POSTPRANDIAL LIPAEMIA IN TYPE 2 DIABETES: RELATIONSHIP TO INSULIN SENSITIVITY

A thesis submitted for the degree of Doctor of Medicine in the University College of London.

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DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed.........................................................(Candidate)
Date...........................23/11/2003

STATEMENT 1

This thesis is the result of my own investigation, except where otherwise stated.

Other sources are acknowledged by footnotes giving explicit references.

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Date.................................3/11/2003
Signed.........................................................(Supervisor)
Date.................................19/12/2003

STATEMENT 2

I hereby give consent for my thesis, if accepted to be available for photocopying and inter-library loan, and for the title and summary to be made available to outside organisations.

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Date.................................23/11/2003
ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Cooper for the help for setting the assays for: the isolation of plasma lipoproteins retinyl palmitate, lipases, LCAT and CETP. I would like to express my gratitude to him for his supervision kindness, patience and support throughout the course of this work. I would also like to thank Sister Maria Adiseshiah for the support throughout the course of this work.

My special thanks to Dr. Bruce Griffin at the Institute of Biochemistry, Surrey University for performing LDL subfractions. I also like to thank Professor C. N. Hales at the Department of Clinical Biochemistry, Addenbrookes Hospital for the measurements of plasma insulin and proinsulin and split proinsulin, Professors B Staels and Gerard Luc from University of Lille, France for the measurements of phospholipid transfer protein.

I am also grateful to Professor D. J. Betteridge for his supervision and guidance throughout this project. I would like to thank Takeda UK Limited for sponsoring my fellowship.

Finally, I am indebted to my dear mother for her encouragement, advice and help. Thanks to my brothers Fayez, Fawaz and to my beloved sister Rajaa’ for their moral support.

My sincere thanks go to my closest friend Karima who cheered me up a lot.

This work was carried out at the Department of Medicine, Royal Free and University College London Medical School and supported by a project grant from Takeda pharmaceutical company.
SUMMARY

In a detailed study, the effect of sensitizing tissues to insulin with a thiazolidinedione (pioglitazone) on postprandial lipid metabolism was compared to a group of type 2 diabetic patients treated with sulphonylurea (glibenclamide) in a treatment regime designed to achieve equivalent glycaemic control (HbA1c) in both groups. An oral fat tolerance test was administered after a 6 week treatment with diet and after 20 weeks treatment with glibenclamide or pioglitazone.

Glycaemic control in both groups was unchanged by either treatment but in the fasting state, insulin sensitization caused a significant reduction in plasma total triglyceride content due to a significant reduction in total VLDL and, in particular, VLDL-2 present. The overall triglyceride response to the fat test showed a significant improvement to control clearance levels in the pioglitazone group that was not observed in the glibenclamide group. Chylomicron and chylomicron remnant clearance improved with insulin sensitization. IDL and LDL-TG was significantly higher in the diabetic patients than the controls and after treatment with thiazolidinedione the triglyceride content of these lipoproteins was reduced by contrast to glibenclamide treatment. Cholesterol content did not change significantly over the postprandial period either overall or in lipoproteins. There were only small effects on the enzymes of lipoprotein remodelling (LCAT, CETP, PLTP, HL or LPL) by insulin sensitization.

Insulin release was reduced over the postprandial period by insulin sensitization and a significant reduction of proinsulin and 32-33 split-proinsulin also occurred. The results of this study show that sensitizing tissues to the action of insulin causes a reduction in insulin resistance (HOMA) and a more rapid clearance of postprandial lipoproteins. In addition there is an apparent beneficial effect on β-cell function which is manifested by lower levels of incompletely processed insulin species probably caused by a less prolonged period of glycaemia.
PUBLICATIONS ARISING FROM THE THESIS

• Felicity C, Al-Majali K, Betteridge DJ. PPARs Insulin Resistance and type 2 Diabetes (Cardiovascular Research Feb 2001).


• M. Cooper, K. Al Majali, M. Adiseshiah, J. Betteridge; The effect of pioglitazone on fasting and postprandial insulin and insulin precursor species in Type 2 diabetes (Abstract presentation for the 40th EASD meeting, Germany Sept 2004).
CHAPTER 1 INTRODUCTION

1.1 LIPOPROTEINS- STRUCTURE AND FUNCTION

1.1.1 Postprandial Lipoproteins (Intestinal) .................................................... 22
1.1.1.1 Exogenous Cholesterol Metabolism ....................................................... 27
1.1.2 Hepatic Lipoproteins ............................................................................... 28
1.1.2.1 Very Low Density Lipoprotein (VLDL) .................................. 28
1.1.2.2 Intermediate Density Lipoprotein (IDL) .................................. 29
1.1.2.3 Low Density Lipoprotein (LDL) ............................................. 30
1.1.2.4 Lipoprotein (a) ........................................................................... 30
1.1.2.5 High Density Lipoprotein ......................................................... 31

1.2 LIPOPROTEIN REMODELLING

1.2.1 Lecithin Cholesterol Acyl Transferase (LCAT) ................................... 33
1.2.2 Cholesterol Ester Transfer Protein (CETP) ........................................... 33
1.2.3 Phospholipid Transfer Protein (PLTP) .................................................. 33
1.2.4 Lipoprotein Lipase (LPL) ....................................................................... 34
1.2.5 Hepatic lipase (HL) ................................................................................. 35

1.3 EFFECTS OF TYPE 2 DIABETES ON LIPOPROTEIN METABOLISM

1.3.1 Pathophysiology of Type 2 Diabetes ..................................................... 37
1.3.2 Molecular mechanism of insulin action ............................................... 38
1.3.3 Receptor signalling pathways ............................................................... 38
1.3.4 Glucose uptake and disposal ................................................................. 40
1.3.5 Fatty acid and insulin action ................................................................. 41
1.3.6 Insulin resistance syndrome .................................................................. 46
1.3.7 Assessment of Insulin Resistance ......................................................... 47

1.4 LIPID METABOLISM IN TYPE 2 DIABETES
1.4.1 Effects on VLDL .................................................................................... 48
1.4.2 Effects on LDL ...................................................................................... 50
1.4.3 Effects on HDL ...................................................................................... 51
1.4.4 LPL- dependent effects .......................................................................... 52
1.4.5 Hepatic lipase dependent effects .......................................................... 52

1.5 TRIGLYCERIDE-RICH LIPOPROTEINS IN ATHEROSCLEROSIS
1.5.1 TRLs and haemostatic abnormalities-relation to CHD ...................... 55
1.5.2 Diabetes mellitus and Coronary Heart Disease ................................ 56

1.6 MANAGEMENT OF TYPE 2 DIABETES
1.6.1 PPAR and Thiazolidinediones.......................................................... 60
1.6.2 Function of PPAR at cellular level ...................................................... 61
1.6.3 Thiazolidinediones ................................................................................. 63
1.6.4 Mechanism of action of TZDs............................................................. 64
1.6.4.1 Increasing glucose transporters and uptake ................................................. 65
1.6.4.2 Enhancement of insulin signalling ....................................................... 66
1.6.4.3 Lowering plasma NEFA content .......................................................... 67
1.6.4.4 Reduction of hepatic glucose output .................................................. 67
1.6.4.5 Adipocyte differentiation ................................................................... 68
1.6.4.6 Reduction of Tumour Necrosis Factor-alpha (TNFα) ..................... 68
1.6.4.7 Increase of adiponectin/ACRP30/adipoQ ..................................... 69
1.6.4.8 Decrease expression of tissue resistin ............................................. 70

1.7 AIMS OF THE STUDY ................................................................................. 71
CHAPTER 2 MATERIAL AND METHODS

2.1 SUBJECTS, INSULIN SENSITIZATION AND POSTPRANDIAL LIPID METABOLISM PROTOCOLS

2.1.1 Diabetic Patients ................................................................. 73
2.1.2 Control subjects ................................................................. 74
2.1.3 Double Blind, Double Dummy protocol for sensitizing the diabetic subjects to insulin with TZD ........................................... 74
2.1.4 Oral fat tolerance test ......................................................... 76
  2.1.4.1 The high-fat content meal ............................................... 76
  2.1.4.2 Blood sampling ............................................................. 77

2.2 ISOLATION OF LIPOPROTEINS AND LIPOPROTEIN SUBFRACTIONS

2.2.1 Intestinal lipoproteins ....................................................... 79
  2.2.1.1 Chylomicrons ............................................................... 79
  2.2.1.2 Chylomicrons Remnants ................................................. 79

2.2.2 Hepatic lipoproteins ........................................................ 79
  2.2.2.1 Very Low Density Lipoproteins 1 & 2 ............................. 79
  2.2.2.2 Intermediate Density Lipoproteins ................................. 80
  2.2.2.3 Low Density Lipoproteins .............................................. 81
  2.2.2.4 Low Density Lipoprotein subfractions ........................... 81
  2.2.2.5 High Density Lipoprotein and subfractions ................... 82

2.2.3 Lipid determinations ........................................................ 83
  2.2.3.1 Retinyl palmitate .......................................................... 83
  2.2.3.2 Determination of cholesterol ......................................... 84
  2.2.3.3 Determination of triglyceride ....................................... 85
  2.2.3.4 Determination of non-esterified fatty acids (NEFA)........ 85
  2.2.3.5 Determination of plasma phospholipid .......................... 86

2.3 TECHNIQUES FOR ASSESSING LIPOPROTEIN REMODELLING

2.3.1 Plasma cholesteryl ester transfer .................................... 86
  2.3.1.1 Assay of total HDL total cholesterol and free cholesterol .... 87
2.3.1.2 HDL cholesteryl ester content and calculation of CETP activity..............................88

2.3.2 Phospholipid transfer protein (PLTP).................................................................88

2.3.3 Lecithin:cholesterol acyl transferase (LCAT)....................................................89

2.3.4 Hepatic and lipoprotein lipase activity.................................................................89
    2.3.4.1 Preparation of post-heparin plasma.........................................................89
    2.3.4.2 Hepatic Lipase Activity..............................................................................90
    2.3.4.3 Lipoprotein Lipase Activity.......................................................................91
    2.3.4.4 Lipase activity normalisation and calculation............................................92

2.4 PLASMA INSULIN, PROINSULIN & 32-33 SPLIT PROINSULIN CONCENTRATIONS............................................................................................92

2.5 GLUCOSE DETERMINATION..................................................................................93

2.6 INSULIN RESISTANCE............................................................................................93

2.7 APOLIPOPROTEINS (APO-AI & APO-B)...............................................................93

2.8 LIPOPROTEIN (A) MEASUREMENT (LPA)..........................................................94

2.9 HbA1C....................................................................................................................94

2.10 STATISTICAL ANALYSIS.......................................................................................95

CHAPTER 3 THE EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY IN TYPE 2 DIABETIC SUBJECTS: PLASMA LIPID CONCENTRATIONS IN THE FASTING AND POSTPRANDIAL STATE

3.1 THE DEMOGRAPHIC DETAILS OF THE STUDY GROUPS........................................96

3.2 THE EFFECT OF INSULIN SENSITIZATION ON FASTING LIPID CONCENTRATIONS IN TYPE 2 DIABETIC SUBJECTS................................................97
    3.2.1 Baseline Fasting plasma lipids in the diabetic groups compared to control subjects.................................................................97
    3.2.2 Fasting plasma lipid and lipoprotein content after treatment..............100

3.3 THE EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY ON POSTPRANDIAL LIPAEMIA IN TYPE 2 DIABETIC PATIENTS AND CONTROL SUBJECTS
3.3.1 Postprandial triglyceride metabolism .................................................101
3.3.2 Chylomicron retinyl palmitate clearance ............................................104
3.3.3 Chylomicron remnant retinyl palmitate clearance .............................105
3.3.4 Postprandial IDL and LDL triglyceride clearance .............................106
3.3.5 Postprandial HDL-TG clearance .........................................................107
3.3.6 Postprandial cholesterol metabolism ..................................................108
3.3.7 Postprandial Non Esterified Fatty Acid metabolism .......................111
3.3.8 Postprandial phospholipid metabolism ..............................................111
3.3.9 LDL subfractions and relation to postprandial lipaemia ...................112
   3.3.9.1 Correlation between LDL subfractions and fasting and
           postprandial measurement .............................................................114

3.4 DISCUSSION
3.4.1 The fasting state: the effect of insulin sensitization on plasma lipid concentration in type 2 diabetic patients compared to normal subjects .....................................................................................116
3.4.2 The postprandial state ............................................................................119
   3.4.2.1 Baseline postprandial lipaemia in type 2 diabetic patients
           compared to normal subjects ..................................................119
   3.4.2.2 The effect of insulin sensitization on lipid clearance in type 2
           diabetic patients compared with normal subjects ..................121
   3.4.2.3 LDL subfractions .....................................................................122

CHAPTER 4 EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARY RECEPTOR PATHWAY IN TYPE 2 DIABETIC AND CONTROL SUBJECTS: EFFECTS ON GLYCAEMIA, INSULIN RELEASE AND BETA-CELL FUNCTION AND INSULIN RESISTANCE IN THE FASTING AND POSTPRANDIAL STATES

4.1 SECRETION AND PROCESSING OF INSULIN .............................................124
4.2 THE EFFECT OF INSULIN ON LIPID METABOLISM ................................125
4.3 AIMS AND OBJECTIVES ............................................................................126
4.4 EFFECT OF INSULIN SENSITIZATION ON FASTING GLUCOSE, INSULIN AND INSULIN PRECURSOR SPECIES IN TYPE 2 DIABETIC SUBJECTS

4.4.1 Baseline Fasting plasma glucose, insulin and insulin precursor in the diabetic groups compared to control subjects........................................127
4.4.2 Assessment of insulin resistance using the HOMA model..............129

4.5 EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY ON POSTPRANDIAL GLUCOSE, INSULIN AND INSULIN PRECURSOR IN TYPE 2 DIABETIC PATIENTS AND CONTROL SUBJECTS

4.5.1 Postprandial glucose clearance.........................................................130
4.5.2 Postprandial insulin clearance.........................................................131
4.5.3 Postprandial proinsulin clearance......................................................132
4.5.4 Postprandial 32-33 split proinsulin clearance.................................134

4.6 RELATIONSHIP OF BASAL BETA-CELL FUNCTION TO FASTING AND POSTPRANDIAL LIPAEMIA.....................................................................................134

4.7 DISCUSSION

4.7.1 The effect of insulin sensitization on the fasting glycaemia, insulin and precursor species.................................................................138
4.7.2 The effect of insulin secretion on postprandial glycaemia and β-cell function.........................................................................................139
4.7.2.1 Baseline postprandial glycaemia β-cell function in type diabetic patient compared to normal subjects.................................139
4.7.2.2 The effect of insulin sensitization on the postprandial glycaemia β-cell function in type 2 diabetic patient compared to normal subjects........................................140
4.7.2.3 The effect of insulin sensitization on insulin resistance as estimated by the HOMA model in type 2 diabetic patient compared to normal subjects........................................141
4.7.2.4 The relationship between fasting and postprandial β-cell function and fasting and postprandial lipaemia

CHAPTER 5 EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY IN TYPE 2 DIABETIC AND CONTROL SUBJECTS: EFFECTS ON LIPASES, LCAT, CETP AND PLTP IN THE FASTING AND POSTPRANDIAL STATE COMPARED TO CONTROL

5.1 Lecithin:cholesterol acyl transferase

5.2 Lipid Transfer Protein; CETP and Phospholipid Transfer Protein

5.3 Relationship of CETP and PLTP activity to lipid and glucose metabolism

5.3.1 Cholesteryl ester transfer protein and atherogenesis

5.3.2 CETP activity and insulin resistance

5.4 Lipoprotein lipase and Hepatic lipase

5.5 Aims of the study

5.6 Results

5.6.1 The effect of insulin sensitization on fasting and postprandial LCAT activity in type 2 diabetic compared to control subjects

5.6.2 The effect of insulin sensitization on fasting and postprandial CETP activity in type 2 diabetic compared to control subjects

5.6.3 The effect of insulin sensitization on fasting and postprandial PLTP activity in type 2 diabetic compared to control subjects

5.6.4 The effect of insulin sensitization on fasting LPL and HL activities in type 2 diabetic compared to control subjects

5.7 DISCUSSION

5.7.1 Lecithin: cholesterol acyl transferase activity

5.7.2 Cholesteryl ester transfer protein

5.7.3 Phospholipid transfer protein

5.7.4 Lipoprotein and hepatic lipase activity
CHAPTER 6 CONCLUDING REMARKS

6.1 PPARγ ACTIONS .................................................................................................. 162

6.2 MAJOR FINDINGS OF THE EFFECTS OF INSULIN SENSITIZATION
BY PIOGLITAZONE IN THIS STUDY

Effects on Triglyceridaemia .............................................................................. 168

Management and clearance of intestinal lipoproteins .............................. 170

Improved β-Cell Function .............................................................................. 171

Limitations of the study .................................................................................. 172

REFERENCES ..................................................................................................... 173
TABLES
Table 1: Major Apolipoproteins: location and function ........................................22
Table 2: Lipoprotein Classification ....................................................................26
Table 3: Metabolic Effects of TZDs ....................................................................64
Table 4: Inclusion and exclusion criteria ..............................................................73
Table 5: Demographic characteristic of controls and diabetic subjects ...............96
Table 6a: Fasting lipid and lipoproteins ...............................................................98
Table 6b: Fasting Lp(a), NEFA and Apolipoproteins ...........................................99
Table 7: Postprandial clearance of lipoproteins triglyceride measured as Area- Under-the Curve (AUC) ...............................................................103
Table 8: Postprandial clearance of total and lipoprotein cholesterol measured as Area-Under-the Curve (AUC) .................................................109
Table 9: Percentage distribution of LDL subfractions ..........................................113
Table 10: Relationships between LDL subfractions and fasting, postprandial lipaemia, CETP, lipases and insulin parameters in all diabetic group at baseline ......................................................................................................................115
Table 11: Results of plasma fasting insulin, proinsulin, 32-33 split proinsulin and insulin resistance by HOMA model ........................................128
Table 12: Integrated responses (AUC) of plasma insulin, proinsulin and 32-33 split proinsulin after the test meal ................................................132
Table 13: Relationship between fasting insulin, proinsulin and split proinsulin levels and fasting and postprandial lipaemia ..................................................135
Table 14: Relationship between integrated response of insulin level and postprandial lipaemia.................................................................136

Table 15: Relationship between insulin resistance and postprandial lipaemia....
........................................................................................................................................137

Table 16: Fasting and postprandial LCAT activity in diabetic and control
subjects before and after treatment.................................................................152

Table 17: Fasting and postprandial CETP activity in diabetic and control
subjects before and after treatment.................................................................153

Table 18: Fasting and postprandial PLTP activity in diabetic and control
subjects before and after treatment.................................................................155

Table 19: Post-heparin lipoprotein lipase and hepatic lipase measured as (μmol
NEFA released/ml/hr)....................................................................................156

Table 20: Relationship between LPL and HL with postprandial lipaemia......156
FIGURES

Figures 1: Remnant lipoprotein uptake by LDL, HSPG-LRP pathway ...........25
Figures 2: HDL cycle and reverse cholesterol transport ..................................32
Figures 3: Lipoprotein remodelling by lipoprotein lipase ..................................34
Figures 4: The functions of hepatic lipase ..........................................................35
Figures 5: Overview of insulin action; proposed insulin signaling cascade ........39
Figures 6: Features of the glucose fatty acid cycle .............................................42
Figures 7: Potential interaction between lipids and insulin signaling .................43
Figures 8: Molecular mechanism of TZDs ............................................................65

Figures 9: The response of VLDL-TG, and subfractions to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ..........................................................101

Figures 10: The postprandial TG response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ...........................................................................102

Figures 11: The postprandial chylomicron RP response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ..........................................................104

Figures 12: The postprandial chylomicron remnant RP response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ..........................................................105

Figures 13: The postprandial IDL-TG response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ...........................................................................106
Figures 14: The postprandial LDL-TG response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ................................................................. 107

Figures 15: The postprandial HDL-TG response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ........................................................................ 108

Figures 16: The postprandial cholesterol response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ........................................................................ 109

Figures 17: Postprandial changes of IDL-cholesterol pre and post-treatment ................................................................................................................. 110

Figures 18: Postprandial changes of LDL-cholesterol pre and post-treatment ................................................................................................................. 110

Figure 19: Postprandial changes of HDL-cholesterol pre and post-treatment ................................................................................................................. 110

Figure 20: The postprandial NEFA response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ........................................................................ 111

Figure 21: The postprandial plasma phospholipid response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ................................................................. 112

Figure 22: The distribution of LDL subfractions before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects ................................................................. 114
Figure 23: HOMA estimate of insulin resistance before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects

Figure 24: The postprandial glucose response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects

Figure 25: The postprandial insulin response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects

Figure 26: The postprandial proinsulin response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects

Figure 27: The postprandial 32-33 split proinsulin response to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects

Figure 28: Model for TZDs action on PPARγ to explain different effects

18
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CHO</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>C-RP</td>
<td>Chylomicron fraction retinyl palmitate</td>
</tr>
<tr>
<td>C-TG</td>
<td>Chylomicron fraction triglyceride</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CR-RP</td>
<td>Chylomicron remnant fraction retinyl palmitate</td>
</tr>
<tr>
<td>CR-TG</td>
<td>Chylomicron remnant fraction triglyceride</td>
</tr>
<tr>
<td>FVII</td>
<td>Factor VII coagulant activity</td>
</tr>
<tr>
<td>HbA1C</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic lipase</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment method</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate Density Lipoprotein</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Resistance</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyltransferase</td>
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<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Lipoprotein a</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL-receptor–related protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-Esterified Fatty Acids</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PHP</td>
<td>Post-heparin plasma</td>
</tr>
<tr>
<td>RA</td>
<td>Retinyl acetate</td>
</tr>
<tr>
<td>RP</td>
<td>Retinyl palmitate</td>
</tr>
<tr>
<td>r</td>
<td>Spearman rank correlation coefficient</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S_{r}</td>
<td>Sverdberg unit of flotation</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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CHAPTER 1

1.1 LIPOPROTEINS – STRUCTURE AND FUNCTION

The insolubility of lipid in aqueous media has led to the evolution of complex mechanisms that allow the distribution of lipid to tissues for anabolic or catabolic purposes. In the blood, the majority of lipids are transported to the site of their uptake by tissues in the form of lipoprotein complexes (Table 1). The lipoproteins are spherical particles containing protein (usually one or more of the apolipoprotein family) and neutral lipids, such as cholesterol and triglyceride, and polar phospholipids. The lipids are packaged together in such a way that hydrophobic lipids (triglyceride and cholesteryl ester) form a central core surrounded by a monolayer consisting of polar phospholipids, free cholesterol and apolipoproteins.

The apolipoproteins have three main functions:

- They solubilize insoluble lipids such as cholesterol, cholesteryl esters and triglyceride.
- They regulate enzyme activity such as lecithin:cholesterol acyl transferase (LCAT) and lipoprotein lipase (LPL).
- They act as ligands for cell surface receptors for the uptake of lipoproteins and lipid exchange.

The different types of lipoproteins present in plasma are characterised by their hydrated buoyant density or for lipoproteins with a density less than 1 by their flotation rate defined in terms of Svedberg units of flotation ($S_f$).
Table 1: Major Apolipoproteins: Location and Function

<table>
<thead>
<tr>
<th>Apolipoproteins</th>
<th>Lipoproteins</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>A-I</td>
<td>HDL</td>
<td>Structural and activator of LCAT</td>
</tr>
<tr>
<td>A-II</td>
<td>HDL</td>
<td>Not known</td>
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<tr>
<td>B-100</td>
<td>VLDL, IDL, LDL, Lp(a)</td>
<td>Structural and ligand for LDL receptor</td>
</tr>
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<td>B-48</td>
<td>Chylomicrons</td>
<td>Structural</td>
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<tr>
<td>C-II</td>
<td>Chylomicrons, VLDL</td>
<td>LPL ↑</td>
</tr>
<tr>
<td>C-III</td>
<td>Chylomicrons, VLDL</td>
<td>LPL ↓</td>
</tr>
<tr>
<td>E</td>
<td>Chylomicron remnants, IDL, LDL, HDL, VLDL</td>
<td>Ligand for LDL receptor;</td>
</tr>
</tbody>
</table>

1.1.1 Postprandial (Intestinal, Gastric or Alimentary) Lipoproteins

Chylomicrons are the largest of the lipoprotein particles present in plasma varying in size from 100 to 1000 nm diameter, and consist mainly of triglyceride, together with lesser amounts of phospholipid, free cholesterol (2%), cholesterol ester (1%) and protein (apo-B48) (Table 2). After a meal, the ingested triglyceride is hydrolysed in the stomach to non-esterified fatty acids (NEFA), di- and monoglycerides by gut lipases and taken up by the enterocytes where they are used to synthesise triglyceride. Apolipoprotein B48 is the major protein component of chylomicrons and is unique to these lipoproteins. Apo-B48 is a short ended protein product generated from a full length apo-B m-RNA by the introduction of a premature stop codon in triplet 2153 by a specific apo-B100 m-RNA enzyme (Scott et al., 1994). The polypeptide consists of the amino-terminal half of apolipoprotein B100 and has a molecular weight about 48% of that of the apolipoprotein B100 polypeptide but,
importantly, it is not a ligand for the B/E receptor. In intestinal cells, the transfer of lipids into nascent chylomicrons (or VLDL in hepatocytes) is mediated by microsomal triglyceride transfer protein (MTP) (Wetterau et al., 1992).

Newly-synthesised chylomicrons are secreted into the lymphatic system and enter the systemic circulation through the thoracic duct. Once in the blood they receive apo-C and apo-E from HDL in exchange for apo-AI and are acted on by lipases that hydrolyse the triglyceride component releasing NEFA with the result that the particles become smaller, less buoyant particles known as chylomicron remnants (Erkelens et al., 1981). The rate of removal of chylomicrons from plasma is rapid, with a half-life of less than 1 hour, and normally they are undetectable after a 12-hour fast. Peak chylomicronaemia normally occurs between 3 to 6 hours after ingestion of a fatty meal.

In normal subjects, chylomicron remnants clearance starts within 30 minutes after the meal (Berr et al., 1992) and they are usually taken by the liver, through a process mediated primarily by receptors that recognise apolipoprotein E (apo-E) (Windler et al., 1996). Apo-E is also found in VLDL, IDL, and HDL. It serves several functions, including the receptor-mediated transfer of cholesterol between tissues and plasma. It is the principal apolipoprotein in the central nervous system (CNS), and it serves as the major apolipoprotein that is capable of lipid transport and regulation of lipid metabolism through known receptor-mediated processes. There are 3 common isoforms of the protein: E2, E3 and E4, arising from 2 alleles. The most common phenotype is apo-E3/3 (55%), with apolipoprotein E3/4 the next most common (26%). The least frequently occurring phenotype is apo-E2/2 (1%), which in some instances is associated with type III hyperlipidaemia. Apo-E mediates
binding of chylomicron remnants to the hepatic B/E receptor where its greater affinity for the receptor in part ensures their rapid removal from the circulation.

In patients with type III hyperlipoproteinaemia, chylomicron remnants accumulate in the plasma and have been linked to the development of accelerated atherosclerosis (Hussain., 1995, 1997; Mahley et al., 1999, 1997, 1994, 1991). The clearance of chylomicron remnants by the liver is a process that involves three steps. It begins with sequestration of the remnants within the space of Disse (Figure 1). Chylomicron remnant enter the space of Disse through the fenestrated sinusoidal endothelium, that allows the smaller remnants to enter (Fraser et al., 1995). The space of Disse is rich in heparan sulphate proteoglycan (HSPG) that has an important role in remnant catabolism by attaching the triglyceride-rich lipoproteins and bringing them in close proximity to lipases (Williams et al., 1997). Treatment of a variety of cells with heparinase, which removes heparan oligosaccharides from HSPG in the liver, significantly inhibits the binding and uptake of apo-E enriched remnant lipoproteins (Ji et al., 1993, 1995). HSPG may serve as a reservoir for apo-E, allowing the particles to be enriched in apo-E and so enabling their interaction with receptors (Hamilton et al., 1990; Huang et al., 1998; Raffai et al., 2003). Several approaches have shown that apo-E is critical ligand for the clearance of remnant lipoproteins in animal and human studies (Mahley et al., 1989, 1998; Shimano et al., 1994). More recently, it has been demonstrated that over expression of human apo-E prevented diet-induced remnant accumulation and also accelerated remnant clearance from the plasma (Fan et al., 1998).
Figure 1: Remnant lipoprotein uptake by the LDL receptor, HSPG-LRP pathway, and HSPG alone. HSPG are abundant in the space of Disse and on the surface of hepatocyte. Apo-E and HL, secreted by the hepatocytes, appear to bind to the HSPG and be available to enrich the remnant lipoproteins. The HSPG/apo-E appear to fulfill a critical role in the sequestration or capture of the remnants. Three major pathways for internalization are illustrated: 1) direct uptake by LDL receptor; 2) HSPG-LRP pathway: (a) remnants transferred to LRP for uptake or (b) HSPG-LRP complex internalized; and 3) HSPG alone mediating direct uptake. (Adapted from Atherosclerosis, Vol. 141 suppl 1. Dec 1998).

The space of Disse contains hepatic lipase (Sanan et al., 1997) and also LPL, which is carried to the space of Disse with the remnants (Skottova et al., 1995). The LDL receptor is a critical participant in the uptake and endocytosis of remnants, but other molecules are involved, including LDL Related Protein (LRP). LRP a very large molecule, expressed predominantly on hepatocytes and has multiple ligands, including apo-E, HL, LPL, a2-macroglobulin (Herz et al., 1995). LRP is synthesised in the cell and transported with receptor-associated protein (RAP), which serve as a
chaperone to usher LRP to the cell surface (Willnow et al., 1996). Interestingly, when RAP is isolated and injected intravenously, it blocks the binding of all the ligands to the LRP (Kounnas et al., 1995) and can also bind to cell-surface HSPG (Mahley et al., 1994). On the other hand, RAP, which is known to be a heparin-binding protein and the hepatic HSPG are rich in heparin-like domains. Therefore, it is likely that RAP binds to hepatic HSPG in vivo and that RAP can inhibit remnant lipoprotein binding to the LRP and HSPG as well as LDL receptor. In summary, under normal physiological conditions, the LDL receptor and the LRP mediate endocytosis by the liver of chylomicron remnant.

Table 2: Lipoprotein Classification

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (g/ml)</th>
<th>Flotation (Sf) (^1)</th>
<th>Major Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt; 0.95</td>
<td>&gt; 400</td>
<td>Transport of exogenous triglyceride.</td>
</tr>
<tr>
<td>Chylo. Remnants</td>
<td>&lt; 0.95</td>
<td>40-400</td>
<td>Transport of exogenous triglyceride.</td>
</tr>
<tr>
<td>VLDL-1</td>
<td>0.95-1.006</td>
<td>60-400</td>
<td>Transport of endogenous triglyceride.</td>
</tr>
<tr>
<td>VLDL-2</td>
<td>0.95-1.006</td>
<td>20-60</td>
<td>Transport of endogenous triglyceride.</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>12-20</td>
<td>Intermediate in the transformation of VLDL to LDL.</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>0-12</td>
<td>Transport cholesterol and phospholipid to peripheral cells.</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>2</td>
<td>Transport cholesterol and other lipids from peripheral tissues to the liver.</td>
</tr>
</tbody>
</table>

1 Expressed in Svedberg units for solvent density of 1.006.

2 HDL sediment at 1.063g/ml salt solution.
1.1.1.1 Metabolism of exogenous cholesterol

Exogenous cholesterol arising from the diet or bile, contributes, in part, to the plasma cholesterol concentration. The remainder is endogenously synthesized mainly by the liver. A daily western diet contains around 400 mg of cholesterol, derived from animal sources, and about 200-400 mg of non-cholesterol sterols, derived mostly from plants (Nair et al., 1984). On average, about 55% of the dietary cholesterol is absorbed and retained on a daily basis, but almost none of the non-cholesterol sterol are retained (sterol, sitosterol, campesterol, etc.) (Salen et al., 1970).

In the intestinal lumen, esterified cholesterol is cleaved by pancreatic cholesteryl ester hydrolase into free cholesterol and fatty acid. Free cholesterol, along with other lipids and fat-soluble vitamins, is then solubilized by bile acids into micelles which interact with the intestinal brush border and enter the enterocyte by a transport process the mechanism of which is not completely understood. After absorption, the free cholesterol is re-esterified by acyl CoA:cholesterol acyltransferase (ACAT) and is packaged along with other lipids, into chylomicrons which are secreted into the lymph and ultimately into plasma, as described previously. The excess remaining free cholesterol and plant sterols are presumed to be pumped out of the enterocyte back to the intestinal lumen by the complex of ATP-binding cassette transporters (ABCG5/ABCG8). These transporters somehow work as pumps in the intestine and liver to move selectively cholesterol and non-cholesterol sterols from the enterocyte/bile canaliculus back to the lumen (Small et al., 2003; Wittenburg et al., 2002).

Ezetimibe, a novel, potent and selective inhibitor of cholesterol absorption (Van Heek et al., 1997). It reduces plasma cholesterol concentrations by up to 15-20% (Davidson et al 2002) and has been found to lower plasma plant sterol
concentrations in sitosterolaemic subjects. This suggests that this drug may be inhibiting the activity of a putative sterol permease in the brush border membrane of the enterocyte (Klett et al., 2003). It does not, however, affect the absorption of bile acids, fatty acids, triglyceride and fat-soluble vitamins (Van Heek et al., 2000).

1.1.2 HEPATIC LIPOPROTEINS

1.1.2.1 Very Low Density Lipoprotein (VLDL)

VLDL is similar to chylomicrons in structure and composition but the particles are smaller in size ranging from 25-100 nm. Compared to intestinal lipoproteins, it contains relatively less triglyceride but more cholesterol, phospholipid and protein. Apo-B100 is the main structural protein present in VLDL and remains with the particles from secretion into and clearance from plasma through the apo-B/E receptor. Other lipoproteins present in VLDL are a mixture of apo-C and apo-E. VLDL is mainly synthesised in the liver and its chief function is the transport of endogenously synthesised triglyceride to peripheral tissues where they are a substrate for the lipases that release NEFA for storage or oxidization as an energy source. As the triglyceride is removed the particle becomes smaller and denser ultimately becoming intermediate-density lipoprotein (IDL). Further lipolysis leads to the production of low-density lipoprotein (LDL) ultimately taken up from the circulation by specific hepatic receptors that recognise the apo-B100 (LDL receptor or apo-B/E receptor). Partially lipolysed VLDL and IDL can also be taken up by the so-called remnant receptors which are structurally different from the apo-B/E receptors.

One of the substrates for VLDL-triglyceride synthesis is NEFA released from adipose tissue stores by the action of a hormone sensitive lipase. This enzyme is inhibited by insulin and thus in the fasting state, when insulin levels are low,
lipolysis proceeds at a maximum rate, releasing NEFA into the plasma. VLDL synthesis is promoted by an increase in the supply of NEFA to the liver or in an increase in the rate of hepatic synthesis of endogenous fatty acids as occurs during periods of high carbohydrate intake.

VLDL exists in two forms differing in flotation rate (S<sub>f</sub>): VLDL-1 (S<sub>f</sub> 60-400) and VLDL-2 (S<sub>f</sub> 20-60), according to size and composition. VLDL-1 are larger particles and contain relatively more triglyceride than VLDL-2. The key observation has been made that the large VLDL-1 particles (S<sub>f</sub> 60-400) and small VLDL-2 (S<sub>f</sub> 20-60) particles exhibit distinct pattern of metabolic behaviour and that their production is independently regulated (Demant et al., 1996; Packard et al., 1997). There is evidence that a greater proportion of VLDL-2 particles are converted into LDL via IDL as compared with VLDL-1, which are converted to IDL that is removed from plasma before undergoing conversion to LDL. The VLDL-1 production rate shows a close positive correlation with plasma triglyceride levels and the current theory is that VLDL-1 overproduction causes elevation of plasma triglyceride whilst VLDL-2 overproduction results in raised LDL concentrations (Packard et al., 1997).

1.1.2.2 Intermediate Density Lipoprotein (IDL)

IDL is the intermediate density particle formed during lipolytic conversion of VLDL to LDL. Apo-B100 is the predominate apolipoprotein present but IDL contain relatively less triglyceride and more cholesterol ester than VLDL and is present in about one tenth of the concentration of LDL in plasma. IDL particles have one of two fates: the particles can be removed from blood, by virtue of apo-E binding to the hepatic apo-B/E receptor or they can be further hydrolysed to LDL.
1.1.2.3 Low Density Lipoprotein (LDL)

LDL is the major carrier of cholesterol, and cholesterol ester, triglyceride and phospholipid in plasma. A typical LDL molecule is composed of 75-80% lipid and 20-25% protein. Apolipoprotein B100 constitutes more than 95% of the protein content of LDL. LDL shows some variation in particle size and composition and can be separated by density gradient ultracentrifugation or gradient gel electrophoresis into 3 main subclasses dependant on their size and density (see Chapter 3 for a detailed discussion of LDL sub classes). LDL is taken up by hepatic and extrahepatic tissues by a specific receptor mediated process. This receptor, known as the LDL receptor (apo-B/E), recognises both apo-B100 and apo-E as ligands with the latter having a greater affinity than apo-B100 and is, in part, responsible for the clearance of the VLDL-derived lipoproteins from the blood (Hersberger et al., 2003; Slowik et al., 2003).

1.1.2.4 Lipoprotein (a)

Another lipoprotein, very similar to LDL, is also found in plasma and known as Lipoprotein (a) (Lp(a)). This lipoprotein is slightly denser than LDL because of the presence of an extra protein called apolipoprotein ‘a’ that is covalently attached to the apolipoprotein B100 by a disulphide bond. This extra protein is synthesised in the liver, and has a close structural homology to plasminogen. The function of Lp(a), if any, is not known but excess levels in blood are a risk factor for CHD probably caused by similarities of apo (a) to plasminogen that interferes with clot dispersion (Buchler et al., 2003).
1.1.2.5 High Density Lipoprotein

HDL are the smallest and densest of the lipoproteins and is also the most heterogeneous. HDL is mainly synthesised by the liver and to a lesser extent by the small intestine. Apolipoprotein AI is the major apolipoprotein of HDL and its functions include mediating removal of cholesterol from cells, including macrophages through interaction with ATP–binding cassette protein (ABCA1); promoting selective uptake of cholesteryl ester in liver, binding to SR-B1 receptor on the surface of liver and steroidogenic tissues, such as, adrenal, and ovary (Remaley et al., 2001). Apo-AI also serves as cofactor for the enzyme, lecithin:cholesterol acyl transferase (LCAT) responsible for the esterification of free cholesterol derived from peripheral tissues with a fatty acid from phospholipid to yield cholesteryl ester. This ester-rich form of HDL is known as HDL2. Newly synthesised ester is exported from the HDL to other lipoproteins by a specific transfer protein (cholesteryl ester transfer protein; CETP) in exchange for triglyceride. As the triglyceride progressively accumulate it causes the HDL to become a less dense more spherical form called HDL3. Triglyceride-rich HDL3 is lipolysed by the hepatic lipase and results in the formation of small HDL2 particles available for further esterification of free cholesterol (Rader et al., 2003).

The mechanism of the protective effect of HDL against the development of CHD is complex. Much research in this field has centred on the lipid transport function of HDL, particularly reverse cholesterol transport, and whether any component of HDL aids the efflux of cholesterol from the artery wall and peripheral tissues. Another area of interest, however, has emerged in recent years concentrating on antioxidant function and how HDL protects LDL and cell membranes against
lipid peroxide-induced damage, which is thought to mediate the initiation and progression of atheromatous lesions. The chief mechanism is through the action of the enzyme paraoxonase which detoxifies the product of lipid peroxidation such as hydroperoxide. This prevents further oxidation of lipids and protects against the formation of oxidized LDL which is known to be atherogenic (Mackness et al, 2002).

Figure 2: HDL cycle and reverse cholesterol transport. The triglyceride present in HDL₃ is acted on by hepatic lipase releasing NEFA into the blood & returning the particle to a small dense HDL₂ form ready to receive more free cholesterol via the ABC1 receptor or cholesterol efflux regulatory protein (CERP) to be esterified by LCAT. The ester formed is exchanged for triglyceride from TRL by the action of CETP. The HDL accumulate the TG and is then acted on by the HL and the cycle continue. (Adapted from Lipids and Lipid Disorders, MD Feher & W Richmond, Excerpta Medica Publications, 2003).
1.2 LIPOPROTEIN REMODELLING, ROLE IN CHOLESTEROL HOMEOSTASIS AND RECEPTOR-MEDIATED UPTAKE

Three enzymes: lipoprotein lipase (LPL), hepatic lipase (HL) and lecithin:cholesterol acyl transferase (LCAT), are of physiological importance in lipoprotein metabolism. In addition, two specific transfer proteins: cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), facilitate the interchange of these lipids between lipoproteins (they will be discussed in detail in Chapter 5).

1.2.1 Lecithin Cholesterol Acyltransferase (LCAT)

LCAT enzyme is secreted by the liver and is present in plasma, lymph, and cerebrospinal fluid and is tightly associated with HDL. It is responsible for the formation of most of the esterified cholesterol present in plasma. The action of LCAT is described in the preceding section (1.1.2.5).

1.2.2 Cholesterol Ester Transfer Protein (CETP)

CETP mediates the transfer of cholesteryl ester from HDL to triglyceride-rich lipoproteins (TRLs) and of triglyceride in the reverse direction. By this means an exit route is provided for cholesterol ester, accumulating in HDL as a result of the action of LCAT, thus maintaining continuity of movement of cholesterol from cells into plasma.

1.2.3 Phospholipid Transfer Protein (PLTP)

Phospholipid transfer protein (PLTP) plays an important role in plasma lipoprotein metabolism. PLTP facilitates the in vivo transfer of phospholipids from triglyceride-rich lipoproteins to HDL during lipolysis by LPL. These phospholipids are released
from chylomicrons and VLDL during lipolysis, and are important precursors of plasma HDL (will be discussed in Chapter 5).

1.2.4 Lipoprotein Lipase (LPL)

Lipoprotein lipase is a key enzyme that is present in a number of tissues, including muscle, adipose tissue, lung, brain and breast tissue. LPL is present primarily on the luminal surface of endothelial cells in tissue capillary beds, where it is bound to heparan-sulphate proteoglycan component of plasma membrane. It is also present in very low concentrations as a free form in the plasma.

Figure 3: Lipoprotein remodelling by lipoprotein lipase. The primary role of LPL is to hydrolysed TG in TRL releasing NEFA and forming smaller remnant particles that can be taken up by hepatic receptors or in the case of VLDL remnants further lipolysed to form LDL. (Adapted from Lipids and Lipid Disorders, MD Fehér & W Richmond, Excerpta Medica Publications, 2003).

LPL has several distinct physiological functions including 1) hydrolysis of the triglyceride component of triglyceride-rich lipoproteins, chylomicron and VLDL. 2) LPL has a bridging function in which it serves to anchor the lipoprotein to the vessel wall and facilitate triglyceride hydrolysis and particle uptake. 3) LPL acts as a ligand
for the LDL receptor, VLDL receptor and LDL related protein. 4) LPL can mediate the uptake of lipophilic vitamins (vitamin A, E) and the uptake of lipoprotein associated lipid (Stein et al., 2003).

1.2.5 Hepatic Lipase (HL)

Hepatic lipase is a glycoprotein that catalyzes the hydrolysis of lipoprotein triacylglycerols and phospholipids in LDL, HDL and triglyceride-rich lipoproteins remnant particles (see Figure 4). The majority of hepatic lipase is synthesised and secreted by the liver. Its catalytic activity contributes to the remodelling of LDL.
HDL resulting in smaller, denser particles. In addition to its lipolytic activity, hepatic lipase participates with surface proteoglycans, the scavenger receptor B1 (SR-B1) and the LDL receptor-like-protein in promoting hepatic uptake of lipoproteins, including triglycerides-rich lipoprotein remnants, LDL and HDL particles and nascent HDL particles. Unlike lipoprotein lipase human HL does not require the presence of apo-CII for its function. HL is known to be important in the generation of small dense LDL (Cheung et al., 2003).
1.3 EFFECTS OF DIABETES AND INSULIN ON LIPOPROTEIN METABOLISM

1.3.1 Pathophysiology of Type 2 Diabetes

The pathophysiology of type 2 diabetes involves characteristic defects in three main organ systems that act together to produce abnormal glucose metabolism i.e., liver, skeletal muscle, and pancreas.

A characteristic trait of type 2 diabetes is the overproduction of glucose by the liver. Increased hepatic glucose output correlates with fasting glucose levels and is the main cause of fasting hyperglycaemia in type 2 diabetic patients. Increased levels of NEFA supplied to the liver contribute to elevated hepatic glucose output. The second defect occurs in glucose disposal by skeletal muscle that accounts for 80-90% of insulin-stimulated glucose uptake and is significantly reduced in type 2 diabetic subjects. Finally, impaired pancreatic β-cell function is commonly found in established type 2 diabetes, usually coincident with fasting hyperglycaemia. Prospective epidemiological studies indicated that insulin resistance may be primary defect in type 2 diabetes, since it can be detected long before deterioration of glucose tolerance occurs, often at a time when insulin secretion is increased. Thus, in many patients, insulin resistance and hyperinsulinaemia precede the development of type 2 diabetes and can be identified in most pre-diabetic individuals. Moreover, insulin resistance can be further exacerbated during the progression of the disease because of abnormal lipid and carbohydrate metabolism. The β-cells respond to peripheral insulin resistance by increasing basal and postprandial insulin secretion in an attempt to maintain normal glucose homeostasis. Eventually, the β-cells are no longer able to compensate for insulin resistance by secreting increased amounts of insulin. At this stage, glucose-induced insulin secretion falls, allowing glucose homeostasis to
deteriorate and leading to the subsequent development of frank diabetes (De Fronzo et al 1997; Chen et al; 1999; Cherrington et al; 1999).

1.3.2 Molecular mechanism of insulin action
Insulin stimulates the cellular uptake of glucose, amino acids and fatty acids, promotes the storage of carbohydrate, lipids and proteins and inhibits their degradation and release into the circulation.

1.3.3 Receptor signalling pathways
The signal transduction pathway is very complex and only partly understood in spite of great progress in the last ten years (Saltiel et al., 2001) (Figure 5). Insulin action begins with its binding to highly specific receptors on the plasma membrane of the target tissue e.g., muscle or adipose tissue. The insulin receptor is a large transmembrane protein consisting of α- and β-subunits. Insulin initiates its cellular effects by binding to the α-subunit which leads to phosphorylation of specific tyrosine residue of the cytoplasmic tail of the β-subunit (Cheatham et al., 1995). The β-subunits themselves possess tyrosine kinase activity and autophosphorylate multiple tyrosine residues on the β-subunits. A number of proteins bind with high affinity to phosphotyrosine residues present in specific sequence motifs on the insulin receptor β-subunit. These proteins include the insulin receptor substrate (IRS) proteins, She and Gab-1, which, when bound, became phosphorylated by the insulin receptor tyrosine kinase activity.

The IRS serve as docking sites for protein ligands that contain specific recognition domains. The divergence of insulin signalling pathways within the cell
Figure 5: Simplified scheme for intracellular action of insulin. The insulin receptor is a tyrosine kinase. Binding of insulin activates intracellular autophosphorylations of the receptors and catalyses the phosphorylation of insulin receptor substrate 1 and 2 (IRS 1,2) cellular proteins of the insulin signalling pathway. The various pathways following act in a concerted action to co-ordinate translocation of Glut-4 containing vesicles into the plasma membrane, enzyme activation involved in the regulation of glucose, lipid, and protein metabolism and gene expression (involving GRB2 growth factor receptor-bound protein and the Ras complex).
may reside at the level of the IRS docking proteins that have been referred to as the metabolic switches of the cell.

Insulin receptor activation is upstream of diverse intracellular signalling pathways including activation of phosphoinositide-3-kinase (PI-3-kinase), Akt B and the MAP kinase (Cheatham et al., 1995; Kim et al., 1999; White et al., 1997). The effect of insulin on glucose uptake involves activation of the enzyme PI-3 kinase which is necessary for insulin action on glucose transport (Hara K et al., 1994. Okada T et al., 1994), glycogen synthesis, protein synthesis (Mendez et al., 1996), antilipolysis (Okada et al., 1994), and gene expression (Sutherland et al., 1998).

Although activation of PI-3 kinase is crucial for transducing the metabolic effects of insulin, it is not sufficient in itself to account for the glucose uptake process. A number of other growth factors stimulate PI-3 kinase to the same extent as insulin, but they do not stimulate glucose transport.

In summary, current evidence suggests that IRS proteins in their phosphorylated form, act as a docking site for a variety of second messenger kinases that allow the insulin signal to diverge throughout the target cell

1.3.4 Glucose uptake and disposal

PI-3 kinase activation ultimately leads to the translocation of cytoplasmic Akt to the plasma membrane where it is phosphorylated to an activated form responsible for the translocation of vesicles containing glucose transport proteins (GLUT) to the cell membrane where they are incorporated and allow the entry of glucose into the cell. Glucose transporters consist of at least four transmembrane proteins (Glut-1, -2, -3, -4). These GLUT proteins have distinct specificities, kinetic properties, and tissue distribution that define their role (Shepherd et al., 1999). Two major glucose transporters (Glut-1 and 4) have been identified in skeletal muscle. Glut-1 is mainly involved in basal
glucose uptake. Glut-4 is predominantly expressed in insulin target tissue (skeletal muscle, adipose tissue, and cardiac muscle), and it is the major insulin responsive glucose transporter. In muscle Glut-4 is recycled between the plasma membrane and an intracellular storage pool with approximately 90% sequestered intracellularly in the absence of insulin. This polarised intracellular distribution is unique to Glut-4. After insulin stimulation, Glut-4 moves from intracellular stores to the plasma membrane, resulting in glucose transport into the cell (Shepherd et al., 1999). The fate of glucose entering the cell is determined too by Akt phosphorylation. In its activated form, Akt inhibits glycogen synthase 3 (GSK-3) and so directs the glucose to catabolic energy-producing pathways.

1.3.5 Fatty acids and insulin action

A role for fatty acids in the regulation of insulin sensitivity has been proposed by Randle and termed the “fatty acid-glucose cycle” (Randle et al., 1963). They speculated that it is increased fat oxidation that causes the insulin resistance associated with obesity. The proposed mechanism is that increase in fatty acids causes an increase in the intramitochondrial acetyl CoA with subsequent inactivation of pyruvate dehydrogenase (Figure 6). This in turn would cause the intracellular citrate concentration to increase, leading to inhibition of phosphofructokinase, a key rate-controlling enzyme in glycolysis. Subsequent accumulation of glucose-6-phosphate would inhibit hexokinase activity resulting in an increased intracellular glucose concentration and decreased glucose uptake.

A recent series of studies proposed an alternative mechanism for fatty acid induced insulin resistance in human skeletal muscle (McGarry et al., 2002).
Figure 6: Features of the glucose fatty acid cycle. Fatty acids are taken up from the plasma either as the NEFA or by action of LPL and carried to the mitochondria (FABPs). The NEFA are transported into the mitochondria by the carnitine palmitoyl transferase (CPT) system, where they undergo B-oxidation to produce acetyl CoA that enters the krebs cycle. Accumulation of acetyl CoA and citrate inhibits PDH and phosphofructokinase (PFK), respectively. This leads to buildup of glucose-6-P and inhibition of hexokinase, resulting in reduced glucose uptake. FABP (fatty acyl binding protein), F-Ac (fatty acyl); HADH (hydroxyacyl-CoA dehydrogenase cytosolic fatty acid binding protein). (Adapted from Diabetes, Vol. 49, May 2000).

It has been proposed that the excess triglyceride or NEFA in muscle in insulin resistant states might lead to increased long-chain acyl CoA (LCCoA) concentrations. An increase in fatty acyl CoAs can lead to increased diacylglycerol (DAG) concentrations, which could also result from partial lipolysis of intracellular triglyceride. DAG, in turn, activates many isoforms of protein kinase C (PKC). Activation of PKC can phosphorylate and inhibit tyrosine kinase activity of the insulin receptor as well as tyrosine phosphorylation of insulin receptor substrate IRS-1 and thereby inhibit its interaction with PI-3 kinase (Figure 7). As a consequence insulin mediated glucose uptake becomes impaired. The relevant concentration of LCCoA is on the other hand dependent on malonyl-CoA, which regulates the activity of carnitine
palmitoyltransferase 1 and thereby the rate of fatty-acid oxidation. The synthesis of this important regulator and "metabolic sensor" is mediated by AMP-kinase. Phosphorylation and activation of this enzyme causes an inhibition of acetyl-CoA carboxylase, but an activation of malonyl-CoA decarboxylase resulting in a fall of malonyl-CoA and an acceleration of fatty-acid oxidation. Up-regulation of relevant phosphatases as observed in experimental diabetes leads to inactivation of AMP-kinase and subsequently to an increased level of malonyl-CoA, a reduced rate of fatty-acid oxidation and accumulation of LCCoA and triglycerides favouring the development of an insulin-resistant state.

Figure 7: Potential interaction between lipids and insulin signalling. Potential inhibitors; potential activators. ACC, acetyl-CoA carboxylase; PKB, protein kinase B. (Adapted from Diabetes, VOL. 49, May 2000).
There are likely to be multiple factors that contribute to impairment of both glucose and fatty acid metabolism. An impaired functional capacity of mitochondria is one factor that might contribute to perturbations in the metabolism of both substrates. A reduction in the activity of marker enzymes of oxidative pathways has been observed in skeletal muscle obtained from individuals with obesity and type 2 diabetes and correlates with the severity of insulin-resistant glucose metabolism (Simoneau et al., 1997).

Reduced mitochondrial capacity for fat oxidation during fasting conditions, as noted in obesity and type 2 diabetes (Kelley et al., 2000), could lead to insulin-resistance through the accumulation of lipid intermediates (Schmitz-Peiffer et al., 1999). It has also been postulated that impaired mitochondrial function could directly contribute to insulin-resistance due to insufficient provision of ATP for the phosphorylation of glucose by hexokinase as well as other reactions requiring phosphorylation (Gerbitz et al., 1996).

Skeletal muscle is richly endowed with mitochondria and strongly reliant on oxidative phosphorylation for energy production. In a previous study using electron microscopy to assess skeletal muscle mitochondrial size and morphology as a monitor of the activity of electron transport chain demonstrated perturbations in type 2 diabetes and, to a lesser degree, in obesity. Activity of rotenone-sensitive NADH:O₂ oxidoreductase was found to be reduced by 40% in skeletal muscle from patients with type 2 diabetes. Skeletal muscle mitochondria were also smaller in obesity and type 2 diabetes, and in some instances, particularly in type 2 diabetes, there was evidence of severely damaged mitochondria. Both findings correlated with the degree of insulin resistance (Kelley et al., 2002). Others had earlier reported reduced activity of mitochondrial tricarboxylic acid cycle enzymes in skeletal muscle.
from type 2 diabetic subjects (Vodra et al., 1977; Lithell et al., 1981). Disturbances of mitochondrial function in muscle and other tissue can lead to lipid accumulation (Vogel et al., 1999), which in turn can cause or aggravate insulin resistance.

There are at least several potential mechanisms by which impaired mitochondrial function might contribute to insulin resistance of skeletal muscle.

- Insulin resistance could be related to lipid accumulation within myocytes. Previous studies have found that increased lipid accumulation in muscle is associated with insulin resistance and that, in turn, lipid accumulation in skeletal muscle in obesity and type 2 diabetes is related to reduced oxidative enzyme capacity (He et al., 2001; Kelly et al., 2002).

- Disturbed oxidative phosphorylation capacity has been proposed to be a direct cause of insulin resistance (Gerbitz et al., 1996).

- An excess of long-chain fatty acid CoA, could damage the mitochondria either directly or by channeling of palmitoyl-CoA to increased ceramide synthesis (Basu et al., 1998).

- Mitochondria DNA (mtDNA) is more susceptible than nuclear DNA to damage and has a less efficient repair mechanism. Increased mtDNA mutations have been reported in type 2 diabetic patients (Liang et al., 1997). One source of injury to mtDNA could be reactive oxygen species, and increased free radical damage to mtDNA has been reported for other tissues in type 2 diabetic patients (Nishikawa et al., 2000).

In summary current evidence suggest that there is an impaired bioenergetic capacity of skeletal muscle mitochondria in type 2 diabetes.
1.3.6 Insulin resistance syndrome

Insulin resistance is characterised by an abnormally low response of the target cells to insulin. The sensitivity and the responsiveness, or both, of the cell to the hormone can be decreased depending on the severity of the syndrome (Olefsky et al., 1981). The term 'Insulin resistance' was originally used to indicate impaired insulin action on glucose uptake and metabolism in skeletal muscle, adipose tissue, and the liver. More recently the term has been used to indicate abnormal insulin activation or inhibition of other pathways such lipolysis, LPL activity and hepatic metabolism (Frayn et al., 1993). The term Syndrome X was introduced in 1988 by Reaven who suggested that insulin resistance and compensatory hyperinsulinaemia, underlie clustering of cardiovascular risk factors including impaired glucose tolerance, type 2 diabetes, hypertension and dyslipidaemia. Several other components have subsequently been added, including obesity especially abdominal obesity, microalbuminuria, abnormalities in fibrinolysis and coagulation and the presence of small dense atherogenic LDL particles (Bjorntorp et al., 1994; Groop et al., 1993; Yudkin et al 1999). It has also been described as the Insulin Resistance Syndrome (IRS) (Haffner et al., 1997). A positive association between insulin resistance and CHD has been shown in different studies. (Reaven et al., 1988; DeFronzo et al., 1991; Ferrannini et al., 1991; Frayn et al., 1992; Howard et al., 1996).

A defect in the insulin signalling transduction cascades, will lead to a reduced biological activity of the hormone i.e. insulin resistance. In vivo and in vitro studies suggest that only those parts of the signalling cascade involved in glucose disposal are affected in insulin resistance, whereas the MAP-kinase pathways appear to be undisturbed. Thus insulin resistance is most often described as a functional defect causing an inappropriate disposal of glycaemia as a consequence.
1.3.7 Assessment of Insulin Resistance

A variety of procedures have been developed to detect the presence of clinical insulin resistance. The most widely accepted and specific measure is a technique called the euglycaemic hyperinsulinaemic clamp; however, this technique has a number of limitations, primarily in the procedure's complexity and expense. A second, less invasive method is the frequently sampled intravenous glucose tolerance test (FSIVGTT) and can be applied to larger populations (Del Prato et al., 1999; Ferrannini et al., 1998). Both of these procedures require a well-staffed clinical research setting and are unrealistic for clinical practice or large population-based studies (Del Prato S, 1999; Ferrannini et al., 1998).

The homeostasis model assessment (HOMA) of insulin sensitivity is a mathematical model that provides a measure of insulin resistance based on the fasting glucose and insulin content of the plasma. (Bonora et al., 2000; Mathews et al., 1985). This parameter was proposed 10 years ago as a simple, inexpensive alternative to more sophisticated techniques. Specifically, an estimate of insulin resistance by HOMA score is calculated with the formula: \([\text{fasting serum insulin (µU/ml)} \times \text{fasting plasma glucose (mM)}]/22.5\) (Mathews et al., 1985). The HOMA method has been shown to correlate strongly to glucose disposal methods as assessed by clamp studies (Bonora et al., 2000). Furthermore, the HOMA model of insulin resistance showed a significant association with risk of CHD over 8-year follow-up of the San Antonio Heart Study, after adjustment of multiple covariates (Anthony et al., 2002). The convenience of the HOMA models has led to its being used in numerous large scale studies and research projects in which the clamp methods are impractical because of the nature of the study.
1.4 LIPID METABOLISM IN TYPE 2 DIABETES

The most common lipid abnormality present is moderate hypertriglyceridaemia associated with reduced levels of HDL cholesterol and is seen in up to one third of type 2 diabetes patients, including those with good glycaemic control (Barrett-Connor et al., 1982; Taskinen et al., 2003). By contrast, total plasma cholesterol concentrations and in particular LDL cholesterol concentrations are little different from normal subjects (Ginsberg et al., 2000).

1.4.1 Effects on VLDL

A variety of factors have been implicated in the aetiology of diabetic dyslipidaemia including: hyperinsulinaemia (Reaven et al., 1981, 2003) insulin resistance (Malmstrom et al., 1997) hyperglycaemia and disturbed fatty acid metabolism (Lewis et al., 1995).

An important function of insulin is to maintain a state of balance between intestinally derived triglyceride containing lipoproteins (chylomicron) and triglyceride containing lipoproteins of hepatic origin (VLDL). In type 2 diabetes, insulin resistance impairs the normal suppression of NEFA release from adipose tissue by a failure in the mechanisms that lead to suppression of hormone sensitive lipase activity. Consequently plasma concentrations of NEFA are greater and the influx of NFFA to liver is increased (Bjorntorp et al., 1994). This is coupled with diminished inhibitory effect of insulin on hepatic apo-B100 synthesis which results in excess VLDL production. It is this excess VLDL which is responsible for hypertriglyceridaemia in particular large VLDL-1 (Malmstrom et al., 1997).

The key observation has been made that the large VLDL-1 particles (Sf 60-400) and small VLDL-2 (Sf 20-60) particles exhibit distinct patterns of metabolic behaviour and that their production is independently regulated (Demant et al., 1996; Packard et al., 1997). VLDL-1 production rate shows a close positive correlation with plasma
triglyceride levels and VLDL-2 overproduction results in raised LDL concentrations (Packard et al., 1997).

VLDL synthesis appears to be regulated by insulin since it correlated positively with plasma insulin concentration (Chirieac et al., 2000; Reaven et al., 1987) and with insulin resistance as measured by the euglycaemic hyperinsulinaemic clamp technique (Abbott et al., 1987). In this scenario, insulin resistance results in compensatory hyperinsulinaemia, which in turn stimulates further VLDL production in the liver and the end result is elevation of plasma triglyceride. The opposite effect has been shown in in vitro studies showing that insulin acutely suppresses VLDL apo-B production in cultured hepatocytes and also increases the intracellular degradation of newly synthesised Apo-B (Sparks et al., 1992). Furthermore, it has been shown that in healthy men acute hyperinsulinaemia suppresses VLDL apo-B and triglyceride production (Cumming et al., 1995). In Type 2 diabetes, the principal finding was that, by contrast to the suppression of VLDL-1 apo-B production observed in normal subjects, insulin failed to decrease VLDL-1 apo-B production (Malmstrom et al., 1997). This lack of inhibition of VLDL-1 production by insulin is a feature of insulin resistance (Taskinen et al., 1998).

In the postprandial state insulin has a physiological action to regulate the balance between liver-derived and intestinally-derived triglyceride-rich particles, and should suppress the secretion of large VLDL-1 particles. This would relieve postprandial competition between chylomicrons and VLDL-1 for lipolysis and other remodelling processes and prevent prolonged alimentary lipaemia. The failure of insulin to suppress VLDL-1 particle release in type 2 diabetes is likely to contribute to a major cause of the raised triglyceride levels and exaggerated postprandial lipaemia (Malmstrom et al., 1997).

The metabolism of the remnant particles may also be influenced by low LPL activity associated with type 2 diabetes and insulin resistance. Evidence from in vitro studies
showed that LPL enhances the binding of lipoprotein remnants to the LRP (Beisiegel et al., 1991). In insulin resistance, the binding of remnant particles to LPL and/or LRP is diminished, resulting in decreased elimination of remnants by peripheral tissues (Beisiegel et al., 1996). This leads to a greater exposure of the vessel wall to triglyceride-rich lipoproteins remnants, secondary to reduced elimination which is thought to promote atherogenesis.

1.4.2 Effects on LDL

Hypertriglyceridaemia in diabetic subjects results in compositional changes in all lipoprotein particles. There is increased neutral lipid exchange resulting in triglyceride enrichment of both HDL and LDL (Bagdade et al., 1990). This is probably a consequence of both CETP over-activity and the expanded VLDL pool (Elchebly et al., 1996). Furthermore, triglyceride-rich HDL and LDL particles undergo increased lipolysis due to the over-activity of hepatic lipase present in type 2 diabetes (De-Man et al., 1996; Syvanne et al., 1995, 1997). This results in generation of smaller, more dense lipoprotein particles with altered physiological function.

Small dense LDL particles are a characteristic of the dyslipidaemia of type 2 diabetes (Taskinen et al., 1996) and are related to hypertriglyceridaemia rather than diabetes per se (Syvanne et al., 1997). The Strong Heart Study Shows observed a stepwise decrease in LDL-size according to diabetic status from normal through impaired glucose tolerance, to full diabetes. This association is more striking in women than in men (Howard et al., 1998). Small dense LDL is mainly generated when there is an elevation of triglyceride-rich lipoproteins, both VLDL and chylomicron particles, that remain in the circulation for longer periods than normal; this allows for an increased exchange of cholesterol ester from LDL in return for triglyceride, resulting in triglyceride-rich LDL, that are then lipolysed by hepatic lipase to small dense particles.
Small dense LDL particles are a strong risk factor for coronary heart disease and is considered to be highly atherogenic (Austin et al., 1998). It is easily oxidised and binds more readily to arterial wall proteoglycans. It has been shown to be an independent determinant of intima-media thickness (IMT) and an independent predictor of coronary artery disease in healthy men (Skoglund-Andersson et al., 1999). Small dense LDL also seems to be associated with very early signs of endothelial dysfunction, particularly in diabetic patients (Tan et al., 1999). The size of small, dense LDL correlates with flow mediated dilatation independent of other variables (HbA1C, blood pressure). It has also been shown to exhibit an increased toxicity to endothelial cells, increased tendency to absorption by macrophages to form foam cells and increased susceptibility to oxidative and glycate modification (Sattar et al., 1998). Furthermore, small LDL particles have an increased tendency to induce free radical production (Heinecke et al., 1998).

1.4.3 Effects on HDL

In diabetic dyslipidemia, not only is the concentration of HDL reduced, but also the distribution and the composition is changed. Levels of HDL$_2$ are reduced and there is a shift towards smaller HDL-particles that are reported to have diminished reverse cholesterol transfer capacity and reduced antioxidant potential hence reducing the antiatherogenic potential of HDL (Syvanne et al., 1995). The mechanisms underlying the lowering of HDL in diabetes may be due to any one or combination of the following. 1) Small HDL particles, which are a product of the lipolysis of triglyceride-enriched HDL are cleared more rapidly from the circulation. 2) Triglyceride-enriched HDL are more unstable in the circulation, with apo-AI loosely bound. 3) Lipolysis of triglyceride-enriched HDL may lower HDL particle number by causing apo-AI to be shed from the HDL particles and cleared from the circulation through the kidney. 4) LPL activity is impaired in diabetes and may contribute to the lowering of HDL levels by reducing the
availability of the surface components (phospholipid and apolipoproteins) of triglyceride-rich lipoproteins for transfer to the HDL fraction (Rader et al., 2002).

1.4.4 Lipoprotein lipase dependent effects

Decreased activity of LPL has been observed in type 2 diabetes compared with age matched non-diabetic subjects (De-Man et al., 1996). The reduction of LPL activity is more striking in diabetic patients with cardiovascular disease (De-Man et al., 1996). In vitro and in vivo studies have suggested a regulatory role for NEFA in LPL-mediated lipolysis since high concentrations of NEFA have been shown to inhibit LPL activity by weakening the binding of the enzyme to its substrate and to the endothelium-bound heparan sulphate (Lewis et al., 1995).

1.4.5 Hepatic lipase dependent effects

Increased activity of hepatic lipase is a feature in type 2 diabetes (Syvanne et al., 1997) and results in generation of small dense LDL particles with altered physiological function (De-Man et al., 1996) and which are a strong risk factor for cardiovascular disease (Austin et al., 1998) as discussed previously.
1.5 TRIGLYCERIDE-RICH LIPOPROTEINS IN ATHEROSCLEROSIS

Triglyceride-rich lipoproteins have been implicated in clinical coronary disease events for over 40 years. Gofman was the first to show an increase in triglyceride-rich lipoproteins (Sf 12-400) in men with myocardial infarction (Gofman et al., 1954). Grundy proposed two hypotheses to understand the atherogenicity of triglyceride-rich lipoproteins, applicable to both diabetic and non-diabetic subjects (Grundy et al., 1991).

The first view is that triglyceride-rich lipoproteins (such as VLDL remnants), are directly atherogenic. The second hypothesis is that triglyceride-rich lipoproteins act indirectly by influencing the concentration and composition of other lipoproteins. There is considerable evidence, both clinical and experimental, supporting the role of triglyceride-rich lipoproteins in atherogenesis. This evidence includes in vitro and animal experiments showing that remnants can induce foam-cell formation, and can infiltrate the arterial wall (Karpe et al., 1995; Rapp et al., 1994). Lipoprotein analysis from the Monitored Atherosclerosis Regression Study (MARS) demonstrate that VLDL remnants and IDL are strong predictor of carotid intima-media thickness, implying the importance of triglyceride-rich lipoproteins in the early stages of atherogenesis (Hodis et al., 1998; Mack et al., 1996).

Miesnbok and Patsch have proposed the concept of "triglyceride or lipid intolerance" whereby ineffective triglyceride removal for any reason prolongs the plasma residence time of triglyceride-rich lipoproteins. These lipoproteins are atherogenic because of their potential to extract choleseryl ester from HDL and LDL, resulting in cholesterol deposition into the arterial wall by these particles whose normal function is to carry triglyceride into the peripheral tissue (Miesnbok et al., 1992; Patsch et al., 2000; Tannock et al., 2002).

The third hypothesis proposed that the effect of hypertriglyceridaemia also renders LDL itself more atherogenic because it is associated with presence of small LDL
Small dense LDL are reported to be more susceptible to oxidative modification (Chait et al., 1993; Chmura et al., 2002). The presence of this small, dense LDL is closely associated with coronary heart disease (Austin et al., 1998; Hokanson et al., 2002).

An expanded pool of circulating cholesteryl ester may be an important factor in the formation of small, dense LDL because of an increased transfer of cholesteryl ester from LDL to those lipoproteins. A similar increase in the transfer of cholesteryl ester from HDL to triglyceride-rich lipoproteins also contribute to the low HDL cholesterol, frequently associated with hypertriglyceridaemia.

HDL particles in type 2 diabetes also have some special characteristics. In a cross sectional study (Syvanne et al., 1995), low levels of apo-AI: AII (particles carrying both apo-AI and apo-AII) particles were a striking feature of in type 2 diabetic subjects, especially in those who had coronary disease. This finding is supported by in vitro studies; diabetic sera had low capacity to induce cholesterol efflux from cells, and this related to apo-AI: AII concentration (Syvanne et al., 1996). These results suggest that impaired reverse cholesterol transport may also play an important role in the enhanced atherogenesis in type 2 diabetes.

There is considerable evidence, both clinical and experimental, supporting the role of triglyceride-rich lipoproteins in atherogenesis, as Zilversmit postulated in 1979 (Zilversmit et al., 1979). The rise in plasma triglycerides and triglyceride-rich lipoproteins concentrations after a test meal is significantly greater in type 2 diabetic patients than in controls (De Man et al., 1996). Fasting hypertriglyceridaemia is predictive of exaggerated postprandial lipid excursions (Lewis et al., 1991; Tan et al., 1995). Several studies suggested that the postprandial increase in hepatic triglyceride-rich VLDL particles result from an increased flow of substrates to the liver as a result of insulin resistance (Heath et al., 2003; Francone et al., 1992). A second explanation is a
postprandial saturation of the lipolysis system, due to competition of VLDL particles with chylomicrons or their remnants for the common removal pathway. The close correlation between the increment in apo-B48 and apo-B100 containing triglyceride-rich lipoproteins in the postprandial state confirms that the clearance of chylomicrons and VLDLs is closely related (Demant et al., 1998; Packard et al., 2000).

Remnant particles have also been shown to be toxic to both endothelial cells and macrophages, and triglyceride-rich apolipoprotein B containing lipoproteins have been isolated in excess from atherosclerotic plaque (Rapp et al., 1994). Recently, it has been noted that the postprandial state is associated with impaired endothelium-dependent vasodilatation, which is generally considered to be an early marker of cardiovascular disease (Anderson et al., 2001; Evans et al., 1999; Muntwyler et al., 1999).

1.5.1 Triglyceride-rich lipoproteins and haemostatic abnormalities-relation to CHD

Several lines of evidence suggests that triglyceride-rich lipoproteins may be directly or indirectly thrombogenic and/or antifibrinolytic. An increase in factor VII coagulant activity (VII c) has been observed in type IIb and type IV hyperlipidaemia. The Nothwick Park Heart Study demonstrated an independent association between factor VII activity and CHD in middle aged men. Both cholesterol and triglyceride concentrations are related to factor VII (Meade et al., 1993).

Demonstrable changes have been observed in postprandial haemostasis including an increase in factor VII when fat is added to the diet (Miller et al., 2002). In addition factor VIIc and factor VII antigen levels are strongly correlated with each other and both were significantly increased in patients who exhibited abnormal postprandial triglyceride level after a test meal, compared to those who displayed normal triglyceridaemia (Miller et al., 1998). A possible explanation for these observations might be that the increase in activated VII activity represents an acute response of the haemostatic system to
postprandial lipolytic activity, and in particular the appearance of negatively charged surface lipoproteins, such as remnant particles that activate the intrinsic pathway (Silveira et al., 1996).

Elevated plasminogen activated inhibitor 1 (PAI-1) levels have recently been included as a component of the insulin resistance syndrome and are strongly associated with body mass index, insulin and triglyceride levels (Dichtil, et al., 2000; Juhan-Vague et al., 1996). Plasma PAI-I activity is considered to be a major determinant of plasma fibrinolytic activity, it is a specific inhibitor of t-PA and is synthesised in the liver and endothelial cells. The concentration of PAI-I is partly dependent on a common polymorphism discovered in the promoter region of the PAI-I gene (Panahloo et al., 1996). Fatty acids derived from triglycerides in VLDL can interact with the promoter region of the PAI-1 gene, leading to increased transcription of PAI-I in human endothelial cells. The molecular mechanism is related to a VLDL-responsive element in the promoter region in PAI-1 gene (Eriksson et al., 1998). Importantly, the influence of VLDL triglycerides on PAI-1 seems to be genotype-dependent. PAI-1 levels are more strongly correlated with levels of triglycerides in 4G/G subjects than in 5G/G subjects (Ossei-Gerning et al., 1997; Fu et al., 2001; Stegnar et al., 1998). Genotypic-specific regulation of plasma PAI-1 by VLDL is due to competition of these two proteins for overlapping binding sites.

1.5.2 Diabetes mellitus and Coronary Heart Disease

Elevated levels of plasma triglyceride have long been associated with an increased risk of cardiovascular disease in diabetic patients. Type 2 diabetes is associated with high risk of premature morbidity and mortality from atherosclerosis-related disease including coronary heart disease (Sniderman et al., 2001), cerebrovascular and peripheral vascular disease (Letho et al., 2000; Manolio et al., 1996). Diabetic patients without previous
myocardial infarctions have been found in same studies to have a high risk of myocardial infarction as non-diabetic patients who have had a previous myocardial infarction (Haffner et al., 1998). Diabetic patients also have high mortality rate from first myocardial infarction.

The aetiology of vascular disease is likely to be multifactorial with factors associated with the diabetic state such as hyperglycaemia and factors associated with concomitant metabolic syndrome particularly dyslipidaemia, hypertension, thrombotic and inflammatory factors. In addition the glycosylation of proteins may also affect arterial wall physiology and risk of disease (Zhang et al., 1998).

In a review of previous studies that examined the role of triglyceride as a risk factor for cardiovascular disease, it was noted that most analyses showed a relation between triglyceride and coronary artery disease (Austin et al., 1991). However, a number of studies found that this association did not remain statistically significant after controlling for other risk factors, especially HDL cholesterol. Data from Lipid Research Clinics Follow-up Study demonstrated that triglyceride was related to 12-year coronary artery disease mortality both in men and women (Criqui et al., 1993) and after adjustment for covariates, including HDL, the relative risk was no longer statistically significant. This finding led the National Institute of Health Consensus Panel to question whether triglyceride is a risk factor for coronary artery disease, independent of HDL.

To address the role of triglycerides in cardiovascular disease using all available data, meta-analysis of the existing epidemiological literature has shown that increased plasma triglyceride is associated with significant increase in risk of incident cardiovascular disease among both men and women. In men, an increase of 1 mmol/L was associated with 32% increase in risk of disease. A greater increase in risk (76%) was found in women. After adjustment for HDL cholesterol the risks were decreased but remained statistically significant (Hokanson et al., 1996).
Recent studies have demonstrated the importance of triglyceride as a risk factor for atherosclerosis. The Physician Health Study showed that both triglyceride and LDL particle size were significant predictors of future coronary artery disease.

In summary both cross sectional and prospective studies have highlighted the strong association between increasing plasma triglyceride concentration and cardiovascular disease and knowledge is increasing concerning the potential atherogenic effects of hypertriglyceridaemia. It is likely that some triglyceride-rich lipoproteins particles such as remnant particles are directly atherogenic and contribute to arterial lipid deposition. Furthermore, postprandial hypertriglyceridaemia is prolonged because of competition for lipoprotein lipase between chylomicrons and continued hepatic VLDL secretion which is not suppressed postprandially in type 2 diabetes. Hypertriglyceridaemia is also associated with important alterations in other lipoproteins. HDL is reduced and the distribution of LDL is shifted to smaller denser particles which are thought to be more atherogenic partly through increased susceptibility to oxidation. Important additional determinants of these changes are increased lipid exchange between lipoproteins via cholesterol ester transfer protein and increased activity of the enzyme hepatic lipase. It is likely that triglyceride-enriched LDL and HDL are substrates for hepatic lipase which hydrolyses triglyceride and phospholipid leading to smaller denser LDL and HDL. Small dense HDL is more rapidly catabolised, contributing to reduced plasma HDL concentrations.
1.6 MANAGEMENT OF TYPE 2 DIABETES

Over the last four decades, oral therapies for type 2 diabetes have focused on sulphonylureas and metformin, with acarbose introduced in 1990.

Sulfonylureas

This class of drugs lowers blood glucose by stimulating insulin secretion from pancreatic \( \beta \)-cells. Their molecular effect is characterised by binding to and blocking an ATP-sensitive potassium (\( K^+ \)) channel (\( K^+(ATP) \)) of the \( \beta \)-cells. The precise binding target is the sulfonylurea receptor (SUR1) subunit of this channel. Reduced \( K^+ \) conductance causes membrane depolarization followed by an influx of calcium through a voltage sensitive calcium channel. The increased intracellular calcium concentration then triggers insulin secretion. Glibenclamide is a member of this group, it blocks channels containing SUR1 and SUR2 (in heart and in smooth muscle cell). However, glibenclamide administration has no effect on the insulin sensitivity of peripheral tissues.

Metformin

Metformin belongs to the biguanide class. It acts as an antihyperglycaemic by decreasing hepatic glucose secretion through inhibition of gluconeogensis and delaying enteric resorption of glucose. Metformin does not influence the secretion of insulin, but it appears to increase insulin action in peripheral tissues.

Acarbose

Acarbose competitively and reversibly inhibits \( \alpha \)-glucosidase, a brush border enzyme in the lumen of the small intestine which hydolyszes disaccharides. This inhibition causes a decrease in carbohydrate absorption, because disaccharides are not broken down into absorbable monosaccharides.
In summary the benefits of these drugs on glycaemic levels, as monotherapy and in combination are well documented. However, current antidiabetic agents can claim only a modest impact against the progressive nature of diabetes and its chronic microvascular and macrovascular complications.

**Insulin Sensitizers**

Thiazolidindiones (TZDs) are a new class of oral antidiabetic agents. They selectively enhance or partially mimic certain effects of insulin on carbohydrate and lipid metabolism in type 2 diabetes mellitus and other conditions of insulin resistance. The next section will discuss the detailed mechanism of action of this new class of drug.

**1.6.1 Peroxisome Proliferator-Activated Receptors and Thiazolidinediones**

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and are members of the nuclear hormone receptor superfamily. Activated PPARs form a heterodimer with the retinoid receptor (RXR) and bind to specific response elements in the promoter region (PPREs) of target genes (Figure 8). An additional "co-repressor" protein in this complex may serve to maintain the receptor in an inactivated state (Desverne and Whali et al., 1999).

PPAR ligands may be natural such as saturated and unsaturated fatty acids or synthetic such as fibric acid derivatives and thiazolidinedione (TZDs). After binding with ligand, the activated PPAR undergoes conformational change, displacing the co-repressor or allowing for the binding of a coactivator that leads to enhanced transcription of the target gene (Auwerx et al., 1999). PPARs control several genes involved in carbohydrate and lipid metabolism (Table 3) (Desvergne and Wahli et al., 1999; Schoonjans et al., 1996).
Three isotypes of PPARs α, β/δ and γ have been described. They are a subgroup of the family of nuclear receptor which include retinoid receptor (RXR), thyroid receptor and vitamin D receptor (Schoonjans et al., 1996). In humans, each PPAR gene is located on different chromosomes and is expressed differently in various organs (Braissant et al., 1996). PPARα is located on chromosome 22 and expressed predominantly in the liver and less in the heart, kidney, skeletal muscle and large intestine, PPAR β/δ is on chromosome 6 and is found at moderate levels in all tissues tested, with high expression in placenta, large intestine and heart.

The PPARγ gene located on chromosome 3, can encode for two proteins by the use of an alternative splice site. PPARγ2 contains an additional 28 amino acids at the amino terminus. Both isoforms PPARγ1, γ2 are expressed primarily in adipose tissue and to a lesser extent in colon, the immune system, skeletal muscle, and the retina.

1.6.2 Function of PPARs at the cellular level

PPARs play key roles in different aspects of lipid metabolism and homeostasis. PPARγ influences the storage of fatty acids in adipose tissue, and is part of the adipocyte differentiation program that induces the maturation of pre-adipocytes into mature adipocyte. PPARγ target genes encode enzymes involved in peroxisomal mitochondrial B-oxidation, ketone body synthesis, lipoprotein lipase (LPL), adipocyte fatty acid binding protein (A-FABP or Ap2), Acyl- CoA synthase and fatty acid transport protein (FATP).

In adipose tissue, even under high catabolic conditions such as fasting, lipogenesis continues and is dependent upon PPARγ while some of the released fatty acids are re-esterified to triglycerides in a reaction which requires synthesis of glycerol in a rate-limiting step which requires phosphoenolpyruvate carboxykinase, whose transcription is positively controlled by PPARγ.
A heterozygous knockout mouse for PPARγ deficiency has been generated by gene targeting. Surprisingly these animals were shown to have enhanced insulin sensitivity in peripheral tissues (measured by glucose disposal rate) and liver (suppression of glucose production) and lower insulin concentrations during glucose tests than wild type mice (Miles et al., 2000). The investigators concluded that the genetic reduction in PPARγ gene expression is associated with increased insulin sensitivity and under normal conditions full PPARγ activity may have a role in the development of insulin resistance. In other studies heterozygous PPARγ deficient mice were shown to be resistant to the effects of a high fat intake on glucose homeostasis (Kubota et al., 1999).

These animals, unlike wild type mice, did not become obese and insulin resistant and maintained expression of Glut-4 in white adipose tissue when fed a high fat diet. Furthermore leptin expression was increased in the PPARγ deficient mice due to partial suppression of PPARγ-mediated inhibition of leptin gene expression, possibly accounting for the protective effect. Results from these studies in heterozygous PPARγ -/+ mice are consistent with a prime role for PPARγ in adipocyte differentiation with secondary effects on insulin sensitivity.

Further insights into the role of PPARγ have come from genetic studies. A mutation in the PPARγ2 that resulted in the conversion of proline to glutamine at position 115 was described in 4 out of 121 obese German subjects (Ristow et al., 1998). All the subjects with the mutant allele were markedly obese. Over expression of the mutant gene in murine fibroblasts led to the production of a protein, which accelerated differentiation of the cells into adipocytes, and greater accumulation of triglyceride. Of interest, insulin resistance was less in these subjects. The mean fasting insulin concentration was lower in these subjects compared to the obese subjects. Furthermore two different heterozygous mutation in the ligand-binding domain of PPARγ have been
reported in three subjects with severe insulin resistance. In addition to insulin resistance, all three subjects developed type 2 diabetes and hypertension at an unusually early age.

These above findings provide compelling genetic evidence that this receptor is important in the control of insulin sensitivity, glucose homeostasis and blood pressure in man. PPARγ is likely to be an important regulator of monocyte/macrophage function with relevance for human atherosclerotic disease (Roberts et al., 2003). PPARγ is present in macrophages and in human atherosclerotic lesions and may regulate expression and activity of matrix metalloproteinases (MMPs) particularly MMP-9, an enzyme implicated in plague rupture (Marx et al., 1998). PPARγ has also been demonstrated in high concentrations in atherosclerotic lesions localised with oxidised LDL in both experimental animal and human atherosclerotic lesions (Ricote et al., 1998; Tontonoz et al., 1998).

1.6.3 Thiazolidinediones

Thiazolidinediones represent a new class of oral antidiabetic drugs, that are chemically and functionally unrelated to other oral antidiabetic agents. A thiazolidine-2,4-dione nucleus is common to all agents of this class, but they differ in side-chain modification that influences their pharmacological actions and potential for adverse effects.

The TZDs: troglitazone, rosiglitazone and pioglitazone have already been used in clinical practice (troglitazone has been withdrawn in the UK and USA because of liver toxicity). TZDs have been shown to be insulin sensitizers acting particularly in muscle and adipose tissue, TZDs reduce hyperglycaemia and insulin levels through a variety of mechanisms, and they increase insulin mediated glucose disposal.
1.6.4 Mechanism of action of thiazolidinediones

Thiazolidinediones appear to enhance insulin action without directly stimulating insulin secretion. They reduce hyperglycaemia in genetically insulin-resistant animals such as KKA, ob/ob, and db/db mouse (Chang et al., 1983; Fujiwara et al., 1995). This has been confirmed by glucose clamp procedures in non genetic models of insulin resistance, including both fructose-fed and high fat diet-adapted rats (Khouriheed et al., 1995; Lee et al., 1994;)

Table 3: Metabolic Effects of TZDs

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Adipose tissue</th>
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<tbody>
<tr>
<td>↑GLUT-I</td>
<td>↑Glucose uptake</td>
</tr>
<tr>
<td>↑GLUT-4</td>
<td>↑Glucose uptake</td>
</tr>
<tr>
<td>↑Glucokinase</td>
<td>↑Glucose utilisation</td>
</tr>
<tr>
<td>↓Phosphoenolpyruvate Carboxykinase</td>
<td>↓Gluconeogensis</td>
</tr>
<tr>
<td>↑Phosphodiesterase 3B</td>
<td>↓Intra-adipocyte lipolysis</td>
</tr>
<tr>
<td>↑Lipoprotein lipase</td>
<td>↑TG clearance</td>
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</tbody>
</table>

The improvement in insulin sensitivity seen with TZD treatment generally involves both increased peripheral glucose disposal and reduced hepatic glucose production in models with hyperinsulinaemia (Bowen et al., 1991; Oakes et al., 1994). In severely insulin-dependent diabetic states induced by streptozotocin or 90% pancreatectomy, TZDs have little or no effect on hyperglycaemia, confirming that their glucose-lowering activity is dependent upon the presence of adequate amounts of insulin (Fujii et al., 1997; Inoue et al 1995). However, chronic administration of TZDs caused regranulation of islet beta-cells in 90% pancreatectomised rats and ob/ob and db/db mice, without increasing plasma insulin concentrations (Fujiwara et al., 1995; Inoue et al., 1995). This may be
related to the sparing effect of TZDs which indirectly assist restoration of the beta-cell insulin reserve.

Figure 8: Molecular mechanism of thiazolidinedione TZDs. TZDs bind to PPARγ, one of the family of transcription factors. PPARγ is known to heterodimerize with another transcription factor, the RXR, enabling PPAR to interact with DNA sequences controlling insulin-sensitive genes. The effects of TZDs appear also to require that insulin-regulated transcription factors bind to insulin response elements. PPARγ activation is linked to increased levels of CCAAT/enhancer binding protein-a (C/EBP-a), a transcription factor involved in the differentiation of preadipocytes to adipocytes.

1.6.4.1 Increasing glucose transporters and uptake

In vitro studies have demonstrated that TZDs act directly on adipocyte and skeletal muscle to increase glucose transport. In the case of pioglitazone, this has been attributed
to increased production of the glucose transporter (Table 3) isoforms Glut-1 and Glut-4 and translocation of Glut-4 into the plasma membrane (Ciaraldi et al., 1995, 2002; El-Kebbi I et al., 1994; Sandouk et al., 1993). This effect was evident without added insulin, and it is possible that TZDs mimic certain effects of insulin and directly increase muscle glycogen synthesis. In cultured hepatocytes (HepG2 cells), TZDs increased glycogen synthesis and decreased gluconeogenesis (Ciaraldi et al., 1991).

KKA mice is a model of insulin resistance with reduced Glut-4 in adipose tissue and skeletal muscles. Treatment of these mice with pioglitazone for 4 days increased Glut-4 in skeletal muscle and adipose tissue (Hofmann et al., 1991).

In humans, euglycaemic hyperinsulinaemia clamp studies have demonstrated an increase in insulin-stimulated glucose disposal in patients with type 2 diabetes treated with pioglitazone (Mahankali et al., 2000).

1.6.4.2 Enhancing Insulin Signalling

TZDs do not appear to act directly on insulin receptor binding in cultured cells, although the improved metabolic environment after administration of TZDs in vivo can increase the number of insulin receptors without altering affinity (Bonini et al., 1995). Wistar fatty rats are an animal model of insulin resistance. They have decreased activity of IRS-1 and low activity of PI-3-kinase (Hayakawa et al., 1996). Pioglitazone administration for 2 weeks altered the activity of the β-subunit of the insulin receptor, increasing autophosphorylation and tyrosine kinase activity with improvement of hyperglycaemia and hyperinsulinaemia.

KKA mice have low levels of PI-3-kinase and IRS protein in adipose tissue and liver. There is also evidence that pioglitazone can increase PI-3-Kinase activity which increases the responses of the cell to insulin (Bonini et al., 1995; Kobayashi et al., 1992).
6.4.3 Lowering plasma NEFA content

TZDs generally reduce circulating concentrations of triglyceride and NEFA in a number of diabetic rodent models (Oakes et al., 1994; Young et al., 1995). These effects are observed in both insulin resistant and insulin deficient animals, thus the lipid-lowering effect of TZDs appears to be independent of their glucose-lowering and their insulin-lowering effects. The lipid lowering effect is usually greater in the hyperlipidaemic animals, this has been attributed to decreased hepatic VLDL synthesis and increased peripheral clearance, together with reduced lipolysis (Oakes et al., 1994; Lee et al., 1994).

Insulin resistance is often associated with increased concentrations of plasma NEFA due to increased intra-adipocyte lipolysis caused by decreased inhibition of the hormone sensitive lipase (Reaven et al., 1988). Insulin exerts its anti-lipolytic effect by activating phosphodiesterase (PDE-3B), which in turn inactivates hormone sensitive lipase activity. Pioglitazone treatment in people with type 2 diabetes can restore both PDE-3B level, in this way result in decreased lipolysis and lower plasma NEFA (Bajaj et al., 2003; Kaneko et al., 1997; Miyazaki et al., 2000).

1.6.4.4 Reduction of hepatic glucose output

KKA mice have an increased activity and expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis. Treatment of these mice with pioglitazone normalised the activity of PEPCK and decreased blood glucose concentration (Ikeda et al., 1990).

In fructose-fed insulin-resistant rats, pioglitazone treatment for 2 weeks reduced glucose output by perfused liver, and insulin perfusion caused a greater decrease in glucose output in the pioglitazone treated group (Ikeda and Fujiyama, et al., 1998). In a perfused rat liver model, addition of pioglitazone decreased gluconeogenesis by around...
70% after 20 minutes (Nishimura et al., 1997). These studies indicate that pioglitazone inhibits gluconeogenesis in the liver.

### 1.6.4.5 Adipocyte Differentiation

PPARγ is a pivotal coordinator of the adipocyte differentiation process enhancing the expression of adipocyte-specific genes and promoting adipocyte differentiation (Hallakou et al., 1997; Hutley et al., 2003; Fajas et al., 1998). Treatment of preadipocyte cultures for 7 days with pioglitazone and insulin resulted in nearly 100% differentiation of cells to lipid-accumulating adipocytes, and such adipocytes showed a markedly increased expression of cellular glucose transporter (Glut-1, Glut-4).

When 3T3-L cells are treated with pioglitazone, a marker of adipocyte differentiation glycerol 3-phosphate increases, with an increase in insulin receptor m-RNA and total number of insulin receptors (Swanson et al., 1995; van Harmelen et al., 2002).

### 1.6.4.6 Reduction of Tumour Necrosis Factor-alpha (TNFα)

Tumour Necrosis Factor-alpha has been shown to have certain catabolic effects on fat cells. Recent studies in animal models indicated that TNFα may play a role in mediating insulin resistance in obesity and diabetes through its ability to inhibit PI-3-kinase downstream in the insulin signalling pathway the tyrosine kinase activity of the insulin receptor (IR). TNF-α causes phosphorylation of serine sites in the IRS and thereby reduces the interaction between the insulin receptor and its ligands (Hotamisligil et al., 1999).

Incubation of 3T3-L1 cells with TNFα completely inhibits adipocyte conversion and expression of fatty acid-binding protein m-RNA. However, co-incubation of TNFα-
treated cells with pioglitazone blocked these effects (Souza et al., 2003; Szalkowski et al., 1995).

1.6.4.7 Increase of adiponectin/ACRP30/adipoQ

Adiponectin is also known as an adipose-specific plasma protein adipoQ (Hu et al., 1996). It is synthesised in adipose tissue, but acts primarily on skeletal muscle by increasing the expression of proteins involved in fatty acid transport, combustion and energy dissipation (CD36, acyl CoA oxidase). By increasing the influx and combustion of fatty acids it reduces the muscle content of triglycerides, the amount of free fatty acids in serum, and lowers liver triglycerides. The adiponectin gene has been mapped to a diabetes susceptibility locus at the human chromosome 3q27. This locus is strongly linked to the “metabolic syndrome” (Berg et al., 2002).

Circulating adiponectin concentrations are decreased in obese individuals (Arita et al., 1999) and the reduction was proposed to have a role in the pathogenesis of arteriosclerosis and cardiovascular disease associated with obesity and other components of the metabolic syndrome (Kishida et al., 2003; Kojima et al., 2003; Matsuzawa et al., 1999).

Thiazolidinedione administration significantly increased plasma adiponectin concentration in insulin resistant humans and rodents. The expression of adiponectin mRNA was normalized or increased by TZDs in the adipose tissue of obese mice, and in the 3T3 L1 adipocyte. These effects were mediated through the activation of the promoter by TZDs. On the other hand, TNF-α, which is produced more in insulin-resistance, reduced the expression of adiponectin in adipocytes by suppressing its promoter activity. TZDs restored this inhibitory effect by TNF-α (Ruan et al., 2003).
1.6.4.8 Decreased expression of tissue resistin

Resistin was recently identified as a hormone secreted by adipocytes, which leads to insulin resistance *in vivo* and *in vitro* (Fasshauer et al., 2001; Steppan et al., 2001). It has been reported recently that resistin expression is significantly increased in adipose tissue in several different animal models of obesity (Ahima et al., 2001; Banerjee et al., 2003). In response to TZDs, adipose tissue resistin expression is decreased in both ob/ob mice and Zucker rat (Arner et al., 2003).
1.7 AIMS OF THE STUDY

The overall aim of this study was to establish if any beneficial metabolic effects are evident in type 2 diabetic patients after 20 weeks tissue sensitisation to insulin through the PPARy nuclear receptor with pioglitazone compared to augmentation of insulin output by sulphonylurea in regimes designed to maintain glycaemic control constant. Maintenance of glycaemic control stabilised this factor known to perturb metabolism by a variety of mechanisms and so allow a less complicated analysis of the results. A group of control subjects was also studied to assess if any changes that occur in diabetic patients improve known defects in metabolism such as delayed clearance of dietary fat to that of normal situation.

Specifically, the following parameters were investigated in a detailed study of the fasting and postprandial state:

**Fasting State**

The effect of insulin sensitisation with pioglitazone on:

- Fasting dyslipidaemia including analyses of total triglyceride, subfractionation of fasting plasma lipoproteins into VLDL and its subfractions (VLDL-1 & VLDL-2) IDL, LDL, HDL (HDL2 & HDL3), apolipoproteins A-I & B, Lp(a) and NEFA.
- Fasting glycaemia and β-cell function as measured by plasma insulin content and the presence of insulin precursors.
- Assessment of insulin resistance using the HOMA model.
- Proteins and enzymes responsible for lipoprotein remodelling processes: LPL, HL, LCAT, CETP and PLTP.
Postprandial State

- Postprandial lipaemia after a high fat meal including analyses of total triglyceride and the cholesterol and triglyceride content of subfractions of plasma lipoproteins including chylomicrons and chylomicron remnants labelled with retinyl palmitate, IDL, LDL, HDL (HDL$_2$ & HDL$_3$), and the insulin-dependent suppression of NEFA release from adipose tissue.

- The effect of insulin sensitisation with pioglitazone on postprandial glucose and β-cell function as measured by the release of insulin and insulin precursors (proinsulin and 32-33 split proinsulin).

- The effects of insulin sensitisation with pioglitazone on postprandial cholesterol esterification by LCAT and lipid exchange by CETP and PLTP.
CHAPTER 2: MATERIALS AND METHODS

2.1 SUBJECTS, INSULIN SENSITISATION AND POSTPRANDIAL LIPID METABOLISM PROTOCOLS

2.1.1 DIABETIC PATIENTS

Twenty-two patients with type 2 diabetes were recruited from the diabetic clinic at University College London and North Middlesex Hospital. All patients exhibited modest glycaemic control, (mean HbA1C 7.3%), with diagnosis of type 2 diabetes based on WHO criteria. The group consisted of 17 males and 5 females with mean age of 56.2 year (age range 30-75 years). All of these patients had a total plasma triglyceride level > 1.7 mmol/L and were receiving no lipid lowering treatment, or taking drugs known to directly affect lipid metabolism. Mean duration of diabetes was 2.5 years. Patients with abnormal hepatic, thyroid or renal function test, and excessive consumption of alcohol were excluded., inclusion and exclusion criteria shown in Table 4. All females in the study were not taking any hormonal therapy. All subjects were euthyroid and had normal liver and kidney functions.

Table 4: Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>INCLUSION CRITERIA</th>
<th>EXCLUSION CRITERIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TG &gt; 1.7-5 mmol/l</td>
<td>Insulin therapy, acarbose</td>
</tr>
<tr>
<td>HbA1C &lt; 9%</td>
<td>Statin, fibrate, hormonal therapy.</td>
</tr>
<tr>
<td>Glibenclamide &lt; 10 mg/d</td>
<td>ALT, AST &gt; 2.5 above normal</td>
</tr>
<tr>
<td>Metformin &lt; 2500 mg/d</td>
<td>LDL &gt; 4.1 mmol/L</td>
</tr>
<tr>
<td>Gliclazide &lt; 240 mg/d</td>
<td>C-peptide &lt; 1 ng/ml</td>
</tr>
<tr>
<td>Age 25-75 years</td>
<td>Creatinine &gt; 150 μmol/l, macroalbuminuria</td>
</tr>
<tr>
<td>BMI 25- 35 kg/m²</td>
<td>Heart failure, uncontrolled blood pressure</td>
</tr>
</tbody>
</table>
2.1.2 CONTROL SUBJECTS

Ten healthy control subjects (6 male, 4 female) matched for age and body mass index (BMI) with the diabetic subjects were recruited into the study. No control subject was taking any medication, and no family history of diabetes mellitus.

2.1.3 DOUBLE-BLIND, DOUBLE-DUMMY PROTOCOL FOR SENSITIZING THE DIABETIC SUBJECTS TO INSULIN WITH THIAZOLIDINEDIONE

Diabetic patients recruited into the study were, at that time, treated with either sulphonylurea or metformin as a mono-therapy. Drug therapy was withdrawn from the subjects for a period of 6 weeks and during which they were given dietary advice. Subjects were monitored during that period for fasting glucose and HbA1C to check that they were suitable to continue on the study.

One week prior to the end of the wash out period subjects attended for preparation of post-heparin plasma (PHP) lipase measurements (see Chapter 5). At the completion of the washout phase the subjects attended for the baseline test meal (see section 2.1.4.1). Patients then were allocated to either receive glibenclamide 5 mg/d or pioglitazone 45 mg/d in a double-blind double-dummy protocol designed to maintain the same glycaemic control for 20 weeks. To achieve the same glycaemic control an open label glibenclamide 5 mg could be added to any subjects (only one patient required this intervention and at the end of the study, when the code was broken, was found to have been in the group taking glibenclamide therapy).

During the treatment period all subjects were monitored regularly for their glycaemic control, liver and kidney function and full blood count. One week prior to the end of the treatment period subjects attended for preparation of another post-heparin plasma (PHP) for lipase measurements. At the completion of the treatment period the
subjects received an identical test meal as described previously. Two weeks after the end of the study, patients attended for a final full clinical and biochemical check up visit.

Control individuals participating in the study received no drug therapy nor placebo prior to the test meal. Post-heparin plasma was prepared one week before the test meal.

The study was fully approved by local ethics committee and all subjects gave informed written consent prior to commencing the study.

**VISIT 1 (week – 8 to –6): Consent and Admission to Trial.** Physical examination (height, weight, blood pressure BP), screening for fasting lipid profile, full blood count (FBC), liver function test (LFT), urea and electrolyte, C-peptide, glucose, HbA1C, urine protein dipstick test.

**VISIT 2 (week –6): Washout period started.** Patients’ diabetic medication withdrawn and replaced with placebo tablets and instructed concerning their diet.

**VISIT 3 (week –1):** Physical examination (body weight, BP), fasting lipid profile, FBS, HbA1C, urine protein dipstick test.

**VISIT 4A (4 days after visit 3):** Post heparin HL/ LPL.

**VISIT 4 (day 0):** Baseline test meal. FBC, LFT, urea & electrolyte, urine protein dipstick test. (Randomization start next day).

**VISIT 5 (week 4):** Body weight, documentation of adverse events or changes in concomitant medication.

**VISIT 6 (week 8):** Vital signs, body weight, documentation of adverse events or changes in concomitant medication. Fasting lipid profile, FBS, HbA1C, FBC, LFT, urea & electrolyte, dipstick urine analysis.
VISIT 7 (week 12): Vital signs, body weight, HbA1C, FBS, fasting lipid profile, dipstick urine analysis. Documentation of adverse events or changes in concomitant medication.

VISIT 8A (4 days before visit 8): Post-Heparin HL/ LPL.

VISIT 8 (week 20): Post-treatment test meal. FBC, LFT, urea & electrolyte, urine protein dipstick test.

VISIT 9 (week 22): Vital signs, body weights. FBC, LFT, urea & electrolyte, urine protein dipstick test.

2.1.4 ORAL FAT TOLERANCE TEST

2.1.4.1 The high-fat content meal

A mixed-meal with a high-fat content was administered to the patients for the measurement of postprandial lipid and glucose metabolism after a 6 week washout period during which the subjects received placebo. A second mixed-meal with a high-fat content was administered following 20 weeks treatment with pioglitazone or glibenclamide.

Patients were asked to refrain from alcohol for the preceding 24 hours and to fast for 14 hours prior to the test. The meal was eaten within 20 minutes and the subject remained fasting for the following 8 hours but was allowed water *ad libitum*. No smoking was allowed during the test. The test meal was given at 8.00 am and consisted of 110 g of fat (P/S ratio 0.4), 46 g of protein, 70 g of carbohydrate, and 729 mg of cholesterol. The ingredients were incorporated as a milkshake, scrambled eggs and cheese sandwich. 300,000 IU of vitamin A (retinyl palmitate - Roche Ltd) was included
with the milkshake to label the intestinally derived lipoprotein. After ingestion, vitamin A is incorporated into the newly-synthesised chylomicrons as retinyl palmitate (RP) (Hazzard and Bierman et al., 1976; Goodman et al., 1980) and is finally taken up by the liver after lipolysis to chylomicron-remnant particles. Retinyl palmitate is then hydrolysed to retinol and either released into the plasma bound to retinol-binding protein or re-esterified and stored in the liver. Since RP is not re-secreted by the liver (Lenich and Ross et al., 1987), and the exchange of RP among lipoproteins is minimal (Berr et al., 1985; Cortner et al., 1987; Sprecher et al., 1991; Wilson et al., 1983) the measurement of RP content reflects the concentration of chylomicrons and their remnants in the circulation.

2.1.4.2 Blood sampling

Blood samples were drawn from an indwelling venous catheter into an appropriate anticoagulant (plasma) or plain tube (serum); the first sample was taken immediately before eating the test meal and then at 30 minutes intervals for the first 2 hours and then hourly for the further 6 hours. Plasma or serum was separated by centrifuge at 3000 x g for 10 minutes. Cholesterol, triglyceride, NEFA, phospholipid, insulin, proinsulin and split proinsulin were measured on these samples. Individual lipoproteins (chylomicron, chylomicron remnants, IDL, LDL, HDL$_2$, HDL$_3$) were isolated from plasma sample taken at 0, 1, 2, 4, 6, 8 hr. LCAT, CETP and PLTP activities were measured on the plasma samples taken in the fasting state and 4 hr postprandial. Apo-AI, apo-B, LP(a), VLDL and LDL subfractions were measured only in the fasting state of each test meal.

Blood sampling for measurements of total cholesterol, triglyceride and their subfractions (VLDL, IDL, LDL, HDL), chylomicron and chylomicron remnants retinyl palmitate, NEFA, phospholipid, glucose, CETP, LCAT, LPL, HL were done by me.
Measurements of serum insulin, proinsulin and 32-33 split proinsulin were done in the department of Clinical Biochemistry, Addenbrookes Hospital (Professor C. N. Hales).

Measurements of LDL subfractions were performed at the Institute of Biochemistry, Surrey University (Dr. B. Griffin). Measurements of PLTP mass were done at the University of Lille, France (Professors B. Staels and G. Luc).
LABORATORY TECHNIQUES

2.2 ISOLATION OF LIPOPROTEINS AND LIPOPROTEIN SUBTRACTIONS

2.2.1 INTESTINAL LIPOPROTEINS

2.2.1.1 Chylomicrons

Chylomicrons (Sf > 1,000) were separated from plasma by sequential flotation, (Dole and Hamlin et al., 1962; Grundy and Mok et al., 1976; Lindgren et al., 1972).

2.5 ml of plasma was overlaid with 0.5 ml of 0.15 M sodium chloride solution. Tubes were balanced and centrifuged at 417,000 x g_average for 5 minutes at 16°C in a Beckmann TLA 110 rotor. Under these conditions, chylomicrons migrate into the saline layer, which was carefully aspirated, and the volume made up to 1 ml with saline. Fractions were stored at -20°C for subsequent lipid analyses and the assay of retinyl palmitate content.

2.2.1.2 Chylomicron Remnants

0.5 ml of saline was overlaid into the plasma infranatant obtained after removal of intact chylomicrons described above. The tubes were centrifuged at 417,000 x g_average for a further 30 minutes at 16°C. The upper saline layer containing the chylomicron-remnants was aspirated and made up to 1 ml with saline. Fractions were stored at -20°C for subsequent lipid analyses and the assay of retinyl palmitate content.

2.2.2 HEPATIC LIPOPROTEINS

2.2.2.1 Very Low-density Lipoproteins 1 and 2 (VLDL-1 and VLDL-2)

Fasting levels of VLDL-1 and VLDL-2 were determined by sequential flotation of the lipoproteins from plasma at densities of 2.006 g/ml and 2.019 g/ml respectively (Hatch
and Lees et al., 1968). Total VLDL was isolated from 2.5 ml of plasma overlaid with 0.5 ml sodium chloride (0.15 M). Samples were ultra-centrifuged at 417,000 x g_{average} for 30 minutes at 16°C. Under these conditions total VLDL (VLDL-1 and VLDL-2) floats into the saline layer which was aspirated and made up to 1 ml with saline. The remaining plasma infranatant was retained and used for IDL and LDL separation.

To subfractionate the total VLDL into VLDL-1 and VLDL-2, 170 mg/ml of solid sodium chloride was added to the VLDL preparation and mixed thoroughly. The samples were overlaid with 1 ml of a KBr solution (density 2.0852 g/ml). The sample was centrifuged at 417,000 x g_{average} for 10 minutes at 16°C. The upper supernatant containing VLDL-1 was removed and made up to a volume of 1 ml with saline. The infranatant was overlaid with 0.5 ml saline and centrifuged at 417,000 x g_{average} for 45 minutes at 16°C and the VLDL-2 was aspirated from the top of the tubes and then made up to a volume of 1 ml with saline for VLDL-2 measurement. Samples were stored at -20°C prior to lipid analyses.

2.2.2.2 Intermediate Density Lipoproteins (IDL)

IDL was isolated from the infranatant obtained after removal of VLDL as described above 127 μl of a high-density KBr/NaCl solution, (KBr 2.97 mol/L, NaCl 2.62 mol/L, EDTA 240 μmol/L; 2.33 g/ml density) was added and mixed well. An aliquot (0.5 ml) of KBr solution (2.019 g/ml density) was overlaid on to the samples and centrifuged at 417,000 x g_{average} for 45 minutes at 16°C. Under these conditions IDL floats into the upper non-plasma layer which was then aspirated and made to 1 ml volume with saline. Samples were stored at -20°C prior to lipid analyses.
2.2.2.3 Low-Density Lipoproteins (LDL)

LDL was isolated from the infranatant obtained after removal of IDL. 525 µl of KBr heavy solution, (KBr 2.97 mol/L, NaCl 2.62 mol/L, EDTA 240 µmol/L; 2.33 g/ml density) was added and mixed well. An aliquot (0.5 ml) of KBr solution (2.063 g/L density) was overlaid on the samples which were centrifuged at 417,000 x g_{average} for 45 minutes at 16°C. Under these conditions LDL floats into the upper non-plasma layer which was then aspirated and made to 1 ml volume with saline. Samples were stored at -20°C prior to lipid analyses.

2.2.2.4 Low-Density Lipoprotein subfractions (LDL)

Isolation of LDL subfractions was achieved by density gradient ultracentrifugation using a discontinuous salt gradient (Griffin et al., 1990). Plasma was adjusted to a density of 2.09 g/ml with solid KBr (0.38 g per 3 ml plasma). The samples and 6-step salt gradient were introduced sequentially into polyvinyl alcohol-coated polyallomer SW-40 tubes by peristaltic pump. The gradient was prepared and centrifugation was carried out at 23°C. All densities were checked using a digital densitometer (PAAE Scientific Ltd, UK). The rotor was accelerated to 170 r.p.m over 4 minutes in Beckman L8-60 ultracentrifuge and then centrifuged at 40,000 r.p.m for 24 hours. The gradient containing the separated LDL fractions was displaced upwards from the tube in an apparatus similar to that devised by Groot et al (1982). A dense, hydrophobic material (Maxidens, 2.9 g/ml, Nyegaard Ltd) was introduced under the plasma layer by a constant infusion pump at a flow rate of 0.69 ml/min. The elute was passed through a UV detector (MSE/Fisons, UK) and continuously monitored at 280 nm. The elution times of the first, least dense, LDL fraction and the appearance of plasma proteins were
identified by peak maxima that occurred between hydrated density interval of 2.025-2.034 g/ml (LDL-I), 2.034-2.044 g/ml (LDL -II), or 2.044-2.060 g/ml (LDL-III).

The individual subtraction areas beneath the LDL profiles were quantified using a Beckman "Data Graphics" software (Beckman "Data Leader", Beckman, UK). Integrated areas were adjusted by specific extinction coefficients calculated previously for LDL-I, II, and III to give their percentage abundance (% LDL). The total LDL mass (lipid and protein mass) of sequentially prepared LDL (density 2.019-2.063) was then subdivided in proportion to the adjusted percentage values. This gave rise to concentration values for each LDL subfraction in mg of lipoprotein per 100 ml of plasma. LDL subfractions were therefore expressed in terms of adjusted percentage abundance and plasma concentration.

2.2.2.5 High-Density Lipoproteins and subfractions (HDL2 and HDL3)

It is well established that the traditional heparin/Mn²⁺ method used for determination of HDL in fasting plasma samples cannot be used for lipaemic samples (Demacker et al., 1980). This is because of the presence of lipid rich particles that do not react or sediment with heparin/Mn²⁺ treatment and lead to an overestimation of HDL cholesterol. The polyethylene glycol technique, which correlates well with results obtained on fasting plasma with the heparin/Mn²⁺ technique, relies instead on dehydrating the lipoproteins causing them to become insoluble. The addition of polyethylene glycol solution of different concentrations and pH results in the selective precipitation of different lipoproteins fractions.

To remove triglyceride-rich lipoproteins, which would otherwise interfere with HDL determination, samples of fasting and postprandial plasmas (1 ml) were overlaid with a 0.5 ml saline and centrifuged at 204,000 x gaverage at 16°C for 45 minutes.
Triglyceride-rich lipoproteins float into the saline layer, which was carefully aspirated and discarded.

Total HDL cholesterol content of the remaining plasma was determined by selective precipitation of VLDL and LDL (Quantolip Reagent A; Technoclone, UK). Followed by determination of the cholesterol content of the supernatant (see section 2.2.3.2). HDL\textsubscript{3} cholesterol content was determined by selective precipitation of VLDL + LDL + HDL\textsubscript{2} (Quantolip Reagent B; Technoclone, UK). Followed by determination of the cholesterol content of the supernatant (see section 2.2.3.2).

2.2.3 LIPID DETERMINATIONS

2.2.3.1 RETINYLM PALMITATE MEASUREMENTS

Sample extraction and High Pressure Liquid Chromatography (HPLC)

Retinyl palmitate present in chylomicron and chylomicron-remnant fractions was extracted by Folch's procedure using an internal standard of retinyl acetate (RA) to monitor and correct for variations in recovery (Folch et al., 1957). An aliquot of the preparation (600 \mu l) was mixed with 50 \mu l of RA (48.68 \mu mol/L) internal standard followed by 7 ml of CHCL\textsubscript{3}/CH\textsubscript{3}OH (2:1 v/v) in a 10 ml screw-top glass tube. After vigorous shaking at room temperature for 10 minutes, the sample was filtered through a glass fibre filter (Whatman glass microfibre filter Grade GF/A), into a 10 ml stoppered conical glass centrifuge tube. The filter was washed twice with 0.7 ml CHCL\textsubscript{3}/CH\textsubscript{3}OH (2:1 v/v) and distilled water (2.6 ml) was added to the filtrate and thoroughly mixed. After centrifugation at 2000 x g for 5 minutes, the upper aqueous phase was aspirated and the lower organic phase evaporated to dryness under nitrogen with gentle warming (50°C) under subdued light. The residue was dissolved in 150 \mu l of CHCL\textsubscript{3}/CH\textsubscript{3}OH (1:4
v/v) and an aliquot (100 μl) of the extract was injected on to a HPLC system (Waters Ltd) equipped with a 150 × 4.6 mm reversed phase column (3 μ octadecyl Spherisorb). The column was eluted with HPLC grade methanol (1 ml/min). The effluent was monitored at 340 nm for RA and RP with a UV detector (Waters Ltd). The RA eluted after about 3 minutes followed by the RP, 25 minutes later. The detector response for RP was linear between 0.05 and 2.2 mmol (DeRuyter and Leenheer et al., 1978; Wilson et al., 1983).

Quantification of Chylomicron and chylomicron remnants retinyl palmitate

A standard solution containing RP (3.8 μmol/L) and RA (48.68 μmol/L) in CHCL3/CH3OH (1:4 v/v) was injected on to the column monitored at 340 nm and eluted as described for the chylomicron and chylomicron remnant extracts. The peak heights were accurately measured for the standards and for the samples. By comparing the ratio of RA present in the standard and the samples to that of the RP present in standard and samples, the concentration of RP in the samples can be calculated using the following formula:

\[
[\text{RP sample}] = [\text{RP standard}] \times \left( \frac{\text{RP}_{\text{sample}}}{\text{RA}_{\text{sample}}} \right) \times \left( \frac{\text{RA}_{\text{std}}}{\text{RP}_{\text{std}}} \right)
\]

where RP_{sample} = peak height of RP in sample, RA_{sample} = peak height of RA in sample, RP_{std} = peak height of RP standard, RA_{std} = peak height of RA in standard. The inter-assay coefficient of variation was 7.0%.

2.2.3.2 Determination of cholesterol

The total cholesterol content of plasma and lipoprotein fractions were measured by an enzyme-linked calorimetric method (CHOD-PAP; Sigma Infinity Cholesterol reagent, Sigma Chemical Co., UK). The inter-assay coefficient of variation was 2.6%. In brief,
an aliquot of sample was measured with a reagent containing: detergent, cholesterol esterase, and cholesterol oxidase. \( \text{H}_2\text{O}_2 \) produced as a result of cholesterol oxidase activity was used to oxidise aminoantipyrene to give a pink product the absorption of which was measured at 500 nm.

### 2.2.3.3 DETERMINATION OF TRIGLYCERIDE

The total triglyceride contents of plasma and lipoprotein fractions were measured by an enzymatic-calorimetric method (GPO-PAP, Sigma Infinity Triglyceride reagent, Sigma Chemical Co. UK). The inter-assay coefficient of variation was 3.7% for triglyceride. In brief, an aliquot of sample was measured with a reagent containing a lipoprotein lipase causing the liberation of glycerol from triglycerides present, which was then determined after enzymatic conversion to glucose-1-phosphate and its subsequent oxidation by glucose phosphate oxidase. The resultant \( \text{H}_2\text{O}_2 \) was coupled to the formation of a coloured dye with horseradish peroxidase to give a pink product the absorption of which was measured at 500 nm.

### 2.2.3.4 DETERMINATION OF NON-ESTERIFIED FATTY ACIDS (NEFA)

NEFA concentrations in plasma were measured using an enzyme-linked calorimetric assay (Roche Diagnostics, UK). In brief, NEFAs were quantitatively converted to Acyl CoA (Reaction 1) that were then quantitatively oxidised by Acyl CoA oxidase causing the liberation of \( \text{H}_2\text{O}_2 \) (Reaction II). The \( \text{H}_2\text{O}_2 \) was used to oxidise 2, 4, 6-tribromo-3-hydroxy-benzoic acid (TBHB) to yield a red dye the absorbance of which was measured at 546 nm (Reaction III). The inter-assay coefficient of variation was 4.6%.

\begin{align*}
&\text{(I)}\quad \text{NEFA} + \text{CoA} + \text{ATP} \rightarrow \text{Acyl-CoA} + \text{AMP} + \text{pyrophosphate} \\
&\text{(II)}\quad \text{Acyl-CoA} + \text{O}_2 \rightarrow \text{enoyl-CoA} + \text{H}_2\text{O}_2 \\
&\text{(III)}\quad \text{H}_2\text{O}_2 + 4\text{-AA} + \text{TBHB} \rightarrow \text{red dye} + 2\text{H}_2\text{O} + \text{HBr76}
\end{align*}
2.2.3.5 DETERMINATION OF PLASMA PHOSPHOLIPID

A sample of heparin plasma was mixed with a reagent containing phospholipase D (Roche Diagnostic, UK). Phospholipase D cleaves the choline from phosphatidylcholine which is acted on by choline oxidase releasing $\text{H}_2\text{O}_2$ which is used to oxidise a chromogenic dye to give a coloured product catalysed by peroxidase. The absorbance at 500 nm was measured and the concentration of phospholipid present in the sample was determined from a standard curve prepared with pure phosphatidylcholine. The inter-assay coefficient of variation was 4.3 %.

2.3 TECHNIQUES FOR ASSESSING LIPOPROTEIN REMODELLING

2.3.1 CHOLESTERYL ESTER TRANSFER PROTEIN (CETP)

The rate of net cholesteryl ester transfer in plasma was determined by measuring the rate of decrease of cholesteryl ester present in HDL after inhibition of LCAT activity. Ten ml of blood was collected into citrate (0.2 M citrate, pH 7.2) at a ratio of 1 part citrate to 19 parts blood and centrifuged at 3000 x g for 15 minutes, at 4°C to obtain the plasma. An aliquot (25 $\mu$l) of 1M Tris-HCl buffer (pH 7.4) was added to 2.5 ml of plasma and LCAT activity in the plasma inhibited by the addition of N-ethylmaleimide (NEM) at a final concentration of 2.5 mmol/L.

Ester transfer activity was measured over 3 hours at 37°C. After 5 min equilibration at 37°C, an initial (0 hour) sample (100 $\mu$l) was taken and added to 200 $\mu$l PEG (Quantolip HDL Reagent A-Technoclone Ltd, UK), mixed and incubated 10 min at room temperature. Non-HDL lipoproteins precipitated by this procedure and were then sedimented by centrifugation at 3000 x g for 10 minutes. The HDL-containing supernatant was carefully aspirated and retained for analysis for free and esterified
cholesterol. Further samples were taken from the NEM-treated plasma at hourly intervals and treated in an identical fashion (Fielding et al., 1981).

2.3.1.1 Assay of HDL total cholesterol and free cholesterol:

HDL free and esterified cholesterol content was measured by a fluorometric method using a 96 well plate. For the assay of free cholesterol present, the samples were diluted 1:10 with saline and aliquots (10 µl) added to 100 µl of a solution containing: 50 mmol/L NaH₂PO₄ pH 7.4, 0.17 mmol/L polyethylene Glycol 6000 (mol. wt); 5 mmol/L sodium taurocholate; 5 mmol/L EDTA (tetrasodium salt); 0.25 v/v Triton X-100; 2.5 µmol/L p-hydroxyphenyl acetic acid; 30 µ/ml horseradish peroxidase; 0.08 µ/ml cholesterol oxidase and incubated at room temperature for 30 minutes with occasional mixing. The fluorescence of the oxidized p-hydroxyphenyl acetic acid was stabilised by the addition of 0.2 ml of Tris buffer (0.3 mol/L, pH 9.0) and left for 5 minutes and then fluorescence determined using a fluorimetric plate-reader set to an excitation wavelength of 325 nm and 415 nm emission. All samples were assayed in quadruplicate. The amount of free cholesterol in the sample was quantitatively derived from the standard curve (0 µmol/L, 25 µmol/L, 50 µmol/L, 100 µmol/L cholesterol) obtained in the same test run. The assay was linear up to 100 µmol/L and the inter-assay coefficient of variation of free cholesterol was 3.8%. The mean of quadruplicate measurements of plasma free cholesterol sampled at 0, 1, 2, 3 hours were determined and plotted against time. LCAT activity was calculated as the gradient determined by least-squares method. In general the correlation obtained by regression analysis was $r^2 > 0.998$. The total cholesterol (free cholesterol + cholesteryl esters) content of the HDL was similarly determined with the exception that cholesterol esterase (0.8 U in 10µl) was added to the reagent.
2.3.1.2 HDL cholesteryl ester content and calculation of CETP activity

At each time point, HDL cholesteryl ester content was obtained by calculating the difference between total and free cholesterol concentrations and plotted against time. The gradient of the curve was determined by the least-squares method and taken to be the rate of transfer of cholesteryl ester from HDL to endogenous plasma triglyceride-rich lipoproteins. The inter-assay coefficient of variation of HDL free cholesterol and HDL total cholesterol were 6% and 4.8% respectively. The reaction was linear over 3 hours ($r^2 = 0.85$); the inter-assay coefficient of variation was 6.5%.

2.3.2 PHOSPHOLIPID TRANSFER PROTEIN (PLTP)

PLTP activity was measured in total plasma as the transfer of radio-labelled phosphatidylcholine from $[^{14}\text{C}]$-phosphatidylcholine liposomes to an excess of exogenous human HDL$_3$ (Damen et al., 1982). Briefly, 10 μmol of $[^{14}\text{C}]$-L-phosphatidylcholine (PerkinElmer Life Sciences) and 0.1 μmol of butylated hydroxytoluene were mixed, and the lipids were dried under nitrogen, suspended in 1 ml of 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4), sonicated, and centrifuged to remove lipid aggregates and metallic debris. Plasma samples were incubated for 1 hour at 37°C with radiolabeled liposomes (50 nmol of phospholipid) and isolated human HDL$_3$ (250 μg of protein) in a final volume of 400 μl with 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4). At the end of the incubation, liposomes were precipitated with dextran sulphate. The supernatant (0.5 ml) was used for radioactivity determinations. Plasma volumes were chosen to keep PLTP activity in the linear range of the assay. PLTP activity was expressed as μmoles of phosphatidylcholine transferred from liposomes to exogenous human HDL$_3$ per ml of plasma (Bouly et al., 2001).
2.3.3 LECITHIN:CHOLESTEROL ACYL TRANSFERASE (LCAT)

The decrease in free cholesterol content of incubated plasma, (Heider and Boyett et al., 1978) reflects the rate of plasma cholesterol esterification by LCAT and was determined as follows. Blood (9.5 ml) was collected into 0.5 ml of sodium citrate (0.2 mol/L sodium citrate pH 7.2) and centrifuged at 3000 x g for 15 minutes at 4°C to obtain plasma. An aliquot (25 µl) of 1M Tris buffer (pH 7.4) was added to 2.5 ml of fresh plasma and incubated for 3 hours at 37°C. Aliquots (250 µl) were taken from the incubation at hourly intervals and N-ethylmaleimide (15 mmol/L saline) was added to stop the reaction. Samples were kept at 4°C until analysed.

The free cholesterol content of the samples was determined as described in the assay of CETP activity (see section 2.3.1). The mean of quadruplicate measurements of plasma free cholesterol sampled at 0, 1, 2, 3 hours were determined and plotted against time. LCAT activity was calculated as the gradient determined by least-squares method. In general the correlation obtained by regression analysis was $r^2 > 0.998$; the inter-assay coefficient of variation was 3.9%.

2.3.4 LIPASE ACTIVITIES

2.3.4.1 Preparation of post-heparin plasma (PHP)

Both lipoprotein lipase and hepatic lipase are released from the proteoglycans by an injection of heparin. Baseline and post-treatment post-heparin plasma samples were collected from the subjects for measurement of lipoprotein lipase and hepatic lipase activity. Heparin (60 U/Kg) was administered intravenously and blood was taken 15 minutes later into heparin tube. Plasma was separated immediately and stored at – 86°C until assayed for lipase activities.
2.3.4.2 Hepatic Lipase Activity

Hepatic Lipase (HL) activity in post-heparin plasma was carried out by the method of Henderson et al 1993. HL activity was discriminated from LPL activity by conducting the assay at the pH optima for HL (pH 8.8) in the presence of high salt concentrations and in the absence of apo-CII (an essential co-factor for LPL activity). Twenty-five µCi of glycerol tri [9, 10 (n)-3H] olate (specific activity 1 µCi/nmol; TRA 191 Amersham Ltd., UK) was added to 100 mg (113 µmoles) of triolein (Sigma Ltd) in 1 ml of toluene. This was dried under nitrogen and then washed with 2 ml heptane. After evaporation, 17.5 ml of gum arabic (5% w/v in 0.2 M Tris buffer, pH 8.8) and 8.25 ml fatty acid free BSA (200 mg/ml 0.2 M Tris pH 8.8) were added and the mixture sonicated for 4 minutes in an ice bath until it was milky with no visible oil droplets. The salt concentration was raised by the addition of 7.74 ml 3.24 M in 0.2 M Tris buffer, pH 8.8.

Samples of PHP (30 µl) were incubated with 0.5 ml of the substrate for 1 hour at 37°C. A blank incubation was performed to compensate for non-specific hydrolysis. The reaction was stopped by the addition of 8.125 ml of Belfrage reagent (Methanol 141: chloroform 125: Heptane 100) and Borate extraction buffer (2.87 ml of 0.14 mol/L, pH 10.5) was added to extract selectively the free fatty acids released by the HL and mixed for 5 minutes. The phases were separated by centrifugation for 10 minutes at 3000 x g and 1 ml of upper phase added to 4 ml scintillation fluid (Aquasafe 300+, Zinsser Analytic, UK) and mixed well. The radioactivity was determined in a liquid scintillation counter with quench correction made by automated external standard (AES) to obtain the results as d.p.m (Henderson et al., 1993).
2.3.4.3 Lipoprotein Lipase Activity

Lipoprotein lipase (LPL) activity in post-heparin plasma was carried out by the method of Henderson et al. (1993). LPL activity was discriminated from HL activity by conducting the assay at the pH optima for LPL (pH 8.2) in the presence of apo-CII (an essential co-factor for LPL activity) and of sodium dodecyl sulphate (SDS - an inhibitor of HL). Twenty-five μCi of glycerol tri [9, 10 (n)-3H] oleate (specific activity 1 μCi/nmol) (TRA 191 Amersham Ltd., UK) was added to 100 mg (113 μmoles) of triolein (Sigma Ltd) in 1 ml of toluene. This was dried under nitrogen and then washed with 2 ml heptane. After evaporation, 17.5 ml of gum arabic (5% w/v in 0.2 M Tris buffer, pH 8.2) was added and the mixture were sonicated for 4 minutes in an ice bath until it was milky with no visible oil droplets. Bovine serum albumin (8.25 ml of 200 g/L defatted BSA) and 0.8 ml of 2.42 mol/L sodium chloride (both in 0.2 mol/L Tris buffer pH 8.2) was added and mixed. Aliquots (0.42 ml) of substrate emulsion were pre incubated for 90 min at 37°C with 80 μl of heat-inactivated human serum as the source of apo-CII. HL activity was inhibited by incubation of the plasma samples for 30 min at room temperature with SDS (1:1 v/v PHP:70 mmol/L SDS in 0.2 mol/L Tris buffer, PH 8.2). An aliquot (60 μl) of the incubated plasma was added to the pre incubated substrate and mixed. Following incubation at for 1 hour at 37°C the reaction was stopped by the addition of Belfrage reagent and the assay continued as per for HL (Henderson et al., 1993).
2.3.4.4 Lipase activity normalisation and calculation

All assays of the same subject’s PHP for LPL and HL determinations, at both baseline and after treatment, were carried out at the same time and using the same batch of substrate in order to compensate for variations in the micelle size of the substrate emulsion. Inter assay variation was accounted for by assaying a PHP sample from a control batch prepared from a healthy volunteer with each group of assays performed. Results from the assays were normalised using the control values as standard. The specific activity of the substrate was determined and expressed as pmoles triolein per d.p.m. This figure was multiplied by 3 to account for the 3 acyl groups on triolein and the d.p.m present in the samples were converted to pmoles triolein and the total amount present in the upper phase of the free fatty acid extraction step was calculated. A correction factor for the efficiency of the Belfrage extraction technique was determined using $[^3]H$-oleic acid was applied to the result. After correction for dilutions, the results were expressed as pmoles fatty acid released/h/L plasma. The inter-assay coefficients of variation was $< 7\%$ for both assays.

2.4 PLASMA INSULIN AND INSULIN PRECURSOR MEASUREMENTS

Plasma insulin, intact proinsulin and 32-33 Split proinsulin concentrations were determined by two-site immunoradiometric assays using highly-specific antibodies (Sobey et al., 1989). The insulin assay cross-reacted 5.3% and 5.0% with intact proinsulin and 32-33 split proinsulin respectively. The proinsulin assay did not cross-react with insulin or 32-33 split proinsulin. The inter-assay coefficients of variation was $< 15\%$ for both assays.
2.5 **GLUCOSE DETERMINATION**

Glucose present in EDTA plasma samples was determined using the hexokinase reaction in which glucose-6-phosphate formed by the hexokinase reaction is oxidised by glucose-6-phosphate dehydrogenase in the presence NAD\(^+\) yielding NADH and 6-phosphogluconate. NADH produced was measured at 340 nm (Sigma Infinity glucose reagent). Glucose present in the plasma was determined from a standard curve that was linear up to 20 mmol/L. The inter-assay coefficients of variation was 3.4 %.

2.6 **HOMA INSULIN RESISTANCE and β-CELL FUNCTION**

Insulin resistance was calculated from fasting plasma glucose and insulin concentrations using the computer-solved homeostasis model assessment (HOMA) method as previously described by Mathews et al. (1985). The estimate of insulin resistance derived from this method has been shown to correlate with those obtained by the use of euglycaemic and hyperglycaemic clamps.

\[
\text{Insulin resistance} = \frac{\text{fasting insulin (µU/ml)} \times \text{fasting glucose (mmol/L)}}{22.5}
\]

2.7 **APOLIPOPROTEIN MEASUREMENTS (APO-AI & APO-B)**

Apo-AI and apo-B were determined separately by immunoturbidometric methods (Apo-AI Direct, Apo-B Direct; Technoclone, UK). Plasma samples were mixed with the reagents and mixed briefly. An initial reading of the absorbance was made at 340 nm and the sample left to incubate for 10 minutes at 37\(^\circ\)C without further stirring. A second reading was made at 340 nm. The initial reading was subtracted from the second reading and the difference in absorbance determined. A standard curve was prepared using apo-AI and apo-B preparations treated identically to the plasma and the
concentration of apo-AI and apo-B in the plasma were determined from these curves.

The inter assay coefficient of variation was < 5 %.

2.8 LIPOPROTEIN (a) (Lp(a)) MEASUREMENTS

Lipoprotein (a) was determined on diluted plasma samples using an Elisa kit in 96-well plate format (Immuno-UK). The inter assay coefficient of variation was < 8 %.

2.9 HbA1C

Liver and kidney function tests, full blood count and HbA1C were assayed by at the chemical pathology lab at University College London Hospital.
2.10 **POWER OF THE STUDY AND STATISTICAL ANALYSES**

The statistical power of the study was greater than 0.85 to detect differences of 20% in glucose, triglyceride, insulin, proinsulin, split proinsulin, HOMA, cholesterol, NEFA at a level of significance of 0.05.

Statistical analyses were performed using program SPSS version 11 (SPSS UK Ltd, Chertsey, UK). Between-group characteristics were compared by unpaired t-test. Within group changes were compared by paired t-test. The effect of glibenclamide and pioglitazone on postprandial lipoprotein, NEFA, glucose, insulin and insulin precursor were analysed using repeated ANOVA (Ludbrook, et al., 1994). Associations between variables were analysed using Spearman’s correlation analysis. Variables with skewed distribution were log-transformed before the analyses, but the untransformed data are shown in the text, figures and tables for ease of interpretation. Postprandial parameters over the 8-hours period were calculated as areas under the curve (AUC). The p value <0.05 (two tailed) was considered statistically significant Data are presented as mean ± S.D (standard deviation) in text and tables and mean ± S.E.M. (standard error of the mean ) in figures.
CHAPTER 3

THE EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY IN TYPE 2 DIABETIC AND CONTROL SUBJECTS: PLASMA LIPID CONCENTRATIONS IN THE FASTING AND POSTPRANDIAL STATE.

The objective of this part of the study was to determine the effect of insulin sensitization through the PPARγ receptor pathway with pioglitazone and compare it with sulphonylurea treatment (glibenclamide) on the fasting lipid concentrations and postprandial clearance of lipoproteins in type 2 diabetic patients compared with a group of control subjects.

3.1 THE DEMOGRAPHIC DETAILS OF THE STUDY GROUPS

Table 5: Demographic characteristic of controls and diabetic subjects

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.4 ± 8.9</td>
<td>58.5 ± 9.2</td>
<td>54.5 ± 12.2</td>
</tr>
<tr>
<td>Sex</td>
<td>6M/4F</td>
<td>8M/3F</td>
<td>9M/2F</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.7 ± 1.5</td>
<td>28.4 ± 2.8</td>
<td>28.2 ± 3.1</td>
</tr>
<tr>
<td>Smoker</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Alcohol (unit/week)</td>
<td>8</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Duration of DM (years)</td>
<td>-</td>
<td>0.5 - 4.8</td>
<td>0.5 - 5.2</td>
</tr>
<tr>
<td>Treatment</td>
<td>-</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Diet &amp; sulphonylurea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet &amp; Metformin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>-</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>
The clinical and physical characteristics of the patients and control subjects are shown in Table 5. It can be seen that the groups of subjects were matched for age and sex and no statistical differences were found (ANOVA, p = NS). However, the diabetic patients had a trend to have a higher BMI when compared to controls but this was not statistically significant. Both diabetic groups had the same duration of diabetes and treated with either metformin or sulphonylurea prior to the study. No changes in the anti-hypertensive treatment were made during the study period.

3.2 THE EFFECT OF INSULIN SENSITIZATION ON FASTING LIPID CONCENTRATIONS IN TYPE 2 DIABETIC SUBJECTS

3.2.1 Baseline fasting lipids in the diabetic groups compared to control subjects

Fasting lipid and lipoprotein concentrations are shown in Table 6a and 6b. Total plasma triglycerides were significantly higher in both diabetic groups in comparison with the control group (p < 0.05). Plasma cholesterol content had a trend to be greater in the diabetic subjects compared to control subjects but only in the pioglitazone group did this achieve statistical significance.

Total VLDL -TG concentrations were significantly higher in both groups of diabetic patients compared to control values (p < 0.05). VLDL-1 and VLDL-2-TG were also significantly greater in both diabetic groups than in control subjects (p < 0.05) see Figure 9. Neither total VLDL cholesterol content nor that in its subfractions were statistically different between the study groups.

IDL cholesterol levels were also higher in the diabetic subjects in comparison with the control group (p < 0.05). This was not the case for triglyceride content which was only significantly higher in the glibenclamide group.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>Pioglitazone</td>
<td>Control</td>
</tr>
<tr>
<td>Baseline</td>
<td>5.02 ± 0.75</td>
<td>5.40* ± 0.94</td>
<td>4.44 ± 0.58</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>5.15 ± 1.04</td>
<td>4.87 ± 1.57</td>
<td></td>
</tr>
<tr>
<td><strong>VLDL (T) CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.72 ± 0.42</td>
<td>0.58 ± 0.22</td>
<td>0.46 ± 0.13</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.72 ± 0.35</td>
<td>0.58 ± 0.46</td>
<td></td>
</tr>
<tr>
<td><strong>VLDL-1 CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.36 ± 0.24</td>
<td>0.29 ± 0.12</td>
<td>0.22 ± 0.11</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.32 ± 0.24</td>
<td>0.29 ± 0.34</td>
<td></td>
</tr>
<tr>
<td><strong>VLDL-2 CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.35 ± 0.20</td>
<td>0.28 ± 0.11</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.40* ± 0.24</td>
<td>0.29 ± 0.15</td>
<td></td>
</tr>
<tr>
<td><strong>IDL-CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.10* ± 0.75</td>
<td>0.94* ± 0.22</td>
<td>0.50 ± 0.21</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.99 ± 0.68</td>
<td>0.91 ± 0.63</td>
<td></td>
</tr>
<tr>
<td><strong>LDL-CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.19 ± 0.51</td>
<td>2.13 ± 0.48</td>
<td>2.0 ± 0.44</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>1.77 ± 0.51</td>
<td>1.83 ± 0.63</td>
<td></td>
</tr>
<tr>
<td><strong>HDL-CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.31 ± 0.54</td>
<td>1.08 ± 0.23</td>
<td>1.31 ± 0.29</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>1.06* ± 0.39</td>
<td>1.01* ± 0.25</td>
<td></td>
</tr>
<tr>
<td><strong>HDL(_2)-CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.95 ± 0.13</td>
<td>0.72 ± 0.12</td>
<td>0.89 ± 0.24</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.66* ± 0.28</td>
<td>0.67* ± 0.15</td>
<td></td>
</tr>
<tr>
<td><strong>HDL(_3)-CH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.35 ± 0.85</td>
<td>0.35 ± 0.20</td>
<td>0.41 ± 0.24</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.40 ± 0.26</td>
<td>0.34 ± 0.21</td>
<td></td>
</tr>
<tr>
<td><strong>Triglyceride</strong></td>
<td></td>
<td></td>
<td>0.82 ± 0.34</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.18* ± 0.33</td>
<td>1.98* ± 1.69</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>1.35* ± 0.52</td>
<td>1.10 ± 0.68</td>
<td></td>
</tr>
<tr>
<td><strong>VLDL (T) TG</strong></td>
<td></td>
<td></td>
<td>0.39 ± 0.25</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.68* ± 0.38</td>
<td>0.86* ± 0.50</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.66* ± 0.34</td>
<td>0.55* ± 0.51</td>
<td></td>
</tr>
<tr>
<td><strong>VLDL-1 TG</strong></td>
<td></td>
<td></td>
<td>0.39 ± 0.25</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.46* ± 0.25</td>
<td>0.54* ± 0.28</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.46* ± 0.22</td>
<td>0.37 ± 0.39</td>
<td></td>
</tr>
<tr>
<td><strong>VLDL-2 TG</strong></td>
<td></td>
<td></td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.21* ± 0.14</td>
<td>0.32* ± 0.28</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.22* ± 0.09</td>
<td>0.16* ± 0.11</td>
<td></td>
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<tr>
<td><strong>IDL- TG</strong></td>
<td></td>
<td></td>
<td>0.15 ± 0.08</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.30* ± 0.22</td>
<td>0.41 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.27* ± 0.14</td>
<td>0.26 ± 0.26</td>
<td></td>
</tr>
<tr>
<td><strong>LDL- TG</strong></td>
<td></td>
<td></td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.16 ± 0.06</td>
<td>0.21 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.16 ± 0.05</td>
<td>0.15 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><strong>HDL- TG</strong></td>
<td></td>
<td></td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.12 ± 0.06</td>
<td>0.14 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.14 ± 0.06</td>
<td>0.11 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><strong>HDL(_2)-TG</strong></td>
<td></td>
<td></td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.10 ± 0.03</td>
<td>0.02 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.12 ± 0.05</td>
<td>0.09 ± 0.04</td>
<td></td>
</tr>
<tr>
<td><strong>HDL(_3)-TG</strong></td>
<td></td>
<td></td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.01 ± 0.03</td>
<td>0.12 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>0.02 ± 0.02</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Data show the mean ± SD. 
Patients groups vs control subjects: * p < 0.05. 
Baseline vs post-treatment for each group: † p < 0.05. 
Glibenclamide vs pioglitazone treated patients: ‡ p < 0.05.
LDL cholesterol and triglyceride were also slightly higher in the diabetic groups than in the control subjects but, in this case, the difference did not reach statistical significance. As shown in Table 6b, apo-B concentrations were also higher in the diabetic groups but this did not reach statistical significance. Lp(a) concentration were significantly higher in the glibenclamide group compared to the control (p<0.05). Lp(a) was also higher in the pioglitazone group but did not reach statistical significance (see Table 6b).

Total HDL cholesterol and triglyceride content did not differ between the diabetics and control subjects. This was also the case when HDL was subfractionated to HDL$_2$ and HDL$_3$. Likewise, no differences were observed for apo-AI.

Both groups of diabetic subjects had a higher concentration of NEFA than controls but this did not achieve statistical significance (Table 5b).

<table>
<thead>
<tr>
<th>Table 6b: Fasting plasma Lp(a), NEFA and apolipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Lp(a) (mg/dl)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>NEFA (µmol/L)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Apo-AI (mg/dl)</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Apo-B (mg/dl)</td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HbA1C %</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Data show the mean ± SD.
Patients groups vs control subjects: * p< 0.05.
Baseline vs post-treatment for each group: † p< 0.05.
Glibenclamide vs pioglitazone treated patients: ‡ p< 0.05.
3.2.2 Fasting plasma lipid and lipoprotein content after treatment

Neither treatment had an effect on the glycaemic control assessed by HbA1C (see Table 6b). Fasting plasma lipid and lipoproteins after treatment with glibenclamide or pioglitazone are shown in Tables 6a and 6b. Neither the total cholesterol nor VLDL-1, VLDL-2, IDL, LDL lipoprotein cholesterol content showed any significant changes from the fasting baseline data. There was a fall in total HDL cholesterol and HDL\textsubscript{3} cholesterol in both groups which both became significantly lower than the control subjects, but this was not the case for HDL\textsubscript{2} which did not change after treatment.

Total plasma triglyceride showed a significant fall in the pioglitazone treated group to a level not significantly different from that of the control subjects. By contrast, there was no change in the total plasma triglycerides in the glibenclamide treated group which remained significantly higher than the control individuals. In the pioglitazone group, there was a significant fall in the total VLDL triglyceride and also in VLDL-2 triglyceride; there was also a fall in VLDL-1 triglyceride but this did not achieve statistical significance. By contrast treatment with sulphonylurea made no changes to triglyceride present in total VLDL and its subfractions. There was a trend for a fall in the triglyceride content of IDL, LDL in the pioglitazone group but it did not achieve a statistical significance. Total HDL triglyceride and its subfractions showed no significant changes with either treatment.

The concentration of Lp(a) showed a significant fall from the baseline after treatment with glibenclamide, but this was not the situation with the pioglitazone group (Table 6b). No effect of either treatment was observed on the plasma content of apo-AI and apo-B (Table 6b). There was a trend for a fall in the fasting plasma NEFA content in the pioglitazone treated group but this was not statistically
significant. In the glibenclamide group, fasting NEFA concentrations increased and became significantly higher than the control subjects.

**Figure 9:** The response of total VLDL-TG and its subfractions to treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

3.3 THE EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY ON POSTPRANDIAL LIPAEMIA IN TYPE 2 DIABETIC PATIENTS AND CONTROL SUBJECTS

3.3.1 POSTPRANDIAL TRIGLYCERIDE METABOLISM

Postprandial triglyceride clearance in diabetic groups and controls subjects is shown in Figure 10. In the control subjects the effect of the meal was to cause an increase in
plasma triglyceride reaching a peak at 4 hr postprandial after which triglyceride concentration declined but did not return to the starting value after 8 hours.

In the diabetic subjects at baseline triglyceride levels increased but the peak was not achieved until 5-6 hr postprandial and remained elevated till the end of the study period. At the baseline condition the AUC for postprandial plasma TG (mmol/L/hr) was similar in both groups of diabetic patient (Table 7), and significantly higher than controls.

![Figure 10: The postprandial triglyceride response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.](image-url)
After the 20 week treatment period, the pioglitazone group showed a significant reduction in both the magnitude and duration of postprandial hypertriglyceridaemia. By contrast, the glibenclamide treated group had no significant changes in postprandial plasma triglyceridaemia.

<table>
<thead>
<tr>
<th>Table 7: Postprandial clearance of lipoprotein triglyceride measured as Area-Under-the Curve (mmol/L/8 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glibenclamide</td>
</tr>
<tr>
<td>Triglyceride Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>Corrected TG Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>Chylomicron RP Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>CR-RP Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>IDL-TG Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>LDL-TG Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>HDL-TG Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>HDL(_2)-TG Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>HDL(_2)-TG Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
<tr>
<td>NEFA Baseline</td>
</tr>
<tr>
<td>Post-treatment</td>
</tr>
</tbody>
</table>

Data show the mean ± SD. Patients groups vs control subjects: * p< 0.05. Baseline vs post-treatment for each group: † p< 0.05. Glibenclamide vs pioglitazone treated patients: ‡ p< 0.05.

In order to assess the impact of the fat load present in the test meal in the absence of the endogenous triglyceridaemia present in the subjects, a correction was made by subtraction of the fasting triglyceride concentrations from the postprandial
data. When the data was corrected in this fashion, the glibenclamide treated group still showed an exaggerated lipaemia in response to the test meal (Table 7). By contrast a significant reduction in the lipaemic response from that in the baseline state was seen in the pioglitazone group and was no longer significantly different from control values.

3.3.2 Chylomicron Retinyl Palmitate Clearance

![Figure 11: The postprandial chylomicron RP response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.](image)

The chylomicron RP content of postprandial plasma samples from diabetic patients and control subjects at baseline and post-treatment is shown in Figure 11. In the control subjects, chylomicron RP reached a peak after 4 hours and began to decline thereafter. By contrast, both groups of diabetic subjects showed a progressive
increase in chylomicron RP over the entire 8 hour postprandial period. After treatment the postprandial chylomicron-RP content in the glibenclamide treated group showed a peak around 5 hours and then started to decline slowly. Nevertheless the clearance of chylomicrons was still severely abnormal when compared to the control subjects. Pioglitazone treatment, however, caused a significant reduction of postprandial chylomicron RP concentrations to that of the controls. This is reflected in the AUC for chylomicron RP (see Table 7). Paired t-test analysis showed that the reduction in chylomicron RP clearance from baseline was statistically significant (p <0.05).

3.3.3 CHYLOMICRON REMNANT RETINYL PALMITATE CLEARANCE

Figure 12: The postprandial chylomicron remnant RP response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.
At baseline, chylomicron remnant RP clearance showed a similar pattern to chylomicron RP, with the diabetic subjects having an exaggerated and delayed clearance of the chylomicron remnant-RP (Figure 12 & Table 7). Glibenclamide treatment had no effect on the clearance of chylomicron remnants where as pioglitazone treatment caused a significant reduction such that there was no longer any difference in the clearance (AUC) from that of the control subjects.

3.3.4 Postprandial IDL and LDL Triglyceride Clearance

![Graph showing postprandial IDL-TG response](image)

**Figure 13:** The postprandial IDL-TG response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

In both groups of diabetic patients the IDL and LDL was significantly enriched with triglyceride when compared to control (see Table 7; Figures 13 & 14). The effect of the test meal was to cause an apparent progressive increase over the 8 hours in the
content of triglyceride but this did not achieve statistical significance. Treatment with glibenclamide had no effect on the postprandial triglyceride content, expressed as AUC, of both IDL and LDL, but treatment with the pioglitazone reduced the triglyceride AUC content of both IDL and LDL to the level seen in controls (Table 7).

![Graph showing baseline and post-treatment LDL-TG response](image)

**Figure 14:** The postprandial LDL-TG response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

### 3.3.5 Postprandial HDL Triglyceride Clearance

HDL triglyceride content in both diabetic groups were similar to that of control subjects. The changes observed after treatment with either glibenclamide or pioglitazone were small and did not reach statistical significance (Table 7). HDL\(_2\) and HDL\(_3\) triglyceride also showed no effects of either treatment (Table 7).
3.3.6 Postprandial Cholesterol Metabolism

There were no significant postprandial changes in plasma total cholesterol content in any of the group studied (Table 8). No statistically significant changes were observed in the plasma concentrations of IDL and LDL cholesterol in the diabetic patients or control group (Table 8).

At baseline, the clearance (AUC) of cholesterol in total HDL, HDL$_2$ and HDL$_3$ were not statistically different from that of the control group (Table 8). After 20 weeks treatment, the HDL$_3$ AUC was reduced in both diabetic groups and became significantly less than the control values. There were no significant differences in the AUC of HDL$_2$ between the control subjects and both diabetic groups patients after treatment.

Figure 15: The postprandial HDL-TG response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.
Table 8: Postprandial clearance of total and lipoprotein cholesterol measured as Area-Under-the Curve (mmol/L/8 hr)

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Baseline</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.0 ± 6.4</td>
<td>36.6 ± 12.0</td>
<td>34.4 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>37.6 ± 6.8</td>
<td>35.9 ± 10.0</td>
<td></td>
</tr>
<tr>
<td>IDL-CHO</td>
<td>Baseline</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.1 ± 1.3</td>
<td>6.6 ± 1.9</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>6.8 ± 3.1</td>
<td>5.6 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>LDL-CHO</td>
<td>Baseline</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.2 ± 2.5</td>
<td>15.4 ± 4.1</td>
<td>13.1 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>12.6 ± 2.7</td>
<td>13.2 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>HDL-CHO</td>
<td>Baseline</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.1 ± 3.1</td>
<td>7.7 ± 2.1</td>
<td>10.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>7.4 ± 1.9</td>
<td>7.6 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>HDL\textsubscript{3}-CHO</td>
<td>Baseline</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.2 ± 1.5</td>
<td>5.8 ± 1.8</td>
<td>7.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>4.7* ± 1.0</td>
<td>4.7* ± 1.4</td>
<td></td>
</tr>
<tr>
<td>HDL\textsubscript{2}-CHO</td>
<td>Baseline</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.3 ± 2.1</td>
<td>2.5 ± 1.8</td>
<td>1.22 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>2.7 ± 0.9</td>
<td>2.9 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

Data show the mean ± SD. Patients groups vs control subjects: * p< 0.05.

Figure 16: The postprandial cholesterol response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.
Figure 17: Postprandial changes of IDL-Cholesterol pre- and post-treatment.

Figure 18: Postprandial changes of LDL-Cholesterol pre- and post-treatment.

Figure 19: Postprandial changes of HDL-Cholesterol pre- and post-treatment.
3.3.7 **POSTPRANDIAL NON-ESTERIFIED FATTY ACID METABOLISM**

In control subjects there was a fall in plasma NEFA concentrations reaching a minimum between 30 minutes and one hour after the test meal. In the diabetic groups this fall was not as pronounced and the minima occurred later between 1-2 hours. After treatment with pioglitazone the AUC for NEFA was reduced by 20% from the baseline. By contrast glibenclamide treatment increased the AUC for NEFA by 17% (Table 7).

![Graphs showing baseline and post-treatment NEFA levels](image)

**Figure 20:** The postprandial NEFA response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

3.3.8 **POSTPRANDIAL PHOSPHOLIPID METABOLISM**

The phospholipid content of postprandial plasma samples from diabetic patients and control group at baseline and in the post-treatment condition are shown in Figure 21.
Figure 21: The postprandial plasma phospholipid response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

At baseline, the AUC of total plasma phospholipid content in the diabetic groups were not statistically different from the control individuals. The changes after treatment with either glibenclamide or pioglitazone were minimal and did not reach statistical significance and neither did they differ from the control subjects.

3.3.9 LDL SUBFRACTIONS AND THEIR RELATION TO POSTPRANDIAL LIPAEMIA

The purpose of this part of the study was to assess the effect of pioglitazone treatment on the LDL density profile in the diabetic subjects. Furthermore, the significance of postprandial lipaemia, lipolytic enzymes, CETP, plasma insulin and insulin resistance as determinants of LDL heterogeneity was examined by statistical
means. The subfractions are defined by their hydrated buoyant density as follows: LDL-I (1.025-1.034 g/ml), LDL-II (1.034-1.044 g/ml) and LDL-III (1.044-1.060 g/ml). The percentage distribution of the major LDL subfractions in the diabetic group are shown in Table 9. At baseline, LDL-II was the predominant subfraction present in both groups of diabetic patients and the control subjects. There was a trend for small dense LDL (LDL III) to be a greater percentage of total LDL than in the control subjects but this did not achieve statistical significance. Treatment with pioglitazone or glibenclamide made no difference to the distribution (Figure 22).

Table 9: Percentage distribution of LDL subfractions

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-treatment</td>
<td>Baseline</td>
</tr>
<tr>
<td>LDL I %</td>
<td>12 ± 6</td>
<td>11 ± 10</td>
<td>10 ± 9</td>
</tr>
<tr>
<td>LDL II %</td>
<td>55 ± 14</td>
<td>52 ± 9</td>
<td>55 ± 22</td>
</tr>
<tr>
<td>LDL III %</td>
<td>33 ± 16</td>
<td>36 ± 16</td>
<td>34 ± 27</td>
</tr>
</tbody>
</table>

Data show the mean ± SD.
3.3.9.1 Correlation between LDL subfractions and fasting and postprandial measurement

The relationships between the percentage distribution of LDL subfractions with fasting and postprandial lipid measurements, CETP, PLTP, LCAT and lipases activities are shown in Table 10. LDL-I levels correlated negatively with fasting triglyceride, overall triglyceride clearance (AUC), total VLDL and VLDL-I triglyceride. The opposite was found for LDL-III and correlated positively with these parameters. Percentage LDL-III correlated inversely with HDL cholesterol, when data for all subjects were analysed together. There was no significant correlation between any of the LDL subfractions and HL, LPL activity nor with CETP, LCAT and PLTP.

A significant correlation were found between LDL subfractions and fasting insulin and proinsulin and split proinsulin levels. LDL-I correlated negatively with fasting insulin, proinsulin, split proinsulin, and LDL-III correlated positively with fasting insulin. Insulin resistance (estimated by the HOMA model) correlated
negatively with LDL-I and positively with LDL-III when the data from all subjects were analysed together (Table 10).

Table 10: Relationships between % LDL subfractions and fasting, postprandial lipaemia, CETP, LCAT, lipases and insulin parameters in all diabetic patients at baseline

<table>
<thead>
<tr>
<th></th>
<th>LDL-I</th>
<th>LDL-II</th>
<th>LDL-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TG (mmol/L)</td>
<td>-0.46*</td>
<td>-0.08</td>
<td>0.27</td>
</tr>
<tr>
<td>Total VLDL TG (mmol/L)</td>
<td>-0.43*</td>
<td>-0.45*</td>
<td>0.52*</td>
</tr>
<tr>
<td>VLDL-1 TG (mmol/L)</td>
<td>-0.55*</td>
<td>-0.24</td>
<td>0.43*</td>
</tr>
<tr>
<td>VLDL-2 TG (mmol/L)</td>
<td>-0.29</td>
<td>-0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>TG AUC (mmol/L/8 hr)</td>
<td>-0.49*</td>
<td>-0.07</td>
<td>0.24</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.30</td>
<td>0.37</td>
<td>-0.43*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.65**</td>
<td>-0.30</td>
<td>0.46*</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>-0.57**</td>
<td>-0.37</td>
<td>0.52*</td>
</tr>
<tr>
<td>Proinsulin (pmol/L)</td>
<td>-0.43**</td>
<td>-0.19</td>
<td>0.29</td>
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<tr>
<td>32-33 split proinsulin (pmol/L)</td>
<td>-0.47*</td>
<td>-0.32</td>
<td>0.40</td>
</tr>
<tr>
<td>CETP (µmole cholesterol ester transferred/L/hr)</td>
<td>-0.02</td>
<td>-0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>PLTP (Arbitrary unit)</td>
<td>-0.19</td>
<td>0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>LCAT (µmole free cholesterol esterified/L/hr)</td>
<td>0.14</td>
<td>0.33</td>
<td>-0.34</td>
</tr>
<tr>
<td>HL (µmol NEFA released/ml/hr)</td>
<td>-0.28</td>
<td>-0.09</td>
<td>0.23</td>
</tr>
<tr>
<td>LPL ((µmol NEFA released/ml/hr)</td>
<td>0.15</td>
<td>-0.19</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Values are Spearman rank correlation coefficients. *P < 0.05, **p < 0.01.
3.4 THE EFFECT OF INSULIN SENSITIZATION WITH PIOGLITAZONE ON PLASMA LIPID AND LIPOPROTEIN CONCENTRATION IN TYPE 2 DIABETIC PATIENTS COMPARED TO CONTROL SUBJECTS.

3.4.1 The Fasting State

Dyslipidaemia is common in type 2 diabetes and in the fasting state, is normally manifest as hypertriglyceridaemia and reduced HDL cholesterol content. Previous reports have shown that TZDs can modify lipid metabolism in insulin resistant animals and humans (Nozue et al., 1999; Sunayama et al., 2000; Crawford et al., 1999). In this study insulin sensitization with pioglitazone caused a fall in the total plasma triglyceride which was most evident in the VLDL triglyceride and subfractions, in particular the smaller VLDL-2 species. Glibenclamide treatment had no effect on total circulating triglyceride nor on VLDL and its subspecies which remained present in significantly higher concentrations than in the control subjects. No changes were seen in the cholesterol content of VLDL and its subfractions suggesting that it is the incorporation of triglyceride into lipoprotein in the liver that is reduced by treatment by pioglitazone. Reduced VLDL triglyceride content could be brought about by a restriction in the supply of NEFA to the liver for a use as a substrate for triglyceride synthesis. Some evidence in support of this hypothesis is given by the finding that apo-B100 concentrations did not alter and that it can be inferred that the particle number was not changed by either treatment. However, other mechanisms involved in the assembly of VLDL such as the microsomal triglyceride transfer protein can not be excluded (Zoltowska et al., 2003).
We have found that insulin sensitization caused a fall in the plasma concentration of NEFA of a similar order of magnitude (18%) as other reports (Gomez-Perez et al., 2002). However in our study the fall was not statistically significant probably because of the small sample size of the patient groups studied and the large variation in the normal range of NEFA concentration 250-700 μmol/L (Perseghin et al., 2001).

Less VLDL in plasma would be thought to be reflected in reduced IDL and LDL content but this was not the situation and we did not find any significant changes in either the triglyceride or cholesterol concentrations of IDL. This suggests that the fall in VLDL and its sub species is associated with a more rapid uptake of by the hepatic remnant receptors rather than them proceeding down the lipolytic cascade to IDL and LDL. Pioglitazone treatment has not been reported to cause an increase in LDL cholesterol content by contrast to treatment using a different TZD (rosiglitazone) that is reported to increase in LDL cholesterol (Lacivita et al., 2002; Van Wijk et al., 2003).

Insulin resistance is reported to be associated with lower plasma concentrations of Lp(a) (Gomez-Perez et al., 2002). In agreement with this finding we have found that treatment with glibenclamide caused an increase in insulin resistance as estimated by the HOMA model (see Chapter 4) and was associated with a significant fall in Lp(a) concentrations. By contrast, treatment with pioglitazone which reduced the insulin resistance showed a trend to increase the plasma Lp(a) concentrations but this did not achieve statistical significance. These finding need to be interpreted with caution because of the relatively small number of patients studied and also because the subjects were not genotyped for Lp(a) polymorphism. The mechanism by which these changes are brought about by insulin sensitization are not known and likewise the physiological importance of the results.
We did not find that sensitization to insulin through the PPARγ pathway increased the amount of HDL cholesterol present in the fasting state. However, from the outset, the patient groups did not have a significantly lower HDL cholesterol level than the control subjects. This is likely also to be the reason why we did not observe any differences in the distribution of HDL into HDL$_2$ and HDL$_3$ particles when compared to control subjects. Previous studies with TZDs have reported a small but significant increase in total HDL cholesterol (Aronoff et al., 2000; Gomez-Perez et al., 2002; Herz et al., 2003; Nolan et al., 2000). In these studies a larger number of patients were recruited and were more severely hypertriglyceridaemic than those in our study. In addition it may be that long term treatment with the insulin sensitizer is required to induce significant changes in total HDL and its subfractions.

The synthesis of apo-AI is thought to be directed through the PPARα receptor (Linden et al., 2002) and not PPARγ. Beneficial effects of TZDs are thus likely to be through indirect mechanisms that lead to a decrease in the turnover of HDL species or a more general effect caused by the improvement in insulin resistance. Recent evidence has been presented that suggest that the increase in plasma HDL content is mediated, at least in part, through PPARγ activators that induce expression of the gene encoding for the ABCA1 transporter (Chinetti et al., 2001). The effect of this is to allow a greater efflux of free cholesterol from peripheral tissues to nascent HDL and hence accelerate the reverse cholesterol transport without increasing the amount of apo-AI present. While this mechanism may improve the effectiveness of HDL in reverse cholesterol transfer it does not explain why the overall amount of HDL (apo-AI) is increased. Our finding that apo-AI concentration did not change with treatment with pioglitazone, is in agreement
with this hypothesis, but additional studies are required for describing the detailed effects of TZDs on HDL kinetics.

Both diabetic group were found to have fasting triglyceride > 1.7 mmol/l on screening. However after the washout period and on the baseline test meal patients founds to have triglyceride level within normal range (< 1.7mmol/l), and those patient were mainly in the glibenclamide group (see table 6A, 6B), also their NEFA were not significantly different from the control subjects and this is not unexpected since this group are not very hypertriglyceridaemic.

3.4.2 The Postprandial State

3.4.2.1 Baseline postprandial lipaemia in type 2 diabetic patients compared to normal subjects

This study is the first to investigate the effect of insulin sensitization through the PPARγ/TZD pathway using pioglitazone on postprandial lipid clearance compared to the effect of sulphonylurea treatment (glibenclamide) in a group of patients with type 2 diabetes. The treatment regimes were designed to maintain the long-term glycaemic control of the patients, as measured by HbA1C, at the same level. The reason for this was to be able to rule out the impact of any change in glycaemic control on the metabolism of lipids.

In the baseline test both groups of diabetic subjects showed the well-established delayed clearance of dietary lipid when compared to normal individuals in agreement with previous studies (Malmstrom et al., 1997; Mero et al., 2000; Tan et al., 1995). This was despite there being only modest fasting hypertriglyceridaemia in the diabetic subjects. There are at least four possible mechanisms, acting singly or in combination, that may explain the exaggerated postprandial lipaemia. These are:
(1) an effect on the receptor-mediated uptake of triglyceride-rich lipoproteins remnants particles, 2) competition between intestinal and hepatic lipoproteins for the available lipase activity caused by abnormal VLDL production in type 2 diabetic dyslipidaemia, 3) insulin-dependent effects on VLDL synthesis, 4) abnormal lipoprotein remodelling processes and apolipoprotein trafficking.

Results from previous studies, and the present one, suggest that delayed clearance of dietary fat is due to a combined effect of both increased VLDL production by the liver and competition between chylomicron remnants and VLDL remnants for the hepatic uptake receptors (Cooper et al., 1996). It has been shown that suppression of hepatic apo-B100 production by insulin is impaired in type 2 diabetes and results in increased synthesis and secretion of VLDL particles in the fasting and postprandial phase (Packard et al., 2000). The normal suppression of NEFA release by insulin from the adipose tissue is also impaired in the insulin-resistant state. This increases the substrate available to the liver for triglyceride synthesis which results in continued VLDL synthesis and secretion at a rate greater than would be expected if the adipose tissue lipase was inhibited to the normal degree (will be discussed in Chapter 4) (Arner et al., 2002; Staehr et al., 2003).

In the postprandial phase, the increase in chylomicron and chylomicron remnants causes abnormal competition with VLDL and its remnants particles for the available lipoprotein lipase activity and uptake by common hepatic receptor (will be discussed further in Chapter 5). The delayed clearance of intestinal and hepatic triglyceride-rich lipoprotein remnant particles increases the potential for lipoprotein remodelling mediated by LCAT / CETP / PLTP (Jones et al., 1996).
The effect of insulin sensitization on lipid clearance in type 2 diabetic patients compared with normal subjects

After treatment with the insulin sensitizer (pioglitazone) major changes were observed in postprandial clearance of total plasma triglyceride, chylomicron, chylomicron remnants and the triglyceride content of IDL, and LDL to levels not longer significantly different from control subjects.

The mechanism by which chylomicron and chylomicron remnant clearance could be improved by insulin sensitization may involve a decrease in the competition for the lipolytic processes caused by the reduced extent of VLDL synthesis after treatment. Some evidence in favour of this is given by our finding that the responses of plasma NEFA content, as judged by AUC, showed a trend to decrease after treatment with pioglitazone which was not seen with glibenclamide treatment. Thus a restriction of a supply of NEFA may well reflect the overall reduction in plasma triglyceride seen after pioglitazone treatment but not after glibenclamide treatment. Other possible mechanisms involving abnormal assembly, secretion and constitution of chylomicron which are corrected by TZDs treatment can not be ruled out. Recent studies in mice lacking adipose tissue have shown that TZD treatment reduced plasma triglyceride concentration (Chao et al., 2000). From this it may be inferred that TZD treatment does not only act on adipose tissue to control the supply of NEFA to the liver but has other complementary actions that may contribute to the reduced hepatic triglyceride synthesis.

Indirect mechanisms arising from an overall reduction in insulin resistance may cause the improved clearance of dietary lipid associated with e.g., an increase of mass or activity of lipoprotein lipase (this will be discussed in Chapter 5).
The postprandial increase in IDL and LDL triglyceride, which is not reflected in an increase in IDL or LDL cholesterol, implies that there is either a failure of lipolysis or other lipoprotein remodelling mechanisms. This is likely to be an indirect effect of the competition between intestinal and hepatic lipoprotein for e.g., lipases and lipid transfer protein. The effect of pioglitazone is to reduce this competition by reducing the VLDL output by the liver and hence permit the normal processing of IDL and LDL by hydrolysis of the triglyceride content. It seems unlikely that the reduction of IDL and LDL triglyceride is due to a fall in particle numbers because we did not observe any postprandial changes in the cholesterol content of these lipoproteins and no change in apolipoprotein B.

Our data showed that the administration of glibenclamide had no apparent effect on postprandial triglyceride metabolism. It has been reported in one study that glibenclamide reduced postprandial triglyceridaemia due to fall in triglyceride of intestinal origin compared to placebo. This may be related to the amelioration of postprandial glycaemia rather than to a direct action of the drug itself (Skrapari et al., 2001). In this study we did not find any significant improvement in the chylomicron and chylomicron remnant clearance with a treatment regime designed to maintain glycaemic control constant.

3.4.2.3 LDL subfractions

In our study we have found that in general, when a negative correlation was found between any of the fasting and postprandial metabolic indices and % LDL-I and LDL-II, a positive correlation was found between LDL-III and these parameters. The opposite occurred when positive correlation was found for LDL-I and LDL-II.
Fasting triglyceride concentrations had a significant negative correlation with % LDL-I, one of the main constituents of the non-atherogenic type A phenotype; it had a positive correlation with LDL-III. Thus the lower the fasting triglyceride content the greater the % LDL-I. This is consistent with the correlation found with the clearance of postprandial triglyceride. In confirmation of this is our finding that one of the main aspects of fasting diabetic hypertriglyceridaemia is excess VLDL which correlated negatively with % LDL-I and % LDL-II and positively with % LDL-III.

We also found a negative correlation between insulin resistance estimated by HOMA and % LDL-I and a positive correlation between HOMA and %LDL-III. This pattern is reflected in the correlation between insulin, proinsulin and split proinsulin showing that any improvement in insulin sensitivity and β-cell function, as gauged by release of partially processed insulin species, will favour type-A phenotype. No significant correlation in the enzymes and transfers proteins responsible for lipoprotein remodelling. The negative correlation between % LDL-III and HDL may well be due to the overall diabetic dyslipidaemia.

The result of abnormal lipolytic remodelling processes leads to the presence of triglyceride-rich lipoprotein relatively enriched of triglyceride which are precursors of small dense LDL. The presence of atherogenic phenotype was not an inclusion requirement in this study and too few of the patients had a predominance of small dense LDL, presumably because the patients studied had only modest hypertriglyceridaemia, to permit an assessment of the effect of insulin sensitization. Our results, however, indicate that any improvement of fasting hypertriglyceridaemia by insulin sensitization will be expected to favour a greater %LDL-I and a lower % of LDL-III.
CHAPTER 4

EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY IN TYPE 2 DIABETES AND CONTROL SUBJECT: EFFECTS ON GLYCAEMIA, INSULIN RELEASE AND PROCESSING IN THE FASTING AND POSTPRANDIAL STATE.

4.1 SECRETION AND PROCESSING OF INSULIN

Type 2 diabetes is associated with both deficient pancreatic islet β-cell function and resistance of peripheral tissues to the action of insulin (insulin resistance). Pancreatic β-cells normally compensate for insulin resistance by increasing basal and postprandial insulin secretion. As long as the hyperinsulinaemia is adequate to overcome the insulin resistance, glucose tolerance remains normal. In patients that are destined to develop type 2 diabetes, the β-cell compensatory response declines and relative and absolute insulin deficiency develops. At this point, insulin secretion cannot keep pace with the underlying insulin resistance and consequently glucose intolerance and eventually type 2 diabetes occur. Chronic stimulation of the β-cells by the requirement to secrete more and more insulin leads to the release of immature secretory vesicles containing abnormally high amounts of insulin precursors e.g. proinsulin. The increase in proinsulin levels with worsening glucose tolerance is greater than the increase in insulin concentration, suggesting a worsening defect in proinsulin processing with worsening glucose tolerance (Haffner et al., 1994; Wareham et al., 1999). Pancreatic function in type 2 diabetes can be affected by gross histological changes such as a loss in the mass of secretory cells present and amyloidosis.
The processing of proinsulin to active insulin proceeds via the action of endopeptidases which cleave proinsulin between residues 32-33 and 65-66 to yield the active insulin molecule. With the availability of highly specific insulin assays recognising proinsulin, it has become possible to study the contribution of “true” insulin relative to its precursors (intact and 32-33 split proinsulin) to various components of the insulin resistance syndrome. Under physiological conditions, only small amounts of proinsulin and 32-33 split proinsulin are co-secreted with insulin. It is now thought that true hyperinsulinaemia in type 2 diabetes is not as severe as previously suggested; instead there is an increase in plasma content of insulin precursors with 32-33 split proinsulin being the predominant species and 65-66 split proinsulin present at only trace concentrations (Temple et al., 1989).

Several studies have suggested that proinsulin concentration may be more strongly related to cardiovascular risk factors such as increased blood pressure and higher triglyceride levels, than are insulin concentrations (Haffner et al., 1994, 2002).

4.2 THE EFFECT OF INSULIN ON LIPID METABOLISM

One of the most important functions of insulin is to inhibit the release of NEFA from adipose tissue. This is brought about by inhibition of hormone-sensitive lipase (HSL) and through an increase in the re-esterification of fatty acids within adipose tissue (Coppack et al., 1997). In the postprandial period when insulin is secreted in response to the meal, inhibition of HSL is most marked with a substantial fall in plasma NEFA concentrations (Ferrannini et al., 1997). In a state of insulin resistance, such as type 2 diabetes, the suppressive effect of insulin on the mobilisation of NEFA from adipose tissue by the hormone-sensitive lipase is impaired, and there is also reduced NEFA re-esterification. This leads to increased supply of NEFA to the liver and abnormally
high hepatic VLDL secretion during the postprandial period (Adeli et al., 2001; Byrne et al., 1997).

Insulin acutely inhibits hepatic VLDL secretion in hepatocyte preparations and also insulin increases the degradation of newly synthesised apo-B100 (Carpentier et al., 2001; Lewis et al., 2002; Duerden et al., 1989; Bartlett et al. 1988). Thus during the early postprandial period, the rise in the portal concentration of insulin enhances the hepatic degradation of apo-B100, reducing VLDL particle secretion. In euglycaemic hyperinsulinaemic clamp studies designed to raise insulin concentrations to postprandial levels, a 50% reduction in VLDL apo-B100 production was observed in control individuals, which was not seen in the hyperinsulinaemic obese subjects (Lewis et al. 1993). This suggests that hyperinsulinaemic subjects are resistant to the effect of insulin on inhibition of apo-B100 synthesis.

4.3 AIMS AND OBJECTIVES

The objective of this part of the study was to determine the effect of insulin sensitization through the PPARγ receptor pathway with pioglitazone and compare it with sulphonylurea treatment (glibenclamide) on the fasting glycaemia, and resultant release of insulin and precursor species in type 2 diabetic patients compared with a group of control subjects.
4.4 THE EFFECT OF INSULIN SENSITIZATION ON FASTING GLUCOSE, INSULIN AND INSULIN PRECURSOR SPECIES IN TYPE 2 DIABETIC SUBJECTS

4.4.1 Baseline fasting plasma glucose, insulin and insulin precursor concentrations in the diabetic groups compared to control subjects

Fasting plasma glucose, insulin, proinsulin and 32-33 split proinsulin concentrations are shown in Table 11. By definition fasting plasma glucose in the baseline condition was significantly greater in the diabetic subjects than in controls. After treatment with pioglitazone fasting glucose concentration fell significantly from the baseline level but remained significantly higher than the control values; glibenclamide treatment did not reduce fasting glycaemia.

Baseline fasting insulin levels were higher in the diabetic group compared to control subjects. After treatment with pioglitazone there was a reduction in insulin levels to values not significantly different from the control subjects. As expected glibenclamide treatment increased fasting insulin concentration from baseline level.

In the baseline fasting state, both groups of diabetic patients had significantly higher levels of proinsulin than control subjects. The effect of glibenclamide treatment was to increase proinsulin significantly from the baseline value. Pioglitazone treatment showed a trend for proinsulin concentrations to fall but this was not statistically significant. Control subjects had significantly lower proinsulin levels than either the pioglitazone or glibenclamide treated group.

Baseline fasting 32-33 split proinsulin concentrations were significantly higher in both diabetic groups than in control subjects. Glibenclamide treatment slightly increased 32-33 split proinsulin from the baseline. Pioglitazone treatment
reduced concentrations of 32-33 split proinsulin significantly to a level that did not
differ from the controls values.

Table 11: Fasting insulin, proinsulin, 32-33 split proinsulin and insulin resistance
estimated by HOMA model

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9.39* (2.0)</td>
<td>9.5* (1.8)</td>
<td>5.28 (0.8)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>9.84* (1.8)</td>
<td>7.5† (1.5)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>4.80%</td>
<td>-21%</td>
<td></td>
</tr>
<tr>
<td><strong>HbA1C%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.21* (0.6)</td>
<td>7.40* (0.5)</td>
<td>5.30 (0.2)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>7.28* (0.5)</td>
<td>7.29* (0.5)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin (pmo/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.06*(31.5)</td>
<td>79.6 (58.3)</td>
<td>44.1(12.9)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>93.9*** (41.6)</td>
<td>62.35 (33.5)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>32.90%</td>
<td>-21.70%</td>
<td></td>
</tr>
<tr>
<td><strong>Proinsulin (pmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>12.05* (7.5)</td>
<td>9.58* (6.9)</td>
<td>3.31 (2.0)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>19.4*† (17.5)</td>
<td>7.57* (5.8)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>60.80%</td>
<td>-20.90%</td>
<td></td>
</tr>
<tr>
<td><strong>32-33 Split proinsulin (pmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16.66** (7.9)</td>
<td>15.91** (12.0)</td>
<td>3.9 (0.6)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>20.8** (13.7)</td>
<td>10.6† (10.6)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>24.40%</td>
<td>-33%</td>
<td></td>
</tr>
<tr>
<td><strong>Insulin Resistance (IR)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.04*** (1.4)</td>
<td>4.96* (3.8)</td>
<td>1.54 (0.53)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>5.97*** (2.7)</td>
<td>2.99† (1.6)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>32.30%</td>
<td>-39.80%</td>
<td></td>
</tr>
</tbody>
</table>

Patient groups vs control subjects: * p < 0.05, **p < 0.005, ***p < 0.0005.
Baseline vs post-treatment for each group: † p < 0.05.
Glibenclamide treated vs pioglitazone treated patients: ‡ p < 0.05.
4.4.2 The effect of glibenclamide and pioglitazone on insulin resistance assessed by the HOMA model

In the baseline state, both groups of diabetes were significantly more insulin resistance than the control subjects using the HOMA model. Treatment with glibenclamide slightly increased insulin resistance in these patients in contrast to pioglitazone which significantly reduced insulin resistance but, nevertheless remained higher than in the control group.

* P < 0.05 patient group versus control subject.

□ P < 0.05 Baseline versus post-treatment

Figure 23: HOMA estimate of insulin resistance before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.
4.5 EFFECT OF INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY ON POSTPRANDIAL GLUCOSE, INSULIN AND INSULIN PRECURSORS IN TYPE 2 DIABETIC PATIENTS AND CONTROL SUBJECTS

4.5.1 Postprandial Glucose Clearance

Plasma glucose increased slightly after the high fat mixed meal in control subjects. By contrast, in both groups of diabetics there was pronounced glycaemia with peak concentrations being about 3-5 mmol/L higher than in the fasting state. In control subjects, peak glycaemia occurred within the first hour postprandial but peak glycaemia in both diabetic groups occurred later. At baseline, both diabetics groups had significantly greater area-under curves (AUC) than that of the control subjects (Table 12).

![Graph showing baseline and post-treatment postprandial glucose levels](image)

Figure 24: The postprandial glucose response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.
After treatment with glibenclamide there was no reduction in the AUC for glucose but, by contrast, treatment with pioglitazone caused a significant decrease in the AUC indicating an improvement in the ability to handle postprandial glucose but still not as effectively as by control subjects.

### 4.5.2 Postprandial Insulin Clearance

![Graph showing baseline and post-treatment postprandial insulin levels](image)

**Figure 25**: The postprandial insulin response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

In control subjects, plasma insulin rose to a peak 30 minutes after the test meal and then declined to the fasting level. In contrast both diabetic groups had an exaggerated insulin response. Peak insulin concentrations occurred approximately 2 hours after the meal and declined only slowly to baseline levels. This was reflected in the area-under curves (AUCs) which were significantly greater than in the control subjects. Not surprisingly the effect of glibenclamide treatment was to increase insulin levels. By
contrast, treatment with pioglitazone reduced the AUC but this was still significantly higher than for the control subjects.

Table 12: Integrated responses (AUC) of plasma insulin, proinsulin and 32-33 split proinsulin after the test meal

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose AUC (mmol/l/8hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>76.7*** (15.3)</td>
<td>81.8*** (18.8)</td>
<td>40.2 (6.9)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>73.4*** (14.8)</td>
<td>61.4*** (11.8)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>-4.30%</td>
<td>-24.90%</td>
<td></td>
</tr>
<tr>
<td><strong>Insulin AUC (pmol/l/8hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1449** (510)</td>
<td>1254** (550)</td>
<td>639 (119)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>1906*** (735)</td>
<td>988‡ (463)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>31%</td>
<td>-21.20%</td>
<td></td>
</tr>
<tr>
<td><strong>Proinsulin AUC (pmol/l/8hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>279** (156)</td>
<td>165* (144)</td>
<td>50 (23)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>345** (236)</td>
<td>113‡* (99)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>23.60%</td>
<td>-31.50%</td>
<td></td>
</tr>
<tr>
<td><strong>32-33 Split proinsulin AUC (pmol/l/8hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>344*** (145)</td>
<td>251** (163)</td>
<td>70 (17)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>362*** (179)</td>
<td>167‡ (149)</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>11%</td>
<td>-33.50%</td>
<td></td>
</tr>
</tbody>
</table>

Patient groups vs control subjects: * p < 0.05, **p < 0.005, ***p < 0.0005
Baseline vs post-treatment for each group: † p < 0.05
Glibenclamide treated vs pioglitazone treated patients:‡ p < 0.05

4.5.3 Postprandial Proinsulin Clearance

Proinsulin concentrations in control subjects showed a small peak coinciding with the peak of insulin secretion. In both groups of diabetic patients maximal concentrations of proinsulin occurred about 2 hours postprandial. After glibenclamide treatment the AUC for proinsulin increased whereas after pioglitazone treatment, there was a significant fall in the proinsulin AUC to that of the control subjects.

132
Figure 26: The postprandial proinsulin response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.

Figure 27: The postprandial 32-33 split proinsulin response to the test meal before and after treatment with pioglitazone or glibenclamide in type 2 diabetic patients compared to control subjects.
4.5.4 Postprandial 32-33 Split Proinsulin Clearance

Plasma 32-33 split proinsulin in control subjects showed a similar pattern to proinsulin (Figure 27). In both groups of diabetic patients after treatment with glibenclamide or pioglitazone the AUC showed the same pattern as for proinsulin. With pioglitazone the amount of 32-33 split proinsulin secreted fell such that it no longer differed significantly from control subjects (Table 12).

4.6 Relationship of Basal Beta-Cell Function to Fasting and Postprandial Lipaemia

To investigate possible relationships between fasting insulin, proinsulin, 32-33 split proinsulin level, insulin resistance, fasting lipoproteins and postprandial lipaemia, Spearman rank correlation coefficients were calculated. No correlation was found between fasting insulin levels and postprandial lipaemia (Table 13). Both fasting proinsulin and 32-33 split proinsulin levels correlate with fasting triglyceride, triglyceride AUC and negatively with plasma HDL cholesterol (Table 13) when the data from all subjects were included in the analyses. However, within each study group (diabetic and control subjects) this correlation did not reach statistical significance.

The integrated response of insulin over the postprandial period correlated positively with chylomicron RP (AUC) and chylomicron remnant RP (AUC) in the diabetic groups when the data from all subjects were included in the analyses (Table 14). Both the integrated proinsulin and 32-33 split proinsulin response over the postprandial period correlated with triglyceride AUC (Table 14). Both fasting triglyceride, proinsulin and split proinsulin correlate with insulin resistance estimated by HOMA model when data from all subjects were included in the analyses (Table 15).
Table 13: Relationships between fasting insulin, proinsulin and 32-33 split proinsulin levels and fasting and postprandial lipaemia

<table>
<thead>
<tr>
<th>INSULIN</th>
<th>Diabetics</th>
<th>Control</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TG (mmol/L)</td>
<td>-0.02</td>
<td>0.21</td>
<td>0.16</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>-0.16</td>
<td>0.13</td>
<td>-0.17</td>
</tr>
<tr>
<td>TG AUC (mmol/L/8hr)</td>
<td>-0.2</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>C-RP AUC (mmol/L/8hr)</td>
<td>-0.36</td>
<td>-0.26</td>
<td>-0.31</td>
</tr>
<tr>
<td>CR-RP AUC (mmol/L/8hr)</td>
<td>-0.13</td>
<td>-0.36</td>
<td>-0.21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PROINSULIN</th>
<th>Diabetics</th>
<th>Control</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TG (mmol/L)</td>
<td>0.21</td>
<td>0.53</td>
<td>0.52**</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>-0.36</td>
<td>-0.33</td>
<td>-0.35*</td>
</tr>
<tr>
<td>TG AUC (mmol/L/8hr)</td>
<td>0.28</td>
<td>0.53</td>
<td>0.55**</td>
</tr>
<tr>
<td>C-RP AUC (mmol/L/8hr)</td>
<td>-0.25</td>
<td>0.21</td>
<td>-0.11</td>
</tr>
<tr>
<td>CR-RP AUC (mmol/L/8hr)</td>
<td>-0.33</td>
<td>0.57</td>
<td>0.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>32-33 SPLIT PROINSULIN</th>
<th>Diabetics</th>
<th>Control</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TG (mmol/L)</td>
<td>0.37</td>
<td>0.09</td>
<td>0.54**</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>-0.41</td>
<td>-0.21</td>
<td>-0.39*</td>
</tr>
<tr>
<td>TG AUC (mmol/L/8hr)</td>
<td>0.23</td>
<td>0.25</td>
<td>0.56*</td>
</tr>
<tr>
<td>C-RP AUC (mmol/L/8hr)</td>
<td>-0.36</td>
<td>0.36</td>
<td>-0.15</td>
</tr>
<tr>
<td>CR-RP AUC (mmol/L/8hr)</td>
<td>-0.33</td>
<td>0.71*</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Spearman rank correlation coefficient *p < 0.05, ** p< 0.01.

C-RP Chylomicron Retinyl Palmitate.
CR-RP Chylomicron Remnant Retinyl Palmitate.
Table 14: Relationships between integrated response of insulin, proinsulin and 32-33 split proinsulin levels and postprandial lipaemia

<table>
<thead>
<tr>
<th></th>
<th>Diabetics</th>
<th>Control</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin (AUC) (mmol/L/8hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (AUC) (mmol/L/8hr)</td>
<td>-0.32</td>
<td>-0.03</td>
<td>0.32</td>
</tr>
<tr>
<td>C-RP (AUC) (mmol/L/8hr)</td>
<td>-0.57**</td>
<td>0.24</td>
<td>-0.29</td>
</tr>
<tr>
<td>CR-RP (AUC) (mmol/L/8hr)</td>
<td>-0.45*</td>
<td>-0.09</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Proinsulin (AUC) (pmol/L/8hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (AUC) (mmol/L/8hr)</td>
<td>0.11</td>
<td>0.53</td>
<td>0.54**</td>
</tr>
<tr>
<td>C-RP (AUC) (mmol/L/8hr)</td>
<td>-0.31</td>
<td>0.19</td>
<td>-0.13</td>
</tr>
<tr>
<td>CR-RP (AUC) (mmol/L/8hr)</td>
<td>-0.34</td>
<td>0.38</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Split Proinsulin (AUC) (pmol/L/8 hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (AUC) (mmol/L/8hr)</td>
<td>0.17</td>
<td>0.16</td>
<td>0.56**</td>
</tr>
<tr>
<td>C-RP (AUC) (mmol/L/8hr)</td>
<td>-0.41</td>
<td>0.47</td>
<td>-0.15</td>
</tr>
<tr>
<td>CR-RP (AUC) (mmol/L/8hr)</td>
<td>-0.4</td>
<td>0.18</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Spearman rank correlation coefficient *p < 0.05, **p< 0.01.
C-RP Chylomicron Retinyl Palmitate.
CR-RP Chylomicron Remnant Retinyl Palmitate.
Table 15: Relationships between insulin resistance (HOMA model) and postprandial lipaemia

<table>
<thead>
<tr>
<th></th>
<th>Diabetics</th>
<th>Control</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(Fasting)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>-0.04</td>
<td>0.22</td>
<td>0.35*</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>-0.21</td>
<td>0.18</td>
<td>-0.21</td>
</tr>
<tr>
<td>Proinsulin (pmol/L)</td>
<td>0.52*</td>
<td>0.33</td>
<td>0.73**</td>
</tr>
<tr>
<td>32-33 split proinsulin (pmol/L)</td>
<td>0.71**</td>
<td>0.07</td>
<td>0.81**</td>
</tr>
<tr>
<td><strong>(AUC)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mmol/L/8 hr)</td>
<td>-0.04</td>
<td>0.22</td>
<td>0.34</td>
</tr>
<tr>
<td>C-RP (mmol/L/8 hr)</td>
<td>-0.44</td>
<td>-0.22</td>
<td>-0.28</td>
</tr>
<tr>
<td>CR-RP (mmol/L/8 hr)</td>
<td>-0.44</td>
<td>-0.07</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Spearman rank correlation coefficient *p < 0.05, **p < 0.01.

C-RP Chylomicron Retinyl Palmitate.
CR-RP Chylomicron Remnant Retinyl Palmitate.
DISCUSSION

4.7.1 The effect of insulin sensitization on the fasting plasma glucose, insulin, proinsulin and 32-33 split proinsulin plasma concentration.

Insulin resistance plays a major role early in the development of type 2 diabetes. It is associated with clusters of cardiovascular risk factors (e.g. hypertension and dyslipidaemia) that contribute to the increased risk of coronary heart disease in these patients. Directly targeting insulin resistance in the peripheral tissues by novel pharmacological agents is a relatively new approach for treating type 2 diabetes.

In the present study, both diabetic groups had the same glycaemic control as shown by HbA1C at baseline (Table 11). In the fasting state there was a significant fall in fasting glucose levels after treatment by sensitization to insulin through the PPARγ receptor pathway with pioglitazone. This may will be due to decreased hepatic gluconeogenesis (Fujiwara et al., 1995; Ikeda et al., 1998) coupled with increased glucose disposal by peripheral tissues. Treatment with glibenclamide, which augments insulin secretion, caused no effect on the fasting glycaemia. This is because its chief pharmacological effect is to overcome relative insulin deficiency and promote glucose disposal rather than reducing hepatic gluconeogenesis.

In the fasting state, both groups of diabetic patients were not significantly hyperinsulinaemic when compared to control subjects but had disproportionate amounts of intact proinsulin and 32-33 split proinsulin present. Thus our results confirm the finding that hyperinsulinaemia in type 2 diabetic patients is due to the secretion of greater amounts of insulin precursor species. This demonstrates the presence of an impairment of β-cell function with the result that the contents of immature vesicles are released into the circulation. The major immature species of insulin present in the circulation is 32-33 split proinsulin.

138
Following treatment with pioglitazone there was a significant improvement in β-cell function as manifested by a significant reduction in proinsulin and 32-33 split proinsulin concentrations in the circulation. As expected sulphonylurea treatment with glibenclamide treatment caused a significant increase in basal insulin concentrations and also in proinsulin and split proinsulin secretion. Over stimulation of the β-cell could be a mechanism whereby immature secretory vesicles are released into the circulation thus explaining the increased amount of insulin precursor present after glibenclamide treatment, and the reduced amount present when the stress on the pancreas is reduced by improving insulin resistance with thiazolidinedione.

4.7.2 The effect of insulin sensitization on postprandial glycaemia and β-cell function in type 2 diabetic patient compared to normal subjects.

4.7.2.1 Baseline postprandial glycaemia and insulin release in diabetic and control subjects

Postprandial glycaemia in control subjects was far less than that in the diabetic patients. Prolonged glycaemia in the diabetic subjects cause chronic stimulation of the β-cells as shown by the greatly prolonged secretion of insulin, proinsulin and split proinsulin that did not occur in the control subjects. This clearly demonstrates a defect in the glucose uptake by the peripheral tissues caused by the presence of insulin resistance and of inadequate insulin secretion.

The effect of test meal was to induce a severe hyperinsulinaemic state in the postprandial period in both groups of diabetic subjects which was associated with the presence of a four-fold increase in the amounts of insulin precursor species released. In this respect it is important to note that non-diabetic subjects secrete approximately 15% of the total pancreatic insulin output as proinsulin and 32-33 split proinsulin. In
type 2 diabetic subjects this increases to approximately 30%. This difference could be caused by chronic glycaemia in the diabetic subjects that stimulates the pancreas to secrete insulin for longer periods than would occur in control subjects. However, it cannot be ruled out that the development of type 2 diabetes is associated with a change in the protein processing and export apparatus.

4.7.2.2 The effect of treatment on postprandial glycaemia and insulin release in diabetic and control subjects

The effect of insulin sensitization with pioglitazone in type 2 diabetic patients was far more evident in the postprandial state when compared to the fasting results. Hyperinsulinaemia and hyperglycaemia were greatly reduced after insulin sensitisation. The overall β-cell response was reduced to that of control subjects showing a significant reduction in the glycaemic stimulus experienced by the β-cell. A plausible explanation for this could be that a reduction of insulin resistance in the peripheral tissues and improved glucose disposal lowers the glycaemic stimulus to secrete insulin. In addition, indirect effects of improved clearance of postprandial lipid could result in reduced glycaemia as predicted by the Randle cycle (Randle et al., 1963). Not surprisingly, stimulation of insulin release by glibenclamide showed the opposite effect to treatment with pioglitazone in that the glycaemic stimulation caused a very large release of insulin and insulin precursors.

The results suggest that the use of a pioglitazone induces qualitative and quantitative changes in insulin secretion. These effects cannot be explained solely by the elimination of chronic stimulation by hyperglycaemia on β-cell secretion because similar results have been reported in normoglycaemic insulin resistant subjects (Berkowitz et al., 1996; Cavaghan et al., 1997). Other possible explanations could be
the elimination of toxic effects of free fatty acids on \(\beta\)-cell secretion, and direct
effects of the thiazolidinedione on \(\beta\)-cells (Saltiel et al., 1996; Kaiyala et al., 1999).

4.7.2.3 The effect of insulin sensitisation on the HOMA model estimate of insulin
resistance in type 2 diabetic patients compared to normal subjects.

The HOMA model estimate gives a usable indication of an individual’s insulin
resistance based on fasting insulin and fasting glucose measurements. Although this
is not the best technique for measuring insulin resistance, it is the most practical for
detailed study where clamps or insulin infusion sensitivity tests would be
impractical. However, despite the drawbacks of the model, when performed on the
subjects pre- and post-treatment it provide a valid index of any changes that might
occur.

Diabetic patients in the baseline state were more insulin resistant than the
controls as judged by the HOMA estimate. Treatment with the insulin sensitizer
pioglitazone was effective in reducing insulin resistance, which clearly had an effect
on \(\beta\)-cells causing them to secrete less insulin to cope with the glycaemic stimulus
and decreased the secretion of partially processed insulin species. HOMA scores
were reduced by 39% in the pioglitazone treated patients. Whereas by contrast
glibenclamide treatment did not improve insulin resistance. The magnitude of the
change in the HOMA scores caused by treatment with pioglitazone in this study is in
agreement with those reported in the European Multicenter Trial using troglitazone
(Gomez-Perez et al., 2002).
4.7.2.4 The relationships between fasting and postprandial β-cell function and fasting and postprandial lipaemia.

The basal fasting plasma insulin concentration had no strong correlation with the extent of the lipaemia in the fasting and the postprandial state when data from all subjects were combined. By contrast, when fasting proinsulin and 32-33 split proinsulin concentrations were subjected to similar analyses, a significant correlation with fasting and postprandial lipaemia were found. The reason for this is most likely due to the much greater half-life of proinsulin and 32-33 split proinsulin (40 min) in the circulation compared to that of insulin (5 min) (Temple et al., 1992). This allows for a more stable measurement of the insulin precursors than for insulin itself. It would seem from our result that measurement of proinsulin and 32-33 split proinsulin have some predictive value for the occurrence of abnormal postprandial lipaemia.

The results of this study clearly demonstrate an association between insulin resistance and the clearance of dietary lipid that can be improved by sensitization to insulin. Both overall triglyceride and in particular the clearance of triglyceride-rich remnant particles are improved by sensitization to insulin through PPARγ pathway. This could have an important therapeutic role in the prevention of atherosclerosis, since remnant particles clearance has been implicated in atherogenesis and this could be important in atherogenesis (Reaven et al., 1987; Zilversmit et al., 1979).

The improvement in insulin sensitivity seen with pioglitazone treatment in this study could involve a general action of TZDs causing increased peripheral glucose disposal and reduced hepatic glucose production acting together to reduce fasting glycaemia and consequently lowering the HOMA estimate of insulin resistance. This effect would be reinforced by the improvement in β-cell function and reduced insulin secretion to deal with the same stimulus.
CHAPTER 5

INSULIN SENSITIZATION THROUGH THE PPARγ RECEPTOR PATHWAY IN TYPE 2 DIABETIC AND CONTROL SUBJECTS: EFFECTS ON LIPASES, LCAT, CETP AND PLTP IN THE FASTING AND POSTPRANDIAL STATE.

5.1 Lecithin:cholesterol acyl transferase

LCAT is a key component in the reverse cholesterol transport pathway. This pathway serves to remove excess free cholesterol from peripheral tissues and transport it to the liver or to steroidogenic tissues in the form of cholesteryl ester for excretion as bile salts or steroid hormone synthesis (Jonas et al., 2000; Dobiasova et al., 1999). Phospholipid serves as the donor of the acyl group from the C2 position of phosphatidylcholine while free cholesterol originates from tissues and is passed to HDL by the action of the ABCA1 transporter. The importance of LCAT in lipoprotein metabolism is shown in individuals with mutations of the LCAT gene. Established mutations are associated with partial or complete absence of plasma LCAT activity, leading to fish eye disease (FED) or familial LCAT deficiency (FLD). FLD is characterized by hypertriglyceridaemia, markedly reduced HDL levels, cloudy corneas, haemolytic anemia and renal disease. Both groups of patients have reduced plasma concentrations of apo-AI and apo-AII but synthesis of these proteins is normal and the decreased plasma content results from increased catabolism of nascent HDL particles (Peelman et al., 2000; Santamarina et al., 2000).

Of particular interest is a recent report that, in addition to having an effect on the metabolism of HDL, LCAT modulates the metabolism of the apo-B100 containing lipoproteins by upregulating the LDL-receptor pathway. Thus it is proposed that LCAT
beneficially modifies the plasma lipid profile by raising HDL and lowering LDL concentrations (Brousseau et al., 1997). However, studies in LCAT transgenic animal models have demonstrated that raising plasma HDL does not always confer protection against the development of atherosclerosis and, in the absence of CETP, LCAT overexpression in mice leads to the formation of abnormal HDL with an impaired facility for reverse cholesterol transport (Tall et al., 1998). Expression of CETP, as well as apo-AI, corrects the dysfunctional properties of HDL from LCAT transgenic mice. However, in humans the effect of LCAT expression on plasma levels of LDL has not been extensively studied.

5.2 Lipid Transfer Proteins: CETP and Phospholipid Transfer Protein

Cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) play a central role in HDL metabolism. PLTP and CETP belong to a family of lipid transfer/lipopolysaccharide-binding proteins (LBP) and share sequence homologies, (Bruce et al., 1998). Their function is to facilitate the redistribution of lipids and by doing so enable cholesterol to be returned to the liver from peripheral tissues.

PLTP also plays an important role in plasma lipoprotein metabolism. It mediates the transfer of phospholipids to HDL from triglyceride-rich lipoproteins undergoing lipolysis by lipoprotein lipase. Surface fragments from chylomicrons and VLDL during lipolysis are important precursors of plasma HDL. PLTP can also remodel HDL particles. The interaction of PLTP with typical HDL results in the release of small, lipid-poor pre-β-HDL particles, and at the same time produces HDL₂ particles by a process
that involves particle fusion (Fielding et al., 2001). Pre-β-HDL particles are efficient acceptors of free cholesterol from peripheral cells.

In fasting plasma, CETP is associated mainly with HDL and redistributes LCAT-derived cholesteryl esters from HDL₃ to the apo-B100, containing lipoproteins (such as VLDL) and apo-B48 containing lipoproteins (chylomicrons and their remnants) in exchange for triglycerides (Eisenberg et al., 1985). The redistribution of cholesteryl ester by CETP to lipoproteins destined for hepatic uptake provides a mechanism for the transfer of peripheral tissue free cholesterol from the plasma to the liver. In addition to this role, CETP activity in conjunction with the hepatic lipase, provides a mechanism for the remodelling of LDL and HDL into smaller particles (Hopkins et al., 1986; Zambon et al., 1993).

PLTP also has an indirect role in the reverse cholesterol transfer pathway. An overexpression of PLTP in transgenic mice increases the pre β-HDL particle number which can then act as a source of ester synthesized from free cholesterol acquired from peripheral tissues through the ABCA1 transporter (Francone et al., 2003; Schlitt et al., 2003). Variation in the plasma level of PLTP activity would be expected to be associated with variations in the cholesterol efflux capacity from the tissues.

5.3 Relationship of CETP and PLTP activity to lipid and glucose metabolism

Plasma CETP activity is highly variable being affected by a variety of metabolic conditions and dietary factors. Both CETP activity and mass are increased in obesity but not in weight-matched type 2 diabetic patients, and these levels correlate with total and LDL cholesterol concentrations (Morton et al., 2003). Earlier studies have shown that
CETP mass and activity levels are increased in diet-induced hypercholesterolaemia. The CETP promoter gene contains a sterol regulatory element presumed to be responsible for sterol induction of CETP expression.

A partial understanding of these complex regulatory events has been gained in recent studies of type 2 diabetes patients and in Type IIa and IIb hyperlipidaemic individuals, and through comparisons with phospholipid transfer protein. PLTP activity and mass are elevated in obese individuals with or without type 2 diabetes (Desrumaux et al., 1999). It has been reported that PLTP values correlate with insulin resistance, fasting glycaemia and HbA1C levels but not with plasma lipid parameters, however the mechanisms that underlie these elevated levels in diabetic patients are not well understood (Riemens et al., 1998). Elevated PLTP activity is often associated with increased VLDL turnover rate and inhibition of VLDL synthesis by insulin infusion results in decreased PLTP activities (Dullaart et al., 2001). Hypertriglyceridaemic patients have higher PLTP activity, but also a lower PLTP mass (Jonkers et al., 2003). During a hyperinsulinaemic euglycaemic clamp, PLTP, but not CETP activity, decreases acutely (Riemens et al., 1998; Maclean et al., 2001). Collectively, these studies illustrate that PLTP activity is related in some fashion to glucose metabolism whereas CETP is related to lipid metabolism (Morton et al., 1999). A linkage of PLTP activity to glucose metabolism is consistent with the finding that PLTP is universally expressed in the plasma of all species studied, whereas CETP expression is highly species specific.
5.3.1 Cholesteryl ester transfer protein and atherogenesis

Much attention has been focused on the potential benefit or liability of CETP activity in the process of atherogenesis since it is clear from numerous studies that CETP activity strongly influences HDL levels, its lipid and protein content, and the size and concentration of individual subfractions (Bruce et al., 1998). For example, recent studies in transgenic mice and individuals with low levels of CETP have clearly illustrated that under some conditions CETP activity is anti-atherogenic, even though the expression of CETP is accompanied by reductions in HDL cholesterol (Bruce et al., 1998; Hirano et al., 1997; Inazu et al., 2000; Tall et al., 1997). Similarly individuals with a CETP amino acid polymorphism (Bruce et al., 1998) associated with lower plasma CETP activity and higher HDL levels were found, to have an increased prevalence of CHD. Together these studies suggest that CETP can have a protective role. However, CETP deficiency is not necessarily pro-atherogenic if very high levels of HDL exist (Tall et al., 1998; Moriyama et al., 1998). In a survey of Japanese with high HDL levels (>2 mmol/L), it was found that 25-40% of these individuals were CETP deficient, but the prevalence of CHD was the same regardless of the presence or absence of CETP (Moriyama et al., 1998).

Inhibition of CETP has been proposed as a strategy to raise HDL cholesterol levels. The effects of torcetrapib, a potent inhibitor of CETP, on plasma lipoprotein levels in patients with low levels of HDL cholesterol (<1.0 mmol/L) increased plasma concentrations of HDL cholesterol by up to 106% depending on the dose and also reduced LDL cholesterol levels by 17% (Brouseau et al., 2004). This finding is consistent with the fact that patients with homozygous defects in the CETP gene have reduced levels of LDL cholesterol (Inazu et al., 1990) and this is related to the increased
clearance of LDL cholesterol from the plasma (Ikewaki et al., 1995) suggesting that the LDL receptor pathway may be up-regulated in CETP deficiency. The mean particle size of both HDL and LDL was significantly increased by torcetrapib and this in agreement with recent report that CETP polymorphism (replacement of isoleucine with valine at position 405) that is linked to reduced CETP activity is significantly associated with longevity and large HDL and LDL particle size.

The relationship of CETP activity to the risk of coronary heart disease remains controversial (Barter et al., 2003). It is not clear whether CETP-deficient persons are protected from CHD; they may even be at increased risk (Zhong et al., 1996). In the Honolulu Heart program, a subgroup of subjects with a heterzygous CETP mutation who had HDL cholesterol levels in the range of 1-1.6 mmol/L appeared to be at increased risk for coronary heart disease (Zhong et al., 1996). However, a recent analysis of seven year prospective data from this study did not reveal a significant relation between heterozygosity for CETP mutations and CHD or stroke (Barter et al., 2003). At the population level, that a common CETP genetic variant (Taq B2) is associated with reduced CETP activity, increased HDL cholesterol levels, and a reduced risk of CHD (Ordovas et al., 2000; Brousseau et al., 2002). Moreover, inhibition of CETP in rabbits has been found to result in reduced atherosclerosis.

Just as CETP expression is protective in some cases, in others it appears proatherogenic. In mice, where expression of either the apolipoprotein E or the LDL receptor gene has been knocked out, concomitant expression of high levels of CETP accelerates lesion development (Plump et al., 1999). By contrast rabbits fed on an atherogenic diet showed a suppression of CETP activity, concomitant decrease in LDL
and VLDL cholesterol levels, increases in HDL cholesterol concentrations and decreased aortic content of cholesterol (Sugano et al., 1998). The results of these studies suggest that under conditions where VLDL remnants and LDL accumulate (either by reduced clearance and/or increased production), CETP activity enhances atherogenesis. These and other studies illustrate that the balance of individual HDL species, which results from the complex interaction of cholesterol esterification and lipid transfer proteins, and not necessarily absolute HDL levels, define the protection generally afforded by high HDL levels (Berard et al., 1997; Hoeg et al., 1996).

Overall, these data strongly suggest that the dynamics of cholesterol movement through HDL underlie its anti-atherogenic potential (Bruce et al., 1998). This process, a key component of reverse cholesterol transport, is protective under normal conditions, but appears to be injurious when catabolism of the acceptor lipoproteins, VLDL and LDL, is compromised. In instances where HDL levels are very high, the absence of CETP activity appears to be adequately compensated by other mechanisms.

5.3.2 CETP activity and insulin resistance

The results of several studies of type 2 diabetic patients have suggested a link between plasma CETP activity and insulin resistance. When undergoing euglycaemic clamps type 2 diabetics showed a decrease in plasma CETP in response to exogenous hyperinsulinemia than did healthy control subjects (Sutherland et al., 1994, 2001; Riemens et al., 1998).

Animal studies have shown that CETP from hamster adipose tissue is increased during fasting and decreased with feeding (Jiang et al., 1991). These results suggest that
insulin action in adipose tissue and muscle may be controlling CETP levels by indirect effects. It is known that insulin activates lipoprotein lipase and this enhances remnant clearance. Another possible explanation is that insulin action in adipose tissue and muscle decreases CETP and PLTP gene expression in these tissues. In turn, CETP expression in peripheral tissues could increase the effectiveness of insulin signal. CETP action on HDL promotes efflux of cholesterol from plasma membranes, improving insulin signalling by maintaining membrane cholesterol:phospholipid ratio in an appropriate range (Jonas et al., 1998).

5.4 Lipoprotein lipase and Hepatic lipase

Lipoprotein lipase (LPL) plays an essential role in the hydrolysis of plasma triglyceride. LPL activity is impaired in diabetes (Eckel et al., 1989; Pulawa et al., 2002), in obesity and other states of insulin resistance (Cominacini et al., 1993). Direct estimation of LPL action by measuring the extraction of plasma TG in subcutaneous fat showed that the enzyme is increased postprandial, but this response is blunted or absent in obese subjects (Coppack et al., 1992) and it has been suggested that the postprandial activation of LPL results from an increase in the specific activity of the enzyme and not from increased transcription and secretion (Coppack et al., 1992; Sakayama et al., 1992).

Impaired postprandial activation of LPL has several consequences. First, it prolongs the postprandial elevation of plasma triglyceride concentrations. Second, lipolysis of triglyceride-rich lipoproteins is impaired; this inhibits the entry of cholesterol into HDL pool, which occurs via the transfer of excess redundant surface material (including cholesterol) to HDL during the lipolysis of triglyceride-rich
lipoproteins. Third, the prolonged residence time of triglyceride-rich lipoproteins in the circulation leads to increased exchange of their triglyceride with cholesterol ester in HDL through the action of CETP.

Hepatic lipase does not seem to be concerned with the supply of NEFA to the tissues to the same extent as the LPL. Its role would seem to be more involved with the remodelling of lipoproteins particularly HDL and LDL. In the case of HDL, HL is active in the hydrolysis of triglyceride enriched HDL species, remodelling them to a small particles possibly ready to participate in further cholesteryl ester synthesis or alternatively be excreted as apo-AI through the kidney. In addition to these effects, LDL remodelling by HL can give rise to small dense particles which are known to be atherogenic (see Chapter 1).

5.5 Aims of the study

The aims of this part of the study were to assess the effect of insulin sensitization and postprandial lipaemia on the rates of plasma cholesterol esterification by LCAT, cholesteryl ester transfer by CETP, phospholipid transfer by PLTP, LPL and HL activity. LCAT, CETP and PLTP were measured in the fasting state and 4 hours after the test meal (according to the methods described in Chapter 2). And before and after treatment with pioglitazone in order to establish the extent to which postprandial lipaemia influences their activities.
5.6 Results

5.6.1 The effect of insulin sensitization on fasting and postprandial LCAT activity in type 2 diabetic patients and control subjects

Result of LCAT measurement in diabetic subjects compared to the control subjects are shown in Table 16. It can be seen that there were no differences in LCAT activity between the diabetic patients and the control subjects at baseline and after either treatment. Four hours after the test meal, plasma cholesterol esterification by LCAT increased significantly from the fasting level in both diabetic groups in the baseline conditions, but not in the control subjects (Paired t-test glibenclamide p<0.04, pioglitazone p<0.01, controls p = NS).

Table 16: Fasting and postprandial plasma LCAT activity in diabetic and control subjects before and after treatment

<table>
<thead>
<tr>
<th></th>
<th>LCAT activity (µmole free cholesterol esterified/L/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glibenclamide</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>49.0 ± 18.1</td>
</tr>
<tr>
<td>4-hr postprandial</td>
<td>58.6± ± 13.3</td>
</tr>
<tr>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>50.5 ± 13.3</td>
</tr>
<tr>
<td>4-hr postprandial</td>
<td>48.4 ± 20.8</td>
</tr>
</tbody>
</table>

Values are mean ± S.D

Fasting vs 4 hr: † p < 0.05.
After treatment with either pioglitazone or glibenclamide there was no change in the fasting activity of LCAT from the baseline conditions, but 4 hours postprandial, by contrast to the baseline condition, there was no longer statistically significant increase in LCAT activity in either group of patients.

5.6.2 The effect of insulin sensitization on fasting and postprandial CETP activity in type 2 diabetic compared to control subjects

Results of CETP analyses in diabetic subjects compared to the control group are shown in Table 17.

At baseline in all three groups, there was a significant increase in the postprandial rates of net cholesteryl ester transfer by CETP (Paired t-test glibenclamide P<0.01, pioglitazone p<0.003, control p< 0.002). There was no significant difference in CETP activity between the 3 groups in the fasting state or 4 hour after the test meal.

Table 17: Fasting and postprandial CETP activity in diabetic and control subjects before and after treatment

<table>
<thead>
<tr>
<th>CETP activity (µmole cholesterol ester transferred/L/hr)</th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Fasting</td>
<td>10.5 ± 9.7</td>
<td>8.0 ± 6.1</td>
<td>8.2 ± 3.7</td>
</tr>
<tr>
<td>4-hr postprandial</td>
<td>24.0† ± 15.7</td>
<td>23.0† † ± 13.2</td>
<td>20.4† † ± 8.9</td>
</tr>
<tr>
<td>Post-treatment Fasting</td>
<td>6.4 ± 2.9</td>
<td>8.7 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>4-hr postprandial</td>
<td>20.1† † ± 9.8</td>
<td>18.2† † ± 11.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D.

Fasting vs 4 hr. † p < 0.02; † † p < 0.002
Both groups of diabetic subjects after treatment showed the same pattern of increase in cholesteryl ester transfer by CETP after the test meal (Glibenclamide p < 0.0005, pioglitazone p < 0.002, control p < 0.002). As at baseline Insulin sensitization did not change the activity of CETP and neither did glibenclamide treatment.

No correlation's were found between the fasting rate of plasma cholesterol esterification and fasting triglyceride, total cholesterol, HDL cholesterol and measurements of glycaemic control. The fasting rate of net cholesteryl ester transfer did not correlate with fasting lipids, fasting glucose or HbA1C concentrations in the diabetic subjects (data not shown). Δ LCAT, Δ CETP did not correlate with the AUC of triglyceride, chylomicron RP, and chylomicron remnant RP.

5.6.3 The effect of insulin sensitization on fasting and postprandial PLTP mass in type 2 diabetic compared to control subjects

The result of PLTP analyses in diabetic subjects compared to the control group are shown in Table 18.

Fasting PLTP mass in the baseline condition was significantly less in both groups of diabetic subjects when compared with control subjects. There were no significant changes in PLTP mass postprandially in the diabetic groups or control subjects.

After treatment with the insulin sensitizer pioglitazone, fasting PLTP mass was increased significantly from the baseline and became not significantly different from the control subjects. Glibenclamide treatment showed no effect on fasting PLTP mass and remained significantly lower than the control subjects.
Table 18: Fasting and postprandial PLTP activity in diabetic and control subjects before and after treatment

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>1.64* ± 0.24</td>
<td>1.56* ± 0.22</td>
<td>1.86 ± 1.72</td>
</tr>
<tr>
<td>4-hr postprandial</td>
<td>1.52 ± 0.27</td>
<td>1.48 ± 0.28</td>
<td>1.62 ± 0.08</td>
</tr>
<tr>
<td><strong>Post-treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>1.59 * ± 0.33</td>
<td>1.69† ± 0.17</td>
<td></td>
</tr>
<tr>
<td>4-hr postprandial</td>
<td>1.52 ± 0.27</td>
<td>1.51† ± 0.25</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D.
Patient groups vs control subjects: *p < 0.05.
Fasting vs 4 hr †p < 0.05. Baseline vs post-treatment for each group: †p < 0.05

There was a significant correlation between PLTP mass and HDL cholesterol content when analysed by Spearman rank (r = 0.58, p < 0.05), no correlation were found with fasting and triglyceride AUC, NEFA, insulin resistance (HOMA), glucose and HbA1C.

5.6.4 The effect of insulin sensitization on fasting LPL and HL activities in type 2 diabetic compared to control subjects

The result of lipase analyses in diabetic subjects compared to the control group are shown in Table 19.

Post-heparin plasma LPL activity was similar in all three groups both at baseline and after treatment. Hepatic lipase activity was higher in the diabetic groups than the control subjects. No statistically significant changes in the plasma concentration of hepatic lipase were observed after either treatment and remained significantly higher in both diabetic groups of patients compared to control subjects (Table 19).
No significant correlation's between LPL or HL with the postprandial parameters was observed at baseline or after treatment with glibenclamide or pioglitazone (Table 20).

**Table 19: Post-heparin lipoprotein lipase and hepatic lipase activities (μmol NEFA released/ml/hr)**

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LPL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9.6 ± 2.6</td>
<td>9.5 ± 4.3</td>
<td>8.8 ± 3.5</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>11.4 ± 4.5</td>
<td>8.3 ± 2.7</td>
<td></td>
</tr>
<tr>
<td><strong>HL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>21.2* ± 11.8</td>
<td>24* ± 10.8</td>
<td>23.4* ± 12.2</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>20.6* ± 9.8</td>
<td>23.4* ± 12.2</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD
Diabetic group vs controls: * P < 0.05.

**Table 20: Relationships between LPL and HL activities (μmol NEFA released/ml/hr) with postprandial lipaemia AUC (mmol/L)**

<table>
<thead>
<tr>
<th></th>
<th>Glibenclamide</th>
<th>Pioglitazone</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LPL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (AUC)</td>
<td>-0.48</td>
<td>-0.1</td>
<td>-0.10</td>
</tr>
<tr>
<td>C-RP (AUC)</td>
<td>-0.01</td>
<td>-0.34</td>
<td>-0.29</td>
</tr>
<tr>
<td>CR-RP (AUC)</td>
<td>-0.08</td>
<td>-0.09</td>
<td>-0.09</td>
</tr>
<tr>
<td><strong>HL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (AUC)</td>
<td>0.35</td>
<td>0.25</td>
<td>0.53</td>
</tr>
<tr>
<td>C-RP (AUC)</td>
<td>-0.02</td>
<td>-0.34</td>
<td>0.64*</td>
</tr>
<tr>
<td>CR-RP (AUC)</td>
<td>0.41</td>
<td>0.38</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Spearman rank correlation: * P < 0.05.
C-RP Chylomicron retinyl palmitate.
CR-RP Chylomicron remnant retinyl palmitate.
DISCUSSION

5.7.1 Lecithin: cholesterol acyl transferase activity

In this study I have demonstrated that the rate of fasting free cholesterol esterification is similar in diabetic and control subjects. This is in agreement with previous findings (Durlach et al., 1996). Four hours postprandial there was a significant increase in LCAT activity in the diabetic patients but not in the control subjects. This finding supports a direct or indirect role for triglyceride on LCAT activity. One possible explanation is that lipolysis of triglyceride-rich lipoproteins gives rise to an excess of phospholipid which is then transferred to HDL by PLTP and so providing additional acyl group-containing substrates as donors for LCAT (Marcel et al., 1980). Postprandial LCAT activity did not increase in control subjects which may be due to a much smaller rise in the concentration of postprandial triglyceride-rich lipoproteins. In this study I did not find any correlation between LCAT activity and triglyceride. However, previous studies have reported that LCAT activity correlates positively with the concentration of plasma triglyceride (Durlach et al., 1996). It has also been reported that LCAT activity is greater in type 2 diabetic subjects (Riemens et al., 1998) in contrast to my results. The reasons for these differences are uncertain but would seem to be related to the extent of hypertriglyceridaemia. If the data from hypertriglyceridaemic subjects are excluded, there was no longer a significant difference in LCAT activity in the diabetic patients than from the controls as I found in my study. The patients in my study were not as severely hypertriglyceridaemic.
No significant relationship was found between LCAT activity and the HOMA estimate of insulin resistance (Data not shown). Given this lack of association, it is not surprising that insulin sensitization through the PPARγ pathway had no effect on the LCAT activity. Similarly glibenclamide had no effect.

5.7.2 Cholesteryl ester transfer protein

In this study fasting CETP activity was similar in the diabetic and control subjects. This finding is at variance with the earlier findings of Fielding et al (Fielding et al., 1984; Riemens et al., 1998) which showed a lowered fasting rate of net cholesteryl ester transfer in diabetic subjects compared to normal individuals. One possible explanation for this discrepancy is the difference in glycaemic control in the subjects studied by Fielding et al. The patients in their study were poorly controlled with fasting glucose in the range of 13-16 mmol/l, whereas the patients in the present study had fasting glucose < 9.0 mmol/L.

In this study, a highly statistically-significant postprandial increase in the rate of CETP activity was demonstrated in control and diabetic subjects. The postprandial increase was of a similar order of magnitude (2-3 fold increase) in the diabetic patients, before and after treatment, and the control subjects. The effect of postprandial lipoaemia on CETP activity could depend on the balance between the transfer of surface components from chylomicrons to make new HDL that subsequently esterify free cholesterol by LCAT and export it by CETP.

The relative proportion of both donor and acceptor lipoprotein particles are known to significantly influence cholesteryl ester transfer mediated by CETP (Guerin et al., 1996,
An earlier study has reported increased cholesteryl ester transfer from HDL to VLDL in patients with hypertriglyceridaemia. Similarly, other studies have reported elevated cholesteryl ester transfer from HDL to LDL in patients with hypercholesterolaemia (Mann et al., 1991). It has also been shown that during postprandial lipaemia in normolipidaemic subjects, CETP mediated an enhanced rate of cholesteryl ester transfer from HDL to both chylomicron and VLDL-I but LDL remained the major cholesterol acceptor of the apo-B containing lipoproteins in the fasting state (Lassel et al., 1999). In addition to these observations, an increase in the relative triglyceride content of the lipoprotein acceptor particles has been shown to increase its capacity to accept cholesteryl ester transferred by CETP (Liu et al., 1995).

Treatment with pioglitazone or glibenclamide had no effect on CETP activity either in the fasting state or 4 hour postprandial. No correlations were found between β-cell function, HOMA or lipaemia. It cannot be ruled out that glycaemic control exerts an influence on CETP activity because in this study it was deliberately maintained by the treatment regime.

5.7.3 Phospholipid transfer protein

In this study fasting PLTP mass was found to be significantly lower in diabetic patients compared with control subjects at baseline and did not change postprandial. This finding is in agreement with previous reports (Elchebly et al., 1996; Jonkers et al., 2003). In agreement with other reports we found only a correlation between PLTP mass and HDL and not with HOMA or indices of beta-cell function and lipaemia. However other reports have indicated that PLTP activity, but not mass, is related to insulin resistance in
type 2 diabetic patients, but in the present study PLTP activity was not measured (Jonkers et al., 2003).

Sensitization to insulin had the effect of increasing PLTP mass present to that of the control subjects despite there being no correlation with the HOMA estimate. By contrast glibenclamide treatment had no effect on fasting and postprandial PLTP mass. The positive correlation between PLTP mass and HDL cholesterol is not unexpected given that the PLTP protein is intimately associated with HDL particles.

5.7.4 Lipoprotein and hepatic lipase activity.

No differences were found in lipoprotein lipase activity before and after treatment with glibenclamide or pioglitazone and neither did the activity in the patient groups differ from that of the control subjects. The values obtained were in good agreement with published values using this method. In addition, the pre- and post-treatment samples were assayed at the same time using the same batch of substrate and should have revealed any changes when analysed by paired t-test. The activities of HL and LPL in post-heparin plasma were assayed under condition of substrate excess and it cannot be ruled out that this does not reflect substrate availability in vivo. Similarly the availability of apo-CII (LPL activator) and apo-CIII (LPL inhibitor) may modulate in vivo activity that is not reflected in the in vitro activity measured in the presence of excess apo-CII as in this study. Further studies on the concentration of these co-factors in post- heparin plasma from patients pre- and post-treatment may reveal differences not seen in in vitro measurements.
Previous studies have reported an increase in LPL activity after treatment with TZDs (Kageyama et al., 2003; Kletzien et al., 1992; Lefebvre et al., 1997). This is not unexpected since the synthesis of the protein is thought to be one of the targets of PPARγ and also influenced by insulin. The reasons why our result are at variance with previous report could be because of: 1) differences in the severity of lipaemia, 2) differences in the severity of insulin resistance. Our data does not support any relationship between postprandial lipaemia and lipase activity but again an assessment of lipase activity in vivo may well demonstrate a relationship. Further studies are required to resolve this issue.

Unlike LPL, hepatic lipase activity is not known to require the presence of apolipoprotein to regulate activity. Post-heparin plasma samples were assayed as before at the same time and with the same substrate and were found to be significantly higher in the diabetic patients compared with control subjects in agreement with previous reports (Rashid et al., 2003; Tan et al., 2001). No effects of TZDs on reducing the HL activity have been reported in agreement with our results. Indirect evidence is however available from studies on the effect of TZDs treatment on small dense LDL. Reduced concentrations of these particles have been reported with TZDs and HL activity is thought to be a major determinant for the production of small dense LDL. However, since the extent of hypertriglyceridaemia is a critical determinant for the generation of small dense LDL and the patients in this study were only modestly hypertriglyceridaemic with only few showing the atherogenic profile, it is perhaps not surprising that we found no effect of treatment with pioglitazone that are evident in more severely hypertriglyceridaemic patients.
CHAPTER 6
CONCLUDING REMARKS

6.1 PPARγ ACTIONS

Thiazolidinediones are a class of oral anti-diabetic agents that selectively enhance or partially mimic certain actions of insulin. These effects have been documented in a variety of genetic and animal models of insulin resistance, as well as in numerous clinical trials in patients with insulin resistance. Increasing sensitivity to insulin is achieved by modulating the activity of the PPARγ receptor. In the nucleus, PPARγ exists as a heterodimer with the nuclear retinoid X receptor (RXR). The heterodimer binds to PPAR response elements within the promoter regions of target genes. In the unliganded state, the PPARγ-RXR heterodimer is associated with a multiprotein corepressor complex that has histone deacetylase activity. Deacetylated histone keeps the nucleosome in a state in which transcription is inhibited. This histone is important in chromatin remodelling, which facilitates gene transcription (Glass et al., 2000). When ligand binds to the receptor, the co-repressor complex dissociates and a co-activator complex with histone acetylase activity is recruited to the PPAR-RXR heterodimer (Saltiel et al., 1996). The activation of PPARγ by ligands correlates well with the antidiabetic action of TZDs. The physiological role of PPARγ is to maintain an appropriate level of expression of key glucose and liporegulatory molecules, as well as other proteins that are involved in the transduction of the insulin signal, which ultimately facilitates a state of normal insulin sensitivity.

The reversal of insulin resistance with PPARγ activation could be explained by a variety of different mechanisms:
• PPARγ up regulates the synthesis of signalling proteins in mature fat cells both *in vitro* and *in vivo* (Baumann et al., 2000; Ribon et al., 1998) that are linked to the insulin receptor. Tyrosine phosphorylation then initiates a signaling pathway that is essential for insulin-stimulated glucose transport (Baumann et al., 2000).

• Thiazolidinedione treatment has been associated with a decrease in circulating non-esterified fatty acid concentrations (Saltiel et al., 1996). However, it is unclear whether the lowering of NEFA by TZDs represents a direct action on adipocytes or whether this is secondary to a general improvement in insulin sensitivity due to the enhancement of the anti-lipolytic effects of insulin. Given the relatively high level of expression of PPARγ in fat cells and that TZDs are strongly adipogenic, it is possible that the primary actions of TZDs are exerted on fat cells that in someway feedback to stimulate the skeletal muscle and improve insulin action. This could involve a TZD-mediated effect on adipocytes such as decrease in circulating free fatty acid levels, or altered of peptide secretion such as TNF-α (Shibasaki et al., 2003; Marx et al., 2002) and they have been shown to increase adiponectin (Satoh et al., 2003; Stumvoll et al., 2003).

• It has also been proposed that TZDs act directly on skeletal muscle to increase glucose uptake, rather than the effects being secondary to an action on adipocytes. *In vitro* studies have revealed that TZDs directly enhance glucose transport in cultured muscle cells (Vidal-Puig et al., 1997). Studies in transgenic mice in which deficient in adipose tissue have shown that these animals are insulin resistant and treatment with TZDs resulted in a striking
improvement in insulin sensitivity (Burant et al., 1997). This suggests that TZDs act directly on skeletal muscle.

In an attempt to elucidate the role of PPARγ in glucose homeostasis and insulin resistance, the gene encoding PPARγ has been knocked out in mice but complete deletion of both alleles (PPARγ−/−) is not compatible with life and the animals die in utero. However heterozygous knockout mice PPARγ+/−, which lack one PPARγ allele are viable. These PPARγ+/− mice were expected to be insulin resistant and possibly diabetic but, on the contrary, studies on these animals, including oral glucose tolerance tests and euglycaemic clamp, revealed that a 50% reduction in PPARγ expression led to enhanced peripheral and hepatic insulin sensitivity (Barak et al., 1999; Miles et al., 2000). These results have led to the hypothesis that the normal role of PPARγ, through its natural ligands, might be to reduce or dampen insulin action, thereby promoting a state of insulin resistance.

The decreased expression of PPARγ that is seen in these mice would partially alleviate this effect and lead to increase insulin sensitivity. This also implies that inhibiting PPARγ function might render animal less susceptible to the endogenous and exogenous causes of insulin resistance. This finding is supported by studies of a mutation in the human PPARγ gene in which the patient exhibited decreased plasma content of insulin, enhanced insulin sensitivity and had lower body mass index (Deeb et al., 1998). Other reported mutations in the ligand-binding domain of PPARγ have been described in three patients but these patients were insulin resistant and hypertensive (Barroso et al., 1999) presumably because the natural ligand does not have a high affinity for the
mutated receptor. These data provide strong evidence that PPARγ is involved in the control of insulin sensitivity, glucose homeostasis and hypertension – important factors in CHD risk.

It is a paradox that PPARγ expression increases insulin resistance while TZDs ligands for the PPARγ receptor reduce insulin resistance. The concept of TZDs as partial agonist/antagonist has been supported by several studies and it is clear, from experimental studies that there is likely to be considerable heterogeneity of actions within the TZDs class (Olefsky et al., 2000). This depends on whether the compounds are full or partial agonists for the PPARγ receptor. In studies using a prompter/ reporter assay troglitazone behaved as a partial agonist whereas pioglitazone and rosiglitazone behaved as full agonists (Camp et al., 2000). As discussed above, studies in knockout mice heterozygous for PPARγ deficiency surprisingly showed enhanced insulin sensitivity in peripheral tissue and liver. The TZDs may displace endogenous ligands, which presumed to act as pure agonists. Therefore, TZD-induced insulin sensitivity may reflect the ability of these compounds to inhibit the dampening effect on insulin action of endogenous ligands (Olefsky et al., 2000). In addition to differences between TZDs in term of full or partial agonist, differences have been reported in the sets of genes induced or suppressed by troglitazone, pioglitazone and rosiglitazone together with overlapping effects (Camp et al., 2000). For these reasons, each compound is likely to have individual effects as well as common effects of the class. Each TZD has different binding affinity, with different residence times on the receptor. This indicate that the conformation of the TZD-PPARγ complex differ according to which ligand is bound (Fig 27).
A recent study has compared the effects of pioglitazone and rosiglitazone monotherapy and combination therapy on fasting blood lipid levels and HbA1c in patients with type 2 diabetes (Olansky et al., 2003). In a multi-centre retrospective chart review of 1115 records of patients with type 2 diabetes who received pioglitazone or rosiglitazone, alone or in combination with other antidiabetic agents, between August 1, 1999, and August 31, 2000.

The demographic characteristics, co-morbidities, and concomitant drug use were similar in both treatment groups. Of the patients who received pioglitazone, 83% also received anti-hyperglycaemic agents and 59% received some form of anti-hyperlipidaemic therapy. Among those who received rosiglitazone, 81% received concomitant anti-hyperglycaemic medication and 60% received some form of anti-hyperlipidaemic therapy. With pioglitazone, mean concentrations of serum triglyceride, total cholesterol, and LDL-cholesterol decreased and HDL cholesterol increased in most patients, with or without concomitant anti-hyperglycaemic medications. Treatment with rosiglitazone, with or without other antidiabetic agents, decreased plasma triglyceride and HDL cholesterol concentrations, whereas total cholesterol and LDL cholesterol levels increased in most patients. Reductions in HbA1c levels and increases in body weight related to each study drug were comparable. It was concluded that in both monotherapy and combination treatment regimens, pioglitazone was associated with greater beneficial effects on lipids than was rosiglitazone. Similar results suggesting greater beneficial effects of pioglitazone on fasting lipids when compared to rosiglitazone have been observed in other independent comparative studies (Gegick et al., 2001; Khan et al., 2001; King et al., 2000; 2001). Further
dedicated studies comparing the long term effects of both drugs may provide more information on their effectiveness with regard to cardiovascular effect.

Figure 28: A model for TZDs action on PPARγ to explain different effects. Different ligands (1, 2, 3) bind to the ligand-binding domain of PPARγ. Each ligand-receptor complex assumes a different conformation that leads to unique and differential interactions with co-factors, histones, other transcription factors, etc. As a result of these differential interactions, each PPARγ ligand-receptor complex leads to a differential, but overlapping, pattern of gene expression, i.e. each ligand activates (or represses) a certain set of genes, some of which are common to other ligands and some of which are not. Adapted from TEM Vol. 11, No. 9, 2000.
6.2 MAJOR FINDINGS OF THE EFFECTS OF INSULIN SENSITISATION BY PIOGLITAZONE IN THIS STUDY

This study is the first to investigate the effect of pioglitazone on postprandial lipid clearance compared to the effect of sulphonylurea treatment (glibenclamide) in a group of patients with type 2 diabetes in a treatment regimes designed to keep the glycaemic control the same.

The most striking effects found by treatment TZD patients with the insulin sensitizer pioglitazone were evident in:

1) Triglyceride management: a reduction in fasting triglyceride, VLDL and improved postprandial triglyceride clearance.

2) Improved management of intestinal lipoproteins leading to a more rapid clearance of dietary fat.

3) Improved β-cell function with less insulin secreted to deal with the same meal and a reduction in the amount of immature secretory granules released.

Effects On Triglyceridaemia

In this study we set out to examine the effect of insulin sensitization on postprandial lipid metabolism at the same level of glycaemic control. This was achieved as assessed by the fact that there were no significant change in HbA1C by either glibenclamide or pioglitazone treatment. Pioglitazone treatment generally reduces the circulating concentrations of fasting and postprandial triglyceride. This has been attributed to decreased hepatic VLDL synthesis and increased peripheral clearance of remnants particles, together with reduced lipolysis.
That insulin sensitization did not have a marked effect in decreasing insulin-dependent NEFA release from adipose tissue but made a significant improvement in glucose disposal provides some evidence in favour of the theory that thiazolidinediones act directly both on skeletal muscle and adipose tissue. While PPARγ expression in skeletal muscle is only 10% of that in adipose tissue, the larger bulk of muscles could make this a significant target for TZDs. In addition, low affinity binding of TZDs to other PPARs (PPARα and PPARδ) might also occur in muscle and liver resulting in different biological effects to those seen in adipocytes (Forman et al., 1995).

A recent study has suggested that pioglitazone can have PPARα activity (Ikeda et al., 2001) however our results did not support involvement of PPARα since we did not find any changes in the plasma concentrations of apo-AI, known to be under the control of PPARα. Supporting this conclusion is our finding that HDL cholesterol concentrations were also unchanged by treatment with pioglitazone. Previous reports of an effect of pioglitazone on raising HDL levels are thus more likely to occur as a secondary effect arising from overall improvement in insulin resistance leading to improved HDL remodelling processes rather than a direct stimulation of apo-AI synthesis.

A major effect of TZDs is to stimulate adipogenesis (Adams et al., 1997; Hallakou et al., 1997). Such an effect is reported to cause significant weight gain but this was not observed in the patients treated with pioglitazone in the current study (Spiegelman et al., 1998; Tordjman et al., 2003).

In this study pioglitazone significantly lowered circulating triglyceride and showed a trend to reduce NEFA concentrations. This could cause an increase in insulin sensitivity by correcting imbalances in glucose fatty acid cycle (Randle...
cycle). NEFA can impair insulin action and glucose metabolism in several ways. A high NEFA level impairs hepatocyte insulin sensitised function, decreased hepatic insulin uptake and enhances gluconeogenesis (Storlien et al., 1996). In skeletal muscle when NEFA concentrations are high, fatty acids are oxidised by muscle cells in preference to glucose. Finally, NEFA may also influence insulin production since it is known that long term exposure of β-cells to insulin production impairs the insulin secretory response to glucose.

In summary sensitization of the tissue to the action of insulin with pioglitazone modifies fasting and postprandial lipid and lipoprotein metabolism in type 2 diabetes, resulting in reduced fasting plasma triglyceride concentrations and attenuated postprandial lipaemia.

**Management and Clearance of Intestinal Lipoproteins**

Sensitization to insulin improved the clearance of chylomicron and chylomicron remnants. The mechanism by which this occurs is not known but is unlikely to involve effects of glycaemia since glycaemic control was unchanged from the baseline test when delayed clearance of intestinal lipoproteins was evident. The results of this study indicate that lipolysis by LPL is an unlikely factor in the reduced clearance since pioglitazone treatment apparently made no effect on the lipase activity. However, these results must be interpreted with caution because the activity expressed *in vitro* under optimal conditions may not reflect the situation *in vivo* caused either by the supply of regulatory apolipoproteins such as apo-CII and apo-CIII. Further studies in the apolipoprotein complement of chylomicron and chylomicron remnants may well reveal changes in the nature of the particles after sensitization to insulin. By attenuating the magnitude and
duration of postprandial lipaemia, pioglitazone therapy may limit the vascular exposure to a potentially damaging environment.

**Improved β-Cell Function**

Sensitization of peripheral tissue to the action of insulin had a beneficial effect on β-cell function. Less insulin was secreted in response to the post-treatment test meal compared to the baseline test and, in addition, the fasting concentrations was also significantly reduced. While it may be that more effective glucose uptake by peripheral tissues and a reduced hepatic glucose output is responsible for providing a smaller stimulus to the β-cell resulting in a smaller amount of insulin secretion, it can not be ruled out that there is a direct effect of pioglitazone on the β-cell causing it to secrete less insulin. In this study pioglitazone caused an improvement in β-cell function in both a qualitative and quantitative fashion. These results are supported by an earlier trial, the TRIPOD study (Troglitazone in prevention of Diabetes), that showed an improvement of β-cell function after treatment with troglitazone (Buchanan et al, 2002). It was found that chronic administration of troglitazone 400 mg for 30 months reduced the incidence of diabetes by > 50% in high-risk Hispanic women with previous history of gestational diabetes. These subjects had insulin resistance and poor β-cell compensation during pregnancy (Buchanan et al, 1999). The treatment preserved β-cell function, for at least 8 months after the drug was stopped as assessed by oral glucose tolerance test and intravenous glucose tolerance tests. The increase in the whole body tissue insulin sensitivity was most evident in women who showed a large reduction in insulin output. Taken together, these findings provide strong support for the concept that type 2 diabetes results from
progressive β-cell dysfunction that is caused at least in part by high secretory demands.

Insulin sensitization with pioglitazone did not only reduced the amount of insulin secreted but improved the processing of insulin precursor resulting in a reduction of the amounts of proinsulin and 32-33 split proinsulin released both in the fasting state and postprandial after the test meal. This suggests that there has been less pressure on the need to secrete insulin-containing granules or that the proteolytic processing mechanisms are more effective after insulin sensitization.

These beneficial therapeutic effects of pioglitazone treatment described in this thesis may reduce the risk of vascular disease in type 2 diabetic patients. However, this can be only be fully evaluated by further detailed studies and by large, long-term, well-designed intervention trials.

Limitations of the study

This study has certain limitations brought about by the strict entrance criteria which precluded recruiting patients who were receiving statin therapy and the need to bring the study to a conclusion within three years. The result of these restraints led to the study groups of diabetic patients being small and of different ethnic origin. The control subjects however had no family history of type 2 diabetes which otherwise have confounded the interpretation of the data.
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