DIETARY FAT AND INSULIN SENSITIVITY

This thesis has been submitted for the degree of

Doctor of Philosophy

by

Karen Aoife Slevin BSc SRD

at the

School of Biomedical and Health Sciences, University of Surrey,
Guildford, GU2 5XH

September 2000
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ALA</td>
<td>α-Linolenic acid</td>
</tr>
<tr>
<td>ALP</td>
<td>Atherogenic lipoprotein phenotype</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>EGIR</td>
<td>European Group of Insulin Resistance</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FSIVGTT</td>
<td>Frequently sampled intravenous glucose tolerance test</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GI</td>
<td>Glycaemic index</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Glucose transporter-4</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>Homeostatic model assessment</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenously</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin-cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acids</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acids</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRL</td>
<td>Triglyceride-rich lipoproteins</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low-density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

In executing and completing this piece of work I am indebted to many. Firstly I would like to thank my supervisor Professor Joe Millward who made it possible for me to have the opportunity to complete a PhD and guided me throughout its lifetime. In addition, I am extremely grateful to Dr. John Wright who supervised all of the metabolic investigations and never appeared to find any Sunday or early morning an inconvenience – his Irish jokes I can also forgive! In addition, I would like to thank the sponsors of this work – MAFF. Thanks also goes to Dr. Amelia Fereday who provided much guidance as well as Dr. Eric Ah Sing, and Andy Irvine who also worked on this MAFF project and helped with study days and with the analysis of the samples generated. I am also extremely grateful to all those who gave of their time to teach me a variety of laboratory techniques and these include, Dr. Eric Ah Sing, Dr. Caitriona Brooks, Dr. Clare Chapman, Dr. Amelia Fereday, Dr. Syrah Khan, Beatrice Messenger and Dr. Margaret Murphy,

The most important element of this work however is the volunteers that participated and I am especially grateful to all of them who so willingly gave up their spare time and remained patient and interested throughout.

I would also like to thank all of my fellow researchers in the Nutrition laboratory and other laboratories in School of Biological Sciences who helped in any way and made my time in Guildford an enjoyable time. Special mention goes to the following who helped to keep me (reasonably) sane, chatty and happy throughout: Richard Barnes, Sian Clarke, Marion Faughnan, Wendy Hall, Syrah Khan, Dave Ribeiro, Lindsay Mitchell, Paul Wilkinson, Nor-Haizan Mohammed Isa, Rohini Naidoo & Suhur Saeed.

Finally, deep felt thanks goes to my parents and my sister Yvonne and my brother Donall who have given me unquestioning support and encouragement always. Ultimately and without doubt however I would not have been able to complete this work without the very steadfast support of my Fiancée Derek who has sacrificed much in order to make my life easier – I am forever grateful.
ABSTRACT

Insulin resistance is associated with a number of metabolic abnormalities that increase the risk of developing coronary heart disease. Dietary fat has been linked with insulin resistance, with alterations in the quality as opposed to the quantity of dietary fat now thought to be more important in instigating improvements in insulin resistance. The aim of this work was to investigate the effect of alterations in the dietary fat intakes of middle-aged men (n = 32) on the insulin sensitivity of glucose disposal and postprandial lipid metabolism and to explore the mechanistic links between these insulin responsive pathways. Three separate dietary interventions were conducted; the first involved an increase in the intake of n-3 polyunsaturated fat, the second a decrease in saturated fat and an increase in carbohydrate and the third a decrease in saturated fat and an increase in monounsaturated fat intake. Compliance was monitored by the measurement of red blood cell phospholipid fatty acid composition, postprandial lipid metabolism was measured over 9 hours following a high-fat breakfast (80 g fat), and insulin resistance was measured using the short insulin tolerance test. The results of the study showed that while insulin sensitivity was inversely correlated with red blood cell saturated fatty acid concentration at baseline, the insulin sensitivity of glucose disposal was unaffected by any of the dietary interventions conducted. In measurements of postprandial lipaemia, improvements were observed following the low-saturated fat / high-monounsaturated fat diet and the n-3 polyunsaturated enriched diet, however the low-saturated fat/ high-carbohydrate diet was associated with a worsening of postprandial lipaemia through an increase in the concentrations of triglyceride-rich-lipoproteins. Changes in fasting biochemical measurements were most evident in the low-saturated / high-monounsaturated diet, with an 11 % reduction in total cholesterol and a 15.4 % reduction in fasting triglycerides. There were no observed changes in the activity levels or the gene expression of lipoprotein lipase. There was an unexpected positive association between the degree of insulin sensitivity and the extent of postprandial lipaemia, indicating that the link between these pathways is complex and warrants further investigation. Overall this work supports the view that dietary guidelines should be directed towards a change in the composition of fat, to a lower saturated fat intake, a higher monounsaturated fat intake and a lower n-6 : n-3 ratio through an increase in the intake of long chain n-3 polyunsaturated fatty acids.
# TABLE OF CONTENTS

## INTRODUCTION................................................................. 19

### 1.1 INSULIN RESISTANCE ......................................................... 19
#### 1.1.1 DEFINITION OF INSULIN RESISTANCE ....................... 19
#### 1.1.2 THE INSULIN RESISTANCE SYNDROME ....................... 20
#### 1.1.3 RISK FACTORS FOR INSULIN RESISTANCE ................... 21
#### 1.1.4 MEASUREMENT OF INSULIN RESISTANCE ....................... 22
#### 1.1.5 INSULIN RESISTANCE AND LIPOPROTEIN METABOLISM ........ 24
#### 1.1.6 INSULIN RESISTANCE AND PLASMA NEFA LEVELS .............. 27
#### 1.1.7 TREATMENT OF INSULIN RESISTANCE ......................... 28

### 1.2 N-3 FATTY ACIDS ....................................................... 29
#### 1.2.1 SOURCE AND FUNCTION OF N-3 FATTY ACIDS ................. 29
#### 1.2.2 N-3 FATTY ACIDS AND LIPOPROTEIN METABOLISM ............... 32
#### 1.2.3 N-3 FATTY ACIDS AND INSULIN ACTION ......................... 34

### 1.3 SATURATED FATTY ACIDS ............................................. 37
#### 1.3.1 DESCRIPTION OF SATURATED FATTY ACIDS .................... 37
#### 1.3.2 SATURATED FAT AND CHD ........................................... 38
#### 1.3.3 SATURATED FAT AND INSULIN ACTION ......................... 40

### 1.4 MONOUNSATURATED FATTY ACIDS ............................... 42
#### 1.4.1 THE MEDITERRANEAN DIET ......................................... 42
#### 1.4.2 MONOUNSATURATED FAT AND CHD ............................... 43
#### 1.4.3 MONOUNSATURATED FAT AND INSULIN ACTION ................. 46

### 1.5 CARBOHYDRATES AND INSULIN RESISTANCE .................. 46
#### 1.5.1 COMPONENTS OF DIETARY CARBOHYDRATE .................... 47
#### 1.5.2 CARBOHYDRATES AND INSULIN SENSITIVITY ................... 48

### 1.6 LIPOPROTEIN LIPASE .................................................. 49
#### 1.6.1 STRUCTURE AND FUNCTION OF LPL ........................... 49
#### 1.6.2 REGULATION OF LPL ................................................ 50
#### 1.6.3 LPL AND INSULIN RESISTANCE .................................. 51

### 1.7 GENETICS OF INSULIN RESISTANCE ............................. 53
#### 1.7.1 DIET AND GENE INTERACTIONS ................................. 54
1.8 AIMS AND OBJECTIVES OF THE STUDY ..................................................58

CHAPTER 2.0 .......................................................................................... 61

MATERIALS & METHODS ........................................................................ 61

2.1 MATERIALS...................................................................................... 61

2.2 METHODS ........................................................................................ 63

2.2.1 COLLECTION AND TREATMENT OF BLOOD SAMPLES ............. 63

2.2.2 PREPARATION OF BLOOD SAMPLES FOR THE ANALYSIS OF RED
BLOOD CELL FATTY ACIDS .................................................................... 64

2.2.3 AUTOMATED ANALYSIS ON THE COBAS MIRA PLUS .......... 64

2.2.3.1 Measurement of plasma Glucose ................................................. 64

2.2.3.2 Measurement of total plasma cholesterol ................................. 65

2.2.3.3 In-direct measurement of HDL-cholesterol ................................. 65

2.2.3.4 Direct measurement of HDL-cholesterol ................................ 65

2.2.3.5 Measurement of LDL-cholesterol ............................................. 66

2.2.3.6 Measurement of plasma NEFA ............................................... 67

2.2.3.7 Measurement of Plasma Triglyceride ........................................ 67

2.2.3.8 Variability of the Cobas Mira automated spectrophotometric techniques .................................................. 68

2.2.4 SEPARATION OF TRL AND TPL FRACTIONS FROM PLASMA USING
ULTRACENTRIFUGATION .................................................................. 68

2.2.5 MEASUREMENT OF APO-B48 ..................................................... 68

2.2.6 MEASUREMENT OF PLASMA INSULIN CONCENTRATION ....... 69

2.2.7 MEASUREMENT OF POST-HEPARIN PLASMA LIPASE ACTIVITY... 71

2.2.7.1 Preparation of $^3$H-triolein-glycerol emulsion .......................... 72

2.2.7.2 Preparation of Tris buffer .......................................................... 72

2.2.7.3 Preparation of the substrate emulsion ........................................ 72

2.2.7.5 Calculation of Enzyme Activity .............................................. 74

2.2.8 OPTIMISATION OF LPL ASSAY .................................................. 74

2.2.8.1 Collection of post-heparin blood samples ................................. 75

2.2.8.2 Method of preparing substrate emulsion ................................. 77

2.2.8.4 Source of Apo C-II in the LPL assay ........................................ 78

2.3 HUMAN METABOLIC INVESTIGATIONS ......................................... 79
5.6.1 DIETARY INTERVENTION DATA ..........................................................161
5.6.2 RED BLOOD CELL PHOSPHOLIPID DATA ..........................................161
5.6.3 FASTING BIOCHEMICAL DATA .............................................................162
5.6.4 POSTPRANDIAL DATA ......................................................................163
5.6.5 POST –HEPARIN LPL ACTIVITY DATA ................................................167
5.6.6 INSULIN TOLERANCE DATA ..............................................................169
5.7 DISCUSSION ......................................................................................173

CHAPTER 6.0 ............................................................................................177
MEASUREMENT OF LPL GENE EXPRESSION USING A COMPETITIVE
RT-PCR METHOD ......................................................................................177
6.1 BACKGROUND ....................................................................................177
6.2 METHODS ..........................................................................................181
  6.2.1 RNA EXTRACTION .........................................................................181
  6.2.1.1 Total RNA extraction from fresh human adipose tissue .................181
  6.2.1.2 Preparation of human frozen tissue for RNA extraction ...............182
  6.2.1.3 Total RNA Extraction procedure for Frozen Human Adipose Tissue, 182
  6.2.1.4 Measurement of concentration and A260/A280 ratio of total RNA
isolated .............................................................................................................184
  6.2.2 GENERAL RT-PCR METHOD AND CONDITIONS ................................184
  6.2.2.1 PCR primers ..................................................................................186
  6.2.2.2 Internal Standards ..........................................................................186
  6.2.3 SEPARATION AND ANALYSIS OF PCR PRODUCTS .........................187
  6.2.3.1 Agarose gel preparation and gel electrophoresis .............................187
  6.2.3.2 Quantification of PCR products .....................................................188
  6.2.4 STATISTICAL ANALYSIS ..................................................................191
6.3 RESULTS ..............................................................................................191
6.4 DISCUSSION .......................................................................................198

CHAPTER 7.0 ............................................................................................201
OVERALL FINDINGS .....................................................................................201
7.1 STUDY DESIGN ....................................................................................201
  7.1.1 STUDY DESIGN LIMITATIONS .......................................................202
LIST OF TABLES

TABLE 1.1. RISK FACTORS ASSOCIATED WITH INSULIN RESISTANCE .................. 21
TABLE 1.2. BENEFICIAL ACTION OF N-3 FATTY ACIDS IN PREVENTING CHD ........ 38
TABLE 1.3. DESCRIPTION AND SOURCE OF SATURATED FATTY ACIDS ............. 38
TABLE 1.4. BENEFICIAL EFFECTS OF MUFA CONSUMPTION .......................... 43
TABLE 1.5. COMPOSITION OF MONOUNSATURATED-RICH OILS ......................... 45
TABLE 2.1. OUTLINE OF THE INSULIN ASSAY PROCEDURE ...................... 70
TABLE 2.2. OUTLINE OF LPL ASSAY PROCEDURE ........................................ 74
TABLE 2.3. NUTRITIONAL CONTENT OF TEST MEAL ....................................... 81
TABLE 3.1. COMPOSITION OF N-3 ENRICHED AND CONTROL MARGARINE AND COOKING FAT ............................................................... 86
TABLE 3.2. WEIGHT AND BMI OF SUBJECTS DURING THE DIETARY INTERVENTION 91
TABLE 3.3. THE MEAN DAILY MACRONUTRIENT, AND EPA+ DHA INTAKES OF VOLUNTEERS CONSUMING HABITUAL, CONTROL AND N-3 ENRICHED DIETS ......... 93
TABLE 3.4. FATTY ACIDS (% AREA) MEASURED AT BASELINE AND AT THE END OF THE CONTROL AND N-3 ENRICHED DIET PERIODS ...................................................... 95
TABLE 3.5. MEAN FASTING PLASMA CONCENTRATIONS OF TOTAL CHOLESTEROL, HDL CHOLESTEROL, LDL CHOLESTEROL, TRIGLYCERIDE, NEFA, GLUCOSE AND INSULIN OF MALE VOLUNTEERS (N=11) ON THEIR HABITUAL DIET AND AFTER CONSUMING THE CONTROL DIET AND THE N-3 ENRICHED DIET ........................................ 97
TABLE 4.1. BASELINE CHARACTERISTICS OF THE STUDY VOLUNTEERS .......... 123
TABLE 4.2. WEIGHT AND NUTRIENT INTAKES BEFORE AND AFTER LOW SFA INTERVENTION .................................................................................. 127
TABLE 4.3. FASTING CONCENTRATIONS OF PLASMA LIPIDS, GLUCOSE (MMOL/L) AND INSULIN (PMOL/L) PRE, MID AND POST-INTERVENTION ......................... 130
TABLE 4.4. POSTPRANDIAL TRIGLYCERIDE DATA, PRE- AND POST-INTERVENTION 132
TABLE 5.1. BASELINE CHARACTERISTICS OF STUDY VOLUNTEERS ................ 157
TABLE 5.2. FATTY ACID COMPOSITION OF RAPESEED OIL .............................. 158
TABLE 5.3. LEVELS OF FATTY ACIDS IN RED BLOOD CELL PHOSPHOLIPIDS MEASURED PRIOR TO AND AT THE END OF THE MUFA ENRICHED DIET .............................. 162
TABLE 6.1. LPL GENE EXPRESSION EXPRESSED AS NO. OF MOLECULES OF LPL mRNA PER MOLECULE OF β2-MICROGLOBULIN mRNA FOR EACH INTERVENTION STUDY ...... 194
TABLE 7.1. OVERALL FASTING BIOCHEMICAL MEASUREMENTS OBTAINED PRIOR PRE- AND POST-INTERVENTIONS .............................................................. 205
TABLE 7.2. POSTPRANDIAL INSULIN, TG AND APO B-48 AUC FOR EACH INTERVENTION STUDY AND FOR THE COMBINED DATA SET ........................................ 207
TABLE 7.3. ANALYSIS OF QUARTILES OF INCREASING INSULIN SENSITIVITY IN RELATION TO OTHER MEASURES OF INSULIN ACTION ................................... 209
TABLE 7.4. ANALYSIS OF QUARTILES RANKED IN ORDER OF INCREASING INSULIN SENSITIVITY IN RELATION TO MEASURES OF LIPID METABOLISM IN THE PRE-INTERVENTION DATA (N=32) 212
TABLE 7.5. ANALYSIS OF QUARTILES RANKED IN ORDER OF DECREASING INSULIN SENSITIVITY IN RELATION TO MEASURES LPL ACTIVITY AND GENE EXPRESSION (N=32) ........ 213
TABLE OF FIGURES

**Figure 1.1.** Lipoprotein associations of the ALP .............................................................26

**Figure 1.2.** Effects of PUFA on membrane fluidity...........................................................36

**Figure 1.3.** Components of carbohydrate in the diet ..........................................................48

**Figure 2.1.** Comparison of post-heparin LPL activity levels using different methods of heparin administration ..........................................................................................76

**Figure 2.2.** Comparison of LPL activity levels measured in individual subjects using a homogenised emulsion and a sonicated emulsion ......................................................78

**Figure 2.3.** Post-heparin LPL activity of 8 samples assayed with and without serum as the source of apo C-II ........................................................................................................ 79

**Figure 3.1.** Intake of SFA, MUFA and PUFA as a percentage of total fat intake during the habitual, control and N-3 enriched diet .................................................................93

**Figure 3.2.** EPA + DHA levels individual subjects at week 0 and week 4 of the N-3 enriched diet ....................................................................................................................95

**Figure 3.3.** Total TG concentrations following the test meal measured at the end of the control and N-3 enriched diet periods..............................................................98

**Figure 3.4.** Postprandial TG response in individual subjects ..............................................99

**Figure 3.5.** The association between fasting TG concentration measured during the control diet and the change in postprandial TG ......................................................99

**Figure 3.6.** Postprandial concentrations of TG in the TG-rich lipoprotein (TRL) fraction after the consumption of a test meal measured at the end of the control and N-3 enriched intervention periods ........................................ 100

**Figure 3.7.** Apo B-48 concentration in individual subjects as measured by the AUC .................................................................................................................................101

**Figure 3.8.** The association between the change in apo B-48 AUC and the apo B-48 AUC during the control diet ..........................................................................................101

**Figure 3.9.** The concentrations of NEFA following the test meal measured at the end of the control and N-3 enriched diet period..............................................................102

**Figure 3.10.** Postprandial insulin levels, following an 80-g fat meal .................................103

**Figure 3.11.** Change in the glucose and cholesterol total AUC measurements from the control diet to the N-3 enriched diet .................................................................103
Figure 3.12. 5-minute post-heparin LPL activity levels in individual subjects measured at the end of the control diet and the end of the N-3 enriched diet .............................................................................................................. 104

Figure 3.13. 15-minute post-heparin LPL activity levels in individual subjects measured at the end of the control diet and the end of the N-3 enriched diet ........................................................................................................... 105

Figure 3.14. Mean total lipase, HL & LPL activity levels, 15 minutes following the administration of 7500 IU of heparin to 11 subjects. .. 105

Figure 3.15. The association between fasting TG concentration and LPL activity levels measured during the control diet .............................................................. 105

Figure 3.16. Fall in blood glucose concentrations over 15 minutes following the administration of a bolus dose of insulin at the end of the control diet and at the end of the N-3 enriched diet. .................................................. 106

Figure 3.17. Effect of the N-3 enriched diet on insulin sensitivity as measured by glucose KITT in individual subjects .......................................................... 107

Figure 3.18. The association between fasting insulin concentration (pmol/l) and insulin sensitivity measured during the control diet ......................... 108

Figure 3.19. Difference in the fall in log transformed NEFA from the control diet to the N-3 enriched diet. ................................................................. 109

Figure 3.20. The association between levels of hepatic lipase activity and insulin sensitivity measured during the control diet. ............ 110

Figure 3.21. The relationship between the change in insulin sensitivity and the change in postprandial lipaemia measured by the TG AUC (N = 11) as a result of the N-3 enriched diet .................................................. 111

Figure 3.22. The association between measures of insulin sensitivity and postprandial lipaemia during the control diet in the 7 least insulin sensitive subjects ................................................................. 112

Figure 4.1. Relationship between the change in energy intake and the change in weight following the low-SFA / high-CHO diet .................... 127

Figure 4.2. The association between the percentage reduction in total fat intake and the percentage reduction in weight following the low-SFA / high-CHO intake ................................................................. 128

Figure 4.3. Levels of principal red blood cell phospholipid fatty acids measured pre-intervention and at the end of the intervention diet. .... 129
**Figure 4.4.** Correlation between weight (Kg) and red blood cell saturated fatty acids (% area) measured prior to the intervention diet ..................129

**Figure 4.5.** Relationship between fasting insulin concentration and SFA intake measured during the pre-intervention diet .............................................131

**Figure 4.6.** Postprandial TRL TG concentrations following the test meal measured prior to and at the end of the low-SFA / high-CHO diet ......133

**Figure 4.7.** Apo B-48 concentration in individual subjects following the pre-intervention and low-SFA / high-CHO diet. .......................................................134

**Figure 4.8.** NEFA concentrations following the test meal (80-g fat) measured prior to and at the end of the intervention diet .........................135

**Figure 4.9.** Changes in energy intake and the association with change in the NEFA AUC measurements following the low-SFA / high-CHO diet ......135

**Figure 4.10.** Insulin (pmol/l) and glucose (mmol/l) responses, following the test meal prior to and at the end of the intervention period ..........136

**Figure 4.11** The relationship between the intake of saturated fat (% energy) and glucose AUC measurements (mmol/l.min) prior to the intervention diet .............................................................................................................................................137

**Figure 4.12.** LPL activity measured 5 and 15 minutes after heparin administration, prior to and at the end of the intervention diet. ......138

**Figure 4.13.** The relationship between fasting TG concentration and plasma LPL activity following the low-SFA / high-CHO diet. ...............138

**Figure 4.14.** The association between post-heparin LPL activity levels and the % increase in TG AUC following the low-SFA / high-CHO diet ......139

**Figure 4.15.** Mean fall in blood glucose concentrations over 15 minutes following insulin administration. .................................................................140

**Figure 4.16.** Glucose KITT in individual subjects prior to and following the low-SFA / high-CHO diet. .........................................................................................................................141

**Figure 4.17.** The relationship between the reduction in saturated fat intake and the improvement in insulin sensitivity as a result of the intervention diet ..........................................................................................................................142

**Figure 4.18.** The association between the reduction in total fat intake and the change in insulin sensitivity as a result of the intervention diet...142

**Figure 4.19.** Decline in plasma NEFA concentration over 15 minute following a bolus dose of insulin ..........................................................................................................................143
**Figure 4.20.** Individual NEFA $K_{IT}$ levels measured prior to and at the end of the low-SFA / high-CHO dietary intervention. ................................................144

**Figure 4.21.** The relationship between the change in postprandial lipaemia and the change in insulin sensitivity following the low-SFA / high-CHO diet. .........................................................................................................................................144

**Figure 5.1.** Nutritional intake measured during the habitual and intervention diet. ........................................................................................................................................161

**Figure 5.2.** Postprandial triglyceride response following the test meal during the habitual diet and following the low SFA / high MUFA diet. .... ........................................................................................................................................164

**Figure 5.3.** Postprandial TRL TG response following the test meal during the habitual diet and following the low SFA / high MUFA diet. ....165

**Figure 5.4.** Concentrations of apo B-48, measured in individual subjects prior to and at the end of the intervention diet, following the test meal. ..165

**Figure 5.5.** Postprandial NEFA concentrations following the test meal measured prior to and at the end of the low SFA / high MUFA diet. .....166

**Figure 5.6.** Plasma glucose and insulin levels following the test meal measured prior to and at the end of the low SFA / high MUFA diet. .....167

**Figure 5.7.** LPL plasma activity levels in individual subjects, 5 minutes after heparin administration. .................................................................168

**Figure 5.8.** Plasma LPL activity in individual subjects 15 minutes, following the administration of heparin. .................................................................168

**Figure 5.9.** Mean LPL activity in 9 subjects, 5 minutes following the administration of heparin. .................................................................169

**Figure 5.10.** Mean plasma glucose concentrations following the administration of a bolus dose of insulin, measured during the habitual diet and following the low SFA / high MUFA diet. ........................................170

**Figure 5.11.** $K_{IT}$ measurements in individual subjects during the habitual diet and following the low SFA / high MUFA diet. ........................................170

**Figure 5.12.** Mean plasma NEFA levels measured over 15 minutes following the administration of a bolus dose of insulin (0.1U / Kg). .......................171

**Figure 5.13.** Change in mean calculated NEFA $K_{IT}$ following the low SFA / high MUFA diet. .................................................................172
**Figure 5.14.** The relationship between changes in postprandial lipaemia and changes in insulin sensitivity following the low SFA / high MUFA diet...

.........................................................................................................................................172

**Figure 6.1.** Schematic of the reverse-transcription polymerase chain reaction...........................................................................................................................................179

**Figure 6.2.** Conditions of PCR amplification .................................................................................................................................185

**Figure 6.3.** Example of LPL and β2-μglob standard and target products visualised under UV following the PCR reaction.................188

**Figure 6.4.** Example of plot of log of ratio of corrected target to standard band density to log no. of molecules of RNA standard ..........189

**Figure 6.5.** Mean LPL mRNA expression measured in adipose tissue biopsy samples obtained at the end of the control diet and at the end of the fish oil diet (n = 11). ..........................................................192

**Figure 6.6.** Mean LPL mRNA expression measured in adipose tissue biopsy samples obtained prior to and at the end of the low SFA / high CHO diet (n = 11). .................................................................................................192

**Figure 6.7.** Mean LPL mRNA expression measured in adipose tissue biopsy samples obtained prior to and at the end of the low SFA / high MUFA diet (n = 9). ........................................................................................................193

**Figure 6.8.** The association between post-heparin LPL activity levels and the level of LPL gene expression following the low SFA / high MUFA diet .............................................................................................................195

**Figure 6.9.** The relationship between fasting insulin levels and LPL gene expression measured prior to the low SFA / high MUFA diet. ............196

**Figure 6.10.** The association between the change in fasting TG concentration and the change in LPL gene expression following the n-3 PUFA enriched diet .................................................................196

**Figure 6.11.** The association between the change in postprandial TG AUC and the change in LPL gene expression following the n-3 PUFA enriched diet ........................................................................................................197

**Figure 6.12.** The association between the change in postprandial insulin AUC and the change in LPL gene expression following the n-3 PUFA enriched diet ........................................................................................................197
**Figure 7.1.** The correlation between fasting insulin and glucose $K_{ITT}$ in the group overall (N = 32) measured pre-intervention ........................................ 210

**Figure 7.2.** The association between glucose $K_{ITT}$ and the change in glucose $K_{ITT}$ .................................................................................................................. 210

**Figure 7.3.** The relationship between the dietary intake of SFA and the level of SFA in RBC membrane phospholipids in individual subjects measured pre-and post-intervention ................................................................. 214

**Figure 7.4.** The relationship between the dietary intake of MUFA and the level of MUFA in RBC membrane phospholipids in individual subjects measured pre-and post-intervention ................................................................. 214

**Figure 7.5.** The relationship between the dietary intake of PUFA and the level of PUFA in RBC membrane phospholipids in individual subjects measured pre-and post-intervention ................................................................. 215

**Figure 7.6.** The relationship between RBC phospholipid palmitate content and $K_{GLUCOSE}$ .................................................................................................................. 216

**Figure 7.7.** Insulin sensitivity ($K_{GLUCOSE}$) examined in quartiles of increasing RBC n-3 PUFA content (N = 32) ................................................................. 217

**Figure 7.8.** HDL-cholesterol ranked by increasing RBC C18:0 (stearic acid) content. .................................................................................................................. 217

**Figure 7.9.** The relationship between HDL-cholesterol and RBC phospholipid n-3 PUFA content (N = 32). ................................................................. 218

**Figure 7.10.** The relationship between TG AUC and RBC phospholipid n-3 PUFA content (N = 32) ................................................................. 219

**Figure 7.11.** The inverse association between RBC n-3 PUFA content and LPL activity ................................................................. 219
CHAPTER 1.0

INTRODUCTION

1.1 INSULIN RESISTANCE

1.1.1 DEFINITION OF INSULIN RESISTANCE

A composite definition, which describes insulin resistance (IR) in its entirety, has not yet been agreed. What is evident however, is that IR plays a major role in the aetiology of many metabolic disorders. Obesity and type II diabetes are two of the most common morbidities associated with IR, but notably around 25% of the apparently healthy population are also thought to be insulin resistant and therefore considered to be at greater risk of coronary heart disease (CHD) (Reaven, 1993). This has lead to a great deal of interest in increasing our understanding of the mechanisms involved in developing IR and its associated metabolic complications.

In broad terms, IR can be defined as an impaired biological response to either exogenous or endogenous insulin (American Diabetes Association: Consensus Development Conference on Insulin Resistance, 1997). The term insulin insensitivity is used synonymously with IR. Insulin promotes a wide variety of actions in the human body and has effects on lipid, glucose and protein metabolism, vascular endothelial function and gene expression. An impaired biological response to one or more of these could, in theory, be classed as a resistance to insulin. In practice however, most definitions of IR have been confined to parameters of glucose metabolism. The most classic definitions of IR refer to an impairment in the ability of insulin to lower plasma glucose both by stimulating glucose uptake in insulin-sensitive tissues (predominantly skeletal muscle) and by inhibiting hepatic glucose output (Tooke & Hannemann, 2000). As stated by Kruszynska & Olfesky (1996) IR should be understood to represent most fundamentally an impairment in insulin signalling, evoked by an impairment in the binding of insulin to the receptor. Among
the first to characterise IR was Himsworth & Kerr (1939) who noted considerable differences in the glucose response to insulin administered to young lean male diabetics and obese older non-diabetic males.

1.1.2 THE INSULIN RESISTANCE SYNDROME

The ‘insulin resistance syndrome’ is a term that has been used to describe a number of metabolic abnormalities associated with IR including:

- Elevated plasma triglyceride (TG) concentration.
- Decreased plasma high-density lipoprotein (HDL) cholesterol concentration.
- Elevated plasma non-esterified fatty acid (NEFA) concentration.
- Increased small dense low-density lipoprotein (LDL) cholesterol concentration.
- Increased plasminogen activator inhibitor 1 (PAI-1) concentration.
- Hyperuricaemia.
- Impaired vasodilation of the endothelium.
- Hypertension.

Reaven (1988) was the first to describe the insulin resistance syndrome, which he coined ‘Syndrome X’. He postulated that the syndrome was central to the development of CHD and was characterised by glucose intolerance, hyperinsulinaemia, decreased HDL-cholesterol concentration, increased plasma TG and high blood pressure. A World Health Organisation (WHO) expert committee has recently proposed that the insulin resistance syndrome be referred to as ‘The Metabolic Syndrome’ and that its definition should focus mainly on its relationship to cardiovascular disease (Alberti & Zimmet for the WHO consultation group, 1998). Bonora et al. (1998) examined the prevalence of IR in the most common metabolic disorders. They observed that higher rates of IR were found in those with type II diabetes, hypertriglyceridaemia and low levels of HDL-cholesterol (~85 % of subjects), whereas lower prevalence rates were found with hypercholesterolaemia, hyperuricemia and hypertension (30-60 % of subjects). They concluded that IR is extensive when several metabolic abnormalities cluster within the same individual, whereas it is rarer when the metabolic disorders (in particular hypertension and
hyperuricemia) are isolated. These findings highlight the strong association between disorders of lipid metabolism and IR and lend credence to the concept of a metabolic syndrome.

1.1.3 RISK FACTORS FOR INSULIN RESISTANCE

In Reaven's original paper it was acknowledged that while there was evidently a genetic component to the development of IR, environmental influences were also important (Reaven, 1988). In particular he observed that the more obese and sedentary an individual, the greater the degree of IR. Since then a growing number of studies have tried to examine the effect of various environmental factors, including diet, on the development and progression of IR. The risk factors thus far associated with IR are outlined in Table 1.1.

Table 1.1. Risk factors associated with insulin resistance

<table>
<thead>
<tr>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity - in particular central adiposity</td>
</tr>
<tr>
<td>Low levels of physical activity</td>
</tr>
<tr>
<td>High fat diets</td>
</tr>
<tr>
<td>Low birth weight</td>
</tr>
<tr>
<td>Genetic factors</td>
</tr>
</tbody>
</table>


It is not currently known whether IR is the result of obesity, the cause of obesity, or whether the two conditions arise independently from each other (Bessesen, 2000). It is known that the prevalence of IR is greater among the obese, however there are normal weight individuals who are equally insulin resistant (Ruderman et al. 1998). Without question reduction in weight is associated with improvements in insulin sensitivity (Torjesen et al. 1997; Markovic et al. 1998). It is also clear that regular physical activity improves insulin action, although the exact mechanisms involved are not clear. It is thought that exercise effects on insulin action, while related to changes in body composition, may also work independently of decreases in fat mass and increases in muscle mass (DeFronzo et al. 1987). The pioneering work by Barker and
colleagues suggests that low birth weight was associated with many of the features of IR in adult life (Barker et al. 1993). Data are also available to indicate a strong genetic contribution to the determination of insulin action in any given individual (Pedersen, 1999). However, attempts to identify the relevant genes have not been successful to date. It is also difficult to separate genetic factors from environmental influences. Several studies have pointed to the detrimental effects of a high fat intake on IR (Lovejoy & DiGirolamo, 1992; Mayer et al. 1993). However, there is a paucity of human dietary studies that have provided clear evidence that alterations in the intake of dietary macronutrients alter insulin action. A number of studies have focused on the ratio of carbohydrate to fat effects, however greater interest in the types of dietary fat rather than total fat intakes per se has developed (Mann, 2000).

1.1.4 MEASUREMENT OF INSULIN RESISTANCE

Most investigators measure insulin action by its ability to modulate glucose metabolism. A variety of techniques have been employed including, the euglycaemic clamp, the insulin suppression test, the frequently sampled intravenous glucose tolerance test (FSIVGTT) with minimal modelling, the homeostatic model assessment (HOMA) and the insulin tolerance test (ITT). The most widely accepted research method or ‘gold standard’ is the euglycaemic insulin clamp technique originally developed by DeFronzo et al. (1979). With this procedure, exogenous insulin is infused, to maintain a constant plasma insulin level (above fasting), while glucose is fixed at basal level by infusing glucose at variable rates. The total amount of glucose infused over time (M value) is an index of insulin action on glucose metabolism. The more glucose that has to be infused per unit time, the more sensitive the individual is to insulin. Conversely, the insulin resistant patient requires much less glucose to maintain basal plasma glucose levels. This technique has generated the largest amount of reliable information regarding IR. However, the clamp technique has a number of limitations, not least the complexity and cost of the procedure, which limits its use as a clinical diagnostic tool.

A more practical method of measuring IR, which could be applied to a larger population, is the ITT. The ITT involves intravenous administration of a bolus dose of
insulin, generally calculated on the basis of body weight, with the subsequent rate of decline in blood glucose taken as a measure of insulin sensitivity. Two major disadvantages to the ITT have been proposed. The first is that pharmacological doses of insulin are used and therefore there is a threat of insulin-induced hypoglycaemia (Del Prato, 1999). Secondly, counter-regulatory hormones are secreted in response to hypoglycaemia blunting the fall in plasma glucose (Harrison & King-Roach, 1976). However, as pointed out by Akinmokun et al. (1992), following intravenous insulin injection, blood glucose reaches its lowest point by 20 minutes and counter regulatory hormone response occurs only after this time. The ITT has been found to compare favourably with other measures of IR including the euglycaemic clamp (Akinmokun et al. 1992) and in practice most investigators have found the method to be both safe and reliable (Chen, 1998). Several studies have examined the reproducibility of the test, with the intra-subject coefficient of variation (CV) varying from 6 to 13 % among the studies conducted (Hirst et al. 1993). In contrast however, a recent study by Hermans et al. (1999) concluded that the short ITT performs less well against other methods of measuring insulin sensitivity including the FSIVGTT, the HOMA and the 2-h continuous infusion of glucose with model assessment due to it's poor reproducibility however, there was no comparison with more complicated measures of insulin sensitivity including the euglycaemic clamp technique. The majority of the studies conducted thus far (with the exception of the study of Hermans and colleagues) have concluded that the test is adequately reproducible in normal subjects and recommended its use particularly in large-scale epidemiological studies.

Studies involving the measurement of IR have highlighted the fact that there is a great deal of variation in insulin sensitivity within population groups. Hollenbeck & Reaven (1987) demonstrated a wide range of insulin sensitivities in 100 individuals with normal glucose tolerance. On the basis of rates of glucose uptake in response to insulin, subjects were divided into quartiles. They observed that those falling into the lowest quartile had insulin sensitivities as low as those observed in the most insulin resistant category of patients with type II diabetes. In the database collected by the European Group of Insulin Resistance (EGIR), approximately 1500 euglycaemic hyperinsulinaemic clamp studies in non-diabetics were performed across 9 European countries. A similar pattern was observed and even in the lean segment of the cohort
Body mass index (BMI) < 25, insulin sensitivity was found to vary over a wide range (Beck-Nielsen, 1999). Ultimately it is the response to IR and the accompanying metabolic abnormalities that determines the impact of the defect and the question remains as to whether measurement of IR is a definitive marker with which to predict future development of diseases such as diabetes and CHD.

From a clinical perspective, the most practical way of assessing IR would seem to be the measurement of insulin concentration in the plasma. However debate exists as to whether fasting plasma insulin levels are a useful predictor of IR. In the EGIR, Beck-Nielsen (1999) found that the degree of IR was positively correlated with fasting plasma insulin levels. One prospective study conducted by Haffner et al. (1992) showed that elevated fasting insulin concentrations were predictive of multiple metabolic disorders. Eight years following the measurement of fasting insulin, those individuals occupying the upper quartile were 5.6 times more likely to develop type II diabetes, 3.5 times more likely to develop hypertriglyceridaemia, 1.6 times more likely to develop low levels of HDL-cholesterol, and 2 times more likely to develop hypertension than those in the lower quartile. In addition, as pointed out by Reaven (1999) insulin-resistant individuals can only remain glucose tolerant if the pancreas is able to respond to this defect by secreting large amounts of insulin. Type II diabetes will inevitably develop when insulin-resistant individuals can no longer sustain the compensatory state of hyperinsulinemia. It is important to remember that fasting hyperinsulinaemia is not synonymous with IR, however its measurement may be of value in risk factor analysis (Jensen, 2000). Therefore, while fasting insulin level alone is an inadequate method of measuring IR it may prove useful in the predicting the development of IR related abnormalities such as type II diabetes and CHD. Genetic phenotyping, combined with measures of fasting insulin may prove to be more predictive of IR.

1.1.5 INSULIN RESISTANCE AND LIPOPROTEIN METABOLISM

The relationship between lipids and IR has been intensively investigated. It is presumed that the association between CHD risk and IR is to a large extent mediated via its effects on lipoprotein metabolism. However, other components of the
metabolic syndrome including raised PAI-1 levels, alterations in endothelial function and hypertension also contribute to the atherosclerotic risk (Festa et al. 1999). In recent years the description of the Atherogenic Lipoprotein Phenotype (ALP) has aided our understanding of the link between IR and lipoprotein metabolism. Central to the ALP is a moderately raised fasting TG level. Hypertriglyceridaemia has also been consistently found to correlate with measures of IR, with much weaker associations for hypercholesterolaemia. The traditional characteristics of the ALP include a normal to marginally raised cholesterol level, a TG level above 2.0 mmol/l and a HDL-cholesterol level below 1.0 mmol/l (Packard, 1996). The lipoprotein profile described in the ALP has been shown to be associated with a preponderance of small dense LDL- cholesterol (LDL subclass III, particle size < 26 nm, density > 1.04 g/ml), an increase in very-low-density lipoprotein (VLDL), TG (particle size > 35 nm, density < 1.006 g/ml) and an increase in small HDL₃ (particle size < 8.8 nm) as outlined in fig 1.1. More recently it has been recognised that increased LDL particle number as well as postprandial TG metabolism is important in the portrayal of the ALP (Griffin, 1999). Small dense LDL are unfavourable in terms of CHD risk and they have been shown to be as present in 40-50 % of all patients with CHD (Griffin, 1999). The correlation between CHD incidence and small dense LDL is explained by the fact that small dense LDL is highly atherogenic. Small dense LDL exhibit a reduced binding to the LDL receptor and have a greater susceptibility to oxidation. Reduced receptor-binding leads to an increase in the residence time of small dense LDL in serum, allowing greater filtration across the endothelium. Oxidised LDL is more easily taken up by macrophages to create foam cells (Parthasarathy et al. 1989), thereby aiding in the formation of an atheromatous plaque.
Atherogenic VLDL & chylomicron remnants

TG > 2.0 mmol/l

ALP

HDL < 1.0 mmol/l

Small non-protective HDL

Small dense LDL

Figure 1.1. Lipoprotein associations of the ALP

It has been found that the measurement of LDL particle size and distribution is more predictive of CHD risk than measurement of serum total cholesterol alone and the key determinant to the generation of small dense LDL is the TG concentration (Austin et al. 1988). A fasting serum TG value of 1.5 mmol/l is thought to represent a threshold value, beyond which LDL becomes small and dense (Griffin, 1999). The importance of TG in the generation of atherogenic small dense LDL is most clearly understood by recognising the postprandial metabolic effects of a raised TG. At serum TG values > 1.5 mmol/l, VLDL synthesized in the liver, become enriched with TG and are secreted into the circulation as larger lighter particles known as VLDL. In the postprandial state, VLDL is a weak competitor (with chylomicrons) for removal from the plasma by lipoprotein lipase (LPL) resulting in a greater residence time of VLDL in plasma. The residence time of chylomicrons in the blood is normally about 5 min, whereas that of VLDL-TG is several fold longer (Havel, 1997). Lipoproteins are continually exchanging their lipid components, catalysed by cholesterol ester transfer protein (CETP). The accumulation of VLDL leads to a greater transfer of TG to LDL and HDL. The TG-enriched LDL and HDL act as good substrates for hepatic lipase (HL) resulting in the formation of small dense LDL and small dense HDL. VLDL is typically over-produced in insulin resistant states such as obesity and type II diabetes. It is obvious therefore that treatment strategies must aim to reduce the TG level in
states of IR in order to reduce the risk of CHD. Reduction in TG concentration can lead to a more favourable shift in the LDL profile of an individual, leading to the generation of larger and denser LDL particles, which are less of an atherogenic threat (Griffin, 1999). While reductions in TG can lead to an improvement in CHD risk, the question remains as to what leads to an increased TG concentration in IR in the first instance?

1.1.6 INSULIN RESISTANCE AND PLASMA NEFA LEVELS

It has been suggested that the link between hypertriglyceridaemia and IR is mediated by a defect in the suppression of NEFA. NEFA, also known as free fatty acids (FFA), are the form in which stored body fat is transported from adipose tissue to its sites of utilization (Frayn, 1998). Plasma NEFA concentrations are normally at their highest after an overnight fast or during aerobic exercise, as it is at these times that the body needs to draw upon its stored fuel. Conversely plasma NEFA concentrations are suppressed after a meal. Insulin is the hormone responsible for suppressing plasma NEFA levels. Suppression of plasma NEFA allows free glucose utilization by peripheral tissues, removes an important stimulus for hepatic glucose production, and likewise removes the major stimulus for hepatic VLDL-TG secretion (Frayn, 1998). Failure to suppress NEFA concentrations after a meal, will result in impaired glucose utilization at a time when it should be stimulated to increase glucose clearance, will stimulate hepatic glucose production at a time when it should be suppressed, and will drive hepatic VLDL-TG secretion so that VLDL will be competing with chylomicrons for peripheral clearance by lipoprotein lipase (LPL).

Higher fasting and postprandial NEFA concentrations are associated with IR (Frayn, 1993). It has been shown that IR within adipose tissue causes failure of NEFA suppression to occur in subjects resistant to insulin’s glucoregulatory action. Randle et al. (1963) suggest that an increase in NEFA supply, by inhibiting glucose uptake and oxidation in muscle cells, causes IR by substrate competition. In other words NEFA will compete with glucose for utilization by insulin-sensitive tissues such as skeletal muscle, leading to impaired glucose utilization. Interaction between NEFAs and glucose at a hepatic level is another proposed mechanism, as an increase in the
delivery rate of NEFAs to the liver leads to stimulation of gluconeogenesis (Girard, 1995; Saloranta & Groop, 1996). It has also been suggested by several investigators that the link between IR and central adiposity (or visceral fat) could be explained by an increase in the liberation of NEFA from intra-abdominal adipose into the portal vein (Arner, 1997; Frayn, 1998). An increased supply of NEFA in the portal vein leads directly to an increased supply of NEFA in the liver. The association is strengthened by the fact that intra-abdominal adipose tissue seems to be more lipolytically active than subcutaneous adipose tissue. The idea that IR, or at least many of its deleterious features, could arise from visceral adipose tissue has attracted much attention and has been called the 'Portal Theory' (Arner, 1997). However, at present it is no more than a theory and as recently discussed by Frayn (2000) there is a lack of evidence in vivo to support the theory. He suggests that an alternative link between visceral adiposity and IR is that both are associated with subcutaneous adipose tissue accumulation. The greater the subcutaneous depot the greater the potential for release of NEFA and subsequently the development of IR as a result of greater competition with glucose. A ‘toxic’ effect of high NEFA concentration on pancreatic cells has also been implicated as contributing to the development of IR however this has not been confirmed (Unger, 1995). It is clear that inappropriately elevated NEFA levels are central to the metabolic syndrome, however further work is needed to understand more about the mechanistic links between increases in NEFA concentration, TG metabolism, IR and the development of CHD.

1.1.7 TREATMENT OF INSULIN RESISTANCE

The treatment of the metabolic syndrome has to be targeted at improving insulin sensitivity and correcting or preventing the associated metabolic and cardiovascular abnormalities. Weight reduction and increased exercise improve insulin sensitivity and have been shown to delay the onset of impaired glucose tolerance to diabetes (Pan et al. 1997). Most of the individuals affected by the metabolic syndrome are overweight and there is almost universal agreement that weight loss and regular exercise should form the cornerstone of the treatment of the metabolic syndrome (Riccardi & Rivellese 2000). Weight loss in the region of 5-10% is sufficient, in most instances, to induce a clinically relevant effect (Weinstock et al. 1998). However,
sustained weight loss is notoriously difficult to achieve and few interventions have proved successful in the long term (Kendall et al. 1991). Additional treatments could focus on the finding that altering the composition of the diet can influence insulin sensitivity, independent of weight loss. In particular, the specific effects of the components of dietary fat are of great interest. There are few dietary components other than dietary fat, which have been shown to influence insulin sensitivity, and for most of these the available evidence is inconclusive. In short there are some indications that alcohol might be beneficial if consumed in limited amounts and detrimental when the intake exceeds 30g/day (Facchini et al. 1994; Kiechl et al. 1996). Also, it has been suggested that a very high sodium intake might impair insulin sensitivity (Donovan et al. 1993). In the following sections, the effect of various dietary components including, n-3 fatty acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and carbohydrate (CHO) on IR and its related metabolic abnormalities including CHD and postprandial lipoaemia will be discussed. Also discussed is the way in which alterations in dietary fat intake can influence the membrane fatty acid composition of various tissues, thereby influencing insulin action.

1.2 N-3 FATTY ACIDS

1.2.1 SOURCE AND FUNCTION OF N-3 FATTY ACIDS

A vast amount of literature, studying the importance of n-3 fatty acids in human nutrition, has been assembled over the last 30-40 years. n-3 fatty acids are essential fatty acids, necessary from conception, which are derived from both marine and plant sources. They are so named because the first of the several double bonds occur three carbons away from the terminal end of the carbon chain. The two major n-3 fatty acids provided by marine sources are eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3). α-Linolenic acid (ALA, 18:3ω3) is derived from plant sources and although present in small amounts in unhydrogenated soybean oil and rapeseed oil it is most abundantly found in flaxseed and perilla seed oils (Harris, 1997). ALA is a metabolic precursor to EPA and DHA. N-3 fatty acids may
be consumed in gram quantities in foods (predominantly fish) or in larger quantities in fish oil capsules. The reason why \( n-3 \) fatty acids have attracted so much attention is for their role in the prevention and modulation of certain disorders including:

- CHD and stroke
- Rheumatoid arthritis
- Autoimmune disorders e.g. lupus and nephropathy
- Cancers of the breast, colon and prostate
- Mild hypertension
- Crohns disease
- Essential fatty acid deficiency in infancy (retinal and brain development)

(Connor, 2000)

The greatest amount of work has focused on the cardiovascular effects of the ingestion of \( n-3 \) fatty acids. The beneficial effects of \( n-3 \) fatty acids in modulating CHD risk first became apparent in epidemiological studies of Greenland Eskimos, who despite having a high intake of dietary fat, had a low incidence of CHD. The majority of fat consumed by the Eskimos was polyunsaturated in nature, rich in EPA & DHA, the main sources of which were fish, shellfish and whale meat. Compared to Danish matched subjects, Eskimos were found to have lower levels of total cholesterol and TG and higher levels of HDL-cholesterol (Bang et al. 1971). Other epidemiological studies seemed to support the suggestion that fish consumption was associated with a lower incidence of CHD (Norell et al. 1986; Kromhout et al. 1995; Stone, 1996). In the Seven Countries study there was an inverse relation between CHD and fish consumption (Keys, 1980). Protective effects of fish consumption were also observed in the Zutphen study, where men who consumed on average 30g of fish daily had a 50 % decrease in coronary mortality compared with men who did not include fish in their diet (Kromhout et al. 1985). Subsequently, it has been found that dietary \( n-3 \) fatty acids have cardio-protective actions through a variety of mechanisms as outlined in table 1.2.
Table 1.2. *Beneficial actions of n-3 fatty acids in preventing CHD*

| Prevent ventricular arrhythmia and cardiac arrest |
| Hypolipidaemic properties with effects on plasma VLDL and TG concentrations |
| Antithrombotic effects – n-3 fatty acids inhibit synthesis of thromboxane A2 |
| Inhibit atherosclerosis |
| Promote nitric oxide induced endothelial relaxation |
| Prostaglandin and leukotriene precursors |

Adapted from Connor & Connor (1997)

In animal models and in tissue culture studies n-3 fatty acids have been shown to prevent the development of ventricular fibrillation (Charnock, 1994; Leaf & Kang, 1996). Results from the recent physicians health study in the United States showed that the consumption of one or more fish meals a week was associated with 52% lower risk of sudden cardiac death compared with consumption of < 1 fish meal per week (Albert *et al.* 1998). In the Diet and Reinfarction Trial (DART), overall mortality was decreased by 29% in men with a positive history of CHD who consumed n-3 fatty acids from fish or fish oil, presumably because of the reduction in cardiac arrests (Burr *et al.* 1989). Recently, the results of the GISSI-prevention trial showed a clear reduction in fatal and non-fatal cardiovascular events in men who received fish oil supplementation following myocardial infarction compared with those receiving vitamin E supplements (Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocardio, 1999).

Thrombosis is a major complication of CHD that can lead to myocardial infarction. n-3 fatty acids have been shown to have strong anti-thrombotic properties. Thromboxane A2 is a prostaglandin that causes platelet aggregation and vasoconstriction in platelets. EPA has been shown to inhibit the synthesis of Thromboxane A2 from arachadonic acid in platelets thereby causing an increase bleeding time and a decrease the stickiness of the platelets for aggregation (Goodnight *et al.* 1981). In addition, the ingestion of fish oil enhances the production of prostacyclin, a prostaglandin that produces vasodilation and less sticky platelets (Goodnight *et al.* 1981). In the original Eskimo studies, Dyerberg & Bang (1979) also showed that n-3 fatty acids were incorporated into the phospholipids of the Eskimos'
platelet membranes leading to a prolonged bleeding time and decreased *in vitro* platelet aggregation.

The role of fish oils in the prevention and retardation of atherosclerosis has been reviewed by Israel & Gorlin (1992). They deliberate that EPA and DHA contained in fish oil produce a number of effects that inhibit the development of atherosclerosis, with evidence derived from animal studies, *in vitro* studies, epidemiological studies and some human clinical studies. Atherosclerosis is a term used to describe the thickening (atherosclerosis) and reduction in the elasticity (sclerosis) of vessel walls in the arteries of the heart (Nettleton, 1995). Evidence suggests that leukotrienes contribute to the acute inflammatory response produced by vascular injury and therefore aid in the formation of atheromatous plaques (Mullane *et al.* 1987). Fish oils exert beneficial effects on leukotriene metabolism by suppressing the formation of leukotriene B4, a powerful chemoattractant for neutrophils and monocytes. Furthermore, *n*-3 fatty acids may affect the synthesis of growth factors by platelets, endothelial cells and monocytes, including platelet-derived growth factor, interleukin-1 and tumour necrosis factor (TNF) (Fox & DiCorleto, 1991). The suppression of these growth factors by fish oil is thought to reduce atherosclerotic plaque formation. In addition, nitric oxide (NO) acts to enhance endothelial vasodilation and fish oil has been shown to stimulate the production of NO (Chaeet *et al.* 1994). Animal studies have so far provided the most direct evidence that fish oil reduces atherosclerosis with the most notable results in pigs and monkeys (Weiner *et al.* 1986; Davis *et al.* 1987). In summary, *n*-3 fatty acids act to halt or reverse many of the deleterious processes associated with the development and progression of atherosclerosis and CHD. Another coronary-protective mechanism of *n*-3 fatty acids relates to their effects on lipoprotein metabolism.

### 1.2.1 N-3 FATTY ACIDS AND LIPOPROTEIN METABOLISM

The effect of *n*-3 fatty acids on lipoprotein metabolism has been investigated in normal healthy individuals, those with CHD, those with type II diabetes and in those with a family history of CHD. The most remarkable finding is that *n*-3 fatty acids have potent hypotriglyceridaemic properties and as little as 3 g (1 % energy) can
reduce serum TG levels by 30 % (Harris, 1997). There is evidence also of a dose response relationship (Blonk et al. 1990). The minimum effective dose of n-3 fatty acids appears to be a little over 1 g/d. In a review by Harris (1997) the results of a total of 36 placebo-controlled studies were combined. The data showed that a daily intake of approximately 4 g of n-3 fatty acids, TG concentrations decreased by 25 % in normal subjects and by 34 % in hypertriglyceridaemic (> 2 mmol/l) subjects. High TG concentrations are now widely recognised as an independent risk factor for CHD, however postprandial concentrations of TG have also been shown to be important in modulating CHD risk (Patsch et al. 1992). In addition to their effects on fasting TG n-3 fatty acids have also been shown to reduce postprandial lipaemia (Harris et al. 1988; Weintraub et al.1988). Fish oils are thought to affect lipoprotein metabolism through a variety of mechanisms including:

- A reduction in VLDL-TG secretion as demonstrated by kinetic studies in humans and animals (Harris et al. 1990).
- An increase in VLDL apolipoprotein B secretion leading to an impairment in VLDL assembly (Nestel et al. 1984).
- Reduced TG transport, resulting in smaller VLDL, largely converted to LDL (Harris et al. 1990).
- An increase in VLDL clearance or in chylomicron clearance due to reduced competition with VLDL (Harris et al. 1990).

It is also suggested that reduced TG synthesis following fish oil feeding could reflect a reduction in NEFA availability (Dagnelie et al. 1994). Studies have shown that fish oil may lead to reduction in fatty acid synthesis and fish oil also diverts fatty acids to phospholipids (Nestel, 2000).

With regard to the effect on other lipoproteins, supplementation with n-3 fatty acids has in some instances lead to an increase in LDL-cholesterol concentration, particularly in hypertriglyceridaemic individuals (Harris, 1989). This may be due to a reduction in LDL removal mediated via a defect in the LDL receptor, although this has not been confirmed. HDL-cholesterol is essentially unaffected by n-3 fatty acid treatment (Harris, 1997) however some reports indicate a favourable effect of fish oil in increasing the number of large cholesterol rich HDLs. Serum total-cholesterol
levels are generally not reduced by n-3 fatty acid supplementation (Wilt et al. 1989). While the effect of n-3 fatty acids on lipoprotein metabolism has been studied extensively further research is needed to clarify the associated mechanisms of action (Harris, 1997; Toft et al. 1997). Issues that need to be resolved include:

1) Whether the TG lowering effect of n-3 fatty acids can be ascribed more to EPA or DHA? To date no trials comparing the effect of EPA to DHA have been conducted.

2) The effect of n-3 fatty acids on other lipoprotein classes such as intermediate density lipoproteins (IDL), VLDL, HDL, and LDL subclasses.

3) There is a suggestion that LDL rich in n-3 fatty acids is more easily oxidised. In vivo studies are needed to explore this issue.

4) What is the exact role of n-3 fatty acids in the primary prevention of CHD?

In summary n-3 fatty acids exert a variety of effects that are beneficial in modulating the risk of CHD however further studies are needed to explore all of the associated mechanisms of action.

1.2.3 N-3 FATTY ACIDS AND INSULIN ACTION

Interest in the effects of n-3 fatty acids on insulin action are centred on the observation that n-3 fatty acids can induce changes in the fatty acid composition of membranes, particularly within skeletal muscle. Skeletal muscle is the principal site of insulin-mediated glucose disposal therefore, the fatty acid composition of membranes is one cellular factor that may influence the action of insulin within skeletal muscle. Increasing the content of n-3 fatty acids within cell membranes in cultured cells and in animal models has been shown to increases membrane fluidity, the number of insulin receptors and the action of insulin (McCaleb et al. 1981; Ginsberg et al. 1982; Gould et al. 1982). Animal studies have also demonstrated that IR induced by high fat feeding can be prevented by the inclusion of n-3 fatty acids in the diet, shown to have been incorporated into the phospholipid component of muscle cells (Storlein et al. 1991). Further evidence comes from human studies and the investigations of Borkman et al. (1993) showed that a high concentration of long
chain n-3 PUFA in the skeletal-muscle phospholipids was associated with an increased insulin sensitivity. Data from Baur et al. (1998) also showed that the degree of IR in human subjects was negatively correlated to the amount of DHA within skeletal muscle phospholipid. The way in which PUFA may induce changes in membrane fluidity has been carefully described by Malasanos & Stacpoole (1991) and is depicted in figure 1.2. The improvement in glucose uptake that occurs when membranes are enriched with long chain PUFA could be due to the fact that glucose transporter-4 (GLUT-4) protein resides within the plasma membrane for a prolonged period of time (Clandinin et al. 1993). The increase in residence time of GLUT-4 is associated with an enlargement of the intracellular pool of glucose-6-phosphate (Podolin et al. 1998). Clarke (2000) hypothesises that the enlargement of the glucose-6-phosphate pool could potentially explain the increase in skeletal muscle glycogen synthesis that is observed in rats fed fish oil. Dietary fish oils have also been shown to reduce the intra-muscular content of triglyceride (Storlein et al. 1991). A decrease in lipid droplet size and number has been found to be associated with an improved insulin sensitivity, presumably due to the fact that fish oils induce skeletal muscle fatty acid oxidation (Power & Newsholme, 1997; Baillie et al. 1999).
Enrichment of membrane phospholipids with PUFA leads to increased fluidity because the irregular shape of PUFA keeps phospholipids from packing tightly against each other. The increase in fluidity allows membrane proteins (A, B, and C) to move more readily, thus potentially changing rates of signal transduction, hormone-receptor interaction or substrate transport.
Despite the intense interest in $n$-3 fatty acids relatively few studies have examined the effect of $n$-3 fatty acids on insulin sensitivity. Animal studies have convincingly shown that supplementation with $n$-3 fatty acids enhances insulin sensitivity (Behme, 1996; Luo et al. 1996). Podolin et al. (1998) also showed that as little as 6 % fish oil in the diets of rats prevented the development of IR. Such convincing evidence is yet to emerge from human studies. To date, few have measured insulin sensitivity directly but several have examined the effect of $n$-3 fatty acids on glucose and insulin levels (Zak et al. 1989; Fasching et al. 1991). In addition, the studies have tended to concentrate on the effect of $n$-3 fatty acids in those with type II diabetes. There is a lack of evidence therefore of the effect of fish oil feeding on insulin sensitivity in normal healthy individuals. Of the studies that have been conducted several have demonstrated an improvement in insulin sensitivity with $n$-3 fatty acid ingestion (Popp-snijders et al. 1987) with others showing either no response (Pelikanova et al. 1993) or a worsening of insulin sensitivity (Stacpoole et al. 1989). It is evident that the effect of $n$-3 fatty acid supplementation on insulin sensitivity has not yet been determined and further studies are imperative.

1.3 SATURATED FATTY ACIDS

1.3.1 DESCRIPTION OF SATURATED FATTY ACIDS

SFA contain the maximum hydrogen atoms that they can hold. A fatty acid becomes unsaturated when a pair of hydrogen atoms is removed, thereby creating a double bond between the adjacent carbon atoms. Saturated fatty acids are associated mainly with animal foods such as meat and dairy products. They are also abundantly found in tropical oils such as coconut, palm and palm kernel oil. Saturated fats vary in their constituent fatty acids. Individual SFA vary in carbon chain length and the various types of SFAs found in foods are shown in table 1.3. The average daily consumption of SFA in the UK is approximately 16 % of energy and expert bodies have advised that this level is reduced to 10 % of dietary energy (Department of Health, 1994).
Table 1.3. *Description and source of saturated fatty acids.*

<table>
<thead>
<tr>
<th>Common name</th>
<th>Systematic name</th>
<th>Carbon atoms</th>
<th>Melting point (°C)</th>
<th>Typical fat source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric</td>
<td>Butanoic</td>
<td>4:0</td>
<td>-7.9</td>
<td>Butterfat</td>
</tr>
<tr>
<td>Caproic</td>
<td>Hexanoic</td>
<td>6:0</td>
<td>-3.4</td>
<td>Butterfat</td>
</tr>
<tr>
<td>Caprylic</td>
<td>Octanoic</td>
<td>8:0</td>
<td>16.7</td>
<td>Coconut oil</td>
</tr>
<tr>
<td>Capric</td>
<td>Decanoic</td>
<td>10:0</td>
<td>31.6</td>
<td>Coconut oil</td>
</tr>
<tr>
<td>Lauric</td>
<td>Dodecanoic</td>
<td>12:0</td>
<td>44.2</td>
<td>Coconut oil</td>
</tr>
<tr>
<td>Myristic</td>
<td>Tetradecanoic</td>
<td>14:0</td>
<td>54.4</td>
<td>Butterfat, coconut oil</td>
</tr>
<tr>
<td>Palmitic</td>
<td>Hexadecanoic</td>
<td>16:0</td>
<td>62.9</td>
<td>Most fats and oils</td>
</tr>
<tr>
<td>Stearic</td>
<td>Octadecanoic</td>
<td>18:0</td>
<td>69.6</td>
<td>Most fats and oils</td>
</tr>
<tr>
<td>Arachidic</td>
<td>Eicosanoic</td>
<td>20:0</td>
<td>75.4</td>
<td>Peanut oil</td>
</tr>
<tr>
<td>Behenic</td>
<td>Docosanoic</td>
<td>22:0</td>
<td>80.0</td>
<td>Peanut oil</td>
</tr>
</tbody>
</table>

Adapted from Dupont *et al.* (1991)

1.3.2 SATURATED FAT AND CHD

The effects of consuming saturated fats on levels of plasma lipids and lipoproteins and on the incidence of CHD have been extensively studied since the 1950's. Interest in the total fat content and SFA composition of the diet in the pathogenesis of atherosclerosis originated from the observed decline in mortality from CHD that occurred during periods when the consumption of milk, butter, cheese and eggs fell such as a during the rationing of World War II (Malmros, 1970). The Seven Countries study was one of the first epidemiological studies that attempted to relate the incidence of CHD to SFA intake. In that study it was found that there was a high correlation between the average percentage of energy from SFA and the 10-year incidence of, and mortality from CHD (Keys, 1980).
It is thought that the association between the level of SFA in the diet and atherosclerosis is mediated by an increase in circulating total and LDL-cholesterol levels. In the Seven Countries study there was a significant correlation between percentage of calories from SFA and median serum cholesterol. Of the individual fatty acids it was observed that myristic acid (14:0) had a potent hypercholesterolaemic effect, with stearic acid (18:0) neutral and palmitic acid (16:0) intermediate. This confirmed the findings of a previous study where it was shown that SFA with 12-14 carbon atoms in chain length had slightly greater serum cholesterol-raising effects, than SFA with 16-18 carbon atoms (Grande et al. 1961). The principal SFA in most human diets is palmitic acid, followed by stearic, myristic and lauric acids (12:0). The view that palmitic acid may have neutral or intermediate cholesterol-raising properties has been contested by the results of several well-controlled studies in which palmitic acid was shown to raise LDL cholesterol relative to the effects of oleic acid (Denke & Grundy, 1992; Zock et al. 1994). It is thought that individual SFAs, notably lauric, myristic and palmitic acids, raise total and LDL-cholesterol by decreasing LDL-receptor-mediated clearance as a result of a down-regulation of LDL receptor activity (Dietschy et al. 1993).

The postprandial effects of SFA have also been investigated. In a study by Weintraub et al. (1988) it was shown that postprandial levels of lipoproteins in addition to fasting lipoprotein levels were raised following a diet (25 days duration) consisting of 67 % SFA, 28 % MUFA and 5 % PUFA as fat. Shepherd et al. (1980) examined the composition of LDL-cholesterol particles following high SFA feeding and found that the relative proportion of cholesterol increased and the relative percentage of phospholipid decreased in the LDL particle with the high SFA diet.

Several primary and secondary drug trials have demonstrated the clear benefit of plasma cholesterol reduction in both the prevention and treatment of CHD (Scandinavian Simvastatin Survival Group, 1994; Shepherd et al. 1995). It is therefore not disputed that reduction in total and LDL-cholesterol has beneficial effects on CHD mortality. Nor is it disputed that individual SFAs have the ability to raise serum cholesterol concentration and several studies have shown that the development and progression of CHD may be related to the intake of SFA (Watts,
What is lacking however is the evidence from long-term clinical primary and secondary intervention trials that a reduction in total and SFA results in a reduction in CHD mortality. Oliver (1997) reviewed the outcomes of several large trials where total and saturated fats were reduced. He concluded that controlled clinical trials of the primary prevention of CHD (six trials in total) showed no significant reduction in either all-cause or CHD mortality. Exceptions were in trials in which the polyunsaturated to saturated ratio was increased to 1.5 (Turpeinen et al. 1979) or where current cigarette smoking was almost halved (Hjermann et al. 1981).

There have been only two small trials of the secondary prevention of CHD with diets low in SFA and cholesterol alone (Research Committee to the Medical Research Council, 1965; Woodhill et al. 1978). In neither trial was there a significant effect on the rate of recurrence of CHD. In contrast in secondary prevention trials where SFA has been replaced by unsaturated fats, CHD and to a lesser extent all-cause mortality have been reduced (Dayton & Pearce, 1969; Leren, 1970; Burr et al. 1989; Singh et al. 1992; de Lorgeril et al. 1994). There is evidence to suggest therefore that it is more beneficial to change the proportion of dietary fat so that the amount of unsaturated fat is increased than to decrease the amount of saturated fat alone. Keys et al. (1984) and Hegsted et al. (1965) have developed predictive equations based on the results of a variety of feeding studies in order to predict serum cholesterol responses to changes in the saturated and polyunsaturated components of the diet. The equations have since been employed in a wide range of studies. However, the use of standard equations to estimate effects of SFA must be cushioned by the realisation that there is considerable variation in response. Some people appear to be hyper-responders, whereas others are hypo-responders to SFA (Denke, 1994). In addition, the absolute response may depend on an individual's baseline cholesterol concentration (Hayes & Khosla, 1992). Possible explanations for hyper- and hypo-cholesterol responses to a reduction in SFA intake are discussed in greater detail in section 1.7.1.

1.3.3 SATURATED FAT AND INSULIN ACTION

There is considerable evidence from experimental animal studies that SFA in the diet may lead to IR. It has been reported that IR is greater in rat adipocytes obtained from animals with diets high in SFA compared with those from animals with diets rich in
MUFA- or PUFA (Ip et al. 1976; Olefsky, 1978; van Amelsvoort et al. 1988). Fewer insulin receptors have also been found in animals fed a high SFA diet (Nagy et al. 1990). Work by Clandinin et al. (1993) demonstrated that in rats, in which the SFA content of the diet has been reduced, adipocyte insulin binding is significantly increased. Several mechanisms for the adverse effect of high SFA feeding have been suggested including, down regulation of the insulin receptor in muscle and adipose tissue (Hedestkov et al. 1992), a decrease in the expression of glucose transporters (GLUT 4) in fat cells (Pedersen et al. 1991) and reduced oxidative glucose disposal (Grundldeger & Thenen, 1982).

In man there is indirect evidence for the same effect i.e. that a higher SFA intake is associated with impaired insulin action (Maron et al. 1991; Marshall et al. 1997) however, since the classic study of Himsworth (1935) in which it was concluded that high fat feeding induced IR, human studies have produced conflicting reports as to the effect of dietary fat on insulin sensitivity. Earlier studies seemed to confirm the finding that individuals fed a high-fat diet had lower insulin action compared to those consuming a low-fat diet. Subsequently, as methods for measuring insulin action improved several studies showed no effect of high fat feeding on insulin sensitivity in subjects both with and without diabetes (Nestel et al. 1984; Borkman et al. 1991). A study by Riccardi & Parillo (1992) also showed that insulin sensitivity was higher on a high-fat diet (38% energy) compared to a diet high in CHO and dietary fibre in a group of subjects with diabetes. In contrast however, Lovejoy & DiGirolamo (1992) showed that insulin sensitivity was inversely associated with total fat and SFA intake and positively associated with fibre intake in a group of lean and obese men and women. Similarly Marshall et al. (1997) found that habitual consumption of SFA and total fat was positively related to hyperinsulinaemia in a sample of 1069 Hispanic and non-Hispanic white persons living in Southern Colorado. Intervention studies have proved less conclusive and when the degree of saturation of fatty acids is increased or decreased, the effect on insulin sensitivity is equivocal (Fasching et al. 1996). Also contested is the effect of replacing SFA with either and unsaturated fat or CHO on insulin sensitivity. Fukagawa et al. (1990) demonstrated that high-CHO, high-fibre diets improve glucose disposal rates in young individuals with a stronger effect observed by Chen et al. (1988) in older subjects but with much higher levels of CHO.
feeding. In contrast, Ginsberg and colleagues reported no difference in substituting either CHO or MUFA for SFA on postprandial glucose and insulin excursions (Ginsberg et al. 1994). However, other studies have shown that replacing SFA with either MUFA- or PUFA improves insulin sensitivity to a greater extent than high-CHO feeding (Riccardi & Parillo, 1992; Garg, 1998). These issues are explored in greater detail in both chapters 4.0 and 5.0.

1.4 MONOUNSATURATED FATTY ACIDS

1.4.1 THE MEDITERRANEAN DIET

The ‘Mediterranean diet’ has received a great deal of attention since the 1950’s and is principally a term used to represent the food patterns of those living in Greece and Southern Italy. The evidence for the beneficial effect of consuming diets based on the Mediterranean pattern of eating were founded on the observation that adult life expectancies for populations in these areas was among the highest in the world, and rates of CHD and certain cancers were among the lowest. This was despite a high incidence of smoking among this population group and also an inferior health care system compared to that available in Northern Europe or in the US (Kushi et al. 1995). In general the Mediterranean diet can be described by the following characteristics.

- An abundance of fresh fruit and vegetables.
- Considerable consumption of breads, cereals, beans, nuts and seeds.
- Dairy products consumed in low to moderate amounts.
- Red meat consumed in low amounts.
- Red wine consumed in low to moderate amounts, normally with meals.
- Olive oil as the principal source of fat.
Of the many dietary patterns that prevail in the Mediterranean region, the consumption of olive oil occupies a central position in all of them. The greatest amount of research has therefore focused on the beneficial effects of olive oil consumption. Olive oil contains a large proportion of monounsaturated fat, is relatively low in saturated fat and is a source of the antioxidant vitamin E (Ascherio & Willett, 1995). Monounsaturated fats are characterised by having one double bond in their fatty acid carbon chain. The most common monounsaturated fatty acid is oleic acid (18:1ω9). Its single double bond is located nine carbons from the methyl end of the fatty acid chain giving rise to an n-9 fatty acid. The main sources of oleic acid apart from olive oil are rapeseed oil, peanut oil and high-oleic containing sunflower and safflower oil. Other food sources include avocados, meat, rice bran, soybeans and olives as well as processed foods such as margarine’s and shortenings. The reasons why monounsaturated fats are thought to be beneficial in the prevention of CHD are summarised in table 1.4.

Table 1.4. Beneficial effects of MUFA consumption

<table>
<thead>
<tr>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid is considered to be antithrombotic compared with SFA.</td>
</tr>
<tr>
<td>Diets rich in MUFA are less likely than diets high in PUFA to cause oxidation of LDL.</td>
</tr>
<tr>
<td>MUFA can cause HDL-cholesterol levels to increase, when substituted for CHO.</td>
</tr>
<tr>
<td>Evidence of TG lowering properties.</td>
</tr>
<tr>
<td>MUFA have been shown to lower total cholesterol concentrations.</td>
</tr>
<tr>
<td>Olive oil facilitates the consumption of large amounts of vegetables by enhancing taste.</td>
</tr>
</tbody>
</table>

(Ascherio & Willett, 1995)

1.4.2 MONOUNSATURATED FAT AND CHD

The Seven Countries Study initially ascribed the lower incidence of CHD to a lower intake of SFA in the Mediterranean region, however it is now clear that the higher intake of MUFA is a more likely explanation for the reduction in CHD mortality. MUFA have been shown to be effective in lowering both total cholesterol and LDL-
cholesterol concentration (Mensink, 1994) as well as TG concentrations (Garg et al. 1992). MUFA are also thought to have antithrombotic effects (Storlein et al. 1997). Blades & Garg (1995) investigated the mechanisms of TG lowering with a high MUFA diet in patients with type II diabetes and reported that TG lowering was not due to increased lipolysis or postprandial clearance of triglyceride-rich-lipoproteins (TRL) but was primarily due to a reduced hepatic secretion of VLDL-TG. Further studies are needed to explore the proposed mechanisms of TG-lowering with high MUFA diets.

There has been some controversy as to whether MUFA is as effective at lowering cholesterol as PUFA. Increasing the PUFA content of diets has been consistently shown to decrease total and LDL-cholesterol levels (Beynen & Katan, 1985). In addition, long chain n-3 PUFA have clear hypotriglyceridemic properties. Linoleic acid is the predominant fatty acid of the PUFAs. Recent analysis of available data suggests that linoleic acid lowers total and LDL-cholesterol concentrations slightly more than does oleic acid but not as much as was previously reported (Grundy, 1997). There is therefore, negligible difference in the cholesterol lowering effects of MUFAs and PUFAs (Nydhall et al. 1994), however there are greater concerns about increasing PUFAs than MUFAs above current amounts. Chiefly, no populations have consumed large amounts of linoleic acid with any proven safety. This contrasts with high intakes of oleic acid in the Mediterranean region, where people have used olive oil for hundreds of years with no evidence of harm. The main cause for concern is that high intakes of linoleic acid are associated with an increased susceptibility of LDL to oxidation, a process thought to increase the risk of atherogenesis and CHD (Schwab et al. 1998). Also in laboratory animals', high intakes of linoleic acid can promote chemical carcinogenesis (Rose, 1997); the same has not been found with oleic acid. Finally, limited epidemiological data further suggest that high PUFA consumption can increase the risk for human cancer although no firm conclusions have been reached (Zock & Katan, 1998).

It is also thought that among the MUFA-rich oils there are some differences in their cholesterol lowering properties (Truswell & Choudhury, 1998). Animal studies have addressed this question and in a study of hamsters, rapeseed oil was found to be as
effective at lowering cholesterol concentration as sunflower oil, whereas olive oil failed to produce a cholesterol lowering effect (Trautwein et al. 1997). Another animal study using guinea pigs also demonstrated that rapeseed and olive oil differed in their effect on plasma total and LDL cholesterol (Fernandez et al. 1997). A number of human studies have also indicated that rapeseed oil (Valsta et al. 1992) as well as high-oleic sunflower oil (Perez-Jimenez et al. 1995) caused lower plasma cholesterol concentrations than olive oil. This finding is further endorsed by the observation that not all studies using an olive oil intervention have had effects on serum cholesterol concentration. The differing cholesterol-lowering effects of monounsaturated oils could be related to differences in their overall fatty acid composition. As shown in table 1.5, rapeseed oil, high-oleic sunflower and high-oleic safflower oil contain only 7-8 % SFA as compared to 14 % in olive oil. In addition rapeseed oil contains a relatively high amount of ALA, which could also contribute to its cholesterol-lowering potential. Differences in nonsaponifiable lipids within the MUFA-rich oils e.g. the squalene and phytosterol content, may also account for their diverse effects on plasma cholesterol. Squalene is a precursor to cholesterol synthesis while phytosterols can reduce cholesterol absorption (Westrate & Meijer, 1998). Olive oil contains more squalene, while rapeseed oil and oleic-rich sunflower oils have higher concentrations of phytosterols than olive oil (Truswell & Choudhury, 1997).

Table 1.5. Composition of monounsaturated-rich oils.

<table>
<thead>
<tr>
<th></th>
<th>Olive oil</th>
<th>Rapeseed oil</th>
<th>High-oleic sunflower oil</th>
<th>High-oleic safflower oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1 cis (% total FAs)</td>
<td>76</td>
<td>63</td>
<td>80</td>
<td>73</td>
</tr>
<tr>
<td>SFA (% total FAs)</td>
<td>14</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>PUFA (% total FAs)</td>
<td>10</td>
<td>30</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Squalene (mg/100g)</td>
<td>500</td>
<td>Small amount</td>
<td>30</td>
<td>Small amount</td>
</tr>
<tr>
<td>Phytosterols (mg/100g)</td>
<td>110</td>
<td>250</td>
<td>350</td>
<td>440</td>
</tr>
</tbody>
</table>

Adapted from Truswell & Choudhury (1997)
Few studies have examined the effects of increasing MUFA intakes on insulin sensitivity. Several studies have reported improvements in the metabolic abnormalities associated with IR such as raised TG level and LDL-cholesterol concentration, however only a small number have measured insulin sensitivity directly. Furthermore, the studies that have been conducted have concentrated on those with type II diabetes or impaired glucose tolerance. Therefore, a consensus has not been reached as to the effect of high-MUFA feeding on insulin sensitivity with some studies reporting no effects (Garg et al. 1992b) and other studies reporting an improvement in insulin mediated glucose disposal with consumption of a high-MUFA fat diet (Parillo et al. 1992). These studies involved a small number of subjects and were essentially designed to compare the effects of high-carbohydrate diets with high-MUFA diets on insulin sensitivity. Neither study supported the notion that high-CHO diets improve insulin sensitivity and in the study of Parillo and colleagues superior improvements in insulin mediated glucose disposal was reported with the high-MUFA diet. It is evident therefore that more investigations are required to confirm whether high-MUFA diets improve insulin sensitivity in those who are insulin resistant.

1.5 CARBOHYDRATES AND INSULIN RESISTANCE

The cornerstone of many national and international dietary guidelines is a reduction in dietary fat and an increase in dietary CHO. The main reason why low-fat, high-CHO diets are recommended is because of their cholesterol lowering effects and in the case of diabetes because of improvements in glycaemia. Coupled with this low-fat, high-CHO diets are thought to promote weight loss, although long term studies have generally reported minor reductions in weight which are not easily maintained (Katan, 1997). The disadvantages of replacing dietary fat with CHO is that a high-CHO diet may increase plasma TG and decrease HDL-cholesterol concentrations. Numerous studies have confirmed the finding that high-CHO feeding induces
hypertriglyceridaemia (Ginsberg et al. 1976; Coulston et al. 1983; Borkman et al. 1991; Katan, 1997; Parks et al. 1999). Several studies have also demonstrated the detrimental effects of high-CHO feeding in the postprandial state (Garg et al. 1988; Garg et al. 1994; Chen et al. 1995). An increased TG and a decreased HDL-cholesterol concentration are central to IR, and treatment with a high-CHO diet could potentially exacerbate the condition rather than affect an improvement. Garg (1998) has recently completed a meta-analysis of all studies comparing a high-CHO and a high-MUFA diet in diabetics and concluded that high-MUFA diets are preferable in the treatment of IR. High-CHO diets have also been shown to be associated with an increase of PAI-1 levels, thereby causing a decrease in fibrinolysis (Lopez-Segura et al. 1996). Despite this there is a reluctance to promote high-unsaturated fat diets over low-fat, high-CHO diets.

1.5.1 COMPONENTS OF DIETARY CARBOHYDRATE

Measurement of carbohydrate and its components is more difficult than measurement of fat, as carbohydrate in the diet is not a single entity. This may account for the discrepancy in the results of studies, which have examined the effects of CHO feeding on IR. There is increasing awareness that the examination of total carbohydrate is not sufficiently accurate to detect relations between CHO components and disease. Figure 1.4. outlines the components of CHO in the diet. Sugars, dietary fibre and starch have been the major focus of studies in which CHO has been examined. Interest in the ‘sugars’ group has tended to focus on sucrose, glucose, and fructose. Studies in humans suggest that moderate amounts of sucrose and fructose have no deleterious effect on insulin resistance (Daly et al. 1997).
Figure 1.3. Components of carbohydrate in the diet

The terms ‘available’ and ‘unavailable’ CHO relate to the availability for digestion. Adapted from Stephen et al. (1995)

1.5.2 CARBOHYDRATES AND INSULIN SENSITIVITY

There is discrepancy among the results of dietary trials that have attempted to address the question of whether replacement of dietary fat with CHO has a beneficial effect on insulin sensitivity. The studies in non-diabetic subjects generally show beneficial effects of a high-carbohydrate diet on insulin sensitivity (Chen et al. 1988; Fukagawa et al. 1990; Borkman et al. 1991), while those in diabetics show a deleterious effect (Parillo et al. 1992; Garg et al. 1992a). The new classification of carbohydrate rich foods based on their glycaemic index (GI) however, has aided our understanding of the specific effects of CHO on insulin sensitivity. GI represents the plasma glucose
response to a food, expressed as a percentage of the glucose response to a reference food. A growing number of studies have confirmed the finding that the detrimental effects of a high-CHO diet on plasma glucose, insulin, and lipoproteins occur only when CHO foods with a high GI index are consumed, while they are abolished if the diet is based largely on fibre-rich, low-GI foods (Riccardi & Rivellese, 1991; Frost et al. 1994; Rivellese et al. 1994; Jarvi et al. 1999). Classification of foods using GI have more predictive physiological effects than classifications based on the fibre content of CHO-rich foods (Riccardi & Rivellese, 2000). Further work needs to be conducted before a firm conclusion can be drawn as to the optimal amount and type of dietary CHO for the treatment of IR.

1.6 LIPOPROTEIN LIPASE

1.6.1 STRUCTURE AND FUNCTION OF LPL

LPL is an insulin-sensitive hormone, which plays a central role in the regulation of lipid metabolism in the body. LPL is expressed in many human tissues including, adipose tissue (white and brown), skeletal muscle, and cardiac muscle and in the mammary glands (Bensadoun, 1991). Other members of the lipase family include pancreatic lipase and HL and all three enzymes have a number of structural features in common including their lipid-binding regions (Wion et al. 1987). Comparison of the coding sequences of the LPL, HL and pancreatic lipase genes indicate that each originated from a common ancestral gene (Raisonnier et al. 1992). Lipases are involved in the hydrolysis of endogenous and exogenous TG molecules. LPL is responsible for the hydrolysis of TG in the circulation, while pancreatic lipase acts principally in the small intestine. The site of action of LPL is on the surface of the capillary endothelium, however LPL is not synthesised by the endothelial cells but within the parenchymal cells of tissues such as adipose tissue (Olivecrona et al. 1987). LPL is dependent on apolipoprotein C-II (Apo C-II) for its activation. Heparin administered intravenously (IV) causes the rapid release of LPL into the surrounding fluid, which stimulates synthesis of the lipase (Quinn et al. 1983). The exact mechanism of how heparin affects the release of LPL is unclear. In the endothelium,
LPL is bound by heparan sulfate proteoglycans integrated into the glycocalyx on the plasma membrane (Olivecrona et al. 1987). The enzyme is found near the luminal end of the proteoglycan complex where it can best bind and hydrolyze circulating TG. Binding to heparan sulfate is thought to stabilise the enzyme but does not activate it (Eckel, 1989) In the circulation TG are carried within chylomicrons or VLDL particles, collectively known as TRL. The presence of LPL on the capillary endothelium allows the TG in the TRL particles to be hydrolysed into FFA and glycerol. FFA are either esterified for storage in adipose tissue or oxidised for fuel as in skeletal tissue. It has been shown that about forty LPL molecules may act on a TRL particle simultaneously to achieve the rates of TG hydrolysis observed (Scow et al. 1977). The lipolysis mediated by LPL also promotes the exchange of lipids between lipoproteins.

The synthesis and secretion of LPL has been studied most extensively in adipose tissue. As observed by Frayn et al. (1995) not all of the fatty acids released by LPL are directed into adipose tissue for esterification and storage; a proportion leaves in the venous plasma as NEFA. The generation and inhibition of NEFA is part of a co-ordinated system between LPL and hormone-sensitive lipase (HSL) both of which are effected by circulating insulin levels. Acylation stimulating protein (ASP), a potent stimulator of fatty acid uptake and esterification in adipocytes is another component of this highly co-ordinated system for the regulation of the deposition of dietary fatty acids (Sniderman et al. 1997). Thus, while LPL is important in the regulation of fatty acid metabolism it is only part of a highly co-ordinated pathway.

1.6.2 REGULATION OF LPL

The regulation and expression of LPL has been investigated in a range of in vivo and in vitro models. It has been found that the extent of LPL expression in different tissues is dictated by the metabolic state of the tissue. In the fed state LPL is activated in adipose tissue and down-regulated in skeletal muscle and heart, thereby allowing fatty acids to be directed to adipose tissue for re-esterification and storage in a time of energy surplus. In contrast in the fasting state LPL is up-regulated in muscle tissue and suppressed in adipose tissue so that fatty acids are directed to the tissue in which
they are needed as an oxidative fuel (Enerbäck & Gimble, 1993). The highest levels of tissue LPL activity are seen in the mammary tissue of the lactating female (Kinnunen et al. 1980). The LPL gene has been observed at the molecular biology level in a number of species including, chicken, bovine, guinea pig, rat and human (Raisonnier et al. 1995). In each of these species, the complementary deoxyribonucleic acid (cDNA) has been sequenced and the amino acid sequence described (Raisonnier et al. 1995). The gene is highly conserved across species. The human genomic LPL gene spans approximately 30Kb and contains 10 exons coding for a 475 amino acid protein. The gene for LPL has been located to the short arm of chromosome 8 (Sparkes et al. 1987). Studies in cultured rat adipocytes show that increases in LPL-specific messenger ribonucleic acid (mRNA) precede increases in the activity of LPL (Dani et al. 1990). Insulin is known to increase the synthesis of LPL but exactly how insulin increase the level of mRNA is unclear (Ranganathan et al. 1995). It is also unclear whether the rapid effect of insulin on mRNA levels is due to changes in gene transcription, to an enhancement of the stability of LPL mRNA or both (Ranganathan et al. 1995). It is thought that the effect of insulin on adipocyte LPL levels is mediated at the post-transcriptional level (Enerbäck & Gimble, 1993).

1.6.3 LPL AND INSULIN RESISTANCE

Since LPL is central to TG metabolism, decreases in LPL activity will inevitably contribute to hypertriglyceridaemia. There are a number of conditions in which a decrease in the level of post-heparin plasma or tissue LPL activity can lead to a defective catabolism of TRL in humans (Taskinen & Kuusi, 1987). These conditions include, diabetes mellitus, hypothyroidism, chronic renal failure, and nephrotic syndrome. Excessive alcohol ingestion is also associated with decreases in LPL activity. Obesity is associated with increased levels of LPL, mainly due to an increase in the number of fat cells. Both genetic and acquired deficiencies of LPL activity are associated with low levels of HDL-cholesterol (Karpe et al. 1993). This suggests that the low HDL levels associated with CHD and IR may be in part due to LPL deficiency. In the presence of hypertriglyceridaemia, the reduction in HDL-cholesterol could represent a reduction in the number of HDL particles (i.e. fewer HDLs generated by the lipase reaction), a change in HDL composition such as TG
enrichment to generate small dense HDL₃, or both. Low levels of LPL in post-heparin plasma and adipose tissue have been found in both type I and type II diabetes. In general the more severe the insulin deficiency or resistance, the greater the likelihood that decreases in LPL contribute to the associated hypertriglyceridaemia, as opposed to increases in VLDL synthesis (Taylor et al. 1980; Levy et al. 1990). The association between plasma lipid levels and LPL activity has been investigated in several studies. In a recent study by Henderson et al. (1999) levels of post-heparin LPL were found to be lower in a group of patients with existing CHD compared with normolipidaemic control subjects. Furthermore they were able to demonstrate that post-heparin LPL activity in male CHD patients was inversely correlated with fasting TG level and positively correlated with HDL-cholesterol levels. The apolipoprotein (apo) E genotype was found to be of little significance in determining the relationship between post-heparin LPL levels and lipid levels in this group of subjects.

Levels of LPL increase with exercise, certain drugs, or with insulin treatment in the case of type I diabetes, and lead to a reduction in TG and an increase in HDL-cholesterol. Of interest also is the effect that alterations in diet composition, particularly dietary fat, can have on LPL activity and expression. Studies of LPL activity after an oral high fat load have shown that LPL activity is increased in response to an increase in chylomicron TG levels (Karpe et al. 1993). Animal studies have shown that meal fatty acid composition effects on postprandial lipaemia may depend in part on differences in chylomicron size, with PUFA producing chylomicrons of larger size, which appear to act as better substrates for the LPL enzyme (Groot et al. 1988; Levy et al. 1991). Animal studies have also suggested that PUFA and MUFA can enhance the activity of LPL and thereby improve TG clearance. Several animal studies have demonstrated clearly that increases in the intake of MUFA or PUFA increase post-heparin LPL activity (Groot et al. 1988; van Heek & Zilversmit, 1990) adipose tissue LPL activity (Levy et al. 1991; Murphy et al. 1993) or skeletal muscle LPL activity (Baltzell et al. 1991). A study by Murphy et al. (1993) has also demonstrated an increase in LPL gene expression in rats fed a fish oil enriched diet. In general, human studies have failed to demonstrate any relationship between alterations in dietary fat composition and LPL activity. One study (Zampelas et al. 1994) showed an increase in post-heparin LPL activity nine
hours following a meal enriched with n-3 PUFA, however studies in which n-3 PUFA were fed as part of the background diet failed to show any effect (Harris et al. 1988; Weintraub et al. 1988; Nozaki et al. 1991; Harris & Muzio, 1993). Williams (1997) deliberates that the reason for the lack of effect observed in these studies may be due to the non-specific measurement of post-heparin LPL activity i.e. LPL measurements were not specific to either adipose tissue or skeletal muscle tissue. Further studies are required to examine the relationship between changes in lipoprotein levels as a result of alterations in dietary fat intake and LPL activity and expression.

1.7 GENETICS OF INSULIN RESISTANCE

IR has a definite genetic component and is known to cluster in families. For example, 45% of first-degree relatives of patients with type II diabetes are insulin resistant compared with 20% of people without a family history of diabetes (Groop & Tuomi, 1997). Abdominal obesity, which is strongly associated with IR, also has a genetic component. Variations in abdominal fat accumulation have been attributed to genetic factors (up to 60%) in post-menopausal women. Furthermore, first-degree relatives of patients with type II diabetes have increased waist to hip ratio compared with their spouse without a family history of diabetes (Groop et al. 1996). This suggests that the inheritance of type II diabetes seems to favour fat accumulation in the intra-abdominal region. Among the genes thought to contribute to the metabolic syndrome, genes regulating lipolysis and thermogenesis remain as prime candidates. The ‘thrifty gene’ hypothesis was put forward by Neel (1962) as an explanation as to why the metabolic syndrome develops in individuals switching from a harsh rural environment to a plentiful urban environment. The hypothesis was that within an austere environment individuals would increase their potential of surviving if they could maximise storage of surplus energy. When exposed to a more plentiful environment these evolved genes could lead to obesity and potentially IR. However the search for ‘thrifty’ genes has yielded disappointing results. Other genes which have been studied in relation to IR include, leptin, β2-and β3-adrenergic receptors, lipases including LPL, uncoupling proteins, TNF-α, peroxisome proliferator-activated receptor γ (PPAR γ), glycoprotein
PC-1, insulin receptor substrate-1 (IRS-1), and glycogen synthase (Groop, 2000). So far our knowledge of the role of these candidate genes and their interaction with environmental factors is limited. Further studies, perhaps involving families with a clearly identifiable genetic risk of IR are required.

1.7.1 DIET AND GENE INTERACTIONS

Among the environmental influences that are thought to enhance the genetic risk of developing IR is diet. It is known that nutrients govern the tissue content of different proteins by functioning as regulators of gene transcription, nuclear RNA processing, mRNA stability and mRNA degradation (Clarke, 2000). The nutrient, which has a strong influence on cell differentiation, growth and metabolism, is fat. The fatty acid component of dietary lipid not only influences hormonal signalling events by modifying membrane lipid composition but fatty acids have a direct influence on the molecular events governing gene expression (Clarke et al. 1997). It is believed that the regulation of gene expression by dietary fats has an impact on the development of IR and obesity. In a review by Clarke (2000) it is summarised that dietary n-3 PUFA may exert greater benefit in the prevention and treatment of IR than MUFA and n-6 PUFA. The author attributes this to a genomic influence at the level of gene transcription. It is proposed that n-3 PUFA inhibit lipid synthesis and decrease malonyl-CoA production by suppressing the expression of lipogenic genes. Concomitantly, n-3 PUFA induce the genes of lipid oxidation and induce thermogenesis by increasing the expression of uncoupling proteins.

It has also become evident that the relationship between responses to dietary manipulations and lipoprotein levels is influenced at least in some part by genetic factors (Williams et al. 1995; Ordovas & Schaefer, 2000). It is important to consider this when interpreting the results of dietary intervention studies. Other factors such as age, gender and baseline lipid levels will influence an individual's response to a dietary change however gene-environment interactions are thought to play a significant role. To date studies have focused on genes known to play a central role in lipoprotein metabolism. The candidate genes include, apo E, apo A1, apo A4, apo B, apo C3, LPL and CETP (Ordovas & Schaefer, 2000). The apo A, apo E and apo C
genes are located on chromosome 11 and are closely linked (Bruns et al. 1984). APOB is found on chromosome 2 (Law et al. 1985), LPL on the short arm of chromosome 8 (Oka et al. 1990), while CETP has been located on chromosome 16, adjacent to the lecithin-cholesterol acyl transferase (LCAT) gene (Ordovas & Schaefer, 2000). Apo A and apo E genes have secured the greatest attention and they have been examined under a variety of different experimental conditions.

Apo A1 is the major protein of HDL, the major activator of the enzyme LCAT and constitutes a key component of the reverse cholesterol transport process (Reichl & Miller, 1989). A common variant due to adenine (A) to guanine (G) transition has been described from the apo A1 gene transcription site. It has been reported that individuals with the A allele, have HDL-cholesterol levels that are higher than those homozygous for the most common G allele (Pagani et al. 1990; Jeenah et al. 1990). Several studies have demonstrated that the A allele appears to be associated with a hyper HDL-response to changes in the amount and saturation of dietary fat in normolipidaemic subjects (Lopez-Miranda et al. 1994; Mata et al. 1998). Genetically determined isoforms of apo A4 have also been detected in human subjects. The two most common isoforms are apo A4*1 and apo A4*2 (Menzel et al. 1990; de Knijff et al. 1992). The effect these isoforms on dietary response has been examined by several investigators. Studies by Mata et al. (1994) have shown that the apo A4*2 allele is associated with hypo-responsiveness of LDL-cholesterol to dietary therapy consisting of reductions in total fat and cholesterol. In addition, it was observed that subjects with the apo A4*2 allele tended to have greater decreases in HDL-cholesterol following a low fat low, cholesterol diet. Jansen et al. (1997) have also examined the effect of this polymorphism on HDL-cholesterol response in 41 healthy male subjects. They observed that after the consumption of a high saturated fat diet, carriers of the apo A4*2 allele had a greater decrease in HDL cholesterol. They also observed that compared to carriers of the apo A4*1 allele, these subjects showed a greater increase in HDL-cholesterol with a high MUFA diet than a diet low in fat and high in CHO. These results suggest that these subjects may benefit particularly from a diet high in monounsaturated fat as opposed to a diet low in fat and high in CHO.
Apo E in serum is associated with chylomicrons, VLDL and HDL, and serves as a ligand for the LDL receptor and the LDL-receptor-related protein (Mahley, 1988; Beisiegel et al. 1989). When apo E deficiency is present, there is a marked accumulation of cholesterol-enriched lipoproteins of density < 1.006 g/ml (Schaefer et al. 1986). Moreover, with this disorder there is delayed clearance of both apo B-100 and apo B-48 within TRL (Schaefer et al. 1986). Genetic variation at the apo E locus results from three common alleles in the population, E*4, E*3, and E*2 (Davignon et al. 1988). Population studies have shown that plasma cholesterol, LDL-cholesterol and apoB levels are highest in subjects carrying the apo E4 isoform, intermediate in those with the apoE3 isoform and lowest in those with the apo E2 isoform (Ordovas et al. 1987; Wilson et al. 1994). The most common apo E allele is the apo E3 allele with a relative frequency of 78% (Davignon et al. 1988; Hixson et al. 1991). The relationship between LDL-cholesterol and apo E genetic variation is not independent of environmental and ethnic factors. The association of the apo E4 isoform with elevated cholesterol is greater in populations consuming diets rich in saturated fat and cholesterol than in other populations (Williams et al. 1995; Ordovas & Schaefer, 2000). These findings indicate that the higher LDL-cholesterol levels observed in subjects carrying the apo E4 isoform are manifested primarily by the consumption of a high fat diet, and that the response to dietary saturated fat and cholesterol may differ among individuals with different apo E phenotypes. A study by Loktionov et al. (1998) observed that elevated plasminogen activator inhibitor (PAI-1) activity was higher in apo E4 subjects than in apo E3/3 or apo E3/2 subjects, suggesting that elevated PAI-1 activity may be an additional factor involved in the increased CHD associated with the apo 4 allele. The link between apo E4 gene variability and PAI-1 may be mediated by the LDL-receptor-related proteir receptor. It has also been observed that carriers of the apo E2 allele have a hyper TG response to low fat and high CHO diets (Ordovas et al. 1995). The apo E gene has also been implicated as one of the genetic factors responsible for variable postprandial responses to dietary manipulations (Williams et al. 1995). The apo E2 isoform is considered to decrease remnant clearance because of decreased affinity for the receptors (Ordovas & Schaefer, 2000). Conversely the apo E4 isoform should induce a faster clearance. However, studies that have compared postprandial TG responses across different apo E genotypes have produced conflicting reports, especially
regarding the effects associated with the apo E4 allele (Brown & Roberts, 1991; Boerwinkle et al. 1991; Nikkilä et al. 1994). Interestingly in the Multiple Risk Factor Intervention Trial (MRFIT), fasting hyperinsulinaemia was a risk factor for coronary artery disease only in men with apo E 3/2 phenotype (Orchard et al. 1994).

A degree of caution is necessary when interpreting the results of diet-gene interactions. Studies conducted to date have produced conflicting results therefore further studies are still required to reconcile the available information. Most of the studies reported were not initially designed to examine diet-gene interactions, and conclusions were drawn from re-analysis as advancing genetic techniques facilitated this possibility. Some allele effects may be apparent during the postprandial state as compared with the fasting state and future studies of diet and lipoprotein response should take this into consideration. We know that magnitude of plasma lipid responses to diet therapies varies considerably among individuals and future studies may be able to use genetic technology to increase our understanding of the effects of dietary manipulation on lipoprotein and insulin response, within those at greater genetic risk of developing CHD. Further studies are also required to establish the genetic components of IR.
1.8 AIMS AND OBJECTIVES OF THE STUDY

CHD remains a major cause of premature death despite a dramatic decline in many Western countries over the past 25 years. IR is strongly associated with the risk of developing CHD. Treatment of IR is focused on the environmental factors thought to contribute to the development of the condition, including diet. The nutrient, which is thought to have a strong influence on the development and progression of IR, is dietary fat. Thus, both quality and quantity of dietary fat are potential targets for dietary manipulation in the treatment of IR and it’s associated lipid abnormalities. In addition to the lack of clear dietary information, the mechanistic links between improvements in IR and lipid metabolism including postprandial lipaemia are poorly defined. Against this background, dietary intervention studies were planned with the objective of providing a stronger scientific base for advising changes in population dietary fatty acid intake likely to be associated with reduced risk of cardiovascular disease.

The hypotheses to be investigated were:-

- Insulin resistance with respect to glucose disposal also extends to deranged postprandial fat metabolism, with consequent increased risk of CHD.

- The insulin sensitivity of these pathways is improved by either increased dietary n-3 PUFA or reduced SFA in normal adults.

To address these hypotheses a series of intensive metabolic studies were designed and conducted in middle-aged men exhibiting characteristics of IR with the following specific objectives and methodologies employed.

1. To determine the effects of reduced dietary SFA and increased dietary n-3 PUFA on the insulin sensitivity of:
a) fasting glucose disposal and NEFA suppression, by the short ITT test measuring glucose and NEFA disposal rate.
b) adipose tissue LPL by adipose tissue lipoprotein lipase gene expression, and heparin releasable LPL activity during postprandial lipid clearance after a high fat breakfast.
c) glycerol release and NEFA suppression by stable isotope studies of the insulin sensitivity of hormone sensitive lipase as the reduction in the fasting glycerol appearance rate in response to a low level insulin infusion.

To relate all these determinants of insulin sensitivity to:

a) Dietary lipid tolerance in terms of the metabolic and hormonal sequelae of postprandial lipid clearance after a high fat breakfast: i.e. triglyceride, cholesterol fractions, NEFA, apo B-48, glucose and insulin concentrations.
b) Altered membrane FA composition by assessment of the fatty acid composition of RBC membranes.

This research programme was supported by the MAFF lipids programme and involved several investigators working together during two dietary interventions, which formed the basis of the MAFF research contract: i.e.

1: increased fish oil: 2-3 g long chain n-3 fatty acids/d in food.
2: reduced saturated fat by isoenergetic exchange with carbohydrate.

However during the execution of the second intervention, concern was expressed that although most National and International dietary guidelines emphasise the need to reduce total and saturated fat intakes and increase the intake of CHO, too much emphasis on reduction of total fat intake could result in excessive increases in CHO consumption. This is important since high-CHO diets have in some instances been shown to be detrimental in the management of IR Thus, a third intervention was carried out which was additional to the MAFF contract.
3: reduced saturated fat by isoenergetic exchange with monounsaturated fat.

To ensure that the results of the study could be applied to the free-living situation, all of the dietary studies were conducted using normal foods, in which the fatty acid composition had been modified.

In this thesis only those aspects which I was directly involved with are described, namely all the above procedures excepting for the stable isotope studies which formed part of interventions 1 & 2. A brief summary of the main findings from the stable isotope studies is included in appendix 1. In the case of the third intervention I was solely responsible for its design and execution and no stable isotope studies were performed.
CHAPTER 2.0
MATERIALS & METHODS

2.1 MATERIALS

Chemicals, compounds and consumables used are presented under the heading of the company from which they were obtained.

- Antigen Pharmaceuticals Ltd (Roscrea, Co. Tipperary, Ireland).
  Lignocaine hydrochloride injection B.P.

- BDH Laboratory Supplies (Poole, Dorset, BH15 ITD, UK).
  N-Lauroylsarcosine, sodium salt, C_{15}H_{29}NO_{3}Na

- Gibco BRL [Produced By Life Technologies Ltd] (Paisley, UK).
  Guanidine thiocyanate (CH_{5}N_{5}•HSCN)
  Primers

- Greiner Labortechnik Ltd
  (Brunel way, Stonehouse, Gloucestershire, GL10 3SX).
  Falcon tubes, sterile (14 ml)

- LIP Equipment & Services Ltd
  (Dockfield rd., Shipley, W. Yorkshire, BD17 7SJ)
  EDTA tubes (1, 5 & 10 ml)
  Fluoride oxalate tubes (1 ml & 5 ml)
  Glass blood tubes (without anticoagulant)
  Lithium Heparin tubes (5 & 10 ml)
  LP3 tubes

- Nycomed Amersham Plc.
  (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK).
  Glycerol Tri (9, 10(n)-{^3}H) olate
125I-Insulin

- **PROMEGA UK Ltd**  
  (Delta House, Chilworth Research Centre, Southampton, SO16 7NS, UK).
  Access RT-PCR system  
  RNagents® Total RNA isolation system

- **Roche Products Ltd (Welwyn Garden City, Hertfordshire, AL7 3AY).**  
  Calibrator H  
  Cholesterol kit (Unimate 5)  
  Control-serum N  
  Glucose kit (Unimate 5 HK)  
  HDL reagent  
  TAG analysis kit (Unimate 5)

- **Randox Laboratories (Co. Antrim, Northern Ireland, UK).**  
  HDL direct

- **Sarsted Ltd. (Leicester, UK).**  
  Centrifuge tubes, opaque, 15 ml

- **Sigma-Aldrich Company Ltd**  
  (Fancy road, Poole, Dorset, BH12 4QH, UK).
  Acetic acid, glacial (C2H4O2)  
  Albumin, bovine (essentially fatty acid free)  
  Chloroform (CHCl3)  
  Diethyl pyrocarbonate (C6H10O3)  
  Ethidium bromide (C21H20N3BR)  
  Glycerol AR (C3H8O3)  
  Heparin (porcine, sodium salt)  
  Heptane  
  Isoamyl alcohol (C5H12O)  
  DNA ladder, 1 Kb  
  Lauryl sulfate sodium salt (C12H25O4SNa)
L-α-phosphatidyl choline (L-α-Lecithin; 1,2-Diacyl-sn-glycero-3-phosphocholine).

Methanol (CH$_3$OH)

2-mercaptoethanol (C$_2$H$_6$OS)

Phenol (C$_6$H$_5$OH)

Polaroid black and white print film 667

Polaroid positive/negative print film 665

Polyethylene Glycol (PEG)

Potassium carbonate (K$_2$CO$_3$)

Sodium Chloride (NaCl)

Triolein (C$_{18}$:1,[cis]-9)

Trizma base (C$_4$H$_{11}$NO$_3$)

- Wallac UK Supplied By Fisons Chemicals (Leicester, UK).
  Optiphase safe

- WAKO Chemicals supplied by Alpha Laboratories (Eastleigh, UK).
  NEFA C kit

2.2 METHODS

2.2.1 COLLECTION AND TREATMENT OF BLOOD SAMPLES

Blood samples were collected into tubes containing a suitable anticoagulant for the analysis required.

<table>
<thead>
<tr>
<th>Sample Analysis</th>
<th>Collection tube type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Fluoride oxalate tube</td>
</tr>
<tr>
<td>Lipids</td>
<td>EDTA tube</td>
</tr>
<tr>
<td>Insulin</td>
<td>Lithium heparin tube</td>
</tr>
</tbody>
</table>

Blood tubes were centrifuged immediately after sample collection at 553 x g for 10 minutes in a bench top centrifuge. For the analysis of glucose, insulin and lipids,
plasma was removed, aliquoted into LP3 tubes and stored at –20°C. HDL-cholesterol samples, were stored at 4°C and analysis conducted within 48 hours.

2.2.2 PREPARATION OF BLOOD SAMPLES FOR THE ANALYSIS OF RED BLOOD CELL FATTY ACIDS

After the separation of blood by centrifugation, plasma was removed and the red blood cells were washed with 10 ml of 0.9 % saline solution. The washed solution was centrifuged at 553 x g for 10 minutes in a bench centrifuge and the top saline portion removed. The washing procedure was repeated a further two times. Finally the blood cells were transferred to a glass tube, were wrapped in para-film and stored at –20°C until required for analysis.

Another investigator measured the fatty acid composition of red blood cell phospholipids. Lipids were extracted with a mixture of chloroform and methanol (2:1 vol/vol), containing 0.01 % BHT as an antioxidant, according to the Folch method (Folch et al. 1957). The fatty acids were separated and quantitated using gas chromatography, with peak areas expressed as % of total peaks. Fatty acids were identified by comparing retention times with those of a known standard mixture.

2.2.3 AUTOMATED ANALYSIS ON THE COBAS MIRA PLUS

2.2.3.1 Measurement of plasma Glucose

Plasma glucose concentrations were measured using the Unimate 5 GLUC HK in-vitro kit (Roche Diagnostic Products Ltd, UK). The method involves an enzymatic UV test and uses the enzymes, hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH). The principle of the assay is described in the following equations:-

\[
\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-P} + \text{ADP}
\]

\[
\text{D-glucose-6-P} + \text{NAD}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-P} + \text{NADH} + \text{H}^+
\]
The formation of NADH is directly related to the glucose concentration of the sample and is measured photometrically at 340 nm.

2.2.3.2 Measurement of total plasma cholesterol
Plasma total cholesterol was measured using a kit and QC supplied by Roche diagnostic products Ltd, UK. The principle of the system is outlined below:

\[
\begin{align*}
\text{Cholesterol ester} + \text{H}_2\text{O} & \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol} + \text{fatty acids} \\
\text{Cholesterol} + \text{O}_2 & \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-en-3-one} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{-chlorophenol} + 4\text{-aminophenazone} & \xrightarrow{\text{peroxidase}} \text{quinoneimine derivative}
\end{align*}
\]

The colour intensity of the red-coloured quinoneimine derivative is directly proportional to the concentration of cholesterol in the sample.

2.2.3.3 In-direct measurement of HDL-cholesterol
For the fish oil (chapter 3.0) and the low SFA / high CHO (chapter 4.0) interventions HDL cholesterol was measured using a kit and QC supplied by Roche Diagnostic products Ltd, UK. Prior to cholesterol analysis, samples and QCs were treated with a reagent containing phosphotungstic acid and magnesium chloride to precipitate out apoB containing lipoproteins (LDL and VLDL). The samples were centrifuged at 5955 x g for 2 minutes in a microcentrifuge. The supernatant was removed and analysed for cholesterol concentration using the total cholesterol kit described in section 2.2.3.2. The standard used for this assay was diluted (1: 2.5) with 0.15 M NaCl prior to cholesterol analysis, to allow for the diluting effect of the precipitating reagents on the samples.

2.2.3.4 Direct measurement of HDL-cholesterol
For the MUFA intervention (Chapter 5.0) HDL-cholesterol samples were measured using the direct HDL-cholesterol kit, supplied by Randox. The kit allows direct measurement of HDL-cholesterol, without sample pre-treatment. The assay consists of 2 distinct reaction steps catalysed by two enzyme reagents. The first step facilitates the elimination of chylomicron, VLDL-cholesterol and LDL-cholesterol, while the
second step allows the specific measurement of HDL-cholesterol after release of HDL-cholesterol by detergents in reagent 2. The principle of the assay is outlined in the equations below.

**Step 1**

\[
\text{cholesterolesterase} 
\text{Cholesterol ester} \rightarrow \text{cholesterol + fatty acids}
\]

\[
\text{Cholesterol oxidase} 
\text{Cholesterol + O}_2 \rightarrow \text{cholest-4-en-3-one + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O}_2 + \text{O}_2
\]

**Step 2**

\[
\text{cholesterolesterase} 
\text{Cholesterol ester} \rightarrow \text{cholesterol + fatty acids}
\]

\[
\text{Cholesterol oxidase} 
\text{Cholesterol + O}_2 \rightarrow \text{cholest-4-en-3-one + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{AA} + \text{HDAOS} \rightarrow \text{Quinone pigment + 4H}_2\text{O}
\]

The intensity of the quinoneimine dye produced is directly proportional to the cholesterol concentration when measured at 600 nm.

\[4\text{AA} = 4\text{- Aminoantipyrine}\]

\[\text{HDAOS} = (2\text{- hydroxy - 3 - sulfopropyl) - 3,5 - dimethoxyaniline}\]

**2.2.3.5 Measurement of LDL-cholesterol**

LDL-C was calculated from the measurement of total cholesterol, HDL-Cholesterol and triglyceride according to Friedewald’s formula (Friedewald et al. 1972).

\[\text{LDL-C (mmol/L)} = \text{Total serum cholesterol} - \left[\text{HDL-cholesterol} + \frac{\text{total TG}}{2.2}\right]\]
2.2.3.6 Measurement of plasma NEFA

Plasma NEFA concentrations were measured using a kit supplied by WAKO Chemical GmbH, Germany and supplied by Alpha Laboratories Ltd, Eastleigh, UK. The QC employed in the assay was supplied by Randox laboratories Ltd, UK. The principle of the system is outlined below

\[
\text{RCOOH (NEFA) + ATP + CoA} \quad \overset{\text{Acyl-CoA synthetase}}{\longrightarrow} \quad \text{Acyl-CoA + AMP + Ppi}
\]

\[
\text{Acyl-CoA + O}_2 \quad \overset{\text{Acyl-CoA oxidase}}{\longrightarrow} \quad \text{2,3-trans-Enoyl CoA + H}_2\text{O}_2
\]

Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(B-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple colour adduct which can be measured colorimetrically at 550 nm. The intensity of the colour formed is proportional to the NEFA concentration. If left to stand at room temperature, the level of NEFA within a sample will increase. Therefore, samples were prepared for analysis as quickly as possible, working on ice at all times.

2.2.3.7 Measurement of Plasma Triglyceride

Plasma TG levels was measured by an automated, enzymatic, spectrophotometric technique on the Cobas Mira Plus analyser (Roche products Ltd, UK). The principle of the assay is described in the following equations.

\[
\text{Lipase} \quad \rightarrow \quad \text{glycerol + fatty acids}
\]

\[
\text{Glycerol + ATP} \quad \overset{\text{Glycerol kinase}}{\longrightarrow} \quad \text{glycerol-3-phosphate + ADP}
\]

\[
\text{Glycerol phosphate oxidase} \quad \text{Glycerol-3-phosphate + O}_2 \quad \overset{\text{Glycerol phosphate oxidase}}{\longrightarrow} \quad \text{dihydroxyacetone phosphate + H}_2\text{O}_2
\]

In the presence of peroxidase, the hydrogen peroxide formed effects the oxidative coupling of 4-chlorophenol and 4-aminotipyrine to form a red-coloured quinoneimine derivative. The colour intensity measured is directly related to the TG concentration in the sample and is measured photometrically at 500 nm. 2.2.3.8 Variability of the Cobas Mira automated spectrophotometric techniques
The intra- and inter-assay coefficient of variation (CV) for the assays employed on the Cobas Mira plus autoanalyser were as follows:

<table>
<thead>
<tr>
<th>Sample analysis</th>
<th>Mean QC value (mmol/l)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.51</td>
<td>1.56</td>
<td>2.53</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>6.54</td>
<td>2.43</td>
<td>5.61</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- indirect method</td>
<td>1.04</td>
<td>1.89</td>
<td>4.94</td>
</tr>
<tr>
<td>- direct method</td>
<td>0.79</td>
<td>0.69</td>
<td>1.82</td>
</tr>
<tr>
<td>NEFA</td>
<td>1.25</td>
<td>1.62</td>
<td>2.71</td>
</tr>
<tr>
<td>TG</td>
<td>1.19</td>
<td>1.05</td>
<td>3.45</td>
</tr>
</tbody>
</table>

2.2.4 SEPARATION OF TRL AND TPL FRACTIONS FROM PLASMA USING ULTRACENTRIFUGATION

At the time of blood separation, 5 ml of plasma was transferred to an anticoagulant-free glass bottle and stored at 4°C overnight. Using thick-walled polycarbonate tubes (38 ml), 3.5 ml of plasma was overlayered with 3.5 ml of NaCl solution (density =1.006 g/ml) using an automated Finnpipette (Lifesciences International, UK). The tubes were ultracentrifuged in a 50.2 Ti rotor (Beckman, UK.) at 4.83 x 10^6 g_{max}.min in a Beckman L7-65 ultracentrifuge (MSE Scientific Instruments, UK.) at 24°C.

The TRL fraction appears as a fine, cream layer on the surface of the plasma. At least 1.2 ml of the TRL layer was isolated using a glass Pasteur pipette and aliquoted into LP3 tubes. The TPL fraction was mixed and aliquoted. All samples were stored at -20°C until analysis.

2.2.5 MEASUREMENT OF APO-B48

ApoB-48 was measured by another investigator according to a method previously described by Lovegrove et al. (1996). The method is a competitive, enzyme-linked immunosorbent assay (ELISA), employing the use of an antiserum, specific for apo
B-48. An heptapeptide-thyroglobulin conjugate consisting of the terminal residues of the apo B-48 molecule is used as the coating material in this ELISA assay. Chylomicron samples from the postprandial studies were incubated with the specific antisera to apo B-48 before addition to the ELISA plate. An horse radish peroxidase conjugated antibody is used to detect antibodies attached to the coating material through its action with the tetramethylbenzidine substrate. Absorbance was measured at 450 nm. A standard curve was constructed and the amount of apo B-48 within the QCs and samples was determined by reading from this curve.

2.2.6 MEASUREMENT OF PLASMA INSULIN CONCENTRATION

Insulin was measured by a radioimmunoassay method developed at the University of Surrey (Hampton & Withey, 1993). The insulin radiolabel was produced by other researchers in the Nutrition laboratory at the University of Surrey. Antibodies against insulin were raised by immunising guinea pigs with porcine insulin conjugated to egg albumin via carbodimide and aliquots were kindly supplied by Dr. S. Hampton at the University of Surrey. Separation of the assay was carried out by a double antibody plus polyethylene glycol method. The assay procedure is outlined in table 2.1.

The assay was conducted over a three-day period. The reagents were added in the order shown in table 2.1. Tubes were set up in duplicate. R.O. water was used in the preparation of all reagents. At the end of the incubation period on day 3 the tubes were centrifuged in a Beckman J-6B at 1300 x g for 30 minutes at 4°C. The resulting supernatant was aspirated and the tubes counted on the Wallac 1470 Wizard, automatic gamma counter using a pre-set programme. A low (mean, 71 pmol/l) and high (mean, 580 pmol/l) QC was run in each assay. The intra-assay CV was 5.2 % and 4.8 % for the low and high QCs respectively, while the inter-assay CV was 7.6 % for the low QC and 9.2 % for the high QC.
Table 2.1. Outline of the insulin assay procedure.

Standards of 23, 47, 94, 188, 375, 750 and 1500 pmol/l are prepared in buffer.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Totals</th>
<th>NSB Standard</th>
<th>Zero Standard</th>
<th>NSB QC</th>
<th>QC</th>
<th>NSB unknown</th>
<th>unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay buffer</td>
<td>-</td>
<td>350 µl</td>
<td>250 µl</td>
<td>200 µl</td>
<td>350 µl</td>
<td>250 µl</td>
<td>350 µl</td>
</tr>
<tr>
<td>Insulin standard</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CSS</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QC plasma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plasma sample</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Antiserum</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>100 µl</td>
<td>-</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Tubes were vortex-mixed and incubated at 4°C for 24 hours.

| **DAY 2** | 125I-Insulin | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl |
|           |              |        |        |        |        |        |        |        |        |

Tubes were vortex-mixed and incubated at 4°C for 24 hours.

| **DAY 3** | NGPS          | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl |
|           | DAGP          | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl |
|           | 4% PEG        | 700 µl | 700 µl | 700 µl | 700 µl | 700 µl | 700 µl | 700 µl | 700 µl |

Tubes were vortex-mixed, incubated at 4°C for 2 hours and then centrifuged at 1300 × g for 30 min. Tubes were aspirated and the pellet counted for radioactivity.
2.2.7 MEASUREMENT OF POST-HEPARIN PLASMA LIPASE ACTIVITY

LPL and HL activity was measured by a modified method of that described by Nilsson-Ehle & Schotz (1976). The method involves the detection of $^3$H-labeled free fatty acids, formed by hydrolysis of a $^3$H-labelled triolein-substrate emulsion, catalysed by the lipase enzymes LPL and HL as depicted in the equation below.

\[
\text{Lipase enzymes} \quad \text{Glycerol}^{3}\text{H-triolate} \rightarrow \quad \text{$^3$H-oleic acid + glycerol.}
\]

Selective measurement of LPL and HL was achieved by selecting assay conditions favourable to the activity of HL.

1 M NaCl can readily suppress the activity of LPL, and the activity recorded (in the absence of apo CII) is a good measure of HL. Salt inactivation of LPL is due to an irreversible denaturation of the enzyme molecule (Olivecrona & Olivecrona, 1989).

In this assay post heparin samples were assayed simultaneously in the presence of 0.2 M NaCl, to give a measure of total lipase activity and 4 M NaCl, to give a measure of HL activity. The difference in total lipase and HL activity was taken as a measure of LPL activity.

Both LPL and HL are inhibited by fatty acids. It was therefore necessary to include albumin in the lipid emulsion to bind any fatty acids present.

The LPL–heparin complex is more stable and soluble than the enzyme alone and is catalytically active (Olivecrona & Olivecrona, 1989). Heparin was therefore included in the Tris-buffer used for the incubation of the enzyme. With a high concentration of NaCl, the interaction between HL and heparin will be disrupted and the presence of heparin will be of no importance.

Finally, with triglyceride emulsions as substrates the activity of LPL, but not the activity of HL, has been shown to be increased several fold by apo-CII. Purified apo-CII was therefore added to the samples assayed for total lipase activity.
2.2.7.1 Preparation of \(^{3}\text{H}-\text{triolein-glycerol emulsion}\)

50 µl of labelled \(^{3}\text{H}-\text{triolein [9,10(n) \text{H oleate}]}}\) and 250 µl unlabeled triolein were mixed and 80 µl of L-\(\alpha\)-phosphatidyl choline added. The solvents were evaporated under a stream of nitrogen in a darkened fume cupboard. The dried lipids were emulsified in 5 ml of glycerol by homogenisation for 5 minutes continuously, using a Black and Decker drill to which a pestle had been attached. The homogenisation was carried out in a glass homogenising tube, immersed in an ice bath. The emulsion was transferred to a glass tube, wrapped in foil (to prevent photolytic destruction) and double contained in a Perspex box until required. The emulsion, which is optically clear due to the similar refractive indices of the lipid and glycerol, could be stored at room temperature for at least six weeks. It will be referred to as the 'concentrated substrate'.

2.2.7.2 Preparation of Tris buffer

50 mM Trizma base containing 2 IU/ml heparin and either 0.2M or 4M NaCl was made up in a volumetric flask with R.O. distilled water. The pH was corrected to 8.4 using 1 M HCl or NaOH and the solutions stored at 4°C until required for use.

2.2.7.3 Preparation of the substrate emulsion

Substrate emulsions for assay were prepared daily by dilution of 1 volume of concentrated substrate with 4 volumes of 0.2 M or 4 M NaCl-Tris buffer (pH 8.4) containing 3% (w/v) bovine serum albumin. The substrate emulsion was shaken vigorously on a vortex mixer for 10 seconds and incubated at 37°C before being added to the reaction tubes.

For the assay of total lipase activity 1 µl of apo-CII (0.5µM) per sample of post-heparin plasma was added to the substrate emulsion. Apo-CII was not added to the substrate emulsion for the assay of hepatic lipase activity.

2.2.7.4 Assay procedure

An outline of the assay procedure is shown in table 2.2.

Using a total assay volume of 200 µl, 10 µl of post-heparin plasma, 100 µl of the appropriate substrate emulsion and 90 µl of either 0.2 M NaCl-Tris buffer or 4 M
NaCl-Tris buffer was added to the reaction tubes. Outside of the incubation period, the reaction tubes were kept on ice at all times.

In addition, blank incubations (without lipase) and a reference sample of post-heparin plasma were included in the assay procedure as a measure of background free fatty acid levels and to monitor inter-assay variation respectively. All samples were assayed in duplicate.

Incubation of the reaction tubes was carried out in a shaking water bath at 37°C for a period of 30 minutes. The extraction of fatty acids produced during the incubation was by a modification of the method described by Belfrage & Vaughan (1969). The enzymatic reaction was stopped by the addition of 3.25 ml of methanol:chloroform:heptane (1.41:1.25:1.00 v/v/v) followed by 1.05 ml of 0.1 M K$_2$CO$_3$ (adjusted to pH 7.4 with saturated boric acid) in order to extract free fatty acids into the methanolic upper phase.

The tubes were vortex mixed and centrifuged at 1700 x g for 10 minutes in a Beckman J6 centrifuge to separate the upper alkaline/water phase containing the fatty acids from the lower chloroform heptane phase, containing triglycerides and partial glycerides. All procedures subsequent to the extraction were performed at room temperature.

A 0.5 ml sample from the upper phase was added to a scintillation counting vial containing 4 ml of optiphase safe and 75 μl of glacial acetic acid. Glacial acetic acid reduced chemiluminescence by neutralising the alkaline sample.

The vials were inverted several times and left in the dark for a minimum of 1 hour before being counted for specific radioactivity on a Wallac 1410 beta counter. Each vial was counted for 5 minutes using a pre-set programme.
Table 2.2. Outline of LPL assay procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>0.2 M/4 M NaCl-Tris buffer</th>
<th>Substrate Emulsion</th>
<th>Post-heparin sample</th>
<th>Reference sample</th>
<th>K2CO3</th>
<th>Organic solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Totals</td>
<td>2.45 ml</td>
<td>100 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blanks</td>
<td>100 μl</td>
<td>100 μl</td>
<td>-</td>
<td>1.05 ml</td>
<td>3.25 ml</td>
<td></td>
</tr>
<tr>
<td>Samples</td>
<td>90 μl</td>
<td>100 μl</td>
<td>10ul</td>
<td>1.05 ml</td>
<td>3.25 ml</td>
<td></td>
</tr>
<tr>
<td>References</td>
<td>90 μl</td>
<td>100 μl</td>
<td>10 μl</td>
<td>1.05 ml</td>
<td>3.25 ml</td>
<td></td>
</tr>
</tbody>
</table>

2.2.7.5 Calculation of Enzyme Activity

Enzyme activity was calculated according to the formula:

\[
\text{Enzyme activity} = \frac{\text{Net DPM}}{\text{incubation time}} \times \frac{1}{\text{Sp Act.}} \times 3 \times 4.9 \times \frac{1}{0.76}
\]

Net DPM = DPM-blank

Sp Act. = Specific radioactivity of triolein expressed as DPM per nmol of triolein.

3 = Number of fatty acids released per molecule of triglyceride hydrolysed.

4.9 = Dilution factor (0.5 ml taken from 2.45 ml upper phase).

0.76 = Partition of oleic acid in the extraction system.

The results were expressed in units of activity equivalent to μmoles of oleate released per ml per hour.
2.2.8 OPTIMISATION OF LPL ASSAY

The method of measuring LPL in this laboratory was based on the method originally described by Nilsson-Ehle & Schotz (1976). When post-heparin samples collected during the first dietary intervention study (chapter 3.0) were analysed using the established method, it was found that the activity values obtained were below published values using the same method of analysis. LPL activity values were also lower than had previously been attained by other investigators in the laboratory using the same method of analysis.

Several experiments were undertaken in an attempt to optimise the assay. The components of the assay as well as the method of collecting blood samples were examined.

2.2.8.1 Collection of post-heparin blood samples

When heparin is administered IV, to stimulate the release of LPL from the endothelial surface, it is recommended that that blood samples are subsequently collected from a site distal to that from which the heparin is administered. Post-heparin samples were collected at the end of a 9-hour postprandial study during this investigation. Subjects had a cannula inserted at the beginning of the study to facilitate the collection of blood samples. To minimise any further potential discomfort for the subjects it was decided to administer heparin via the cannula and to collect blood samples from the same cannula.

To investigate whether collecting blood from the same site through which heparin has been administered has an effect on levels of LPL activity, a small study was set up. The aim of the study was to establish whether there was any difference in LPL activity, measured in blood samples collected from a distal site or from the same site through which heparin was administered.

Six subjects (4 females and 2 males) participated in the study. Blood samples were collected four hours after consuming a high fat breakfast (60 g fat). Blood samples were collected on two occasions 5 and 15 minutes following heparin administration, one week apart. For each study a cannula was inserted into the antecubital vein of the
non-dominant arm and blood samples collected from here. Heparin (7500 IU) was administered via the cannula on one occasion and via an IV injection in the opposite arm on another occasion. Subjects were randomized in the order of the method of heparin administration.

Four subjects completed the study. For one subject when it was attempted to administer heparin by IV injection in the opposite arm, the vein was missed and blood sample collection was therefore not possible. This served to highlight the disadvantage of administering heparin in this fashion.

The levels of LPL activity measured 5 and 15 minutes following heparin administered via either the cannula or the opposite arm are presented in fig 2.1. It was observed that there was no difference in the method of administering heparin on LPL activity levels, either in the 5 minute or 15 minute sample. However it was noted that the results obtained by either method were still lower than published values for normal control subjects. It was concluded therefore, that the method of administration of heparin did not impact on the levels of LPL activity and was not accounting for the low levels being obtained in the fish oil study samples.

![Figure 2.1. Comparison of post-heparin LPL activity levels using different methods of heparin administration Data are means of 4 subjects ± SE.](image)
2.2.8.2 Method of preparing substrate emulsion

The established method of emulsifying the $^{3}$H-triolein glycerol emulsion in this laboratory was by sonication using a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments). After removal of solvents from the triolein mixture described in section 2.2.7.3, emulsification was carried out by intermittent sonication (10 second bursts of sonication) over a period of 4-5 hours, cooling the emulsion for at least 15 minutes on ice between sonications. The emulsifying procedure was considered complete when no trace of oil was visible on the surface of the emulsion.

An alternative method of emulsifying is by homogenisation as described by Olivecrona et al. (1979). An experiment was set up to investigate the effect on the assay performance when an emulsion was prepared by homogenisation as described in section 2.2.7.4. The samples analysed were those collected during the heparin study described in section 2.2.8.2. The results obtained and a comparison with the values obtained using a sonicated emulsion are presented in figure 4.3. The LPL activity values measured using the homogenised emulsion were within previously published values with a range of 9.8 - 24.2 U and were greater than the results obtained previously using a sonicated emulsion. The intra-assay CV was 6.2% at 47.2 U using the homogenising method and was determined by repeated measure of a bovine milk standard. The inter-assay CV was 30% at 17.1 U and 30.5% at 8.5 U using post-heparin plasma samples.

It was concluded therefore that preparing emulsions by homogenisation as opposed to the established method of sonication would improve the validity of the LPL assay.
2.2.8.4 Source of Apo C-II in the LPL assay

Apo C-II is a co-factor necessary for LPL activation, therefore its inclusion is recommended in assays measuring LPL activity. Traditionally heat-inactivated serum has been used as a source of apo C-II. Previous studies however, have reported variable LPL activity values based on the source of serum, the length of time that substrates are incubated with serum and the amount of serum added (Bengston-Olivecrona & Olivecrona, 1992; Henderson, 1993). In addition, apo C-III present in the serum has the potential of inhibiting the activity of HL, thereby obscuring the measurement of LPL activity. An experiment was therefore set up to examine the effect on LPL activity of the exclusion of serum from the assay system and replacing this with a purified source of apo C-II.

Postheparin plasma samples (5 and 15 minute samples) previously obtained from 4 subjects were analysed in duplicate. Within the assay, one set of samples was analysed using the addition of heat-inactivated serum as the source of apo C-II, while a duplicate set of samples was analysed using a purified source of apo C-II. The results of the experiment are presented in fig 2.3 and show that the removal of serum
from the assay procedure and replacement with a purified source of apo C-II has increased the LPL activity values ($p = 0.05$). All measurements of LPL activity in the intervention studies presented were subsequently conducted without the use of heat-inactivated serum.

Figure 2.3. Post-heparin LPL activity of 8 samples assayed with serum and with pure apo C-II. Data are presented as means ± SE.

2.3 HUMAN METABOLIC INVESTIGATIONS

For each intervention study the following investigations were conducted.

2.3.1 POSTPRANDIAL STUDY

The study was conducted in the Clinical Investigation Unit at the Royal Surrey County Hospital. Each postprandial investigation began between 8:00 am and 9:00 am, following an overnight fast. All subjects had refrained from strenuous exercise and abstained from alcohol on the day prior to the investigation.
On arrival a 19 gauge, 32-mm venous cannula (Abbott Ireland Ltd.) was inserted in the antecubital vein of the non-dominant forearm, and 22 ml of fasting blood was taken. The test meal consisting of a cheese sandwich, orange juice and a chocolate milkshake, providing 80 g of fat was consumed. The nutrient composition of the test meal is provided in table 2.3. The subjects consumed the test meal within 20 minutes. No other food was allowed throughout the 9-hour test period, although energy-free and caffeine-free fluids were allowed *ad libitum*.

Following the test meal 10 ml of blood was drawn every 30 min for the first two hours and hourly thereafter for the remaining 7 hours. The cannula was flushed with 1-2 ml of saline solution after sampling to ensure that the cannula remained patent. At each sampling point the first 1-2 ml of blood was therefore discarded to eliminate any traces of saline solution.

Blood samples were collected for the analysis of triglyceride, cholesterol, NEFA, insulin, glucose, and chylomicron fractions. The collection and treatment of blood samples was as described in section 2.2.1.

Six hours into the postprandial investigation an adipose tissue biopsy was taken from the par-umbilical region of the stomach. The procedure is outlined in detail in, section 2.3.3. Adipose tissue was stored at -80°C until required for analysis of LPL mRNA levels, the results of which are described in chapter 6.0.

At the end of the 9-hour study, volunteers were given a bolus dose of 7500 IU of heparin, via the cannula. Blood samples (5 ml) were collected 5 and 15 minutes following heparin administration for the analysis of plasma LPL enzyme activity as described in section 2.2.7.
Table 2.3. *Nutritional content of test meal.*

<table>
<thead>
<tr>
<th>FOOD</th>
<th>Quantity</th>
<th>Energy (kJ)</th>
<th>Fat (g)</th>
<th>Protein (g)</th>
<th>CHO (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White bread</td>
<td>70 g</td>
<td>630</td>
<td>0.9</td>
<td>5.4</td>
<td>35</td>
</tr>
<tr>
<td>Margarine</td>
<td>10 g</td>
<td>300</td>
<td>8.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>30 g</td>
<td>510</td>
<td>10.4</td>
<td>7.7</td>
<td>-</td>
</tr>
<tr>
<td>Chocolate powder</td>
<td>10 g</td>
<td>170</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Whole milk</td>
<td>130 ml</td>
<td>350</td>
<td>5.1</td>
<td>4.2</td>
<td>6</td>
</tr>
<tr>
<td>Double cream</td>
<td>120 ml</td>
<td>2220</td>
<td>57.6</td>
<td>2.0</td>
<td>4</td>
</tr>
<tr>
<td>Orange juice</td>
<td>200 ml</td>
<td>300</td>
<td>trace</td>
<td>1.0</td>
<td>18</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4480</strong></td>
<td><strong>82.7</strong></td>
<td><strong>20.3</strong></td>
<td><strong>72.5</strong></td>
<td></td>
</tr>
</tbody>
</table>

2.3.2 SHORT INSULIN TOLERANCE TEST

The investigation was conducted in the Clinical Investigation Unit at the Royal Surrey County Hospital under the supervision of the project Clinician. Upon arrival at the investigation unit subjects were asked to place their non-dominant hand into a hand-warming unit. The hand-warming unit was set to a temperature of 60°C and was designed to enable the collection of 'arterialised' venous blood samples. Subjects had fasted overnight following the consumption of a standardised ready meal at 20:00 hours on the evening prior to the investigation. No strenuous exercise had been undertaken or alcohol consumed in the previous 24-hour period.

A long arm butterfly cannula was inserted into an antecubital vein on the dorsum of the hand and a 4-ml fasting blood sample taken. The cannula arm was fixed with a three-way tap and positioned to facilitate the collection of blood samples without
having to remove the hand from the hand-warming unit. The insulin (Human Actrapid, Novo laboratories, Basingstoke, UK) was prepared by diluting 100 units in 100 ml of saline (1 unit/ml) and a bolus dose (0.1U/Kg body weight) was administered via the cannula. Following insulin administration, 1-ml blood samples were drawn at timed one-minute intervals over a period of 15 minutes. The cannula was flushed with a 0.9% saline solution between samples and at each sampling point the first 1 ml of blood was discarded to remove any traces of saline solution from the sample for assay. Giving a concentrated glucose drink (Hycal) terminated the test. Volunteers were provided with a breakfast before leaving the investigation unit.

Blood samples were collected and analysed for glucose and NEFA levels as described section 2.2.3.1 and 2.2.3.6 respectively. Insulin sensitivity was determined from the slope in the fall of log-transformed glucose between 4 and 15 minutes following insulin injection.

2.3.3 COLLECTION OF HUMAN ADIPOSE TISSUE

Adipose tissue samples were collected during the postprandial study day of each dietary intervention study. The tissue samples were collected from the paraumbilical region under local anaesthetic. The area was first cleaned using an alcohol swab and 5 ml of lignocaine injected deep into the paraumbilical region. A small amount of skin was excised using a fine needle to allow the sterile biopsy needle to be inserted into the adipose tissue beneath the skin. Adipose tissue was manipulated using the biopsy needle and removed by aspiration using a 20-ml syringe. The procedure was stopped when sufficient tissue had been collected and the area was cleaned and fixed with a plaster.
CHAPTER 3.0
THE EFFECT OF A LONG CHAIN N-3 PUFA ENRICHED DIET ON INSULIN SENSITIVITY AND POSTPRANDIAL LIPEMIA

3.1 BACKGROUND

As indicated in the introduction while there is little dispute regarding the role of n-3 fatty acids in lowering serum TG and VLDL concentration there is less agreement as to the effect of n-3 supplementation on glycaemic control and insulin action. In terms of glucose tolerance, epidemiological observations suggest that the incidence of diabetes is lower in populations known to consume large amounts of fish and marine mammals (Mouratoff et al. 1969; Kromann & Green, 1980). Intervention studies which have examined the effect of n-3 PUFA on blood glucose levels have generally been conducted in individuals with abnormal glucose tolerance or diabetes and here contradictory results have emerged. While some have found an improvement in the regulation of blood glucose levels (Zak et al. 1989; Fasching et al. 1991), several others report either no change (Kasim et al. 1988; Puhakainen et al. 1995) or a worsening of glycaemic control (Friday et al., 1989; Hendra et al. 1990; Malasanos & Stacpoole, 1991; Zambon et al., 1992). The suggested explanation for the hyperglycaemic response, is an increase in basal hepatic glucose output. Those studies which have reported an adverse effect of fish oil supplementation have been criticised on the grounds that large doses of n-3 fatty acids (5.5 – 8.0 g/d) have been used, that the studies were uncontrolled and involved small numbers. Popp-Snijders et al. (1990) also suggests that the baseline glycaemic control, body weight and the extra dietary energy provided by the fatty acid supplementation may well be responsible for the observed hyperglycaemic effects.

In terms of insulin sensitivity animal studies have clearly demonstrated a beneficial effect of fish oil feeding (Behme, 1996; Chicco et al. 1996; Luo et al. 1996) and it is hypothesised that the enrichment of tissue phospholipids with n-3 PUFA induce changes in membrane fluidity which in turn may enhance both insulin sensitivity and secretion. As with the glucose tolerance studies, results from human studies of insulin
sensitivity are inconclusive. Stacpoole et al. (1989) found that insulin sensitivity was impaired following a period of fish oil feeding while other investigators have found no change (Pelikanova et al. 1993) or an increase in insulin sensitivity with fish oil feeding (Popp-snijders et al. 1987).

In recognising the potential beneficial effects of the ingestion of long chain \( n-3 \) PUFA, questions remains as to optimum levels of intake and how best to achieve such intakes. Bang et al. (1971) reported that it was customary for Eskimos to consume 400-500 g of fish or whale meat daily, containing up to 7 g of \( n-3 \) PUFA. Such levels would be impossible to achieve within normal western style diets, however studies have shown that diets containing 1.6 – 2.8 g of long chain \( n-3 \) PUFA are sufficient to produce a 30 \% reduction in serum triglyceride levels (Williams, 1997). Studies which have looked at the effect of \( n-3 \) PUFA supplementation on glucose tolerance and insulin sensitivity have used varying amounts of fish oil with a suggestion that larger doses of fish oils have lead to greater increases in blood glucose levels. In the UK, the average daily fish consumption is 26 g, providing approximately 0.2 g per day of long chain \( n-3 \) PUFA (Gregory et al. 1990). From this platform achieving potent levels of supplementation would require taking 4-6 fish oil capsules, 10-ml (2 teaspoons) of cod liver oil or 100 g of salmon daily. In the primary prevention of heart disease, manifested through insulin resistance, the implementation of such dietary measures presents a challenge.

Recent technological advances have made it possible to enrich a variety of common foods with long chain \( n-3 \) PUFA. This has opened up the possibility of increasing \( n-3 \) PUFA intakes without increasing intakes of oily fish or without the need to take large amounts of fish oil supplements. Another advantage is that the commercial food industry, keen to promote their food products rich in \( n-3 \) PUFA, may be much more successful at achieving an increase in \( n-3 \) PUFA intakes than that procured by Government health messages alone. As pointed out by Gibney (1997) we have seen how the margarine industry, by providing \( n-6 \)-rich PUFA spreads, has promoted a greater knowledge of the different types of dietary fatty acids than most government-funded interventions could ever achieve.
3.1.1 STUDY OBJECTIVES

At the outset of the work described here the effect of consuming n-3 PUFA enriched diets on lipoprotein metabolism was well established, but less was known about the effect of such diets on glucose metabolism and insulin action. In particular, the mechanistic links between disorders of lipid and glucose metabolism were poorly defined. The aim of this study therefore, was to investigate the effect of ingesting a diet enriched with long chain n-3 PUFA on insulin sensitivity and postprandial lipaemia. The hypothesis of the study was that insulin resistance with respect to glucose disposal also extends to disturbances of lipid metabolism, thus contributing to an increased risk of CHD. It was postulated that the insulin sensitivity of postprandial lipid metabolism as well as glucose metabolism would be improved by an increase in long chain n-3 PUFA intake. The study used a combination of normal foods, which had been enriched with long chain n-3 PUFA, and fish oil capsules in order to increase the intake of EPA and DHA over a twelve-week period. The incorporation into red blood cell membranes, effects on fasting and postprandial lipoproteins and hormone levels including LPL activity and mRNA expression as well as the effects on insulin sensitivity were investigated in 11 middle-aged, insulin-resistant men.

3.2 STUDY DESIGN AND SUBJECTS

3.2.1 EXPERIMENTAL DESIGN

The study design was a randomised, controlled, single blind, crossover study comprising of two 10-week periods of feeding with control or fish-oil enriched foods, separated by a 3-month washout phase. Fish oil was incorporated into the diet of volunteers through a combination of foods made with a long chain n-3 PUFA enriched cooking fat (MD foods, Denmark), a fish oil enriched spread, PACT (MD foods, Denmark) and 1 g Pikasol fish oil capsules (LUBE, Germany). The control phase comprised foods made using an un-enriched cooking fat (MD foods, Denmark), an n-6 spread (MD foods, Denmark) as well as 1 g olive oil capsules (Lube, Germany). The fatty acid composition of the control and enriched cooking fat and margarine are shown in table 1.1. The food items provided were biscuits, cakes, patés
and pasta sauces. A local catering company, who was supplied with the cooking fat prepared the foods. Control foods were prepared and packaged in an identical form to the enriched products to allow a blind protocol. The volunteers were asked to incorporate the foods into their habitual diet, exchanging them for their usual snacks or main meals, in an attempt to maintain their habitual energy intakes.

Table 3.1. Composition of n-3 enriched and control margarine and cooking fat

<table>
<thead>
<tr>
<th></th>
<th>Enriched margarine</th>
<th>Control margarine</th>
<th>Enriched cooking fat</th>
<th>Control cooking fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat (g)</td>
<td>60</td>
<td>60</td>
<td>99.3</td>
<td>99.3</td>
</tr>
<tr>
<td>SFA (g)</td>
<td>15</td>
<td>15</td>
<td>17.5</td>
<td>16.0</td>
</tr>
<tr>
<td>C18:0 (g)</td>
<td>1.5</td>
<td>1.5</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>30</td>
<td>30</td>
<td>26.3</td>
<td>21.3</td>
</tr>
<tr>
<td>C18:1 (g)</td>
<td>27.6</td>
<td>27.6</td>
<td>23.0</td>
<td>21.3</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>15</td>
<td>15</td>
<td>42.5</td>
<td>48.9</td>
</tr>
<tr>
<td>Total n-6 (g)</td>
<td>9</td>
<td>10.5</td>
<td>37.3</td>
<td>48.5</td>
</tr>
<tr>
<td>Total n-3 (g)</td>
<td>6</td>
<td>4.5</td>
<td>4.4</td>
<td>0.4</td>
</tr>
<tr>
<td>n6: n3</td>
<td>1.5</td>
<td>2.3</td>
<td>8.5</td>
<td>121.3</td>
</tr>
<tr>
<td>C20:4 (g)</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>EPA &amp; DHA (g)</td>
<td>1.4</td>
<td>0</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>Trans FA (g)</td>
<td>0</td>
<td>0</td>
<td>13.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

The aim of the study was for volunteers to achieve a mean daily intake of 2-3 g of EPA & DHA per day on the enriched diet. A ‘unit’ system was devised to allow volunteers choice in the foods, that could be consumed, and to ensure that volunteers achieved the desired intake of long chain n-3 PUFA. Each food portion was assigned a unit value where one unit was equivalent to 0.4 g of EPA & DHA. The fish oil enriched-cooking fat provided 4 g of EPA & DHA per 100 g and each food item was made using either 10 g or 20 g of cooking fat i.e. equivalent to 1 or 2 units. The fish oil enriched-margarine, contained 1.4 g of EPA & DHA per 100 g and was packaged
in 15-g portions. One portion of margarine was therefore equivalent to \( \frac{1}{2} \) a unit. Subjects were asked to consume 3 to 4 units per day, depending on their habitual energy intake. In addition to these foods volunteers were requested to consume two capsules per day, with each 1-g fish oil Pikasol capsule providing 310 mg of EPA and 200 mg of DHA.

During the study, volunteer's attended the University of Surrey every two weeks to meet with investigators and to collect foods. This allowed investigators to answer any queries, to resolve any problems encountered with the study foods and to aid volunteer motivation. During the two periods of dietary intervention, volunteers were asked to complete two 4-day food diaries, one at the beginning and one towards the end of the intervention period. These data were analysed using the FOODBASE nutrient analysis programme (Version 1.2; Institute of Brain Chemistry and Human Nutrition, London).

3.2.2 SUBJECTS

Twelve healthy middle-aged male volunteers, who were selected from 35 subjects initially screened, were recruited to the study. Volunteers had responded to advertisements placed with the local media in the Guildford area. The volunteers all satisfied the following inclusion criteria: they were aged between 35 and 60 years, with a BMI less than 35 Kg/m\(^2\) and a waist to hip ratio of less than 1.0. They were all non-smokers and did not take regular exercise i.e. more than three 20-minute aerobic sessions per week. All of the volunteers were free from medication and did not have any previous medical history of coronary artery, endocrine or liver disease. None of the volunteers were following any type of therapeutic or specialised diet or taking any form of dietary supplements.

All subjects had a fasting blood sample collected prior to inclusion in the study for screening. The criteria for inclusion were: glucose < 7.0 mmol/l, total cholesterol < 7.0 mmol/l, TG < 3.0 mmol/l, insulin > 40 pmol/l and haemoglobin > 130g/l. Volunteers were also asked to complete a 4-day food diary, at screening, to determine their habitual energy and fat consumption including their habitual long chain \( n-3 \) PUFA intake.
Ethical approval for the study was obtained from the University of Surrey and the Royal Surrey County Hospital Research Ethics Committees. Informed and witnessed consent was obtained from each subject before participating in the study.

3.3. METABOLIC INVESTIGATIONS

The metabolic investigations at the start and end of each dietary period required volunteers to attend on three study days i.e.

1. Fasting blood biochemistry and the short ITT: 3 subjects per day (work described in this thesis).

2. Postprandial lipid disposal, LPL activity and gene expression during a 9-hour study following a high fat breakfast: 5-6 subjects per day (work described in this thesis).

3. NEFA / glycerol suppression by insulin infusion and fasting proteolysis inhibition by postprandial insulin and amino acids: 9 hr stable isotope study of glycerol production rate and endogenous leucine production rate in response to insulin infusion: 2 subjects per day (work not described in this thesis, but results summarised in appendix 1)

A description of the metabolic investigations conducted are provided in chapter 2.0, section 2.3.

3.3.1 SUBJECT NUMBERS AND POWER CALCULATIONS

The intensity of the metabolic investigations, i.e. the need for subjects to attend on three separate study days at the start and end of each study and especially the study day 3 procedures, which only allowed two subjects to be investigated per day, limited the total numbers of subjects who could be realistically studied. Previous experience at Surrey of the short ITT indicated that an intra-individual variation (6 young men measured on 4 occasions) of 15 %, similar to fasting TG (14.5 %) and cholesterol (10 %), but more than fasting glucose (3 %) and less than fasting NEFA (23 %). This
would allow a 20% difference between diets to be detected by paired analysis with \( n = 12 \) at an error of \( p \leq 0.05 \), with a beta error (possibility of a type 2 error) of < 0.05. Since 12 subjects was identified as the maximum number who could be taken through the complete protocol within a realistic time frame, an a 20% change by the diets was estimated as the minimum likely response, the study number was set at \( n = 12 \).

### 3.4 ANALYTICAL PROCEDURES

Analytical procedures are described in detail in chapter 2.0, section 2.2.3 - 2.2.7 with a brief outline provided in this section.

Plasma glucose concentrations, total cholesterol, HDL cholesterol, and triglycerides were assayed on a COBAS-MIRA autoanalyser (Roche Products Ltd, Welwyn Garden City, Herts) by enzymatic colorimetric methods using commercially available kits supplied by Roche Diagnostic Products Ltd (Welwyn Garden City, Herts). Plasma NEFA concentrations were also measured using the COBAS-MIRA analyser by a standard enzymatic colorimetric method (Wako Chemicals, GmbH, Germany supplied by Alpha laboratories Ltd., London). Appropriate quality controls with predefined reference ranges were run with each assay in order to determine intra and inter assay variability.

Serum insulin levels were measured by radioimmunoassay as described in chapter 2.0, section 2.2.6 Samples from each intervention period were analysed together in the same run at the end of the study period.

The fatty acid composition of red blood cell phospholipids was determined after the extraction of lipids with a mixture of chloroform and methanol (2:1 vol/vol), containing 0.01% BHT as antioxidant, according to the Folch method (Folch et al. 1957). The fatty acids were separated and quantitated using gas chromatography. Fatty acids were identified by comparing retention times with those of a known standard mixture.
Triglyceride-rich and triglyceride poor fractions of plasma were separated using an adapted method of that described by Grundy & Monk (1976). 3.5 ml of plasma was overlaid with an equal volume of saline (1.006g NaCl/ml), and ultracentrifuged for 4.8 X 10^6 g at 24°C in a 10-ml centrifuge tube. The top 1.2 ml of the upper layer, the TRL fraction was removed, aliquoted and frozen at -20°C for later analysis. TG levels were measured in TRL fraction using an enzymatic, colorimetric kit supplied by Roche Diagnostic Products Ltd (Welwyn Garden City, Herts).

Lipoprotein lipase activity was measured in 10 µl portions of plasma taken 5 and 15 minutes after the administration of an intravenous dose of heparin. LPL activity was measured with LPL activity being inhibited with 4 M NaCl by a modified method described by Nilsson-Ehle & Schotz (1976) using the detection of liberated ^3H-labelled free fatty acids from ^3H-labelled triolein substrate emulsion (Amersham International, Bucks, UK) as the index of triolein hydrolysis.

3.5 STATISTICAL ANALYSIS

Statistical analysis was performed using the windows compatible computer package, Statistica (version 5, Statsoft Inc.) and the Excel (Microsoft Excel 97) computer programme.

For most data tabulated results are presented as mean values and standard deviations (SD). Individual time points on graphs are presented as mean values with their standard errors (SE).

The effect of treatment on fasting values was analysed by comparing the results within each individual during the n-3 enriched diet and the control diet using the student’s t test for paired data. Relationships between changes in insulin sensitivity and changes in postprandial lipaemia were analysed by the Pearson’s correlation. The postprandial data was expressed in summary form as an area under the postprandial response curve. Postprandial variations were determined by comparing area under the curve calculations from the control period and the n-3 enriched period. The are under
the postprandial response curves were calculated using the Trapezium rule (Matthews et al. 1990). Values of \( P < 0.05 \) were considered statistically significant.

### 3.6 RESULTS

Eleven subjects of the twelve subjects recruited to the study successfully completed both phases of the dietary intervention. One subject voluntarily withdrew at the end of the first intervention due to personal difficulties. All subjects remained healthy for the duration of the dietary intervention studies. Subjects reported that no difficulties were encountered in the consumption of the study foods or with any of the study day investigations. The results of the study are presented under the following sections.

#### 3.6.1 ANTHROPOMETRY

There was no difference in the weight and subsequently the BMI of subjects at the beginning of the study compared with the end of the control period and the end of the \( n-3 \) enriched dietary period as shown in table 3.2.

<table>
<thead>
<tr>
<th>Table 3.2. Weight and BMI of subjects during the dietary intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Habitual diet</strong></td>
</tr>
<tr>
<td>Weight (Kg)</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD (n = 11).
3.6.2 NUTRITIONAL ANALYSIS

The mean nutrient intakes of the volunteers on their habitual diet and during the n-3 enriched diet period and the control diet period is shown in table 3.3. There were no significant differences in energy on the n-3 enriched and control diet compared with the habitual diet however, there was a significant difference in the protein and carbohydrate intake on the control diet compared to the habitual diet (p< 0.05). The two intervention periods did not differ in their macronutrient content.

Total fat intakes did not differ between the control and n-3 enriched diet however the % of energy from fat was increased from 39.8 (SD 5.0) % during the habitual diet to 43.6 (SD 4.3) % and 44.8 (SD 4.4) % during the n-3 enriched and control diets respectively (p = 0.07; p = 0.01). The proportion of fat from SFA, MUFA and PUFA was significantly different from the habitual to the control and n-3 enriched diets as shown in figure 3.1. The percentage of total fat composed of SFA was significantly reduced (p < 0.001) from 38.1 (SD 5.2) % to 28.0 (SD 1.8) % during the control diet and 28.9 (SD 2.7) % during the n-3 enriched diet. Intakes of PUFA were significantly increased (p < 0.001) from 16.0 (SD 5.1) % of total fat during the habitual diet to 25.0 (SD 2.3) % during the control diet and 24.3 (SD 2.9) % during the n-3 enriched diet. The ratio of n-3 to n-6 polyunsaturated fatty acids was also significantly increased as a result of the n-3 enriched diet when compared to the control and habitual diet (p < 0.001).

The amount of EPA and DHA provided by the n-3 enriched diet was significantly greater (p < 0.0001) when compared to both the control and habitual diet. There was no difference in the amount of EPA and DHA ingested during the habitual diet and the control diet. The estimated average intake of EPA & DHA during the n-3 enriched period was 3.1 g/d, assessed by two 4-day food records.
Table 3.3. The mean daily macronutrient, and EPA + DHA intakes of volunteers (n=11) consuming habitual, control and n-3 enriched diets.

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Habitual diet</th>
<th>n-3 Enriched diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>11975 ± 1430.2</td>
<td>11183 ± 1231.0</td>
<td>11317 ± 1340.0</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>105.6 ± 16.1</td>
<td>91.4 ± 12.9*</td>
<td>89.6 ± 18.7*</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>306.4 ± 33.0</td>
<td>271.6 ± 54.4</td>
<td>269.3* ± 45.6</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>127.8 ± 26.0</td>
<td>129.7 ± 20.0</td>
<td>134.7 ± 20.0</td>
</tr>
<tr>
<td>EPA + DHA (g)</td>
<td>0.2 ± 0.2</td>
<td>3.1 ± 0.3**†</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>n3 : n6 ratio</td>
<td>0.14 ± 0.05</td>
<td>0.23 ± 0.05**†</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD

*P < 0.05; ** P<0.01 Mean values were significantly different from those for the habitual diet.
†P < 0.01 Mean values were significantly different from those for the control diet.

Figure 3.1. Intake of SFA, MUFA and PUFA as a percentage of total fat intakes during the Habitual, Control and n-3 Enriched diet.

P < 0.01 Mean values were significantly different from n-3 enriched and control diet compared to habitual diet.
3.6.3 PHOSPHOLIPID FATTY ACID COMPOSITION OF ERYTHROCYTE MEMBRANES

The phospholipid fatty acid composition of erythrocytes at baseline and at the end of the control and n-3 enriched diet periods are shown in table 3.4. Fatty acids are expressed as a percentage of the total area of all fatty acids measured. Saturated, monounsaturated and polyunsaturated fatty acids did not change between the control and n-3 enriched diets, a finding that is in agreement with the results of the nutritional analysis.

The amount of EPA and DHA was increased from the control diet to the n-3 enriched diet however, this did not reach statistical significance. There was a significant increase in saturated fat and a significant decrease in polyunsaturated fat between baseline and the end of the control and the end of the n-3 enriched periods. Arachidonic acid levels were reduced from baseline both at the end of the control diet and the end of the n-3 enriched diet. There was also a significant decrease in EPA and DHA levels from baseline to the end of the control diet (p = 0.03).

Fatty acids were measured at week 0, week 4 and week 8 of the intervention periods. Figure 3.2. shows the increase in EPA & DHA in individual subjects from week 0 to week 4 of the n-3 enriched diet. The 0 week measurement in one individual was not available and in another individual the 0 week measurement was greater than 2 standard deviations above the mean as highlighted in figure 3.2. using a shaded bar. When those individuals are excluded from the data there was a significant increase (p = 0.01) in the amount of EPA and DHA from 6.35 (SD 2.01) % to 11.31 (SD 5.2) %. The increase in EPA and DHA was not sustained through to week 8 of the intervention period. At week 0, week 4 and week 8 of control diet there was no change in any of the fatty acids measured.
Table 3.4. Fatty acids (% area) measured at baseline and at the end of the control and n-3 enriched diet periods.

<table>
<thead>
<tr>
<th>Fatty acid (% area)</th>
<th>Baseline</th>
<th>Control</th>
<th>n-3 enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td>30.7 ± 1.5</td>
<td>40.8 ± 7.2**</td>
<td>43.1 ± 6.2†</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>15.1 ± 1.1</td>
<td>16.8 ± 6.7</td>
<td>18.8 ± 3.9</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>51.6 ± 2.3</td>
<td>39.2 ± 12.9**</td>
<td>34.5 ± 11.0†</td>
</tr>
<tr>
<td>EPA + DHA</td>
<td>7.4 ± 1.8</td>
<td>4.9 ± 2.7*</td>
<td>6.4 ± 3.8</td>
</tr>
<tr>
<td>Polyunsaturated w-3</td>
<td>7.7 ± 1.8</td>
<td>5.4 ± 2.4*</td>
<td>6.9 ± 3.9</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20.6 ± 3.4</td>
<td>12.9 ± 6.3**</td>
<td>8.6 ± 4.3†</td>
</tr>
</tbody>
</table>

Values are expressed as the percentage of the total area of all fatty acids measured and are presented as means of 11 subjects ± SE.

*P < 0.05, **P < 0.01, mean control values are significantly different from baseline
†P < 0.01, mean n-3 values are significantly different from baseline.

Figure 3.2. EPA + DHA levels individual subjects at week 0 and week 4 of the n-3 enriched diet.

Subjects are ranked in order of increasing levels of EPA + DHA at week 0. The shaded bar highlights an individual with a high level of EPA + DHA at week 0.
3.6.4 FASTING BIOCHEMICAL DATA

The fasting plasma concentrations of lipids, glucose and insulin are shown in table 3.5. There were no significant differences in the concentration of total cholesterol, HDL cholesterol, LDL cholesterol, glucose and insulin when measured at the outset of the study and at the end of the control diet and the \(n\)-3 enriched diet.

The concentration of triglyceride was reduced by an average of 23.2 % from 2.05 (SD 0.65) mmol/l on the habitual diet to 1.58 (SD 0.51) mmol/l at the end of \(n\)-3 enriched period (\(p = 0.06\)). The reduction in the concentration of triglyceride becomes significant (\(p = 0.015\)) on the removal of the result of one individual who’s TG level increased as a result of the \(n\)-3 enriched diet (2.05 ± 0.69 mmol/l Vs 1.44 ± 0.22 mmol/l, \(n = 10\)). Comparing the triglyceride levels at the end of the control period and the end of the \(n\)-3 enriched period in the group overall, TG concentration was lowered but does not become significant (\(p = 0.049\)) until removal of the individual with a raised TG as a result of the intervention diet (1.91 ± 0.73 Vs 1.44 ± 0.22, \(n = 10\)).

There was a significant increase in the concentration of NEFA from 0.35 (SD 0.14) mmol/l on the habitual diet to 0.57 (SD 0.18) mmol/l at the end of the control diet and 0.53 (SD 0.20) mmol/l after the \(n\)-3 intervention period (\(p < 0.01\)).
Table 3.5. Mean fasting plasma concentrations of total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, NEFA, glucose and insulin of male volunteers (n=11) on their habitual diet and after consuming the control diet and the n-3 enriched diet.

<table>
<thead>
<tr>
<th></th>
<th>Habitual diet</th>
<th>Control diet</th>
<th>n-3 Enriched diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.23 ± 1.09</td>
<td>6.15 ± 1.04</td>
<td>5.96 ± 0.89</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.94 ± 0.17</td>
<td>0.93 ± 0.14</td>
<td>0.96 ± 0.14</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.52 ± 1.09</td>
<td>4.46 ± 1.30</td>
<td>4.39 ± 1.11</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>2.05 ± 0.65</td>
<td>1.89 ± 0.70</td>
<td>1.58 ± 0.50</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td>0.35 ± 0.14</td>
<td>0.57 ± 0.18*</td>
<td>0.53 ± 0.20*</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.81 ± 0.64</td>
<td>5.61 ± 0.56</td>
<td>5.69 ± 0.61</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>81.5 ± 25.48</td>
<td>94.32 ± 25.89</td>
<td>95.10 ± 28.87</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD.

* Mean values were significantly different from those for the habitual diet, p<0.01

3.6.5 POSTPRANDIAL DATA

Figure 3.3. shows that the mean postprandial levels of triglyceride were lower following the n-3 enriched diet when compared to the control diet, although this did not reach significance. The postprandial profiles show a mean peak TG at 256 (SD 76.3) min and 229.1 (SD 52.4) min after the control and the n-3 enriched diet respectively. As with the fasting TG results one individual had a marked increase in postprandial TG concentrations following the n-3 enriched diet. When this individual is excluded from the data there was a significant reduction in the peak TG from 4.21 (SD 0.95) mmol/l on the control diet to 3.40 (SD 0.84) mmol/l on the n-3 enriched diet (p = 0.04, n = 10). Fig 3.4. depicts the measurement of TG area under the curve in individual volunteers following the control diet and the n-3 enriched diet. It was observed that in those individuals with the highest area under the TG curve on the control diet (n = 6) there was a significant acceleration in the removal of postprandial TG from the plasma as measured by the AUC following the n-3 enriched diet (1934.4 ± 281.4 Vs 1318.6 ± 274.7, p = 0.008). In contrast, those with the lowest postprandial TG response (n = 5) had no change in the TG area under the curve following the n-3 enriched diet (p = 0.19). Fig 3.5. depicts the relationship between the change
postprandial lipaemia (control TG AUC / n-3 PUFA TG AUC) and the level of fasting TG during the control diet. This indicates that the change in lipaemia following the test meal was dependent on initial fasting TG level (p = 0.005).

Figure 3.3  Total TG concentrations following the test meal (80-g fat) as measured at the end of the control and n-3 enriched diet periods. Data are presented as means ± SE.
Figure 3.4. Postprandial TG response in individual subjects as measured by the area under the curve (mmol/l.min).

Subjects are ranked in order of decreasing lipaemia during the control diet. The patterned bars, highlight a subject who had a marked increase in the TG AUC following the n-3 enriched diet.

Figure 3.5. The association between fasting TG concentration measured during the control diet and the change in postprandial TG ($r = 0.781$, $p = 0.005$).
Fig 3.6. shows the postprandial TRL TG profiles following the n-3 and control intervention periods. As with the total TG profile the peak TG was later on the control diet at 261.8 (SD 90.1) min compared with 234.5 (SD 62.7) min on the n-3 enriched diet. There was no significant difference in the levels of postprandial TRL between the control and n-3 enriched diets when measured by the total area under the curve. However, in the late postprandial phase as measured by the 6-9 hour area under the curve the concentration of TRL TG was significantly attenuated on the n-3 enriched diet from 504.7 (SD 255.9) mmol/l.min during the control diet to 360.4 (SD 186.8) mmol/l.min on the n-3 enriched diet (p = 0.037). Fig 3.7 shows the postprandial apo-B48 concentrations in individual subjects, measured by the total area under the curve (AUC), following the control and the n-3 intervention diet. There was no difference in the mean concentrations of apo B-48 in the group overall. However, as with the postprandial TG response there was a significant attenuation of apo B-48 levels in those with the greatest apo B-48 AUC during the control diet (706.2 ±207.6 μg/ml./hr (control) Vs 460.4 ± 180.5 μg/ml.hr (n-3), n = 7) and is demonstrated by the correlation between apo B-48 AUC during the control diet with the change in apo B-48 concentration following the intervention diet (r = 0.77, p = 0.005) shown in figure 3.8.

Figure 3.6. Postprandial concentrations of TG in the TG-rich lipoprotein (TRL) fraction after the consumption of a test meal containing 80 g fat as measured at the end of the control and n-3 enriched intervention periods.

Data are presented as means of 11 subjects ± SE.
Apo B-48 concentration in individual subjects as measured by the AUC (μg/ml.min).

Subjects are ranked in order of decreasing apo B-48 AUC during the control diet.

The association between the change in apo B-48 AUC and the apo B-48 AUC during the control diet ($r = 0.77$, $p = 0.005$).
Fig. 3.9. shows the postprandial NEFA profiles. They followed an expected contour, dipping sharply after the consumption of the test meal and gradually rising to a concentration almost twice that above fasting levels by 9 hours at the end of the study. From the fasting level the fall in NEFA at 30-min following the test meal was significantly lower (p = 0.02) on the n-3 enriched diet (0.32 ± 0.11 mmol/l) compared to the control diet (0.43 ± 0.02 mmol/l).

![Graph](image-url)

**Fig 3.9.** The concentrations of NEFA following the test meal (80-g fat) as measured at the end of the control and n-3 enriched diet period. Data are presented as means of 11 subjects ± SE.

Fig 3.10. shows the postprandial insulin response at the end of the control diet and at the end of the n-3 enriched diet. As expected the insulin levels increase sharply following the test meal and return to baseline levels at the end of the 9-hour test. There was no significant difference in the insulin AUC at the end of the n-3 enriched period and the end of the control period.

There was no difference in postprandial levels of glucose or total cholesterol between the control diet and the n-3 enriched diet as depicted in fig. 3.11.
Figure 3.10. Postprandial insulin levels, following an 80-g fat meal.
Data are presented as means of 11 subjects ± SE.

Figure 3.11. Change in the glucose and cholesterol total AUC (mmol/l.min)
measurements from the control diet to the n-3 enriched diet.
Data are means of 11 subjects ± SE.
3.6.6 POST-HEPARIN LIPOPROTEIN LIPASE ACTIVITY

The effect of the $n$-3 enriched diet on post-heparin LPL activity 5 and 15 minutes following heparin administration is shown in figs 3.12 and 3.13, respectively. There was a wide range of LPL activity among the study group ranging from 1.41 to 8.21 umole.oleate.ml.hr measured at the 15-minute time point during the control diet. As expected, the amount of LPL released into the plasma was increased from 5 to 15-minutes following the heparin administration. There was no significant difference in plasma post-heparin LPL activity at the 5 or 15-minute measurement between the control diet and the $n$-3 enriched diet.

The mean total lipase activity, HL activity and LPL activity 15 minutes following heparin administration is shown in figure 3.14. There was no difference in the levels of lipase activity following the $n$-3 enriched diet.

There was also no association between fasting TG levels and the level of LPL activity measured during the control diet as shown in figure 3.15.

![Graph showing LPL activity levels](image)

**Figure 3.12.** 5-minute post-heparin LPL activity levels in individual subjects measured at the end of the control diet and the end of the $n$-3 enriched diet.

Subjects are ranked in the order of increasing LPL activity during the control diet.
Figure 3.13. 15-minute post-heparin LPL activity levels in individual subjects measured at the end of the control diet and the end of the *n*-3 enriched diet.

Subjects are ranked in the order of increasing LPL activity during the control diet.

Figure 3.14. Mean total lipase, HL & LPL activity (umole.oleate.ml.hr) levels, 15 minutes following the administration of 7500 IU of heparin to 11 subjects.

The vertical bars represent standard error bars.
3.6.7 INSULIN SENSITIVITY DATA

Linear regression was used to estimate the fall in log transformed blood glucose concentration following the administration of a bolus dose of insulin during the insulin tolerance test. The slope was multiplied by $-100$ to derive the rate constant ($K_{ITT}$) and by $60$ to determine the percentage decline in blood glucose per minute ($\%$ min$^{-1}$). Four minutes following intravenous injection of insulin, blood glucose levels began to decline as shown in fig 3.16. The glucose disappearance rate $K_{ITT}$ was therefore estimated from the slope of the fall in glucose from 4-15 minutes. It was observed that the concentration of glucose was higher following the $n$-3 enriched diet at the 4 minute measurement when compared to the control diet ($5.65 \pm 0.73$ mmol/l Vs $5.18 \pm 0.56$ mmol/l, $p = 0.006$). The mean plasma glucose concentration fell from 5.38 (SD 0.54) mmol/l to 3.78 (SD 0.87) mmol/l during the control diet and from 5.64 (SD 0.54) mmol/l to 3.98 (SD 0.86) mmol/l during the $n$-3 enriched diet following insulin administration.

The results of the short insulin tolerance test in individual subjects at the end of the control diet and at the end of the $n$-3 enriched diet are presented in fig 3.17. Subjects are ranked in the order of increasing insulin sensitivity on the control diet. There was
a wide range of insulin sensitivity in the study group as measured during the control period, with $K_{ITT}$ glucose varying from 1.19 to 6.16 % min$^{-1}$ (mean = 3.19 ±1.77) % min$^{-1}$). While there was no difference in insulin sensitivity as a result of the intervention in the group overall, it was observed that in the 7 least insulin sensitive subjects during the control diet, there was a significant improvement in insulin sensitivity from 2.07 (SD 0.85) % min$^{-1}$ to 3.52 (SD 0.95) % min$^{-1}$ as a result of the $n$-3 enriched diet ($p = 0.009$). There was no change in glucose $K_{ITT}$ measurements in the 4 most insulin sensitive subjects as a result of the $n$-3 enriched diet ($p = 0.106$).

**Figure 3.16.** Fall in blood glucose concentrations over 15 minutes following the administration of a bolus dose of insulin (0.1 U/Kg) at the end of the control diet and at the end of the $n$-3 enriched diet.

Data are means ± SE.
There was an inverse correlation between the fasting concentration of insulin and the degree of insulin resistance measured during the control diet as shown in fig 3.18. This indicates that fasting insulin was a good indicator of insulin resistance in this study group. Those subjects with a fasting insulin level greater than 90 pmol/l group were the least insulin sensitive and had the greatest improvement in insulin sensitivity as a result of the n-3 intervention. Those with the greatest improvement in insulin sensitivity also had an initial $K_{ITT}$ of less than 4.7 % min$^{-1}$. 

**Figure 3.17.** Effect of the n-3 enriched diet on Insulin sensitivity as measured by glucose $K_{ITT}$ (% min) in individual subjects.

Subjects are ranked in order of the least insulin sensitive during the control diet.
Figure 3.18. The association between fasting insulin concentration (pmol/l) and insulin sensitivity (Glucose $K_{ITT}$ % min$^{-1}$) measured during the control diet ($r = -0.0779$, $p = 0.005$).

The fractional rate of fall in plasma NEFA levels between 4 and 15 minutes following insulin administration served as a measure of insulin mediated inhibition of hormone sensitive lipase. The rate at which plasma NEFA levels fell was accelerated following the $n$-3 enriched diet as shown in fig 3.19, however this did not reach statistical significance.

In comparing lipase activity with measurements of insulin sensitivity, no association between LPL and activity and insulin sensitivity was observed, however as shown in fig 3.20, there was an inverse correlation between levels of HL activity and the degree of insulin sensitivity.
Figure 3.19. Difference in the fall in log transformed NEFA (%/min⁻¹) from the control diet to the n-3 enriched diet. Data are presented as means of 11 subjects ± SE.

Figure 3.20. The association between levels of hepatic lipase activity and insulin sensitivity measured during the control diet (r = -0.613, p = 0.045).
3.6.8 POSTPRANDIAL LIPAEMIA AND INSULIN SENSITIVITY

In comparing the results of the insulin tolerance test and the postprandial test, it was shown that there was no association between changes in insulin sensitivity and changes in postprandial lipaemia. Fig 3.21 shows the lack of association between the change in TAG AUC and the change in glucose $K_{ITT}$ following the $n$-3 enriched diet.

There was also no association between the extent of postprandial lipaemia and measures of insulin sensitivity i.e. those individuals who were most insulin resistant were not the subjects with the greatest degree of postprandial lipaemia. When the individuals who had the greatest improvement in insulin resistance ($n = 7$) are analysed separately there was in fact an inverse correlation between the extent of postprandial lipaemia and the degree of insulin resistance as shown in fig 3.22. Since insulin resistance has been shown to be associated with high fasting TG values this finding is contradictory to the expected relation between measures of lipid metabolism and insulin sensitivity.

![Graph](image-url)  

**Figure 3.21.** The relationship between the change in insulin sensitivity and the change in postprandial lipaemia measured by the TG AUC ($n = 11$) as a result of the $n$-3 enriched diet ($r = 0.0656$, $p = 0.84$).
Figure 3.22. The association between measures of insulin sensitivity and postprandial lipaemia during the control diet in the 7 least insulin sensitive subjects ($K_{ITT} < 4.74 \% \text{ min}^{-1}$) ($r = 0.743$, $p = 0.035$).
3.7 DISCUSSION

This study is one of the few studies to investigate the effect of foods supplemented with $n$-3 PUFA on postprandial lipaemia and one of the first to analyse the effect of consuming such foods on insulin sensitivity as measured by the short insulin tolerance test. The use of enriched foods as a means of increasing the EPA and DHA content of diets was used in conjunction with a modest fish oil capsule supplementation (two 1-g capsules per day). This offered an alternative to the use of an essentially pharmacological intervention where fish oil capsules alone are provided as a means of increasing EPA & DHA intakes. The data could also provide valuable information as to the impetus of such foods on improving insulin resistance if available for consumption in the free-living situation. Previous work in this research group in which foods alone had been used as a means of increasing EPA & DHA intakes highlighted that ‘a greater level of incorporation of EPA & DHA into the foods would be preferable for future studies’ (Lovegrove et al. 1997). This observation was based on the finding that a moderate intake (1.4 g/d) of EPA & DHA given in the form of enriched foods did not yield the expected hypotriglyceridaemic effect in a group of male volunteers. The aim of the present study was to achieve an intake of between 2-3 g EPA & DHA daily, of which 1.02 g was to be provided from two 1-g fish oil capsules. The addition of fish oil capsules therefore ensured that a potent dose of EPA & DHA would be consumed.

Analysis of the volunteers’ diets, calculated from a 4-day food record, revealed that the average intake of EPA & DHA achieved during the $n$-3 enriched intervention was 3.1 g/d. There was no change in average energy intakes between the habitual and the control and intervention diets, a finding reflected in the stability of the volunteers’ body weight throughout the study. The lack of change in energy intakes also indicates that volunteers were successful in substituting ‘study’ foods for the foods normally consumed in order to achieve an increase in the levels of EPA & DHA.

The phospholipid fatty acid composition data is somewhat contradictory to the findings of the dietary analysis. Dietary analysis showed a decrease in the intake of SFA and an increase in the intake of dietary PUFA from the habitual to the intervention diets, whereas the analysis of membrane phospholipids demonstrated a
fall in the levels of polyunsaturated fatty acids and an increase in the levels of saturated fatty acids. The erythrocyte membrane phospholipid levels of EPA & DHA were greater but not significantly increased at the end of the n-3 enriched period compared to the end of the control period. However, it was observed that EPA & DHA levels were significantly higher at week 4 compared to week 0 of the n-3 intervention period, indicating that the enriched foods were being consumed. The fact that the increase in the enrichment of EPA & DHA was not sustained through to week 8 of the intervention may be a reflection of diminishing volunteer compliance. However, this possibility is not supported by the analysis of food records. Harris et al. (1991) have reported a peak level of fish-oil incorporation into platelet phospholipids after supplementation for one month, with various doses of fish oil, with no increase for the subsequent 5 months of supplementation. Measurement of membrane phospholipids also showed that there was a significant reduction in arachidonic acid levels from the habitual to the n-3 enriched diet. Carlson et al. (1987) have shown that feeding fish oil to n-3 deficient animals, reduces the plasma and red cell phospholipid content of arachidonic acid and there has been a suggestion that fish oil may inhibit n-6 fatty acid metabolism (Innis et al. 1991). Inhibition of arachidonic acid production and incorporation into membrane phospholipids may have a potentially antithrombotic effect (Heemskerk et al. 1996).

The n-3 intervention did not result in the expected reduction in triglyceride levels in the group overall. Other studies have shown a hypotriglyceridaemic effect at lower doses of EPA & DHA in both normotriglyceridaemic and hypertriglyceridaemic subjects, however none of the studies have used foods as the vehicle of supplementation. Our findings are in agreement with other studies in which functional foods have been used, in that they also demonstrated an overall lack of effect on triglyceride levels, although the types of foods used and the level of supplementation was lower in both studies (Lovegrove et al. 1997; Roche & Gibney, 1994). One subject had an increase in triglyceride levels following the n-3 enriched diet, which has masked the finding that triglyceride levels were significantly reduced in the remainder of the group (n = 10) at the end of the n-3 enriched diet when compared to the habitual and the control diet. The reduction in plasma triglyceride levels associated with fish oil supplementation is thought to be as a result of a decrease in hepatic VLDL synthesis and/or an accelerated rate of VLDL removal. Animal studies
have demonstrated that n-3 fatty acids inhibit fatty acid esterification to glycerol (Wong et al. 1989) and blunt the stimulatory action of insulin on hepatic lipogenesis (Topping et al. 1987). Human studies appear to agree with these findings and lipoprotein kinetic studies in normal subjects indicate that VLDL synthesis and secretion are decreased by fish oil administration (Harris et al. 1990). The reduction in VLDL synthesis is thought to be due to an inhibitory effect of n-3 fatty acids on the esterification of glycerol.

No differences in the fasting concentration of other lipoproteins such as total, LDL and HDL-cholesterol levels as a result of the fish oil supplementation was observed, a finding consistent with other dietary interventions of this nature. Studies observing effects on LDL and HDL-cholesterol levels as a result of fish oil feeding have been criticised by a failure to control the intake of other dietary fatty acids (Malasanos & Stacpoole, 1991). Increases in LDL cholesterol level have been reported but as Harris (1989) summarises, when saturated fat intake was held constant in dietary trials, LDL-cholesterol rose by 5% but when fish oil replaced saturated fats LDL cholesterol fell by 5%. It has been reported that fish oil intervention in placebo-controlled trials generally increases HDL-cholesterol levels by 5-10% (Harris, 1989). The response of HDL-cholesterol appears to be dependent on the quantity of fish oil administered because higher doses were associated with an increase of HDL-cholesterol levels whereas lower doses had a negligible effect.

Several studies with glucose intolerant and type II diabetic subjects have reported an increase in blood glucose levels following fish oil supplementation. Lovegrove et al. (1997) also reported an increase in fasting glucose levels following the consumption of manufactured foods enriched with EPA & DHA. In the present study there was no effect of fish oil feeding on fasting blood glucose levels however glucose measurements taken during the insulin tolerance test showed an increase in glucose levels at the 4 minute time point following insulin administration. Fasting insulin levels were also unchanged as a result of the intervention. In studies reporting plasma insulin concentrations, fish oils have not usually altered fasting levels. Few studies reporting an increase in blood glucose levels have reported a concomitant decline in the fasting levels of insulin making a direct inhibitory action of n-3 fatty acids on the β-cell an unlikely explanation for the observed rise in blood glucose concentration.
However, the failure of insulin to respond to elevated glucose levels could be interpreted as a deficiency in the response of the $\beta$-cell. Alternatively Glauber et al. (1988) and Friday et al. (1989) suggest that $n$-3 fatty acids may impair the insulin secretory response to hyperglycaemia.

While a large number of studies have looked at the effects of fish oil supplementation on fasting TG levels, fewer although a continually increasing number have investigated the postprandial effects of fish oil feeding (Harris et al. 1988; Weintraub et al. 1988). In this study it appears that the improvements in postprandial triglyceride concentrations as a result of the $n$-3 enriched diet occurred in those subjects who had the greatest degree of postprandial lipaemia at the outset of the study. Postprandial response was also closely related to the baseline fasting TG concentration. Studies examining the effect of fish oils in NIDDM have also shown variable triglyceride responses, and in a study by Kasim et al. (1988) a significant hypotriglyceridaemic effect was observed in a group of NIDDM patients only when the subjects with the highest baseline TG levels were isolated. Harris et al. (1988) also demonstrated that in a group of non-diabetic hypertriglyceridaemic subjects, the higher the initial TG level the greater the fall as a result of fish oil administration. Similarly Connor et al. (1993) found that fish oil incorporated into the diet of hypertriglyceridaemic subjects had a more profound hypolipaemic effect than had been observed in normal subjects.

In this study while the concentration of TRL TG was not significantly attenuated over the entire 9-hour postprandial study there was a significant lowering of TRL TG in the late (6-9 hour) postprandial phase. Elevations in late postprandial triglyceride levels have been identified as a significant risk factor for CHD in a number of case control studies (Groot et al. 1991; Patsch et al. 1992) and in a study of the offspring of subjects with CHD (Uiterwaal et al. 1994). Karpe (1997) and other investigators have highlighted that an accentuated profile in the late postprandial phase is potentially more atherogenic. Measurement of apo B-48 concentration showed no difference over the nine-hour postprandial study as a result of fish oil feeding. Studies in which fish oil was fed to healthy volunteers have demonstrated that $n$-3 fatty acids are incorporated into chylomicrons. The decrease in the late TRL-TG concentrations following fish oil administration could have resulted from slower synthesis of
chylomicrons, and slower entry of chylomicrons into the circulation or, alternatively, from a more rapid removal of the chylomicrons in the circulation.

Post-heparin LPL activity was unaffected by the fish oil intervention in the present study. It is unlikely therefore that the decrease in TG concentrations were as a result of an increase in LPL mediated lipolysis. Studies, which have examined the effect of fish oil feeding on LPL activity, have shown variable results. In general earlier studies have shown no effect in humans (Harris et al. 1988; Weintraub et al. 1988; Nozaki et al. 1991). A study by Zampelas et al. (1994) reported an increase in post-heparin LPL activity following the consumption of a fish oil enriched meal. Kasim et al. (1995) also reported an elevation in post-heparin LPL activity with fish oil enrichment of the background diet and Harris et al. (1997) have shown increases in endogenous lipase activities following 3 weeks of fish oil feeding. A deeper understanding of the effects of fish oil enriched diets on LPL activity is required before any firm conclusions on the mechanisms of n-3 TG lowering are made.

Plasma NEFA concentrations are usually unchanged as a result of fish oil feeding. In this study while fasting levels of NEFA were not altered there was a significant lowering of NEFA levels at 30 min postprandially following the n-3 enriched intervention. Elevation of NEFA and in particular postprandial NEFA levels is recognised as being unfavourable in terms of increasing atherogenic risk (Frayn, 1998). The reduction in NEFA could indicate that in the early postprandial phase there was a greater suppression of insulin induced lipolysis following the n-3 enriched diet however, the mechanisms of action of NEFA are not fully understood and the effect of fish oil feeding requires greater investigation.

Stronger evidence of the benefits of fish oil feeding on insulin sensitivity are provided by animal studies as opposed to human studies. The mechanism by which insulin sensitivity is improved by fish oil feeding in animals is thought to be via the incorporation of n-3 PUFA into membrane phospholipids. It is postulated that the induction of changes in membrane structure and fluidity of cells as a result of fish oil feeding could influence insulin receptor binding as well as insulin action. Alternatively a change in the conformation of diacylglycerols, as a result of fish oil feeding, could increase their potency as secondary messengers to insulin (Luo et al.
In addition, the association of n-3 fatty acids with prostaglandin and leukotriene production could secure changes in insulin secretion (Mori et al. 1992). The discrepancy between human and animal studies with regard to changes in insulin sensitivity may be as a result of the way in which fatty acids are incorporated into the various classes of phospholipids. In humans, fish oil intake does not affect the phosphatidyl-inositol composition. As phosphoinositides have been implicated as secondary messengers in the insulin signal transduction this may explain the lack of a clear beneficial effect of fish oil consumption on insulin sensitivity in humans (Malasanos & Stacpoole 1991).

In the measurement of insulin sensitivity in this study the principal observation was that insulin sensitivity was improved only in individuals with the greatest degree of insulin resistance during the control diet. This indicates that fish oil feeding may be of greatest benefit to those with the lowest insulin sensitivity. In this study subjects were selected on the basis of fasting insulin levels. Fasting levels of insulin were subsequently shown to be a good indicator of the degree of insulin resistance in this group. The subject selection criteria included a fasting insulin greater than 40 pmol/l however the subjects who had the lowest insulin sensitivity all had a fasting insulin concentration greater than 90 pmol/l. In a recent study by Graci et al. (1999) a $K_{ITT}$ value of less than or equal to 4.8 % min$^{-1}$ identified a cluster of insulin resistance related abnormalities with an accuracy of 82 %. In this study we identified that the greatest improvement in insulin sensitivity as a result of the n-3 enriched diet occurred in subjects with a $K_{ITT}$ value of less than 4.7 % min$^{-1}$. Our findings contrast to that of Graci et al. (1999) in that in this study we could find no association between improvements in insulin related lipid abnormalities and improvements in insulin sensitivity. From the results of this study it would appear that the link between pathways involved in the improvement of insulin sensitivity of glucose disposal and lipid tolerance as a result of increasing n-3 PUFA intake, are not clear cut and warrant further investigation. Baseline assessment of insulin sensitivity or selection of subjects with higher fasting insulin levels would be advisable in future studies to examine the effect of fish oil feeding on insulin sensitivity. Also a greater number of studies, involving direct measurements of insulin sensitivity are required to gain a clearer picture of the effect of fish oil feeding on improving insulin tolerance in humans.
CHAPTER 4.0

EFFECT OF A REDUCED SFA AND INCREASED CHO INTAKE ON INSULIN SENSITIVITY AND POSTPRANDIAL LIPAEMIA

4.1 BACKGROUND

Dietary recommendations for the general public and in particular for those with type II diabetes advocate a reduction in total and SFA and an increase in the intake of complex CHO. Low-fat, high-CHO diets have been recommended for several reasons but were primarily based on the effect of such diets on total plasma cholesterol concentrations. Epidemiological observations have further endorsed the benefit of consuming such diets in that countries with higher intakes of fat and lower intakes of CHO have a higher prevalence of diabetes and obesity (West & Kalbfleisch, 1971).

Two main arguments have been used against the use of a high-CHO diet. The first relates to the effect of low-fat, high-CHO diets on serum lipoproteins. While there is little doubt that low-fat diets can lower total plasma cholesterol concentrations, it is now known that part of this reduction includes a fall in HDL-cholesterol levels. Lowering of HDL-cholesterol by a high-CHO diet was reported by Levy et al. (1966) and has been a continual finding in such dietary interventions since (Turley et al. 1998; Vélez-Carrasco et al. 1999). Katan (1998) combined data from 27 trials and showed that HDL-cholesterol fell by 0.012 mmol/l for every 1% of energy that was replaced by carbohydrates. HDL-cholesterol has been shown to be a protective factor against CHD (Miller & Miller, 1975). A reduction in HDL-cholesterol levels would therefore be unfavourable in terms of CHD risk. In addition, an abundance of evidence from metabolic trials has consistently pointed to the hypertriglyceridaemic effect of low-fat and high-CHO feeding (Mensink & Katan, 1992). It is thought that increasing the CHO content as a percentage of total calories in isocaloric diets increases TG levels as a result of increased synthesis of hepatic VLDL-TG particles. It has also been shown that low-fat, high-CHO diets can accentuate postprandial TG levels (Chen et al. 1995). Higher fasting and postprandial TG levels are associated
with an increased risk of atherosclerotic disease (Mamo et al. 1995; Hokanson & Austin, 1996).

The second main argument relates to the effect of high-CHO feeding on insulin and glucose responses. As pointed out by Reaven (1997) it would be expected that if dietary CHO intake is increased a greater postprandial insulin excursion must be secreted in order to maintain glucose homeostasis and thus may lead to impairment in insulin action in vulnerable individuals. While some early clinical studies suggested that high-CHO diets increased basal insulin concentrations (Grey & Kipnis, 1971) there has been little further evidence to support the view that high-CHO feeding is detrimental to insulin and glucose function. In a study by Ginsberg et al. (1994) no significant differences in 8-hour excursions of glucose or insulin were demonstrated between individuals receiving a high-MUFA diet and those receiving a high-CHO diet. Similarly in a group of NIDDM patients no significant differences in fasting glucose or insulin levels following a high-CHO diet (55% energy) was observed (Garg et al. 1994). In addition, greater insulin levels have been observed in those habitually consuming diets high in SFA compared to individuals consuming low-fat, high-CHO diets (Maron et al. 1991; Marshall et al. 1996).

Recommendations for Western countries advise that fat intakes be reduced in most cases to 30% of energy, and that CHO intakes be increased to at least 50% of energy. Emphasis is generally placed on the increase being in the form of starchy foods or complex CHO. In developing countries CHO intakes contribute 60-70% of total energy with corresponding low intakes of fat. It is well known that the incidence of CHD and diabetes is lower in developing countries but as pointed out by Katan (1997) the type of foods consumed in these countries is very different to the low-fat products available in supermarkets today. Dietary studies, which have looked at the metabolic effects of low-fat, high-CHO diets, have varied in the amounts and types of CHO ingested and in the duration of the low-fat, high-CHO feeding. Data is also limited by the fact that measurement of CHO and its components is more difficult than measurement of fat, as CHO in the diet is not a single entity. Increases in CHO intake have varied from 50% to 70-80% of total energy intake and have included CHOIs of varying composition. It has been suggested that improvements in insulin action are observed only in diets extremely rich in CHO (70-80% energy) however these diets
are not practical (Garg et al. 1992b). When changes in CHO intake are restricted to a range typically consumed in a Western society there is less agreement as to the effect on insulin sensitivity. Variations in the type and composition of dietary CHO consumed may therefore account for some of the differences that have been observed in the metabolic outcomes of these types of dietary interventions.

4.1.1 STUDY OBJECTIVES

Although epidemiological studies have suggested that a high-fat diet results in obesity and IR, few studies have measured insulin sensitivity directly. In addition, it is difficult to extract firm conclusions about the effects of low-fat and high-CHO diets on insulin sensitivity from previous studies as they have varied considerably in terms of subject characteristics, dietary components altered and the techniques employed to measure insulin sensitivity. The purpose of this study therefore was to investigate the metabolic consequences, in particular the effect on insulin sensitivity, of replacing energy derived from SFA with energy derived from dietary CHO in 12 middle-aged insulin resistant men. The aim of the dietary intervention was to reduce SFA intakes to the recommended amount of less than 10% of energy intake, while increasing the intake of CHO from all dietary sources in order to maintain habitual energy intakes. Investigations included measurement of red blood cell membrane phospholipids, fasting and postprandial levels of plasma lipoproteins, insulin and glucose levels as well as measurement of insulin sensitivity using the short insulin tolerance test.

4.2 STUDY DESIGN AND SUBJECTS

4.2.1 EXPERIMENTAL DESIGN

The study design was a single dietary intervention with the subjects’ pre-intervention diet acting as a control phase. Thus a set of metabolic investigations was conducted on recruitment into the trial, prior to any dietary advice. On completion of these investigations advice was given on how to reduce the intake of SFA. Subjects then adhered to a low-saturated-fat diet for a period of 8 weeks, at the end of which the second set of metabolic investigations was conducted. The metabolic studies conducted pre- and post-diet were identical to those described in the fish-oil trial
(chapter 3.0) i.e. three study days involving 1) Fasting blood biochemistry and the short ITT; 2) a 9 hr study postprandial study of lipid disposal and 3) a 9hr stable isotope study of NEFA/glycerol suppression by insulin infusion and fasting proteolysis inhibition by postprandial insulin and amino acids. The stable isotope results are presented in appendix 1 but are not discussed further here.

In addition fasting blood samples were collected during the study at weeks 0, 4 and 8 and were analysed for TG, total cholesterol, HDL cholesterol, glucose, insulin and red blood cell phospholipid fatty acids.

4.2.1.1 Subject numbers and power calculations

As with the fish oil trial a cohort of 12 was established on the basis of feasibility and the short ITT variability (see section 3.2)

4.2.2 SUBJECTS

The study was approved by The Ethics Committee of the Royal Surrey County Hospital and the University of Surrey, Guildford. The study days were carried out in the Clinical Investigation Unit of the Royal Surrey County Hospital and all volunteers provided informed consent before participating in the trial.

30 subjects were recruited to the study via advertisements in the local media in the Guildford area for screening on the basis of a fasting blood sample, anthropometric and dietary/lifestyle criteria i.e. glucose < 7.0 mmol/l, total cholesterol < 7.0 mmol/l, TG < 3.0 mmol/l, insulin > 40 pmol/l and haemoglobin > 130g/l. Volunteers also had a waist circumference greater than 100 cm. The screening biochemical and anthropometric data are shown in table 4.1.

Twelve healthy male volunteers were selected, all non-smokers with a low level of physical activity and they all habitually consumed at least 15% of energy as SFA. All of the volunteers were free from medication and did not have any previous medical history of coronary artery, endocrine or liver disease. None of the volunteers were
following any type of therapeutic or specialised diet or taking any form of dietary supplements.

Table 4.1. *Baseline Characteristics of the study volunteers.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>44.2 ± 4.8</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>27.9 ± 2.6</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>104.3 ± 4.9</td>
</tr>
<tr>
<td>Total plasma cholesterol (mmol/l)</td>
<td>5.62 ± 1.11</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.16 ± 0.97</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.57 ± 0.17</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.97 ± 0.59</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>71.2 ± 44.2</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.44 ± 0.36</td>
</tr>
</tbody>
</table>

Data are presented as the mean of 12 subjects ± SD

4.3 DIETARY INTERVENTION

Upon entry into the study each volunteer completed a seven-day food record, as used in the Epic study, to determine usual dietary intakes of energy, CHO and fat (Bingham *et al.* 1997). Nutritional analysis was completed using the FOODBASE 1.2 nutrient analysis programme (Institute of Brain Chemistry and Human Nutrition, London). On the basis of their habitual intakes volunteers were given individual dietetic advice on how to reduce SFA and increase CHO in their diet, whilst maintaining their usual energy intake. SFAs were derived predominantly from dairy products, meat and meat products, and convenience foods. Commonly eaten foods were used in the intervention, and the volunteers were provided with low-fat dairy products and commercially available low-fat convenience snacks. Volunteers were provided with a list of CHO-rich food portions, each equivalent to 10 g of CHO.
Volunteers were asked to consume 3-4 portions of these foods daily in addition to their normal food intake in an attempt to maintain habitual energy levels. The amount of CHO which volunteers consumed was adjusted throughout the intervention on the basis of volunteers weight measurements.

During the intervention volunteers attended the Nutrition Department at the University of Surrey weekly for the first two weeks and once every two weeks thereafter. Weight measurements were taken at each visit and volunteers were provided with low-fat foods.

At week 3 and week 7 of the intervention volunteers were asked to complete a 7-day food record in order to monitor compliance and to analyse the nutrient composition of the intervention diet.

### 4.4 ANALYTICAL PROCEDURES

All procedures are described in detail in Chapter 2.0 with a brief outline of the general procedures provided here.

Plasma glucose concentrations, total cholesterol, HDL cholesterol, and TGs were assayed on a COBAS-MIRA autoanalyser (Roche Products Ltd, Welwyn Garden City, Herts) by enzymatic colorimetric methods using commercially available kits supplied by Roche Diagnostic Products Ltd (Welwyn Garden City, Herts). LDL cholesterol concentration was estimated using the Friedewald equation (Friedewald, 1972). Plasma NEFA concentrations were also measured using the COBAS-MIRA analyser by a standard enzymatic colorimetric method (Wako Chemicals, GmbH, Germany supplied by Alpha laboratories Ltd., London). Appropriate quality controls with pre-defined reference ranges were run with each assay in order to determine intra and inter assay variability. The inter and intra assay CV are outlined in Chapter 2.0 section 2.1. Serum insulin levels were measured by radioimmunoassay. Samples from each intervention period were analysed together in the same run at the end of the study period.
The fatty acid composition of red blood cell phospholipids was determined after the extraction of lipids with a mixture of chloroform and methanol (2:1 vol/vol), containing 0.01% BHT as antioxidant, according to the Folch method (Folch et al. 1957). The fatty acids were separated and quantitated using gas chromatography. Fatty acids were identified by comparing retention times with those of a known standard mixture.

Triglyceride-rich and triglyceride poor fractions of plasma were separated using an adapted method of that described by Grundy & Monk (1976). 3.5 ml of plasma was overlaid with an equal volume of saline (1.006 g NaCl/ml), and ultracentrifuged for 5.0 X 10^6 g at 24°C in a 10-ml centrifuge tube. The top 1.2 ml of the upper layer, the TRL fraction was removed, aliquoted and frozen at -20°C for later analysis. The remaining TPL fraction was mixed, aliquoted and stored at -20°C for future analysis. TG levels were measured in TRL fractions using an enzymatic, colorimetric kit supplied by Roche Diagnostic Products Ltd (Welwyn Garden City, Herts). Apo B-48 was assayed in the TRL fraction using an antiserum specific to apo B-48. The method used was as previously described by Lovegrove et al. (1996).

Lipoprotein lipase activity was measured in 10 µl portions of plasma taken 5 and 15 minutes after the administration of an intravenous dose of heparin. LPL activity was measured by a modified method described by Nilsson-Ehle & Schotz (1976).

4.5 STATISTICAL ANALYSIS

For most data tabulated results are presented as mean values and standard deviations. Individual time points on graphs are presented as mean values with their standard errors.

Statistical analysis was performed using the windows compatible computer package, Statistica (version 5, Statsoft Inc.) and the Excel (Microsoft Excel 97) computer programme.
The effect of the low SFA diet on fasting and postprandial values was analysed using the student's t test for paired data. The postprandial data was expressed in summary form as an area under the postprandial response curve. The areas under the postprandial response curves were calculated using the Trapezium rule (Matthews et al. 1990). Relationships between study variables were analysed by the Pearson's correlation. Values of $P < 0.05$ were considered statistically significant.

4.6 RESULTS

4.6.1 NUTRITIONAL ANALYSIS

Data for weight, energy and macronutrient intake are presented in table 4.2. A significant weight loss (mean of 1.7 Kg) was observed within the study group following the low-SFA intervention ($p < 0.01$).

Energy intakes were lower following the low-SFA / high-CHO. There was a significant reduction in the proportion of energy derived from fat ($p < 0.0001$) and a significant increase in the proportion of energy derived from CHO ($p < 0.0001$). In conjunction with the increase in CHO there was also a significant increase in the intake of dietary fibre (Englyst method) as a result of the intervention diet. SFA levels fell from an average of 16.3 (SD 3.0) % to 7.9 (SD 1.6) % of total energy intake ($p < 0.0001$). There was also a significant reduction in MUFA levels from 14.3 (SD 2.3) % to 8.9 (SD 1.7) % of energy intake as a result of the intervention ($p < 0.0001$) with no change in the intake of PUFA. No difference in the percentage of energy from alcohol was observed during the intervention.

The change in weight (pre-intervention weight/ post-intervention weight) as a result of the intervention diet was positively correlated with the change in energy intake (pre-intervention/ post-intervention) as well as the extent of the reduction in total fat intake as shown in figs 4.0, and 4.1, respectively.
Table 4.2.  *Weight and nutrient intakes before and after low SFA intervention*

<table>
<thead>
<tr>
<th></th>
<th>Pre-intervention diet</th>
<th>Intervention diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (Kg)</td>
<td>90.2 ± 9.7</td>
<td>88.5 ± 10.1**</td>
</tr>
<tr>
<td>Energy (MJ)</td>
<td>13.1 ± 2.2</td>
<td>11.9 ± 2.4</td>
</tr>
<tr>
<td>Protein (% energy)</td>
<td>13.0 ± 1.9</td>
<td>16.6 ± 2.0**</td>
</tr>
<tr>
<td>Carbohydrate (% energy)</td>
<td>41.0 ± 7.5</td>
<td>49.2 ± 5.2***</td>
</tr>
<tr>
<td>Alcohol (% energy)</td>
<td>3.7 ± 5.6</td>
<td>4.2 ± 4.8</td>
</tr>
<tr>
<td>Total fat (% energy)</td>
<td>42.0 ± 4.4</td>
<td>29.7 ± 4.0***</td>
</tr>
<tr>
<td>Saturated fat (% energy)</td>
<td>16.3 ± 3.0</td>
<td>7.9 ± 1.6***</td>
</tr>
<tr>
<td>Monounsaturated fat (% energy)</td>
<td>14.3 ± 2.3</td>
<td>8.9 ± 1.7***</td>
</tr>
<tr>
<td>Polyunsaturated fat (% energy)</td>
<td>6.5 ± 2.1</td>
<td>6.7 ± 2.0</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>14.4 ± 3.7</td>
<td>17.5 ± 5.5*</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD. Significance of difference following intervention diet *p < 0.05, **p < 0.01; *** P < 0.0001.

![Graph](image)

**Figure 4.1.** Relationship between the change in energy intake and the change in weight following the low-SFA / high-CHO diet ($r = 0.6979$, $p = 0.012$).
Figure 4.2. The association between the percentage reduction in total fat intake and the percentage reduction in weight following the low-SFA / high-CHO intake ($r = 0.7097, p = 0.010$)

4.6.2 RED BLOOD CELL PHOSPHOLIPID FATTY ACID COMPOSITION

The phospholipid fatty acid compositions of erythrocytes measured prior to the intervention and at the end of the intervention period are presented in fig 4.3. The results are in agreement with nutritional analysis data in that there was a significant reduction in saturated ($p = 0.015$) and MUFA levels ($p = 0.002$) following the low SFA diet. There was also an increase in the PUFA content of phospholipids following the intervention ($p = 0.01$).

Pre-intervention data showed that there was a significant relationship between the level of SFA in phospholipid membranes and the weight of subjects as shown in fig 4.4.
Fig 4.3. Levels of principal red blood cell phospholipid fatty acids measured pre-intervention and at the end of the intervention diet.

Values are expressed as a percentage of the total area of all fatty acids measured and are presented as means of 12 subjects ± SE.

Figure 4.4. Correlation between weight (Kg) and red blood cell saturated fatty acids (% area) as measured prior to the intervention diet ($r = 0.6454$, p = 0.023)
4.6.3 FASTING BIOCHEMICAL DATA

The fasting plasma concentrations of lipids, glucose and insulin are presented in table 4.3. No consistent alterations were observed in plasma cholesterol, HDL-cholesterol, LDL-cholesterol, TG, glucose or insulin levels as a result of the low SFA diet. NEFA concentrations were significantly reduced from the pre-intervention to the post-intervention period.

In comparing the fasting biochemical data with the results of the nutritional analysis it was shown that there was a positive correlation between fasting insulin levels and SFA intake during the pre-intervention diet \((r = 0.59, p = 0.042; \text{Fig } 4.5.)\). No other significant correlations were observed between biochemical measurements and the results of the nutritional analysis.

<table>
<thead>
<tr>
<th>Biochemical Measurement</th>
<th>Pre-intervention</th>
<th>Mid-intervention</th>
<th>Post-intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>1.85 ± 0.40</td>
<td>1.84 ± 0.64</td>
<td>1.93 ± 0.49</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4.71 ± 1.16</td>
<td>4.76 ± 1.16</td>
<td>4.89 ± 0.90</td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>0.95 ± 0.21</td>
<td>0.94 ± 0.23</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>2.92 ± 1.08</td>
<td>2.99 ± 1.22</td>
<td>3.12 ± 0.79</td>
</tr>
<tr>
<td>CHOL:HDL ratio</td>
<td>5.1 ± 1.4</td>
<td>5.3 ± 1.6</td>
<td>5.7 ± 1.3</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.58 ± 0.17</td>
<td>0.39 ± 0.17†</td>
<td>0.43 ± 0.13*</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.47 ± 0.45</td>
<td>5.59 ± 0.30</td>
<td>5.49 ± 0.28</td>
</tr>
<tr>
<td>Insulin</td>
<td>92.81 ± 47.40</td>
<td>87.50 ± 33.98</td>
<td>91.84 ± 46.92</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD.

*\(p < 0.01\) Significance of difference pre and post intervention.

†\(p < 0.01\) Significance of difference pre and mid intervention.
Figure 4.5. Relationship between fasting insulin concentration and SFA intake measured during the pre-intervention diet \( (r = 0.592, p = 0.042) \).

4.6.4 POSTPRANDIAL DATA

Postprandial TG data are presented in table 4.4. There was a 20% increase in TG AUC over the 9-hour postprandial study following the intervention diet although this was not statistically significant \( (p = 0.057) \). Taking account of the fasting TG values there was an increase in the TG incremental AUC \( (p = 0.025) \) as well as an augmentation in the 6-9 hour TG profile \( (p = 0.043) \) as a result of the low-SFA / high-CHO intervention diet. No differences were observed in the peak TG and the time to peak over the 9-hour study between the pre-intervention and the post-intervention diet.
Table 4.4. *Postprandial Triglyceride data, pre- and post-intervention*

<table>
<thead>
<tr>
<th></th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG AUC (mmol/l.min)</td>
<td>1665.66 ± 406.02</td>
<td>1990.45 ± 646.73</td>
<td>0.057</td>
</tr>
<tr>
<td>Incremental TG AUC (mmol/l.min)</td>
<td>645.51 ± 285.35</td>
<td>920.35 ± 400.29</td>
<td>0.025*</td>
</tr>
<tr>
<td>6-9 hour TG AUC (mmol/l.min)</td>
<td>517.9 ± 200.46</td>
<td>653.70 ± 242.74</td>
<td>0.043*</td>
</tr>
<tr>
<td>Peak TG (mmol/l)</td>
<td>4.28 ± 1.05</td>
<td>5.07 ± 1.61</td>
<td>0.094</td>
</tr>
<tr>
<td>Time to peak (hours)</td>
<td>4.75 ± 1.22</td>
<td>4.58 ± 1.24</td>
<td>0.551</td>
</tr>
</tbody>
</table>

Data are presented as means of 12 subjects ±SD.
Significance of difference pre and post intervention * p < 0.05.

Figure 4.6. shows the postprandial TRL TG profile prior to and at the end of the intervention diet. Concentrations of TRL TG were significantly augmented following the 80-g test meal as a result of the low-SFA and high-CHO diet as measured by the TRL TG AUC (p = 0.028). Incremental measurement of the TRL TG AUC and the late postprandial (6-9 hour) TRL TG phase also showed a significant reduction in the clearance of TRL TG following the low-SFA / high-CHO diet compared to the subjects habitual diet (p = 0.027; p = 0.047 respectively).
Figure 4.6. Postprandial TRL TG concentrations (mmol/l.hr) following the test meal (80-g fat) measured prior to and at the end of the low-SFA / high-CHO diet.

Data are presented as means of 12 subjects± SE.

Concentrations of apo B-48 are shown in fig 4.7. There was no variation in apo B-48 concentrations as a result of the low-SFA/high-CHO diet. Apo B-100 concentrations were calculated by subtracting apo B-48 from total Apolipoprotein B concentrations. There was no difference in Apo B-100 AUC between the pre-intervention (7387.8 ± 48457.7 µg/ml.min) and intervention diets (62301.9 ± 13521.5 µg/ml.min).
Figure 4.7. Apo B-48 concentration (µg/ml) in individual subjects following the pre-intervention and low-SFA / high-CHO diet. The subjects are ranked in order of decreasing apo B48 concentration prior to the intervention diet.

Figure 4.8. shows the postprandial NEFA profile. As expected there was an inhibition of NEFA release directly after the consumption of the test meal with NEFA levels gradually increasing to a level almost twice that above baseline at the end of the 9-hour study. In agreement with the fasting NEFA data, there was a significant reduction in the total area under the NEFA curve following the low-SFA / high-CHO diet (303.3 ± 45.3 mmol/l.min Vs 280.5 ± 51.5 mmol/l.min, p = 0.01). In separating the NEFA response curve into two phases, it was shown that there was a significant reduction in NEFA release during the later (4-9 hour) postprandial phase (232.0 ± 31.3 mmol/l.min Vs 210.4 ± 37.05 mmol/l.min, p = 0.004) and no significant change in NEFA concentrations in the early (0-4 hour) postprandial phase (71.2 ± 19.1 mmol/l.hr Vs 70.1 ± 20.1 mmol/l.hr, p = 0.85). This would indicate that the intervention affected late postprandial NEFA release to a greater extent than the inhibition of NEFA levels immediately following the test meal. The reduction in NEFA release was positively correlated with the change in energy intakes following the low-SFA / high-CHO diet as shown in fig 4.9. The greater the reduction in energy levels, the greater the fall in NEFA levels as a result of the intervention.
Figure 4.8. NEFA concentrations following the test meal (80-g fat) measured prior to and at the end of the intervention diet.

Data are means of 12 subjects ± SE.

Figure 4.9. Changes in energy intake and the association with change in the NEFA AUC measurements following the low-SFA/high-CHO diet (r = 0.576, p = 0.050).
Fig 4.10. shows the glucose and insulin levels following the test meal. They follow an expected contour with glucose rising, following ingestion of the test meal and insulin released in response to the rise in glucose. There was no change in overall glucose and insulin concentrations as measured by the area under the curve over the 9-hour study period. However, there was a significant reduction in the 60-min glucose measurement from $6.9 \pm 1.5$ mmol/l during the pre-intervention diet to $6.2 \pm 0.9$ mmol/l during the intervention diet ($p = 0.04$). The fall in blood glucose was matched by a significant reduction in insulin release ($p = 0.02$) at the 60-min time point following the low-SFA / high-CHO diet. Glucose and insulin responses were also compared to the results of the nutritional analysis. As shown in fig 4.11, there was a positive association between the level of SFA in the subjects’ diets and the extent of the glucose response prior the low-SFA / high-CHO diet.

![Graph showing insulin (pmol/l) and glucose (mmol/l) responses](image)

**Figure 4.10.** Insulin (pmol/l) and glucose (mmol/l) responses, following the test meal (80-g fat) prior to and at the end of the intervention period. Data are presented as means of 12 subjects ± SE.
4.6.5 POST-HEPARIN PLASMA LPL ACTIVITY

The effect of the low SFA and high-CHO diet on post-heparin LPL activity is shown in fig 4.12. As expected, the amount of LPL released into the plasma was increased from 5 to 15 minutes following the heparin administration. There was no significant difference in plasma post-heparin LPL activity at the 5 or 15-minute measurement between the pre-intervention diet and the post-intervention diet.

In comparing plasma LPL activity and TG levels it was shown that there was an association between LPL activity at the 15-minute measurement and fasting TG levels at the end of the intervention diet. Figure 4.13. shows that those subjects with the highest fasting TG concentration also tended to have the highest enzyme activity (p = 0.009). There was also a positive correlation between the extent of the increase in the area under the TG response curve and the level of post-heparin LPL activity (r = 0.9320, p < 0.0001) following the low-SFA / high-CHO diet as shown in fig 4.14.
Figure 4.12. LPL activity measured 5 and 15 minutes after heparin administration, prior to and at the end of the intervention diet.

Data are presented as means of 12 subjects with standard error bars.

Figure 4.13. The relationship between fasting TG concentration and plasma LPL activity following the low-SFA / high-CHO diet (r = 0.7118, p = 0.009).
Figure 4.14. The association between post-heparin LPL activity levels and the % increase in TG AUC following the low-SFA / high-CHO diet (r = 0.9320, p < 0.0001).

4.6.6 INSULIN SENSITIVITY DATA

The short insulin tolerance test determined insulin sensitivity in the study group. Linear regression was used to estimate the slope of the fall in log transformed blood glucose concentration following the administration of a bolus dose of insulin. The slope was multiplied by -100 to derive the rate constant ($K_{ITT}$) and by 60 to determine the percentage decline in blood glucose per minute (% min$^{-1}$). Following intravenous injection of insulin, blood glucose levels started to fall after 4 minutes as shown in fig 4.15. The glucose disappearance rate $K_{ITT}$ was therefore estimated from the slope of glucose disappearance from 4-15 minutes. Following the injection of insulin, the mean plasma glucose concentration fell from 5.84 (SD 0.43) mmol/l to 4.18 (SD 0.54) mmol/l during the habitual diet and from 5.73 (SD 0.11) mmol/l to 3.96 (SD 0.54) mmol/l during the intervention diet.

The results of the short insulin tolerance test in individual subjects prior to and at the end of the low-SFA / high-CHO diets are presented in fig 4.16. Subjects are ranked in
the order of increasing insulin sensitivity as measured during the pre-intervention period. There was a wide range of insulin sensitivity in the study group, with $K_{ITT}$ glucose varying from 1.79 to 4.96 % min$^{-1}$ (mean = 3.25 ± 0.87 % min$^{-1}$) prior to the intervention. While there was no difference in insulin sensitivity as a result of the intervention in the group overall, it was observed that in the 7 least insulin sensitive subjects during the control diet, there was a significant improvement in insulin sensitivity as a result of the low-SFA/high-CHO diet ($p = 0.005$). In the 4 most insulin sensitive subjects there was a significant decline in insulin sensitivity as measured by the rate of fall in blood glucose, as a result of the low-SFA/high-CHO diet ($p = 0.042$).

![Figure 4.15. Mean fall in blood glucose concentrations over 15 minutes following insulin administration.](image)
Pre-intervention diet
Post-intervention diet

Figure 4.16. Glucose $K_{ITT}$ (% min$^{-1}$), a measure of insulin sensitivity, in individual subjects prior to and following the low-SFA / high-CHO diet. Subjects are ranked in order of the least insulin sensitive subjects prior to the intervention.

In comparing the results of the insulin tolerance test with nutritional intakes it was observed that there was significant association between the reduction in SFA intake and improvement in insulin sensitivity ($r = 0.785$, $p = 0.005$) as shown in fig 4.17. This would indicate that the greater the reduction in SFA the greater the improvement in insulin sensitivity as measured using the short insulin tolerance test in the group overall. The association was also observed with total fat intake in that there was a positive correlation between the reduction in total fat intake following the intervention diet and the extent of the improvement in insulin sensitivity as a result of the low-SFA / high-CHO diet ($r = 0.634$, $p = 0.027$) as shown in fig 4.18.
Figure 4.17. The relationship between the reduction in saturated fat intake and the improvement in insulin sensitivity as a result of the intervention diet ($r = 0.785, p = 0.005$).

Figure 4.18. The association between the reduction in total fat intake and the change in insulin sensitivity as a result of the intervention diet ($r = 0.635, p = 0.027$).
The fractional rate of fall in plasma NEFA levels between 4 and 15 minutes following insulin administration served as a measure of insulin mediated inhibition of hormone sensitive lipase. Fig 4.19 shows the mean decline in NEFA levels following the administration of a bolus dose of insulin. The NEFA $K_{ITT}$ data for individual subjects prior to and at the end of the intervention diet are shown in figure 4.20. There was no difference in the rate at which plasma NEFA levels fell following insulin administration as a result of the low-SFA / high-CHO diet.

As shown in fig 4.21, there was no association between changes in postprandial lipaemia as measured by the TG response and insulin sensitivity of glucose disposal as measured by the short insulin tolerance test ($r = 0.011$, $p = 0.971$).

**Figure 4.19.** Decline in plasma NEFA concentration over 15 minute following a bolus dose of insulin.
Figure 4.20. Individual NEFA $K_{ITT}$ (% min$^{-1}$) levels measured prior to and at the end of the low-SFA / high-CHO dietary intervention. Subjects are ranked in order of increasing $K_{ITT}$ values during the pre-intervention diet.

Figure 4.21. The relationship between the change in postprandial lipaemia and the change in insulin sensitivity following the low-SFA / high-CHO diet ($r = 0.011$, $p = 0.971$).
4.7 DISCUSSION

This study was designed to evaluate the effects of reducing intakes of SFA and increasing the intake of dietary CHO on various metabolic outcomes including insulin sensitivity and measures of postprandial lipaemia. The study was conducted in a group of middle-aged men exhibiting characteristics of the insulin resistance syndrome and therefore considered to be at greater risk of developing CHD. Dietary advice directed at reducing risk of CHD has emphasised the need to reduce intakes of SFA and increase intakes of dietary CHO. The rationale for this approach appear to be based on the premise that such diets result in a decrease in total and LDL cholesterol and ultimately reduce the risk and incidence of CHD. Less emphasis has been placed on the effect of such diets on insulin sensitivity and the association with postprandial lipoprotein metabolism.

Results of the nutritional analysis demonstrated that the intervention was successful in reducing the intake of SFA from an average of 16% to less than 8% of energy intakes. This was achieved by volunteers substituting high SFA foods in their diets for lower-fat, high-CHO alternatives. The intervention also led to a reduction in the intake of both total fat and of MUFA. It is expected that decreasing saturated fat intake without increasing the intake of unsaturated oils will produce a reduction in total and MUFA intake because of the reduction in high fat dairy products, meats and high fat snack foods. There was also a moderate increase in the intake of CHO from 41% to 49% of energy, as a result of the intervention diet. Intakes of energy were lower but not significantly decreased as a result of the intervention.

Consumption of the reduced SFA diet was accompanied by a significant weight loss (mean 1.7 Kg) and is a reflection of the difficulty of the isocaloric substitution of dietary fat for dietary CHO. It was observed that the decrease in body weight was associated with a reduction in total energy intakes. Subjects commented throughout the intervention on the quantity of foods they had to consume in order to prevent weight loss, which was not unexpected as in order to match their habitual energy intakes, a lower-fat, higher-CHO diet would have a greater weight value. Assuming that all of the weight loss was fat loss it is estimated that a deficit of approximately 890 kJ (213 Kcal) per day is required to shed 1.7 Kg of body fat in 8 weeks. Our
findings are in agreement with other studies that have reported weight loss with the consumption of a low-fat and high-CHO diet. Higher fat diets have a higher energy density and it appears that under ad libitum conditions individuals will not compensate for the change in energy density when asked to consume a diet, which is lower in fat and higher in CHO. (Dougherty et al. 1988; Kendall et al. 1991; Sheppard et al. 1991). Apart from the decrease in energy intake caused by consuming a diet low in fat there are indications that fat and CHO may be different in the regulation of body fat. The storage of dietary carbohydrates in adipose tissue is a less-energy efficient process requiring 23% of energy intake, as compared with only 3% of energy required to store dietary fat as fat (Schaefer et al. 1995). Any dietary fat eaten in excess is therefore more likely to be stored as fat, whereas excess CHO intake is mostly oxidised (Acheson et al. 1982; Flatt et al. 1985). However, the long term weight loss associated with consuming diets lower in fat and higher in carbohydrates is disappointing, producing decreases in body weight from 0.4 to 2.6 Kg (Katan, 1998) with much larger decreases needed to affect improvements in fasting lipoprotein levels.

Results of the red blood cell phospholipid analysis were in agreement with the results of the nutritional analysis data and therefore served as a good indicator of volunteer compliance. There was a significant reduction in the level of both SFA and MUFA with an increase in the PUFA component of membrane phospholipids. Data indicate that peripheral insulin sensitivity may be influenced by phospholipid fatty acid composition and that changes in the fatty acid composition of the cell membranes may modulate the action of insulin (Borkman et al. 1993). Increasing the content of PUFA within cell membranes in cultured cells increases membrane fluidity, the number of insulin receptors, and the action of insulin (Thomson et al. 1987); converse effects occur when the concentration of SFA in the membranes is increased (Ginsberg et al. 1982). In a study by Folsom et al. (1996) fasting serum insulin was associated positively with the percentage of SFA in fasting plasma phospholipids. The detected association was strong even after adjustment for BMI. In this study while there was no association between the levels of SFA in phospholipids and the levels of fasting insulin a positive association between the subjects weight and phospholipid SFA content was observed. This supports the perception that long term exposure to high
levels of fat in the diet is associated with a greater degree of obesity and overweight and that chronic lifetime habits may modulate insulin action.

Despite the reduction in SFA intakes, the anticipated reduction in total and LDL-cholesterol levels was not observed. Levels of HDL-cholesterol were also unchanged following the intervention diet. Levels of total cholesterol were not clinically raised in this group of individuals (mean 4.71 mmol/l) and therefore a reduction in cholesterol concentration may have been more evident had cholesterol levels been above normal values. Furthermore, studies reporting a reduction in plasma cholesterol levels following the consumption of a low-SFA diet have often been accompanied by a considerable amount of weight loss. Other studies provide no information as to the effect of the consumption of low-SFA / high-CHO on body weight, thereby making it difficult to draw firm conclusions as to the effect of the diet alone on improvements in lipoprotein metabolism (Vélez-Carrasco et al. 1999). Katan (1998) has remarked on the detrimental effect of low-fat and high-CHO diets on levels of HDL-cholesterol. The mean HDL-cholesterol concentration in this group was below 1.0 mmol/l, a concentration associated with IR. An increase in CHO intake may therefore have elicited little effect on the already lowered levels of HDL-cholesterol. In addition, no alterations in fasting TG were observed as a result of the low-SFA and high-CHO diet. Our findings are not in agreement with other studies that have reported an increase in fasting TG levels as a result of an increase in the CHO content of the diet (Ginsberg et al. 1976; Coulston et al. 1987; Borkman et al. 1991; Vélez-Carrasco et al. 1999). However, the amount of CHO consumed in this intervention (49 % of energy) is not as extreme as the level of CHO in other interventions where a hypertriglyceridaemic effect has been observed. In a large study conducted by Retzlaff et al. (1995) the effect of four ranges of CHO intake varying from 45 % to 60 % of energy on TG levels in a group of hypercholesterolaemic individuals was examined. The study was conducted over a period of 24 months and the authors concluded that a moderate but not extremely high-CHO diet (> 60% energy) can be used long term without deleterious effects on plasma TG levels. It has also been suggested that increases in the plasma TG level as a result of an increase in dietary CHO can be avoided if the increase in CHO is introduced gradually (Ullman et al. 1991). Other studies of low-fat, high-CHO diets have not found increases in plasma TG levels, however the majority of these studies did not control for energy intake and

147
were therefore accompanied by weight loss (Thuesen et al. 1986; Brown et al. 1984). Another study conducted by Sacks et al. (1986) reported that TG levels remained unchanged in a group of normolipidaemic men following 3 months feeding of a 60% CHO diet, however the baseline levels of CHO intake were already high at 51% of energy in this group of semi-vegetarian men. Although the weight loss observed in the present study was small it may have been enough to offset any deleterious effect on fasting TG levels of the increase in CHO feeding.

While fasting levels of TG remained unchanged there was a 20% increase in postprandial TG concentrations following the low-SFA / high-CHO diet. The greatest increase in the postprandial TG concentration was observed between 6 and 9 hours following the consumption of the high fat breakfast. The postprandial investigation conducted in this study consisted of one test meal only, whereas the consumption of two meals and one snack is more representative within a nine-hour time frame. It would be expected that had more than one meal been ingested over the 9 hour period that the day-long TG level would have been raised as a result of the intervention diet. The accumulation of TG-rich lipoproteins was also greater following the low-SFA / high-CHO diet. TRL consist of chylomicrons, chylomicron remnants and VLDL TGs. To distinguish between chylomicrons of intestinal and hepatic origin, analysis of apo B-48 particles was conducted with subsequent indirect measurement of apo B-100 concentration. Concentrations of apo B-48 were significantly lowered as a result of the intervention diet indicating that the increase in postprandial TG concentration was not as a result of an increase in exogenous TG-rich lipoproteins. This would confirm the findings of several other studies that have suggested that the increase in plasma TG concentration in response to low-fat, high-CHO diets is due to an increase in hepatic VLDL production. It has been argued that the CHO-induced hypertriglyceridemic effect of low-fat, high-CHO diets can be attenuated if such diets are enriched with dietary fibre. However, it is thought that the amount of dietary fibre needed to stunt the hypertriglyceridemic effect of high-CHO feeding is five to seven times the amount currently consumed in the UK and as such would be an amount that most individuals would find unacceptable (Simpson et al. 1982; Hollenbeck et al. 1983). It has also been argued that CHO-induced hypertriglyceridemia is a transient phenomenon however, several studies do not support this conclusion. In the present
study postprandial TG increases were evident after two months of the low-fat, high-CHO diet suggesting that the increase in TG was persistent.

Increasingly it is thought that higher concentrations of both fasting and postprandial plasma NEFA are risk factors associated with CHD and IR (Frayn, 1998) and for the development of NIDDM (Paolisso et al. 1995). In this study there was a significant reduction in the levels of fasting NEFA and also a greater suppression of postprandial NEFA release following a high fat meal as a result of the low-SFA / high-CHO diet. The reduction in NEFA levels was more pronounced during the late postprandial period, which is somewhat contradictory to the observed concomitant increase in TG levels. It would be expected that the rise in TG would be accompanied by a rise in NEFA levels as plasma NEFAs are the major substrate for hepatic TG synthesis, and it has been shown that there is a close correlation between NEFA and VLDL-TG secretion (Kissebah et al. 1974). In a study by Byrne et al. (1997) the relationship between TG and NEFA concentrations was examined and it was shown that the most important determinants of plasma TG concentrations in a group of 1122 subjects was the area under the non-esterified fatty acid suppression curve. Plasma NEFA concentrations are largely determined by the action of insulin to suppress adipocyte lipolysis. Postprandial suppression of NEFA availability is one of the mechanisms through which insulin enhances glucose disposal and Roust & Jensen (1993) showed that postprandial release of NEFAs in upper-body obese women is not suppressed normally compared to that in lower-body obese and non-obese women. In this study was observed that fall in NEFA levels was associated with a reduction in the intake of energy following the low-SFA / high-CHO diet. No association between measures of fasting insulin or insulin sensitivity and NEFA levels was observed. The reduction in energy following the intervention diet was associated with a reduction in body weight. It is possible that the reduction in weight which occurred during the intervention may have led to a decrease in the number of fat cells resulting in a decline in the rate of lipolysis and therefore the concentration of plasma NEFA. Certainly in obese individuals the converse effect has been observed and it has been shown that as the net rate of lipolysis increases with increasing fat mass, there is an increase in the plasma NEFA concentrations. In addition, it has been shown by Lillioja et al. (1986) that it is the size of the fat depot rather than the basal rate of lipolysis per se that determines NEFA availability. Less is known about the effect of
individual fatty acids in the diet on NEFA. It is known that if rat adipocytes are cultured with (non-esterified) SFA, a reduction in the sensitivity to insulin of glucose transport is observed (Storlein et al. 1991). More information needs to be obtained from human studies in order to have a greater understanding of the effects of changes in the fatty acid composition of diets on the regulation of NEFA.

Hyperinsulinaemia is frequently associated with hypertension (Modan et al. 1985), hyperlipidaemia (Zavaroni et al. 1989), CHD (Fontbonne et al. 1988) and the development of type II diabetes (Stout, 1985). In the present study the mean fasting insulin concentration was 92.8 pmol/l. From the analysis of the food records a positive correlation between levels of fasting insulin and habitual intake of SFA was found. This finding concurs with the study of Marshall et al. (1997) that showed that diets high in total and SFA and low in starch and fibre were significantly associated with fasting hyperinsulinaemia. In a study of men with type II diabetes Maron et al. (1991) also showed that the intake of SFA was significantly related to elevated fasting insulin concentration independently of body mass index. Mayer et al. (1993) also demonstrated that the usual intake of total dietary fat was positively related to fasting insulin concentration in a group of healthy women twins. In the present postprandial study, while the overall concentrations of glucose and insulin in response to the test meal were unchanged, there was a significant reduction at the 60 min time point in both insulin and glucose levels following the low-SFA, high-CHO diet. In recent studies the 30-min insulin concentration during the oral glucose tolerance test has been shown to be a good marker of insulin secretion (Phillips et al. 1994). In the present study there was also a positive association between the extent of the postprandial glucose response and the level of SFA consumed during the subjects' habitual high fat diet. A study conducted in Italy also showed that increased consumption of butter, a food rich in SFA, was associated with higher glucose concentrations in both men and women (Gatti et al. 1992). In healthy men, glucose stimulated insulin secretion is increased after the acute ingestion of SFA, and the authors suggest that this may be due to a rise in plasma gastric inhibitory polypeptide (Collier et al. 1988). Another suggestion is that the increase in free fatty acids due to the consumption of a high SFA diet leads to an increase in glucose levels caused by the glucose-fatty acid cycle as originally proposed by Randle et al. (1963). In this study the levels of circulating NEFA were higher during the subjects' habitual SFA.
diet and may have played a role in the observed positive association between the usual intake of SFA and postprandial glucose concentrations.

No changes in post-heparin LPL or HL activities were observed following the low-SFA / high-CHO diet. Little consistent information is available from previous studies regarding the effects of high-CHO and low-fat diets on LPL and HL activities. It has been proposed that high-CHO diets may reduce post-heparin LPL and HL activities and thus contribute to the rise in TG concentrations following these type of diets (Blades & Garg, 1995). In a study by Jackson et al. (1990), a high-CHO diet was reported to lower post-heparin LPL activity in a group of healthy individuals within 1 week. In contrast, in a group of NIDDM patients Chen et al. (1995) reported an increase in post-heparin LPL activity but not HL activity, following a high-CHO diet. The increase in LPL activity occurred alongside an increase in fasting TG concentration. The effects of high-CHO diets on adipose tissue LPL activity have not been studied. Studies looking at the effects on skeletal muscle LPL activity report a lowering of activity following high-CHO feeding (Lithell et al. 1982; Kiens et al. 1987). In terms of a mechanistic effect of CHO induced triglyceridaemia, it is generally thought that a reduction in total plasma post-heparin LPL activity does not contribute to the increase in plasma TG concentrations associated with high-CHO diets during long-term studies (Blades & Garg, 1995). In this study there was a positive association between levels of fasting TG and LPL activity. A higher level of LPL activity was also associated with a greater increase in the postprandial area under the TG curve following the low-SFA / high-CHO diet. This suggests that in this study the increase in postprandial TG concentration was not as a result of a decrease in the activity of plasma LPL activity. Greater evidence for an increase in hepatic VLDL production as an explanation for CHO-induced triglyceridaemia exists. However, more information regarding the effect of low-fat and high-CHO diets on LPL activity in plasma and adipose tissue is required to make firm conclusions as to the role of LPL in modifying lipoprotein response.

Previous work has implicated that a higher habitual intake of dietary fat is associated with a worsened insulin sensitivity (Feskens & Kromhout, 1990; Lovejoy & DiGirolamo, 1992). In contrast to work that has been done in animals, studies that have looked at the effect of reducing intakes of dietary fat on insulin sensitivity in
humans, are equivocal. Few of those studies have measured insulin sensitivity using
the short insulin tolerance test and related this to changes in the dietary intake of SFA
and CHO. In this study subjects were selected on the basis of fasting insulin levels.
Fasting insulin levels were shown to be associated with the intake of SFA in this
group of subjects. As was observed with the fish oil study (chapter 3.0) insulin
sensitivity was improved following the intervention diet in the individuals with the
greatest degree of IR at the outset of the study. It was also observed that there was an
association between the extent of the reduction in the intake of SFA and the
improvement in insulin sensitivity. This indicated that the individuals with the
greatest improvement in insulin sensitivity were also the individuals with the greatest
reduction in the intake of SFA. The reduction in dietary SFA was confirmed in this
study by a reduction in red blood cell phospholipid SFA content. The hypothesis of
this study was that insulin sensitivity would improve as a result of a reduction in the
intake of SFA. This was based on the finding that the fatty acid composition of
phospholipids influence insulin sensitivity and that a reduction in the SFA content of
phospholipids would induce positive changes in insulin responsiveness. It would
appear therefore, that the improvement in insulin sensitivity in this study was related
firstly to the degree of IR at the outset of the study and secondly to the extent of the
reduction in the intake of SFA. As with the fish oil study (chapter 3.0) there was no
association between measures of insulin sensitivity and postprandial lipoprotein
metabolism in this study.
CHAPTER 5.0
REPLACEMENT OF SATURATED FAT WITH MONOUNSATURATED FAT AND THE EFFECT ON POSTPRANDIAL LIPAEMIA AND INSULIN SENSITIVITY

5.1 BACKGROUND

Since the 1950s evidence has been growing that Mediterranean countries display rates of chronic disease, particularly CHD, that are among the lowest in the world and life expectancies that are among the highest (Nestle, 1995). The Mediterranean diet is characterised by large intakes of MUFA in the form of olive oil, with correlating low intakes of SFA. As discussed in chapter 4.0 dietary recommendations aimed at reducing the risk of CHD however, are still heavily directed towards a decrease in total and SFA and an increase in CHO intake. While there is little doubt that there is a positive association between the intake of SFA and CHD there is as yet little trial evidence to support the notion that decreasing intakes of SFA alone will lead to a reduction in CHD (Oliver et al. 1997). As yet only those trials with parallel interventions such as reduction of cigarette smoking (Hjermann et al. 1981), or those in which the unsaturated components of dietary fat was altered have had an effect on the incidence of CHD (Frantz et al. 1989).

A diet rich in CHOs and fibre but low in fat has also been widely recommended for those with type II diabetes and impaired glucose tolerance. However, debate exists as to whether a high-CHO, low-fat diet is the optimal diet therapy for all subjects with type II diabetes. Garg (1998) has recently conducted a meta-analysis of the metabolic effects of diets high in MUFA or high in CHOs in the management of diabetes. On the basis of the meta-analysis the author concludes that it is clear that compared with high-CHO diets, high MUFA diets improve lipoprotein profiles as well as glycaemic profiles. Overall it was estimated that high MUFA diets reduce fasting TG and VLDL-cholesterol concentrations by 19% and 22% respectively, and cause modest increases in HDL-concentrations without adversely affecting LDL-cholesterol concentrations. The meta-analysis also revealed a significant net lowering of plasma glucose concentrations by 0.23 mmol/l. Fewer studies have looked at the effect of
high monounsaturated diets in individuals who do not have diabetes but may be insulin resistant.

Since obesity is a strong risk factor for CHD, lowering the intake of total and SFA has also been promoted on the basis that such dietary measures will lead to a reduction in body weight through a decrease in energy intakes. Long-term trials of low-fat diets are however discouraging, in that weight loss is generally achieved in the first few months of an intervention but typically cease thereafter (Katan, 1997). This is usually as a result of poor compliance to this type dietary advice. Also, as discussed by Katan (1997) while the emphasis of dietary guidelines on diets low in total rather than just SFA rest partly on scientific data, non-scientific issues may also have played a role. The author comments that firstly the low-fat concept is simple to communicate and secondly a campaign against total fat will receive wider support in society because various economic interests are more or less equally affected. However, with intakes of fat in the region of 30-40% of total energy, low-fat diets are considered by many as unpalatable and unsustainable in today's affluent society. Decreasing the intake of SFA by increasing the intake of MUFA may be a more acceptable and effective dietary change.

5.1.2 STUDY OBJECTIVES

Studies looking at the effect of high MUFA feeding on insulin sensitivity are inconclusive with some reporting no effects (Garg et al. 1992b) and others reporting an improvement in insulin mediated glucose disposal with consumption of a high-MUFA diet (Parillo et al. 1992). Studies have tended to focus on effects in glucose intolerant or diabetic subjects. The aim of this intervention was to examine the effect of lowering the intake of SFA by increasing the intake of MUFAs in healthy subjects with indications of insulin resistance. MUFA intakes were to be increased via supplementation of the volunteers' diets with rapeseed oil. Most studies that analyse the effects of dietary MUFA in humans have used olive oil or oleic acid-rich variants of sunflower or safflower oil. None of these oils are widely consumed in northwestern Europe or America where there is greater availability of the less expensive rapeseed oil (Valsta et al. 1992). In addition, rapeseed oil differs from olive oil in its content of the n-3 PUFA, α-linolenic acid: 10-13 % in rapeseed oil versus 0% in olive
oil (Valsta et al. 1992). This dietary intervention was designed to lower SFA intakes to less than 10% of energy and increase MUFA intakes to approximately 18% of energy intakes. The metabolic investigations conducted included, measurement of insulin sensitivity using the short insulin tolerance test and measurement of postprandial lipaemia following a high fat meal. Compliance was determined from the measurement of red blood cell phospholipids. Fasting measurements of glucose, insulin, cholesterol fractions and TG were also analysed. To explore the mechanism by which an alteration in dietary fat composition may improve insulin sensitivity, LPL activity and gene expression was also measured.

5.2 STUDY DESIGN AND SUBJECTS

5.2.1 EXPERIMENTAL DESIGN

The study consisted of a single dietary intervention with the subject’s pre-intervention diets acting as a control phase. Prior to the subjects receiving advice on how to reduce their intake of SFA and increase the consumption of MUFA the first set of metabolic investigations was conducted. Subjects then adhered to the intervention diet for a period of 4 weeks, at the end of which the second set of metabolic investigations was conducted. From our previous studies 4 weeks was considered sufficient to detect changes in the fatty acid composition of membrane phospholipids following the introduction of a fatty acid altered diet. The metabolic studies conducted pre and post-diet included two study days, the measurement of postprandial lipaemia and insulin sensitivity using the short ITT. No stable isotope studies were carried out in this study. All subjects had refrained from strenuous exercise and abstained from alcohol on the day prior to each investigation. A description of each of the metabolic investigations conducted is provided in chapter 2.0, section 2.3.

Fasting blood samples were also collected during the study at week 0 and week 4 of the intervention. Samples were analysed for triglyceride, total cholesterol, HDL-cholesterol, glucose, insulin and red blood cell phospholipid fatty acids.
5.2.1.1 Subject numbers and power calculations
As with the fish oil and SFA-CHO exchange trials, the initial intention was to establish a cohort of 12 on the basis of feasibility and the short ITT variability (see section 3.2). However, for a variety of reasons it proved difficult to recruit sufficient numbers for screening to yield 12 subjects and some who were recruited dropped out of the trial at an early stage. For this reason only 9 completed the trial.

5.2.2 SUBJECTS

The Ethics Committee of the University of Surrey, Guildford, approved the study. The study days were carried out in the Clinical Investigation Unit in the School of Biological Sciences at the University of Surrey. All volunteers provided witnessed informed consent before participating in the trial.

Nine healthy male volunteers were recruited to the study from a cohort who were identified via advertisements in the local media as well as posters placed with companies in the Guildford area. The volunteers had a mean age of 47 (SD 4.8) years (range 41-55 years) and a mean body mass index of 28.7 Kg/m². The subjects were all non-smokers with a low level of physical activity and they all habitually consumed at least 13% of energy as saturated fat. All of the volunteers were free from medication and did not have any previous medical history of coronary artery, endocrine or liver disease. None of the volunteers were following any type of therapeutic or specialised diet or taking any form of dietary supplements.

All subjects had a fasting blood sample collected prior to inclusion in the study for screening. The criteria for inclusion were glucose <7.0 mmol/l, total cholesterol <7.0 mmol/l, TG> 1.9 mmol/l, insulin > 40 pmol/l and haemoglobin> 130g/l. Volunteers also had a waist circumference greater than 90 cm. The screening biochemical and anthropometric data are shown in table 5.1.
Table 5.1. *Baseline characteristics of study volunteers.*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47 ± 4.8</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>28.7 ± 2.8</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>104 ± 7.6</td>
</tr>
<tr>
<td>Total plasma cholesterol (mmol/l)</td>
<td>5.67 ± 1.01</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.70 ± 0.98</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.82 ± 0.11</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>2.55 ± 0.60</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>103.6 ± 53.0</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.31 ± 0.60</td>
</tr>
</tbody>
</table>

Data presented as means ± Standard deviation (n = 9).

5.3 DIETARY INTERVENTION

Upon entry into the study each volunteer completed a seven-day food record, as used in the Epic study, to determine usual dietary intakes of energy and fat (Bingham *et al.* 1997). Nutritional analysis was completed using Dietplan 5 for windows (Forestfield Software Ltd.). The aim of the study was for volunteers to increase the intake of MUFA from an average of 10 to 18 % of energy and reduce the intake of SFA from 16 to less than 10 % of energy.

On the basis of their habitual intakes volunteers were given individual advice on how to reduce SFA. SFAs were derived predominantly from dairy products, meats and meat products and convenience foods. Volunteers were also asked to increase their intake of MUFA by changing to monounsaturated based margarine, using rapeseed oil (Goldenfields Ltd.) for cooking and by consuming specially prepared foods which were made using rapeseed oil as the source of fat. The fatty acid composition of the rapeseed oil is shown in table 5.2. The food items provided were biscuits, cakes, breads and pasta sauces. A local catering company, who was supplied with rapeseed
oil, prepared the foods. The volunteers were asked to incorporate the foods into their habitual diet, exchanging them for their usual snacks or as part of their main meals, in an attempt to maintain their habitual energy intakes. A ‘unit’ system was devised to aid the volunteers in aiming for a daily intake of the MUFA-enriched foods. Each food portion was assigned a unit value where one unit was equivalent to 10 g of rapeseed oil. Each food item was made using either 10 g or 20 g of rapeseed oil i.e. equivalent to 1 or 2 units and volunteers were asked to consume 2-3 units daily on the basis of their habitual energy and fat intake. The amount of MUFA-enriched foods that volunteers consumed was adjusted throughout the intervention on the basis of the volunteers’ weight measurements.

During the study, volunteers attended the University of Surrey weekly to meet with investigator and to collect foods. This allowed investigator’s to answer any queries, to resolve any problems encountered with the study foods and to aid volunteer motivation. Weight measurements were also taken at each visit. At week 2 of the intervention volunteers were asked to complete a 7-day food record in order to monitor compliance and to analyse the nutrient composition of the intervention diet.

Table 5.2 Fatty acid composition of rapeseed oil.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>% content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SFA</td>
<td>5.6</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>68.1</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>26.3</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>15.7</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>10.6</td>
</tr>
</tbody>
</table>
5.4 ANALYTICAL PROCEDURES

Plasma glucose concentrations, total cholesterol, HDL cholesterol, and triglycerides were assayed on a COBAS-MIRA autoanalyser (Roche Products Ltd, Welwyn Garden City, Herts) by enzymatic colorimetric methods using commercially available kits supplied by Roche Diagnostic Products Ltd (Welwyn Garden City, Herts) and Randox Laboratories (Co. Antrim, N.Ireland). LDL-cholesterol concentration was estimated using the Friedewald equation (Friedewald, 1972). Plasma NEFA concentrations were also measured using the COBAS-MIRA analyser by a standard enzymatic colorimetric method (Wako Chemicals, GmbH, Germany supplied by Alpha laboratories Ltd., London). Appropriate quality controls with pre-defined reference ranges were run with each assay in order to determine intra and inter assay variability. The within and between CV's are outlined in Chapter 2.0 section 2.1. Serum insulin levels were measured by radioimmunoassay. Samples from each intervention period were analysed together in the same run at the end of the study period.

The fatty acid composition of red blood cell phospholipids was determined after the extraction of lipids with a mixture of chloroform and methanol (2:1 vol/vol), containing 0.01% BHT as antioxidant, according to the Folch method (Folch et al, 1957). The fatty acids were separated and quantitated using gas chromatography. Fatty acids were identified by comparing retention times with those of a known standard mixture.

Triglyceride-rich and triglyceride poor fractions of plasma were separated using an adapted method of that described by Grundy & Monk (1976). 3.5 ml of plasma was overlaid with an equal volume of saline (1.006g NaCl/ml), and ultracentrifuged for 5.0 X 10^6 g at 24°C in a 10-ml centrifuge tube. The top 1.2 ml of the upper layer, the TRL fraction was removed, aliquoted and frozen at -20°C for later analysis. TG levels were measured in the TRL fraction using an enzymatic, colorimetric kit supplied by Roche Diagnostic Products Ltd (Welwyn Garden City, Herts).
Lipoprotein lipase activity was measured in 10 μl portions of plasma taken 5 and 15 minutes after the administration of an intravenous dose of heparin. LPL activity was measured by a modified method described by Nilsson-Ehle & Schotz (1976).

5.5 STATISTICAL ANALYSIS

For most data tabulated results are presented as mean values and standard deviations; individual time points on graphs are presented as mean values with their standard errors. Statistical analysis was performed using the windows compatible computer package, Statistica (version 5, Statsoft Inc.) and the Excel (Microsoft Excel 97) computer programme.

The effect of the intervention diet on fasting values was analysed using the student’s t test for paired data. The postprandial data was expressed in summary form as an area under the postprandial response curve. The areas under the postprandial response curves were calculated using the Trapezium rule (Matthews et al. 1990). Differences in postprandial metabolism were calculated by comparing areas under the curve pre- and post-intervention. Values of P < 0.05 were considered statistically significant.

5.6 RESULTS

5.6.1 DIETARY INTERVENTION DATA

Data for energy and macronutrient intakes are presented in Figure 5.1. There was an increase in energy intake as a result of the intervention diet (p = 0.037) although this was not reflected in an increase in body weight, which remained stable for the duration of the study (91.4 ± 10.4 Kg Vs 91.2 ± 10.4 Kg). The increase in energy intake was as a result of an increase in CHO intake from 39.6% to 43.8% of total energy intake (p = 0.002). Total fat intakes were unchanged as a result of the intervention however the proportion of energy derived from individual fatty acids was altered. The aim of the dietary intervention was achieved, in that there was a significant decrease in SFA from and average of 15% to 8.2% of energy (p = 0.0003).
and a significant increase in MUFA from 13 % to 18.4 % of energy intake ($p = 0.0026$). There was also an increase in the percentage of energy derived from PUFA from 6.6 % to 8.8 % of energy ($p = 0.022$). The intakes of protein were unchanged as a result of the low SFA / high MUFA diet.

![Graph showing nutritional intake comparison between baseline and intervention diets.](image)

**Figure 5.1.** Nutritional intake measured during the habitual and intervention diet. Data are means of 9 subjects ± SE.

Significance of difference following the intervention diet * $p < 0.05$, ** $p < 0.001$.

### 5.6.2 RED BLOOD CELL PHOSPHOLIPID DATA

Table 5.3. shows the results of the red blood cell phospholipid data. There was no difference in the levels of any of the major fatty acids as a result of the dietary intervention. Therefore the change in the composition of fat intakes to a decrease in SFA and an increase in MUFA was not reflected in a change in red blood cell phospholipids.
### Table 5.3

Levels of fatty acids in red blood cell phospholipids measured prior to and at the end of the MUFA enriched diet.

<table>
<thead>
<tr>
<th>Fatty acid (% area)</th>
<th>Habitual diet</th>
<th>MUFA-enriched diet</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td>46.7 ± 8.8</td>
<td>44.8 ± 7.3</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>23.5 ± 3.9</td>
<td>28.9 ± 8.5</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>28.8 ± 11.5</td>
<td>25.1 ± 5.7</td>
<td>NS</td>
</tr>
<tr>
<td>Total n-6 fatty acids</td>
<td>22.5 ± 7.6</td>
<td>19.8 ± 5.9</td>
<td>NS</td>
</tr>
<tr>
<td>Total n-3 fatty acids</td>
<td>3.9 ± 4.6</td>
<td>2.9 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>9.0 ± 5.7</td>
<td>8.3 ± 4.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are expressed as the percentage of the total area of all fatty acids measured and are presented as means of 11 subjects ± SD.

NS = non significant.

### 5.6.3 Fasting Biochemical Data

The fasting plasma concentrations of lipids, glucose and insulin are presented in table 5.4. As a result of the low SFA / high MUFA diet there was a significant reduction in the fasting levels of TG and cholesterol. There was a 13% reduction in LDL-cholesterol levels although this did not reach a level of significance. There was no change in HDL-cholesterol levels. The ratio of total cholesterol to HDL cholesterol was lowered following the intervention diet although again this did not reach a level of significance. No alterations in fasting NEFA, glucose or insulin measurements were observed as a result of the consumption of the MUFA-enriched diet.
Table 5.4. *Fasting concentrations of plasma lipids (mmol/l), glucose (mmol/l) and insulin (pmol/l) measured during the habitual diet and at the end of the intervention diet.*

<table>
<thead>
<tr>
<th>Biochemical Measurement</th>
<th>Habitual diet</th>
<th>Intervention diet</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>2.43 ± 0.87</td>
<td>2.06 ± 0.53</td>
<td>0.021*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.42 ± 1.27</td>
<td>4.81 ± 0.97</td>
<td>0.03*</td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>0.76 ± 0.08</td>
<td>0.77 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>3.55 ± 1.45</td>
<td>3.10 ± 1.04</td>
<td>NS</td>
</tr>
<tr>
<td>CHOL: HDL ratio</td>
<td>7.1 ± 1.4</td>
<td>6.2 ± 0.86</td>
<td>NS</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.28 ± 0.11</td>
<td>0.29 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.24 ± 0.67</td>
<td>5.19 ± 0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin</td>
<td>110.87 ± 69.78</td>
<td>100.76 ± 63.33</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD.

Significance of difference pre and post intervention * p < 0.05. NS = non-significant.

5.6.4 POSTPRANDIAL DATA

Postprandial TG data are presented in figure 5.2. There was a significant reduction in the TG response, measured by the total area under the curve, from 2088.5 (SD 565.9) mmol/l.min during the habitual diet to 1726.6 (SD 485.2) mmol/l.min following the low SFA / high MUFA diet (p = 0.013). There was also a significant attenuation in the 6-9 hour TG profile, measured by the 6-9 hour area under the curve as a result of the low SFA / high MUFA intervention diet (p = 0.018). Taking account of the fasting TG values there was no difference in the incremental area under the curve during the habitual diet and the intervention diet (p = 0.162). No significant differences were observed in the peak TG and the time to peak over the 9-hour study between the pre-intervention and the post-intervention diet.
Postprandial Triglyceride response following the test meal (80-g fat) during the habitual diet and following the low SFA / high MUFA diet. Data are presented as means of 9 subjects ± SE.

Figure 5.3. shows the postprandial TRL TG profile prior to and at the end of the intervention diet. There was no significant change in the clearance of TRL TG following the low SFA / high MUFA diet compared to the subjects habitual diet.

Concentrations of apo B-48 were attenuated following the low SFA / high MUFA intervention diet (p = 0.0001) as measured by the total AUC and shown in fig 5.4. The incremental AUC was also lower as a result of the intervention diet (p = 0.0059). There was also a significant variation in apo B-48 concentrations during the late postprandial phase (6-9 hour) with a reduced response following the low SFA / high MUFA diet (p = 0.0008).

Figure 5.5. shows the postprandial NEFA profile. As expected there was an inhibition of NEFA release directly after the consumption of the test meal with NEFA levels gradually increasing to a level almost twice that above baseline at the end of the 9-hour study. There was no change in the total area under the NEFA curve following the low SFA / high MUFA diet.
Fig 5.3. Postprandial TRL TG response following the test meal (80-g fat) during the habitual diet and following the low SFA / high MUFA diet.

Data are presented as means of 9 subjects ± SE.

Fig 5.4. Concentrations of apo B-48, measured in individual subjects prior to and at the end of the intervention diet, following the test meal (80-g fat).

Subjects are ranked in order of decreasing apo B-48 AUC on the control diet.
Figure 5.5. Postprandial NEFA concentrations following the test meal (80g fat) measured prior to and at the end of the low SFA / high MUFA diet. Data are presented as means ± SE.

Fig 5.6. shows the glucose and insulin levels following the test meal. They follow an expected contour with glucose rising following ingestion of the test meal and insulin released in response to the rise in glucose. There was no change in overall glucose and insulin concentrations as measured by the total area under the curve over the 9-hour study period. Two hours following the consumption of the test meal, the concentration of insulin was lower following the low SFA / high MUFA diet (p = 0.039). The reduction in insulin at this time point was not reflected in a reduction in glucose concentration at the same time point.
Figure 5.6. Plasma glucose and insulin levels following the test meal (80g fat) measured prior to and at the end of the low SFA / high MUFA diet. Data are presented as means ± SE.

5.6.5 POST–HEPARIN LPL ACTIVITY DATA

Plasma levels of LPL activity in individual subjects, measured 5 and 15 minutes following the administration of heparin are shown in figs 5.7. and 5.8. respectively. There was a wide range of LPL activity among the study group, ranging from 0.62 to 13.22 umol.oleate.ml.hr measured at the 15-minute time point during the habitual diet. At the 5 minute measurement there was a significant increase in LPL activity in the group overall from 3.39 (SD 3.49) umol.oleate.ml.hr during the habitual diet to 5.46 (SD 3.62) umol.oleate.ml.hr following the low SFA / high MUFA diet (p = 0.03) as shown in fig 5.9. The increase in LPL activity was not sustained through to the 15-minute measurement and there was no change in LPL activity in the group overall at the 15-minute time point as a result of the low SFA / high MUFA diet. Despite repeated analysis, two subjects had a greater level of hepatic lipase than total lipase...
activity, resulting in negative levels using the method of measuring LPL activity employed in this study.

Figure 5.7. LPL plasma activity levels in individual subjects, 5 minutes after heparin administration.

Figure 5.8. Plasma LPL activity in individual subjects 15 minutes, following the administration of heparin.
5.6.6 INSULIN TOLERANCE DATA

Figure 5.10. shows the fall in blood glucose concentration over 15 minutes following the administration of a bolus dose of insulin (0.1U / Kg of body weight) on both the habitual diet and the intervention diet. Insulin sensitivity was calculated from the slope of the fall in log-transformed glucose between 4 and 15 minutes following insulin administration and the range of insulin sensitivity measured in the study group is show in figure 5.11. There was a wide range of insulin sensitivity among the study group with glucose $K_{ITT}$ varying from 2.8 ($\%$/min $^{-1}$) to 5.33 ($\%$/min $^{-1}$) during the habitual diet. It was observed that there was no alteration in insulin sensitivity as measured by the short insulin tolerance test following the low SFA / high MUFA diet in the group overall.
Figure 5.10. Mean plasma glucose concentrations following the administration of a bolus dose of insulin (0.1U/Kg), measured during the habitual diet and following the low SFA / high MUFA diet.

Figure 5.11. $K_{ITT}$ measurements in individual subjects during the habitual diet and following the low SFA / high MUFA diet. Subjects are ranked in order of increasing insulin sensitivity measured during the habitual diet.
The fractional rate of fall in plasma NEFA levels between 4 and 15 minutes following insulin administration served as a measure of insulin mediated inhibition of hormone sensitive lipase. The fall in NEFA concentrations following the administration of insulin is shown in figure 5.12. There was an acceleration in the rate at which plasma NEFA levels fell following insulin administration as a result of the low SFA / high MUFA diet as shown in fig 5.13, although this did not reach a level of statistical significance (p = 0.068).

Figure 5.14. shows the lack of association between changes in postprandial lipaemia and changes in insulin sensitivity measured by the glucose KITT as a result of the low SFA / high MUFA diet.

**Figure 5.12.** Mean plasma NEFA levels measured over 15 minutes following the administration of a bolus dose of insulin (0.1U / Kg).
Figure 5.13. Change in mean calculated NEFA $K_{ITT}$ following the low SFA / high MUFA diet.
Data are presented as the mean of 9 subjects ± SE.

Figure 5.14. The relationship between changes in postprandial lipaemia and changes in insulin sensitivity following the low SFA / high MUFA diet ($r = 0.563$, $p = 0.114$).
This study is novel in its use of rapeseed oil enriched foods as a means of increasing the MUFA content of subjects’ diets. Analysis of the food records showed that the volunteers were successful in reducing the intake of SFAs to less than 10% of energy and increasing the intake of MUFAs to 18% of energy intake. Substitution of dietary SFAs with MUFAs resulted in a 11% decrease in total cholesterol and a 15.4% reduction in the levels of TG. Body weight was unaffected by the dietary intervention indicating that the subjects were successful in adapting their habitual energy intakes. The dietary intervention was well tolerated by the subjects and suggests that as a dietary strategy this may be more acceptable than the low-fat, high-CHO diet currently recommended in most national and international nutritional guidelines. However, red blood cell phospholipid measurement pre- and post-intervention did not reflect the change in the intake of dietary fatty acids.

The purpose of the study was to evaluate the effects of MUFAs on plasma lipoprotein levels and insulin sensitivity. The data confirm that MUFA substitution for SFA is an effective dietary approach for reducing plasma cholesterol and TG concentrations. The 13% reduction in LDL-cholesterol did not reach a level of significance and differs from other studies which have shown convincing reductions in plasma LDL-cholesterol levels following a high MUFA enriched diet (Vasta et al. 1992; Roche et al. 1998). Plasma HDL-cholesterol concentration was unaffected by the diet. Levels of fasting and postprandial NEFA were also unchanged as a result of the low-SFA / high-MUFA diet. It is widely accepted that MUFA, and especially oleic acid when substituted for SFA, decrease total cholesterol levels. The habitual high SFA intakes of the subjects was halved during the intervention diet and is thought to have induced the fall in plasma cholesterol levels. This is supported by the findings of other studies, which have shown that substitution of SFA with MUFA results in cholesterol lowering whereas, supplementation of diets with MUFA alone has not procured a lowering of cholesterol concentrations (Vicario et al. 1998). While the aim of this intervention was to maintain habitual energy intake and weight interestingly Gumbiner et al. (1998) reported that weight loss therapy can more effectively ameliorate cardiovascular risk in obese type II diabetes patients if MUFAs are incorporated into a hypocaloric diet. It would appear therefore, that increasing the
MUFA content of diets' has favourable effects on lipoprotein response in conditions of both low fat and high fat feeding.

Alongside the reduction in fasting TG levels this study also showed a significant decrease in postprandial TG concentrations. The TG attenuation was more pronounced during the late postprandial phase i.e. 6-9 hours following the high fat meal. This study was of relatively short duration however, the effect of long term ingestion of MUFAs on postprandial lipaemia was highlighted in a study by Zampelas et al. (1998) who showed a more advantageous postprandial pattern in response to a high fat meal in Southern Europeans compared with Northern Europeans. Blades & Garg (1995) investigated the mechanisms of TG lowering with high MUFA diets in subjects with type II diabetes and reported that TG lowering was due to reduced hepatic production of VLDL TG and not due to increased lipolysis or greater postprandial clearance of TRL. In this study levels of TRL-TG were unchanged as a result of the high MUFA intervention diet. The reduction in TG levels during the late postprandial phase was therefore not due to a greater clearance of postprandial TRL. However, the concentration of apo B-48 within the TRL fraction was significantly attenuated following the high MUFA diet. This would suggest that the reduction in TG was due to a decrease in the secretion rate of intestinally derived chylomicrons. This is in contrast to the outcomes reported by Roche et al. (1998) who found an increase in levels of apo B-48 following 8 weeks of a low SFA / high MUFA diet using olive oil as the source of MUFA. Other studies have reported reductions in apo B levels following high MUFA feeding although they have not looked specifically at apo B-48 content (Valsta et al. 1992; Becker et al. 1983). A greater number of studies are required to learn more about the effects of high MUFA feeding on apo B-48 concentration.

Measurement of LPL activity was conducted to provide insight into the possible mechanisms for the TG lowering observed with the high MUFA feeding in this study. Few studies have examined the effects of a greater MUFA intake on plasma LPL activity. A study by Williams et al. (1999) failed to show any effect on plasma LPL activity with high MUFA feeding, however TG levels were also unchanged following their intervention diet. In the present study there was a suggestion of an increase in LPL activity following the high MUFA diet through a significant increase in the 5-
minute measurement however this was not sustained through to the 15-minute measurement. There is no direct evidence therefore that the reduction in fasting and postprandial TG observed in the present study was due to an increase in the rate of lipolysis, mediated through an increase in plasma LPL activity.

There was no effect of the MUFA enriched diet on glucose or insulin concentrations measured in the fasting state. Two hours after the consumption of the high fat meal there was a significant reduction in insulin levels following the low SFA / high MUFA intervention diet. This was not matched by a significant reduction in plasma glucose levels at the 2-hour time point. Studies in those with type II diabetes have reported lower 2-hour postprandial plasma glucose concentrations with consumption of a high MUFA diet compared with a high CHO diet (Parillo et al. 1992; Parillo et al. 1996). Sarkinen et al. (1996) have also observed improvements in glucose tolerance in glucose-impaired subjects following a high MUFA diet. A similar finding has been reported in subjects of normal glucose tolerance (Pelikanova et al. 1989; Uusitupa et al. 1994) as well as in those with type II diabetes (Parillo et al. 1992). Garg (1998) reported a significant net lowering of fasting plasma glucose concentrations by 0.23 mmol/l with consumption of a high MUFA diet in a meta-analysis of studies centred on subjects with diabetes. He found no change in plasma insulin levels. Few studies have measured insulin sensitivity directly and related this to changes in the intake of MUFA. Studies in diabetics have in some cases reported improvements in insulin mediated glucose disposal with consumption of a high MUFA diet (Parillo et al. 1992). In this study there was no observed effect on insulin sensitivity following the MUFA enriched diet. The MUFA enriched diet was not associated with an increase in the unsaturated component of red blood cell phospholipids, which may have accounted for the lack of improvement in insulin action. In common with the previous dietary intervention studies discussed in chapter 4.0 & chapter 5.0 there was no association between measures of insulin sensitivity and lipoprotein metabolism both before and following the intervention diet. Further studies however, are needed to confirm whether high MUFA diets can improve insulin sensitivity.

In conclusion this study, albeit short-term and of limited power has shown a clear benefit of substituting MUFA for SFA in the diets of overweight middle-aged men.
Fasting and postprandial lipid levels were improved as a result of 4 weeks feeding with a rapeseed oil-enriched diet. There was a significant reduction in the intake of SFA and the study confirms that this the replacement of SFA for MUFA is a more favourable alternative to the strategy of replacing SFA for CHO recommended by most Western countries. The study is novel in its approach of using rapeseed oil enriched foods as a means of increasing the MUFA component of diets. The study has proved that it is possible to increase MUFA intake to levels comparable to that consumed in Mediterranean countries using foods commonly consumed in this country. The findings of the study could provide valuable information to the food industry keen to promote the 'health benefits' of particular foods. This study was conducted in a small group of individuals and for a relatively short duration. A greater number of studies, of greater duration are needed to confirm the long-term effects of consuming a rapeseed oil-enriched diet. Further work is also needed to examine the effect on insulin sensitivity and also to explore the associated mechanisms between postprandial lipid metabolism and insulin sensitivity.
CHAPTER 6.0
MEASUREMENT OF LPL GENE EXPRESSION USING A
COMPETITIVE RT-PCR METHOD

6.1 BACKGROUND

The polymerase chain reaction (PCR), originally described by Saiki et al. (1985), has
devolved from a highly specialised research tool into a routine procedure, which is
now widely employed in many laboratories. In essence, PCR is a powerful copying
system, which is used to target a specific nucleotide sequence within a sample of
tissue and then produce millions of identical copies of this sequence. These copies
provide ample nucleic material for further analysis, or can be used simply to confirm
the presence of the target sequence in the sample. Deoxyribonucleic acid (DNA),
extracted from a specimen e.g. blood or a sample of tissue, is the usual template in
PCR. RNA can also be examined following reverse transcription by employing a
reverse transcriptase enzyme. Reverse transcriptase PCR (RT-PCR) therefore,
involves an initial reverse transcription step in order to produce a complimentary
DNA (cDNA) copy of the original RNA under investigation. This technique can be
used to detect or quantify the presence of specific messenger RNA (mRNA).

The key components of PCR are the oligonucleotide primers. For a standard PCR
there are usually two primers. One primer matches one strand of the target DNA and
the second primer matches the complementary strand. The 5' ends of the primers
mark the boundaries of the DNA sequence to be investigated therefore primers are
sometimes labelled as up-stream primer and down-stream primers. Primers can be
designed against a specific target DNA of known sequence or they can be designed in
order to amplify unknown sequences. The PCR procedure entails adding small
amounts of extracted DNA/RNA to a reaction mixture containing a buffer solution,
carefully optimised levels of ions (especially magnesium), single nucleotide bases and
primers. If RT-PCR is being conducted a reverse transcription step is included to
produce a double stranded cDNA sequence, otherwise only a DNA polymerase
enzyme is added. PCR amplification is then achieved by repeated cycles of heating
(denaturing), cooling (annealing) and extension using either a solid temperature
cycling block or less often using a water bath. The components of the RT-PCR
reaction are described in fig 6.1. Following the transcription of RNA to cDNA, the temperature is raised to 95°C, allowing the double-stranded template cDNA to denature into two single strands. This is followed by cooling to around 50-55°C which facilitates annealing of the primers to the appropriate cDNA strands. Finally, heating to 68-72°C allows the extension of a complementary strand of cDNA along each strand of substrate DNA in the 5'-to-3' direction. The temperature then reverts to 95°C and the cycle is repeated. There is an exponential increase in cDNA and after $n$ cycles there are theoretically $2^n$ copies of the sequence being amplified. However, in practice only the first 20 or so cycles result in exponential increase of product.

Competitive PCR and competitive RT-PCR is used to obtain the most precise quantitation of DNA and RNA. This assay is based on competitive co-amplification of a specific target template (of unknown concentration), together with known concentrations of an internal standard in one reaction tube. The internal standard has to share recognition sites with the target template. The target template and internal standard must be amplified with the same efficiency and separate analysis of the PCR products of both should be possible. Quantitation is then performed by comparing the PCR signal of the target template with the PCR signals obtained with the known concentrations of the internal standard. Several dilutions of standards are used to quantify the target DNA. The PCR signal or relative amount of target DNA to the internal standard can be measured by densitometric analysis. The easiest way to distinguish between the target template and internal standards is by differences in the size of the two products. This can be achieved, for example, by constructing standards having the same sequence as the specific target but containing a deletion or insertion. The construction of suitable standards is extremely important, as this will determine the accuracy of the measurement of the target template. It is also essential that the concentration of the internal standard is determined accurately.

PCR is hampered by a number of variations that can occur during sample preparation or in the course of the reaction, and minor variations in the reaction conditions are greatly magnified during the amplification process. Such variability may be partly overcome by relating the amount of PCR products of the target template to an internal reference template (‘housekeeping’ gene) such as the cellular gene $\beta$-globulin.
Figure 6.1. Schematic of the reverse-transcription polymerase chain reaction. Single stranded RNA is reversed transcribed to cDNA, which is then denatured. Primers anneal to their complementary sequences in the target DNA and extend. The process is repeated for each cycle of PCR. P1 = primer 1, P2 = primer 2.
The PCR technique is of interest in Human Nutrition because it can be employed to investigate the effect of nutrients at the level of gene expression. The technology can potentially provide us with more information as to the mechanisms involved in, for example, improvements in lipoprotein metabolism following changes in dietary fat intakes. Various genes play a role in lipoprotein metabolism, one of which is the LPL gene. As previously described LPL is an enzyme produced predominantly by adipose tissue and muscle and is a central enzyme in TRL metabolism. Although there is only one gene for LPL, there are important differences between adipose tissue and muscle LPL gene expression (Ranganathan et al. 1994). The regulation of LPL in different tissues is due to nutritional as well as hormonal influences. LPL gene expression has been measured in a number of studies in both animals and humans, however the effect of changes in nutrient intakes in relation to LPL gene expression has been relatively under-explored. In a study by Murphy et al. (1993) male rats who were fed a diet enriched with fish oil were shown to have a greater amount of LPL mRNA in epididymal adipose tissue compared with maize-oil fed rats. In an extension of that work to human studies Murphy and colleagues examined the effects of the consumption of fish oils on the expression of LPL (Murphy et al. 1999). They found no significant difference in the levels of LPL expression following an n-3 PUFA enriched diet, however changes in LPL expression were significantly correlated with changes in TG concentrations as a result of the intervention. As yet no such similar studies have been reported in the literature. Studies involving dietary n-3 PUFA are of interest because of their effect on TG metabolism. However, no studies have to date explored the effect of other dietary fat components such as SFA and MUFA on adipose tissue LPL gene expression.

In this laboratory a competitive RT-PCR method had been developed by Brooks (1998) in order to facilitate the measurement of LPL mRNA from samples of human adipose tissue. That method was subsequently employed in this study to measure the effect of the dietary interventions described in chapters 3.0, 4.0 and 5.0 on the expression of LPL mRNA within adipose tissue samples, obtained by needle biopsy from the human subjects. The aim of this work therefore was to examine the effect of changes in SFA, MUFA and PUFA intakes on the expression of LPL within subcutaneous adipose tissue using a competitive RT-PCR method.
6.2 METHODS

6.2.1 RNA EXTRACTION

6.2.1.1 Total RNA extraction from fresh human adipose tissue

Adipose tissue samples from the fish oil study (chapter 3.0) were extracted at the time of biopsy described in section 2.2.9 using a technique based on a method reported by Chomczynski & Sacchi (1987). All solutions and pipette tips were autoclaved before use and all glassware was baked at 180°C for at least 12 hours to destroy RNases. The denaturing and phenolic solutions used in the extraction procedure were autoclaved before use.

The denaturing solution was prepared by dissolving 50 g of Guanidine isothyocyanate (GTC) in 63.4 ml of DEPC water and 3.52 ml of 0.75 M sodium citrate (pH 7.0) to which 528 μl of Sarkosyl had been added (10 % v/v). The reagents were mixed and held at 65°C to completely dissolve the GTC.

The required amount of phenolic solution was prepared by combining Chloroform : Isoamyl alcohol (24:1), 2 M Sodium acetate (pH 4.0) and Phenol in the ratio of 1:2:10 respectively.

Immediately upon extraction as described in section 2.2.9 the human adipose tissue was placed into a falcon tube containing 3 ml of denaturing solution and 21.6 μl of β-mercaptoethanol. 3.9 ml of pre-chilled phenolic solution was added. The tube was shaken vigorously for 10 seconds in order to ensure that the tissue was completely disrupted.

The tubes were kept on ice for 30 minutes before centrifuging in a Camlab refrigerated high-speed centrifuge (model number 4239R) at 6595 x g for 20 min at 4°C. Following centrifugation, the aqueous top layer containing the RNA, was transferred into a sterile falcon tube and an equal volume of isopropanol added. The tubes were stored at −20°C overnight to precipitate the RNA.
The following day the tubes were re-centrifuged at 6595 x g for 20 min at 4°C and the alcohol was poured off. The remaining pellet was dissolved in 300 µl of the denaturing solution and transferred to a sterile 1 ml eppendorf tube. 600 µl of ethanol was added and the tubes stored at −20°C for one hour.

The tubes were centrifuged in a microcentrifuge at 4135 x g for 20 minutes at room temperature and the supernatant discarded. The RNA-containing pellet was washed with 50 µl of 75% (v/v) ethanol and centrifuged as before in the microcentrifuge. The ethanol was removed and the tubes left to air-dry on a clean surface for 5 minutes. The RNA pellet was re-suspended in 50 µl of 0.5% (w/v) sodium lauryl sulphate (SDS) and stored at −80°C until required for analysis.

6.2.1.2 Preparation of human frozen tissue for RNA extraction
Adipose tissue samples, not extracted at the time of collection, and stored in plastic tubes at −80°C were placed in liquid nitrogen immediately prior to the extraction procedure. Tissues samples collected by needle biopsy contained a mixture of blood and lignocaine, making it difficult to obtain an accurate weight of adipose tissue. Attempts to weigh the recommended amount of tissue (50-100 mg) led to unsuccessful RNA extraction’s as the actual amount of adipose tissue may have been too little to obtain the required amount of RNA. Therefore, an unweighed amount of material, amounting to approximately half the contents of the tube (approx. 250 mg) was removed using a spatula in order to ensure that a sufficient amount of adipose tissue was obtained. Standardisation of the tissue weight used for RNA extraction was therefore not possible.

6.2.1.3 Total RNA Extraction procedure for Frozen Human Adipose Tissue.
RNA was extracted from frozen adipose tissue collected during the SFA study as described in section 2.2.9 using the RNAgents® Total RNA Isolation System, (Promega Ltd, UK). The system contains:

- 120 ml Denaturing solution
  - 26mM sodium citrate, pH 4.0
  - 0.5% N-lauryl sacrosine
- 0.125M β-mercaptoethanol
- 4M guanidine thiocyanate
♦ 10 ml 2M Sodium Acetate, pH 4.0
♦ 100 ml Phenol:Chloroform:isoamyl Alcohol (125:24:1), pH 4.7
♦ 100 ml Isopropanol
♦ 25 ml Nucleas free water

600 µl of denaturing solution was dispensed into a pre-baked glass homogeniser tube and chilled on ice for 5 minutes. The tube containing the tissue sample was removed from the liquid nitrogen and a section of material placed into the denaturing solution using a pre-baked-cooled spatula. The tissue was then disrupted using a glass homogeniser, ensuring that no clumps of tissue were visible.

60 µl of 2M Sodium Acetate, pH 4.0 was added and mixed thoroughly by inverting the tube 4-5 times. 600 µl of phenol:chloroform:isoamyl alcohol was added care having been taken to remove only from the lower organic phase of the bottle. The tube was capped, and carefully mixed by inverting 3-5 times and then shaken vigorously for 10 seconds. The samples were chilled on ice for 15 minutes and centrifuged at 2065 x g for 20 minutes at 4°C using a refrigerated bench top centrifuge.

The top aqueous phase containing the RNA was carefully removed and transferred to an autoclaved plastic eppendorf tube. As DNA and proteins remain in the organic phase and at the interface care was taken to avoid taking material from this area. An equal volume of isopropanol was added to the aqueous phase and the sample was incubated at -20°C overnight to precipitate the RNA.

The next day the sample was removed from the freezer and the RNA pelleted by centrifuging at 2065 x g for 10 minutes at 4°C. The pellet formed was washed using 1 ml of ice-cold 75% ethanol (95% or 100% ethanol diluted to 75% with DEPC-treated water). The sample was then centrifuged again at 2065 x g for 10 minutes at 4°C.
The pellet was air-dried in an RNase-free environment for 5 minutes. Care was taken not to dry the pellet completely as desiccated RNA is extremely difficult to re-suspend. The RNA isolated was dissolved in 250μl of nucleas-free water and stored at −20°C.

6.2.1.4 Measurement of concentration and A260/A280 ratio of total RNA isolated.

Pure RNA exhibits an A260/A280 ratio of 2.0. However, due to variations between individual starting materials and in performing the procedure, the range of RNA by A260/A280 ratio was expected to be 1.7-2.0.

Total RNA concentration was measured by absorption at 260nm using a Gene Quant II microspectrophotometer (Pharmacia Biotech, St Albans, UK). A 70 μl cell was used to measure samples and the background reading of the spectrophotometer was set with nucleas-free water. To measure RNA the dilution factor was set at 2 (35 μl of nucleas free water and 35 μl of sample). The cell was inserted and removed when indicated and the concentration, ratio and absorbance of each sample read and recorded. Yields of total RNA from the adipose tissue biopsies ranged from 38.6 ng/μl. The absorption ratio (260:280) was between 1.79 and 2.0 for all preparations.

6.2.2 GENERAL RT-PCR METHOD AND CONDITIONS

Adipose tissue RNA samples were amplified in an Omn-E thermal cycler (Hybaid, Middlesex, UK) using the Access RT-PCR System kit (Promega Ltd, UK). The kit is designed for the reverse transcription and PCR amplification of a specific target RNA from either total RNA or mRNA. The system uses AMV Reverse Transcriptase (AMV RT) from Avian Myeloblastosis Virus for first strand cDNA synthesis, and the thermostable Tfl DNA Polymerase from Thermus Flavus for second strand cDNA synthesis and DNA amplification.

Working on ice, the reaction mixture, labelled as a mastermix, was prepared by combining the components of the kit in the following ratio.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleas-Free Water</td>
<td>xμl</td>
</tr>
<tr>
<td>AMV/Tfl 5x Reaction Buffer</td>
<td>10μl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1μl</td>
</tr>
</tbody>
</table>

(to a final volume of 49μl)
downstream primer 1 µl (50 pmol)
upstream primer 1 µl (50 pmol)
25 mM MgSO₄ 2 µl
AMV Reverse Transcriptase 1 µl
Tfl DNA Polymerase 1 µl

RNA sample x µl (volume equivalent to 100 ng/µl)

The components were mixed by vortexing gently for 10 seconds and 49 µl of mastermix was transferred into each of 7 thermocycler tubes.

1 µl of standard was added to each reaction tube (in a separate area to PCR set-up area) to cover a range of concentrations i.e. 10-fold, 5-fold or 2-fold dilution of standard concentration. The tubes were then transferred to a Hybaid Omn-E thermal cycler and cDNA synthesis amplified under the conditions shown in fig 6.2.

---

**Reverse transcription step**

48°C – 45 min 1 cycle
94°C – 1 min

**Amplification step**

94°C – 30 seconds (denaturing)
62°C – 1 min (annealing) 27-35 cycles
68°C – 2 min (extension)

---

**Figure 6.2.** Conditions of PCR amplification.
6.2.2.1. **PCR primers**
The primers described by Laville et al. (1996) were supplied by Gibco BRL custom primers, Life Technologies Ltd. The primers used to amplify the LPL and \( \beta_2 \)-microglobulin (\( \beta_2 \)-\( \mu \)glob) target genes as well as standards are listed below.

♦ LPL Upstream oligonucleotide primer (CBLPLU).

\[
5' \text{CACTGGGTAATGCTCCTGAG} 3'
\]

♦ LPL downstream oligonucleotide primer (CBLPLD). Sequence 5' to 3'.

\[
5' \text{ACACAGCTGAGGACACTTG} 3'
\]

♦ \( \beta_2 \)-\( \mu \)glob upstream oligonucleotide primer (CBGLOBU).

\[
5' \text{GATGCTGCTTACATGTCTCG} 3'
\]

♦ \( \beta_2 \)-\( \mu \)glob downstream oligonucleotide primer (CBGLOBU).

\[
5' \text{CCAGCAGAGAATGGAAAGTC} 3'
\]

6.2.2.2 **Internal Standards**
The internal standard for LPL and \( \beta_2 \)-\( \mu \)glob was prepared by another investigator as described in Murphy et al. (1999). Briefly a vector (containing the multispecific internal standard) was cloned in E-Coli, cultured in LB-media and ampicillin and the resulting culture extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, Southampton, UK). The concentration of the synthetic RNA standard was measured spectrophotometrically by absorbance at 260 nm. The size of the standard was confirmed by running the RNA on an agarose gel (1.8 % w/v) alongside a known marker.
6.2.3 SEPARATION AND ANALYSIS OF PCR PRODUCTS

6.2.3.1 Agarose gel preparation and gel electrophoresis

RT-PCR reaction products were visualised using ethidium bromide stained agarose gels. In this work 3% (w/v) agarose gels were used. The materials used in the preparation and running of gels are outlined below.

1 x Tris acetate (TAE) buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma base</td>
<td>48.4 g</td>
</tr>
<tr>
<td>0.5M EDTA, PH 8.0</td>
<td>20 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>11.42 ml</td>
</tr>
</tbody>
</table>

Made up to 10 litres with distilled water. PH adjusted to 8.3.

Orange G loading dye

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange G dye</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Formamide</td>
<td>10 ml</td>
</tr>
<tr>
<td>0.5M EDTA, PH 8.0</td>
<td>200 μl</td>
</tr>
</tbody>
</table>

Mixed and stored at room temperature in a foil-covered container. 3 μl of Orange G loading dye per 15μl of sample was used for gel electrophoresis.

1 Kb DNA ladder mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Kb DNA ladder (1 μg/μl)</td>
<td>10 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>90 μl</td>
</tr>
<tr>
<td>Orange G loading dye</td>
<td>30 μl</td>
</tr>
</tbody>
</table>

The agarose gel was made by adding the appropriate amount of agarose to 100 ml of 1 x TAE buffer and heating in a microwave for 3-4 minutes until completely dissolved. The molten agarose was left to cool slightly before adding 3 μl of ethidium bromide (10 mg/ml), swirling to mix. The agarose was cooled to a hand-hot temperature and poured into a gel mould. The gel was allowed to set at room temperature, wrapped in Clingfilm, and stored at 4°C until required for use.
When PCR products were to be loaded the gel cast was inserted into an electrophoresis gel tank and over-layered with TAE buffer. 15 μl of each amplified PCR product was mixed with 3μl of the orange G loading dye and loaded using a finpipette. A 1 Kb DNA ladder was run in an adjacent lane to confirm the size of the PCR product. The tank was filled with sufficient TAE buffer and gel was run at a constant current of 100mV over a period of 2-3 hours.

6.2.3.2 Quantification of PCR products

The agarose gels were visualised under UV light and a positive and negative photograph (665 film; Polaroid) of the gel was obtained. An example of a typical photograph obtained following competitive RT-PCR is shown in figure 6.3. The density of the standard and target template was measured on the negative film using a dual-wavelength, flying spot scanner (Shimadzu Europa GMBH, Duisberg, Germany). To determine the point of equivalence (the point at which the density of the target and internal standard products were equal), the log of the ratio of the internal standard to the target template was plotted against the log of the internal standard concentration. Figure 6.4 shows that the resultant curve was rectilinear with a slope of one demonstrating that the internal standard was amplified with the same efficiency as the target mRNA. The point of equivalence lies at log (standard/target) = 0.

![Figure 6.3. Example of LPL and β2-μglob standard and target products visualised under UV following the PCR reaction.](image-url)
Figure 6.4. Example of plot of log of ratio of corrected target to standard band density to log no. of molecules of RNA standard.

The majority of the RNA samples were analysed using either a 2-fold or a 5-fold serial dilution of the standards. A series of 2-fold and 5-fold dilutions of standard were prepared in advance of the PCR procedures and ranging in a starting concentration of between 1 ng/μl to 1 fg/μl. In order to compare samples as accurately as possible, the target mRNAs from both the pre-intervention and post-intervention studies were measured in the same run, using the same mastermix of reagents.

The specific calculations used to estimate the number of molecules of LPL mRNA per molecule of β₂-μglob mRNA in each sample is outlined below.

1. The target and standard RNA templates differed in size i.e. in the number of base pairs (BP) that they contain. The greater the number of base pairs the more ethidium bromide that is taken up. To correct for this the ratio of the size of the
target to standard was calculated for each gene to determine a correction factor as shown.

**LPL correction factor**

LPL target (BP = 227) : LPL standard (BP = 267) = 0.85

**β₂-μglob correction factor**

β₂-μglob target (BP = 269) : β-MG standard (BP = 306) = 0.88

Having measured the target and standard band densities (using the dual wave scanner) it was necessary to correct for the difference in the size of the mRNA templates by multiplying the target band densities by the appropriate correction factor as calculated above.

2. The number of molecules of mRNA in each concentration of standard was determined from the calculations below.

1 mole of LPL/ β₂-μglob target = 6.023 X 10^{23} molecules (i.e. Avogadro's number)

Molecular weight (MW) of 1 RNA base = 343

Weight 1 mole of LPL target = 343 X 267 (number of BP) = 91581 (g)
Weight 1 mole of β₂-μglob target = 343 X 306 (number of BP) = 104958 (g)

1 (g/mole) LPL target = 6.58 X 10^{18} molecules mRNA
1 (g/mole) β₂-μglob target = 5.74 X 10^{18} molecules mRNA

3. The log of the ratio of the corrected target to standard band density was plotted against the log of the number of molecules of target RNA used in the PCR reaction.
4. The number of molecules of target mRNA was calculated from the equation of the line i.e. $Y = MX + C$, where $y = 0$.

5. Finally the results were expressed as the number of molecules of LPL mRNA per molecule of $\beta_2$-µglob mRNA.

6.2.4 STATISTICAL ANALYSIS

The paired Students $t$ test was used to compare levels of target LPL mRNA expression in adipose tissue obtained prior to and following each dietary intervention study. The Pearson's correlation was used to compare measures of LPL mRNA expression with all other parameters measured during the intervention studies.

6.3 RESULTS

The mean level of LPL mRNA expression (number of molecules of LPL mRNA / ng mRNA) following the n-3 PUFA, low SFA / high CHO and low SFA / high MUFA intervention studies, measured using the competitive RT-PCR method are shown in figs. 6.5., 6.6. and 6.7. respectively. There was a wide variation in the level of LPL mRNA expression both between and within each intervention diet. In the fish oil intervention the mean LPL expression value was $1.61 \times 10^4$ molecules of LPL mRNA per ng total RNA on the control diet and $2.51 \times 10^4$ molecules of LPL mRNA per ng total RNA on the n-3 PUFA enriched diet. There was no significant difference in the level of LPL gene expression as a result of the n-3 PUFA enriched diet ($p = 0.10$). In the second intervention mean LPL expression values were higher than the fish oil intervention at $1.11 \times 10^6$ molecules of LPL mRNA per ng total RNA on the habitual diet and $2.95 \times 10^6$ molecules of LPL mRNA per ng total RNA on the low SFA / high CHO diet. No significant difference in LPL gene expression was detectable following the low SFA / high CHO diet ($p = 0.58$). In the MUFA intervention LPL gene expression was higher as a result of the low SFA / high MUFA diet at $9.05 \times 10^4$ molecules of LPL mRNA per ng total RNA compared with $2.79 \times 10^4$ molecules of LPL mRNA per ng total RNA on the habitual diet, however this did not reach a level of significance ($p = 0.07$).
Figure 6.5. Mean LPL mRNA expression (molecules LPL mRNA / ng total mRNA) measured in adipose tissue biopsy samples obtained at the end of the control diet and at the end of the fish oil diet (n = 11).

Figure 6.6. Mean LPL mRNA expression (molecules LPL mRNA / ng total mRNA) measured in adipose tissue biopsy samples obtained prior to and at the end of the low SFA / high CHO diet (n = 11).
Figure 6.7. Mean LPL mRNA expression (molecules LPL mRNA / ng total mRNA) measured in adipose tissue biopsy samples obtained prior to and at the end of the low SFA / high MUFA diet (n = 9).

To control for variations in the PCR technique the results were expressed as the number of molecules of LPL mRNA per molecule of β2-μglob and these results are presented for each intervention study in table 6.1. There was no change in the expression of LPL mRNA expressed as the number of molecules of LPL mRNA per molecule of β2-μglob as a result of any of the dietary interventions.
Table 6.1.  LPL gene expression expressed as no. of molecules of LPL mRNA per molecule of β2-microglobulin mRNA for each intervention study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Control diet</th>
<th>Intervention diet</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-3 PUFA</td>
<td>0.28 ± 0.21</td>
<td>0.47 ± 0.36</td>
<td>0.17</td>
</tr>
<tr>
<td>Low SFA / high CHO</td>
<td>1.18 ± 1.54</td>
<td>0.76 ± 1.50</td>
<td>0.27</td>
</tr>
<tr>
<td>Low SFA / high MUFA</td>
<td>0.54 ± 0.48</td>
<td>0.86 ± 1.15</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD.

There was no association with the level of mRNA LPL gene expression and post-heparin LPL activity in either the n-3 PUFA intervention or the low SFA/ high CHO intervention. In the MUFA intervention study there was a positive association between the level of post-heparin LPL activity and the level of LPL gene expression following the low-SFA / high-MUFA diet as shown in figure 6.8. In the MUFA intervention study there was also a positive association between levels of fasting insulin and levels of LPL gene expression prior to the intervention as shown in fig 6.9. It is interesting to note that in the low-SFA / high-CHO intervention the levels of LPL activity as well as LPL mRNA expression were higher when compared to the n-3 PUFA enriched diet and the low SFA / high MUFA intervention diet.

An association between TG concentrations and LPL gene expression was detectable only in the n-3 PUFA intervention. As shown in figure 6.10, the change in fasting TG concentration was negatively correlated with the change in LPL gene expression ($r = -0.6918, p = 0.02$). In addition, the change in postprandial TG concentrations was also associated with the change in LPL gene expression following the n-3 PUFA enriched diet as shown in fig 6.11. ($r = -0.6031, p = 0.05$). This would indicate that the greater the improvement in TG concentrations following the n-3 PUFA enriched diet the greater the level of LPL gene expression. In the n-3 PUFA study the change in LPL
gene expression was also associated with the change in the postprandial insulin response as shown in fig 6.12. (r = -0.6179, p = 0.04). Those subjects therefore, with the greatest reduction in postprandial insulin concentrations also had the greatest increase in LPL gene expression levels following the n-3 PUFA enriched diet. There was no association between the level of LPL gene expression and either TG or insulin concentrations in the low SFA / high CHO diet.

![Graph showing the association between LPL activity and gene expression](image)

**Figure 6.8.** The association between post-heparin LPL activity levels (μmole.oleate.ml.hr) and the level of LPL gene expression (no. of molecules of LPL mRNA per ng total RNA) following the low SFA / high MUFA diet (r = 0.6874, p = 0.04).
Figure 6.9. The relationship between fasting insulin levels and LPL gene expression measured prior to the low SFA / high MUFA diet ($r = 0.774$, $p = 0.01$).

Figure 6.10. The association between the change in fasting TG concentration and the change in LPL gene expression following the $\nu$-3 PUFA enriched diet ($r = -0.6918$, $p = 0.02$).
Figure 6.11. The association between the change in postprandial TG AUC and the change in LPL gene expression following the n-3 PUFA enriched diet ($r = -0.6031$, $p = 0.05$).

Figure 6.12. The association between the change in postprandial insulin AUC and the change in LPL gene expression following the n-3 PUFA enriched diet ($r = -0.6179$, $p = 0.043$).
6.4 DISCUSSION

The development of a competitive RT-PCR method in this laboratory, facilitated the measurement of LPL gene expression in small amounts of subcutaneous adipose tissue. In this study, three separate dietary intervention studies were conducted and adipose tissue biopsy samples were obtained prior to and following each dietary intervention. The aim of the study was to measure LPL gene expression using the RT-PCR method to investigate whether changes in lipid metabolism as a result of the dietary interventions were related to the regulation of LPL in adipose tissue.

In order to correct for any imprecision's in the RT-PCR method the results were expressed in relation to a reference gene i.e. β2-microglobulin. There was no difference in the expression of LPL gene, expressed as the number of molecules of LPL mRNA per molecule of β2-µglob mRNA following any of the dietary interventions. There was also no change in the absolute levels of LPL mRNA (expressed without the reference gene) as a result of any of the dietary intervention studies. LPL gene expression was therefore unaffected by either an increase in n-3 PUFA intake, a decrease in SFA intake or an increase in CHO or MUFA intake in this group of middle aged men. The expression of the results against a reference gene (β2-µglob) was designed to account for variations in the RT-PCR method however, as pointed out by Murphy et al. (1999) there is no universally accepted reference gene for adipose tissue. In addition, it is expected that the reference gene will remain at a constant level of expression regardless of cell cycle state, age of cell or external stimuli, however as pointed out by Zimmerman & Mannhalter (1996) it cannot be assumed that the housekeeping gene will maintain a steady state of expression under all circumstances. While the β2-µglob gene has been utilised as a reference gene in many studies the expression of β2-µglob has not been tested under conditions where dietary fat has been altered and it is recommended that future studies should address this.

Post-heparin LPL activity levels were not associated with measurements of LPL gene expression in either the n-3 PUFA or the low-SFA / high-CHO dietary studies. In the MUFA intervention while no changes occurred in either the level of post-heparin LPL
activity or adipose tissue LPL gene expression as a result of the intervention there was a positive association between each measure of LPL following the low SFA / high MUFA diet. This would indicate that any change in post-heparin LPL activity was reflected in a change in the level of LPL gene expression. Few studies have related measurement of post-heparin LPL activity to LPL gene expression, however it accepted that measurement of post-heparin LPL activity alone is a rather crude indication of the regulation of LPL in the human body. For example a number of tissues, including muscle, contribute to the LPL released into plasma in response to an injection of heparin. Post-heparin LPL activity measurements alone therefore, do not provide information as to tissue specific effects in the regulation of LPL.

The significant negative relationships between the change TG levels (fasting and postprandial) and the change in LPL gene expression in the n-3 PUFA intervention is in common with the finding of Murphy et al. (1999). This association was not detected in the other intervention studies conducted in this project and suggests that the relationship between the consumption of fish oil and changes in TG concentration are in part determined by changes in the expression of LPL in adipose tissue. The fact that the changes in LPL expression were correlated to changes in plasma TG and not changes in post-heparin LPL activity suggests that LPL expression is a longer term regulator of plasma lipid levels. Further studies involving larger numbers of subjects are required to provide more information as to the effect of fish oil feeding on LPL gene expression.

In conclusion the evidence provided from this work suggests that changes in plasma TG in response to an alteration in the intake of dietary fatty acids are related to changes in LPL gene expression only when the intake of long chain n-3 PUFA are increased. We found no relation between the expression of adipose tissue LPL and changes in any of the lipid parameters measured following the low-SFA / high-CHO or the low-SFA / high MUFA interventions suggesting that alternative mechanisms are involved in the observed changes in lipid metabolism following a reduction in SFA. The method of increasing long chain n-3 PUFA intake in this intervention was via foods enriched with fish oil. Greater TG lowering effects have been observed in interventions using capsule supplementation. To explore the relationship between LPL gene expression and alterations in n-3 PUFA intake it is recommended that
future studies involving a greater number of subjects and an intervention using fish oil capsules alone are conducted. In addition these studies were conducted in genetically uncharacterised men and it is now known that individuals differ in their response to alterations in the intake of fatty acids as a result of genetic influences e.g. those with an apo E4 phenotype have a greater cholesterol lowering response. Greater insight into the regulation of LPL gene expression following alterations in the intake of dietary fatty acids could be obtained in studies involving groups of individuals that have been genetically characterised.
CHAPTER 7.0

OVERALL FINDINGS

7.1 STUDY DESIGN

Derangements in postprandial lipid metabolism are increasingly recognised as a risk factor for CHD, primarily due to the association between an enhanced postprandial response and the risk of developing atherosclerosis (Boquist et al. 1999). Our understanding of the factors involved in the regulation of postprandial lipaemia is continually evolving however many aspects of the postprandial process remain to be elucidated. As discussed in the introduction, the relationship between insulin resistance with respect to glucose disposal and disturbances in postprandial lipid metabolism has been relatively under-explored (Guerci et al. 2000). It has long been recognised that resistance to the action of insulin is associated with various metabolic abnormalities including; hypertriglyceridaemia, a greater number of small dense LDL particles, low levels of HDL-cholesterol, elevated NEFA concentrations, and in some instances hyperinsulinaemia (Frayn, 1993). Few studies however, have measured the insulin sensitivity of glucose disposal directly and compared this to measures of fasting and postprandial lipid metabolism. The majority of the studies conducted have relied on fasting insulin concentrations as a surrogate measure of insulin resistance (Jeppesen et al. 1995; Boquist et al. 2000). While many studies have demonstrated that there is an association between fasting insulin concentrations and lipoprotein fractions including VLDL, HDL and LDL levels the value of using measures of fasting insulin and glucose levels to interpret the interrelation between insulin sensitivity and lipoprotein metabolism is questionable (Jensen, 2000). In consideration of this observation, the focus of this study was to investigate the relations of insulin sensitivity measured using the short insulin tolerance test to postprandial lipaemia, when dietary fat composition is altered in the diets of middle-aged men.
7.1.1 STUDY DESIGN LIMITATIONS

Three separate dietary intervention studies were conducted over the course of this project. The first intervention was designed to investigate the effect of an n-3 PUFA enriched diet. The second intervention involved a decrease in SFA intakes and an increase in CHO intakes. The final intervention was designed to examine the effect of replacing SFA with MUFA. The studies conducted were intense, expensive metabolic studies involving 3 days of investigation conducted pre-and post-intervention (2 of which are reported on in this thesis) some of which could only be applied to two subjects at any one time. The effect of this is that the number of subjects that could be studied was limited in each intervention: 32 were studied in all.

In addition, these subjects were genotypically uncharacterised. When this study was originally planned the importance of genotype was not considered in the selection of subjects. As discussed in chapter 1.0 population studies have shown that not only do genetics play a role in determining an individual’s lipid profile, but it can also influence their response to a lipid-altering intervention (Williams et al. 1995; Ordovas & Schaefer, 2000). For example, it has been shown that plasma cholesterol, LDL-cholesterol and apo B levels are highest in subjects carrying the apo E4 isoform, intermediate in those with the apo E3 isoform, and lowest in those with the apo E2 isoform and that the response to dietary fat intake may differ among individuals with different apo E phenotypes (Schaefer et al. 1986; Ordovas et al. 1987; Wilson et al. 1994). In particular, it is thought that subjects with an apo E4 isoform have a greater response to cholesterol-lowering diets. The effects of TG-lowering interventions across different apo E genotypes have produced conflicting reports, however it has been observed that carriers of the apoE2 isoform have a hyper-TG response to low-fat and high-CHO diets (Ordovas et al. 1995). The genetic factors influencing insulin sensitivity and changes in insulin sensitivity are less well defined. Within this study we observed a great deal of individual variation both in response to the high fat meal and the insulin tolerance test. Genotypic heterogeneity may have accounted at least in part for these observed differences. Had genotyping been incorporated into the screening programme for subject selection more information could have been obtained as to the effect of manipulating dietary fat intakes on the insulin sensitivity of glucose and lipid metabolism in individual’s within specific genotypes. In addition,
it may have been possible to identify clearer relationships between the insulin sensitivity of glucose disposal and postprandial lipaemia.

Finally the method of measuring insulin sensitivity in this study was with the short ITT and while several studies have found that the short ITT compares favourably to other more sophisticated methods of measuring insulin sensitivity a recent study conducted by Hermans et al. (1999) found that the ITT performed less well than other tests due to it’s poor reproducibility. This differs from the work of Hirst et al. (1993) who found that the short ITT compared favourably with other measures of insulin sensitivity including the euglycaemic clamp technique. Hirst et al. (1993) found that the intra CV varied from between 6 and 13 % across a number of studies, which had used the ITT to measure insulin sensitivity. The intra-individual variation of glucose K_{ITT} measured using the short ITT, was determined by another investigator within this laboratory in 6 young men on 4 occasions (unpublished data). The CV was 15 %, similar to the variation in fasting TG (14.5 %) and total cholesterol (10 %). Thus while some reports have indicated that the ITT performs poorly against other measures of insulin sensitivity others have not found this however, more studies are necessary to confirm that using the short ITT does not present a methodological problem.

7.2 OVERALL RESULTS

The findings from the n-3 PUFA, low-SFA / high-CHO, and low-SFA / high-MUFA interventions have been discussed separately in chapters 3.0, 4.0, and 5.0 respectively. Since the same set of measurements were conducted in each intervention study it is possible to combine the data sets to compare overall findings in order to test the original hypotheses which were that:

- Insulin resistance with respect to glucose disposal also extends to deranged postprandial fat metabolism, with consequent increased risk of CHD.

- The insulin sensitivity of these pathways is improved by either increased dietary n-3 PUFA or reduced SFA in normal adults.
7.2.1 INFLUENCE OF DIETARY INTERVENTIONS ON FASTING BLOOD BIOCHEMISTRY

There were few overall changes in the mean values for any of the blood biochemistry measures obtained prior to and following each dietary intervention study. The mean fasting blood biochemistry results for the combined data set is shown in table 7.1. Fasting TG was marginally lower in the post-intervention phase at 1.85 (SD 0.53) mmol/l compared with 2.03 (SD 0.69) mmol/l in the pre-intervention phase (p = 0.09). This was mainly as a result of the highly significant reduction in TG as a result of the low-SFA / high-MUFA trial (p = 0.02) and the mean (non-significant) fall in TG levels in the fish oil group. While a TG lowering effect was most evident with the low-SFA / high-MUFA diet, this group of subjects also had a higher mean baseline fasting TG concentration and may therefore have been more responsive to a change in dietary fat intake. In addition, the non-significant reduction in mean fasting TG in the fish oil group was influenced by one individual with an un-explained increase in fasting TG following the fish oil intervention. Fasting NEFA concentration was significantly reduced for the post-intervention groups overall (p = 0.01) mainly due to the significant fall in the low SFA / high CHO intervention (p = 0.01) and the non-significant fall in the fish oil group. There were no changes in insulin, glucose, HOMA, total-cholesterol, HDL-cholesterol or LDL-cholesterol levels as a result of any of the dietary interventions.
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.03 ± 0.69</td>
<td>1.85 ± 0.53</td>
<td>0.09</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.40 ± 1.28</td>
<td>5.23 ± 1.03</td>
<td>0.36</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.63 ± 1.39</td>
<td>3.55 ± 1.13</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.88 ± 0.18</td>
<td>0.88 ± 0.17</td>
<td>1.00</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td>0.48 ± 0.20</td>
<td>0.39 ± 0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>96.9 ± 56.0</td>
<td>92.7 ± 43.9</td>
<td>0.54</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.39 ± 0.54</td>
<td>5.41 ± 0.45</td>
<td>0.79</td>
</tr>
<tr>
<td>HOMA</td>
<td>3.19 ± 0.54</td>
<td>3.02 ± 1.49</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD.

7.2.2 INFLUENCE OF DIETARY INTERVENTIONS ON POSTPRANDIAL LIPAEMIA

In each intervention study postprandial lipaemia was studied over a 9h period following a standardised high fat breakfast (80 g fat). The results for each intervention and for the group overall are shown in table 7.2. It was observed that there was a wide degree of variation in initial dietary lipid tolerance among the study group. While there was variability in the TG and apo B-48 postprandial levels within the group overall, it was also clear that the responses to the three dietary interventions were quite different. Both the n-3 PUFA and the low-SFA / high-MUFA interventions were associated with improvements in dietary lipid tolerance in terms of both TG AUC and Apo B-48 AUC. In contrast the low-SFA / high-CHO dietary intervention was associated with a worsening of TG AUC but not with a worsening of apo b-48 AUC indicating that there was an additional adverse influence of CHO on VLDL which negates any benefit when SFA is replaced by carbohydrates. This would indicate that either an increase in n-3 PUFA intake or a decrease in SFA intake is
associated with an improvement in postprandial lipaemia at the level of chylomicron clearance, however replacement of SFA with CHO has an adverse effect on VLDL TG levels which is not observed when SFA is replaced by MUFA.

Since postprandial TG clearance can be assumed to reflect insulin levels and action, the TG AUC/insulin AUC ratio is to some extent a measure of the insulin sensitivity of TG clearance. A low ratio would indicate increasing insulin sensitivity, especially where it reflects a lower TG in relation to the insulin level rather than an increasing insulin in relation to the TG level. Table 7.2. shows the change in TG AUC/insulin AUC ratio as a result of the interventions. Thus, in the fish-oil trial insulin sensitivity increased since the TG AUC/insulin AUC ratio fell by 24 % (p = 0.02) with no change in the insulin AUC. In contrast, in the low SFA / high CHO intervention, the TG AUC/insulin AUC ratio increased by 27 % (p = 0.01) with no change in the insulin AUC indicating a fall in insulin sensitivity. In the low-SFA / high-MUFA intervention the mean value fell indicating an increase in insulin sensitivity but the change was not significant.
Table 7.2.  Postprandial insulin, TG and apo B-48 AUC for each intervention study and for the combined data set.

<table>
<thead>
<tr>
<th></th>
<th>n-3 PUFA</th>
<th>Low SFA/ high CHO</th>
<th>Low SFA/ high MUFA</th>
<th>Studies combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>post</td>
<td>pre</td>
<td>post</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Apo B-48 AUC</td>
<td>27.0 ± 13.3</td>
<td>23.6 ± 12.0</td>
<td>28.6 ± 7.0</td>
<td>19.0 ± 8.7*</td>
</tr>
<tr>
<td>(µg.ml.min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG AUC</td>
<td>1575 ± 481</td>
<td>1391 ± 406</td>
<td>1666 ± 406</td>
<td>1990 ± 647</td>
</tr>
<tr>
<td>(mmol.l.min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin AUC</td>
<td>116564 ± 55716</td>
<td>122799 ± 38240</td>
<td>120872 ± 48523</td>
<td>116757 ± 52423</td>
</tr>
<tr>
<td>(pmol.l.min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGAUC/InsulinAUC</td>
<td>1.60 ± 0.66</td>
<td>1.21 ± 0.40*</td>
<td>1.54 ± 0.55</td>
<td>1.96 ± 0.89**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD.  pre = pre-intervention, post = post-intervention

Significant difference from pre to post intervention, * p < 0.05, ** p < 0.01.
As with the fat tolerance test, there was a wide degree of variation in insulin sensitivity among the study groups, with glucose $K_{ITT}$ varying between 1.20 and 6.17 %/min$^{-1}$ prior to the dietary intervention studies. Table 7.3 shows the interrelations between glucose $K_{ITT}$ and fasting glucose and insulin. The data are organised into quartiles of increasing insulin sensitivity and shows that in subjects at baseline glucose $K_{ITT}$ correlated with fasting glucose ($r = -0.39$, $p = 0.03$), fasting insulin ($r = -0.36$, $p = 0.04$) and HOMA ($r = -0.38$, $p = 0.03$). In addition, glucose $K_{ITT}$ was associated with Insulin AUC following the high fat meal ($r = -0.37$, $p = 0.04$). NEFA $K_{ITT}$ was unrelated to glucose $K_{ITT}$ at baseline in the group overall ($r = 0.14$, $p = 0.44$). ANOVA of quartiles showed no significant differences for any of these parameters. Initial fasting insulin was used as a predictor of insulin sensitivity in this study however caution is necessary when using fasting insulin alone as a predictor of insulin sensitivity. The relationship between fasting insulin and glucose $K_{ITT}$ is shown in figure 7.1.

In terms of improvements in the insulin sensitivity of glucose disposal no intervention induced a significant change. Overall, 15 individuals improved, 5 worsened and 12 varied by less than 15% (the intra-individual variation in this test measured by another investigator in this research group). Improvements in glucose $K_{ITT}$ were related to the initial glucose $K_{ITT}$ and those individuals with the lowest insulin sensitivity at the outset had the greatest improvements in insulin sensitivity following the intervention diets. The association between the change in glucose $K_{ITT}$ (pre-intervention glucose $K_{ITT}$ / post-intervention glucose $K_{ITT}$) and the initial glucose $K_{ITT}$ measurements is shown in fig 7.2. Recommendations for future studies would therefore be to include measurements of glucose $K_{ITT}$ at baseline in order to ensure that a more homogenous group, in terms of the insulin sensitivity of glucose disposal, is studied.

The fall in NEFA was used as a measure of the insulin sensitivity of HSL inhibition. Pre-intervention NEFA $K_{ITT}$ values predicted post-intervention NEFA $K_{ITT}$ values ($r^2 = 0.35$) however, there were no significant changes in NEFA $K_{ITT}$ following any of the dietary interventions.
Table 7.3. Analysis of quartiles of increasing insulin sensitivity in relation to other measures of insulin action.

<table>
<thead>
<tr>
<th>Quartile</th>
<th>$K_{\text{glucose}}$ (%.min$^{-1}$)</th>
<th>Fasting insulin (pmol/l)</th>
<th>Fasting glucose (mmol/l)</th>
<th>HOMA</th>
<th>Insulin AUC (pmol/l.min)</th>
<th>$K_{\text{NEFA}}$ (%.min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.84</td>
<td>113.8</td>
<td>5.80</td>
<td>3.95</td>
<td>142983.1</td>
<td>4.13</td>
</tr>
<tr>
<td>2</td>
<td>3.06</td>
<td>110.8</td>
<td>5.32</td>
<td>3.57</td>
<td>132051.2</td>
<td>5.28</td>
</tr>
<tr>
<td>3</td>
<td>3.85</td>
<td>88.0</td>
<td>5.31</td>
<td>2.88</td>
<td>114717.0</td>
<td>4.14</td>
</tr>
<tr>
<td>4</td>
<td>5.05</td>
<td>74.8</td>
<td>5.13</td>
<td>2.36</td>
<td>95058.34</td>
<td>4.66</td>
</tr>
<tr>
<td>ANOVA</td>
<td>0.46</td>
<td>0.07</td>
<td>0.37</td>
<td>0.32</td>
<td>0.32</td>
<td>0.52</td>
</tr>
<tr>
<td>R</td>
<td>-0.36</td>
<td>-0.39</td>
<td>-0.38</td>
<td>-0.37</td>
<td>-0.37</td>
<td>0.14</td>
</tr>
<tr>
<td>P</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.44</td>
</tr>
</tbody>
</table>
Figure 7.1. The correlation between fasting insulin and glucose $K_{ITT}$ in the group overall ($n = 32$) measured pre-intervention ($r = -0.36$, $p = 0.04$).

Figure 7.2. The association between glucose $K_{ITT}$ ($\%$/min$^{-1}$) and the change in glucose $K_{ITT}$ i.e. ratio of glucose $K_{ITT}$ post-intervention to pre-intervention (Delta glucose $K_{ITT}$). $r = -0.78$, $p < 0.0001$. 
7.2.4. INTERRELATION BETWEEN INSULIN SENSITIVITY OF GLUCOSE DISPOSAL AND POSTPRANDIAL LIPAEMIA

As previously discussed few studies have measured insulin sensitivity directly and related this to measures of postprandial lipid metabolism. To test the hypothesis that insulin resistance with respect to glucose disposal also extends to deranged postprandial fat metabolism, insulin sensitivity as $K_{glucose}$ was compared with all variables of postprandial lipaemia by correlation across the pre-intervention data set. The data was organised into quartiles ranked in order of increasing insulin sensitivity (i.e. $K_{glucose}$) and the results of the analysis is shown in table 7.4.

TG AUC was positively correlated with $K_{glucose}$ ($r = 0.35, p = 0.05$) with a 35% difference in TG between the top and bottom quartiles. ANOVA of quartiles however showed that the significance of this difference was low for TG ($p = 0.14$). The relationship between $K_{glucose}$ and postprandial TG may have reflected an increased TG clearance due to increasing hyperinsulinaemia with insulin in-sensitivity since there was also a positive relationship of $K_{glucose}$ with the TG AUC/insulin AUC ratio ($r = 0.52, p = 0.02$). There was no association between $K_{glucose}$ and fasting TG, Cholesterol, LDL-cholesterol and HDL-cholesterol. Neither LPL activity nor mRNA LPL level were related to $K_{glucose}$ as shown in table 7.5.

This data shows that in genotypically uncharacterised middle aged men, the insulin sensitivity of glucose disposal as measured by the short ITT does not predict the apparent sensitivity of lipid-related targets of insulin action in a simple way.
Table 7.4. Analysis of quartiles ranked in order of increasing insulin sensitivity in relation to measures of lipid metabolism in the pre-intervention data (n = 32)

<table>
<thead>
<tr>
<th>Quartile</th>
<th>$K_{\text{glucose}}$</th>
<th>Fasting TG (mmol/L)</th>
<th>Fasting CHOL (mmol/L)</th>
<th>HDL-CHOL (mmol/L)</th>
<th>LDL-CHOL (mmol/L)</th>
<th>TG AUC (mmol/L.min)</th>
<th>Apo B-48 AUC (ug/ml.min)</th>
<th>Insulin : TG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.84</td>
<td>1.76</td>
<td>5.82</td>
<td>0.98</td>
<td>3.99</td>
<td>1421.9</td>
<td>594.3</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>3.06</td>
<td>1.99</td>
<td>5.06</td>
<td>0.88</td>
<td>3.50</td>
<td>1783.9</td>
<td>783.5</td>
<td>1.52</td>
</tr>
<tr>
<td>3</td>
<td>3.85</td>
<td>2.09</td>
<td>5.28</td>
<td>0.87</td>
<td>3.41</td>
<td>1888.8</td>
<td>811.7</td>
<td>1.90</td>
</tr>
<tr>
<td>4</td>
<td>5.05</td>
<td>2.26</td>
<td>5.43</td>
<td>0.79</td>
<td>3.61</td>
<td>1919.4</td>
<td>861.1</td>
<td>2.24</td>
</tr>
<tr>
<td>ANOVA</td>
<td>0.55</td>
<td>0.70</td>
<td>0.18</td>
<td>0.86</td>
<td>0.19</td>
<td>0.19</td>
<td>0.47</td>
<td>0.04</td>
</tr>
<tr>
<td>R</td>
<td>0.25</td>
<td>-0.04</td>
<td>-0.17</td>
<td>-0.06</td>
<td>0.35</td>
<td>0.31</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.16</td>
<td>0.79</td>
<td>0.33</td>
<td>0.72</td>
<td>0.05</td>
<td>0.08</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>
Table 7.5. Analysis of quartiles ranked in order of decreasing insulin sensitivity in relation to measures LPL activity and gene expression (n = 32)

<table>
<thead>
<tr>
<th>Quartile</th>
<th>$K_{\text{glucose}}$</th>
<th>LPL activity (umole oleate.ml.hr)</th>
<th>LPL mRNA (no. of molecules)</th>
<th>LPL : $\beta$-glob ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.84</td>
<td>8.59</td>
<td>$2.47 \times 10^5$</td>
<td>0.86</td>
</tr>
<tr>
<td>2</td>
<td>3.06</td>
<td>7.49</td>
<td>$1.51 \times 10^6$</td>
<td>0.26</td>
</tr>
<tr>
<td>3</td>
<td>3.85</td>
<td>7.91</td>
<td>$1.49 \times 10^5$</td>
<td>0.97</td>
</tr>
<tr>
<td>4</td>
<td>5.05</td>
<td>6.58</td>
<td>$2.14 \times 10^5$</td>
<td>0.69</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>r</td>
<td></td>
<td></td>
<td></td>
<td>-0.07</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td></td>
<td></td>
<td>0.69</td>
</tr>
</tbody>
</table>

7.2.5 RED BLOOD CELL PHOSPHOLIPID DATA

7.2.5.1 Relationship between the dietary intake of fatty acids and the fatty acid content of red blood cell phospholipids

Red blood cell fatty acids were measured and the levels of fatty acids compared to the intake of fatty acids from food. Combining the data for each intervention and for each dietary phase it was shown that there was a positive correlation between the dietary intake of SFA, MUFA and PUFA and the corresponding levels of SFA, MUFA and PUFA measured in the RBC phospholipids. These relationships are depicted in figures 7.3., 7.4., and 7.5, respectively. Red blood cell phospholipid measurement was therefore a useful marker of the dietary intake of fatty acids.
Figure 7.3. The relationship between the dietary intake of SFA and the level of SFA in RBC membrane phospholipids in individual subjects measured pre-and post-intervention (n = 64). $r = 0.34$, $p = 0.005$.

Figure 7.4. The relationship between the dietary intake of MUFA and the level of MUFA in RBC membrane phospholipids in individual subjects measured pre-and post-intervention (n = 64). $r = 0.36$, $p = 0.003$. 
7.2.5.2 The relationship between red blood cell fatty acid content and measures of insulin sensitivity and lipid tolerance.

Having established that the red blood cell fatty acid levels were a reliable indicator of the dietary intake of fatty acids we examined the relationship between all outcome variables and RBC fatty acids. The purpose of this was to test the hypothesis that the insulin sensitivity of pathways of both glucose and lipid homeostasis is improved by \( n\)-3 PUFA and impaired by SFA. Analysis was performed both on the pre-intervention data set (n = 32), the post-intervention data set (n = 32) for total \( n\)-3 fatty acids and SFA in RBC membranes.

For the pre-intervention data saturated fat in terms of palmitate (C16:0), the main saturated fatty acid present in the RBC membrane was inversely related to \( K_{\text{glucose}} \) and was the only significant fatty acid \(( r = -0.44, p = 0.01)\) in a forward stepwise multiple regression which accounted for 28 % of the variance in \( K_{\text{glucose}} \) \((p = 0.012)\). The dependent variable used was \( K_{\text{glucose}} \) and the independent variables were C16:0,
C18:0, C18:1, C:18:2, C22:4, C20:3, C20:4, C20:5 & C22:6. This significant correlation ($r = -0.44$) shown in figure 7.6. means that increasing C16:0 in RBC membranes is related to decreasing insulin sensitivity. Stearic acid (C18:0) was also inversely related to HDL-cholesterol indicating that increasing C18:0 is related to decreasing HDL-cholesterol, both adverse influences ($r = -0.52$, $p = 0.002$). These influences when examined in terms of quartiles of variables ranked for either C16:0 or C18:0 indicate a 21 % improvement in $K_{\text{glucose}}$ and a 26 % increase in HDL-cholesterol from between the top and bottom quartiles of the SFAs as shown in figs 7.7. and 7.8. respectively. In the pre-intervention data therefore it is evident that a high level of saturated fat represented as either C16:0 or C18:0 is associated with a decreased insulin sensitivity and a low level of HDL-cholesterol, both adverse influences in terms of CHD risk.

![Figure 7.6.](attachment:image)

The relationship between RBC phospholipid palmitate content and $K_{\text{glucose}}$ ($r = -0.44$, $p = 0.01$).
Figure 7.7. Insulin sensitivity ($K_{\text{glucose}}$) examined in quartiles of increasing RBC $n$-3 PUFA content ($n = 32$).

Figure 7.8. HDL-cholesterol ranked by increasing RBC C18:0 (stearic acid) content.
In the post intervention data measures of lipid tolerance were generally associated with total n-3 PUFA content of RBC membranes. Thus, there was an inverse association between TG AUC (r = -0.37, p = 0.034) and RBC n-3 PUFA and a direct correlation between RBC n-3 PUFA content and HDL-cholesterol (r = 0.51, p = 0.002). The improvement in HDL-cholesterol with increasing RBC n-3 PUFA content is shown in fig 7.9. where the data is organised into quartiles ranked in order of increasing RBC n-3 PUFA content. Figure 7.10. shows the TG AUC ranked by decreasing total RBC n-3 fatty acids and overall there was a 32 % improvement in the TG AUC between the top and bottom quartiles. The improvement in TG AUC with increasing n-3 PUFA content of RBC membrane phospholipids was not mediated by an increase in LPL activity as LPL activity was inversely related to RBC n-3 PUFA (r = -0.345, p = 0.05) as shown in figure 7.11.

Figure 7.9. The relationship between HDL-cholesterol and RBC phospholipid n-3 PUFA content (n = 32). The data is ranked in quartiles of decreasing RBC n-3 fatty acid content (r = 0.51, p = 0.002).
Figure 7.10. The relationship between TG AUC and RBC phospholipid n-3 PUFA content (n = 32). The data is ranked in quartiles of decreasing RBC n-3 fatty acid content (r = -0.37, p = 0.034).

Figure 7.11. The inverse association between RBC n-3 PUFA content and LPL activity (r = -0.35, p = 0.05).
7.3. DISCUSSION OF OVERALL FINDINGS

Understanding the biology of insulin resistance is important to facilitate the development of new therapies, to optimise current therapies and to identify causative genes and their products. The aspect of insulin resistance, which has been most extensively studied in man, is the defective insulin-mediated uptake and utilization of glucose. However insulin is associated with many more processes other than glucose metabolism including lipid and protein metabolism (American Diabetes Association: Consensus Development Conference on Insulin Resistance, 1997). Insulin inhibits adipose tissue lipolysis, muscle protein catabolism, and the synthesis of some hepatic proteins. Insulin also stimulates the synthesis of the enzyme LPL which is known to play a central role in lipoprotein metabolism. Conditions related to insulin resistance therefore abound (Bonora et al. 1998) and research into the factors associated with IR and how to reduce the risk of developing conditions such as diabetes and CHD, morbidities associated with IR, is ongoing.

Both genetic and environmental factors play a role in the development and progression of insulin resistance. Dietary factors undoubtedly influence insulin action (Storlein et al. 2000) however, controversy exists as to the best dietary approach in the treatment of insulin resistance. Type II diabetes is the metabolic disease most commonly associated with insulin resistance and is characterised by the inability to handle carbohydrate. Storlein et al. (2000) therefore ask ‘why advocate a high-carbohydrate diet to individuals whose core problem is that macronutrient’? Part of the answer relates to the macronutrients available to substitute for carbohydrate. There is limited ability to manipulate the intake of protein, and therefore reducing carbohydrate generally comes at the cost of increasing fat. Since obesity is also part of the metabolic syndrome disease cluster, and insulin resistance is closely linked with adiposity, there is a general reluctance to advocate any dietary therapy likely to increase the intake of fat (Katan, 1997). However, dietary fat is not a single entity, with saturated and unsaturated fatty acids exerting very differing metabolic effects in the body. Recently there has been greater focus on the composition of dietary fat rather than total fat intake per se with regard to effects on insulin action. Saturated fat is generally negatively associated with insulin action whereas polyunsaturated fat is thought of as beneficial. Less evidence exists on the impact of monounsaturated fat.
In addition it has been suggested that saturated and unsaturated fats have very differing effects not only on energy storage and utilisation, and in their function as structural components of cell membranes but also in their role as regulators of gene expression (Storlein et al. 2000). There are also gaps in the literature on the optimum type of carbohydrate in the diet, the impact of dietary fibre and the role of the glycaemic index in insulin resistance (Riccardi & Rivellese, 2000). In this study we attempted to identify the effect of changes in the composition of dietary fat through an increase in the intake of n-3 PUFA or a decrease in the intake of SFA on measures of insulin sensitivity. To address the question of whether it is of greater benefit to increase the intake of carbohydrate or increase the intake of MUFA in order to reduce the intake of SFA one intervention was designed to decrease the intake of SFA in favour of an increase in CHO while a second was designed to decrease the intake of SFA in favour of an increase in MUFA.

Nutritional recommendations have placed a greater emphasis on the need to reduce percentage of fat in the diet, rather than a need to decrease the absolute amount of fat or to alter the composition of fat in the diet. Accordingly, this recommendation has taken hold in many sectors of the food industry and the number of low-fat or %-fat-free labelled products has increased on our supermarket shelves. In addition, despite more than a decade of public health messages aimed at the promotion of lower fat diets this has not resulted in a decrease in the prevalence of obesity nor in the occurrence of metabolic disorders. Achievement of a low-fat diet among many populations would require a dramatic shift in eating habits and it is not altogether surprising that in today’s affluent society this has been difficult to achieve. What may be more attainable is to change the composition of that fat intake towards a greater amount of unsaturated fat and a lower intake of saturated fat. In consideration of this the intervention studies discussed in this thesis were designed to focus on altering the composition of fat in the diet and not decreasing the intake of total fat alone. The method of altering the composition of fat in the diets of the volunteers was through foods in which the type of fat used to make the food was altered or in the case of the low-fat/ high-CHO intervention through the use of commercially available low-fat foods. A strength of this study therefore, is that it was conducted under normal free-living conditions, using familiar foods such as sauces, cakes and biscuits. The study demonstrates that it is feasible to achieve alterations in the composition of dietary fat...
intakes in men habitually consuming a western style diet, without the consumption of unfamiliar foods or without the use of liquid formula diets or capsule supplements alone.

Assessing dietary compliance in the free-living population is often difficult and most studies rely on the interpretation of food records (Livingstone, 1995). Participants in this study reported energy and macronutrient intakes that were extremely close to the target intakes. Confidence in the accuracy of the dietary assessment is also increased by the observation that lower energy intakes were associated with a reduction in body weight (mean 1.7 Kg) during the low-SFA/ high-CHO intervention, where a reduction in total fat intakes also occurred. A significant weight loss following the consumption of low-fat diets of this nature has also been observed in other studies (Kasim et al., 1993; Shah et al., 1994). Apart from the analysis of food diaries, a second measure of compliance in this study was the measurement of red blood cell phospholipid fatty acid composition. Across all of the intervention studies pre- and post-intervention there was a positive association between the intake of individual dietary fatty acids and the measurement of individual fatty acids in the red blood cell phospholipids. Measurement of red blood cell phospholipid fatty acid composition therefore proved to be a useful marker of the intake of dietary fatty acids.

Changes in fasting biochemical measurements were most evident in the low SFA/ high MUFA diet where there was an 11 % reduction in total cholesterol and a 15.4 % reduction in the levels of fasting TG. In the n-3 PUFA intervention study the reduction in fasting TG was influenced by one individual who had an increase in fasting TG as a result of the intervention, making the fall in TG non-significant (p = 0.06). The increase in fasting TG, which might have been expected to occur as a result of the increase in CHO during the low-SFA/ high-CHO diet, was not evident however, this did not hold true in postprandial measurements of TG. Overall no changes were observed in the levels of LDL-cholesterol and HDL-cholesterol or in the fasting measures of glucose and insulin as a result of any of the intervention diets. There was however, a significant decrease in the level of fasting NEFA when the results of each intervention were combined, mostly as a result of the highly significant reduction in NEFA concentrations following the low-SFA / high-CHO diet. NEFAs have been shown to be an important stimulus to hepatic TG secretions and that
inadequate suppression of NEFAs may result in increased TG concentrations (Mckeigue et al. 1993; Byrne et al. 1994). Byrne et al. (1995) conducted a study in which individuals were studied using a glucose tolerance test and subsequently divided into quartiles based on 2-hour glucose measurements. They observed that individuals in the upper quartile of glucose tolerance (i.e. most glucose intolerant) also had the highest mean TG concentrations and the highest mean NEFA concentrations. In addition, Paolisso et al. (1995) have also demonstrated that large fat cells and the resulting increased plasma NEFA concentrations are independent risk factors for the development of type II diabetes. In terms of CHD risk, Frayn (1998) speculates that failure to regulate the plasma NEFA concentration normally both in the fasting and postprandial state, might be associated with development of a number of risk factors for CHD, including insulin resistance, hypertriglyceridaemia, hyperapoB and increased coagulant activity. Plasma NEFA concentrations are however inherently variable from subject to subject and also within subjects from day to day (Frayn, 1998). In addition, there are no population derived reference ranges within which to define a raised or normal NEFA level. The reduction in fasting NEFA levels observed in this study were not associated with a reduction in either fasting or postprandial TG and the benefit of reducing NEFA levels alone is not known since few studies have examined the effect of reducing NEFA levels on CHD risk. Therefore, while there seems to be good evidence to suggest that plasma NEFA concentration may play a central role in the risk factors associated with insulin resistance more studies are needed to gain a greater understanding of the effects of alterations in fasting NEFA levels as a result of alterations in dietary fat intake.

The short ITT was the method of measuring insulin sensitivity employed in this study. Across all of the intervention studies there was a wide degree of insulin sensitivity among the subjects, with initial fasting insulin levels predictive of the degree of insulin resistance. Overall, there was no change in insulin sensitivity as a result of any of the dietary interventions, however it was evident that the improvements in insulin sensitivity occurred in individuals with the greatest degree of insulin resistance at baseline. When the pre-intervention data for the combined studies was organised into quartiles based on glucose K_{ITT} it was observed that those in the lowest quartile (i.e. most insulin resistant) also had the highest levels of fasting glucose, fasting insulin and the highest insulin AUC measurements following the high fat meal. The HOMA
model is another measure of insulin sensitivity, which is determined from fasting insulin and glucose levels (Matthews et al. 1985), and there was also an association between insulin sensitivity measured using the short ITT and HOMA. It appears therefore that the short ITT provided a good measure of the insulin sensitivity of glucose disposal in this study. A fasting insulin level greater than 40 pmol/l was included in the screening criteria for the selection of subjects for this study, however those subjects who had a low insulin sensitivity measured using the ITT the mean fasting insulin was 112.4 (SD 58.5) pmol/l. It is recommended for future studies of this nature that a fasting insulin level of at least 90 pmol/l is used to predict insulin resistance or that baseline assessments of insulin sensitivity are included to select a more homogeneous group.

As far as the hypothesis that glucose $K_{\text{ITT}}$ would predict postprandial lipaemia, the opposite was observed with TG AUC positively correlated with glucose $K_{\text{ITT}}$. This indicates that the greatest TG response following the ingestion of a high-fat meal was observed in individuals who were the most insulin sensitive. The TG AUC to insulin AUC ratio, which could be used as a measure of the insulin sensitivity of TG clearance, was also positively associated with glucose $K_{\text{ITT}}$. In addition, there was also a marginal association between glucose $K_{\text{ITT}}$ and apo B-48 AUC ($p = 0.08$). No associations were evident for fasting TG, cholesterol, HDL-cholesterol, LDL-cholesterol, LPL activity or LPL mRNA expression. These findings are in contrast to those of Graci et al. (1999) who found that when subjects ($n = 40$) were divided into quartiles of insulin sensitivity, measured using the short ITT, the concentrations of TG and cholesterol were progressively increased from the most insulin sensitive quartile to the least insulin sensitive quartile. Relationships between fasting insulin levels, as a surrogate measure of insulin resistance, and fasting plasma lipoprotein levels have also been established in many population based studies (Laakso et al. 1990; Ostlund et al. 1990; Haffner et al. 1993). In a recent study by Boquist et al. (2000) fasting insulin was found to be associated with the TG response to a high fat meal independently of BMI, waist-to-hip ratio, blood glucose and fasting TG. The differences between the study of Boquist and colleagues and this study however, is that firstly subjects in that study were selected on the basis of their Apo E genotype (all participants were homozygous for the apo E3 allele) and secondly the range of fasting insulin was much lower in the Swedish study with the majority of the
subjects would being judged as insulin sensitive according to their fasting insulin values (only 15% had a fasting insulin > 60 pmol/l). In addition, the range of mean fasting plasma TG concentrations in quartiles of glucose $K_{ITT}$ was from 1.06 - 1.79 mmol/l in the Swedish study whereas in our study the fasting TG concentrations were higher and ranged from 1.79 - 2.26 mmol/l across quartiles of insulin sensitivity. Fasting concentrations of TG above 1.9 mmol/l have been shown to be associated with an unfavourable lipoprotein profile, the ALP and an ALP has been shown to be strongly associated with the insulin resistance syndrome. Few of the Swedish subjects would therefore have been classified as having an ALP whereas the majority of our subjects would have fallen into this category. It is possible therefore that associations between insulin sensitivity and measures of fasting lipid metabolism are apparent only within relatively normal or low concentrations of fasting TG and low concentrations of fasting insulin, both of which are unlikely to indicate any metabolic disturbances.

The argument against recommending carbohydrate as a source of replacement energy for saturated fat hinges largely on the observation that high-carbohydrate diets are associated with an increase in TG and a reduction in HDL-cholesterol. Several authors have argued strongly in favour of replacing saturated fats with unsaturated fats (Grundy, 1986, Mensink & Katan, 1987; Katan et al. 1997) an argument which is supported by epidemiological studies e.g. the low CHD rates in people habitually consuming a Mediterranean diet (Kushi et al. 1995). In this study while fasting levels of TG and HDL-cholesterol were unchanged following the low-SFA/high-CHO intervention there was a 20% increase in levels of postprandial TG with the most acute increase in the late postprandial phase (6-9 hour TG response). In addition, the levels of TRL-TG were significantly accentuated following the low-SFA / high-CHO diet. The results of this study therefore substantiate the view that a high-CHO diet is detrimental in terms of causing unfavourable changes in lipoprotein levels. In contrast, the low-SFA / high-MUFA diet was associated with an improvement in postprandial TG, TRL-TG and apo B-48 concentrations, further endorsing the findings of previous studies that replacement of SFA with MUFA is more beneficial than replacement with dietary CHO. In the n-3 PUFA intervention, while there was no change in postprandial lipaemia as a result of fish oil feeding in the group overall, it was evident that the greatest improvements in postprandial TG concentrations occurred in subjects who had the greatest degree of postprandial lipaemia at the outset.
of the study. Similarly Harris et al. (1988) demonstrated that in a group of non-diabetic hypertriglyceridaemic subjects, a higher initial TG level was associated with a greater TG reduction as a result of fish oil administration. In addition, Connor et al. (1993) found that fish oil incorporated into the diet of hypertriglyceridaemic subjects had a more profound hypolipaemic effect than had been observed in normal subjects.

In the n-3 PUFA intervention while the concentration of TG was not significantly attenuated over the entire 9-hour postprandial study there was a significant lowering of TRL TG in the late (6-9 hour) postprandial phase. Many investigators have highlighted that an accentuated lipoprotein profile in the late postprandial phase is potentially more atherogenic (Groot et al. 1991; Patsch et al. 1992; Uiterwaal et al. 1994; Karpe, 1997) therefore the reduction in lipaemia in the late postprandial phase observed in this study is favourable in terms of reducing CHD risk.

It is known that peripheral insulin sensitivity can be influenced by phospholipid fatty acid composition and that changes in the fatty acid composition of the cell membranes may modulate the action of insulin (Borkman et al. 1993). Increasing the content of PUFA within cell membranes in cultured cells increases membrane fluidity, the number of insulin receptors, and the action of insulin (Thomson et al. 1987); converse effects occur when the concentration of SFA in the membranes is increased (Ginsberg et al. 1982). Animal studies have shown that in rats made insulin resistant with a high-fat diet, the resistance can be prevented by the inclusion of n-3 PUFA in the diet which have been incorporated into the phospholipid component of muscle cells. In humans, insulin sensitivity has been correlated with the ratio of n-6 PUFA to SFA in serum phospholipids (Field et al. 1988). In this study we examined the association between red blood cell phospholipid fatty acid content and measures of insulin sensitivity and lipid metabolism. In the pre-intervention data it was observed that there was a significant inverse association between palmitate (C16:0), which was the main saturated fatty acid present in the RBC membranes, and glucose K\textsubscript{irr}. In addition there was an inverse relation between stearic acid (C18:0) and HDL-cholesterol levels. This data indicates that there is a detrimental influence of high levels of SFA in membrane phospholipids on both insulin sensitivity and HDL-cholesterol levels. This finding is similar to the finding of other workers who have examined the relationship between phospholipid fatty acid content and insulin sensitivity. For example, in a study by Vessby et al. (1994) insulin sensitivity was
measured by the hyperinsulinaemic clamp technique in a group of 70 year old men and it was found that peripheral insulin sensitivity was significantly and negatively correlated to the proportion of palmitic acid in the skeletal muscle phospholipids. Their study differs from this study in that we examined the effect in RBC membranes and not skeletal muscle, which is the main insulin-sensitive tissue (Borkman et al. 1993). However the majority of fatty acids within membranes are derived from dietary sources and it is assumed that fatty acids present in muscle cell membranes would be reflective of fatty acids present in erythrocyte cell membranes. In the post intervention data the main associations between RBC membrane phospholipid fatty acid content were with measures of lipid metabolism and total n-3 fatty acid content. Total n-3 PUFA content was inversely correlated with TG AUC and positively correlated with HDL-cholesterol. When the data was ranked into quartiles of membrane n-3 PUFA content, it was observed that TG AUC decreased and HDL-cholesterol levels increased with the progressive increase in n-3 PUFA content. The majority of studies conducted have compared membrane n-3 fatty acid composition with measures of insulin sensitivity or fasting insulin level. In this study an association between measures of insulin sensitivity and membrane total n-3 fatty acid content were not detected and one explanation could be that the proportion of total n-3 fatty acids were too low to detect significant relationships between insulin sensitivity and n-3 PUFA content. Only one intervention in this study was designed to increase the dietary intake of n-3 PUFA and in experimental studies, the content of n-3 fatty acids in the diet, as well as in the tissues, has been shown to be critical for the maintenance of good insulin sensitivity. The mechanism by which membrane fatty acids influence insulin sensitivity is thought to be due to the close contact of the insulin receptor and glucose transporter with membrane lipids. Certainly in animal studies, rats fed a high fat diet have a decreased capacity to transport and metabolise glucose. Similarly in human studies, while membrane fatty acids have not generally been measured it has been shown that a higher SFA intake is associated with impaired insulin action (Maron et al. 1991; Marshall et al. 1997). Other studies have shown that decreased insulin sensitivity is associated with decreased concentrations of PUFA in skeletal muscle phospholipids (Borkman et al. 1993). An increase in the unsaturated component of cell membranes is thought to increase membrane fluidity and as a consequence the residence time of glucose transporters is increased with a resultant increase in the disposal of glucose. The mechanism by which an increase in
the n-3 PUFA content is associated with a lower postprandial TG in this study is not thought to be related to increase in the activity of the enzyme LPL, since LPL activity was found to be negatively correlated with membrane n-3 PUFA content. Since the TG to insulin AUC ratio was also inversely correlated with RBC n-3 PUFA it is assumed that this is associated with an improved insulin action, however the exact mechanisms are unclear and warrant further investigation.
7.4 SUMMARY AND RECOMMENDATIONS FOR FUTURE STUDIES

The aim of this study was to investigate the effects of modifying the composition of dietary fat in the diets of a group of middle-aged men on the insulin sensitivity of glucose disposal and postprandial lipid metabolism. To explore some of the mechanisms by which alterations in dietary fat can influence insulin action and lipoprotein metabolism, measurements of the post-heparin activity and the gene expression of the insulin sensitive enzyme LPL were conducted.

The insulin sensitivity of glucose disposal was not affected by any of the dietary interventions conducted in this study. The lack of effect on insulin sensitivity with alterations in dietary fat is similar to the finding of the majority of studies conducted in non-diabetic subjects to date (Schwab et al. 1995; Fasching et al. 1996; Louheranta et al. 1998; Louheranta et al. 1999). In common with other studies we also found an association between the fatty acid composition of phospholipid membranes and the degree of insulin resistance at baseline. It was postulated therefore, that alterations in the intake of dietary fatty acids would influence insulin sensitivity, however this was not the case. One reason for the lack of any observed effect in this study may have been due to the wide variation in insulin sensitivity among the study group at the outset. In addition, the number of subjects studied in each intervention was small and therefore the study may have had too low statistical power to demonstrate a significant effect. It is recommended for future studies that an assessment of insulin sensitivity is conducted at screening in order to ensure that individuals are insulin resistant and secondly that a greater number of subjects are included.

With respect to postprandial lipaemia, improvements were observed following the low-SFA/ high-MUFA diet and the n-3 PUFA enriched diet. In contrast the low-SFA/ high-CHO diet was associated with a worsening of postprandial lipaemia through a increase in TRL-TG following the intervention diet. From the results of this study therefore, it is would appear that it is more beneficial to increase the intake of fat from unsaturated sources as opposed to reducing total fat intakes and increasing the intake of carbohydrate. However as stated in the introduction to this work most Nutritional Recommendations are directed towards decreasing the intake of all fat and increasing the intake of carbohydrate. Low-fat, high-CHO diets have been recommended for
several reasons but were primarily based on the effect of such diets on total plasma cholesterol concentrations (Kromhout et al. 1995). However unsaturated fats have been shown to be equally effective at lowering plasma cholesterol levels (Mensink, 1994). Other workers have also argued strongly against the use of high-CHO diets in the prevention of the development of or attenuation of the manifestations of the insulin resistance syndrome. The main argument against the use of high-CHO diets in both normal and diabetic subjects is that they increase the concentration of both fasting and postprandial TG levels, and that they can lower the concentration of HDL-cholesterol (Reaven, 1997; Grundy, 1999; Katan, 1997). Our work supports the finding that postprandial TG levels are increased as a result of high-CHO feeding and adds to the consensus that dietary fat quality is the important determinant of CHD risk, with SFA increasing risk and MUFA and PUFA posing little risk and in some cases reducing risk. The work suggests that dietary fat composition should be changed towards a lower SFA intake a higher MUFA intake and a lower n-6 : n-3 ratio through an increase in the intake of long chain n-3 PUFA. Recommendations for future studies are that dose response studies are conducted to determine the optimum dietary levels of each of the main fatty acid groups.

There was no change in the measurement of LPL activity or LPL gene expression following any of the dietary intervention studies. Few other studies have shown any effect of n-3 PUFA enrichment on post-heparin LPL activity (Harris et al. 1988; Weintraub et al. 1988; Nozaki et al. 1991). In addition, little consistent information is available from previous studies regarding the effects of high-CHO and low-fat diets on LPL and HL activities (Blades & Garg, 1995) and few studies have examined the effects of a greater MUFA intake on plasma LPL activity (Williams et al. 1999). We also found no change in the levels of mRNA gene expression following any of the intervention diets. Again, few studies have looked at the effect of manipulating intakes of dietary fat and the expression of LPL. Our results are in agreement with those of Murphy et al. (1999) who found no effect of fish oil feeding on the expression of LPL in subcutaneous adipose tissue, however as with our study the number of subjects was small (n = 6). Further studies involving larger numbers of subjects are required before any firm conclusions can be reached as to the effect of altering dietary fat intakes on the activity and expression of the enzyme LPL.
The relationship between insulin sensitivity and postprandial lipaemia was complicated and in the group overall there was a positive association between the insulin sensitivity of glucose disposal and TG AUC at baseline. The impact of insulin resistance on postprandial metabolism has not been extensively analysed however our results are not in agreement with other studies that have indicated that hyperinsulinaemia and/or decreased insulin sensitivity is involved in altered lipoprotein metabolism (Graci et al. 1999; Boquist et al. 2000; Jeppesen et al. 1999). The results of this study warrant further investigation as a sufficient number of similar studies have not been completed from which to make adequate comparisons.

Finally these studies were completed in a group of genetically uncharacterised subjects. The importance of genotype was not considered when these studies were originally planned however, since then it has become clear that not only can genetics play a role in determining an individual’s lipid profile, but it can also influence their response to a lipid-altering intervention. Less is know about the effect of particular genetic phenotypes on insulin sensitivity. Had a larger number of subjects that had been genetically characterised been included in the study it may have been possible to observe clearer associations between alterations in dietary fat intake on measures of lipid metabolism, insulin sensitivity and LPL gene expression.
REFERENCES


Oliver, M.F. (1997). It is more important to increase the intake of unsaturated fats than to decrease the intake of saturated fats: evidence from clinical trials relating to ischemic heart disease. *American Journal of Clinical Nutrition* 66, 980S-986S.


APENDIX: Stable isotope studies conducted by other investigators during dietary interventions (not described in thesis)

INSULIN SENSITIVITY OF GLYCEROL RELEASE AND NEFA SUPPRESSION

i.e. insulin sensitivity of fasting HSL inhibition (stable isotope study of glycerol production rate in response to insulin infusion).
This involved measurements of insulin, glycerol and NEFA concentrations and glycerol production rate during the fasting (3h) and low-insulin infusion phase (3h) of a 6 hr infusion of [1,2,3,5^3H5]glycerol (see protocol Fig 1a).
In each study the insulin infusion resulted in a very small but significant increase in serum insulin and significant reductions in glycerol and NEFA (see Fig 2b appendix).
In the FO study there were no differences in fasting or low insulin glycerol or NEFA concentrations between the FO and the control group. In contrast both fasting and low insulin glycerol and NEFA levels were lowered by the reduced SFA diet compared with the control diet, significantly so during the insulin infusion (glycerol, 29.3 (sd 10.2) v. 20.4 (sd 11.6) mmols/l, p=0.01; NEFA 0.42 (sd 0.10) v. 0.29 (sd 0.08) mmols/l, p=0.01 for the control v. the reduced SFA diet respectively. The insulin-induced fall in glycerol concentrations reflected a significant fall in the glycerol production rate and the extent of the fall in glycerol concentration was highly correlated with the fall in the appearance rate ($R^2 = 0.71$).

Fish oil intervention
There is no effect of the fish oil intervention on glycerol kinetics. Appearance rate was not influenced by the intervention in the fasting or low insulin state. The appearance rate fell significantly with the low level insulin infusion (p=0.015 before and p= 0.034 after reduced fat intake but the extent of the reduction did not change.

Reduced SFA intervention
There was an effect of the intervention on glycerol kinetics since the appearance rate was marginally lowered in fasting (p=0.061), and with insulin (p=0.022) by the dietary intervention. The appearance rate fell significantly with the low level insulin infusion (p=0.01 control, p= 0.001 intervention) but the extent of the reduction did not change.

If the glycerol production rate is assumed to reflect the activity of HSL which is primarily a function of insulin activity then these results suggest that the insulin sensitivity of HSL is not altered by increased FO. However the significant fall in the glycerol Ra during fasting and with insulin suggests that the insulin sensitivity of this step is improved by decreased SFA intake.

<table>
<thead>
<tr>
<th>Glycerol production rate</th>
<th>Fish oil study</th>
<th>SFA study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glycerol Ra (mmols/kg/h)</td>
<td>fall in Ra</td>
</tr>
<tr>
<td>control</td>
<td>Fasting +insulin</td>
<td>Fasting +insulin</td>
</tr>
<tr>
<td>Mean</td>
<td>113</td>
<td>90</td>
</tr>
<tr>
<td>Sd</td>
<td>39</td>
<td>30</td>
</tr>
<tr>
<td>p insulin effects</td>
<td>0.015</td>
<td>0.034</td>
</tr>
<tr>
<td>p diet effects fasting</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td>p diet effects insulin</td>
<td>0.814</td>
<td></td>
</tr>
<tr>
<td>p diet on fall in Ra</td>
<td>0.866</td>
<td></td>
</tr>
</tbody>
</table>
Fig 1 INSULIN SENSITIVITY OF GLYCEROL RELEASE AND NEFA SUPPRESSION

Figure 1a Protocol
Glycerol release protocol
constant infusion of [1,2,3\textsuperscript{3}H\textsubscript{5}] glycerol + insulin
blood sampling: insulin, glycerol and NEFA

<table>
<thead>
<tr>
<th>Fasting after overnight fast</th>
<th>Low insulin Infusion 0.005U.kg\textsuperscript{-1} h\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3hrs</td>
</tr>
<tr>
<td></td>
<td>6hrs</td>
</tr>
</tbody>
</table>

Time (hours)

primed constant infusion of [1,2,3\textsuperscript{3}H\textsubscript{5}] glycerol
glycerol enrichment measured by GCMS
glycerol appearance rate (Ra)
Ra = infusion rate/isotope dilution.

Fig 1b Glycerol and NEFA concentrations with low insulin infusion