**	**(0.01)**	**(289)**	**(548)**	**(0.05)**	**(45)**
Ciprofibrate						
Ciprol 1	2691	0.49	1331	1227	0.18	216
Ciprol 2	674	0.94	633	917	0.16	144
Ciprol 3	1111	0.43	477	443	0.14	63
Ciprol 5	1113	0.51	569	1126	0.17	189
Ciprol 6	1983	0.43	860	1131	0.18	202
Ciprol 8	1547	0.60	922	853	0.20	169
Ciprol 9	2194	0.53	1154	1838	0.18	334
Ciprol 10	1160	0.43	500	1438	0.19	268
mean	**1559**	**0.54**	**806**	**1122**	**0.17**	**198**
(SEM)	**(677)**	**(0.17)**	**(316)**	**(414)**	**(0.018)**	**(29)**
p	**NS**	**NS**	**NS**	**<0.001**	**NS**	**<0.05**

FCR-fractional catabolic rate; synthetic rate (SR) is the product of FCR in pools/d and the plasma pool of apo-LDL in mg.

6.7 Discussion

The results of this study give valuable information not only on the mechanism of action of the second generation fibrates fenofibrate and ciprofibrate but also on the underlying metabolic heterogeneity of LDL.

In the present study, fenofibrate and ciprofibrate produced beneficial perturbations in the levels of all the plasma lipids and lipoproteins. There were reductions in plasma cholesterol (29% FF, 17% Cipro), plasma triglyceride (35% FF, 41% Cipro), VLDL cholesterol (36% FF, 38% Cipro) and LDL cholesterol (36% FF, 22% Cipro) with an increase in HDL cholesterol (8% FF, 11% Cipro). These changes were consistent with and in many cases better than changes previously observed with these and other fibrates. Fibrates are believed to reduce the levels of triglyceride through three main mechanisms, decreased hepatic triglyceride synthesis secondary to diminished free fatty acid flux to the liver (Kissebah *et al*, 1974), suppression of apo CIII synthesis (Staels *et al*, 1995, Haubenwallner *et al*, 1995) leading to a reduction in apo CIII : apo E ratio and so enhancing chylomicron and VLDL clearance and thirdly, increased lipolysis by stimulation of lipoprotein lipase activity (Wolfe *et al*, 1983). Since VLDL and LDL are closely linked in a metabolic cascade it is to be expected that changes in the turnover of the former will have consequences for the latter. However, the perturbances are more subtle than would be expected from a simple precursor-product relationship.

The kinetic results of the present study of apo-LDL turnover in moderate hypercholesterolaemic subjects are concordant with and amplify those reported for other fibrates (Stewart *et al*, 1982; Malmendier *et al*, 1985). In the present study there was a difference in the magnitude of the response of the two fibrates but both increased the catabolic rate of native and receptor-mediated apo-LDL (native: FF 65%, Cipro.19%; receptor-mediated: FF 105%, Cipro 38%). These results are the same as those in an earlier investigation using similar methodology from this laboratory (Stewart *et al*, 1982) that examined the effect of bezafibrate on apo-LDL turnover in type IIa hyperlipidaemic subjects. It was postulated then that the fibrate activated receptors so that the increased clearance of LDL was the result of altered intracellular cholesterol homeostasis in hepatocytes. This metabolic response was similar to that proposed for resins and statins. However in this present study a more detailed kinetic analysis of plasma and urine was employed and it may be concluded that the changes in LDL metabolism were due to a combination of 2 effects - a shift in synthesis, favouring the formation of the rapidly metabolised species (pool A) and an increase in the rate of elimination by the receptor pathway.

In this study there was a decrease in the amount of apo-LDL associated with the slowly metabolised pool B species (FF 2077 to 1180 mg; Cipro 1828 to 1122 mg) due to a fall in its rate of synthesis (FF 422 to 258 mg/d; Cipro 320 to 198 mg/d). Pool A kinetics remained unaltered on ciprofibrate whereas fenofibrate increased both fractional catabolic rate and synthetic rate. There was no change in the elimination rate of pool B. Fig. 6.5 shows the relationship between the kinetic parameters obtained with fenofibrate, ciprofibrate and compared with those obtained from normal subjects with similar plasma triglyceride levels. The apo-LDL masses quoted for normals are approximately 20% lower than those in chapter 5 and Caslake *et al* (1993) as these included a small component due to IDL (d 1.006-1.019 g/ml) which was included in the LDL isolation.

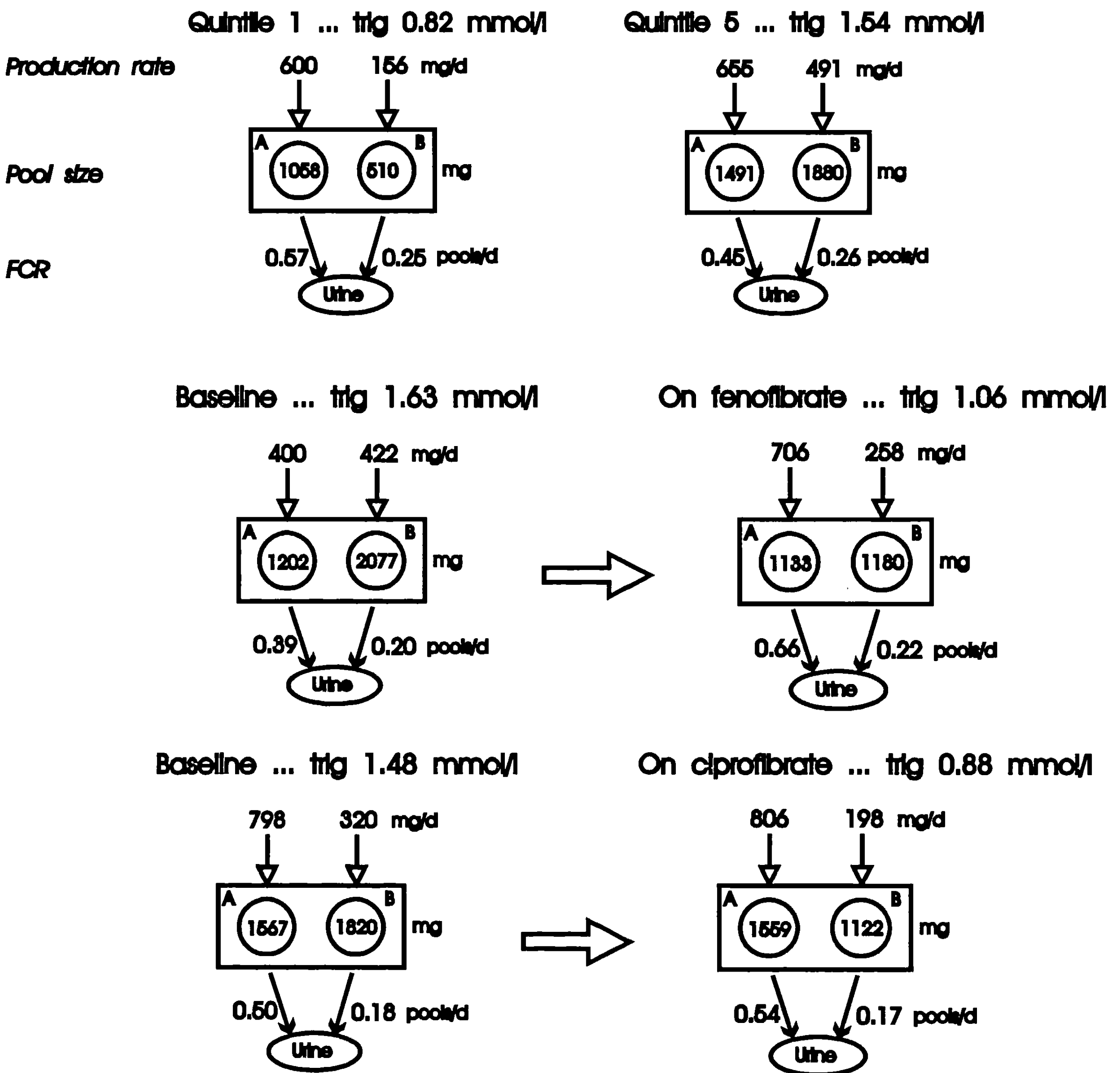

Fig.6.5 Mean LDL Kinetic Parameters in Normal Subjects, Subjects at Baseline and on Fenofibrate and Subjects at Baseline and on Ciprofibrate, at Comparable Plasma Triglyceride Concentrations

This figure shows the mean production rates in mg/d, masses of pool A and B in mg and fractional catabolic rates (FCR) for subjects in quiltiles I and V from chapter 5, subjects at baseline and on fenofibrate therapy and subjects at baseline and on ciprofibrate therapy.

As suggested in chapter 5, the mass of apo-LDL in pool B becomes predominant at higher triglyceride levels. There is a strong relationship ($r = 0.615$, $p<0.0001$) between LDL pool B synthesis and plasma triglyceride. This association (fig 6.6) is consistent across all the subjects so far studied and holds both before and on therapy ($n = 55$).

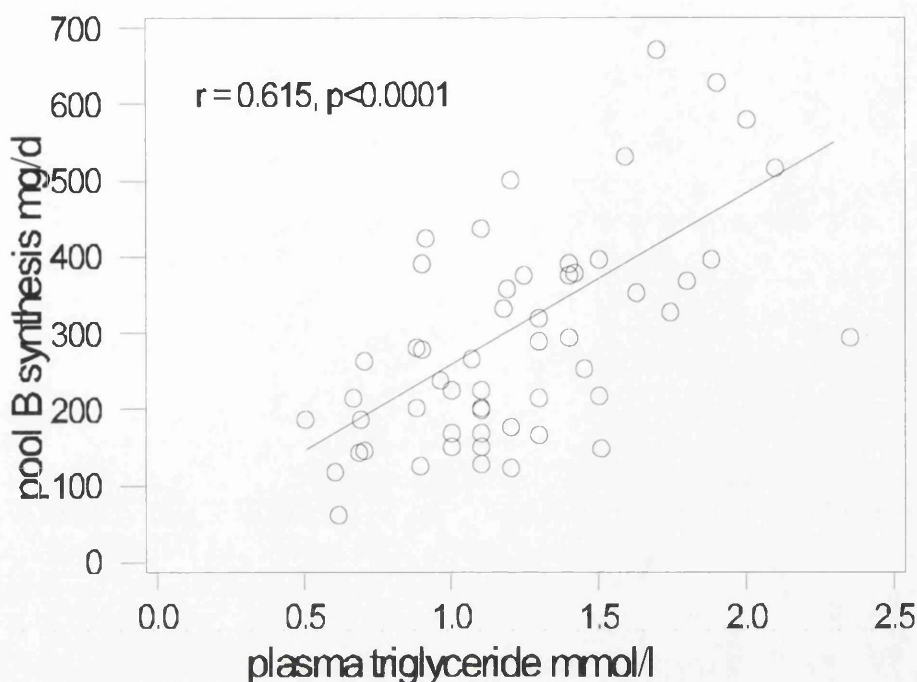

Fig. 6.6 Relationship Between Plasma Triglyceride and Synthesis into Pool B

Pool B synthesis in 25 normal subjects, and 15 hyperlipidaemic subjects at baseline and on fibrate therapy (subjects from chapters 5 and 6). $r = 0.615$, $p<0.0001$

Thus it can be postulated that the main reason for the kinetic alterations observed on treatment with the fibrates is that the LDL particles formed were better ligands for receptors. This occurred because the fibrates caused a reduction in triglyceride levels relative to apoB synthesis in the liver and so promoted the secretion of smaller VLDL precursor particles. Previous studies (Packard *et al*, 1984; Demant *et al*, 1991) have shown that the VLDL to LDL delipidation cascade contains parallel pathways that have the potential to give rise to LDL species with different metabolic properties. Larger VLDL appear to be metabolised to more slowly catabolised LDL (FCR 0.2 pools/d, analogous to pool B), while smaller VLDL are converted to rapidly cleared LDL (perhaps analogous to pool A). Thus it can be postulated that fibrate therapy, by diminishing the production of larger VLDL particles, reduced the formation of slowly metabolised LDL. Evidence to support this hypothesis comes from studies in type III (Packard *et al*, 1986) and type IV (Shepherd *et al*, 1984) hyperlipidaemic subjects that show that fibrate treatment causes a reduction in the synthesis of large triglyceride-rich $VLDL_1$ (S_f 60-400).

The mass of pool B apo-LDL appears to regulate the overall plasma LDL level and hence, it may be argued, the atherogenic potential of this lipoprotein. Clearly it is of interest to determine the nature of the LDL that contains this slowly metabolised apoB. Since its

concentration is directly related to the plasma triglyceride level in the same way as small dense LDL, it is appropriate to speculate that the apo-LDL in pool B resides in the LDL III species. This would be in line with the finding of Thomson *et al* (1987) who suggested that small dense LDL were metabolised more slowly than their larger counterparts because of reduced affinity for the receptor pathway In the present study we examined the subfraction distribution within LDL and attempted to relate changes in it to the altered metabolic properties of the lipoprotein. The data indicate that there is no simple link between the mass of LDL III apoprotein and the estimated amount of pool B apo-LDL. Most subjects prior to therapy had relatively low LDL III since in general the plasma triglycerides were low and, although there was a trend towards a reduction in LDL III, only 3 subjects showed substantial reductions in LDL III but substantial and significant reductions were observed in LDL II. Furthermore the mass of apo-LDL in pool B exceeded substantially that of apoprotein in LDL III.

In conclusion, fibrate therapy in primary, moderate hypercholesterolaemia is associated with highly significant and clinically relevant changes in the lipoprotein profile, associated with changes in the kinetic parameters describing the metabolism of apo-LDL. In the latter, there are changes predominantly in the high-affinity receptor mediated clearance of LDL, which may be explained both by an up-regulation of the receptors and by a drug induced shift in the synthesis of an LDL species which is more readily removed by the LDL receptor.

Chapter 7 Ciprofibrate and LDL Metabolism in Moderate Hypertriglyceridaemia

7.1 Introduction

Fibrates have a complex effect on the concentration, composition and metabolism of LDL, the end product of VLDL lipolysis. In subjects with elevated LDL concentrations they induce a fall in the level of the lipoprotein, while in those with low initial concentrations of LDL (i.e. hypertriglyceridaemics), therapy results in a rise in LDL. In previous studies in moderate hypercholesterolaemia, it was shown that bezafibrate (Stewart *et al*, 1982), fenofibrate (Caslake *et al*, 1993) and ciprofibrate (Gaw *et al*, 1994) increased clearance of LDL by the receptor-mediated pathway through two potential mechanisms : first, alteration of the LDL itself to make it more receptor active and second, upregulation of receptor activity. In the last two studies, described in detail in chapter 7, there was evidence to suggest that the second mechanism was more important i.e. fibrate therapy perturbed the synthesis of LDL in such a way as to form an LDL particle which had increased affinity for the LDL receptor. The basis of this was an observed metabolic heterogeneity in apo-LDL turnover. To explain the disappearance from plasma and the rate of appearance in urine, it was necessary to postulate the existence of two kinetically distinct LDL pools in plasma - pool A which had a rapid clearance and high affinity for receptor-mediated pathway and pool B with a slower catabolic rate with a reduced rate of receptor-mediated catabolism. This kinetic model has been used to describe apo-LDL metabolism in a group of normal subjects with plasma triglyceride ranging from 0.5-1.9 mmol/l (chapter 5) and in a group of moderate hypercholesterolaemic subjects (chapter 6) with plasma triglyceride from 0.61-2.3 mmol/l on and off fibrate therapy. Plasma triglyceride has been shown to have a strong influence on the distribution between these two pools, indicating that the mechanisms underlying the relationship between LDL cholesterol and plasma triglyceride are complex.
In contrast to the situation in hypercholesterolaemia, Shepherd *et al* (1985) showed that in hypertriglyceridaemia fenofibrate suppressed hypercatabolism of LDL by receptor-independent pathways while at the same time activating clearance of LDL by receptors. The outcome of this redirection of LDL metabolism was to normalise the initially low levels of LDL. There were accompanying changes in the composition of LDL so that the particles became enriched in cholesteryl ester and depleted in phospholipid and triglyceride, characteristic of an increase in particle size.
The methodology described in chapter 3 permits examination of the structural heterogeneity of LDL. The three subfractions isolated by density gradient ultracentrifugation are: LDL I-the largest, most buoyant and most lipid rich, LDL II- of intermediate size and density and usually most abundant and LDL III- which is small and dense. The concentration of LDL I is inversely related to fasting plasma triglyceride while that of LDL III is highly positively correlated to plasma triglyceride levels. Austin & Krauss (1988, 1990) named the triad of moderately raised triglyceride, low HDL and a predominance of small dense LDL the 'atherogenic lipoprotein phenotype' (ALP) and demonstrated its association with a threefold increased risk of coronary heart disease. In a case controlled study of male myocardial infarction (MI) survivors versus normals (Chapter

3 and Griffin *et al*, 1994) we have shown a sevenfold increased risk of MI associated with a plasma LDL III concentration >100 mg lipoprotein/100ml plasma. This level of LDL III was usually only seen above a threshold level of fasting plasma triglyceride of 1.5 mmol/l. Since plasma triglyceride has such a profound influence on the structure and metabolism of LDL and fibrates exert action by lowering plasma triglyceride, the object of the present study was to explore further the link between the structural and metabolic heterogeneity of LDL by examining the effects of ciprofibrate in a group of subjects with moderate hypertriglyceridaemia and an ALP profile.

7.2 Method

7.2.1 Protocol

The study was carried out in an identical manner to that described in chapter 6 except for the method of isolation of LDL for radiolabelling. This was prepared by isolation of the S_f 0-12 fraction by density gradient ultracentrifugation as described in 2.4.3. There were three phases to the protocol. In the preliminary screening period a three month standard cholesterol-reducing diet was advised during which time each patient was screened for cardiological, haematological, hepatic, endocrine, renal and metabolic disease by routine clinical laboratory testing. This was followed by a baseline study for two weeks when the subjects underwent measurements of lipids, lipoproteins and lipoprotein subfractions and determination of native and 1,2-cyclohexanedione-treated, radiolabelled LDL turnover. In the active treatment period subjects were commenced on ciprofibrate therapy at a dose of 100 mg/d immediately after the baseline measurement of apo-LDL metabolism. Patients remained on this dose for 10 weeks and during the final 2 weeks of this period each patient underwent a second turnover investigation with lipid, lipoprotein and lipoprotein subfraction measurements.

7.2.2 Recruitment and Conduct of the Study.

Recruitment took place at Lipid Clinic at Glasgow Royal Infirmary by Dr J Hinnie, who was responsible for the clinical aspects of the study. Dr L Forster assisted with one of the subjects. The patients were visited at home each day for venepuncture by nurses Moira Devine, Irene Hendry and Margaret Towland, whose contribution by encouraging the patients to adhere to the protocol was invaluable.

7.2.3 Kinetic Analysis

Kinetic analysis was carried out using both Matthews analysis and multicompartmental modelling in SAAM 30. Both baseline and on-drug plasma and urine radioactivities were modelled together in the same SAAM deck. In an initial analysis, using the two pool model (fig. 5.3) proposed in chapter 5 for normal individuals and applied to apo-LDL metabolism in moderate hypercholesterolaemia in chapter 6, it was not possible to obtain an acceptable fit for the observed and calculated data. The main discrepancy occurred during the first few days of the turnover when there was in the present group of hypertriglyceridaemic subjects a high rate of catabolism of both tracers with the appearance of high levels of radiolabelled breakdown products in the urine. Close examination of the decay curves of plasma and the urine : plasma (U:P) ratio, a daily index of catabolic potential, showed that the cyclohexanedione-labelled apo-LDL was behaving differently to

what had been observed in chapter 6. Fig. 7.1 shows the U:P ratio for this tracer for subjects CiproII 1 and CiproII 10 at baseline. In subject CiproII 1 it rises to a peak at 0.5 and falls rapidly to 0.2 at 6 days, whereas in subject CiproII 10 a level of 0.2 was maintained throughout the turnover (as in the subjects of chapters 5 and 6). Examination of the plasma decay curves for the same subjects (fig. 7.2) shows an initial rapid decay for CHD-apo-LDL at baseline in subject CiproII 1 but not in CiproII 10.

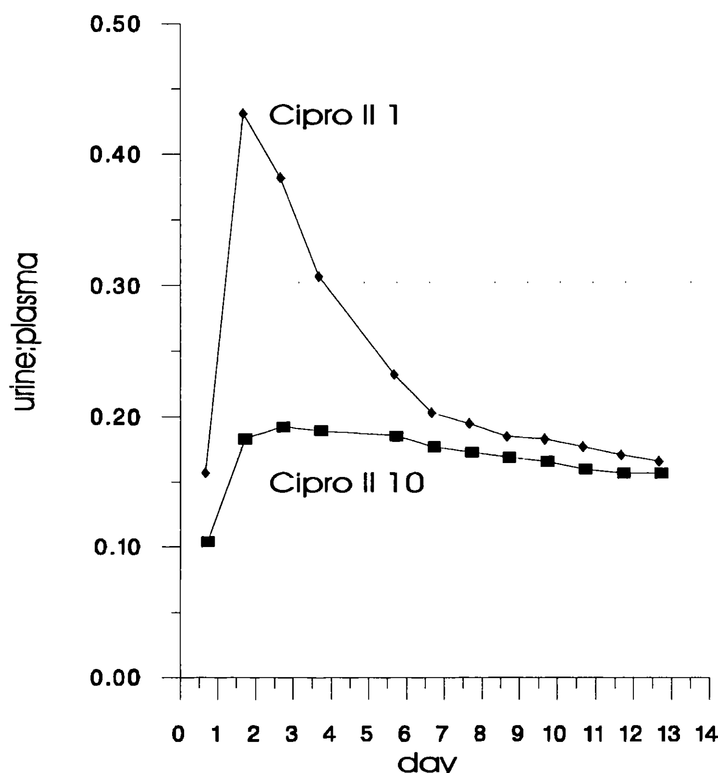

Fig. 7.1 Urine to Plasma Ratios for Native LDL at Baseline
Baseline urine to plasma radioactivity ratios for [125] I-native LDL for subjects CiproII 1 and CiproII 10

It was obvious that the conceptual two pool (AB) model was not adequate to explain LDL kinetics in the subjects in this study and so a modified model was developed. The minimal model which fitted the data consisted of three plasma pools A, B and C (fig. 7.3): the addition of pool C permitted rapid CHD-LDL clearance. In this more complex model there are too many variables to estimate with certainty from the data ie. the model is not identifiable. So, on the basis of previous analysis in chapters 5 and 6, it was decided to fix the elimination rates from each of these pools to population average values (table 7.1).

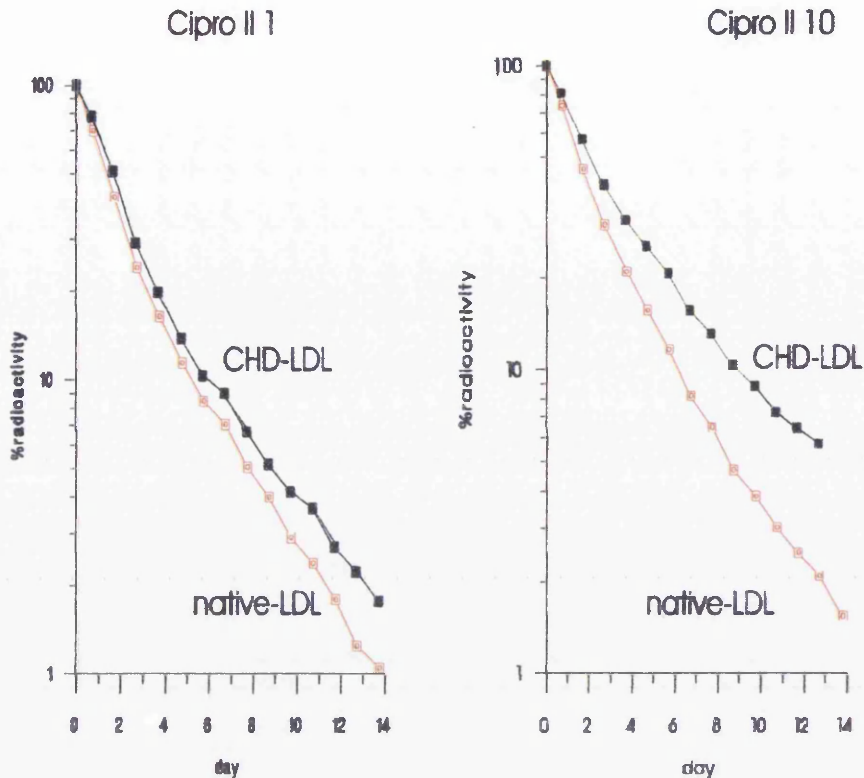

Fig.7.2 Plasma Decay Curves at Baseline
Baseline plasma decay curves for subjects CiproII 1 and CiproII 10. Native ^{125}I-LDL in red and ^{131}I cyclohexanedione-modified LDL in black.

Table 7.1 ABC Model Catabolic Rates

Pool	Receptor-mediated FCR pools/d	Receptor-independent FCR pools/d
A	0.60	0.30
B	0.25	0.15
C	0.00	0.50

As with the AB model, pool B was allowed to exchange with an extravascular pool. There was no requirement for A or C to undergo exchange in order to achieve an acceptable fit. Fitting of observed data was therefore a function simply of distributing LDL between the three pools according to the nature of the plasma and urine radioactivity data. For example, if a subject had a high initial clearance of native but not CHD tracer then more LDL would be placed in pool A (see chapter 6), but if both tracers exhibited rapid initial clearance then a high content of pool C would reflect this.

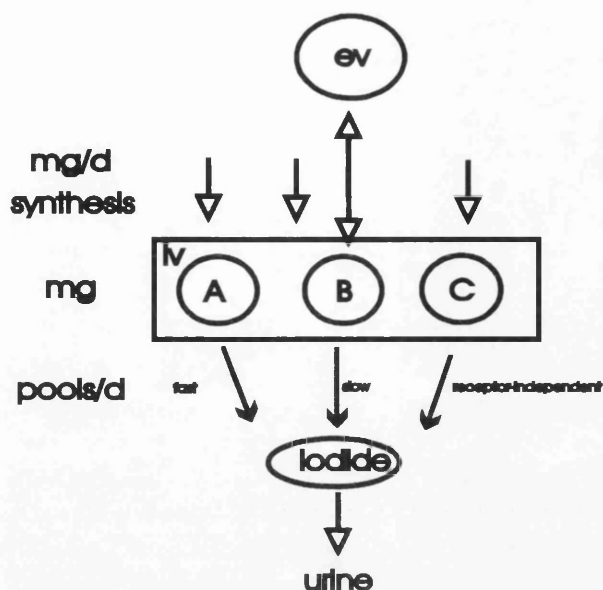

Fig. 7.3 ABC Kinetic Model of LDL Metabolism
The plasma compartment consists of three pools A,B and C each of which has input measured in mg/d and elimination rates in pools /d. Pool B has extravascularvexchange.

7.2.4 Subjects

The characteristics of the subjects are shown in table 7.2. Ten male subjects, aged 34-65 and exhibiting Type IIb hyperlipidaemia with initial plasma triglyceride concentrations of 2.0-5.2 mmol/l and plasma cholesterol 5.2-7.5 mmol/l were recruited into the study. Six of the subjects were 3/3 Apo E phenotype, 2 were 2/3 , one 3/4 and one 2/4.

Table 7.2 Characteristics of the Subjects

Subject	*Age*	*Weight kg*	*ApoE Phenotype*
CiproII 1	**34**	78.2	**4/3**
CiproII 2	**61**	94.9	**3/3**
CiproII 3	**51**	77.9	**3/3**
CiproII 4	**56**	83.5	**3/3**
CiproII 5	**53**	80.0	**3/3**
CiproII 6	**54**	86.7	**3/3**
CiproII 7	**50**	102.1	**2/3**
CiproII 8	**53**	88.3	**3/3**
CiproII 9	**65**	100.8	**2/3**
CiproII 10	**59**	75.2	**2/4**

7.3 Results

7.3.1 Lipids, Lipoproteins, Apolipoproteins and HDL Subfractions

The lipid and lipoprotein levels of the subjects are given in table 7.3 and listed in descending order of fasting plasma triglyceride. The subjects exhibited a lipoprotein profile (mean triglyceride 3.07 mmol/l, mean HDL cholesterol 0.9 mmol/l)with a preponderance (69%, table 7.7) of small dense LDL III, indicative of ALP. Treatment with ciprofibrate significantly ($p<0.02$) reduced fasting plasma triglyceride by 34% and this was reflected in a 41% fall in VLDL cholesterol. Plasma cholesterol and HDL cholesterol remained unchanged.

Table 7.3 Lipid and Lipoprotein Levels

Subject	*Cholesterol*	*Triglyceride*	*VLDLchol mmol/l*	*LDLchol*	*HDLchol*
Baseline					
CiproII 1	5.6	5.2	2.1	2.7	0.8
CiproII 2	7.1	4.3	2.1	4.0	0.9
CiproII 3	7.1	3.5	1.7	4.5	0.9
CiproII 4	5.2	3.3	1.7	2.6	0.9
CiproII 5	5.6	2.8	1.4	3.3	0.8
CiproII 6	7.6	2.5	1.0	5.5	1.1
CiproII 7	5.9	2.4	1.0	4.2	0.8
CiproII 8	5.6	2.3	1.1	3.5	0.8
CiproII 9	6.7	2.3	1.3	4.3	1.1
CiproII 10	5.6	2.0	1.0	3.7	0.9
mean	**6.21**	**3.07**	**1.45**	**3.83**	**0.90**
(SEM)	**(0.36)**	**(0.33)**	**(0.14)**	**(0.28)**	**(0.04)**
Ciprofibrate					
CiproII 1	6.3	2.4	0.9	4.7	0.7
CiproII 2	6.3	2.5	1.2	4.3	0.9
CiproII 3	6.9	2.7	1.3	4.7	1.0
CiproII 4	5.6	1.3	0.6	3.9	1.2
CiproII 5	5.4	2.0	0.9	3.5	1.0
CiproII 6	6.6	2.4	0.9	4.8	0.9
CiproII 7	4.7	2.3	0.8	3.1	0.8
CiproII 8	5.0	2.0	0.7	3.5	0.8
CiproII 9	6.5	1.7	0.7	4.5	1.2
CiproII 10	4.2	1.1	0.4	2.6	1.2
mean	**5.74**	**2.04**	**0.85**	**3.94**	**0.96**
(SEM)	**(0.29)**	**(0.16)**	**(0.08)**	**(0.24)**	**(0.06)**
p	**NS**	**<0.02**	**<0.002**	**NS**	**NS**

There was a varied response in LDL cholesterol with no change overall. For example, in subject CiproII 8, LDL cholesterol fell from 3.7 to 2.6 mmol/l, whereas in CiproII 10 the change was from 4.3 to 4.5 mmol/l and subject CiproII 3 with initial low levels of LDL cholesterol of 2.7 mmol/l exhibited a rise to 4.7 mmol/l. These changes in LDL cholesterol were related to the change in plasma triglyceride i.e. those with an increased basal triglyceride tended to show an increased LDL cholesterol on therapy. There was a small but significant ($p<0.05$) rise in HDL_3 subfraction which was associated with a rise in apolipoprotein A II (table 7.4). The other lipoproteins AI, B and Lp(a) were unaffected by therapy (table 7.4)

Table 7.4 Apoprotein and HDL Subfraction Concentrations

Subject	*Apo AI*	*Apo AII*	*Apo B mg/100ml*	*Lp(a)*	*HDL_2*	*HDL_3*
Baseline						
CiproII 1	166	27	177	23	10	265
CiproII 2	131	28	180	3	13	226
CiproII 3	122	32	164	29	22	257
CiproII 4	90	26	147	10	11	224
CiproII 5	106	26	119	12	21	217
CiproII 6	126	36	150	84	28	303
CiproII 7	98	28	149	44	13	224
CiproII 8	96	26	134	70	16	176
CiproII 9	112	36	147	15	22	374
CiproII 10	118	26	116	46	26	267
mean	**119**	**29**	**148**	**34**	**18**	**253**
(SEM)	**(5)**	**(1)**	**(7)**	**(8)**	**(2)**	**(17)**
Ciprofibrate						
CiproII 1	136	31	127	33	11	293
CiproII 2	134	32	146	9	19	286
CiproII 3	131	36	157	28	29	294
CiproII 4	137	31	105	13	15	326
CiproII 5	103	35	164	8	18	293
CiproII 6	129	36	162	148	12	375
CiproII 7	111	28	99	30	18	234
CiproII 8	114	30	96	46	29	241
CiproII 9	114	43	122	17	31	305
CiproII 10	96	26	62	53	70	401
mean	**114**	**34**	**125**	**39**	**25**	**305**
(SEM)	**(5)**	**(1)**	**(10)**	**(13)**	**(6)**	**(16)**
p	**NS**	**NS**	**NS**	**NS**	**NS**	**<0.05**

7.3.2 ApoB Lipoprotein Subfractions

When VLDL was subdivided into large $VLDL_1$ (S_f 60-400) and small $VLDL_2$ (S_f 20-60) it was observed (table 7.5) that the reduction in VLDL was due to a significant ($p<0.05$) 40% fall in the mass of the larger VLDL particle. There were no changes in the lipoprotein masses of IDL (S_f 12-20) nor LDL (S_f 0-12).

Table 7.5 Masses of Lipoprotein Subfractions (S_f 0-400)

Subject	*$VLDL_1$*	*$VLDL_2$ mg/100ml*	*IDL*	*LDL*
Baseline				
CiproII 1	319	105	37	206
CiproII 2	312	152	67	232
CiproII 3	365	104	60	361
CiproII 4	226	87	42	185
CiproII 5	231	65	65	220
CiproII 6	189	121	73	290
CiproII 7	143	57	58	246
CiproII 8	105	114	62	271
CiproII 9	162	151	61	259
CiproII 10	83	70	61	227
mean	**212**	**107**	**58**	**253**
(SEM)	**(34)**	**(11)**	**(4)**	**(52)**
Ciprofibrate				
CiproII 1	188	82	59	306
CiproII 2	127	109	61	219
CiproII 3	201	103	72	217
CiproII 4	128	52	65	293
CiproII 5	---	---	---	---
CiproII 6	197	89	78	292
CiproII 7	108	68	64	216
CiproII 8	96	42	106	198
CiproII 9	65	178	130	217
CiproII 10	29	43	63	186
mean	**127**	**85**	**76**	**238**
(SEM)	**(20)**	**(14)**	**(8)**	**(46)**
p (n=9)	**<0.05**	**NS**	**NS**	**NS**

There was a significant change in the percentages of free cholesterol (51% decrease, $p<0.005$) of $VLDL_1$ (table 7.6).

Table 7.6 Composition of Lipoprotein Subfractions (S_f 0-400)

	%Protein	*%Free cholesterol*	*%Cholesteryl ester*	*%Triglyceride*	*% Phospholipid*
$VLDL_1$					
Baseline	7.7	5.9	14.5	57.8	15.9
	(0.4)	(0.7)	(1.1)	(0.7)	(0.9)
Ciprofibrate	8.0	2.9	15.4	59.1	14.8
n=9	(0.5)	(0.6)	(1.5)	(1.3)	(0.7)
p (n=9)	**NS**	**<0.005**	**NS**	**NS**	**NS**
$VLDL_2$					
Baseline	13.1	6.6	26.8	35.8	18.8
	(0.6)	(0.6)	(1.8)	(1.4)	(0.6)
Ciprofibrate	13.4	6.8	26.9	34.0	19.2
n=9	(1.3)	(1.5)	(1.7)	(2.0)	(1.0)
p (n=9)	**NS**	**NS**	**NS**	**NS**	**NS**
IDL					
Baseline	19.6	5.8	41.1	14.4	19.1
	(1.5)	(0.4)	(1.0)	(1.3)	(0.9)
Ciprofibrate	19.3	7.1	40.9	15.1	20.9
n=9	(1.3)	(0.8)	(2.5)	(1.9)	(0.9)
p (n=9)	**NS**	**NS**	**NS**	**NS**	**NS**
LDL					
Baseline	26.8	6.9	40.7	7.0	18.6
	(0.8)	(0.6)	(0.8)	(0.6)	(0.6)
Ciprofibrate	26.7	7.9	41.4	7.0	19.3
n=9	(1.6)	(0.9)	(1.3)	(0.5)	(0.5)
p (n=9)	**NS**	**NS**	**NS**	**NS**	**NS**

Values are mean (SEM)

Ciprofibrate significantly altered the distribution of the LDL subfraction profile as seen in table 7.7 .

Table 7.7 LDL Subfraction Concentrations

Subject	*LDL mass*	*LDL I mg/100ml*	*LDL II*	*LDL III*
Baseline				
CiproII 1	221	22	32	166
CiproII 2	191	14	18	160
CiproII 3	356	40	66	250
CiproII 4	202	25	47	130
CiproII 5	354	0	0	354
CiproII 6	401	50	146	205
CiproII 7	256	21	84	151
CiproII 8	305	27	85	193
CiproII 9	323	19	73	230
CiproII 10	302	42	112	149
mean	**291**	**26**	**66**	**199**
(SEM)	**(23)**	**(5)**	**(14)**	**(21)**
Ciprofibrate				
CiproII 1	396	24	192	180
CiproII 2	369	56	146	167
CiproII 3	333	61	220	53
CiproII 4	395	37	124	134
CiproII 5	307	10	37	260
CiproII 6	385	76	195	114
CiproII 7	256	37	117	102
CiproII 8	304	28	148	128
CiproII 9	302	56	162	84
CiproII 10	207	42	149	16
mean	**315**	**38**	**134**	**116**
(SEM)	**(19)**	**(7)**	**(21)**	**(24)**
p	**NS**	**NS**	**<0.02**	**<0.02**

At baseline, 69% of LDL was in the small dense fraction. Therapy significantly ($p<0.002$) reduced this to 38%, while at the same time increasing the proportion of LDL II (22% to 48%, $p<0.001$). The absolute concentrations changed accordingly. LDL I increased from 26 to 38 mg/100ml (44%, NS), LDL II from 66 to 134 mg/100ml (102%, $p<0.02$) and LDL

III fell from 199 to 116 mg/100ml (42%, $p<0.02$). In subject CiproII 4 at baseline only small dense LDL III was detectable (fig. 7.4) with small amounts of LDL I and LDL II appearing on treatment with ciprofibrate. On closer examination of the LDL subfraction profile, it was observed that the LDL in subjects CiproII 1, 2 4 and 5 was very small and dense. In the density gradient ultracentrifugation method we did not distinguish small from very small LDL, but these subjects exhibited the presence of 'LDL IV' as described by Krauss *et al* (1982). On ciprofibrate no 'LDL IV' was observed.

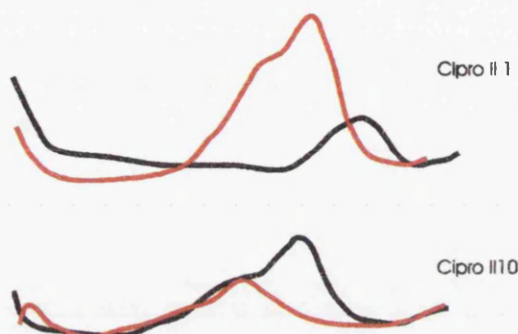

Fig. 7.4 LDL Subfractions at Baseline and on Ciprofibrate
LDL subfraction profiles for subjects CiproII 1 and CiproII 10 at baseline (black) and on ciprofibrate (red)

7.3.3 Receptor-mediated and Receptor-independent Apo LDL Metabolism

The parameters derived from Matthews kinetic analysis are shown in table 7.8.

The overall mean FCR remained unaltered by therapy (0.33 versus 0.35 pools/day) but there was a marked individual variation in response depending on the plasma triglyceride concentration achieved on treatment.

At baseline, 28% of apo-LDL removal was receptor-mediated but ciprofibrate induced significant alterations in route of removal resulting in an equal catabolism of apo-LDL by both receptor-mediated and receptor-independent pathways. The receptor-mediated FCR was significantly ($p<0.002$) increased by 74%, while receptor-independent FCR was significantly decreased by 21% ($p<0.05$). There was no change in the mass of circulating apo-LDL but the amount of apo-LDL removed by these routes was altered in concert such that there was almost a doubling of removal by the receptor-mediated pathway ($p<0.0005$). In these subjects, it was observed that receptor-independent catabolism of apo-LDL was positively correlated ($r = 0.54$, $p<0.02$) to the ratio of the mass of LDL III to LDL II (fig. 7.5), linking structural and metabolic changes. Plasma triglyceride correlated with the ratio of LDL III to LDL II ($r = 0.63$, $p<0.005$), receptor-independent FCR ($r = 0.80$, $p< 0.0001$) and receptor-mediated FCR ($r = - 0.57$, $p< 0.01$). As expected, plasma

with $VLDL_1$ and these parameters: $VLDL_1$ with the ratio of LDL III to LDL II ($r = 0.70$, $p < 0.0001$), $VLDL_1$ with receptor-independent FCR ($r = 0.63$, $p < 0.005$) and $VLDL_1$ with receptor-mediated FCR ($r = -0.54$, $p < 0.02$).

Table 7.8 Matthews Kinetic Parameters of Apo-LDL

Subject	*Apo-LDL mg/100ml*	*FCR pools/d*	*RI FCR pools/d*	*RM FCR pools/d*	*Synthetic Rate mg/kg/d*	*RI ACR mg/kg/d*	*RM ACR mg/kg/d*
Baseline							
CiproII 1	61.6	0.42	0.35	0.07	12.9	10.8	2.1
CiproII 2	58.4	0.39	0.27	0.12	14.8	10.2	4.6
CiproII 3	92.8	0.34	0.23	0.11	10.5	7.1	3.4
CiproII 4	53.6	0.44	0.35	0.09	14.8	11.8	3.0
CiproII 5	95.8	0.30	0.22	0.08	9.5	6.9	2.6
CiproII 6	94.0	0.24	0.22	0.02	8.3	7.7	0.7
CiproII 7	63.5	0.34	0.20	0.14	13.9	8.1	5.7
CiproII 8	82.7	0.29	0.19	0.10	10.4	6.8	3.6
CiproII 9	88.8	0.31	0.19	0.12	12.5	7.7	4.8
CiproII 10	80.2	0.28	0.18	0.09	8.6	5.7	2.9
mean	**77.1**	**0.33**	**0.24**	**0.09**	**11.6**	**8.3**	**3.3**
(SEM)	**(5.1)**	**(0.02)**	**(0.02)**	**(0.01)**	**(0.8)**	**(0.6)**	**(0.5)**
Ciprofibrate							
CiproII 1	103.8	0.26	0.16	0.10	8.2	5.0	3.2
CiproII 2	103.3	0.35	0.22	0.13	13.4	8.5	4.9
CiproII 3	73.0	0.34	0.20	0.14	10.7	6.2	4.4
CiproII 4	77.4	0.39	0.21	0.19	13.1	6.9	6.2
CiproII 5	83.2	0.33	0.20	0.14	10.7	6.3	4.4
CiproII 6	99.5	0.34	0.21	0.13	12.0	7.5	4.6
CiproII 7	56.4	0.40	0.19	0.20	16.4	8.0	8.4
CiproII 8	77.5	0.36	0.18	0.18	12.7	6.3	6.4
CiproII 9	71.8	0.35	0.19	0.16	14.2	7.6	6.6
CiproII 10	48.5	0.43	0.16	0.27	12.6	4.7	7.9
mean	**79.4**	**0.35**	**0.19**	**0.16**	**12.4**	**6.7**	**5.7**
(SEM)	**(5.9)**	**(0.02)**	**(0.01)**	**(0.01)**	**(0.7)**	**(0.4)**	**(0.5)**
p	**NS**	**NS**	**<0.05**	**<0.002**	**NS**	**NS**	**<0.005**

FCR-fractional catabolic rate; RI - receptor independent ie clearance from cyclohexanedione modified LDL; RM - receptor mediated calculated as the difference between native and CHD modified apo-LDL; synthetic rate is the product of FCR in pools/d and the plasma pool of apo-LDL in mg divided by the body weight in kg; ACR is the absolute catabolic rate and is the product of either receptor independent or the receptor mediated FCR and the apo-LDL pool size divided by the body weight. Under steady state conditions synthetic rate and catabolic rate should be equal.

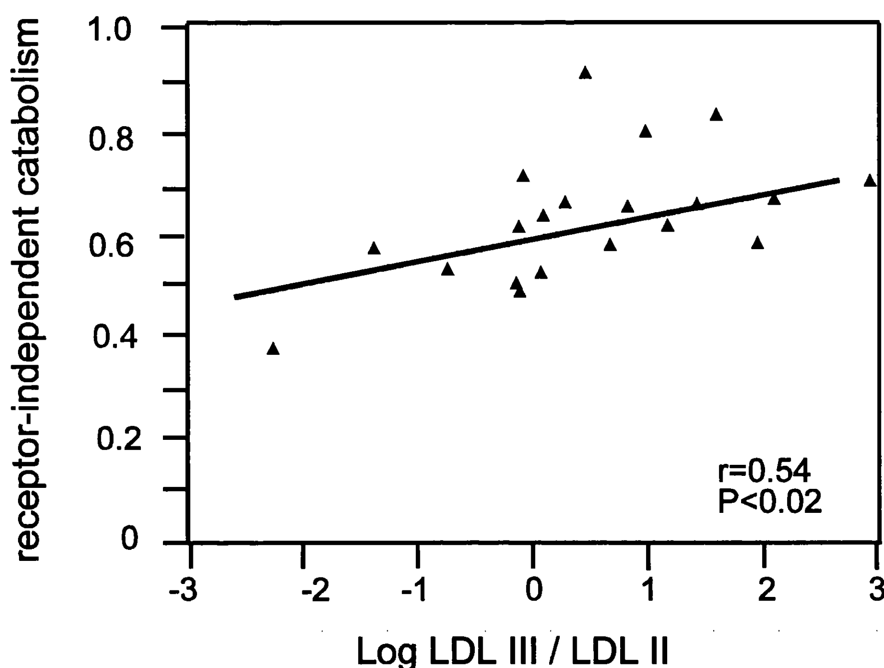

Fig.7.5 Receptor-independent LDL catabolism and LDLIII/LDLII
Receptor independent catabolism versus log LDLIII/LDLII, r=0.54, p<0.02

7.3.4 Three compartmental Model of Apo LDL Metabolism

Multicompartmental modelling of plasma and urine radioactivity led to a distribution of mass of apo-LDL among the three plasma pools A, B and C as shown in table 7.9.
All of the subjects had apo-LDL mass that was in pool B and this was significantly correlated with total apo-LDL mass (r = 0.83, p<0.0001). It was the major component in 8 out of 10 subjects. However there were marked differences as to whether pool A or pool C was the other component. Plasma triglyceride was negatively correlated with pool A mass (r = -0.49, p<0.05), positively with pool B (r = 0.48, p<0.05) and positively with pool C (r =0.81, p<0.0001). For example, subjects CiproII 7-10 at baseline with plasma triglyceride <2.4 mmol/l had no pool C compartment and A and B were in the ratio 3:5. Ciprofibrate treatment altered this to 1:1. In subjects 1 - 6 with higher basal plasma triglyceride levels, there was no apo-LDL in pool A at baseline and pool C was more important. At triglyceride >4.3 mmol/l more than 80% apo-LDL mass was in pool C. Pools A and C were negatively correlated (r= -0.77, p< 0.0001). The synthesis of apo-LDL in each compartment (table 7.10), calculated as the product of the mass and FCR, showed similar relationships. There was a redistribution of the mass in ABC towards pools A and B, with a resultant decrease in the median mass of pool C and its elimination in most individuals. The basis for this change is evident from the changes in the decay curves (fig. 7 2). The change in pool C on fibrate therapy was significant (p<0.05) using a non-parametric test (Mann Whitney). The mass in pool C was positively associated with receptor-independent

catabolic rate (r = 0.60, p=0.006), % LDL III (r = 0.48, p=0.034) and the ratio of LDL III to LDL II (r =0.48, p=0.040). 'LDL IV' was only observed in those subjects with plasma triglyceride >2.5 mmol/l and apo-LDL in pool C.

Table 7.9 Distribution of apo-LDL Mass in Pools A, B and C

Subject	*Apo-LDL mass mg/100ml*	*Pool A %mass*	*PoolB %mass*	*Pool C %mass*	*Pool A mass*	*Pool B mass mg/100ml*	*Pool C mass*
Baseline							
CiproII 1	1927	0	15	85	0	289	1638
CiproII 2	1790	0	20	80	0	358	1432
CiproII 3	2216	14	40	47	310	887	1042
CiproII 4	2890	0	64	36		1849	1040
CiproII.5	3066	0	54	46		1655	1410
CiproII 6	3260	0	74	26		2412	848
CiproII 7	2592	36	64	0	933	1659	0
CiproII 8	3575	23	74	3	822	2645	107
CiproII 9	2918	20	80	0	584	2335	0
CiproII 10	2412	40	59	1	965	1423	24
median	2741	7.0	61.5	31.0	155	1657	944
(SEM)	(184)	(5)	(7)	(10)	(134)	(261)	(209)
Ciprofibrate							
CiproII 1	3247	0	100	0	0	3247	0
CiproII 2	2584	49	46	6	1266	1189	155
CiproII 3	3920	8	62	30	314	2431	1176
CiproII 4	2273	2	71	27	45	1614	614
CiproII 5	2662	28	72	0	745	1917	0
CiproII 6	3451	18	67	15	621	2312	518
CiproII 7	2302	50	50	0	1151	1151	0
CiproII 8	2894	37	63	0	1071	1823	0
CiproII 9	3920	36	64	0	985	1750	0
CiproII 10	1458	74	26	0	1079	379	0
median	2778	32.0	63.5	0.0	865	1786	0.0
(SEM)	(247)	(7)	(6)	(3.8)	(147)	(250)	(127)
p	NS		NS	<0.05		NS	

Table 7.10 Synthesis into Pools A, B and C

Subject	Pool A	Pool B mg/d	Pool C
Baseline			
CiproII 1	0	72	819
CiproII 2	0	89	716
CiproII 3	186	222	521
CiproII 4	0	462	520
CiproII.5	0	414	705
CiproII 6	0	603	424
CiproII 7	560	415	0
CiproII 8	493	661	54
CiproII 9	350	584	0
CiproII 10	579	356	12
median	93	415	472
(SEM)	(80)	(65)	(105)
Ciprofibrate			
CiproII 1	0	812	0
CiproII 2	760	297	78
CiproII 3	188	608	588
CiproII 4	27	403	307
CiproII 5	47	479	
CiproII 6	373	578	259
CiproII 7	691	288	0
CiproII 8	642	456	0
CiproII 9	591	438	0
CiproII 10	648	95	0
median	482	447	0
(SEM)	(97)	(63)	(63)
p	NS	NS	NS

7.4 Discussion

In this study of LDL kinetics in hypertriglyceridaemia, it was necessary to extend the multicompartmental model devised for normals in chapter 5 and applied to hypercholesterolaemia in chapter 6, to allow for the fact that in individuals with moderate to high concentrations of plasma triglyceride, LDL undergoes accelerated catabolism by the receptor-independent pathway (Shepherd *et al*, 1986). This was reflected in the baseline turnovers where there was present a substantial mass of apo-LDL which was attributable to pool C, the LDL pool whose kinetic characteristic is that of a compartment catabolised entirely by the receptor-independent pathway at a fixed rate of 0.5 pools/day. The ABC

model produced highly satisfactory fits to the plasma and urine radioactivity curves for all subjects at baseline. Treatment with ciprofibrate reduced pool C mass with a redistribution towards pools A and B, while the mass of apo-LDL was unchanged at 2.7 g. Again the modified model was required to generate satisfactory fits to the observed and calculated data.

The concept of fixing the elimination rates from pools ABC to population average values suggests that LDL kinetics can be explained by the presence of distinct metabolic pools and that the presence of hypertriglyceridaemia and its correction by therapy with ciprofibrate causes LDL to switch between these pools. The presence of pool C was highly correlated with plasma triglyceride ($r = 0.81$, $p < 0.0001$) and the necessity for pool C in those subjects with plasma triglyceride >2.5 mmol/l at baseline was linked to the presence of an abnormally small dense species on density gradient ultracentrifugation profile i.e. LDL IV. A number of other investigators have shown that in individuals with similar plasma triglyceride levels that LDL is small and dense with reduced affinity for LDL receptors (Eisenberg *et al*, 1984, Kleinman *et al*, 1985, Nigon *et al*, 1991, Chen *et al*, 1994).

In the subjects in this study, we were able to see the relationships between plasma triglyceride, LDL structure and kinetic heterogeneity. LDL subfraction distribution (i.e. the ratio of LDL III to LDL II and % LDL III) was linked to receptor-independent catabolism. This is the first time that a link has been shown between structural and metabolic heterogeneity in humans.

At present it is not entirely clear what the structural basis of the kinetic differences between pools A, B and C are, but from the above discussion it is clear that particle size and density play an important role in determining the properties of the lipoprotein. It is proposed that a density and size shift induced an alteration in the conformation of apo B on LDL and this is the basis of the observations in this chapter. We postulate that the apo B on LDL in pool A is in a conformation that has a high affinity for the LDL receptor, whereas the protein in pool B still binds to the receptor but with lower affinity. In hypertriglyceridaemia where the cycles of lipid exchange and hydrolysis generate an abnormally small particle 'LDL IV', then it is possible that apo B has undergone a further conformational change which activates particle clearance by receptor-independent mechanisms. These proposals are consistent with the known variation of epitope expression of apo B in larger versus smaller LDL and the link between these variations and receptor binding capabilities *in vitro*. The activation of receptor-independent catabolism is conjectural at present.

Chapter 8 Impact of Heterogeneity in LDL Structure and Metabolism

There has traditionally been a lack of consensus on the role of plasma triglyceride as an independent risk factor for CAD but there is an emerging view incorporated in new guidelines for the prevention of coronary disease that moderately elevated plasma triglyceride levels associated with a low HDL cholesterol predispose to an increased risk for coronary disease. Evidence to support this is emerging from clinical trials as well as epidemiological surveys. In the Framingham study (Castelli *et al*, 1986) elevation of triglyceride was a highly significant independent risk factor in women and in the Helsinki Heart study (Manninen *et al*, 1988) the benefit of treatment with gemfibrozil was seen largely in the group of subjects with plasma triglyceride > 2mmol/l and HDL cholesterol < 1.2 mmol/l. The WOSCOPS study also confirmed that the absolute risk reduction was greater in those subjects with triglyceride concentrations > 1.67 mmol/l (CJ Packard, personal communication).

The results of this thesis, presented in chapters 3 to 7, provide evidence to support the contention that plasma triglyceride influences both the structural and metabolic heterogeneity in LDL and the associated risk of coronary disease.

8.1 LDL Heterogeneity and Apo-B Metabolism

The results of the kinetic studies in subjects described in chapter 5 and 6 altered the concepts of the regulation of LDL metabolism. Fig. 8.1 shows that as plasma triglyceride rose from 0.6 to 2 mmol/l, the catabolic rate of LDL fell from 0.45 to 0.25 pools /d due to a fall in the receptor-mediated catabolism, while receptor-independent catabolism remained constant. This was accompanied by a rise in the concentration of LDL. However, as plasma triglyceride rose above 2 mmol/l, there was increased clearance via the receptor-independent pathway while clearance by the receptors remained constant. The classical (Matthews) approach to analysing the data would have attributed to the rise in mass of apo-LDL with increasing plasma triglyceride to a fall in its catabolic rate and the conclusion would have been that the elevated plasma triglyceride levels in some way down-regulated the LDL receptor. However recognition that LDL was not behaving as a homogeneous entity opened up other possibilities, namely that the variation in plasma triglyceride affected the nature of LDL. Two plasma pools for LDL were proposed, pool A having a fast clearance rate and mostly via the LDL receptor while pool B had a slower rate of clearance. Fig. 6.6 shows the close relationship between the synthesis of pool B and plasma triglyceride levels, indicating that at higher plasma triglyceride levels more mass appears in pool B. One way to interpret this association is to note that plasma triglyceride levels are a reflection of $VLDL_1$ concentrations (Tan *et al*, 1995a). It is tempting therefore to speculate that pool B apo LDL is derived from large $VLDL_1$ and there are several lines of evidence from VLDL metabolic investigations to support this. Early studies on the metabolism of trace-labelled VLDL established its precursor relationship to LDL in normal and dyslipidaemic subjects (Berman *et al*, 1978). However, not all VLDL apo B is converted to LDL apo B and not all LDL apo B is derived from VLDL apoB.

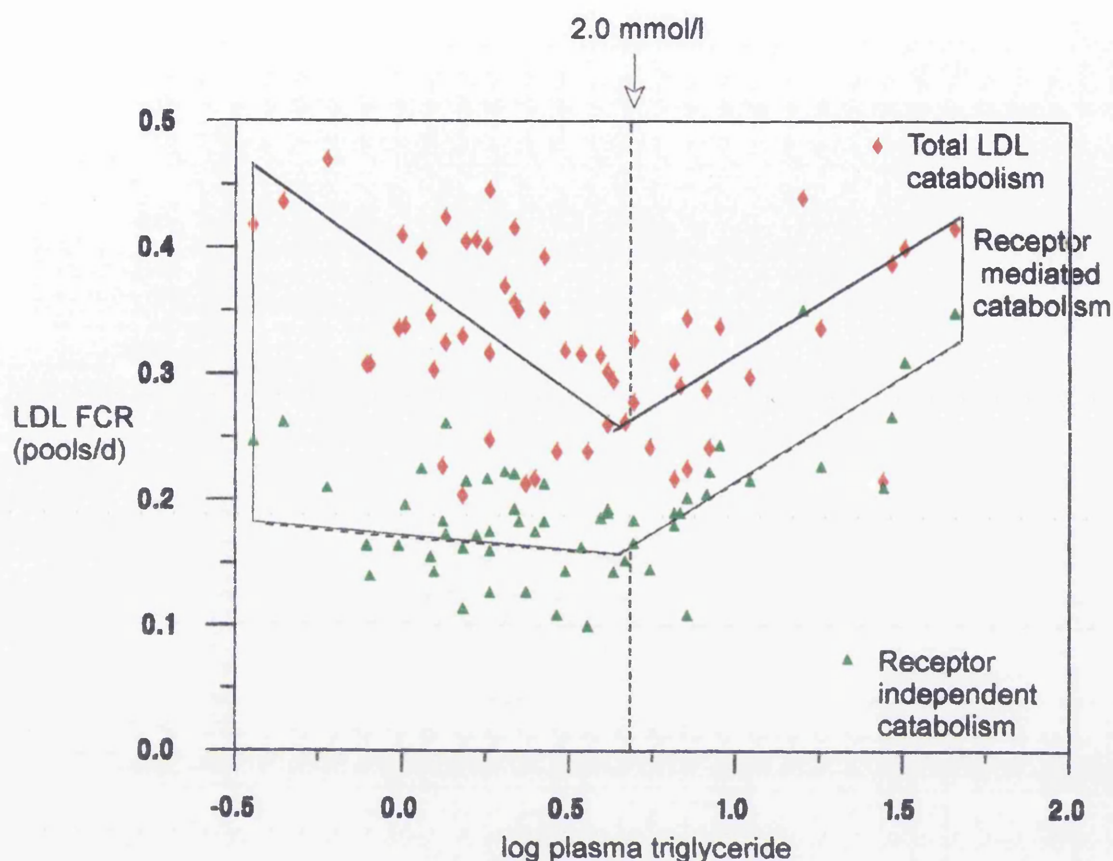

Fig.8.1 Plasma Triglyceride and Fractional Catabolic Rates of LDL
FCR- fractional catabolic rate. Total LDL catabolism is in red, receptor-independent is in green and the difference in grey is receptor-mediated catabolism.

Importantly metabolic channels exist in the VLDL-LDL delipidation cascade (Packard *et al*, 1995) so that different VLDL precursors give rise to products with varying metabolic properties. In a large number of turnover studies (Packard *et al*, 1995) it has been shown that LDL derived from the delipidation of $VLDL_1$ has a catabolic rate of 0.2 pools/d while LDL derived from lipoproteins first secreted into the $VLDL_2$ density range has a catabolic rate of about 0.5 pools/d. (fig.8.2) These are comparable to catabolic rates calculated in chapters 5 and 6 for pool B and pool A apo-LDL respectively and these data suggest that pool B LDL is indeed the product of VLDL1 lipolysis and this is the basis of the association seen in fig. 6.6.

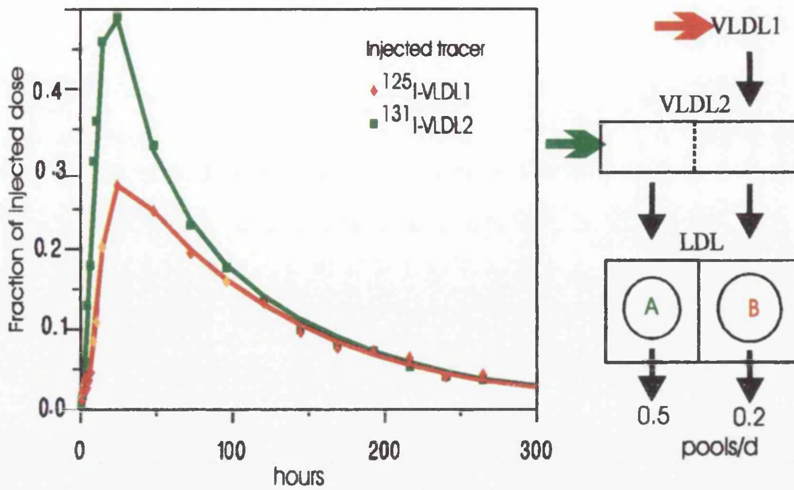

Fig.8.2. Association of $VLDL_1$ and $VLDL_2$ with A and B LDL kinetic Plasma Pools

Turnovers of ^{125}I-$VLDL_1$ and ^{131}I-$VLDL_2$ result in LDL with catabolic rates of 0.2 and 0.5 pools/d, akin to those calculated for pools B and A LDL.

Pool A synthesis on the other hand was poorly related to triglyceride and new evidence from unpublished stable isotope VLDL-LDL studies from this laboratory shows that rapidly removed LDL is also derived from lipoproteins secreted from the liver into the IDL and LDL density interval. This suggests that pool A apo-LDL is derived from particles released by the liver in the S_f 0-60 range. Fig. 8.3 draws this information together and postulates that the liver forms lipoprotein particles of differing sizes depending on the availability of hepatic triglyceride and these lead to the formation of LDL particles with different metabolic fates and different atherogenic potential.

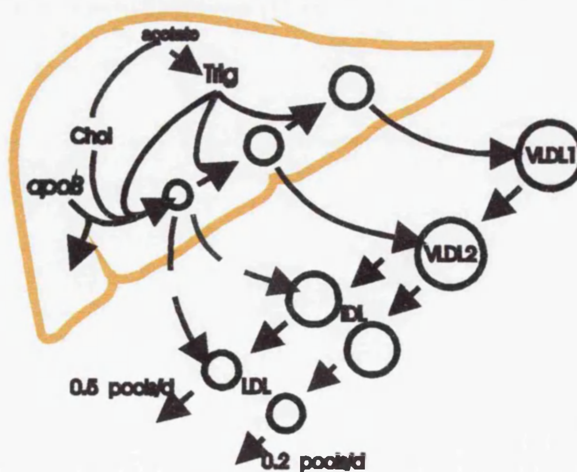

Fig.8.3 Hepatic Lipoprotein Metabolism

8.2 Relating LDL Structure to Metabolism

The results in this thesis provide evidence that the influence of plasma triglyceride is the common link between structural and metabolic heterogeneity in LDL.

Numerous in vitro studies have suggested that apoB found in smaller LDL particles has a reduced receptor-binding affinity which arises from a conformational change in apo B (Kleinman *et al*, 1987, Chen *et al*, 1994, Galeano *et al*, 1994). Can we say therefore that pool B apo-LDL is made up of LDL III? The answer is no for two reasons. First, there is not enough mass in LDL III to account for the mass of apo-LDL in pool B (tables 6.7, 6.9, 6.14, 6.16) and secondly, the kinetic studies in chapters 5 and 6 were carried out using lipoprotein from a narrow density interval as described in the zonal ultracentrifugation procedure and so both A and B pools co-existed in this narrow density range. It is suggested therefore that pool B consists of LDL III and a proportion of LDL II. Pool A on the other hand is thought to be present in LDL I and LDL II because direct synthesis of LDL, which occurs in subjects with low plasma triglyceride, was related to concentrations of LDL I plus LDL II (Gaw *et al*, 1995). This finding is confirmed in the unpublished stable isotope study where a high proportion of direct lipoprotein synthesis in the IDL-LDL range was associated with low plasma triglyceride (< 1.0 mmol/l) and a high content of LDL I and LDL II in plasma.

It is clear therefore, that the source of LDL is very important in determining both its structure and kinetic properties. The longer LDL is resident in plasma the more likely it is to undergo neutral lipid exchange and hydrolysis to form small LDL. It is therefore proposed that pool A is comprised of LDL which is derived from the lipoproteins in the S_f 0-60 range and forms the majority of LDL I and a substantial amount of LDL II, whereas pool B is in LDL II initially and following neutral lipid exchange and lipolysis can form LDL III, and pool C is LDL IV (fig. 8.4).

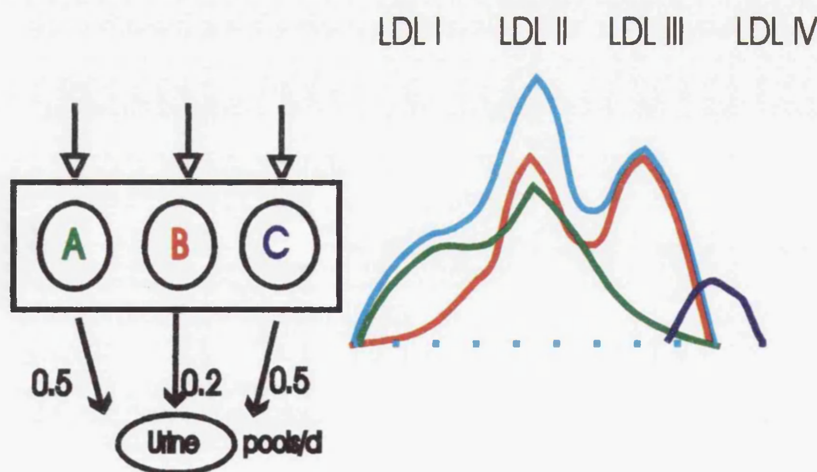

Fig.8.4 Linking Structural and Metabolic Heterogeneity of LDL

Fig. 7.5 links structural and metabolic heterogeneity in LDL in that receptor-independent catabolism is increased as the proportion of very small, dense LDL rises. In the special circumstances of moderate to severe hypertriglyceridaemia a new type of LDL (LDL IV) is formed with an apo B conformation such that it no longer binds to the LDL receptor and is subject to a rapid removal by non-receptor pathways.

8.3 Correction of an Atherogenic Lipoprotein Phenotype by Drug Therapy

The studies in chapter 4 indicate that an undesirable LDL subfraction profile may be corrected by therapy. Those that lower LDL III concentrations principally through reductions in plasma triglyceride such as the fibrates (tables 4.18, 4.20, 4.22, 4.24) may be the drugs of choice in subjects with ALP, principally by their action of altering the quality of lipoprotein assembled in the liver and so reducing the precursors of atherogenic LDL. However it was suggested from the data (fig.4.1) that the statins in combined hyperlipidaemia may accelerate the removal of the large VLDL in such a way that less pool B and hence less LDL III is formed. This is an exciting new prospect in the treatment of ALP and prevention of coronary disease, with drugs that have been proven to be safe and clinically effective.

8.4 Directions for Future Research

A number of questions have arisen from the studies in this thesis, some of which are already being addressed and others which will be the focus of future work.

It is important to learn more about the inter-relationships between the LDL subfractions both in healthy subjects and those presenting with an atherogenic lipoprotein phenotype. Turnovers using radioactively labelled purified LDL I, LDL II and LDL III have already been carried out in three young normal males and incorporation of a tracer amino acid L-(5,5,5-$^2H_3$)-leucine (d_3-leucine) into the three subfractions has been conducted in those subjects in whom VLDL stable isotope turnovers are referred to in section 8.2. At present the data is at the limit of the number of compartments that SAAM 30 can handle and we await the new SAAM II version to analyse this data.

It would be interesting to investigate further the male/female differences in the formation of LDL III observed by Tan *et al* (1995) and examine the LDL distribution profile, hepatic lipase activity and androgen:oestrogen ratio in females with premature coronary disease. Studies are underway to measure these indices in pre- and post-menopausal female diabetics who appear to have lost their 'female protection' against coronary disease.

Further investigations are required to identify the nature of the receptor-independent catabolism of pool C apo-LDL and methodology will need to be developed to observe the binding properties and define the epitope expression to explain conformational differences.

There are only two publications in the literature to date on IDL and its subfractions. Evidence has been presented in this thesis that the precursors of LDL are very important in determining its structure and metabolism. Preliminary gradient gel electrophoresis has

been carried out and different IDL species have been identified. It is the intention to develop a quantitative method for the isolation and characterisation of IDL subfractions it in a similar way that has been described in chapter 3 for LDL subfractions.

References

Aalto-Setala K, Fisher EA, Chen X, Chajek-Shaul T, Hayek T, Zechner R, Walsh A, Ramakrishnan R, Ginsberg HN, Breslow JL. (1992) Mechanism of hypertriglyceridemia in human apolipoprotein (apo) C-III transgenic mice. *Journal of Clinical Investigation*, **90:**1889-1900.

Abbott WGH, Swinburn B, Ruotolo G, Hara H, Patti L, Grundy SM, Howard BV. (1990) Effect of a high carbohydrate diet, low saturated fat diet on apolipoprotein B and triglyceride metabolism in Pima Indians. *Journal of Clinical Investigation*, **86:**632-650.

Adams RF. (1974) Determination of amino acid profiles in biological samples by gas chromatography. *Journal of Chromatography*, **95:**189-212.

Alexander CA, Hamilton RL, Havel RJ. (1976) Subcellular localization of 'B' apoprotein of plasma lipoproteins in rat liver. *Journal of Cell Biology*, **69:**241-263.

Anber V, Griffin BA, McConnell M, Packard CJ, Shepherd J. (1996) Influence of plasma lipid and LDL-subfraction profile on the interaction between low density lipoprotein with human arterial wall proteoglycans. *Atherosclerosis*, **124:**261-271.

Austin MA & Krauss RM. (1986) Genetic control of low-density-lipoprotein subclasses. *Lancet*,**13:**592-594.

Austin MA, Breslow JL, Hennekens CH, Buring JE, Willett WC, Krauss RM. (1988) Low-density lipoprotein subclass patterns and risk of myocardial infarction. *Journal of the American Medical Association*, **260:**1917-1921.

Austin MA, King M-C, Vranzian KM, Newman B, Krauss RM. (1988) Inheritance of low-density lipoprotein subclass patterns: Results of complex segregation analysis. *American Journal of Human Genetics*, **43:**838-846.

Austin MA, King M-C, Vranizan KM, Krauss RM. (1990) Atherogenic Lipoprotein Phenotype A proposed genetic marker for coronary heart disease risk. *Circulation*, **82:**495-506.

Austin MA, Brunzell JD, Fitch WL, Krauss RM. (1990) Inheritance of low density lipoprotein subclass patterns in familial combined hyperlipidaemia. *Arteriosclerosis*, **10:**520-530.

Austin MA. (1991) Plasma triglyceride and coronary heart disease. Arteriosclerosis and *Thrombosis*, **11:**2-14.

Austin MA. (1992) Genetic epidemiology of low-density lipoprotein subclass phenotypes. *Annals of Medicine*, **24:**477-481.

Austin MA, Herowitz H, Wijsman E, Krauss RM, Brunzell J. (1992) Bimodality of plasma apolipoprotein B levels in familial combined hyperlipidemia. *Atherosclerosis*, 92:67-77.

Austin MA, Newman B, Selby JV, Edwards K, Mayer EJ, Krauss RM. (1993) Genetics of LDL subclass phenotypes in women twins. Concordance, heritability, and commingling analysis. *Arteriosclerosis and Thrombosis*, **13:**687-695.

Austin MA (1993) Genetics of low-density lipoprotein subclasses. *Current Opinion in Lipidology*, **4:**125-132.

Austin MA, Jarvik GP, Hokanson JE, Edwards KE. (1993) Complex segregation analysis of low-density lipoprotein peak particle diameter. *Genetics and Epidemiology*, **10:**599-604.

Avila EM, Lopez F, Camejo G. (1978) Properties of low density lipoprotein related to its interaction with arterial wall components. In vitro and in vivo studies. *Artery*, **4:**36-60.

Aviram M, Bierman EL, Chait A. (1988) Modification of low density lipoprotein by lipoprotein lipase or hepatic lipase induces enhanced uptake and cholesterol accumulation in cells. *Journal of Biological Chemistry*, **264:**15416-15422.

Arai H, Kita T, Yokode M, Naramiya S, Kawai C. (1989) Multiple receptors for modified LDL in mouse peritoneal macrophages: different uptake mechanisms for acetylated and oxidised LDL. *Biochemica et Biophysica Research Communications*, **159:**1373-1376.

Armstrong VW, Cremer P, Eberle E, Manke A, Schulze F, Wieland H, Kreuzer H, Siedel D. (1986) The association between serum Lp(a) concentrations and angiographically assessed coronary atherosclerosis: Dependence on serum LDL levels. *Atherosclerosis*, **62:**249-257.

Armstrong VW, Walli AK, Seidal D. (1985) Isolation characterization and uptake into human fibroblasts of an apo(a)-free lipoprotein obtained on reduction of lipoprotein(a). *Journal of Lipid Research*, **26:**13141323.

Assmann G, Schulte H. (1992) Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). *American Journal of Cardiology*, **70:**733-737.

Bainton D, Miller NE, Bolton CH. (1992) Plasma triglyceride and high-density-lipoprotein cholesterol as predictors of ischaemic heart disease in British men. *British Heart Journal*, **68:**60-66.

Barakat HA, Carpenter JW, McLendon VD, Khazanie P, Leggett N, Heath J, Marks R. (1990) Influence of obesity, impaired glucose tolerance, and NIDDM on LDL structure and composition: possible link between hyperinsulinemia and atherosclerosis. *Diabetes*, **139:**1527-1533.

Barbosa DS, Maranhao RC, Araujo FB, Chang YH, Hirata MH, Abdalla DSP. (1995) Sialic acid and oxidizability of low density lipoprotein subfractions of hyperlipidemic patients. *Clinical Biochemistry*, **28:**435-441.

Baumstark MW, Frey I, Berg A, Keul J. (1992) Influence of n-3 fatty acids from fish oils on concentration of high- and low-density lipoprotein subfractions and their lipid and apolipoprotein composition. *Clinical Biochemistry*, **25:**338-340.

Baumstark, MW, Frey I, Berg A. (1993) Acute and delayed effects of prolonged exercise on serum lipoptroteins. II. Concentrations and composition of low- density lipoprotein subfractions and very low-density lipoproteins. *European Journal of Applied Physiology*, **66:**526-530.

Beisegel U, Weber W, Ihrke G, Herz J, Stanley KK. (1989) The LDL-receptor-related protein, LRP, is an apolipoprotein E binding protein. *Nature*, **341:**162-164.

Beisegel U, Weber W, Bengtsson-Olivecrona G. (1991) Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proceedings of the National Academy of Sciences of USA*, **88:**8342-8346.

Beisegal U. (1995) Receptors for triglyceride-rich lipoproteins and their role in lipoprotein metabolism. *Current Opinion in Lipidology*, **6:**117-122.

Belfrage P, Vaughan M. (1969) Simple liquid-liquid partition system for isolation of labeled oleic acid from glycerides. *Journal of Lipid Research*, **10:**341-344.

Berg K. (1963) A new serum type system in man - the Lp system. *Acta Patholigica et Microbiologica Scandinavica*, **59:**369-382.

Berg A, Halle M, Baumstark MW, Frey I, Keul J. (1992) Physical activity, lipids and lipoprotein metabolism. The benefit of exercise and training in hyperlipidemia. In: Watson RR, Eisinger M (eds) *Exercise and disease*. CRC Press, Boca Raton, :26-36.

Berman M, Weiss MF. (1974) SAAM Manual, USPHS publication no 1703 US Government Printing Office, Washington DC.

Berman M, Hall M, Levy RI et al (1978) Metabolism of apo B and apo C lipoproteins in man : kinetic studies in normal and hyperlipoproteinemic subjects. *Journal of Lipid Research*, **19:**38-56.

Bernt J, gaumert R, Still J. (1978) Mode of action of the lipid lowering agents clofibrate and BM15.075 on cholesterol biosynthesis in rat liver. *Atherosclerosis*, **30:**147-152.

Betteridge DJ, Dodson PM, Durrington P, Hughes EA, Laker MF, Nicholls DP, Rees JAE, Seymour CA, Thompson GR, Winder AF, Winocour PH, Wray R. (1993) Management of hyperlipidaemia: guidelines of the British Hyperlipidaemia Association. *Postgraduate Medical Journal*, **69:**359-369.

Bilheimer DW, Stone NJ, Grundy SM. (1979) Metabolic studies in familial hypercholesterolemia. *Journal of Clinical Investigation*, 64:524-533.

Blanche PJ, Gong EL, Forte TM, Nichols AV. (1981) Characterization of human high-density lipoproteins by gradient gel electrophoresis. *Biochimica et Biophysica Acta*, **665:**408-419.

Boston RC, Grief PC, Berman M. (1982) CONSAM In: Berman M, Grundy SM, Howard BV, eds. *Lipoprotein Kinetics and Modeling.* New York: Academic Press Inc,:437-460.

Bottalico LA, Keesler GA, Fless GM, Tabas I. (1993) Cholesterol loading of macrophages leads to marked enhancement of native lipoprotein(a) and, apolipoprotein(a) internalization and degradation. *Journal of Biological Chemistry*, **268:**8569-8573.

Brown MS, Basu SK, Falck JR, Ho YK, Goldstein JL. (1980) The scavanger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. *Journal of Supramolecular Structure*, **13:**67-81.

Brown MS, Goldstein JL. (1984) How LDL receptors influence cholesterol and atherosclerosis. *Scientific American*,**251:**58-66.

Bruckert E, Dejager S, Chapman MR. (1993) Ciprofibrate therapy normalises the atherogenic low-density lipoprotein subspecies profile in combined hyperlipidemia. *Atherosclerosis*, 100:91-102.

Brunzell JD, Hazzard WR, Porte D, Bierman EL. (1973) Evidence for a common, saturable, triglyceride removal mechanism for chylomicrons and very low density lipoproteins in man. *Journal of Clinical Investigation*, **52:**1578-1585.

Bu G, Maksymovitch EA, Nerbonne JM, Schwartz AL (1994) Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian cell neurones. *Journal of Biological Chemistry*, **269:**18521-18528.

Camejo G, Lopez A, Lopez F, Quinones J. (1985) Interaction of low density lipoproteins with arterial proteoglycans. The role of charge and sialic acid content. *Atherosclerosis*, **55:**93-105.

Camejo G, Waich, S, Quintero G, Berrizbeita ML, Lalaguna F. (1976) The affinity of low density lipoproteins for an arterial macromolecular complex. A study in ischemic heart disease and controls. *Atherosclerosis*, **24:**341-354.

Campos H, McNamara JR, Wilson PWF, Ordovas JM, Schaefer EJ. (1988) Differences in low density lipoprotein subfractions and apolipoproteins in premenopausal and postmenopausal women. *Journal of Clinical Endocrinology and Metabolism*, **67:**30-35.

Campos H, Bailey SM, Gussak LS, Siles X, Ordovas JM, Schaefer EJ. (1991) Relations of body habits, fitness lecvel, and cardiovascular rosk factors including lipoproteins and apolipoproteins in a rural and urban Costa Rican population. *Arteriosclerosis and Thrombosis*, **11:**1077-1088.

Campos H, Bilijlevens E, Mc Namara JR, Ordovas JM, Posner BM, Wilson PWF, Castelli WP, Schaefer EJ. (1992) Low density lipoprotein particle size: Results from the Framingham offspring study. *Arteriosclerosis and Thrombosis*, **12:**1412-1419.

Campos H, Genest JJ, Blijlevens E, McNamara JR, Jenner JL, Ordovas JM, Wilson PW, Schaefer EJ. (1992) Low density lipoprotein particle size and coronary artery disease. *Arteriosclerosis and Thrombosis*, **12:**187-195.

Campos H, Sacks FM, Walsh BW, Schiff I, O'Hanesian MA, Krauss RM. (1993) Differential effects of estrogen on low-density lipoprotein subclasses in healthy postmenopausal women. *Metabolism*, **42:**1153-1158.

Campos H, Dreon D, Krauss RM. (1995) Associations of hepatic and lipoprotein lipase activities with changes in dietary composition and low density lipoprotein subclasses. *Journal of Lipid Research*, **36:**462-472.

Campos H, Roederer GO, Lussier-Cacan S, Davignon J, Krauss RM. (1995) Predominance of large LDL and reduced HDL_2 in normolipidemic men with coronary artery disease. *Arteriosclerosis, Thrombosis and Vascular Biology*, **15:**1043-1048.

Campos H, Arnold KS, Balestra ME, Innerarity TL, Krauss RM. (1996) Differences in receptor binding of LDL subfractions. *Arteriosclerosis, Thrombosis and Vascular Biology*, **16:**794-801.

Carew TE, Schwenke DC, Steinberg D. (1987) Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can

selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe hyperlipidemic rabbit. *Proceedings of the National Academy of Sciences of the USA*, **84:**7725-7729.

Caslake MJ, Packard CJ, Series JJ, Yip B, Dagen MM, Shepherd J. (1992) Plasma triglyceride and low density lipoprotein metabolism. *European Journal of Clinical Investigation*, **22:**96-104.

Caslake MJ, Packard CJ, Gaw A, Murray E, Griffin BA, Vallance BD, Shepherd J. (1993) Fenofibrate and LDL metabolic heterogeneity in hypercholesterolemia. *Arteriosclerosis and Thrombosis*, **13:**702-711.

Castelli WP, (1986) The triglyceride issue: A view from Framingham. *American Heart Journal*, **112:**432-437.

Castro GR, Fielding CJ. (1988) Early incorporation of cell derived cholesterol into preβ-migrating high density lipoproteins. *Biochemistry*, **27:**25-29.

Chait A, Foster DM, Albers JJ et al. (1986) Low density lipoprotein metabolism in familial combined hyperlipidemia and familial hypercholesterolemia. Kinetic analysis using an integrated model. *Metabolism*, **35:**697-704.

Chait A, Berzg RL, Tribble DL, Krauss RM. (1993) Susceptibility of small, dense low density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *American Journal of Medicine*, **94:**350-356.

Chapman MJ, Laplaud PM, Luc G, Forgez P Bruckert E, Goulinet S, Lagrange D. (1988) Further resolution of the low density lipoprotein spectrum in normal human plasma : physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifigation. *Journal of Lipid Research*, **29:**442-458.

Chapman MJ, Bruckert E. (1996) The atherogenic role of triglycerides and small, dense low density lipoproteins: impact of ciprofibrate therapy. *Atherosclerosis* 124:S21-S28.

Chappell DA, Fry GL, Waknitz MA, Berns JJ. (1991) Ligand size as a determinant of catabolism by the low density lipoprotein (LDL) receptor pathway. *Journal of Biological Chemistry*, **266:**19296-19302.

Chatterton JE, Phillips ML, Curtiss LK et al. (1991) Mapping apolipoprotein B on the low density lipoprotein surface by immunoelectron microscopy. *Journal of Biological Chemistry*, **266:**5955-5962.

Chen ZM, Peto R, Collins R et al. (1991) Serum cholesterol concentration and coronary heart disease in a population with low cholesterol concentrations. *British Medical Journal*, **303:**276-282.

Cheung MC, Albers JJ. (1982) Distribution of high density lipoprotein particles with different apoprotein composition: Particles with A-I and A-II and particles with A-I but no A-II. *Journal of Lipid Research*, **23:**747-753.

Cheung MC, Albers JJ. (1984) Characterization of lipoprotein particles isolated by immunoaffinity chromatography. *Journal of Biological Chemistry*, **259:**12201-12209.

Cheung MC, Brown BG, Wolf AC, Albers JJ. (1991) Altered particle size distribution of apolipoprotein A-I containing lipoproteins in subjects with coronary artery disease. *Journal of Lipid Research*, **32:**383-394.

Christensen NJ, Rubin CE, Cheung et al (1983) Ultrastructural immunolocalization of apolipoprotein B within human jejunal absorptive cells. *Journal of Lipid Research,***24:**1229-1242.

Cobelli C, Toffolo G, Foster D.. (1992) Tracer-to-tracee ratio for analysis of stable isotope tracer data: link with radioactive kinetic formalism. *American Journal of Physiology,* **262:**E968-E975.

Cohen JC, Wang Z, Grundy SM, et al. (1994) Variation at the hepatic lipase and apolipoprotein A-I/C-III/A-IV loci is a major cause of genetically determined variation in plasma HDL cholesterol levels. *J Clin Invest,* **94:**2377-2384.

Consam User's Manual for Consam Version 30. (1990) Resource Facility for Kinetic Analysis, department of Bioengineering, University of Washington, Seattle.

Coresh J, Kwiterovich P, Smith HH, Bachorik PS. (1993) Association of plasma triglyceride concentration and LDL particle diameter, density, and chemical composition with premature coronary artery disease in men and women. *Journal of Lipid Research,* **34:**1687-1697.

Cremer P, Nagel D, Labrot B, Mann H, Muche R, Elster H, Siedel D. (1994) Lipoprotein Lp(a) as predictor of mypcardial infarction: Results from the prospective Gottingen Risk Incidence and Prevention Study. *European Journal of Clinical Investigation,* **24:**444-453.

Crouse JR, Parks JS, Schey HM, Kahl FR. (1985) Studies of low density lipoprotein molecular weight in human beings with coronary artery disease. *Journal of Lipid Research,* **26:**566-574.

Cummings MH, Watts GF, Umpleby M, Hennessy TR, Quiney JR, Sonksen PH. (1995) Increased hepatic secretion of very low density lipoprotein apolipoprotein B_{100} in heterozygous familial hypercholesterolemia : a stable isotope study. *Atherosclerosis,* **113:**79-89.

Curry MD, Gustafson A, Alaupovic P, McConathy WJ. (1978) Electroimmunoassay, radioimmunoassay and radial immunodiffusion assay evaluated for quantification of human apolipoprotein B. *Clinical Chemistry,* **24:**280-286.

Day JR, Albers JJ, Lofton-Day CE et al. (1994) Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. *Journal of Biological Chemistry,* **269:**9388-9391.

de Graaf J, Hak-Lemmers HLM, Hectors MPC, Demacker PNM, Hendriks JC, Stalenhoef AFH. (1991) Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arteriosclerosis and Thrombosis,* **11:**298-306.

de Graaf J, Hendriks JCM, Demaker P, Stalenhoef AFH. (1993) Identification of multiple dense LDL subfractios with enhanced susceptibility to in vitro oxidation among hypertriglyceridemic subjects. Normalization after clofibrate treatment. *Arteriosclerosis and Thrombosis,* **13:**712-719.

de Graaf J, Swinkels DW, DeHaan, afj, Demacker PNM, Stalenhoef AFH. (1992) Both inherited susceptibility and environmental exposure determine low density lipoprotein subfraction pattern distribution in healthy Dutch families. *American Human Journal of Genetics,* **51:**1295-1310.

de Rikje YB, van Berkel ThJC. (1989) Rat liver Kupffer and endothelial cells express different uptake mechanisms for acetylated and oxidised LDL. *Biochemica et Biophysica Research Communications* ,**159:**1375-

Deckelbaum RJ, Eisenberg S, Oschry Y, et al. (1982) Abnormal high density lipoproteins of abeta-lipoproteinemia: relevance to normal HDL metabolism. *Journal of Lipid Research*, **23:**1274-1282.

Dejager S, Bruckert E, Chapman MJ. (1993) Dense low density lipoprotein subspecies with diminished oxidative resistance predominate in combined hyperlipidemia. *Journal of Lipid Research*, **34:**295-308.

Demant T, Carlson LA, Holmquist L, et al. (1988) Lipoprotein metabolism in hepatic lipase deficiency: studies on the turnover of apolipoprotein B and on the effect of hepatic lipase on high density lipoprotein. *J Lipid Res*, **29:**1603-1611.

Demant T, Bedford D, Packard CJ, Shepherd J. (1991) Influence of apolipoprotein E polymorphism on apolipoprotein B-100 metabolism in normolipemic subjects. *Journal of Clinical Investigation*, **88:**561-568.

Demant T, Packard CJ, Demmelmair H, Stewart P, Bedynek A, Bedford D, Seidel D. (1994) Sensitive mass spectrometry techniques for measuring metabolism of human apolipoprotein B in vivo. *Clinical Chemistry*, **40:**1825-1827.

Demant T, Packard CJ, Stewart P, Bedynek A, Calder AG, Shepherd J, Seidel D, Shepherd J. (1996) Sensitive methods to study human apolipoprotein B metabolism using stable isotope labelled amino acids. *American Journal of Physiology*

Demuth K, Myara I, Chappry B, Vedie B, Pech-Amsellum MA, Haberland ME, Moatti N. (1996) A cytotoxic electronegative LDL subfraction is present in human plasma. *Arteriosclerosis, Thrombosis and Vascular Biology*, **16:**773-783.

Dietschy JM, Wilson JD. (1970) Regulation of cholesterol metabolism. *New England Journal of Medicine*, **282:**1128-1138.

Dixon JL, Ginsberg HN. (1993) Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: Information obtained from cultured liver cells. *Journal of Lipid Research*, **34:**167-179.

Doi T, Higashino K, Kurihara Y, Wada Y, Miyazaki T, Nakamura H, Uesugi S, Imanishi T, Kawabe Y, Itakura H, Yazaki Y, Matsumoto A, Kodama T. (1993) Charged collagen structure mediates the recogntion of negatively charged macromolecules by macrophage scavenger receptors. *Journal of Biological Chemistry*, **268:**2126

Doolittle MH, Ben-Zeev O, Elovson J et al. (1990) The response of lipoprotein lipase to feeding and fasting. Evidence for posttranslational regulation. *Journal of Biochemical Chemistry*, **265:**4570-4577.

Dreon DM, Fernstrom HA, Miller B, Krauss M. (1995) Apolipoprotein E isoform phenotype and LDL subclass response to a reduced-fat diet. *Arteriosclerosis, Thrombosis and Vascular Biology*, **15:**105-111.

Driscoll DM, Cassanova E. (1990) Characterization of the apolipoprotein B mRNA editing activity in enterocyte extracts. *Journal of Biological Chemistry*, **265:**21401-21403.

Durrington PN, Ishola M, Hunt L, Arrol S, Bhatnagar D. (1988) Apolipoproteins (a), AI and B and parental history in men with early onset ischaemic heart disease. *Lancet*,1070-1073.

Duverger N, Rader D, Duchateau P, Fruchart J-C, Castro G, Brewer HB Jr. (1993) Biochemical characterization of the three major subclasses of lipoprotein A-I preparatively isolated from human plasma. *Biochemistry*, **32:**12372-12379.

Eckel RH. (1989) Lipoprotein lipase: a malfunctional enzyme relevant to common metabolic diseases. *New England Journal of Medicine*, **320:** 1060-1068.

Enerback S, Semb H, Tavernier J et al. (1988) Tissue-specific regulation of guinea pig lipoprotein lipase: Effects of nutritional state and of tumor necrosis factor on mRNA levels in adipose tissue, heart and liver. *Gene*, **64:**97-106.

Eisenberg S. (1984) High density lipoprotein metabolism. *Journal of Lipid Research*, **25:**1017-1058.

Eisenberg S, Gavish, D, Oschry Y, Fainaru M, Deckelbaum RJ. (1984) Abnormalities in very low, low, and high density lipoproteinsin hypertriglyceridemia. *Journal of Clinical Investigation*, **74:**470-482.

Eisenberg S, Sehayek E, Olivecrona T, Vlodavsky I. (1992) Lipoprotein lipase enhances binding of lipoproteins to heparan sulphate on cell surfaces and extracellular matrix. *Journal ,of Clinical, Investigation*, **90:**2013-2021.

Egusa GD, Brady DW, Grundy SM, Howard BV. (1983) Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein apolipoproteon B. *Journal of Lipid Research*, **24:**1261-1267.

Ericsson S, Erikssn M, Vitols S, Einarsson K, Berglund L, Angelin B. (1991) Influence of age on the metabolism of plasma low density lipoproteins in healthy males. *Journal of Clinical Investigation*, **87:**591-596.

Ericsson C-G, Hamsten A, Nilson J. (1996) Angiographic assessment of effects of bezafibrate on progression of coronary disease in young male postinfarction patients. *Lancet*, **347:**849-853.

Esterbauer H, Dieber-Rotheneder M, Strigler G, Waeg G. (1991) Role of vitamin E in preventing the oxidation of low-density lipoprotein. *American Journal of Clinical Nutrition*, **53:**314S-321S.

Filipovic I, Schwarzmann G, Mraz W, Wiegant H, Buddecke E. (1979) Sialic -acid content of low-density lipoproteins controls their binding and uptake by cultured cells. *European Journal of Biochemistry*, **93:**51-55.

Fisher WR, (1982) Apoprotein B kinetics in man: concepts and questions. In: Berman M, Grundy SM, Howard BV, eds. *Lipoprotein Kinetics and Modeling*. New York: Academic Press, 43-68.

Fisher WR, (1983) Heterogeneity of plasma low density lipoproteins. Manifestations of the physiologic phenomenon in man. *Metabolism*, **32:**283-291.

Fless GM, Rolih CA, Scanu AM. (1984) Heterogeneity of human plasma lipoprotein(a). Isolation and characterization of the of the lipoprotein sub-species and their apoproteins. *Journal of Biological Chemistry*, **259:**11470-11478.

Frick MH, Elo O, Hapa K, Heinonen OP, Heinsami P, Huttunen JK, Kaitaneimi P, Koskinen P, Manninen V, Maenpaa H, Malkonen M, Manttari M, Norola S,

Pasternack, A, Pikkarainen J, Romo M, Sjoblom T, Nikkila EA. (1987) Helsinki Heart Study: Primary prevevtion trial with genfribrozil in middle-aged men with dyslipidemia. *New England Journal of Medicine*, **317:**1237-1245.

Francheschini G, Bernini F, Michelagnoli S, Bellosta S, Vaccarino V, Fumagalli R, Sirtori CR. (1990) Lipoprotein changes and increased affinity of LDL for their receptors after acipimox treatment in hypertriglyceridemia. *Atherosclerosis*, **81:**41-49.

Francheschini G, Lovati MR, Manzoni C, Michelagnoli S, Pazzucconi F, Gianfranceschi G, Vecchio G, Sirtori C. (1995) Effect of gemfibrozil treatment in hypercholesterolemia on low density lipoprotein (LDL) subclass distribution and LDL-cell interaction. *Atherosclerosis*, **114:**61-71.

Francke U, Brown MS, Goldstein JL. (1984) Assignment of the human gene for the low density lipoprotein receptor to chromosome 19: synteny of a receptor, a ligand and a genetic disease. *Proceedings of the National Academy of Science USA*, **81:**2826-2834.

Frayn KN. (1993) Insulin resistance and lipid metabolism. *Current Opinion of Lipidology*, **4:**197-204.

Freeman DJ, Griffin BA, Holmes AP, et al. (1994) Regulation of plasma HDL cholesterol and subfraction distribution by genetic and environmental factors. *Arteriosclerosis and Thrombosis*, **14:**336-344.

Freeman DJ, Packard CJ, Shepherd J, Gaffney D. (1990) Polymorphisms on the gene coding for cholesteryl ester transfer protein are related to plasma high-density lipoprotein cholesterol and transfer activity. *Clinical Science*, **79:**575-581.

Forster L, Stewart G, Bedford D, Stewart P, Caslake M, Packard C, Shepherd. (1996a) Stable isotope studies of lipoprotein kinetics in combined hyperlipidaemia before and after treatment with simvastatin. *Atherosclerosis*, in press.

Forster LF, Stewart G, Bedford DK, Stewart JP, Caslake MJ, Packard CJK, Shepherd J, Black DM. (1996b) Stable isotope turnover studies of apolipoprotein B in combined hyperlipidaemia before and after treatment with atorvastatin, a new HMG-CoA reductase inhibitor. *Circulation*, in press

Foster DM, Chait A, Albers JJ, Failor RA, Harris C, Brunzell. (1986) Evidence for kinetic heterogeneity among human low density lipoproteins. *Metabolism*, **8:**685-696.

Forte TM, Nordhausen RW. (1986) Electron microscopy of negatively stained lipoproteins. *Methods in Enzymology*, **128:**442-457.

Gaffney D, Reid JM, Cameron IM, Vass K, Caslake MJ, Shepherd CJ. (1995) Independent mutations at codon 3500 of the apolipoprotein B gene are associated with hyperlipidemia. *Arteriosclerosis, Thrombosis and Vascular Biology*, **15:**1025-1029.

Galeano NF, Milne R, Marcel YL, Walsh MT, Levy E, Thanh-Dung Ngu'Yen, Gleeson A, Arad, Y, Witte L, Al-Haider M, Rumsey SC, Deckelbaum RJ. (1994) Apoprotein B structure and receptor recognition of triglyceride-rich low density lipoprotein (LDL) is modified in small LDL but not in triglyceride-rich LDL of normal size. *Journal of Biological Chemistry*, **269:**511-519.

Gamberrt P, Bouzerand-Gambert C, Athias A, Farnier M, Lallemant C. (1990) Human low density lipoprotein subfractions separated by gradient gel electrophoresis: composition, distribution, and alterations induced by cholesteryl ester transfer protein. *Journal of Lipid Research*, **31:**1199-1210.

Gavish D, Oschry Y, Fainaru M, Eisenberg S. (1986) Change in very low-, low-, and high-density lipoproteins during lipid lowering (bezafibrate) therapy: studies in the type IIa and type IIb hyperlipoproteinemia. *European Journal of Clinical Investigation*, **16:**61-68.

Gaw A, Griffin BA, Caslake MJ, Collins SM, Lorimer AR, Packard CJ, Shepherd J. (1990) Effects of acipimox on apolipoprotein B metabolism and low density subfraction distribution in hypercholesterolaemic subjects. *Journal of Drug Development*, **3**(Supplement 1):107-109.

Gaw A, Packard CJ, Murray EF, Lindsay GM, Griffin BA, Caslake MJ, Vallance BD, Lorimer AR, Shepherd J. (1993) Effects of simvastatin on apo B metabolism and LDL subfraction distribution. *Arteriosclerosis and Thrombosis*, **13:**170-189.

Gaw A, Packard CJ, Lindsay GM, Griffin B, Caslake MJ, Lorimer AR, Shepherd J. (1995) Overproduction of small very low density lipoproteins (Sf 20-60) in moderate hypercholesterolemia: relationshps between apolipoprotein B kinetics and plasma lipoproteins. *Journal of Lipid Research*, **36:**158-171.

Gaw A, Packard CJ, Lindsay GM, Murray EF, Griffin BA, Caslake MJ, Colquhoun I, Wheatley, DJ, Lorimer AR, Shepherd J. (1996) Effects of colestipol alone and in combination with simvastatin on apolipoprotein B metabolism. *Arterisclerosis, Thrombosis and Vascular Biology*, **16:**236-249.

Genest J Jr, Jenner JL, McNamara JR, Ordovas JM, Silberman SR, Wilson PWF, Schaefer EJ. (1991) Prevalence of lipoprotein (a) (Lp(a)) excess in coronary artery disease. *American Journal of Cardiology*, **67:**1039-1045.

Genest J Jr, Martin-Munley SS, McNamara JR, Ordovas JM, Jenner JL, Meyers RH, Silberman SR, Wilson PW, Salem DN, Schaefer EJ. (1992) Familial lipoprotein disorders in patients with premature coronary artery disease. *Circulation*, **85:**2025-2033.

Ginsberg HN, Lee N-A, Goldberg IJ, et al. (1986) Apolipoprotein B metabolism in subjects with a deficiency in apolipoproteins C-III and A-I. *Journal of Clinical Investigation*, **78:**1287-1295.

Goebel R, Garnick M, Berman M. (1976) A new model for low density lipoprotein kinetics: evidence for two labeled moieties. *Circulation*, **54** (Supplement II):4.

Gofman JW, Jones HB, Lindgren FT, Lyon TP, Elliott HA, Strisower B. (1950) Blood lipids and human atherosclerosus. *Circulation*, **2:**161-177.

Goldstein JL, Ho YK, Basu SK, Brown MS. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proceedings of the National Academy of Sciences of the USA*, **76:** 333-337.

Goldstein JL, Browm MS. (1984) Progress in understanding the LDL receptor and HMG Co A reductase, two membrane proteins that regulate plasma cholesterol. *Journal of Lipid Research*, **25:**1450-1461.

Gottlicher M, Widmark E, Li Q, Gustafsson JA. (1992) Fatty acids activate chimera of the clofibric acid-activated receptor and glucocorticoid receptor. *Proceedings of the National Academy of Sciences USA*, **89:**4653-4657.

Grainger DJ, Kemp PR, Liu AC, Lawn RM, Metcalfe JC. (1994) Activation of transforming growth factor-β is inhibited in apolipoprotein(a) transgenic mice. *Nature*, **370:**460-462.

Grainger DJ, Kemp PR,Metcalfe JC, Liu AC, Lawn RM, Williams NR, Grace AA, Schofield PM, Cauhan A. (1995) The serum concentration of active transforming growth factor-β is severely depressed in advanced atherosclerosis. *Nature Medicine*, **1:**74-79.

Grainger DJ, Kirschenlohr HL, Metcalfe JC, Weissberg PL, Wade DP, Lawn RM. (1993) The proliferation of human smooth muscle,cells is promoted by lipoprotein(a). *Science*, **260:**1655-1658.

Green PHR, Glickman RM, Saudek CD, Blum CB, Tall AR. (1979) Human intestinal lipoproteins. Studies in chyluric subjects. *Journal of ClinicalInvestigation*, **64:**233-242.

Griffin BA, Caslake MJ, Yip B, Tait GW, Packard CJ, Shepherd J. (1990) Rapid isolation of low density (LDL) subfractions from plasma by density gradient ultracentrifugation. *Atherosclerosis*, **83:**59-67.

Griffin BA, Freeman DJ, Tait GW, Thomson J, Caslake MJ, Packard CJ, Shepherd J. (1994) Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis*, **106:**241-253.

Groot PHE, Scheek LM, Havekes L, Noort WL, van't Hooft FM. (1982) A one-step peparation of human serum high density lipoproteins 2 and 3 by rate-zonal density gradient ultracentrifugation in a swinging bucket rotor. *Journal of Lipid Research*, **23:**1342-1347.

Guerin M, Bruckert E, Dolphin P, Turpin G, Chapman MJ. (1996) Fenofibrate reduces plasma cholesteryl ester transfer from HDL to VLDL and normalizes the atherogenic, dense LDL profile in combined hyperlipidemia. *Arteriosclerosis Thrombosis and Vascular Biology*, **16:**763-772.

Haberland ME, Fong D, Cheng L. (1988) Malondialdehyde-altered protein occurs in atheroma of Watanebe Heritable Hyperlipidemic rabbits. *Science*, **241:**215-218.

Haberland ME, Fless GM, Scanu AM, Fogelman AM. (1992) Malondialdehyde modification of lipoprotein(a) produces avid uptake by human monocyte-macrophages. *Journal of Biological Chemistry*, **267:**4143-4151.

Hamilton RL. (1994) Apolipoprotein-B-containing plasma lipoproteins in health and in disease. *Trends in Cardiovascular Medicine*, **4:**131-139.

Hamilton RL, Havel RJ. (1993) Is microsomal triglyceride transfer protein the missing link in abetalipoproteinemia? *Hepatology*, **18:**460.

Hamilton RL, Moorehouse A, Havel RJ. (1991) Isolation and properties of nascent lipoproteins from highly purified rat hepatocytic Golgi fractions. *Journal of Lipid Research*, **32:**529-543.

Hammond MG, Fisher WR. (1971) The characterization of a discrete series of low density lipoproteins in the disease, hyper-prebetalipoproteinemia. *Journal of Biological Chemistry*, 246:5454-5465.

Hammond MG, Mengel MC, Warmke GL, Fisher WR. (1977) Macromolecular dispersion of human plasma low density lipoproteins in hyperlipoproteinemia. *Metabolism*, **26:**1231-

Hana H, Yokoyama S. (1992) Role of apolipoproteins in cholesterol efflux from macrophages to lipid micro-emulsion. Proposal of a putative model for the pre-β-high density lipoprotein pathway. *Biochemistry*, **31:**2040-2046.

Harpel PC, Gordon BR, Parker TS. (1989) Plasmin catalyzes binding of lipoprotein(a) to immobilized fibrinogen and fibrin. *Proceedings of the National Academy of Sciences of USA*, **86:**3847-3851.

Haubenwallner S, Essenberg AD, Barnett BC, Pape ME, DeMattos RB, Krause BR, Minton LL, Auerbach BJ, Newton RS, Leff T, Bisgaier CL. (1995) Hypolipidemic activity of select fibrates correlates to changes in hepatic apolipoprotein C-III expression: a potential physiologic basis for their mode of action. *Journal of Lipid Research*, **36:**541-2551.

Havekes LM, de Knijff P, Beisegel U, Havinga J, Smit M, Klasen E. (1987) A rapid micromethod for apolipoprotein E phenotyping directly in serum. *Journal of Lipid Research*, **28:**455-463.

Havel RJ, Eder HA, Bragdon JH. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *Journal of Clinical Investigation*, **34:**1345-1353.

Havel RJ, Kane JP, Kashyap ML. (1973) Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man. *Journal of Clinical Investigation*, **52:**32-38.

Hayek T, Azrolan N, Verdery RB. (1993) Hypertriglyceridemia and cholesteryl ester transfer protein interact to dramatically alter high density lipoprotein levels, particle sizes and metabolism. *Journal of Clinical Investigation*, **92:**1143-1152.

Hoeg JM, Demosky Jr SJ, Gregg RE, Dchaefer EJ, Brewer HB. (1985) Distinct hepatic receptors for low density lipoproteins and apolipoprotein E in humans. *Science*, **227:**759-761.

Hofer F, Gruenberger M, Kowalski H, Machat H, Huettinger M, Kuechler E, Blaas D. (1994) Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proceedings of the National Academy of Sciences USA*, **91**:1839-1842.

Hoff HF, Morton RE. (1987) Uptake of LDL sized particles extracted from human aortic lesions by macrophages in culture. In Gallo ed, *Cardiovascular Disease*: 103-119, Plenum Press, New York.

Hokanson JE, Austin MA. (1993) Triglyceride and coronary heart disease: meta- analysis of population-based propecrive studies. *Circulation*, **88**:1-510.

Hokanson JE, Austin MA, Zambon, A Brunzell J. (1993) Plasma triglyceride and LDL heterogeneity in familial combined hyperlipidemia. *Arteriosclerosis and Thrombosis*, **13:**427-434.

Hokanson JE, Krauss RM, Albers JJ, Austin MA, Brunzell JD. (1995) LDL physical and chemical properties in familial combined hyperlipidemia. *Arteriosclerosis, Thrombosis and Vascular Biology*, **15:**452-459.

Holmquist R . (1982) Surface modification of ultraclear centrifuge tubes. *Journal of Lipid Research*, **23:**1249-1250.

Homma Y, Moriguchi EH, Sakane H, Ozawa H, Nakamura H, Goto Y. (1991) Effects of probucol on plasma lipoprotein subfractions and activities of lipoprotein lipase and hepatic triglyceride lipase. *Atherosclerosis*, **88:**175-181.

Homma Y, Ozawa H, Kobayashi T, Yamaguchi H, Sakane H, Mikami Y, Nakamura H. (1994) Effects of bezafibrate therapy on subfractions of plasma low-density lipoprotein and high-density lipoprotein, and on activities of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in patients with hyperlipoproteinemia. *Atherosclerosis,* **106:**191-201.

Homma Y, Ozawa H, Kobayashi T, Yamagushi H, Sakane H, Nakamura H. (1995) Effects of simvastatin on plasma lipoprotein subfractions, cholesterol esterification rate, and cholesteryl ester transfer protein in type II hyperlipoproteinemia. *Atherosclerosis*, **114:**223-234.

Houmard JA, Bruno NJ, Bruner RK, McCammon MR, Israel RG, Baraket HA. (1994) Effects of exercise training on the chemical composition of plasma LDL. *Arteriosclerosis and Thrombosis*, **14:**325-330

Huff MW, Telford DE Woodcroft K, Strong WLP. (1985) Mevinolin and cholestyramine inhibit the direct synthesis of low density lipoprotein apolipoprotein B in miniature pigs. *Journal of Lipid Research, 26:*11175-11186.

Hurt-Camejo E, Camejo G, Rosengren B, Lopez F, Wiklund O, Bondjers G. (1990) Differential uptake of proteoglycan-selected subfractions of low density lipoprotein by human macrophages. *Journal of Lipid Research*, **31:**1387-

Imaizumi K, Fainaru M, Havel RJ. (1978a) Composition of proteins of mesenteric lymph chylomicrons in the rat and alterations produced upon exposure of chylomicrons to blood serum and serum proteins. *Journal of Lipid Research*, 19:712-722.

Imaizumi K, Havel RJ, Fainaru M et al. (1978b) Origin and transport of the AI and arginine-rich apolipoproteins in mesenteric lymph of rats. *Journal of Lipid Research*, **19:**1038-1046.

Innerarity TL, Mahley RW, Weisgraber KH, Bersot TP, Krauss RM, Vega GL, Grundy SM, Friedl W, Davignon J, McCarthy BJ. (1990) Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolaemia. *Journal of Lipid Research*, **31:**1337-1349.

International Task Force for Prevention of Coronary Heart Disease. (1992) Prevention of coronary heart disease: scientific background and new clinical guidelines. Recommendations of the European Atherosclerosis Society. *Nutrition, Metabolism and Cardiovascular Diseases*, **2:**113-156.

Ishida BY, Frolich J, Fielding CJ. (1987) Pre-beta migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmumoassay following electrophoretic transfer. *Journal of Lipid Research*, **28:**778-786.

Jaakkola O, Solakivi T, Yla-Herttuala S, Nikkari T. (1989) Receptor-mediated binding and degradation of subfractions of human plasma low-density lipoprotein by cultured fibroblasts. *Biochimica et Biophysica Acta,* **1005:**118-122.

Jaakkola O, Solakivi T, Tertov VV, Orekhov AN, Miettinen TA, Nikkari T. (1993) Characteristics of low-density lipoprotein subfractions with coronary artery disease. *Coronary Artery Disease,* **4:**379-385.

James RW, Pometta D. (1990) Differences in lipoprotein subfraction composition and distribution between type I diabetic men and control subjects. *Diabetes,* **39:**1158-1164.

James RW, Pometta D. (1994) Postprandial lipaemia differentially influences high density lipoprotein subpopulation LpA-I and LpA-I, A-II. *Journal of Lipid Research,* **93:**1583- 1591.

Jansen H, Hop W, van Tol A, Brusche AVG, Birkenhager JC. (1994) Hepatic lipase and lipoprotein lipase are not major determinants of the low density lipoprotein subclass pattern in human subjects with coronary heart disease. *Atherosclerosis,* **107:**45-54.

Janus ED, Nicoll AM, Turner PR, Magill P, Lewis B. (1979) Kinetic basis of the primary hyperlipidaemias: studies of apolipoprotein B turnover in genetically defined subjects. *European Journal of Clinical Investigation,* **10:**161-172.

Jauhiainen M, Koskinen P, Ehnholm C, Frick MH, Manttari M, Manninen V, Huttunen JK. (1991) Lipoprotein(a) and coronary heart disease risk: A case-control study of the Helsinki Heart Study participants. *Atherosclerosis,* **89:**59-67.

Jokinen EV, Landschulz KT, Wyne KL, Ho YK, Frykman PK, Hobbs HH. (1994) Regulation of the very low density lipoprotein receptor by thyroid hormone in rat skeletal muscle. *Journal of Biological Chemistry,* **269:**26411-26418.

Kane JP, Sata T, Hamilton RL, Havel RJ. (1975). Apoprotein composition of very low density lipoproteins of human serum. *Journal of Clinical Investigation,* **56:**1622-1634.

Kane JP, Hardman DA, Paulus HE. (1980) Heterogeneity of apolipoprotein B : isolation of a new species from human chylomicrons. *Proceedings of the National Academy of Sciences (USA),* **77:**2465-2469.

Kannel WB, Castellli WP, Gordon T, McNamara PM. (1971) Serum cholesterol, lipoproteins and the risk of coronary heart disease. The Framingham Study. *Annals of Internal Medicine,* **74:**1-12.

Karpe F, Tornvall P, Olivecrona T, Steiner G, Carlson L, Hamsten A. (1993) Composition of human low density lipoprotein: effects of postprandial triglyceride-rich lipoproteins, lipoprotein lipase, hepatic lipase and cholesteryl ester transfer protein. *Atherosclerosis,* **98:**33-49.

Kayden HJ, Traber MG. (1993) Absorption, lipoprotein transport and regulation of plasma concentrations of vitamin E in humans. *Journal of Lipid Research,* **34:**343-358.

Katzel LI, Coon PJ, Rogus E, Krauss RM, Goldberg AP. (1995) Persistence of low HDL-C levels after weight reduction in older men with small LDL particles. *Arteriosclerosis and Thrombosis,* **15:**299-305.

Kesaniemi YA, Witzum JL, Steinbrecher UP. (1983) Receptor-mediated catabolism of low density lipoprotein in man. Quantitation using glucosylated low density lipoprotein. *Journal of Clinical Investigation*, **71**:950-959.

Kesaniemi YA, Grundy SM. (1982) Significance of low density lipoprotein production in the regulation of plasma cholesterol level in man. *Journal of Clinical Investigation*, **70:**13-22.

Kissebah AH, Adams PW, Harrigan P, Wynn V. (1974) The mechanism of action of clofibrate and tetranicotinoylfructose (bradilin) on the kinetics of plasma free fatty acid and triglyceride transport in type IV and type V hypertriglyceridemia. *European Journal of Clinical Investigation*, **4:**163-174.

Kissebah AH, Peiris AN. (1989) Biology of regional fat distribution: relationship to non-insulin dependent diabetes mellitus. *Diabetic Metabolism Reviews*, **5:**83-109.

Kleinman Y, Eisenberg, Oschry Y, Gavish D, Stein O, Stein Y. (1985) Defective metabolism of hypertriglyceridemic low density lipoprotein in cultured human skin fibroblasts. Normalizaton with bezafibrate therapy. *Journal of Clinical Investigation*, **75:**1796-1803.

Kleinman Y, Schonfeld G, Gavish D, Oschry Y, Eisenberg S. (1987) Hypolipidemic therapy modulates expression of apolipoprotein B epitopes on low density lipoproteins. Studies in mild to moderate hypetriglyceridemic patients. *Journal of Lipid Research*, **28:**540-548.

Knight BL, Thompson GR, Soutar AK. (1986) Binding and degradation of heavy and light subfractions of low density lipoprotein by cultured fibroblasts and macrophages. *Atherosclerosis*, **59:**301-306.

Kodama t, Freeman M, Roher L, Zabrecky J, Matsudaira P, Krieger M. (1990) Type I macrophage scavanger receptor contains α-helical and collagen-like coiled coils. *Nature*, **343:**531-

Koizumi J, Inazu A, Kunimas Y, Ichiro K, Uno Y, Jakinami K, Miyamoto S, Molin P, Tall AR, Mabuchi H, Takeda R. (1994) serum lipoprotein lipids concentrations and composition in homozygous and heterozygous patients with cholesteryl ester transfer protein deficiency. *Atherosclerosis*, **90:**189-196.

Kondo I, Berg K, Drayna D, et al. (1989) DNA polymorphism at the locus for cholesteryl ester transfer protein (CETP) is associated with high density lipoprotein cholesterol and apolipoprotein levels. *Clinical Genetics*, **35:**49-56.

Krause BR, Sloop CH, Castle CK, Roheim PS. (1981) Mesenteric lymph apolipoproteins in control and ethinyl estradiol-treated rats: a model for studying apolipoproteins of intestinal origin. *Journal of Lipid Research*, **22:**610-619.

Krauss RM, Lindgren FT, Ray RM. (1980) Interrelationships among subgroups of serum lipoproteins in normal human subjects. *Clinical Chimica Acta*, **104:**275-

Krauss RM, Burke DJ (1982) Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *Journal of Lipid Research*, **23:**97-104.

Krauss RM, Williams PT, Lindgren FT, Wood PD. (1988) Coordinate changes in levels of human serum low and high density lipoprotein subclasses in healthy men. *Arteriosclerosis*, **8:**155-162.

Krauss RM. (1991) Low-density lipoprotein subclasses and risk of coronary artery disease. *Current Opinion in Lipidology*, **2:**248-252.

Krauss RM, Blanche PJ. (1992) Detection and quantitation of LDL subfractions. *Current Opinion in Lipidology, 3*:377-383.

Krauss RM. (1994) Heterogeneity of plasma low-density lipoproteins and atherosclerosis risk. Current Opinion in Lipidology, **5**:339-349.

Krauss RM, Dreon DM. (1995) Low-density-lipoprotein subclasses and response to a low-fat diet in healthy men. *American Journal of clinical Nutrition*, **62**:478S-487S.

Krieger M, Acton S, Ashkenas J, Pearson A, Penman M, Resnick D. (1993) Molecular flypaper, host defense, and atherosclerosis. *Journal of Biological Chemistry*, **268**:4569-

Kristensen T, Moestrup SK, Gliemann J, Bendtsen L, Sand O, Sottrup-Jensen L: (1990) Evidence that the newly cloned low-density-lipoprptein receptor related protein(LRP)is the alpha -macroglobulin receptor. *FEBS Lett*, **276**:151-155.

Kuchinskiene Z, Carlson LA. (1982) Composition, concentration and size of low density lipoproteins and of very low density lipoproteins from human serum of normal men and women. *Journal of Lipid Research*, **23**:762-769.

Kunitake ST, Mendel CM, Hennessey LK. (1992) Interconversion between apolipoprotein AI-containing lipoproteins of pre-beta and alpha electrophoretic mobilities. *Journal of Lipid Research*, **33**:1807-1816.

Langer T, Strobber W, Levy RI. (1972) The metabolism of low density lipoprotein familial type II hyperlipoproteinemia. *Journal of Clinical Investigation*, **51**:528-1536

Lahdenpera S, Sane T, Vuorinen-Markkola H, Knudsen P, Taskinen M-R. (1995) LDL particle size in mildly hypertriglyceridemic subjects: no relation to insulin resistance or diabetes. *Atherosclerosis*, **113**:227-236.

Lahdenpera S, Syvanne M, Taskinen M-R. (1996) Regulation of low-density lipoprotein particle size distribution in NIDDM and coronary disease: importance of serum triglycerides. *Diabetologia*, **39**:in press.

Lamon-Fava S, Fischer EC, Nelson ME, Evans WJ, Miller JS, Ordovas JM, Schaefer EJ. (1989) Effect of exercise and menstrual cycle status on plasma lipids, low density lipoprotein particle size, and apolipoproteins. *Journal of Clinical Endocrinology and Metabolism*, **68**:17-21.

Lamon-Fava S, Jimenez D, Christian JC, Fabsitz RR, Reed T, Carmelli D, Castelli WP, Ordovas JM, Wilson PWF, Schaefer EJ. (1991) The NHLBI Twin Study: heritibility of apolipoprotein A-I, B, and low density lipoprotein subclassses and concordance for lipoprotein (a). *Atherosclerosis*, **91**:97-106.

La Belle M, McCall MR, Krauss RM, Forte TM. (1990) Unique structural properties of apolipoprotein B in low-density lipoproteins produced by several human hepatoma-derived cell lines. *Biochimica et Biophysica Acta*, **1046**:288-293.

LaBelle M, Krauss RM. (1990) Differences in carbohyrate content of low density lipoproteins associated with low density lipoprotein subclass patterns. *Journal of Lipid Research*, **31**:1577-1588.

Lagrost L, Gandijini H, Athias A, Guyard-Dangremont V, Lallemant C, Gambert P. (1993) Influence of plasma cholesteryl ester transfer activity on the LDL and

HDL distribution profiles in normolipidemic subjects. *Arteriosclerosis and Thrombosis*, **13:**815-825.

Law MR, Wald NJ, Thomson SG. (1994) By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? *British Medical Journal*, **308:**367-372.

Lawn RM, Scanu AM. (1996) Lipoprotein (a). Atherosclerosis and Coronary Artery Disease V Fuster, Ross R, Topol EJ (eds) Lippincott-Raven publishers, Philadelphia, **9:**151-161.

Lee D, Downs D. (1982) A quick and large-scale density gradient subfraction method f or low density lipoproteins. *Journal of Lipid Research*, **23:**14-27.

Lee DM, Alaupovic P. (1986) Apolipoproteins B, C-III and E in two major subpopulations of low-density lipoproteins. *Biochimica et Biophysica Acta*, **879:**126-133.

Lesnik P, Vonica A, Guerin M, Moreau M, Chapman MJ. (1993) Anticoagulant activity of tissue factor pathway inhibitor in human plasma is preferentially associated with dense subspecies of LDL and LDL and with Lp(a). *Arteriosclerosis and Thrombosis*, **13:**1066-1075.

Liang H-Q, Rye K-A, Barter PJ. (1994) Dissociation of lipid free apoA-I from high density lipoproteins. *Journal of Lipid Research*, **35:**1187-1199.

Linden T, Bondjers G, Camejo G, Bergstrand R, Wilhelmsen L, Wiklund O. (1989) Affinity of LDL to a human arterial proteoglycan among male survivors of myocardial infarction. *European Journal of Clinical Investigation*, 19:38-43.

Lindgren, FT, Jensen LC, Wills RD, Freeman NK. (1969) Flotation rates, molecular weights and hydrated densities of the low-density lipoproteins. *Lipids*, **4:**337-344.

Lindgren FT, Jensen LC, Hatch FT. (1972) The isolation and quantitation analysis of serum lipoproteins. Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism. Nelson GJ, editor. Wiley-Interscience, New York 181-274.

Lipid Research Clinics Program: Manual of Laboratory Operations (1975) Volume 1 : Lipid and Lipoprotein Analysis. US Dept Health, Education and Welfare publication no. (NIH) 75-628. Bethesda, MD, National Institutes of Health

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193:**265-275..

Luc G, Chapman MJ. (1988) Guinea pig low density lipoproteins: structural and metabolic heterogeneity. *Journal of Lipid Research*, **29:**1251-1263.

Lusis AJ, (1988) Genetic factors affecting blood lipoproteins: The candidate gene approach. Journal of Lipid Research, **29:**397-429.

Luo C-C, LI W-H, Moore MN **et al** (1986) Structure and evolution of the apolipoprotein multigene family. *Journal of Molecular Biology*, **187:**325-340.

Luoma J, Hiltunen T, Sarkioja T, Moestrup SK, Gliemann J, Kodoma T, Nikkari T, Yla-Herttuala S. (1994) Expression of alpha2-macroglobulin receptor/low density lipoprotein receptor related protein and scavanger receptor in human atherosclerotic lesions. *Journal of Clinical Investigation*, **93:**2014-2021.

Lupu F, Heim D, Bachman F, Kruithof EKO. (1994) Expression of LDL receptor-related protein/alpha2-macroglobulin receptor in human normal and atherosclerotic arteries. *Arteriosclerosis and Thrombosis*, **14:**1438-1444.

Maartmann-Moe K, Berg K. (1981) Lp(a) lipoprotein enters cultured fibroblasts indepentently of the plasma membrane low density lipoprotein receptor. *Clinical Genetics*, **20**:352-362.

Maeda N, Li H, Lee D, Oliver P, Quarfordt SH, Osada J. (1994) Targeted disruption of the apolipoprotein C-III gene in mice results in hypotriglyceridemia and protection from postprandial hypertriglyceridemia. *Journal of Biological Chemistry*, **269**:23610-123616.

Mahley RW, Weisgraber KH, Melchior GW, Innerarity TL, Holcombe KS. (1980) Inhibition of receptor-mediated clearance of lysine and arginine modified lipoproteins from the plasma of rats and monkeys. *Proceedings of the National Academy of Sciences of the USA*, **77**:225-229.

Mahley RW, Innerarity TL. (1983) Lipoprotein receptors and cholesterol homeostasis. *Biochimica et Biophysica Acta*, **737**:197-222.

Malloy MJ, Zoppo A, Tu AY et al. (1994) A new metabolic disorder: Phospholipid transfer protein deficiency. *Clinical Research*, **42**:85A.

Malmendier C, Berman M. (1978) Endogenously labeled low density lipoprotein triglyceride and apoprotein B kinetics. *Journal of Lipid Research*, **19**:978-984.

Malmendier CL, Delcroix C, Lontie J-F. (1987) The effect of combined fenofibrate and cholestyramine therapy on low-density lipoprotein kinetics in familial hypercholesterolemia patients. *Clinica Chimica Acta*, **162**:221-227.

Malmendier CL, Delcroix C, Lontie J-R. (1989a) Kinetics of a heterogeneous population of particles in low density apolipoprotein B. *Atherosclerosis,* **80**:91-100.

Malmendier CL, Lontie J-F, Delcroix C, Magot T. (1989b) Effect of simvastatin on receptor-dependent low density lipoprotein catabolism in normocholesrerolemic human volunteers. *Atherosclerosis*, **80**:101-109.

Mann JI, Lewis B, Shepherd J, Winder AF, Fenstar S, Rose L, Morgan B. (1988) Blood lipid concentrations and other cardiovascular risk factors: distribution, prevalence and detection in Britain. *British Medical Journal*, **296**:1702-1706.

Mann CJ, Yen FT, Grant AM, Bihain BE. (1991) Mechanism of plasma cholesteryl ester transfer in hypertriglyceridemia. *Journal of Clinical Investigation*, 88:2059-2066.

Manninen V, Tenkanen L, Koskinen P. (1993) Joint efects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease in the Helsinki Heart Study: implications for treatment. *Circulation*, **85**:37-45.

Marsh JB, Diffenderfer MR. (1991) Isolation of nascent high-density lipoprotein from rat liver perfusates by immunoaffinity chromatography: Effects of oleic acid infusions. *Metabolism*, **40**:26-30.

Martin MJ, Hulley SB, Browner. (1986) Serum cholesterol, blood pressure and mortality: implications from a cohort of 261 662 men. *Lancet* **ii**:933-936.

Marz W, Baumstark MW, Scharnagl H, Ruzicka V, Buxbaum S, Herwig J, Pohl T, Russ A, Schaaf L, Berg A, Bohles H-J, Usadel KH, Groβ W. (1993) Accumulation of 'small dense' low density lipoproteins (LDL) in a homozygous patient with familial defective apolipoprotein B-100 results from heterogeneous interaction of

LDL subfractions with the LDL receptor. *Journal of Clinical Investigation*, **92**:2922-2933.

Marzetta CA, Foster DM, Brunzell JD. (1989) Relationships between LDL density and kinetic heterogeneity in subjects with normolipidemia and familial combined hyperlipidemia using density gradient ultracentrifugation. *Journal of Lipid Research*, **30**:1307-1317.

Matthews CME (1957) The theory of tracer experiments with ^{131}I-labelled plasma proteins. *Phys Med Biol*, **2**:36-53

Moestrup SK, Gliemann J, Pallesen G (1992) Distribution of the alpha2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Research*, **269**:375-382.

Mokuno H, Brady S, Kotite L, Herz J, Havel J. (1994) Effect of the 39-kDa receptor-associated protein on the hepatic uptake and endocytosis of chylomicron remnants and low density lipoprotein in the rat. *Journal of Biological Chemistry*, **269**:13238-13243.

Mulder M, Lombardi P, Jansen H et al. (1992) Heparan sulphate proteoglycans are involved in the lipoprotein lipase-mediated enhancement of the cellular binding of very low density and low density lipoproteins. *Biochimica et Biophysica Acta*, **185**:582-587.

Musliner TA, Giotas C, Krauss RM. (1986) Presence of multiple subpopulations of lipoproteins of intermediate density in normal subjects. *Arteriosclerosis*, **6**:79-87.

Musliner TA, McVicker KM, Iosefa JF, Krauss RM. (1987) Metabolism of human intermediate and very low density lipoprotein subfractions from normal and dysbetalipoproteinemic plasma. *Arteriosclerosis*, **7**:408-420.

Musliner TA, Krauss RM. (1988) Lipoprotein subspecies and risk of coronary disease. *Clinical Chemistry*, **34**:B78-B83.

Myant NB. (1993) Familial defective apolipoprotein B-100: a review, including some comparisons with familial hypercholesterolaemia. *Atherosclerosis*, **104**:1-18.

McFarlane AS. (1958) Efficient trace-labelling of proteins with iodine. *Nature*, **182**: 53.

McLean JW, Tomlinson JE, Kuang WJ, Eaton DL, Chen EY, Fless GM, Scanu AM, Lawn RM. (1987) Human apolipoprotein(a): cDNA sequence of an apolipoprotein homologous to plasminogen. *Nature*, **330**:132-137.

McNamara JR, Campos H, Ordovas JM, Peterson J, Wilson PWF, Schaefer EJ. (1987) Effect of gender, age, and lipid status on low density lipoprotein subfraction distribution. Results from the Framingham offspring study. *Arteriosclerosis*, **7**:483-490.

McNamara JR, Jenner JL, Li Z, Wilson PWF, Schaefer EJ. (1992) Change in LDL particle size is associated with change in plasma triglyceride concentration. *Arteriosclerosis and Thrombosis*, **12**:1284-1290.

Nichols AV, Krauss RM, Musliner TA. (1982) Non-denaturing polyacrylamide gradient gel electrophoresis. *Methods in Enzymology*, **128**:417-431.

Nigon F, Lesnik, P, Rouis M, Chapman MJ. (1991) Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor. *Journal of Lipid Research*, **32**:1741-1753.

Nikkila M, Solakivi T, Lehtimaki T, Koivula T, Laippala P, Astrom B. (1994) Postprandial plasma lipoprotein changes in relation to apolipoprotein E phenotypes and low density lipoprotein size in men with and without coronary artery disease. *Atherosclerosis*, **106:**149-157.

Nikkila M, Pitkajarvi T, Koivula T, Solakivi T, Lehtimaki T, Laippala P, Jokela H, Lehtomaki E, Seppa K, Sillanaukee P. (1996) Women have larger and less atherogenic low density lipoprotein particle size than men. *Atherosclerosis*, **119:**181-190.

Nishina PM, Johnson JP, Naggert JK, Krauss RM. (1992) Linkage of atherogenic lipoprotein phenotype to the low density lipoprotein receptor locus on the short arm of chromosome 19. *Proceedings of the National Academy of Sciences of the USA*, **89:**708-712.

Nykjaer A, Bengtsson-Olivecrona G, Lookene A, Moestrupt SK, Petersen CM, Weber W, Beisegel U, Glieman J. (1993) The alpha2-macroglobulin receptor/low density lipoprotein receptor-related protein binds lipoprotein lipase and beta-migrating very low density lipoprotein associated with the lipase. *Journal of Biological Chemistry*, **268:**15048-15055.

Nykjaer A, Nielsen M, Lookne A, Meyer N, Roigaard H, Etzerodt M, Beisegel U, Olivecrona G, Gliemann J. (1994) A carboxyl-terminal fragment of lipoprotein lipase binds to the loe density lipoprotein receptor-related protein and inhibits lipase-mediated uptake of lipoprotein to cells. *Journal of Biological Chemistry*, **269:**31747-31755.

Oka K, Tzung KW, Sullivan M, LIndsay E, Baldani A, Chan L. (1994) Human very-low-density lipoprotein receptor complentary DNA an deducted amino acid sequence and localization of its gene (VLDLR) to chromosome band 9p24 by fluorescence *in situ* hybridization. *Genomics*, **20:**298-300.

Olivecrona T, Bengstsson-Olivecrona G. (1993) Lipoprotein lipase and hepatic lipase. *Current Opinion of Lipidology*, **4:**187-196.

Ordonez JD, Hiatt RA, Killebrew EJ, Fireman BH. (1993) The increased risk of coronary heart disease associated with the nephrotic syndrome. *Kidney International*, **44:**638-642.

Ottnad E, Via DP, Frubis J, Sinn H, Friedrich E, Ziegler R, Dresel HA. (1992) Differentiation of binding sites on reconstituted hepatic scavanger receptors using oxidised low-density lipoprotein. *Biochemical Journal*, **281**:745-749.

Packard CJ, Third JLHC, Shepherd J, Lorimer AR, Morgan HG, Lawrie TDV. (1976) Low density lipoprotein metabolism in a family of familial hypercholesterolemic patients. *Metabolism*, **25:**995-1006

Packard CJ, Shepherd J. (1982) The hepatobiliary axis and lipoprotein metabolism: effects of bile acid sequestrants and ileal bypass surgery. *Journal of Lipid Research*, **23:** 1081-1098.

Packard CJ, Munro A, Lorimer AR, Gotto AM, Shepherd J. (1984) Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects. *Journal of Clinical Investigation*,**74:**2178-2192.

Packard CJ, Clegg RJ, Dominiczak MH, Lorimer AR, Shepherd J. (1986) Effects of bezafibrate on apolopoprotein B metabolism in type III hyperlipoproteinemic subjects. *Journal of Lipid Research*, **27**:930-938.

Packard CJ, Gaw A, Demant T,Shepherd J. (1995) Development and application of a multicompartmental model to study very low density lipoprotein subfraction metabolism. *Journal of Lipid Reserach*, **36**:172-187.

Patsch JR, Sailer S, Kostner G, Sandhofer F, Holansk *orHolasek* A, Brainsteiner H. (1974) Separation of the main lipoprotein classes from human plasma by rate-zonal ultracentrifigation. *Journal of Lipid Research*, **15**:356-366.

Patsch JR, Patsch W. (1986) Zonal ultracentrifugation. *Methods in Enzymology*, **129**:3-26.

Patsch JR, Prapad S, Gotto AM, et al. (1987) High density $lipoprotein_2$. Relationships of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia and to the activity of lipoprotein lipase and hepatic lipase. *Journal of Clinical Investigation*, **80**:341-347.

Patsch W, Patsch JR, Kostner GM, Saller S, Braunsteiner H. (1978) Isolation of subfractions of human very low density lipoproteins by zonal ultracentrifugation. *Journal of Biological Chemistry*, **253**:4911-4915.

Patsch W, Franz S, Schonfeld G. (1983) Role of insulin in lipoprotein secretion by cultured hepatocytes. *Journal of Clinical Investigation*, **71**:1161-1174.

Pearson AM, Rich A, Krieger M. (1993) Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *Journal of Biological Chemistry*,**268**:3546-3551.

Pease RJ, Leiper JM. (1996) Regulation of hepatic apolipoprotein-B-containing lipoprotein secretion. *Current Opinion in Lipidology*, **7**:132-138.

Phillips NR, Havel RJ, Kane JP. (1981) Levels and interrelationships of serum and lipoprotein cholesterol and triglycerides. *Arteriosclerosis*,**1**:13-24.

Phillips ML, Schumaker VN. (1989) Conformation of apolipoprotein B after lipid extraction of low density lipoproteins attached to an electron microscope grid. *Journal of Lipid Research*, **30**:415-422.

Pullinger C, North J, Teng B, et al. (1989) The apolipoprotein B gene is consecutively expressed in HepG2 cells: Regulation of secretion by oleic acid, albumin and insulin, and measurement of the mRNA half-life. *Journal of Lipid Research*, **30**:1065-1077.

Pullinger C, Hennessy LK, Chatterton JE, Liu W, Love j, Mendel CM, Frost PH, Malloy MJ, Schumaker VN, Kane jp. (1995) Familial liagand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *Journal of Clinical Investigation*, **95**:1225-1234.

Reaven GM, Chen Y-D I, Jeppeson J, Maheux P, Krauss RM. (1993) Insulin resistance and hyperinsulinemia in individuals with small, dense, low density lipoprotein particles. *Journal of Clinical Investigation*, **92**:141-146.

Redgrave TG, Small DM. (1979) Quantitation of the transfer of surface phospholipid of chylomicrons to the high density lipoprotein fraction during the catabolism of chylomicrons in the rat. *Journal of Clinical Investigation*, **64**:162-171.

Resnick D, Freedman NJ, Xu S, Krieger M. (1993) Secreted extracellular domains of macrophage scavanger receptors form elongated trimers which specifically bond crocidolite asbestos. *Journal of Biological Chemistry*, **268:**538-541.

Ridker PM, Hennekens CH, Stampfer MJ. (1993) A prospective study of lipoprotein(a) and the risk of myocardial infarction. *JAMA*, **270:**2195-2199.

Ross R. (1986) The pathogenesis of atherosclerosis- an update. *New England Journal of Medicine*, **314:**488-500.

Roy CC, Levy E, Green PHR et al. (1987) Malabsorption, hypocholesterolemia, fat-filled enterocytes with increased intestinal apoprotein B: Chylomicron retention disease. *Gastroenterology*, **92:**390-399.

Rossouw JE, Lewis B, Rifkind BM. (1990) The value of lowering cholesterol after myocardial infarction. *New England Journal of Medicine*, **323:**1113-1119.

Sakai J, Hishino A, Takahashi S, Miura Y, Ishii H, Suzuki H, Kawarabayasi Y, Yamamoto T. (1994) Structure, chromosome location, and expression of the human very low density lipoprotein receptor gene. *Journal of Biological Chemistry*, **269:**2173-2181.

Salonen EM, Jauhianen L, Zardi L, Vaheri A, Enholm C. (1989) Lipoprotein(a) binds to fibronectin and serine proteinase capable of cleaving it. *EMBO J*, **8:**4035-4040.

Scandinavian Simvastatin Survival Study Group (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian survival study. *Lancet*, 344:1383-1389.

Schaefer ER, Wetzel MG, Bengtsson G, Scow RO, Brewer HB, Olivecrona T. (1982) Transfer of human lymph chylomicron constituents to other lipoprotein density fractions during in vitri lipolysis. *Journal of Lipid Research*, **23:**1259-1273.

Schaefer EJ, Foster DM, Zech LA, Lindgren FT, Brewer HB, Levy RI. (1983) The effects of estrogen administration on plasma lipoprotein metabolism in pre-menopausal females. *Journal of Endocrinology and Metabolism*, **57:**262-267.

Schaefer EJ, Lamon-Fava S, Jenner JL, McNamara JR, Ordovas JM, Davis CE, Abolafia JM, Lippel K, Levy RI. (1994) Lipoprotein (a) levels and risk for coronary heart disease in men : The Lipid Research Clinics Primary Prevention Trial. *Journal of American Medical Association*, **271:**999-1003.

Schaefer EJ, Lamon-Fava S, Johnson S, Ordovas JM, Schaefer MM, Castelli WP, Wilson PWF. (1994) Effects of gender and menopausal status on the association of apolipoprotein E phenotype with plasma lipoprotein levels. Results from the Framingham offspring study. *Arteriosclerosis and Thrombosis*, **14:**1105-1113.

Schaffer TE, Lodish HF. (1994) Expression cloning and characteristic of a novel adipocyte lung chain fatty acid transport protein. *Cell*, **79:**427-436.

Schneider WJ, Goldstein JL, Brown MS. (1980) Partial purification and characterisation of the low density lipoprotein receptor from bovine adrenal cortex. *Journal of Biological Chemistry*, **255:**11442-11447.

Schoonjans k, Steals B, Deed S, Auwerx J. (1995) Fibrates and fatty acids induce lipoprotein lipase gene expression via peroxisome proliferator-activated receptor. *Circulation*, **92**(suppl I):I,495.

Schumaker VN, Phillips, ML, Chatterton JE. (1994) Apolipoprotein B and low density lipoprotein structure : Implications for biosynthesis of triglyceride-rich lipoproteins. *Advances in Protein Chemistry*, **45**:205-248.

Shafi S, Brady SE, Bensadoun A et al. (1994) Role of hepatic lipasein the uptake and processing of chylomicron remnants in rat liver. *Journal of Lipid Research*, **35**:709-720.

Shen MMS, Krauss RM, Lindgren FT, Forte T. (1981) Heterogeneity of serum low density lipoproteins in normal human subjects. *Journal of Lipid Research*, **22**:236-244.

Shepherd J, Bedford DK, Morgan HG. (1976) Radioiodination of human low density lipoprotein: a comparison of four methods. *Clinica Chimica Acta*, **66**:97-109.

Shepherd J, Bicker S, Lorimer AR, Packard CJ. (1979) Receptor-mediated low density lipoprotein catabolism. *Journal of Lipid Research*, **20**: 999-1006.

Shepherd J, Packard CJ, Lawrie TDV, Morgan HG. (1980) Cholestyramine promotes receptor-mediated low-density-lipoprotein catabolism. *New England Journal of Medicine*, **302**:1219-1222.

Shepherd J, Caine EA, Bedford DK, Packard CJ. (1984a) Ultracentrifugal subfractionation of high-density lipoprotein. *Analyst*, **109**:347-351.

Shepherd J, Packard CJ, Stewart JM, Atmeh R, Clark JG, Boag DE, Carr K, Lorimer AR, Ballantyne FC, Morgan HG, Lawrie TDV. (1984b) Apolipoprotein A and B (Sf 100-400) metabolism during bezafibrate therapy in hypertriglyceridemic subjects. *Journal of Clinical Investigation*, 74:2164-2177.

Shepherd J, Caslake MJ, Lorimer AR, Vallance BD, Packard CJ. (1985) Fenofibrate reduces low density lipoprotein catabolism in hypertriglyceridemic subjects. *Arteriosclerosis,* **5**:162-168.

Shepherd J, Packard CJ. (1986) An overview of the effects of p-chlorophenoxyisobutynic acd derivatives on lipoprotein metabolism, in Fears R, Prous JR (eds) *Pharmalogical Control of Hyperlipidaemia*. Barcelona, Spain, Science Publishers, SA pp135-144.

Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. (1995) Prevention of coronary heart disease with pravastatin in men with hypercholesterolaemia. *New England Journal of Medicine*, **333**:1301-1307.

Shimano H, Namba Y, Ohsuga J, Kawamura M, Yamamoto K, Shimada M, Gotoda T, Harada K, Yazaki Y, Yamada N. (1994) Secretion-recapture process of apolipoprotein E in hepatic uptake of chylomicron remnants in transgenic mice. *Journal of Clinical Investigation*, **93**:2215-2223.

Sigurdsson G, Nicholl A, Lewis B. (1976) The metabolism of low density lipoprotein in endogeneous hypertriglyceridaemai. *European Journal of Clinical Investigation*, **6**:151-158.

Soutar AK, Knight BI, Myant NB. The characterizationof lipoproteins in the high density fraction obtained from patients with familial lecithin: cholesterol acyltransferase deficiency and their interaction with cultured humen fibroblasts. *Journal of Lipid Research*, **23**:380-390.

Sparks CE, Sparks JD, Bolognino M, et al. (1986) Insulin effects on apolipoprotein B lipoprotein synthesis and secretion by primary cultures of rat hepatocytes. *Metabolism*, **35:**1128-1136.

Sparrow CP, Parthasarathy S, Steinberg D. (1989) A macrophage receptor that recognizes oxidised LDL but not acetylated LDL. *Journal of Biological Chemistry*, **264:**2599-2603.

Spring DJ, Chen-Liu LW, Chatterton JE, et al. (1992) Lipoprotein assembly. Apolipoprotein B size determines lipoprotein core circumference. *Journal of Biological Chemistry*, **267:**14839-14845.

Staels B, Auwerx J. (1992) Perturbation of developmental gene expression in rat liver by fibric acid derivatives: lipoprotein lipase and a-fetoprotein as models. *Development*, **115:**1035-1043.

Staels B, Vu-Dac N, Kosykh VA, Saladin R, Fruchart JC, Dallongeville J, Auwerx J. (1995) Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomal acyl coenzyme A oxidase: a potential mechanism for the hypolipemic action of fibrates. *Journal of Clinical Investigation*, **95:**705-712.

Stamler J, Wentworth D, Neaton JD. (1986) Is the relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? *Journal of the American Medical Association*, **256:**2823-1228.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. (1989) Beyond cholesterol. Modifications of low density lipoprotein that increase its atherogenicity. *New England Journal of Medicine*,**320:**915-924.

Stewart JM, Packard CJ, Lorimer AR, Boag DE, SHepherd J. (1982) Effects of bezafibrate on receptor-mediated and receptor-independent low density lipoprotein catabolism in type II hyperlipoprtoeinaemic subjects. *Atherosclerosis*, **44:**355-365.

Strickland DK,Ashcom JD, Williams S, Burgess WH, Migliorini M, Argraves WS: (1990) Sequence identity between the alpha -macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *Journal of Biological Chemstry,* **265:**17401-17404.

Suehiro T, Ohguro T, Sumiyoshi R, Yasuoka N, Nakauchi Y, Kumon Y, Hashimoto K. (1995) Relationship of low-density lipoprotein particle size to plasma lipoproteins, obesity, and insulin in Japenese men. *Diabetes Care*, **18:**333-338.

Superko, HR, Krauss RM. (1992). Differential effects of nicitonic acid in subjects with different LDL subclass patterns. *Atherosclerosis*, **95:**69-76.

Suzukawa M, Abbey M, Howe PRC, Nestel PJ. (1995) Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. *Journal of Lipid Research*, **36:**473-484.

Swinkels DW, Hak LHL, Demacker PN. (1987) Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions. *Journal of Lipid Research*, **28:**1233-1239.

Swinkels DW, Demacker PNM, Hak-Lemmers HLM, Mol MJTM, Yap SH, van't Laar A. (1988) Some metabolic characteristics of low-density lipoprotein subfractions, LDL-1 and LDL-2: invitro and in vivo studies. *Biochimica et Biophysica Acta*, **960:**1-9.

Swinkels D, Demacker PNM, Hendriks JCM, van't Laar A. (1989) Low density lipoprotein subfractons and relationship to other risk factors for coronary artery disease in healthy individuals. *Arteriosclerosis*, **9**:604-613.

Swinkels DW, Hendriks JCM, Demacker PNM, Stalenhoef AFH,. (1990) Differences in metabolism of three low density lipoprotein subfractions in Hep G2 cells. *Biochimica et Biophysica Acta*, **1047**:212-222.

Tall AR, Green PHR, Glickman RM, Riley JW. (1979) Metabolic fate of chylomicron phospholipids and apoproteins in the rat. *Journal of Clinical Investigation*, **60**:977-989.

Tall AR, Sammett D, Granot E. (1986) Mechanisms of enhanced cholesteryl transfer from high density lipoproteins to appolipoprotein B-containing lipoproteins during alimentary lipemia. *Journal of Clinical Investigation*, **77**:1163-1172.

Tall AR. (1990) PLasma high density lipoproteins. Metabolism and relationship to atherogenesis. *Journal of Clinical Investigation*, **86**:379-384.

Tall AR, Breslow JL. (1996) Plasma high-density lipoproteins and atherogenesis. in *Atherosclerosis and Coronary Disease* Fuster V, Ross R, Topol EJ (eds), Lippincott-Raven, New York :105-128.

Tam S-P, Archer TK, Deeley RG. (1986) Biphasic effects of estrogen on apolipoprotein synthesis in human hepstoma cells: Mechanisms of antagonism by testosterone. *Proceedings of the National Academy of Sciences, USA*, **83**:3111-3115.

The MAAS Investigators. (1994) Effect of simvastatin on coronary atheroma: the Multicentre Anti-Atheroma Study (MAAS). *Lancet*,**344**:633-638.

Tan CE, Forster L, Caslake MJ, Bedford D, Watson, TDG, McConnell M, Packard CJ, Shepherd J. (1995a) Relations between plasma lipids and postheparin plasma lipases and VLDL and LDL subfraction patterns in normolipidemic men and women. *Arteriosclerosis , Thrombosis and Vascular Biology*,**15**:1839-1848.

Tan KCB, Cooper MB, Ling KLE, Griffin BA, Freeman DJ, Packard CJ, Shepherd J, Hales CN, Betteridge DJ. (1995b) Fasting and postprandail determinants for the occurrence of small dense LDL species in non-insulin-dependent diabetic patients with and without hypertriglyceridaemai: the involvement of insulin, insulin precursor species and insulin resistance. *Atherosclerosis*, **113**:273-287.

Teng B, Thompson GR, Sniderman AD, Forte TM, Krauss RM, Kwiterocich. (1983) Composition and distribution of low density lipoprotein fractions in hyperapobetalipoproteinemia, normolipidemia and familial hypercholesterolemia. *Proceedings of the National Academy of Sciences of the USA*, **80**:6662-6666.

Teng B, Sniderman AD, Soutar AK, Thompson GR. (1986) Metabolic basis of hyperapobetalipoptoteinemia. *Journal of Clinical Investigation*, **77**:663-672.

Tertov VV, Orekhov N, Sobenin IA, Morrisett JD, Gotto AM, Guevara JG. (1993) Carbohydrate composition of protein and lipid components in sialic acid-rich and - poor low density lipoproteins from subjects with and without coronary artery disease. *Journal of Lipid Research*, **34**:365-375.

Thomson GR, Teng B, Sniderman AD. (1987) Kinetics of LDL subfractions. *American Heart Journal*, **113**:514-517.

Thrift RH, Drisko J, Dueland S, et al. (1992) Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in a nonhepatic cell line. *Proceedings of the National Academy of Sciences of USA*, **89:**9161-9165.

Thurnhofer H, Schabel J, Botz M et al (1991) Cholesterol transfer protein located in the intestinal brush border membrane. Partial purification and characterization. *Biochimica Biophysica Acta*, **1064:**275-286.

Tikkanen MJ, Nikkila EA, Kuusi T, Sipinen S. (1982) High density lipoprotein and hepatic lipase - reciprocal changes produced by estrogen and norgestrel. *Journal of Clinical Endocrinology*, **54:**1113-1117.

Tilly-Kiesi MK, Tikkanen MJ. (1991) Differential low density lipoprotein hydrated density distribution in female and male patients with familial hypercholesterolemia. *Clinica Chimica Acta*, **201:**65-74.

Tilly-Kiesi M, Kuusi T, Lahdenpera S, Taskinen M-R. (1992) Abnormalities of low density lipoproteins in normolipidemic type II diabetic and nondiabetic patients with coronary artery disease. *Journal of Lipid Research*, **33:**333-342.

Tollefson JH, Ravnik S, Albers JJ. (1988) Isolation and characterisation of a phospholipid transfer protein (LTP-II) from human plasma. *Journal of Lipid Research*, **15:**1593-1602.

Tornvall P, Karpe F, Carlson LA, Hamsten A. (1990) Relationships of low density lipoprotein subfractions to angiographically defined coronary artery disease in young survivors of myocardial infarction. *Atherosclerosis*, **90:**67-80.

Torsvik H, Solaas HM, Gjone E. (1970) Serum lipoproteins in plasma lecithin: cholesterol acyltransferase deficiency, studied by electron microscopy. *Clinical Genetics*, **1:**139-150.

Towbin H, Staehelin T, Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA*, **76:**4350-4354.

Tribble DL, Holl LG, Wood PD, Krauss RM. (1992) Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. *Atherosclerosis*, **93:**189-199.

Tribble DL, van den BEG JJM, Motchnik PA, Ames BN, Lewis DM, Chait A, Krauss RM. (1994) Oxidative susceptibility of low density lipoprotein subfractions is related to their ubiquinol-10 and α-tocopherol content. *Proceedings of the National Academy of Sciences of USA*, **81:**1183-1187.

Tribble DL, Kraus RM, Lansberg MG, Thiel PM, van den Berg JJM. (1995) Greater oxidative susceptibility of the surface monolayer in small dense LDL may contribute to differences in copper-induced oxidation among LDL density subfractions. *Journal of Lipid Research*, **36:**662-671.

Turner RH, Snavely JR, Goldwater WH, Randolph ML, Sprague CC, Unglaub WG. (1951) The study of serum lipoproteins and lipids with the aid of the quantity ultracentrifuge. *Journal of Clinical Investigation*, **30:** 1071-1081.

Van Berkel ThJC, Nagelkerke JF, Harkes L, Kruijt JK. (1982) Processing of acetylated LDL by parenchymal and non parenchymal cells, involvement of calmodulin. *Biochemical Journal*, **208:**493-

Van Berkel ThJC, de Rijke YB, Kruuijt JK. (1991) Different fate in vivo of oxidatively modified LDL and acetylated LDL in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. *Journal of Biological Chemistry*, **266**:2282-.

Vega GL, Grundy SM. (1986) Kinetic heterogeneity of low density lipoproteins in primary hypertriglyceridemia. *Arteriosclerosis*, **6**:395-406.

Von Eckardstein A, Huang Y, Assman G. (1994) Physiological role and clinical relevance of high density lipoprotein subclasses. *Current Opinion in Lipidology*, **5**:404-416.

Wang C-S, McConathy WJ, Kloer HU, Alaupovic P. (1985) Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. *Journal of Clinical Investigation*, **75**:384-390.

Warnick GR, Albers JJ. (1978) A comprehensive evaluation of the heparin/manganese precipitation procedure for estimating HDL cholesterol. *Journal of Lipid Research*, **9**:65-76.

Warshawsky I, Broze GJ Jr, Schwartz AL (1994) The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proceedings of the National Academy of Science USA*, **91**:6664-6668.

Wastney ME, Riemke R, Malmendier CL, Berman M. (1982) Heterogeneity of low-density lipoproteins: kinetic analysis of asialated lipoproteins. In: Berman M, Grundy SM, Howard B V.(eds) *Lipoprotein Kinetics and Modeling*, Academic Press, New York.

Watanabe Y, Inaba T, Shimano H, Gotoda T, Yamamoto K, Mokuno H, Sato H, Yazaki Y, Yamada N. (1994) Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages. *Arteriosclerosis and Thrombosis*, **14**:1000-1006.

Watson TDG, Burns L, Packard CJ, Shepherd. (1992) Selective determination of lipoprotein lipase and hepatic triglyceride lipase in heparinized plasma from horses. *American Journal of Veternary Research*, **53**:771-775.

Watson TDG, Caslake MJ, Freeman DJ, Griffin BA, Hinnie J, Packard CJ, Shepherd J. (1994) Determinants of low-density lipoprotein subfraction distribution and concentrations in young normolipidemic subjects. *Arteriosclerosis*, **14**:902-910.

Watts GF, Naoumova R, Cummings MH, Umpleby AM, Slavin BM, Sonksen PH, Thomson GR. (1995) Direct correlation between cholesterol synthesis and secretion of apolipoprotein B100 in normolipidemic subjects. *Metabolism*, **44**:1052-1057.

Weisser B, Locher R, de Graaf, Vetter W. (1993) Low density lipoprotein subfractios and $(Ca^{2+})_i$ in vascular smooth muscle cells. *Circulation Research*, 73:118-124.

Wetterau JR, Aggerbeck LP, Bouma ME et al (1992) Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science*, **258**:999-1001.

Wiklund O, Bondjers G, Wright I, Camejo G. (1996) Insoluble complex formation betweeen LDL and arterial proteoglycans in relation to serum lipid levels and effects of lipid lowering drugs. *Atherosclerosis*, **119**:57-67.

Williams KJ, Fless GM, Petrie KA et al. (1992a) Mechanisms by which lipoprotein lipase alters cellular metabolism of lipoprotein(s). Low density lipoprotein, and nascent lipoproteins. Roles for low density lipoprotein receptors and heparan sulphate proteoglycans. *Journal of Biological Chemistry*, **267:**13284-13292.

Williams PT, Krauss RM, Wood PD, Lindgren FT, Giotas C, Vranizan KM. (1986) Lipoprotein subfractionsof runners and sedentary men. *Metabolism*, **35:**45-52.

Williams PT, Krauss RM, Vranzin KM, Albers JJ, Terry RB, Wood PDS. (1989) effects of exercise-induced weight loss on low density lipoprotein subfractions in healthy men. *Arteriosclerosis*, **9:**623-632.

Williams PT, Krauss RM, Vranizan KM, Wood PDS. (1990) Changes in lipoprotein subfractions during diet-induced and exercise-induced weight loss in moderately overweight men. *Circulation*, **81:**1293-1304.

Williams PT, Vranizan KM, Krauss RM. (1992b) Correlations of plasma lipoproteins with LDL subfractions by particle size in men and women. *Journal of Lipid Research*, **33:**765-774.

Williams PT, Dreon DM, Krauss RM. (1995) Effects of dietary fat on high-density-lipoprotein subclasses are influenced by both apolipoprotein E isoforms and low-density-lipoprotein subclass patterns. *American Journal of Clinical Nutrition*, **61:**1234-1240.

Witzum JL, Young SG, Elam RL, Carew TE, Fisher M. (1985) Cholestyramine-induced changes in low density lipoprotein composition and metabolism. 1. Studies in the guinea pig. *Journal of Lipid Research*, **26:**92-103.

Wu A-L, Windmueller HG. (1979) Relative contribution by liver and intestine to individual plasma apolipoproteins in the rat. *Journal of Biological Biochemistry*, **254:**7316-7322.

Wojciechowski AP, Farrall M, Cullen P, Wilson TME, Batliss JD, Farren B, Griffin B, Caslake MJ, Packard CJ, Shepherd J, Thakker R, Scott J. (1991) Familial combined hyperlipidaemia linked to the apolipoprotein AI-CIII-AIV gene cluster on chromosome 11q23-q24. *Nature*, **349:**161-164.

Wolfe BM, Kane JP, Havel RJ, Brewster HP. (1973) Mechanism of the hypolipidemic effect of clofibrate on postabsorptive man. *Journal of Clinical Investigation*, **52:**2146-2159.

Xu Z, Bernloher DA, Banaszak IJ. (1992) Crystal structure of recombinant murine adipocyte lipid-binding protein. *Biochemistry*, **31:**3484-3492.

Yao Z, Blackhart BD, Linton MF, et al. (1991) Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. *Journal of Biological Chemistry*, **266:**3300-3308.

Yla-Herttuala S, Palinski W, Rosenfield ME, Parthasarthy S, Carew TE, Butler S, Witzum JL, Steinberg D. (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *Journal of Clinical Investigation*, **87:**1146-1152.

Yla-Herttuala S, Rosenfield ME, Parthasarathy S, Sigal E, Sarkioja T, Witzum JL, Steinberg D. (1991) Gene expression in macrophage-rich human atherosclerotic lesions. 15-lipoxygenase and acetyl low density lipoprotein receptor messenger

RNA colocalize with oxidation specific lipid-protein products. *Journal of Clinical Investigation*, **87:**1146-1152.

Young SG, Witzum JL, Carew TE, Krauss RW, Lingren FT. (1989) Colestipol-induced changes in LDL composition and metabolism.II. Studies in humans. *Journal of Lipid Research*, **30:**225-238.

Zambon A, Austin MA, Brown BG, Hokanson JE, Brunzell JD,. (1993) Effect of hepatic lipase on LDL in normal men and those with coronary disease. *Arteriosclerosis and Thrombosis*, **13:**147-153.

Zhang F, Yang Y, Steinbrecher UP. (1993) Structural requirements for the binding of modified proteins to the scavanger receptor of macrophages. *Journal of Biological Chemistry*, **268:**5535-

Zhao SP, Verhoeven MH, Vink, J, Hollaar l, van der Laarse A, de Knijff P, van't Hooft FM. (1993) Relationship between apolipoprotein E and low density lipoprotein particle size. *Atherosclerosis*, **102:**147-154.

Appendix 1
Manufacturers and Suppliers of Reagents, Hardware and Software

Anachem Limited,
Anachem House,
20 Charles Street,
Luton Bedfordshire, LU2 0EB,UK.

Amersham International plc,
Amersham Place,
Litte Chalfont,
Bucks HP7 9NA, UK.

Amicon Limited,
Upper Mill,
Stonehouse,
Gloucestershire GL10 2BJ, UK.

Baker Instruments Limited,
Rusham Park,
Whitehall Lane,
Egham, Surrey,
TW20 9NW, UK.

Beckman Instruments (UK) Ltd,
Analytical Sales and Service Operation,
Progress Road,
Sands Industrial Estate,
High Wycombe,
Bucks,
HP12 4JL, UK

BDH Laboratory Supplies,
McQuilkin & Co,
21 Polmadie Avenue,
Glasgow G5 0BB, UK.

Bio-Rad Laboratories,
2000 Alfred Nobel Drive,
Hercules,
CA 94547, USA.

Boehringer Mannheim UK (diagnostics and Biochemicals) Ltd,
Bell Lane,
Lewes,
East Sussex,
BN7 1LG, UK.

Bristol Myers Squibb,
Squibb House,.
141-149 Staines Road,
Hounslow, Middx TW3 3JA, UK.

Cricket Software Inc,
40 Valley Stream,
Malvern, PA, USA.

Dynex Technologies,
Daux Road,
Billingshurst,
West Sussex RH14 9SJ, UK.

Farmitilia Carlo Erba Ltd,
Italia House,
23 Grosvenor Road,
St Albans, AL1 3AW, UK.

Fisons Instruments,
Crewe Road,
Wythenshaw,
Manchester,
M23 9BE, UK.

Fournier,
Centre de Recherches de Daix,
50 rue de Dijon,
Daix, 21121 Fontaine les Dijon, France.

Gelman Sciences Limited,
Brackmills Business Park,
Carswell Road,
Northampton NN4 7EZ, UK.

Innogenetics NV,
Canadastraat 21-Haven 1009,
b-2070 Zwijndrecht,
Belgium.

Microsoft Corporation,
1 Microsoft Way,
Redmond, WA, USA.

Minitab Inc,
3081 Enterprise Drive,
State College,
PA 16801-3008, USA.

Merck, Sharp and Dohme Ltd,
Hertford Road, Hoddesdon,
Herts, EN11 9BU, UK.

Nycomed (UK) Ltd,
Nycomed House,
2111 Coventry Road,
Birmingham,
B26 3EA, UK.

Orion Diagnostica
Espoo, Finland

Packard Instrument Company,
Canberra Packard Ltd,
Brook House,
14 Station Road,
Pangbourne,
Berks, RG8 7DT, UK.

Paar Scientific Ltd,
594 Kingston Road,
Raynes Park,
London SW20 8DN, UK.

Parke-Davis,
Warner Lambert,
Lambert Court,
Chestnut Avenue,
Eastleigh, Hampshire, SO53 3ZQ, UK.

Pharmacia Biotech Ltd,
23 Grosvenor Road,
St Albans,
Herts AL1 3AW.

SAAM Institute FL-20,
Resource Facility for Kinetic Analysis,
University of Washington, Seattle,
Washington 98195, USA.

Sanofi-Winthrop
Onslow Street, Guildford,
Surrey, GU1 4YS, UK.

Scottish Antibody Production Unit (SAPU),
Law Hospital
Carluke,
Lanarkshire,
ML8 5ES, UK.

Sigma Chemical Company,
Fancy Road,
Poole,
Dorset,
BH17 7TG, UK.

Upjohn Ltd,
Fleming Way,
Crawley,
West Sussex, RH10 2 NJ, UK.

Glossary

ACR	Absolute catabolic rate
ALP	Atherogenic lipoprotein phenotype
Apo	Apolipoprotein
ACAT	Acyl CoA: cholesterol acyl transferase
BCAIT	Coronary atherosclerosis intervention trial
BHA	British Hyperlipidaemia Association
BMI	Body mass index
CAD	Coronary artery disease
CABG	Coronary artery bypass surgery
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CHD-LDL	Cyclohexanedione modified LDL
CHL	Combined hyperlipidaemia
Ci	Curie
Cipro	Ciprofibrate
CoA	Coenzyme A
d	Density
DGUC	Density gradient ultracentrifugation
EAS	European Atherosclerosis Society
EDTA	Ethylenediaaminetetra-acetate
EV	Extravascular
FCR	Fractional catabolic rate
FDB	Familial defective B lipoproteinaemia
FF	Fenofibrate
FFA	Free fatty acid
FH	Familial hypercholesterolaemia
GGE	Gradient gel electrophoresis
HC	Moderate hypercholesterolaemia
HDL	High density lipoprotein
HL	Hepatic lipase
HMG CoA	3-hydroxy, 3-methylglutaryl coenzyme A
HSL	Hormone sensitive lipase
HTG	Moderate hypertriglyceridaemia
IDL	Intermediate density lipoprotein
IV	Intravascular
LCAT	Lecithin: cholesterol acyl transferase
LDL	Low density lipoprotein
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LRC	Lipid Research Clinics

LRP	LDL receptor related protein
MAAS	Multi-centre anti atheroma study
MI	Myocardial infarction
MTP	Microsomal triglyceride transport protein
NCEP	National cholesterol education program
NIDDM	Non insulin dependent daibetes mellitus
OD_{280}	Optical density at 280 nm
PLTP	Phospholipid transfer protein
PMI	Post myocardial infarction
PPAR	Peroxisome proliferator activated receptors
REGRESS	Regression growth evaluation statin study
rpm	Revolutions per minute
SAAM	Simulation, analysis and modelling
SCRIP	Stanford coronary risk intervention program trial
SD	Standard deviaton
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sf	Svedberg flotation units
SR	Synthetic rate
TFPI	Tissue factor pathway inhibitor
Trig	Triglyceride
Tris	Tris hydroxymethylamino methane
U	Input
U/P	Urine to plasma ratio
VLDL	Very low density lipoprotein
WOSCOPS	West of Scotland Coronary Prevention Study

GLASGOW UNIVERSITY LIBRARY
GLASGOW UNIVERSITY LIBRARY

Bibliography

Griffin BA, Caslake MJ, Yip B, Tait GW, Packard CJ, Shepherd J. (1990) Rapid isolation of low density (LDL) subfractions from plasma by density gradient ultracentrifugation. *Atherosclerosis*, **83:**59-67.

Caslake MJ, Packard CJ, Series JJ, Yip B, Dagen MM, Shepherd J. (1992) Plasma triglyceride and low density lipoprotein metabolism. *European Journal of Clinical Investigation*, **22:**96-104.

Caslake MJ, Packard CJ, Gaw A, Murray E, Griffin BA, Vallance BD, Shepherd J. (1993) Fenofibrate and LDL metabolic heterogeneity in hypercholesterolemia. *Arteriosclerosis and Thrombosis*, **13:**702-711.

Griffin BA, Freeman DJ, Tait GW, Thomson J, Caslake MJ, Packard CJ, Shepherd J. (1994) Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis*, **106:**241-253.

Gaw A, Packard CJ, Caslake MJ, Griffin BA, Lindsay GM, Thomson J, Vallance BD, Wosornu D, Shepherd J. (1994) Effects of ciprofibrate on LDL metabolism in man. *Atherosclerosis*, **108:**137-148.

Watson TDG, Caslake MJ, Freeman DJ, Griffin BA, Hinnie J, Packard CJ, Shepherd J. (1994) Determinants of low-density lipoprotein subfraction distribution and concentrations in young normolipidemic subjects. *Arteriosclerosis*, **14:**902-910.

Tan CE, Forster L, Caslake MJ, Bedford D, Watson, TDG, McConnell M, Packard CJ, Shepherd J. (1995) Relations between plasma lipids and postheparin plasma lipases and VLDL and LDL subfraction patterns in normolipidemic men and women. *Arteriosclerosis , Thrombosis and Vascular Biology*,**15:**1839-1848.