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Molecular Aspects of Human Lipid Metabolism

By

Irene Lucy Black

A thesis for the degree of Doctor of Philosophy (Ph.D.)

Submitted to the University of Dublin, Trinity College

April 2001

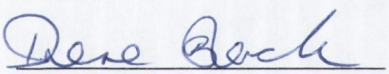
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This thesis is dedicated
to those people who made it possible to complete it,
my husband Christopher,
my Mum, my mother in-law Kathie
and my daughter Orianna.

Declaration

I, the undersigned hereby declare that this thesis is my own work and it has not been previously submitted for any other degree at this or any other University except where otherwise acknowledged. This thesis is submitted to University of Dublin, Trinity College for examination for the degree of Ph.D., and may be made available from the library for consultation or copying.



Irene Black
April 2001

Summary

The postprandial lipaemic response refers to the changes in lipid and lipoprotein concentrations after consumption of a meal containing more than 15g of long-chain fatty acids. The magnitude and duration of the response have been shown to reflect the risk of coronary heart disease (CHD). The postprandial triacylglyceride (TAG) response, which is indicative of postprandial lipaemia, is a quantitative trait since the magnitude and duration of the response are related to an individual's genotype and environment. Such environmental factors include, gender, weight, age, fasting lipid concentrations, exercise, alcohol consumption, background diet and the meal consumed to cause the response. This thesis set out to investigate some of the genetic and dietary factors involved in modulating the postprandial TAG response.

In chapter 2 the effects of some genetics polymorphisms on postprandial lipid metabolism were investigated. Polymorphisms, a term which refers to common variations in the genetic code, in the *apolipoprotein B* (*apoB*), *apolipoprotein E* (*apoE*), *lipoprotein lipase* (*lpl*), *lecithin:cholestol acyltransferase* (*lcat*) and *cholesteryl ester transfer protein* (*cetp*) genes were studied for their possible contribution to modulating lipid and apolipoprotein concentrations in plasma both at fasting and postprandially. The D allele of the signal peptide polymorphism in the *apoB* gene was linked (in some cases non-significantly) to lower body mass index (BMI), postprandial cholesterol and apoB48 levels and higher fasting concentrations of high density lipoprotein 2 (HDL_2)-cholesterol. The ε4 allele in *apoE* was associated with higher fasting and postprandial plasma TAG levels, which may have contributed to the finding that the ε2 allele was associated with lower BMI. One haplotype of the *lpl* gene, H-T, was significantly associated with lower fasting and postprandial plasma TAG concentrations in men. However, in females the H-T haplotype was only associated with lower fasting TAG levels. Individuals homozygous for the B- allele of the *cetp* gene were found to have higher fasting and postprandial cholesterol, HDL_3-cholesterol levels and BMI values but lower CETP mass at fasting than heterozygotes or B+ homozygotes. The conclusion drawn from these findings is that polymorphisms in the *lpl* gene have a major influence on plasma TAG concentrations. However, the relatively small sample size (82 individuals) makes analysis of minor effects of the other polymorphisms on lipid metabolism problematic.

Postprandial triacylglycerolaemia has been found to stimulate the activation of coagulation factor VII (FVII) whose activation (FVIIa) initiates the extrinsic coagulation pathway. Chapter 3 discusses a small study in which carriers of the Q/10bp haplotype (of the *FVII* gene) are compared to R/0bp homozygotes. The findings showed that FVIIa and the ratio of FVIIa to total FVII (FVIIa:FVIIag) are only increased in R/0bp homozygotes during the postprandial response. Q/10bp carriers had lower fasting FVIIa and FVIIag levels than R/0bp homozygotes, and these variables remained at fasting levels throughout the postprandial period. It could be hypothesised that Q/10bp carriers have a reduced risk of diet induced heart attack than R/0bp homozygotes, due to less activation of the coagulation pathway during the postprandial lipid response in Q/10bp carriers.

As stated above, postprandial TAG concentrations are affected by environmental factors including the type of fat regularly ingested and the type of fat ingested just before the response is measured. Cell lines have been used to investigate the effects of one dose (simulating a test meal) of fatty acids on lipoprotein, lipid and apolipoprotein synthesis and secretion levels. No previous studies had used cell lines to investigate the effects that long-term presence of fatty acids (simulating background diet) may have on TAG synthesis and secretion levels. The hypothesis that a low dose of fatty acid administered during cell growth and differentiation could affect how cells synthesise and secrete lipids and apolipoproteins after a high dose of fatty acid was tested.

In chapter 4, Caco-2 cells (cell model of human small intestine) were chronically supplemented with a range of concentrations of eicosapentaenoic acid (EPA), oleic acid (OA) or palmitic acid (PA), characterised by looking at viability and tight junction formation and examined for incorporation of the supplementary fatty acid into Caco-2 cell lipid moieties. Fatty acids supplemented chronically at 0.05mM did not affect cell growth and were incorporated into fatty acid components of cell lipid fractions.

In chapter 5, chronically supplemented Caco-2 cells were treated with acute doses of 0.5mM EPA, OA or PA and primarily studied for presence of an interaction between the chronic and acute fatty acid treatments. Cells given chronic EPA supplementation followed by acute doses of BSA, EPA or PA manifested a significant interaction between the chronic and acute fatty acid treatments for the ratio of non-secreted TAG to secreted TAG compared to cells given no supplementary fatty acids and the same acute fatty acid treatments. However, this interaction appeared to be inconsistent. After the first two acute treatments the EPA supplemented cells had increased *de novo* TAG secretion levels compared to non-supplemented cells. However, after acute PA treatment the EPA supplemented cells had reduced *de novo* TAG secretion levels compared to control cells. Separate acute and chronic effects on lipid and lipoprotein secretion were also observed for the variables measured but no other interaction between chronic and acute fatty acid treatments was observed.

The final study in chapter 6, investigated the effects of the two major isomers (c-9 and t-10) of conjugated linoleic acid (CLA), on lipid and apolipoprotein synthesis and secretion in Caco-2 cells. Consumption of CLA has been found to reduce plasma TAG concentrations in animals and humans. Interestingly the findings of this study suggest that CLA does not reduce plasma TAG levels by altering intestinal lipid metabolism. Instead, CLA isomers appear to behave as pseudosaturated fatty acids. However, chronic CLA supplementation did affect how Caco-2 cells responded to fatty acid treatments. This highlights the fact that CLA concentrations in foetal calf serum may affect results obtained in fatty acid metabolism studies using cell lines. Caco-2 cells given chronic supplementation with linoleic acid (LA), t-10 and c-9 were characterised. During characterisation 0.1mM and 0.05mM of the t-10 isomer were found to retard cell monolayer formation. The c-9 isomer was found to reduce arachidonic acid levels in cellular PL fractions, which may affect eicosanoid synthesis. This suggests that both CLA isomers can retard tumorigenesis, although through different mechanisms.

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Table of Contents

Declaration i

Summary ii

Acknowledgements iv

Table of Contents vi

Abbreviations x

1 Introduction: Postprandial and Molecular Aspects of Lipid Metabolism 1

1.1 Introduction 1

1.2 Postprandial Lipid Metabolism 2
1.2.1 Postprandial Response 2
1.2.2 Postprandial Lipid Metabolism 4
1.2.3 Effects of Fatty Acids on Postprandial Lipaemia 11
1.2.4 Summary 15

1.3 Genetics of Lipid Metabolism 15
1.3.1 Human Genome Project 15
1.3.2 Proteins Involved in Lipid Metabolism 17
1.3.3 Gene-Diet Effects on Fat Metabolism 28
1.3.4 Transgenic Animal Models of Atherosclerosis 32
1.3.5 Summary 34

1.4 Relationship between Coagulation Pathway and Postprandial Lipidaemia 35
1.4.1 Coagulation Cascade 35
1.4.2 Extrinsic Pathway and Postprandial Triacylglycerolaemia 36
1.4.3 Coagulation Factor VII Polymorphisms 38
1.4.4 Summary 38

1.5 Cell Lines for the Study of Fat Absorption and Metabolism 39
1.5.1 Colon Carcinoma Cell Line (Caco-2): A Model for Enterocyte Function 39
1.5.2 Hepatocytes as a Model of Liver Function 42
1.5.3 Summary 44

1.6 Conjugated Linoleic Acid 44
1.6.1 Body Composition Changes Due to CLA Intake 45
1.6.2 Effect of CLA Ingestion on Eicosanoid Synthesis 46
1.6.3 Interaction between CLA and the Cytokine Signal Transduction Mechanism 47
1.6.4 CLA as a Peroxisome Proliferator 48
1.6.5 Summary 48

1.7 Aims 49

References 50

2 Effect of Polymorphisms in Genes Involved in Lipid Metabolism on the Postprandial Lipid Response 59

2.1 Introduction 59
2.1.1 Effect of Five Proteins Involved in Lipid Metabolism 59
2.1.2 Linkage Disequilibrium 60
2.1.3 Polymorphisms of Five Proteins Involved in Lipid Metabolism 61

2.2 Materials and Methods 65
2.2.1 Study Design 65
2.2.2 DNA Isolation from Blood 65
2.2.3 Polymerase Chain Reaction Amplification 65
2.2.4 Restriction Enzyme Digests of Polymerase Chain Reaction Amplification Products and their Visualisation 66
2.2.5 Postprandial Investigation; Isolation of Postprandial Components from Plasma ... 67
2.2.6 Lipid and Protein Analyses 67
2.2.7 Statistical Analysis of Genotype Frequencies 68

2.3 Results 73
2.3.1 Genotype Frequencies and Haplotype Analysis of Genes Involved in Lipid Metabolism 73
2.3.2 Effect of Genotype on Fasting and Postprandial Lipid Metabolism 81

2.4 Discussion 90
2.4.2 Conclusion 94

References 94

3 The Effect of Coagulation Factor VII Polymorphisms on the Relationship Between Postprandial Lipaemia and Coagulation Factor VII Activity 97

3.1 Introduction 97
3.1.1 Association Between Coagulation Factor VII Activity and Risk of Coronary Heart Disease 97
3.1.2 Factor VII Activity and Postprandial Triacylglyceride Concentrations 98
3.1.3 Variation in FVII Activity Associated with Polymorphisms in the *FVII* Gene 98

3.2 Materials and Methods 100
3.2.1 DNA Samples 100
3.2.2 Genotyping the R353Q Polymorphism 100
3.2.3 Genotyping the Decanucleotide Insertion Polymorphism 101
3.2.4 Study Conditions 101
3.2.5 Lipid and Factor VII Analysis 101
3.2.6 Statistical Analysis 102

3.3 Results 102

3.4 Discussion 107
3.4.1 Conclusion 109

References 110

4 Characterisation of the Growth of Caco-2 Cells Under Chronic Fatty Acid Supplementation 112

4.1 Introduction 112
4.1.1 Characteristics of the Colon Carcinoma Cell Line 112

4.1.2 Characterisation of the Colon Carcinoma Cell Line 113

4.2 Materials & Methods 114
4.2.1 Caco-2 Cell Culturing 114
4.2.2 Fatty Acid Supplementation 115
4.2.3 Transepithelial Electrical Resistance 116
4.2.4 Viability (MTT) Assay 117
4.2.5 Proliferation (MTS) Assay 117
4.2.6 Translocation Assay 118
4.2.7 Cell Differentiation 119
4.2.8 Assay of Effect of Fatty Acid Supplementation on mRNA Levels of Microsomal Triglyceride Transfer Protein 122
4.2.9 Gas Chromatography 122

4.3 Results 125
4.3.1 Transepithelial Electrical Resistance 125
4.3.2 Viability (MTT) Assay 126
4.3.3 Proliferation (MTS) Assay 126
4.3.4 Translocation Assay 127
4.3.5 Cell Differentiation 127
4.3.6 Gas Chromatography 132

4.4 Discussion 140
4.4.1 Experimental Design 140
4.4.2 Characterisation of Fatty Acid Supplemented Caco-2 Cells 140
4.4.3 Incorporation of Supplementary Fatty Acids into Cellular and Secretion TAG and PL Fractions 141
4.4.4 Conclusion 143

References 143

5 Caco-2 Cell Model of Intestinal Lipoprotein Metabolism to Investigate the Effect of Dietary Fat Composition on Lipid and Apolipoprotein Synthesis and Secretion 145

5.1 Introduction 145
5.1.1 Effects of Diet in Humans on Postprandial Response 145
5.1.2 Acute Fatty Acid Treatment of Caco-2 Cells 146
5.1.3 Aim of the Study 147

5.2 Materials and Methods 147
5.2.1 Apolipoprotein B Isoform Separation 147
5.2.2 Acute Experiment Set-up 149
5.2.3 Chronic-Acute Experimental Set-up 151
5.2.4 Statistical Analysis 155

5.3 Results 158
5.3.1 Chronic and Acute Effects of Fatty Acid Treatment 158

5.4 Discussion 168
5.4.1 Acute by Chronic Interaction 168
5.4.2 Acute and Chronic Effects of Fatty Acid Treatments on Caco-2 Cell Lipid Metabolism 168
5.4.3 Conclusion 172

References 172

6 Effect of Conjugated Linoleic Acid on Growth and Lipid Metabolism of Caco-2 Cells 175

6.1 Introduction 175

6.2 Materials and Methods 176
6.2.1 Proliferation (MTS) Assay 177
6.2.2 Gas Chromatography 177
6.2.3 Effect of Linoleic Acid and its conjugates on lipid and apolipoprotein synthesis and secretion 178

6.3 Results 179
6.3.1 Transepithelial Electrical Resistance (TEER) 179
6.3.2 Viability (MTT) Assay 179
6.3.3 Proliferation (MTS) Assay 180
6.3.4 Translocation Assay 180
6.3.5 Cell Differentiation 181
6.3.6 Gas Chromatography 187
6.3.7 Acute Effect of Conjugated Linoleic Acid Isomers on Fatty Acid Synthesis and Secretion 194
6.3.8 Chronic Effect of Conjugated Linoleic Acid Isomers on Fatty Acid Synthesis and Secretion 194

6.4 Discussion 203
6.4.1 Effects of LA, t-10 and c-9 on Lipid and Apolipoprotein Synthesis and Secretion by Caco-2 Cells 203
6.4.2 Characterisation of Caco-2 Cells Supplemented with LA, t-10 or c-9 204
6.4.3 Effect of LA and CLA Isomer Supplementation to Caco-2 Cells on the Fatty Acid Composition of their TAG and PL Fractions 205
6.4.4 Conclusion 207

References 208

7 Conclusion 210

7.1.1 Introduction 210
7.1.2 Effects of Polymorphisms in Genes Involved in Lipid Metabolism on Fasting and Postprandial Lipid and Lipoprotein Levels 210
7.1.3 Relationship Between Postprandial Triacylglycerolaemia and Activation of Coagulation Factor VII is Modulated by a Factor VII Haplotype 211
7.1.4 Effects of Chronic Fatty Acid Supplementation on the Response of Caco-2 Cells to Acute Fatty Acid Supplementation 212
7.1.5 Effects of CLA Isomers (t-10 and c-9) on Caco-2 Cells: A Model of the Human Small Intestine 213

References 214

Appendix I i

Appendix II ix

Appendix III xii

Appendix IV xiv

Abbreviations

AA	Arachidonic Acid (20:4n-6)
ANOVA	Analysis of Variance
apoA-I	Apolipoprotein A-I
apoA-II	Apolipoprotein A-II
apoA-IV	Apolipoprotein A-IV
apoB	Apolipoprotein B
apoB100	Apolipoprotein B100
apoB48	Apolipoprotein B48
apoC-I	Apolipoprotein C-I
apoC-II	Apolipoprotein C-II
apoC-III	Apolipoprotein C-III
apoE	Apolipoprotein E
AUC	Area Under the Curve
BHT	Butylated Hydroxy Toluene
BMI	Body Mass Index
bp	base pairs
BSA	Bovine Serum Albumin
Caco-2	Colon Carcinoma Cell Line
CAD	Coronary Artery Disease
cDNA	complementary Deoxyribonucleic Acid
CE	Cholesterol Esters
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
c-9	*cis*-9 Conjugated Linoleic Acid (18:2n-6)
CLA	Conjugated Linoleic Acid
CPT	Carnitine Palmitoyltransferase
CV	Coefficient of Variation
DEPC	Diethyl Pyrocarbonate
df	degrees of freedom
DHA	Docosahexaemoic Acid
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
DPM	Disintegrations per Minute
ELISA	Enzyme-Linked Immunosorbent Assay

EPA	Eicosapentaenoic Acid (20:5n-3)
FCS	Foetal Calf Serum
FED	Fish-Eye Disease
FLD	Familial LCAT Deficiency
FVII	Coagulation Factor VII
FVIIa	Activated Coagulation Factor VII
FVIIag	Coagulation FVII Antigen (Zymogen and Activated Factor VII)
FVIIc	Coagulation Factor VII Activity
GC	Gas Chromatography
GIP	Glucose-Dependent Insulinotrophic Polypeptide
HBSS	Hanks Balanced Salt Solution
HDL	High Density Lipoprotein
HepG2	Human Hepatoma Cell Line
HGP	Human Genome Project
HL	Hepatic Lipase
IAUC	Incremental Area Under the Curve
IDL	Intermediate Density Lipoproteins
IHD	Ischemic Heart Disease
LA	Linoleic Acid (18:2n-6)
LCAT	Lecithin:Cholesterol Acyltransferase
LDL	Low Density Lipoprotein
L-FABP	Liver Fatty Acid Binding Protein
LPL	Lipoprotein Lipase
LSD	Least Significance Difference
MAG	Monoacylglycerol
mRNA	messenger Ribonucleic Acid
MSE	Mean Squared Error
MTP	Microsomal Triglyceride Transfer Protein
MTS	3-(4,5-Dimethylthiazol-2-yl)-3-(3-Carbozymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Dephenyltetrazolium Bromide; Thiazolyl Blue
MUFA	Monounsaturated Fatty Acid
n-3	omega-3 fatty acid
n-6	omega-6 fatty acid
NEFA	Non-Esterified Fatty Acids
OA	Oleic Acid (18:1)

PA	Palmitic Acid (16:0)
P_{app}	Apparent Permeability Coefficient
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PL	Phospholipid
PMS	Phenazine methosulfate
PPAR	Peroxisome Proliferator-Activated Receptor
PUFA	Polyunsaturated Fatty Acid
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
RT	Room Temperature
SA	Stearic Acid (18:0)
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SFA	Saturated Fatty Acid
SNP	Single Nucleotide Polymorphism
TAG	Triacylglycerol
TEER	Transepithelial Electrical Resistance
TLC	Thin Layer Chromatography
t-10	*trans*-10 Conjugated Linoleic Acid (18:2n-6)
TPL	Triacylglycerol Poor Lipoproteins
TRL	Triacylglycerol Rich Lipoprotein
Tween-20	Polyoxyethylenesorbitan Monolaurate
UTR	untranslated region
VLDL	Very Low Density Lipoprotein
16:1	Palmitoleic Acid
18:3n-3	Linolenic Acid

1 Introduction: Postprandial and Molecular Aspects of Lipid Metabolism

1.1 Introduction

The duration and magnitude of the postprandial lipid response are known to be risk factors for development of coronary heart disease (CHD) (section 1.2.1.2). The response is variable; the duration and magnitude of the response are dependent on genetic and environmental factors (sections 1.2.1.3, 1.2.4 and 1.2.3).

Some of the genes encoding proteins involved in lipid metabolisms have polymorphisms, which have been associated with the level of postprandial response that occurs following a test meal (section 1.3.3). Chapter 2 discusses the effects that polymorphisms in the *apolipoproteins B*, *apolipoproteins E*, *lipoprotein lipase* and *cholesteryl ester transfer protein* genes have on the distribution of the triacylglycerol (TAG), triacylglycerol rich lipoprotein triacylglyceride (TRL-TAG), cholesterol, apolipoprotein B48 (apoB48) and cholesteryl ester transfer protein (CETP) concentrations at fasting and postprandially following a test meal high in omega-6 polyunsaturated fatty acids (n-6 PUFA). The findings of this study are that some genotypes are associated with a more anti-atherogenic lipid profile than others (section 2.3.2).

A diet high in fatty acids is known to increase an individual's risk of having a heart attack due to blood clots. Coagulation factor VII (FVII) has been found to be activated by TRL particles. The increased TRL concentrations during the postprandial response increasing the concentration of activated FVII (section 3.1). However, the results of a study discussed in this thesis have shown that activation of FVII by TRL levels occurs only in individuals carrying a certain haplotype (Figure 3.3.3).

The postprandial lipid response is a quantitative trait and so genetic effects are only part of the story. To assess effects of environmental factors on the postprandial response it was decided to analyse the effects of a range of fatty acids on a colon carcinoma cell line (Caco-2), which simulates the human intestine (section 4.1). The use of a cell line removes any genetic variation between samples and so the effect of fatty acids can be examined alone. Much work had been carried out previously on the effects of acute doses of fatty acids (one high dose of fatty acid) on the secretion levels of lipid and apolipoproteins from Caco-2 cells (sections 1.5.1 and 5.1.2). However, previously, no work had been carried

out on the effects of low doses of fatty acid administered to Caco-2 cells over a long period, which would simulate a background diet. The observations discussed in chapter 5 show that oleic acid is associated with increased TAG secretion by Caco-2 cells. Palmitic acid is associated with high concentrations of cellular TAG and eicosapentaenoic acid is associated with increased PL synthesis over TAG synthesis, whether administered chronically or acutely.

Fatty acids, which are of great interest at the moment, are conjugated linoleic acid (CLA) isomers. These isomers have been shown to reduce plasma TAG concentrations when ingested (section 1.6.1). Chapter 6 investigated whether the reduction in plasma TAG levels is due in part to changes in lipid and apolipoprotein synthesis and secretion by the gut using the Caco-2 cell line, which is a model of the human intestine. The findings of this study suggest that in humans, the changes in plasma TAG levels are not at an intestinal level.

1.2 Postprandial Lipid Metabolism

1.2.1 Postprandial Response

1.2.1.1 Usual Postprandial Response

Postprandial lipid metabolism refers to the events that occur following the ingestion, digestion and absorption of dietary fat. Dietary fat is made up primarily of triacylglycerol (TAG), which is broken-down into free fatty acids (long- (>C10:0) and short- (<C12:0) chain), which are absorbed by the intestine. A characteristic of the postprandial lipid response is an increase in plasma TAG levels from 1.0-1.5mM at fasting to 1.5-4.0mM during the postprandial response (Williams 1999). This increase is known to affect the composition of high density lipoproteins (HDL). The postprandial lipid response lasts approximately 6-10 hours. The intestinally derived chylomicrons peak at about 2-4 hours and the hepatically derived very low density lipoproteins (VLDL) appear at 4-6 hours following ingestion of lipid. Human TAG levels have been found to be above fasting values for 16-20 hours per 24 hours (Williams 1999). Therefore, the normal state for humans is the postprandial state and not the fasting state. Postprandial variables may thus be a more appropriate predictor of CHD risk than fasting variables. There is a high degree of variability in fasting plasma TAG levels within and between individuals. This

variability may be the reason that fasting TAG levels do not always emerge as an independent risk factor for CHD. It is known that postprandial TAG concentration and HDL-cholesterol levels have an inverse relationship. Therefore, fasting HDL-cholesterol levels, which are frequently measured in assessing CHD risk, may in fact be an indirect measure of fasting and postprandial TAG metabolism (Roche & Gibney 1999).

1.2.1.2 Postprandial Response and Risk of Developing Coronary Heart Disease

An elevated and prolonged postprandial response has been found to be a risk factor in the development of coronary heart disease (CHD). During an elevated and prolonged postprandial response there is a large decrease in the anti-atherogenic HDL fractions and retarded removal of triacylglyceride rich lipoproteins (TRL). TRL particles consist of chylomicrons secreted by the intestine, VLDL particles secreted by the liver and intermediate density particles (IDL), which are a by-product of VLDL-TAG lipolysis (Williams 1999). Small VLDL particles have been shown to form small dense low density lipoproteins (LDL) after TAG hydrolysis and these small dense LDL particles are known to be pro-atherogenic (Shepherd & Packard 1987). The Zilversmit Hypothesis (1979) proposed that chylomicron remnants, formed by the hydrolysis of TAG from chylomicrons during the postprandial response, are as atherogenic as low-density lipoproteins (LDL). Other studies have shown that there is retarded chylomicron remnant removal in coronary artery disease (CAD) patients, that apolipoprotein B48 (apoB48; apolipoprotein found in chylomicrons) levels are elevated in CAD patients and that the magnitude of the postprandial response is greater in CAD patients than controls (Roche & Gibney, 1995). The atherogenic lipoprotein phenotype is low HDL concentrations (<1.0mM), moderately raised fasting TAG levels (1.5-3.0mM), increased proportion of small dense LDL (>45% LDL3), raised concentrations of remnant particles and an elevated TAG response to a standard meal. This phenotype appears to be driven by elevated TAG and TRL levels causing alterations in the cholesterol containing lipoprotein concentrations (LDL and HDL) due to the relationship between cholesterol and TAG containing lipoproteins (section 1.2.2.9) (Williams 1999).

In addition to increased concentrations of lipid and lipoprotein variables being observed during the postprandial response, postprandial activation of the blood coagulation cascade has been found. Postprandial triacylglycerolaemia has been shown to cause a parallel increase in coagulation factor VII activity, stimulating the extrinsic pathway (section 1.4). Stimulation of the coagulation pathway during the postprandial response increases the risk

of ischemic heart disease (section 1.4) (Ruddock & Meade 1994). This is another method how postprandial lipaemia is an indirect risk factor for the development of CHD.

1.2.1.3 Factors Effecting Postprandial Response

Lipid metabolism is a complex process involving many genetic and environmental factors (Talmud & Waterworth 2000). It is considered to be a quantitative process because changes in any one of these factors affects lipid metabolism, which makes it difficult to assess the importance of each of the other factors involved individually. The most commonly used method of assessing the postprandial response is by measuring fasting plasma TAG levels. High fasting plasma TAG concentrations are associated with high postprandial TAG concentrations (postprandial peak (maximum) and length of the response (magnitude)). Age has also been found to affect the postprandial response; older individuals (>60) have a higher TAG-maximum than younger individuals (<30). Gender also affects the response; premenopausal women have a lower TAG-maximum than males of the same age. This may be due to increased lipoprotein lipase (LPL) activity in premenopausal women than postmenopausal women and men. In addition exercise is known to reduce the TAG-maximum, which may also be due to increased LPL activity in active individuals. Alcohol consumption is known to retard TAG clearance during the postprandial response. Weight is also an affecter of the postprandial response; obese individuals have elevated fasting TAG and reduced HDL-cholesterol levels (Roche & Gibney 1995). An individual's genetics is also known to contribute towards fasting and postprandial lipid levels. Family and population-based studies have shown that approximately 50% of the variance in plasma lipid levels between individuals is due to genetic parameters (Talmud & Waterworth 2000). Effects of genetics on lipid metabolism will be discussed in subsequent sections (1.2.4). Lastly, the actual quantity and type (saturated, monounsaturated, omega-6 polyunsaturated or omega-3 polyunsaturated fatty acid) of dietary fat ingested has been shown to affect the postprandial response (Roche & Gibney 1995). The contribution of dietary fat intake on lipid metabolism will be discussed in section 1.2.3.

1.2.2 Postprandial Lipid Metabolism

About 100g of fat is ingested per person per day in the Western society. 95-98% comprises of TAG, 0.5% of cholesterol and 4-8% of phospholipids (PL) (Frayn 1998). The

postprandial lipid response will occur following the ingestion of >15g of fat containing long-chain (>C10:0) fatty acids (Sanders *et al.* 2000).

Before ingestion of a meal containing fat, the concentration of fasting plasma TAG is influenced by the synthesis and secretion of TAG containing VLDL particles from the liver. Insulin, whose concentration increases during the postprandial response, signals that dietary nutrients are available, stimulating the production of lipoprotein lipase (LPL) in adipose tissue. The secretion of glucose and TAG from the liver, which is associated with a fasting profile, is suppressed (Parkard 1999). During the postprandial lipid response the concentrations of plasma TAG and total cholesterol increase. In contrast, the concentrations of plasma HDL-cholesterol and non-esterified fatty acid (NEFA) decrease. Figure 1.2.3 is an overview of lipid metabolism and its consequences.

1.2.2.1 Lipid Digestion, Absorption and Secretion

The digestion of lipid, allows free fatty acids to become available for absorption by the intestine. A small amount of incomplete fat digestion occurs in the mouth and stomach. Dietary fat digestion is subsequently completely in the small intestine by pancreatic enzymes (Small 1991). Free fatty acids are absorbed by enterocytes, which form the lining of the small intestine. Fatty acids either diffuse across or are actively transported across the cellular lipid membrane by proteins (McArthur *et al.* 1999). Long-chain (>C10) fatty acids are esterified into PL, TAG (section 1.2.2.2) or cholesterol esters (CE) that are assembled into chylomicrons (section 1.2.2.4), which contain the apolipoprotein, apoB48. Chylomicrons, which are comprised of 86-92% TAG in their core, begin to appear approximately 1 hour after fat ingestion and their secretion continues for about 6-8 hours (Roche & Gibney 1995).

1.2.2.2 Synthesis of Triacylglycerol

Triacylglycerol molecules are resynthesised via the monoacylglycerol pathway in enterocytes (Figure 1.2.1) from monoacylglycerol (MAG) formed during digestion in the small intestine. The monoacylglycerol pathway is therefore a restructuring pathway. 2-monoacylglycerol is the preferred substrate over 1-monoacylglycerol for synthesis of 1,2-diacylglycerol from MAG and acyl-coenzyme A. The rate of this first step in the pathway is influenced by the type of fatty acid in the 2 position of the preferred MAG substrate. Short-chain or long-chain unsaturated fatty acids are the best substrates. 1,2-diacylglycerol is metabolised into TAG by the addition of the acyl group from acyl-coenzyme A by the enzyme diacylglycerol acyl transferase. Diacylglycerol acyl transferase has a wide specificity for fatty acids (Gurr & Harwood 1991).

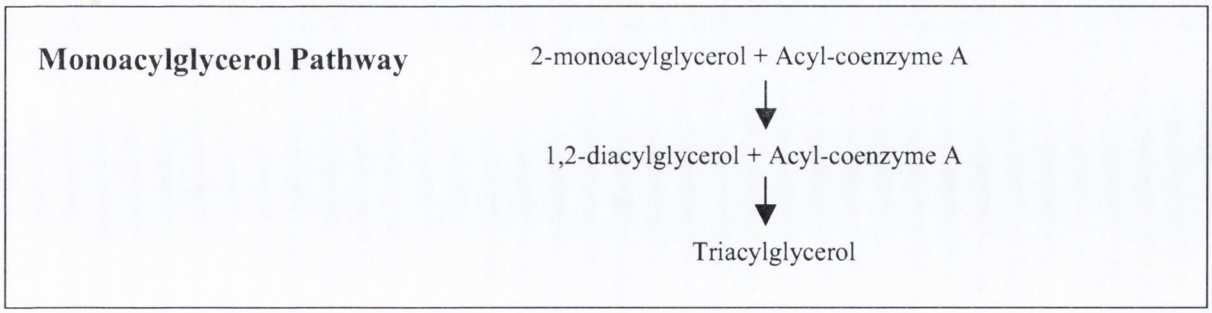


Figure 1.2.1 Monoacylglycerol pathway of triacylglycerol synthesis. Triacylglycerol is synthesised (restructured) from the starting substrate monoacylglycerol.

In other tissues TAG is synthesised from a glycerol backbone, via the glycerol-3-phosphate pathway (Figure 1.2.2), which is the *de novo* pathway for TAG synthesis. The colon carcinoma cell line (Caco-2) discussed in subsequent sections (1.5.1 and chapters 4, 5 and 6) is a model of enterocytes in the human small intestine. However, Caco-2 cells synthesise TAG via the glycerol-3-phosphate pathway. The glycerol backbone is synthesised via the glycolysis pathway. Preferentially a saturated acyl group is transferred from acyl-coenzyme A to glycerol-3-phosphate to form 1-acyl-glycerol-3-phosphate, by the action of glycerol phosphate 1-*O* acyl transferase. Then preferentially an unsaturated acyl group is transferred to the glycerol backbone to form 1,2-diacyl-glycerol-3-phosphate (phosphatidic acid) by the action of 1-acyl glycerol phosphate 2-*O*-acyl transferase. 1,2-diacyl-glycerol-3-phosphate is metabolised into 1,2-diacylglycerol by the action of the rate limiting enzyme phosphatidate phosphohydrolase. The final step in the metabolism of TAG is the addition of an acyl group from acyl-coenzyme A to 1,2-diacylglycerol by the action of diacylglycerol acyl transferase (Gurr & Harwood 1991).

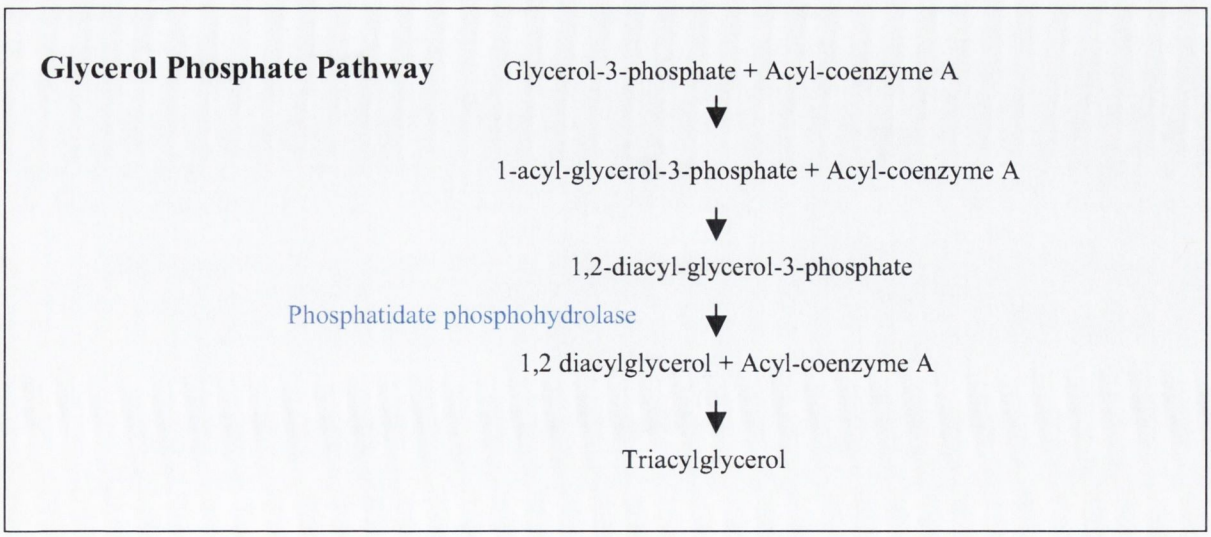


Figure 1.2.2 Glycerol phosphate pathway of TAG synthesis. This is the pathway of *de novo* TAG synthesis. TAG is synthesised from a glycerol precursor.

1.2.2.3 Regulation of Apolipoprotein B Expression by Addition of Lipid by Microsomal Triglyceride Transfer Protein Complex

The *apoB* gene is constitutively expressed. Therefore protein concentrations are regulated at a posttranscriptional level (Innerarity *et al.* 1996). The mRNA is translated by ribosomes bound to the rough endoplasmic reticulum. The translating apoB is translocated into the lumen of the rough endoplasmic reticulum where lipid is transferred to apoB by the action of microsomal triglyceride transfer protein (MTP) (Davis *et al.* 1990). The attachment of lipid to apoB is necessary to stop degradation of the protein. In cell lines the addition of oleic acid has been found to increase apoB secretion levels by decreasing the percentage of apoB degraded from 58% to 29% (Dixon *et al.* 1991). The N-terminal 32% of apoB (apoB32) is necessary for the production of lipoproteins (Rosseneu & Labeur 1995). ApoB17 is strongly associated with the membrane of the rough endoplasmic reticulum (Pease *et al.* 1991). However, apoB32 is the smallest sized apoB peptide necessary for the MTP complex to bind and add lipid to and which results in the secretion of lipoproteins (Rosseneu & Labeur 1995). Therefore, apoB concentrations are regulated at a posttranslational level.

MTP is expressed in the liver, intestine and kidney (Hagan *et al.* 1994). MTP is a heterodimer that consists of a 97kDa large sub-unit and a smaller 58kDa protein disulfide isomerase (PDI) sub-unit. The PDI subunit is responsible for the folding of apoB, establishing a hydrophobic core into which lipid is transferred (Gordon 1997). The large sub-unit is responsible for the transfer of lipid to apoB as it is being translated and folded thus protecting the protein against degradation (Jamil *et al.* 1995). The addition of lipid to apoB during assembly of the lipoprotein particle appears to occur in two steps. The first is the co-translational production of a lipid-poor lipoprotein precursor and the second the addition of the bulk of the dietary lipid to the precursor (section 1.2.2.4). Some studies have shown that MTP activity is necessary for the addition of lipid to apoB in the rough endoplasmic reticulum but is not necessary for the addition of the majority of lipid to the lipoprotein precursor particle in the smooth endoplasmic reticulum (Gordon *et al.* 1996). However, van Greevenbroek *et al.* (1998) observed that MTP activity was necessary for the addition of all lipids attached to the secreted lipoproteins.

1.2.2.4 Independent Pathway and Sequential Assembly Pathway of Chylomicron Assembly

The process of lipoprotein assembly by the intestine during fasting and postprandial states is described in a review by Hussian (2000). Chylomicrons and VLDL lipoproteins are

synthesised by and secreted from intestinal enterocytes (duodenum and jejunum). Chylomicrons contain apoB48 while VLDL particles contain apoB100. Both isoforms of apoB (apoB48 and apoB100) are encoded by the same gene. ApoB48 and apoB100 are thought to represent 84% and 16% respectively of the total quantity of apoB protein in enterocytes (Ordovas *et al.* 1995).

It is hypothesised that gut VLDL production is saturated during the postprandial response by the elevated fatty acid concentrations in the gut, causing chylomicron production to increase exponentially. The mechanism of lipoprotein assembly is hypothesised to occur by either of two pathways: the independent pathway or the sequential assembly pathway. The independent pathway hypothesises that gut VLDL and chylomicrons are formed independently. Chylomicron production is thought to be induced in the postprandial state, while gut VLDL production is thought to be constitutive. Experimental evidence shows that only gut VLDL is produced at fasting and Golgi apparati contain either VLDL or chylomicron particles (Hussain 2000). In the second pathway, the sequential assembly pathway, it is hypothesised that primordial lipoproteins containing apoB and PL are formed in the rough endoplasmic reticulum. Formation is dependent on microsomal triglyceride transfer protein (MTP) activity. Experimental evidence to support the sequential assembly pathway of chylomicron assembly has shown that the PL in chylomicrons is preformed. The expression of MTP is necessary for stabilising apoB mRNA in the rough endoplasmic reticulum and allowing fusion of nascent dietary TAG to apoB protein. Dietary TAG is formed into lipid droplets in the smooth endoplasmic reticulum. The size of the lipid droplet determines the size of the lipoprotein secreted; VLDL, small chylomicrons or large chylomicrons. Subsequently the primordial lipoprotein and the lipid droplet may fuse at the junction of the rough and smooth endoplasmic reticulum. Subsequent to fusion the lipoprotein is transferred to the Golgi apparatus for secretion. Experimental evidence supporting the sequential assembly pathway shows that the VLDL (0.93-1.006g/ml) produced by the gut contains equal quantities of preformed and nascent (dietary) TAG, whereas chylomicrons (<0.93g/ml) contain only nascent TAG. Expression of apoB48 has been found not to induce chylomicron production because apoB is regulated at a posttranslational level (Hussain 2000).

1.2.2.5 Hydrolysis of TAG by Lipoprotein Lipase

Assembled lipoproteins are secreted by enterocytes into mesenteric lymph vessels for transport to peripheral tissues. Absorption of dietary TAG by enterocytes stimulates

glucose-dependent insulinotrophic polypeptide (GIP) secretion, which induces the synthesis and secretion of insulin. In turn, insulin induces adipose tissue lipoprotein lipase (LPL) activity and represses muscle LPL activity (Jackson *et al.* 2000).

Chylomicrons produced by the intestine bind to LPL with apolipoprotein C-II (apoC-II) as a co-factor and the chylomicron TAG core is hydrolysed into MAG and free fatty acids (NEFA). NEFA are either stored (in adipocytes) or oxidised (in muscle tissue). It has been found that 90-95% of the long-chain fatty acids in TAG, released by the action of LPL, enter the tissue to which LPL is attached (McArthur *et al.* 1999). The NEFA that escapes returns NEFA concentrations back to preprandial levels (Roche & Gibney 1999).

1.2.2.6 Removal of Triacylglyceride Rich Lipoprotein Remnant Particles

PL, cholesterol and some apolipoproteins are transferred to and from the TAG depleted chylomicrons by the action of the cholesterol ester transfer protein, to form chylomicron remnants. These remnants are cleared from the blood by hepatocytes using receptor dependent endocytosis. Remnants are defined as CE-rich lipoproteins with high apolipoprotein E (apoE) content and low apoC-II content (Packard 1999). Chylomicrons and VLDL particles are lipolysed by the action of LPL and so form remnants. The liver removes chylomicron remnants and large VLDL remnants using apoE and apoB100 as ligands, while small VLDL remnants form intermediate density lipoproteins and LDL particles (Shepherd & Packard 1987).

1.2.2.7 Synthesis and Secretion of VLDL Particles by the Liver

Short-chain fatty acids (<C12) from the diet, NEFA released from TAG hydrolysis and chylomicron remnants are taken up by the liver and stimulate the liver to synthesise TAG. The TAG is assembled and secreted as VLDL particles at about 4-6 hours post-ingestion.

1.2.2.8 Competition Between VLDL Particles and Chylomicrons for Binding to the LPL Active Site

More VLDL particles than chylomicrons (20:1) are present in the circulation during the postprandial response. Despite this, chylomicrons are cleared at a faster rate from blood. This may be due to chylomicrons having a higher binding affinity for the LPL binding site than VLDL particles. In addition, the binding of one chylomicron is likely to have a larger effect on the pool of unbound chylomicrons than the binding of one VLDL particle to LPL on the pool of unbound VLDL (Xiang *et al.* 1999). It has been found that the apolipoprotein composition of VLDL particles alters during the postprandial response; there is an increase in apolipoprotein C-I (apoC-I) and apoE and a decrease in apoC-II

concentrations. Since apoC-II is the co-factor necessary for lipoprotein binding to LPL, this reduces VLDL's affinity for the LPL binding site (Packard 1999). Thus different binding affinities and the larger proportion of VLDL to chylomicrons may equalise the chances that chylomicrons and VLDL particles have for binding to free LPL binding sites.

1.2.2.9 The Reverse Cholesterol Transfer Pathway

The reverse cholesterol transfer pathway refers to the function of HDL particles in lipid homeostasis. HDL particles remove cholesterol from non-hepatic tissues and transport it to the liver for elimination from the body in the form of bile. Cholesterol is a polar molecule, which remains on the surface of HDL particles. This terminates the removal of cholesterol from cells without the action of lecithin:cholesterol acyltransferase (LCAT), which acts in the following way. The enzyme LCAT acts at the rate regulating step of the pathway by esterifing cholesterol into CE which migrates to the HDL core and so forms a gradient of cholesterol between cells and HDL particles. This gradient regulates the concentration of cellular cholesterol (Hill *et al.* 1993).

Cholesterol is a necessary lipid, but in excess has been shown to be a risk factor for development of atherosclerosis. Cholesteryl ester transfer protein (CETP) maintains the necessary levels of cholesterol in the body by transferring core CE from HDL particles to chylomicrons and VLDL particles in exchange for TAG from the triacylglycerol-rich lipoproteins (TRL = chylomicrons, VLDL and IDL particles). CETP becomes enzymatically active during the postprandial response due to the increase in TRL particles in the plasma. If there is a high concentration of TRL particles in plasma, more CE for TAG exchange can occur, which may lead to both an increased and unnecessary concentrations of cholesterol being maintained in the body, and a reduction in the concentration of cardioprotective HDL_2 particles (Juvonen *et al.* 1995). TAG enriched HDL and LDL particles make very good substrates for hepatic lipase, causing the formation of small dense LDL and HDL_3 particles (Chait *et al.* 1993). Therefore the inverse relationship between plasma TAG concentrations and HDL levels is due to CETP activity. The presence of apoE increases the affinity of lipoproteins for CETP, resulting in more TAG transfer between TRL remnants and HDL particles (Kinoshita *et al.* 1993). An elevated or prolonged postprandial lipid response can increase the risk of developing CHD in the following way. An elevated response causes an increase in the formation of small CE-rich remnants, which like LDL particles can cause influx of cholesterol into arterial walls. It also stimulates the formation of the highly atherogenic small dense LDL particles. These particles have been associated with a 4-6 fold increase in risk of CHD (Roche &

Gibney 1995). A prolonged postprandial response allows more lipid exchange between chylomicrons and cardioprotective HDL_2 particles, while rapid TRL clearance promotes the formation of HDL_2 particles (Patsch 1994). A study looking at differences between postprandial responses in Southern and Northern Europeans, found that Southern Europeans had earlier postprandial TAG peaks, faster clearance of chylomicrons and higher levels of the hormone GIP than Northern Europeans. LPL and hepatic lipase (HL) activity was measured 9 hours after ingestion of the meal and no difference could be seen in their LPL activity. However, HL activity was increased in the Southern European population. Due to the enhanced clearance of dietary TAG in the Southern European population, less CETP activity can occur and the increased HL activity may result in the increased chylomicron remnant removal seen in this population (Jackson *et al.* 2000). The phenotypic result is that Southern Europeans are possibly less likely to develop CHD due to the postprandial response.

1.2.3 Effects of Fatty Acids on Postprandial Lipaemia

1.2.3.1 The Effects of Quantity and Type of Fat Ingested on the Postprandial Response

As mentioned previously, lipid metabolism can be influenced by environmental factors. One important factor is the type and quantity of fat ingested. A meal containing a high proportion of fat will produce a larger postprandial response that a low fat meal. In other words, less fat causes a smaller postprandial response. In addition, the type of fat ingested is extremely influencal on the size and length postprandial response. A meal high in saturated fatty acids (SFA) will cause a larger and/or longer postprandial response that a meal high in monounsaturated (MUFA), omega-6 polyunsaturated (n-6 PUFA) and omega-3 polyunsaturated (n-3 PUFA) fatty acids. The long-term diet of an individual also affects the postprandial response. An SFA diet will cause a larger postprandial response that an MUFA or either of the PUFA diets, independent of the test meal given.

Therefore, there are at least three important dietary factors contributing to the postprandial lipid response: the amount of fat consumed, the type of fat ingested, and the type of fats regularly ingested. A review by this group discusses in detail the effects of fat ingestion on the postprandial lipid response (Roche & Gibney 1995). Dubois *et al.* (1998) and Cohen *et al.* (1988) investigated the effect of the quantity of n-6 PUFA and SFA ingested on postprandial lipid levels. They both concluded that a dose of 30-40g of fat attenuates the

postprandial response compared to a fat load of above 40g. This shows a dose-dependent relationship between dietary fat intake and plasma TAG levels so long as dietary carbohydrate and protein levels remain constant. n-3 PUFA, especially fish oils (20:5n-3 and 22:6n-3), has been shown to be more hypotriglyceridemic than SFA, MUFA or n-6 PUFA test meals, when the quantity of fat ingested remains constant (Williams 1999). This may be because LPL activity is at its lowest 12hr post-ingestion of an n-3 PUFA test meal (Zampelas *et al.* 1994).

1.2.3.2 Effect of Dietary Fatty Acids on Postprandial Lipid Response

A background diet refers to a diet where fatty acid supplements have been administered to a study population over a period of 4-12 weeks. Background diets have been shown to affect the magnitude and duration of the postprandial lipid response, with diets high in n-3 PUFA having the smallest response followed by n-6 PUFA, MUFA and SFA diets (Williams 1998). Weintraub *et al.* (1988) found that individuals taking a background diet consisting of either n-6 or n-3 PUFA who were given a SFA test meal had a reduced postprandial response. This reduction was significant in individuals on the n-3 PUFA diet. In addition, individuals consuming a background diet high in n-6 PUFA followed by an n-6 PUFA test meal showed a more attenuated postprandial lipid response than individuals given a SFA test meal. Because of the n-3 PUFA hypotriglyceridemic effect, many studies have been carried out investigating the necessary dose of fatty acid to achieve attenuation of the postprandial lipid response. The dose and duration of n-3 PUFA supplementation have been found to be important. A dose of approximately 1g of n-3 PUFA given to humans over >15wk reduces their fasting TAG concentrations to equivalent levels of about 4-7g of n-3 PUFA taken by individuals over 4-12wk (Roche & Gibney 1996). It has been shown that the longer the duration that individuals are supplemented with n-3 PUFA, the more their postprandial TAG levels per unit of n-3 PUFA supplement are reduced (Roche & Gibney 1999). n-3 PUFA has been shown to reduce TAG synthesis and secretion from liver, reducing the quantity of plasma VLDL particles (Harris *et al.* 1990). This reduces the competition between dietary chylomicrons and hepatic VLDL particles for LPL binding, causing a faster clearance of postprandial TAG (section 1.2.2.8). In addition, n-3 PUFA test meals have been shown to increase LPL activity compared to SFA test meals (Zampelas *et al.* 1994, Sanders *et al.* 2000). A recent study by Murphy *et al.* (1999) found a significant relationship between fasting TAG concentrations and LPL mRNA levels when individuals consumed an n-3 PUFA enriched diet. However, there was no

significant increase in LPL mRNA levels observed in individuals given the non-enriched and n-3 PUFA enriched diets.

A study into habitual diets, which is the type of fat regularly contained in the diet, of Northern and Southern Europeans found that the two groups responded differently to the same test meal. Southern Europeans had an earlier TAG peak and a more rapid return to fasting plasma TAG concentrations (6 hours), than Northern Europeans who maintained postprandial plasma TAG concentrations for 8-9 hours. The habitual diet of Southern Europeans is known to be high in MUFA, while the habitual diet of Northern Europeans is high in SFA (Zampelas *et al.* 1998).

1.2.3.3 Effect of *Trans* Fatty Acids on Lipid Concentrations

For naturally occurring unsaturated fats the double bonds between the carbon atoms are usually found in the *cis* configuration. Unsaturated fatty acids can also be found naturally in the *trans* configuration. The most common *trans* fat in beef and milk is vaccenic acid (*trans* 11-18:1). The principle source of *trans* fats in the human diet is through the partial hydrogenation of vegetable or marine oils. *Trans* unsaturated fatty acids have a more rigid structure than their *cis* counterparts, which makes them useful in the preparation of margarines, cooking oils and shortenings. *Trans* fatty acid supplements given to rats are known to have no effect on their growth or reproductivity as long as the essential fatty acids are present (linoleic (18:2n-6) and linolenic acid (18:3n-3) (Gurr & Harwood 1991)). In humans, *trans* fatty acids have been found to be more hypercholesterolaemic than their *cis* counterparts but not as hypercholesterolaemic as SFAs. *Trans* fats may also reduce plasma HDL-cholesterol levels. In the above studies elaidic acid (*trans* 18:1n-9) was the *trans* fatty acid usually administered (Kritchevsky 1999). However, fatty acids, which are of growing interest, are the *trans* isomers of linoleic acid (section 1.6). Conjugated linoleic acid (CLA) has two major isomers, the t-10 isomer and c-9 isomer. The t-10 isomer has been found to reduce fasting plasma TAG levels in humans after 8 week supplementation. The c-9 isomer, however, is not thought to reduce fasting plasma TAG levels (Noone *et al.* in press).

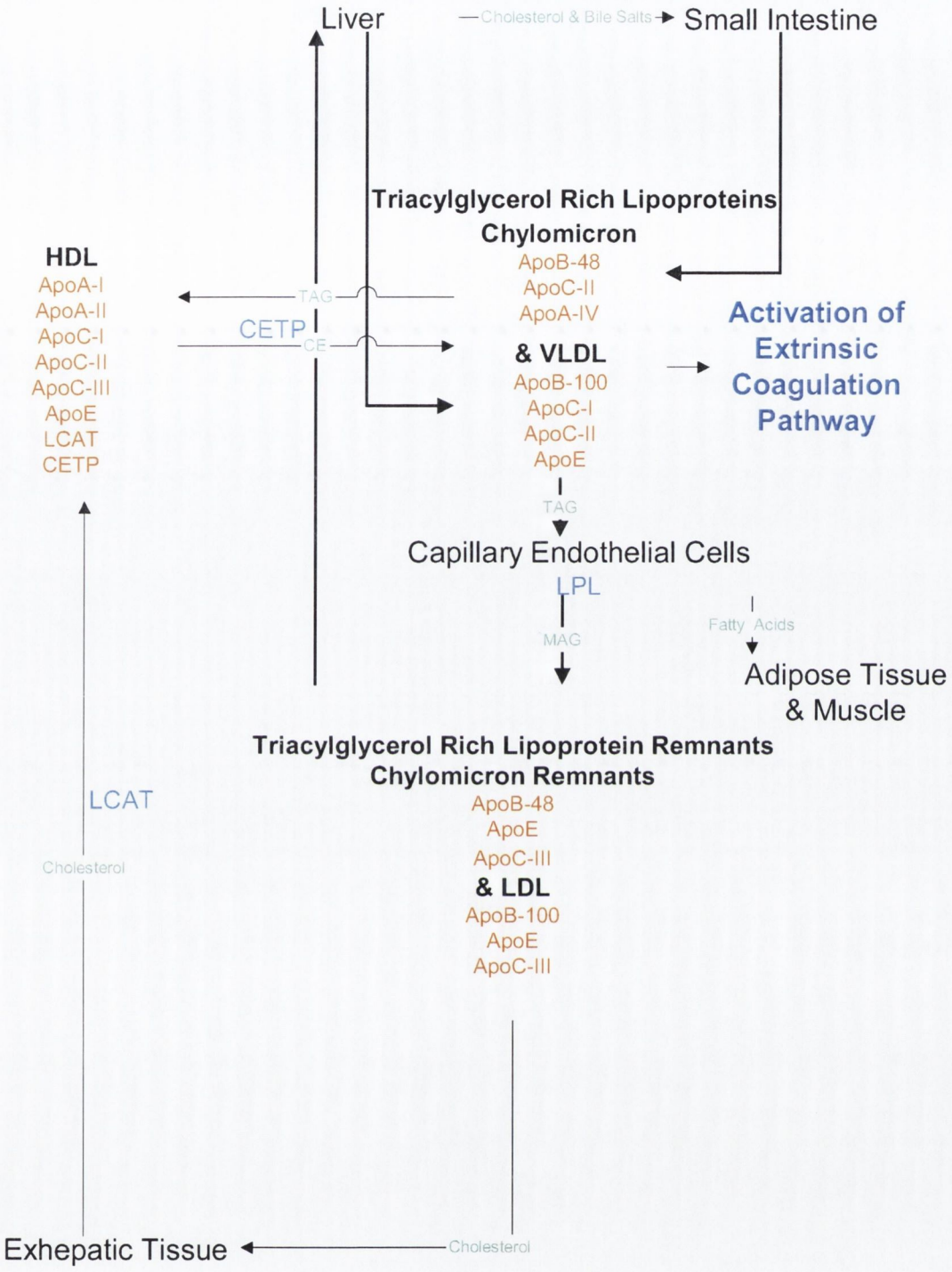


Figure 1.2.3 Diagram of tissues, lipoproteins and enzymes involved in lipid metabolism. Chylomicrons are secreted from the intestine and VLDL from the liver. The TAG in these particles is lipolysed by LPL into MAG and fatty acids. Fatty acids are either stored in adipose tissue or oxidised in muscle, and TRL remnants are formed. LDL particles deposit cholesterol in exhepatic tissues, which is transferred by LCAT into HDL particles. CE from HDL is transported to TRL and TAG from TRL to HDL particles. The liver removes the TRL remnants and cholesterol and bile salts are secreted into the intestine via the gall bladder (section 1.2.2).

TAG in TRL particles has been found to be associated with activation of coagulation factor VII, the first factor involved in the extrinsic coagulation pathway, thus increasing the risk of blood clots during the postprandial response (section 1.4).

1.2.4 Summary

In conclusion, postprandial response not only represents the influx of dietary TAG into the circulation but also represents a very significant period during which the composition of plasma lipoproteins is significantly changed. Efficient postprandial lipaemia has a number of beneficial effects including reverse cholesterol transport, but elevated and prolonged postprandial lipaemia has a number of pro-atherogenic and pro-thrombotic effects (Roche *et al.* 2000).

1.3 Genetics of Lipid Metabolism

1.3.1 Human Genome Project

The genetics of lipid metabolism is complex; many genes are involved and many different interactions take place between the proteins and fats involved in the response. A project such as the Human Genome Project (HGP) gives hope that all the genes and their variations involved in lipid metabolism may be discovered. The HGP is an international effort to understand the structure and function of the human genome. The project has many goals, the first being the provision of a continuous human genomic sequence, accurate to 1 base in 10,000. Another goal is the formation of single nucleotide polymorphism (SNP) maps, which can be used for fine-mapping in linkage studies. In addition, candidate SNPs can be studied for potential contribution to disease risk in polygenic diseases such as atherosclerosis. It has also been suggested that haplotypes (i.e. combinations of various SNPs in an individual) may be predictive of an individual's response to environmental factors, such as diet. An additional goal of the HGP is to characterise all the open reading frames representing all the human genes. The mouse genome, also sequenced in the HGP, will be utilised in identifying the functional regions in both coding and noncoding regions. The instigators of the HGP wish to provide a database with the human genomic sequence, which will provide information on analogous model organisms and web links to phenotypic information. Research into the ethical, legal and social implications of genomic research is also being carried out. This makes the HGP a first in allowing professionals from many disciplines, including mathematics, computer science, genetics, biochemistry, sociology and law to name but a few, to work together to

gather and distribute information which will be useful to researchers and the general population (Collins *et al.* 1998).

In June 2000 Celera Genomics completed and published on the web (www.celera.com) the entire genetic sequence of one individual. The sequence showed the location and order (assembly) of the genetic code on the chromosomes. By December 2000 Celera Genomics had sent an article to Science for publication with the assembly of the consensus genome. The SNP Consortium (www.snp.cshl.org) reported the launch of the first 2,279 mapped SNPs in December 1999. The database can be found at www.ncbi.nlm.nih.gov. The experiments on the effects of genotype on postprandial lipid response were designed prior to these results being published.

The experimental design was to investigate possible effects of a number of polymorphisms in a number of genes of interest on the postprandial response. In addition certain combinations of polymorphisms (i.e. haplotypes) were studied for having possible effects on the postprandial response.

An individual's response to diet may be regulated by genes. Alternatively, diet may regulate gene expression. An example of the latter is how some fatty acids can be natural ligands of peroxisome proliferator-activated receptors (PPAR). PPAR are transcription factors of which 4 different forms have been identified (α, β, γ and δ) (Gibney & Roche 2001). PPARγ ligands inhibit tumour necrosis factor α (TNF-α) mRNA expression in monocytes (Jiang *et al.* 1998). In the colon carcinoma cell line, Caco-2, PPARγ ligands have been shown to maintain NF-κB in an inactive state. NF-κB is a transcription factor for cytokine expression (Su *et al.* 1999).

The following sections discuss the relationship between genetics and lipid metabolism. They focus on how population polymorphisms have been found to be associated with an individual's postprandial response levels to different test meals (section 1.3.3). This therefore suggests that individuals' genetics affects their physiological response to diet. The public health message of 'one-size-fits-all' is therefore inappropriate (Ordovas & Schaefer 1999). One of the goals of the HGP is to use the emerging genetic information and learn what role genetic polymorphisms may have in disease phenotype, drug response and indeed an individual's physiological response to diet.

1.3.2 Proteins Involved in Lipid Metabolism

Proteins involved in lipid metabolism are frequently associated with lipoproteins. Some of these proteins, the lipoproteins they are associated with and the function that the proteins fulfil are contained in Table 1.3.1. Proteins that are not associated with lipoproteins can also be involved in lipid response and the following sections will discuss both types of proteins. Some of these proteins such as apolipoprotein B (apoB), apolipoprotein E (apoE), lipoprotein lipase (LPL), cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT), will be discussed in more detail than others given that they were the focus of research reported in this thesis. Polymorphisms, which are frequently occurring sequence variations in a population (frequency <0.95), in genes involved in lipid metabolism have been found in many populations. Phenotypes associated with polymorphisms in genes involved in lipid metabolism are discussed below. The relationship between lipoproteins and the proteins they are associated with during lipid homeostasis is shown in Figure 1.2.3.

1.3.2.1 Apolipoprotein A-I

Apolipoprotein A-I (apoA-I) is the major apolipoprotein component of HDL particles and activator of lecithin:cholesterol acyltransferase (LCAT). By activating LCAT it matures the HDL lipoprotein by forming a cholesterol ester (CE) core. Low levels of HDL and apoA-I have been associated with premature atherosclerosis, xanthomatosis and corneal opacities. Mutations in the N-terminal region of the protein have been associated with reduced stability of apoA-I. ApoA-I is clustered with apoC-III and apoA-IV on chromosome 11q (Rosseneu & Labeur 1995). The apoA-I gene has a promoter polymorphism at nucleotide position -75G/A, which has been associated with variation in HDL-cholesterol concentrations. The A allele occurs at a frequency of between 0.15-0.2 in the Caucasian population. Individuals carrying the A allele have higher fasting plasma HDL-cholesterol levels than individuals who are homozygous for the G allele (Ordovas *et al.* 1995).

1.3.2.2 Apolipoprotein A-II

Apolipoprotein A-II (apoA-II) associates with HDL particles and is believed to be involved in lipid binding and lecithin:cholesterol acyltransferase (LCAT) activation. Absence of apoA-II has not been found to cause clinical signs of atherosclerosis. ApoA-II is not present in all animal species. Therefore, this apolipoprotein may not be critical for lipid metabolism (Rosseneu & Labeur 1995).

Table 1.3.1 Table of proteins associated with lipoproteins and their functions in lipid metabolism (Rosseneu & Labeur 1995). Chylomicrons, VLDL and intermediate density lipoproteins (IDL) are TAG transporting lipoproteins and LDL and HDL are cholesterol transporting lipoproteins (Williams 1999).

Lipoprotein	Protein	Function
Chylomicron	ApoB-48	Lipid binding
	ApoA-IV	LCAT activation
	ApoC-II	LPL co-factor
Chylomicron Remnant	ApoB-48	Lipid binding
	ApoC-III	LPL inhibitor
	ApoE	ApoB/ApoE receptor ligand
VLDL	ApoB-100	Lipid binding & ApoB/ApoE receptor ligand
	ApoC-I	LPL inhibitor and inhibitor of TRL remnant clearance
	ApoC-II	LPL co-factor
	ApoE	ApoB/ApoE receptor ligand
IDL	ApoB-100	Lipid binding & ApoB/ApoE receptor ligand
	ApoC-I	LPL inhibitor and inhibitor of TRL remnant clearance
	ApoC-II	LPL co-factor
	ApoC-III	LPL inhibitor
	ApoE	ApoB/ApoE receptor ligand
LDL	ApoB-100	Lipid binding & ApoB/ApoE receptor ligand
	ApoC-III	LPL inhibitor
	ApoE	ApoB/ApoE receptor ligand
HDL	ApoA-I	LCAT activation
	ApoA-II	Lipid binding
	ApoC-I	LPL inhibitor and inhibitor of TRL remnant clearance
	ApoC-II	LPL co-factor
	ApoC-III	LPL inhibitor
	ApoE	ApoB/ApoE receptor ligand
	LCAT	Formation of CE from peripheral cholesterol
	CETP	Transfer of CE to TRL and TAG from TRL to HDL

1.3.2.3 Apolipoprotein A-IV

Apolipoprotein A-IV (apoA-IV) is synthesised primarily in the intestine and is involved in dietary fat absorption and chylomicron synthesis. It is thought to mediate the activation of apoC-II by lipoprotein lipase (LPL), may be an activator of LCAT and be a ligand for the putative HDL receptor (Rosseneu & Labeur 1995). The metabolic consequences of polymorphisms in the gene are still unknown, but 8 isoforms of this protein have been identified (apoA-IV*0 – apoA-IV*7). The most common isoform is apoA-IV*1 (0.88-0.95 in the Caucasian population) and the second most common is apoA-IV*2 (0.05-0.12 in the Caucasian population). Some studies have found that the apoA-IV*2 allele is associated with increased HDL-cholesterol concentrations and/or reduced TAG concentrations. Some other common polymorphisms, first identified by polymerase chain reaction (PCR)

amplification, have been found to be linked to some particular apoA-IV isoforms (Ordovas & Schaefer 1999).

1.3.2.4 Apolipoprotein B

Apolipoprotein B (apoB) is found in two forms, apoB100 and apoB48. Both proteins are translated from the same gene transcript. However apoB48 is a truncated form of apoB100, which consists of only the N-terminal 48% of apoB100. The CAA codon at position 2153 of the gene transcript (mRNA) is edited to a UAA stop codon to produce the apoB48 protein. This RNA editing occurs in the small intestine in humans. ApoB48 is assembled into chylomicrons and these lipoproteins are secreted into the circulation. As the TAG of chylomicrons is hydrolysised and chylomicron remnants are formed, apoB48 remains in the remnant lipoproteins. Therefore, apoB48 is found in chylomicrons and their remnants. ApoB48 is necessary for the transport of lipid and fat-soluble vitamins around the body. ApoB100 functions as a lipid transporter in VLDL and LDL particles and also is a ligand for the LDL receptor (Ordovas *et al.* 1995).

The *apoB* gene is located on chromosome 2p24-p23 and is 43kb long containing 29 exons. The gene has no similarity to other known apolipoproteins. Exon 1 encodes the signal peptide; exon 26 is very large, encoding amino acids 1379-3902. Therefore, apoB48 is made up of the first 25 exons plus the first third of exon 26. Exon 29 is also relatively long (Ordovas *et al.* 1995). Mutations resulting in truncated apoB result in hypobetalipoproteinaemia, which manifests itself as a lack of apoB containing lipoproteins. Hypobetalipoproteinaemia is inherited in a codominant manner. Heterozygotes have reduced cholesterol, LDL-cholesterol and apoB concentrations. The extent and severity of this syndrome is related to the size of the apoB protein (Rosseneu & Labeur 1995). An apoB protein translated from 32% of the transcript will result in the presence of some apoB containing lipoproteins but they are of HDL size. This is the minimum length necessary for microsomal triglyceride transfer protein (MTP) to stabilise the apoB protein in the rough endoplasmic reticulum to allow the addition of dietary TAG (section 1.2.2.3). Mutations that cause hypercholesterolaemia, which is reduced uptake of LDL by the LDL receptor, are found at the C-terminus of exon 26, thus effecting VLDL remnant homeostasis. Hypercholesterolaemia is observed as an accumulation of LDL particles. One mutation, an Arg3500Gln mutation, is thought to cause a reduction in the binding affinity of LDL for the LDL receptor resulting in an accumulation of LDL particles (Rosseneu & Labeur 1995).

Many population polymorphisms have been found in apoB, which have small effects on lipid metabolism. Individuals with a polymorphism in the signal peptide of the *apoB* gene, resulting in a deletion of amino acids -14 to -16 (Leu Ala Leu), have been shown to have reduced fasting plasma TAG levels compared to insertion homozygotes (II). The deletion allele (D) seems to have a gene dosage effect. Thus, heterozygotes have intermediate plasma TAG levels and DD individuals have the lowest plasma TAG levels (Renges *et al.* 1992). The effect is less apparent after a low-fat test meal than after a high-fat test meal (Ordovas *et al.* 1995). The I allele, which produces a signal peptide of 27 amino acids, is found at a frequency of approximately 0.69, and the D allele, whose signal peptide is 24 amino acids, at a frequency of 0.31 in the Caucasian population. A study looking at the first 4 hours of a lipid postprandial response found that DD individuals had a smaller VLDL (LpCIII:B) particle fraction, than II and ID individuals who had a peak of VLDL levels at 2 hours (Regis-Bailly *et al.* 1995). Another postprandial study carried out over a 12 hour period, found that during the postprandial response D carriers had a 38% smaller peak in the large chylomicron fraction and a 29% smaller peak in the large VLDL fraction than I homozygotes (Peacock *et al.* 1995, Syvanne *et al.* 1997). Fasting plasma TAG concentrations are known to be influenced by free fatty acid levels. Byrne *et al.* (1996) found that 8 hours after the start of the postprandial response the TRL concentration was determined by the maximum free fatty acid concentration in I homozygotes, but not D carriers. It is proposed in the Byrne *et al.* study that the relationship between maximum free fatty acid levels and 8 hour TRL particle concentrations explains the increased prevalence of ischemic heart disease (IHD) in II individuals. The insertion/deletion polymorphism may affect the intracellular handling and/or secretion of apoB, with the D allele being associated with reduced apoB translocation. Therefore, the D allele appears to be less effected by a low-fat diet than the I allele. This may be because the D signal peptide retards chylomicron secretion to a greater extent when high-fat diets are consumed than low-fat diets (Ordovas *et al.* 1995).

An XbaI polymorphism, which is a silent polymorphism causing no amino acid change (ACC2488ACT), is found at a frequency of X+ 0.6, X- 0.4 in Caucasian populations. The X+ mutation has been associated with increased fasting total cholesterol, LDL-cholesterol and plasma TAG levels. However, the X- allele has been shown to associate with increased risk of coronary artery disease (CAD) (Juvonen *et al.* 1995, Renges *et al.* 1992). X+ carriers were found to respond to a low-fat, low-cholesterol diet better than X-X- individuals (Ordovas *et al.* 1995). X- homozygotes have been shown to have a larger postprandial response in comparison to X+X- and X+X+ individuals (Ordovas & Schaefer

1999). This may explain why the X- allele has been associated with increased risk of CAD. It has been suggested that the XbaI polymorphism is a marker for a functional mutation and is in linkage disequilibrium with that functional mutation, which causes the effects on lipid levels.

An MspI polymorphism (Arg3611Gln) has been found to be in linkage disequilibrium with an EcoRI polymorphism (Gln4154Lys). The MspI Gln allele associates with the EcoRI R+ allele and the MspI Arg allele with the EcoRI R- allele (Glisic *et al.* 1995). The Gln allele has been associated with increased risk of premature CAD. The frequency of the alleles is approximately Arg 0.89, Gln 0.11. The EcoRI polymorphism is located close to the LDL receptor-binding region of the apoB protein (Knott *et al.* 1986). Some studies have found the R-R- genotype to be associated with increased risk of CHD and increased plasma cholesterol concentrations. However, not all studies have found biochemical factors to associate with either of the alleles, which occur at a frequency of R+ 0.8 and R- 0.2 in Caucasian populations (Glisic *et al.* 1995, Juvonen *et al.* 1995, Renges *et al.* 1992).

A study on an Asn4311Ser polymorphism, which occurs at a frequency of Asn 0.8 and Ser 0.2 (Renges *et al.* 1992), has shown SerSer individuals to have lower apoB, TAG and apoB/apoE containing lipoprotein levels and higher apoA-I levels than Asn carriers.

1.3.2.5 Apolipoprotein C-I

Apolipoprotein C-I (apoC-I) has been found to be an inhibitor of LPL activity. Like apoC-III it displaces apoE from TRLs, thus interfering with hepatic clearance of TRL remnants. The presence of apoC-I on migrating β-VLDL lipoprotein particles decreases their binding affinity for remnant receptors and LDL-related receptors in the liver. Its presence also decreases apoE-mediated clearance of VLDL and IDL particles by the LDL receptor (Shachter *et al.* 1996). The *apoC-I* gene is found in a gene cluster with *apoC-II* and *apoE* on chromosome 19q13. It is located 4.3kb downstream of *apoE*, and all 3 genes are transcribed in the same direction (Myklebos & Rogne 1986). A polymorphic restriction site in the *apoC-I* gene has been tightly linked to the ε4 allele in the *apoE* gene. Both alleles occur at the same frequency in Alzheimer patients (0.45) and controls (0.14) (Poduslo *et al.* 1995).

1.3.2.6 Apolipoprotein C-II

Apolipoprotein C-II (apoC-II) is a co-factor for the activation of LPL. It is found in chylomicrons and VLDL particles. ApoC-II contains domains involved in binding to chylomicrons and VLDL particles, activation of LPL and binding to LPL. Mutations in the

lipoprotein binding domain result in formation of no or reduced apoC-II levels. This causes type I hyperlipoproteinaemia or hypertriglyceridaemia, which clinically can be characterised by the presence of high concentrations of fasting chylomicrons (Rosseneu & Labeur 1995).

1.3.2.7 Apolipoprotein C-III

Apolipoprotein C-III (apoC-III) is known to inhibit the activity of LPL and hepatic lipase *in vitro*. ApoC-III and apoE are transferred from HDL particles to lipolysed chylomicrons and VLDL particles thus forming their remnants. Remnants are removed from the circulation via the LDL receptor -related protein receptor pathway, since apoC-III has been shown to inhibit the binding of apoE-containing lipoproteins to the LDL receptor (Ordovas & Schaefer 1999). Mutations in the apoC-III gene can cause enhanced lipolysis, which is called hyperalphalipoproteinaemia (Rosseneu & Labeur 1995). Polymorphisms have been found both in the promoter region and 3' untranslated region (UTR) of the gene. An SstI polymorphism located in the 3' UTR has been associated with an increase in plasma TAG, total cholesterol and apoC-III concentrations (Ordovas & Schaefer 1999). This polymorphism is in linkage disequilibrium with certain mutations in the promoter region. A polymorphism in the promoter has been found in the insulin response element between -490 and -449. Wild type promoters may be downregulated by insulin, while promoters containing the polymorphism are not, resulting in decreased LPL activity (Ordovas & Schaefer 1999).

1.3.2.8 Apolipoprotein E

Apolipoprotein E (apoE) is produced mainly by the liver, and is a constituent of chylomicrons and VLDL, their remnants and HDL particles. It is transferred to the remnants after hydrolysis of TAG by LPL, and acts as a ligand for the LDL-receptor and the LDL receptor-related protein receptor (Larson *et al.* 2000). The presence of apoE has been shown to enhance CE and TAG transfer between VLDL and HDL particles in the presence of CETP. This may be due to enhanced affinity of CETP for apoE containing lipoproteins (Kinoshita *et al.* 1993).

The *apoE* gene is located on chromosome 19, closely linked to the *apoC-I* and *apoC-II* loci and more distantly linked to the *LDL-receptor* locus (Larson *et al.* 2000). Mutations in *apoE* that disrupt its binding to the LDL receptor, can cause type III hyperlipoproteinaemia that manifests itself as an overproduction of TRL particles, appearance of pre-β-VLDL particles and accumulation of remnant particles (Rosseneu & Labeur 1995). Three

isoforms of the *apoE* gene have been identified, ε2 (polymorphism) or apoE2 (protein), ε3 (polymorphism) or apoE3 (protein) and ε4 (polymorphism) or apoE4 (protein). The ε3 allele, which is the wild type, contains a cysteine residue at position 112, and an arginine at position 158. The ε2 allele has a cysteine 112 and a cysteine 158 and the ε4 allele has an arginine 112 and an arginine 158. The frequency of these alleles in the Caucasian population is ε2 0.08, ε3 0.77 and ε4 0.15. Although there is a North-South divide in *apoE* genotype frequency with more ε3ε4 individuals in Northern populations (Finland, Northern Ireland) than in Southern populations (Spain, Portugal, Greece) and more ε3ε3 individuals in Greece (Schiele *et al.* 2000) than in Northern populations. However, it is thought that *apoE* allelic variation may account for approximately 7% of the variation in total cholesterol and LDL-cholesterol concentrations in the general population. ε2 carriers have been shown to have reduced total cholesterol, LDL-cholesterol and apoB levels and increased apoE concentrations. ε3 homozygotes are known to have intermediate levels of these biochemical variables and ε4 carriers to have increased concentrations of total cholesterol, LDL-cholesterol and apoB and low apoE levels (Ordovas & Schaefer 1999). The apoE protein has been shown to be self-regulating; individuals with the ε2 allele have higher apoE levels and ε4 carriers have lower apoE levels than ε3 homozygotes. This may be due to the apoE2 isoform being catabolised more slowly compared to the other two isoforms (Larson *et al.* 2000). The concentration of apoE is found to be lower in Finish and Northern Irish populations than Spanish, Portuguese and Greek populations (Schiele *et al.* 2000).

The apoE receptor-binding site is located between residues 136-158. The difference in charge between the apoE isoforms (apoE2 (cys158), apoE3 (arg158) and apoE4 (arg158)) is thought to be responsible for their different binding affinities for the LDL-receptor (Larson *et al.* 2000). The apoE2 isoform has a lower binding affinity for the LDL-receptor than the other two isoforms, thus causing slower clearance of TRL remnants. Individuals with the apoE2 isoform have lower levels of LDL-cholesterol, slower conversion of VLDL remnants to LDL and increased LDL catabolism, than individuals with either the apoE3 or apoE4 isoforms. The reduction in intracellular cholesterol, due to reduced TRL remnant uptake, causes an up-regulation of the LDL-receptor and increased availability of the receptor due to less competition from remnant particles. This leads to individuals with the apoE2 isoform having lower serum cholesterol levels than individuals with the apoE3 isoform.

The apoE4 isoform is associated with increased serum cholesterol levels, which is caused by increased binding affinity for the LDL-receptor. This results in a higher concentration of intracellular cholesterol, which causes a down-regulation of the LDL-receptor. The apoE4 isoform has a lower affinity for HDL particles than for VLDL particles. Individuals with the apoE4 isoform also convert VLDL particles into LDL particles more efficiently than individuals with the apoE3 isoform. This results in individuals with the apoE4 isomer having elevated LDL-cholesterol levels (Larson *et al.* 2000).

1.3.2.9 Lipoprotein Lipase

Lipoprotein Lipase (LPL) is the rate-limiting enzyme in the hydrolysis of TAG present in chylomicrons and VLDL into NEFA and monoacylglycerol. LPL is active as a homodimer. LPL is bound to the endothelium of adipose tissue, cardiac and skeletal muscle and mammary glands. It is attached to the endothelium by heparin sulphate proteoglycan, which is integrated into the glycocalyx on the endothelial membrane (Ordovas *et al.* 1995). LPL activity appears to be regulated posttranslationally in humans. No increase in LPL mRNA or protein mass has been associated with the fasting or postprandial state. Instead an increase in activity in the heparin releasable enzyme fraction has been found during postprandial lipidaemia. LPL activity is switched on during the postprandial state in adipose tissue, and off during fasting. In muscle tissue it is switched on following a reduction in plasma glucose levels. During the postprandial state insulin activates adipose LPL and inhibits muscle LPL activity (Ong & Kern 1989).

The *lpl* gene is located on chromosome 8p22, and contains 10 exons, which form a 475 amino acid protein including a 27 amino acid signal peptide. The 3-d structure has elucidated that the N-terminal two-thirds of the protein contain a catalytic triad in a hydrophobic pocket. The C-terminus is involved in lipid binding and the N-terminus may be involved in lipid binding. It is thought that residues 290-300 may bind to heparin (Fisher *et al.* 1997). LPL activity is critical for postprandial TRL clearance. Mutations in the LPL gene can cause familial hypertriglyceridaemia, due to reduced hydrolysis of TAG in chylomicrons and VLDL particles (Fisher *et al.* 1997).

Two functional mutations have been found at positions D9N (G280A) and N291S (A1127G) of the *lpl* gene, which both cause increased fasting TAG levels and decreased HDL-cholesterol levels. The mutations seem to cause a reduction in LPL activity, the D9N mutation causing decreased activity by reducing the amount of LPL secreted from the cells and the N291S mutation causing increased LPL dimer dissociation into the inactive monomer form. The effects of these two mutations are enhanced when carriers are obese.

This may be due to the increased secretion of VLDL particles from the liver overwhelming the impaired lipolytic system (Fisher *et al.* 1997).

A number of polymorphisms that may cause a pre-disposition for or against the development of hyperlipidaemia have been characterised. An intronic PvuII polymorphism located in intron 6 of the *lpl* gene has in some studies been shown to have an effect on TAG and HDL levels. Individuals with a P+P+ genotype have lower HDL-cholesterol and higher TAG fasting levels than individuals with P+P- or P-P- genotypes (Ukkola *et al.* 1995). A HindIII polymorphism in intron 8 of the *lpl* gene has also been associated with differences in fasting TAG and HDL-cholesterol levels. The H- allele is associated with low fasting TAG levels and in some studies also with high fasting HDL-cholesterol levels (Fisher *et al.* 1997). These two intronic polymorphisms may directly affect LPL activity by effecting mRNA splicing or stability. Alternatively, the polymorphisms may be in linkage disequilibrium with a mutation in the 3'UTR, promoter region or indeed a functional mutation. A third polymorphism, the S447T polymorphism resulting from a C to G transversion at nucleotide 1595, causes the production of a mature LPL protein, which is truncated by 2 amino acids. The T allele (with a termination codon at residue 447) has been associated with low fasting TAG and high fasting HDL-cholesterol concentrations. A trend has been found for either unchanged or increased LPL protein mass and activity in individuals with the T allele. Increased LPL production has been seen in individuals with the T variant (Zhang *et al.* 1996). In addition, it has been suggested that LPL protein from the T allele may have a reduced or increased affinity for lipids, and thus an altered substrate specificity (Fisher *et al.* 1997).

The PvuII polymorphism has a carrier frequency of approximately 50%, the HindIII polymorphism of approximately 40%, D9N of 2%, N291S of 4.5% and the S447T polymorphism of 20% in the general Caucasian population (Fisher *et al.* 1997).

Strong linkage disequilibrium has been found between the P-, H- and T alleles, and the P-H-T haplotype is thought to be anti-atherogenic (Jemaa *et al.* 1995). A number of linkage disequilibrium studies between LPL polymorphisms have been carried out on EARS populations. EARS populations are offspring of people who developed premature CAD. A study using the EARS I (male and female) and EARS II (male only) populations found a linkage disequilibrium of >0.99 between the H- and T alleles, with only the H+S, H-S and H-T haplotypes being present. The frequency of the H-T haplotype was lower in the EARS I group than a control population. However, the frequency of the H-S haplotype was similar in both the EARS populations and controls. Fasting plasma TAG levels were

examined in the EARS I population and controls, and no gender effect was found. Both H-T males and females had lower fasting plasma TAG levels than H+S and H-S individuals. A postprandial response was measured in EARS II individuals (males only), and again the H-T haplotype was associated with lower TAG concentrations (Humphries *et al.* 1998).

1.3.2.10 Lecithin:Cholesterol Acyltransferase

Lecithin:cholesterol acyltransferase (LCAT) is associated with HDL particles. It esterifies peripheral cholesterol, which is effluxing from cells onto HDL particles, into cholesterol esters (CE), which subsequently migrated to the core of HDL particles. The principle substrate for LCAT is HDL. However, it is thought that it may interact directly with lower density lipoproteins (Karmin *et al.* 1993). A hydrophobic face orientation of the 6 amphipathic helical repeat units in the apoA-I protein is involved in regulating LCAT activation (Navab *et al.* 1998). It is thought that mutations in apoA-I can lower LCAT reactivity by 5- to 6-fold (Navab *et al.* 1998). It has been suggested that there are 2 classes of LCAT. One class is believed to be unable to esterify long-chain polyunsaturated acyl groups, leading to the synthesis of additional saturated cholesterol, which may increase the risk of atherosclerosis (Navab *et al.* 1998).

The *lcat* gene has been found to span a 4.2kb region of chromosome 16q21-22. It is transcribed in the human liver into a 1.55kb mRNA, containing 6 exons. Exon 5 has been found to have homologies with the interfacial active site of several lipases and to contain an amphipathic α-helix resembling the carboxy-terminus of apoE (McLean *et al.* 1986). Fish-eye disease (FED) and familial LCAT deficiency (FLD) are rare recessively inherited disorders, caused by many different single base pair mutations in the *lcat* gene. Both disorders are associated with hypoalphalipoproteinaemia and corneal opacities. Renal insufficiency and anaemia are associated only with FLD. Premature atherosclerosis has been found occasionally in FLD patients. FLD patients have little or no plasma LCAT mass or activity, and FED patients have low plasma LCAT concentrations (Qu *et al.* 1995). As of yet no population polymorphisms have been described in the *lcat* gene.

1.3.2.11 Cholesteryl Ester Transfer Protein

Cholesteryl ester transfer protein (CETP) is the protein that transfers CE from HDL particles to TRL particles in exchange for TRL-TAG. CETP is also involved in the transfer of surface components, such as VLDL-PL, chylomicron remnant-PL and HDL surface components, during lipolysis (Kinoshita *et al.* 1993). The expression of CETP is upregulated in response to increases in dietary cholesterol or endogenous

hypercholesterolaemia. Thus CETP activity is increased during the postprandial lipid response (Navab *et al.* 1998). The result is that there is up to 25% more CE in TRL, and 15% less CE in HDL and LDL during the postprandial response. Delayed postprandial clearance of TRL results in increased CETP activity (Roche & Gibney 1995). CE in TRL is removed more slowly from the circulation by the liver than HDL-CE. CETP and LCAT are closely linked, with high levels of LCAT causing increases in CE concentration for transfer to TRL. The transfer of CE to TRL causes a secondary reduction in HDL size and protein content, which leads to a reduction in HDL_2 levels (Juvonen *et al.* 1995). The role CETP has in reverse cholesterol transport suggests that it may be anti-atherogenic (Navab *et al.* 1998).

The *cetp* gene is located on 16q13 close to the *lcat* gene. Its genomic sequence is 25kb long containing 16 exons, while its mRNA is only 2kb long (Agellon *et al.* 1990). CETP is synthesised in the liver, small intestine, adrenal glands and spleen (probably by macrophages and lymphocytes) (Drayn *et al.* 1987), and circulates with HDL. HDL-cholesterol levels have been shown to have a strong genetic component. Sib-pair linkage studies have linked genes involved in HDL-cholesterol levels tightly to the CETP, apoA-II, hepatic lipase, apoA-I, apoA-IV and apoC-III loci (Zhong *et al.* 1996). Mutations in the CETP gene cause a reduction in plasma CETP levels and an increase in HDL levels. CETP deficiency has been found at its highest frequency in the Japanese population. CETP deficiency results in a lipid profile that is anti-atherogenic, with high HDL and low VLDL-CE and IDL-CE levels. However, CETP deficient patients have been found with premature CAD (Tall 1995). CETP can promote dissociation of apoA-I from mature HDL, to generate pre-β HDL. This could accelerate the transfer of cellular cholesterol to HDL (Fielding & Havel 1996). The effect of partial CETP deficiency (less CETP activity than normal) on prevalence of CHD has been found to depend on HDL concentration. When HDL levels are low, CETP does not remain rate limiting for the production of pre-β HDL. The main activity of CETP may then become transfer of CE to HDL and not to TRL. At normal HDL levels the transport of cholesterol from peripheral tissues to plasma may decrease CETP's effect of increasing TRL cholesterol levels (Zhong *et al.* 1996).

A polymorphism known as TaqIB has been characterised in intron 1 at nucleotide 277 in the *cetp* gene. The B+ allele occurs at a frequency of approximately 0.6 and the B- allele at 0.4, in a Caucasian population (Juvonen *et al.* 1995, Kuivenhoven *et al.* 1998, Bernard *et al.* 1998). In some studies the B- allele has been found to have a gene dosage effect on CETP concentration, with B- homozygotes having lower CETP levels than either

heterozygotes or B+ homozygotes (Juvonen *et al.* 1995, Kuivenhoven *et al.* 1998, Bernard *et al.* 1998). Kuivenhoven *et al.* (1998) and Bernard *et al.* (1998) described an inverse relationship between CETP concentrations and HDL-cholesterol levels, which appear to depend on the TaqIB polymorphism. B+B+ individuals had increased CETP concentration and decreased HDL-cholesterol concentrations, and B-B- individuals had decreased CETP and increased HDL-cholesterol levels. The association between the TaqIB polymorphism and CETP concentration was independent of its association with HDL-cholesterol concentration (Bernard *et al.* 1998).

1.3.3 Gene-Diet Effects on Fat Metabolism

To reduce the risk of acquiring heart disease, dietary changes, regular aerobic exercise and normalisation of body weight are recommended. However, due to genetic differences between individuals, a 'one-size-fits-all' recommendation may not be appropriate. Many studies have been carried out examining the effects that genetic population polymorphisms have on fat metabolism. In addition, some of the effects that diet can have on gene expression both at the mRNA and protein levels, have been investigated. For example, conjugated linoleic acid isomers have been shown to sometimes behave in a similar manner to peroxisome proliferators. Peroxisome proliferators associate with peroxisome proliferator-response elements, which have been found upstream of genes involved in lipid metabolism (Moya-Camarena & Belury 1999). This results in a reduction in body weight, an increase in hepatic lipid concentration and increased hydrolysis of TAG by LPL (Moya-Camarena *et al.* 1999). The effect of CLA on fat metabolism will be discussed in more detail in a subsequent section (1.6.4). Results of some studies showing effects of polymorphisms in seven different genes on lipid metabolism when diets were altered are discussed in the following sections. Diets that were studied included high fat, low fat, high SFA, high PUFA, cholesterol rich or cholesterol poor diets.

1.3.3.1 Apolipoprotein A-I

The common promoter polymorphism –75G/A in the *apoA-I* gene has been associated with variation in HDL-cholesterol concentrations, with A carriers having higher HDL-cholesterol levels. It is believed that 6% of the variation in apoA-I levels between individuals is due to diet (Ordovas *et al.* 1995). Using a male population sample, A allele carriers were found to have lower LDL-cholesterol levels on a high fat diet than individuals who were homozygous for the G allele. In a female population, individuals

who were homozygous A for the –75 polymorphism had lower cholesterol and LDL-cholesterol levels on a diet high in PUFA than individuals with one are more G allele. Another study showed that males with the A allele had lower levels of plasma TAG as well as higher levels of HDL-cholesterol than males without the A allele. However, the study design suggested that the effect of the A allele was independent of total fat and cholesterol intake (Ordovas & Schaefer 1999).

1.3.3.2 Apolipoprotein A-IV

Eight isoforms of apoA-IV have been identified (apoA-IV*0 – apoA-IV*7). Most is known about effects of the apoA-IV*2 (Gln360His) isoform on lipid metabolism. This isoform has been found to be hyporesponsive to LDL-cholesterol concentrations after dietary therapy, which reduces total fat and cholesterol. The hyporesponsiveness of LDL-cholesterol concentrations is thought to be due to the reduction in dietary cholesterol. A reduction in HDL-cholesterol levels has also been associated with a reduced total fat and cholesterol diet (Ordovas & Schaefer 1999). A recent study (Wallace *et al.* 2000) found that when His carriers were switched to a PUFA-rich diet from an SFA-rich diet, they responded with a larger increase in HDL-cholesterol and decrease in the particularly atherogenic dense LDL particles than Gln homozygotes who were switched from the SFA diet. The authors suggested that apoA-IV*2 His carriers may benefit most from a diet high in PUFA, as in these individuals this diet causes a larger reduction in dense LDL particles. The different alleles of a Thr347Ser polymorphism which associates with the apoA-IV*1 isoform have also been found to affect an individuals response to dietary intake. Ser allele carriers have lower levels of total cholesterol, LDL-cholesterol and apoB, when transferred from a low fat and low cholesterol diet to a MUFA diet, than individuals who are homozygous for the Thr allele. Individuals with the Ser allele appear to respond to high fat diets by increasing LDL-cholesterol levels (Ordovas & Schaefer 1999). The apoA-IV affect is modulated through changes in dietary cholesterol.

1.3.3.3 Apolipoprotein B

The 3 amino acid insertion/deletion polymorphism in exon 1 of the *apoB* gene (section 1.3.2.4) has been associated with a variation in plasma TAG levels dependent on diet. It has been established that when a high fat and high cholesterol diet is consumed individuals who are homozygous for the insertion allele (II) have the highest fasting TAG levels and individuals who are homozygous for the deletion allele (DD) have the lowest fasting TAG concentrations. Heterozygotes (ID) are known to have intermediate plasma fasting TAG

levels after consuming a high fat, high cholesterol diet (Xu *et al.* 1990). When a low fat low cholesterol diet is consumed this genotypic effect disappears (Ordovas *et al.* 1995).

The silent XbaI polymorphism (section 1.3.2.4) in the *apoB* gene has been found to be associated with variations in lipid levels and risk of CAD. A postprandial study on a male population found that X-X- individuals had larger postprandial responses than either X+X+ or X+X- individuals (Ordovas & Schaefer 1999). It is therefore thought that the X- allele may be associated with increased risk of CAD; fasting lipid concentrations are less atherogenic for X- carriers than for the X+X+ individuals.

A recent study on the EcoRI polymorphism in the *apoB* gene (section 1.3.2.4) demonstrated that individuals with the R+R- and R-R- genotypes responded differently than R+R+ individuals to being switched from a low SFA, low cholesterol diet to a high SFA, high cholesterol diet. The R- carriers and homozygotes (R-R-) had larger increases in plasma total cholesterol and LDL-cholesterol levels than the R+R+ homozygotes (Friedlander *et al.* 2000).

1.3.3.4 Apolipoprotein C-III

One allele, S2 of the SstI polymorphism (section 1.3.2.7) in the 3' UTR of the *apoC-III* gene, has been associated with increased plasma TAG, total cholesterol and apoC-III concentrations. S1S1 males given MUFA diets had high cholesterol and LDL-cholesterol levels and S1S2 males given MUFA diets had low LDL-cholesterol and apoB levels (Ordovas & Schaefer 1999).

1.3.3.5 Apolipoprotein E

The effects of the ε4 allele of the *apoE* gene (section 1.3.2.8) such as increased TAG and LDL-cholesterol levels are most pronounced in individuals consuming a diet high in SFA (Ordovas & Schaefer 1999). Martin *et al.* (1993) studied a group of individuals consuming either a high or low cholesterol diet over a 35 day period and found that ε2ε3 individuals had a large increase in CETP activity but no increase in HDL-cholesterol levels. However, ε3ε4 individuals had a small increase in CETP activity and a large increase in HDL-cholesterol levels, after consuming either a higher or low cholesterol diet. It was determined with the DELTA study (Dietary Effects on Lipoproteins and Thrombogenic Activity), that in the case of the *apoE* gene the gene-diet interaction is gender specific, with the apoE4 isoform increasing plasma cholesterol levels in males fed an SFA diet but not in females. The increased plasma cholesterol levels associated with the apoE4 isoform

were also observed in individuals susceptible to hypercholesterolaemia, when the diet included changes in fatty acids and cholesterol.

In ε2 carriers polyphenols found in tea, fruit and vegetables, had a beneficial effect on lipid levels and coagulation factors. This apoE affect is modulated through the type and amount of dietary fat administered. During the postprandial response, the ε2 allele is associated with retarded remnant clearance, and the ε4 allele with enhanced clearance. A study was carried out to investigate the effects of long-term increase in dietary soluble fibre on postprandial lipid response. There were insufficient numbers of individuals with the ε2 allele to describe its effect, but ε3ε3 individuals manifested enhanced fat absorption during the postprandial response. However, no effect on fat absorption was seen for ε4 allele carriers (Ordovas & Schaefer 1999).

1.3.3.6 Lipoprotein Lipase

The intronic HindIII polymorphism in the *lpl* gene (section 1.3.2.9) has been associated with variability in lipid response between individuals changing from a high-SFA diet to a low-SFA diet (Ordovas *et al.* 1995). The N291S polymorphism in the *lpl* gene has been found to affect individual's responses to being switched from low SFA to high SFA diets; S carriers have a significant increase in plasma TAG levels and decrease in HDL-cholesterol levels (Friedlander *et al.* 2000). In hypercholesterolaemic individuals the T allele of the Ser447Ter polymorphism has been associated with an increase in their plasma cholesterol levels when they are switched from a low SFA to high SFA diet. Hypercholesterolaemic S homozygotes of the Ser447Ter polymorphism show no such increase (Wallace *et al.* 2000).

1.3.3.7 Cholesteryl Ester Transfer Protein

In a normolipidaemic population, plasma CETP levels can vary over 3-fold due to environmental influences. Smoking is associated with increased CETP concentrations whereas heavy drinking and physical training are associated with decreased plasma CETP concentrations (Bernard *et al.* 1998). The TaqIB polymorphism (section 1.3.2.11) is known to effect an individual's reaction to diet. In one study the B- allele has been found to increase plasma HDL levels in individuals consuming moderate quantities of alcohol (Fumeron *et al.* 1995). However, no association with alcohol was found by Corella *et al.* (2000). Kauma *et al.* (1996) found that male smoking B- carriers had 10% lower HDL-cholesterol levels than male smoking B+B+ individuals, but this was not observed by Fumeron *et al.* (1995). B-B- hypercholesterolaemic individuals have been associated with

a larger increase in cholesterol concentrations when changed from a low SFA diet to a high SFA diet than B+B+ or B+B- individuals (Wallace *et al.* 2000).

1.3.4 Transgenic Animal Models of Atherosclerosis

The science of using genes to correct for inborn mutations may at sometime be used for the treatment or preventative treatment of common diseases such as stroke and heart disease. However, prior to using such gene therapies in humans, genetic drugs will have to be tested in suitable model systems, such as cell culture systems or animal models for both efficacy and safety. Rabbits are good animal models for studying the development of atherosclerosis in, because rabbits synthesise apoB-containing lipoproteins, which are similar to human apoB-containing lipoproteins in chemical composition and apolipoprotein content. Like humans, rabbits do not edit apoB mRNA in their livers, they have abundant CETP activity and are also susceptible to diet-induced atherosclerosis. A review of the effect of human transgenes in rabbits has been written (Brousseau & Hoeg 1999).

1.3.4.1 Human ApoA-I Transgene

The introduction of the apoA-I transgene into New Zealand White rabbits increased their plasma cholesterol levels. The increase was shown to be due to increased HDL-cholesterol levels and not LDL-cholesterol levels. When fed a cholesterol-rich diet over a 14 week period transgenic rabbits maintained HDL-cholesterol levels which were twice that of control animals. This resulted in the aortic surface of transgenic rabbits being covered with fewer atherogenic lesions than the aortic surface of control rabbits. Therefore, at least in rabbits, apoA-I may have a protective effect against the development of atherogenic lesions (Duverger *et al.* 1999).

1.3.4.2 Human LCAT Transgene

When a human LCAT transgene was introduced into New Zealand White rabbits, the transgenic rabbits were protected from diet-induced atherosclerosis. This was due to transgenic rabbits having lower non-HDL-cholesterol lipoproteins than non-transgenic controls. The HDL-cholesterol levels increased similarly in both the experimental and control animals (Brousseau *et al.* 1996). When the human LCAT transgene was introduced into Watanabe Heritable Hyperlipidaemia rabbits, which are a model of familial hypercholesterolaemia, an interesting result was obtained. An increase in the apoA-I production rate, correcting the familial hypercholesterolaemic phenotype of low HDL and

apoA-I concentrations, was observed in the transgenic animals. Therefore, LCAT may potentially be a valuable therapeutic gene for familial hypercholesterolaemia (Brousseau *et al.* 1998).

1.3.4.3 Human ApoB Transgene

A human apoB transgene (hapoB100) was introduced into the liver of New Zealand White rabbits. This resulted in increased plasma total cholesterol and TAG levels and decreased HDL-cholesterol levels compared to non-transgenic animals. The over-expression of hapoB100 caused the secreted hepatic lipoproteins to be of LDL size rather than TRL size. TRL particles of LDL size are less accessible to LPL, resulting in increased plasma TAG concentrations. The hapoB100 was found to associate with LDL particles along with the extra cholesterol, which caused apoC-III and apoE to associate with lipoproteins of LDL size. The risk of developing atherosclerosis has not been investigated in these rabbits (Fan *et al.* 1995).

1.3.4.4 Human ApoE Transgenes

The isoforms of apoE have been associated with inherited lipid metabolism disorders. The apoE2 isoform is associated with type III hyperlipoproteinaemia, some of whose symptoms are the presence of β-migrating VLDL particles and increases in VLDL and intermediate density lipoprotein (IDL) concentrations. A human apoE2 transgene has been introduced into New Zealand White rabbits. Male and female transgenic rabbits were studied separately and a phenotypic difference in their metabolism observed. Female transgenic rabbits had lower plasma β-VLDL, VLDL and IDL concentrations than transgenic male rabbits. Female transgenic rabbits had twice as much HDL-cholesterol than transgenic males, and 40% higher HDL-cholesterol concentrations than non-transgenic females. Spontaneous atherosclerosis was observed after 11 months in rabbits of both genders that were fed normal rabbit chow diets. The atherosclerotic potential was believed to be due to the increased concentration of β-VLDL particles due to the apoE2 isoform (Huang *et al.* 1997).

Transgenic New Zealand White rabbits carrying the human apoE3 gene have also been generated. The apoE3 isoform is the most frequently occurring isoform of apoE (section 1.3.2.8). ApoE3 transgenic animals were found to have reduced concentrations of large LDL particles, which is believed to be due to increased receptor mediated clearance by apoE3 (Fan *et al.* 1998).

An apoE deficient mouse has been developed with a hypercholesterolaemia and dyslipoproteinaemia phenotype. Desurmont *et al.* (2000) showed that the hypercholesterolaemic phenotype could be rescued with a human apoE cDNA transgene. Introduction of the human transgene normalised cholesterol and TAG levels over a 5 month period. The apoE transgene was present for 4 months. After 6 months the numbers of fatty streak lesions in the aorta were examined. It was shown that expression of human apoE resulted in dose-dependent regression of fatty streak lesions in the aorta correcting the dyslipoproteinaemia.

1.3.4.5 Mouse LPL Transgene

An LPL deficient mouse model has been generated. The mouse LPL gene was mutated in the C-terminus. This resulted in mutant homozygotes (knockout) dying within 48 hours of birth and having extremely elevated TAG concentrations. Heterozygote pups and adults had higher TAG concentration and lower LPL activity than wild type control animals. The authors concluded that this mouse model was a model of mild to moderate hypertriglyeridaemia and may be useful in determining the influence of hypertriglyeridaemia on development of atherosclerosis (Coleman *et al.* 1995).

1.3.5 Summary

Lipid metabolism is a complex process involving many proteins, which are dependent on each other. Many polymorphisms have been identified which can effect fasting and postprandial lipid concentrations and how individuals respond to particular fatty acid diets. This information along with the SNPs identified from the HGP will help bring about 'personalised medicine'.

Animal models are extremely useful systems for analysing possible effects of single transgenes. Especially when studying complex disorders such as atherosclerosis, animal systems are likely to be the most accurate models available, because the effects of each gene, mutation or polymorphism can be assessed separately. This removes the confusing issue of whether other genes, mutations or polymorphisms are also involved in the phenotype. Transgenic animal models can be used to investigate the effects of disease causing mutations in genes involved in lipid metabolism as well as polymorphisms in genes involved in lipid metabolism. However, to analyse the effects of fatty acids alone on

the postprandial response cell models may be better, due to reduced between individual variation (section 1.5).

1.4 Relationship between Coagulation Pathway and Postprandial Lipidaemia

1.4.1 Coagulation Cascade

Postprandial TAG concentrations have been found to be associated with activation of factor VII thus stimulating the coagulation cascade (Figure 1.2.3). Blood coagulation occurs to provide an insoluble fibrin matrix for formation of a haemostatic plug. There are two pathways of initiation: intrinsic pathway and extrinsic pathway. The pathways converge with the activation of factor X, the pivotal molecule in the coagulation cascade (Figure 1.4.1). In the intrinsic pathway all the substances necessary for the formation of fibrin are present in precursor zymogen form. The extrinsic pathway accelerates clot formation. The intrinsic and extrinsic pathways are interdependent. Conversion of factor VII (extrinsic pathway) into its active serine protease form is enhanced by activated factor XII, which is generated by the conversion of factor XII (intrinsic pathway) into its active serine protease form (Figure 1.4.1) (Bloom & Giddings 1994).

1.4.1.1 Intrinsic Pathway

The intrinsic pathway (Figure 1.4.1) may be initiated by the cleavage of prekallikrein to kallikrein, with high molecular weight kininogen as a co-factor. Kallikrein then converts factor XII into its active form factor XIIa, which in turn converts factor XI into its active form factor XIa. These steps culminate in the activation of factor IX to factor IXa. These interactions are autocatalytic. Many molecules are involved in the pathway so that prekallikrein alone is unable to activate factor IX. Factor X is activated by the formation of an enzyme complex containing factor IXa, factor X, and factor VIIIa (which is activated by thrombin) bound to phospholipid micelles in the presence of calcium ions. The enzymatic activity is carried out by factor IXa with factor VIIIa as its co-factor. Phospholipids and calcium ions are necessary for optimal activation of factor X.

1.4.1.2 Extrinsic Pathway

The extrinsic pathway (Figure 1.4.1) is believed to initiate coagulation after vascular injury. Tissue factor is expressed on the surface of endothelial cells, perivascular

fibroblasts and leukocytes after exposure to thrombin. A complex consisting of factor VIIa, tissue factor and calcium ions is necessary to activate factor IX and factor X.

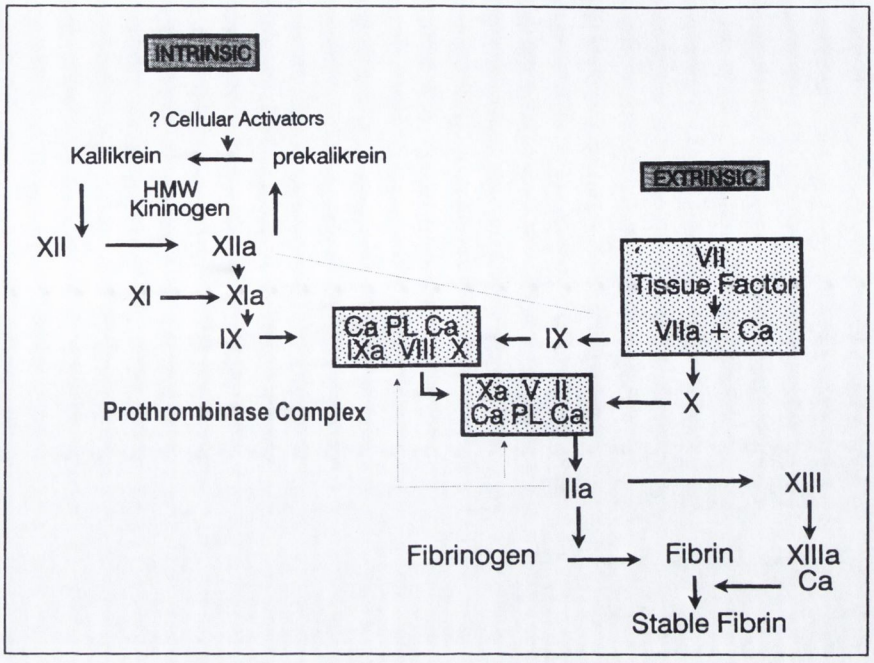


Figure 1.4.1 Diagram of the conventional coagulation cascade. "Sequential activation of zymogens to serine proteases in intrinsic and extrinsic mechanisms. Central holoenzyme complexes involving coagulation proteins, phospholipid and calcium are outlined in shaded rectangles. HMW = high molecular weight." (Bloom & Giddings 1994).
The intrinsic pathway is believed to initiate coagulation by the activation of prekallikrein to kallikrein. The extrinsic pathway initiates coagulation after vascular injury with the formation of the factor VIIa-tissue factor calcium ion complex. Both pathways culminate in the formation of the prothrombinase complex. The prothrombinase complex causes the formation of thrombin (factor II), which cleaves fibrinogen into insoluble fibrin, which forms the matrix of the haemostatic plug.

1.4.1.3 Prothrombinase Complex and the Formation of Stable Fibrin

In the presence of calcium ions and on a phospholipid surface the prothrombinase complex is formed consisting of factor Xa and factor Va, which has been activated by thrombin. Factor Xa converts prothrombin (II in Figure 1.4.1) into the active thrombin (IIa). The rate-limiting step in the coagulation cascade is the transport of membrane bound prothrombin to the prothrombinase complex. Fibrinogen is cleaved by thrombin to form fibrin. Fibrin is then stablised into a fibrin of high tensile strength by factor XIIIa, which is also activated by thrombin (Bloom & Giddings 1994).

1.4.2 Extrinsic Pathway and Postprandial Triacylglycerolaemia

Levels of factor VII activity (FVIIc) have been shown to be predictive of fatal ischemic heart disease (IHD) (Ruddock & Meade 1994). FVII is found in two forms; the majority is

in the inactive zymogen form and 1% is in the active dimer form, FVIIa (Miller 1998). FVIIa is formed by proteolysis of its Arg152Ile153 peptide bond by factor Xa and factor IXa, causing the formation of a disulphide-linked two chain molecule. This dimer alone has catalytic activity (Bloom & Giddings 1994). There are three measures of factor VII: factor VII activity (FVIIc), activated factor VII (FVIIa) and factor VII antigen (FVIIag). FVIIc measures FVIIa concentrations and some of the zymogen. FVIIag is a measure of total factor VII concentration (zymogen and FVIIa) (Miller 1998). FVIIc concentrations have been positively associated with postprandial cholesterol (Meade *et al.* 1980) and TAG (Miller *et al.* 1985) concentrations. In addition, total dietary fatty acid concentrations have been shown to determine FVIIc levels. Consumption of a fatty meal transiently increases FVIIc levels without increasing FVIIag levels (Miller *et al.* 1991). Long-chain fatty acids have been shown to increase FVIIc levels, whereas, test meals consisting of medium-chain fatty acids (C8:0-C12:0) do not increase FVIIc levels (Yahia *et al.* 1995) probably due to TRLs not being formed.

Salomaa *et al.* (1993) showed that test meals consisting of n-6 PUFA or SFA activate factor VII equivalently. A study by this group (Roche & Gibney 1997) investigated effects of MUFA test meals on FVIIc levels. FVIIc postprandial responses induced by test meals high in MUFA, were shorter than FVIIc responses induced by test meals low in MUFA. Thus FVIIc levels may be tightly linked to postprandial triacylglycerolaemia. However, fasting FVIIc concentrations have been found to predict postprandial FVIIc levels most accurately (Roche & Gibney 1997). It is hypothesised that TRLs are capable of converting FVII zymogen into the active dimer form (Miller *et al.* 1991) by the following method. PL rich lipoproteins activate factor XII, which subsequently activates FVII molecules initiating the coagulation cascade (Juhan-Vague 1996). Mitropoulos *et al.* (1994) examined FVIIc levels after consumption of diets high in SFA, n-6 PUFA and a low-fat diet. They found that the high SFA diet increased FVIIc levels compared to the n-6 PUFA diet and the low-fat diet reduced FVIIc levels. This highlights the influence the fatty acid composition of background diets can have on the magnitude of postprandial lipaemia. Consumption of a background diet high in long-chain fatty acids have been shown to cause a long-term increase in FVIIag (total FVII concentration consisting of inactive and active forms) concentrations, and a short-term increase in FVIIa levels during postprandial lipaemia (Miller 1998).

1.4.3 Coagulation Factor VII Polymorphisms

FVIIc concentrations have been found to vary between individuals depending on individuals' genotypes. Two polymorphisms in the *FVII* gene have been well documented in the literature: the R353Q polymorphism (section 3.1.3.1) and a decanucleotide insertion polymorphism in the promoter region of the gene (section 3.1.3.2). The Q allele of the R353Q polymorphism has been associated with lower fasting FVIIc and FVIIa concentrations (Sanders *et al.* 1999) and in some studies with reduced FVIIa levels during the postprandial response (Silveira *et al.* 1994). The Q allele is hypothesised to alter FVII conformation, thus making the FVII zymogen more resistant to activation by TRL particles (Humphries et al. 1996). FVII proteins containing the Q polymorphism have been shown to have reduced secretion from COS-1 monkey kidney cells (Hunault *et al.* 1997). Therefore, human carriers of the Q polymorphism possibly may have a decreased FVIIag concentration due to decreased secretion levels of the FVII protein.

The 10bp insertion allele of the decanucleotide insertion polymorphism has been associated with reduced fasting FVIIc levels. This polymorphism is in the promoter region of the *FVII* gene and thus may effect transcription of the gene. Promoters with the 10bp insertion allele have been shown to be 30% less active than the wild type (0bp) promoters (Pollak *et al.* 1996). The Q and 10bp alleles are in linkage disequilibrium and while a 20% reduction in FVIIc levels is associated with each of these alleles, 80% (16% of total) of this effect has been shown to be due to the decanucleotide insertion polymorphism and 20% (4% of total) due to the R353Q polymorphism (Humphries *et al.* 1996).

1.4.4 Summary

The increase in FVIIa levels during the postprandial response increases the risk of heart attack due to initiation of the coagulation cascade. Tissue factor is present on atherosclerotic plaques (Miller 1998) and formation of the FVIIa-tissue factor complex enhances FVIIa catalytic activity by 1000-fold over FVIIa in solution (Bloom & Giddings 1994). Therefore consuming a diet high in SFA increases the risk of IHD due to an increase in FVIIc levels, which is associated with an atherogenic profile (Roche & Gibney 1995). However, individuals who are carriers of the Q allele and 10bp allele of the *FVII* gene have reduced risk of IHD, because they have lower FVIIc levels at fasting than RR or 0bp0bp individuals (Sanders *et al.* 1999, Humphries *et al.* 1996).

1.5 Cell Lines for the Study of Fat Absorption and Metabolism

Cell lines are frequently useful model systems, for investigating cellular processes at the molecular level. For example, in humans, lipids are synthesised and metabolised in the intestine and liver. Therefore, two types of cell line frequently used in the study of lipids are the colon carcinoma cell line (Caco-2) and hepatocyte cell lines (HepG2 and isolated hepatocytes).

1.5.1 Colon Carcinoma Cell Line (Caco-2): A Model for Enterocyte Function

The lining of the small intestine is composed of cells called enterocytes, which are functionally and morphologically polarised. They have microvilli protruding into the lumen of the intestine, which contain hydrolyases, and their nuclei are found at the opposite (basolateral) end of the cell. Enterocytes are involved in the absorption of fatty acids from the intestinal lumen and the synthesis and secretion of chylomicrons (section 1.2.2.3). The Caco-2 cell line (Fogh *et al.* 1977) is an appropriate model for enterocytes because of their similar functional and morphological polarisation. Studying human Caco-2 cells allows information to be acquired about the composition, structure, distribution and function of intestinal lipoproteins and contributes towards the elucidation of the molecular mechanisms of lipoprotein formation and assembly. However, unlike enterocytes, Caco-2 cells produce more apoB100, secrete less lipid and produce HDL, LDL, VLDL and chylomicron sized particles containing amongst other apolipoproteins apoB100, apoB48 and apoE. Enterocytes do not synthesise apoE, secrete apoB48 containing chylomicrons or apoB100 containing VLDL particles. The ratio of apoB100 to apoB48 produced by Caco-2 cells is dependent on culturing conditions. Growing Caco-2 cells on semipermeable membranes increases the quantity of apoB48 they produce (section 4.1) (Review, Levy *et al.* 1995).

It is important to note that *apoB* transcription is constitutive, and that fatty acid treatments of Caco-2 cells do not effect their apoB mRNA concentrations (Hughes *et al.* 1988). Therefore any affect fatty acids have on apoB protein concentration must be posttranscriptional. Oleic acid (OA) has been shown to reduce apoB degradation in the endoplasmic reticulum by nearly 2-fold. In the presence of OA, degradation of apoB in the endoplasmic reticulum is reduced from 58% to 29% (Dashti *et al.* 1990, Dixon *et al.* 1991). Cotranslational addition of OA lipid is critical to arrest apoB degradation and for

transfer of apoB to lumen of the endoplasmic reticulum, Golgi apparatus and finally for secretion (Sakata *et al.* 1993).

1.5.1.1 Effects of Palmitic Acid and Oleic Acid on Lipoprotein and Lipid Synthesis and Secretion

Many studies have been designed to investigate the effects of physiological doses of different types of fatty acids on lipid and lipoprotein expression by Caco-2 cells, in order to gain information on the molecular mechanisms that cause fatty acid dependent variations in the postprandial lipid response. In one study, Caco-2 cells were treated acutely with palmitic acid (PA), which is an SFA or linoleic acid (LA), which is an n-6 PUFA. Caco-2 cells treated with PA secreted less TAG than cells treated with LA. A dose of 1mM PA over 24 hours, caused a decrease in the number of microvilli formed and an increase in the concentration of PA in phosphatidic acid and diacylglycerol (van Greevenbroek *et al.* 1995). The lipoproteins secreted by Caco-2 cells treated with PA and stearic acid (SA) were either TRL (density<1.006g/ml) or IDL/LDL (density between 1.009 and 1.068g/ml) particles. Caco-2 cells treated with PA or SA secreted less *de novo* apoB and TAG than cells fed with unsaturated fatty acids (van Greevenbroek *et al.* 1996). A later study by van Greevenbroek *et al.* (1998) found that an acute dose of 0.1mM rather than 0.5mM PA given to Caco-2 cells resulted in more secretion of TRL density particles and a reduction in IDL/LDL particle secretion. The authors suggested that the increased ratio of unsaturated to saturated fatty acids might have enhanced TRL density particle secretion. Subsequently van Greevenbroek *et al.* (2000) continued investigating the influence of ratio of saturated to unsaturated fatty acid, on TRL secretion and found the following. When 0.5mM PA or 0.5mM fatty acid (made up of different percentages of OA and PA (total fatty acid concentration 0.5mM)) was added to Caco-2 cells it was found that as the concentration of OA increased, the cells secreted more TRL density particles. The lowest amount of TRL density particles was secreted when Caco-2 cells were treated with 0.5mM PA. Additionally the rate of the lipoprotein particle secretion was recorded. During the first four hours of incubation, the Caco-2 cells only secreted TRL density particles containing unsaturated fatty acid. Subsequently, the cells also secreted an IDL/LDL density fraction and by 22-26 hours only IDL/LDL density lipoproteins were being secreted (van Greevenbroek *et al.* 2000). The TAG in the IDL/LDL density fraction was synthesised from PA. It therefore appears that Caco-2 cells preferentially secrete lipoproteins containing unsaturated TAG and only when all the unsaturated TAG has been secreted do they secrete smaller lipoproteins containing saturated TAG. Van Greevenbroek *et al.*

(2000) then hypothesised that a meal high in unsaturated fatty acids may cause the secretion of large chylomicrons which are rapidly cleared from the circulation by LPL, while a meal high in SFA may cause a secretion peak of large chylomicrons made from the unsaturated fatty acids contained in the meal followed by the secretion of smaller chylomicrons containing SFA. Notably, small chylomicrons are not cleared as efficiently as large chylomicrons from the circulation (section 1.2.2.6). Results from van Greevenbroek's study (2000) show that even small changes in the ratio of SFA to unsaturated fatty acid in media effects the amount of *de novo* TAG cells secrete.

Field *et al.* (1988) examined how the type of fatty acid absorbed by Caco-2 cells regulates their secretion levels of TRL density particles, and found that OA absorption resulted in the highest level of secretion of newly synthesised TAG, followed by LA and SFA such as PA. The rate of TAG synthesis was higher in cells given OA than cells given LA or PA. Caco-2 cells treated with LA or PA had similar rates of TAG synthesis. OA is frequently used as a control in studies where effects of fatty acid treatments on cells are compared. However, when cells given OA treatment are compared to cells not fed any fatty acids, OA treated cells show increased TAG and apoB secretion levels. OA is preferentially incorporated into newly synthesised TAG. Cells fed OA secrete lipoproteins at a TRL particle density ($d<1.006$g/ml). The increase in concentration of secreted apoB by cells given OA treatment is believed to be due to an increase in the apoB48 isomer concentration (van Greevenbroek *et al.* 1996). This increase is therefore associated with the secreted TRL particles containing the newly synthesised TAG (Moberly *et al.* 1990). Dixon *et al.* (1991) suggested that OA must be present in cell culture media for it to enhance apoB secretion. This was indeed observed when addition of OA to media enhanced apoB secretion by cells and subsequent removal of OA caused the cells to return to baseline levels of apoB secretion (Dixon *et al.* 1991).

1.5.1.2 Effect of Eicosapentaenoic Acid on Lipoprotein and Lipid Synthesis and Secretion

Eicosapentaenoic acid (EPA) is believed to be the active component in fish oil, which causes the attenuated postprandial response observed after fish oil ingestion (Rambjor *et al.* 1996). It is hypothesised that the presence of EPA reduces the production of VLDL particles by the liver, by reducing the secretion levels of TAG and apoB (Lin *et al.* 1995). EPA fed hepatic cells are therefore expected to secrete lipoproteins, which contain less TAG than hepatic cells fed other fatty acids (Ranheim *et al.* 1992). Previous studies on EPA have shown that administration of acute doses of EPA to Caco-2 cells reduces their

synthesis and secretion levels of newly synthesised TAG although the mass of secreted TAG remains the same as that of cells fed OA (Murthy *et al.* 1990). However, Ranheim *et al.* (1992) found that while EPA and OA are absorbed and metabolised at similar rates and promote similar secretion levels of newly synthesised TAG, EPA reduces apoB secretion levels and OA increases apoB secretion levels. EPA was found to be preferentially incorporated into the cellular PL fraction over cellular TAG. This is thought to result from a decrease in competition between the TAG synthesis (section 1.2.2.2) and the PL synthesis pathways due to inhibition of diacylglycerol acyl transferase activity in the presence of EPA. When comparing the incorporation of EPA and OA into cell lipids, EPA was preferentially incorporated into phosphatidylinositol, lysophosphatidylcholine and sphingomyelin (Ranheim *et al.* 1992).

1.5.2 Hepatocytes as a Model of Liver Function

After dietary fatty acids have been cleared from the circulation by LPL activity, chylomicron remnants, NEFA and short chain fatty acids are processed into TAG and secreted as VLDL by the liver. Both human hepatocytes and rat hepatocytes have been used to investigate effects of fatty acids on the regulation of lipoprotein synthesis and secretion by the liver. These cells are obtained from healthy livers. HepG2 cells, which are also frequently used as a model for liver function, were derived from a human hepatoma of a 15-year-old Caucasian male (Elul *et al.* 1975). HepG2 cells are epithelial cells, which like normal liver cells, synthesise and secrete apoA-I, apoA-II, apoA-IV, apoB100, apoC-I, apoC-II, apoC-III and apoE. However, unlike hepatocytes they produce VLDL and smaller density lipoproteins, while hepatocytes produce VLDL density particles alone. This is believed to be due to HepG2 cells having high concentrations of hepatic lipase, which lipolyses TAG in VLDL sized particles to form LDL and smaller density particles (Arrol *et al.* 2000).

Many studies have investigated the effects of fish oil and MUFA on HepG2 cells as these fatty acids have been associated with a reduction in CAD risk (Roche & Gibney 1995). The active component of fish oil has been shown to be eicosapentaenoic acid (EPA) (Rambjor *et al.* 1996). EPA is known to reduce VLDL synthesis and secretion levels of HepG2 cells. Lin *et al.* (1995) compared effects of EPA and OA feeding to no fatty acid treatment in HepG2 cells and human and rat hepatocytes. They discovered that OA enhanced TAG synthesis and secretion levels in all three cell lines, whereas EPA

stimulated TAG synthesis alone. EPA did not alter TAG secretion levels of rat hepatocytes or HepG2 cells but inhibited TAG secretion by human hepatocytes. The rate of diacylglycerol synthesis after EPA feeding was similar to that after OA feeding, although the increase in TAG synthesis was less high after EPA feeding than OA feeding. Treatments of OA and EPA given to hepatocytes did not influence their cellular apoB levels. However, EPA feeding did decrease their apoB secretion rate. In contrast, the addition of OA and EPA to HepG2 cells increased both their cellular apoB levels and their rate of apoB secretion. The effect of OA on apoB secretion rate was larger than EPA. Thus in general, OA induces TAG synthesis and secretion levels, whereas EPA decreased TAG secretion levels and reduces the rate of apoB secretion into the circulation. This corroborates the hypothesis that fewer VLDL particles are secreted by human hepatocytes in the presence of EPA. It is important to note that the results obtained from experimentation on HepG2 cells and human hepatocytes were slightly different. This highlights the fact that cell lines, which are transformed or cancerous cells, such as HepG2 cells, can have different properties to normal cells or indeed *in situ* organs.

HepG2 cells produce high concentrations of hepatic lipase causing the TAG from VLDL to be lipolysed and smaller density lipoprotein particles (eg. LDL) to be formed. Arrol *et al.* (2000) postulated that, in HepG2 cells, apoB secretory concentrations may be a better measure of lipoprotein secretion than the TRL density fraction. To test this hypothesis the authors cultured HepG2 cells under continuous perfusion of media with fatty acid treatment and found that higher concentrations of VLDL particles than had been previously reported were secreted by the cells. All SFA treatments that were examined increased cells' rate of apoB secretion except stearic acid. Both MUFAs (OA and palmitoleic acid (16:1)) and PUFAs (LA, EPA and arachidonic acid (20:4n-6)) given to cells increased their rate of apoB secretion. However, linoleinic acid (18:3n-3) treatment did not enhance apoB secretion in cells. A correlation was found between cellular TAG concentration and apoB secretion rate, which suggests that the ability of fatty acids to stimulate TAG synthesis might be a major determinant of their ability to stimulate apoB secretion.

A study was undertaken in rats, which examined effects of diet on the secretion level of VLDL from isolated rat hepatocytes. The rats were fed a low fat, OA, or fish oil diet over a 14 day period. Subsequently, their livers were harvested and liver cells (hepatocytes) subjected to a dose of BSA (as control) or OA and BSA. Hepatocytes from rats fed fish oils had lower VLDL-TAG and VLDL-apoB48 secretion rates than hepatocytes from rats fed the other two diets (Brown *et al.* 1997). Humans do not edit apoB mRNA in

hepatocytes but rats do edit apoB mRNA in their livers to produce apoB48. Nutritional variations that result in changes in the hepatic lipid balance, including TAG secretion levels, are accompanied by changes in apoB48 metabolism in rats. A study in monkeys, chronically fed a fish oil diet, found that decreased levels of VLDL-TAG production were not accompanied by decreases in apoB100 secretion levels from the liver (Parks *et al.* 1990). The monkey study may therefore be a more appropriate comparison to the human system.

1.5.3 Summary

Studies on the effects of physiological doses of fatty acids in Caco-2 and hepatocyte cell lines have found that the type of fatty acid treatment the cells receive can effect the synthesis and secretion levels of TAG and apoB and lipoprotein formation by the cells. EPA treatments appear to reduce TAG and apoB secretion levels in Caco-2 cells and hepatocytes. OA treatments increase TAG and apoB secretion levels and promote the formation and secretion of chylomicron and VLDL sized lipoproteins. However, PA treatments of Caco-2 and HepG2 cells result in an increase in cellular TAG concentration and promote the formation and secretion of IDL and LDL density lipoproteins.

1.6 Conjugated Linoleic Acid

At present, much of the focus of studies on lipid metabolism in human intestinal or hepatic cells lines has been on all *cis* unsaturated fatty acids. In recent years, there has been a huge increase in interest in the area of conjugated unsaturated fatty acids, such as conjugated linoleic acid (CLA), which in this thesis, features alongside palmitic, oleic and eicosapentaenoic acid. This section reviews some of the relevant literature on CLA.

CLA refers to the *trans* stereoisomers of linoleic acid (LA). There are two major *trans* isomers, the c-9 (*cis*-9, *trans*-11-octadecanoic acid) and the t-10 isomer (*trans*-10, *cis*-12-octadecanoic acid), which are found in beef and dairy fat (Figure 1.6.1). c-9 is more common than t-10 (Park & Pariza 1998). LA is bihydrogenated into both the c-9 and t-10 isomers in the ruminant intestine (Pariza *et al.* 1999). The t-10 found in ruminant tissues originates from the gastrointestinal tract, but the c-9 isomer can be synthesised from *trans*-11 vaccenic acid, by the action of Δ9-desaturase in the gastrointestinal tract and other

tissues. Trace amounts of CLA occur naturally in plants, but various isomers are produced during the hydrogenation of vegetable fats (Pariza *et al.* 1999). A study by Salminen *et al.* (1998) found that in humans CLA can be derived from the dietary *trans* fatty acids. When a regression line was plotted of diet score (i.e. amount of CLA ingested, x-axis) versus percentage molar ratio of CLA found in the body (y-axis), the line did not pass through zero (above zero on the y-axis). This suggests that humans synthesise some of their CLA (Britton *et al.* 1992). The structure and geometrical positioning of LA and the c-9 and t-10 isomers can been seen in Figure 1.6.1.

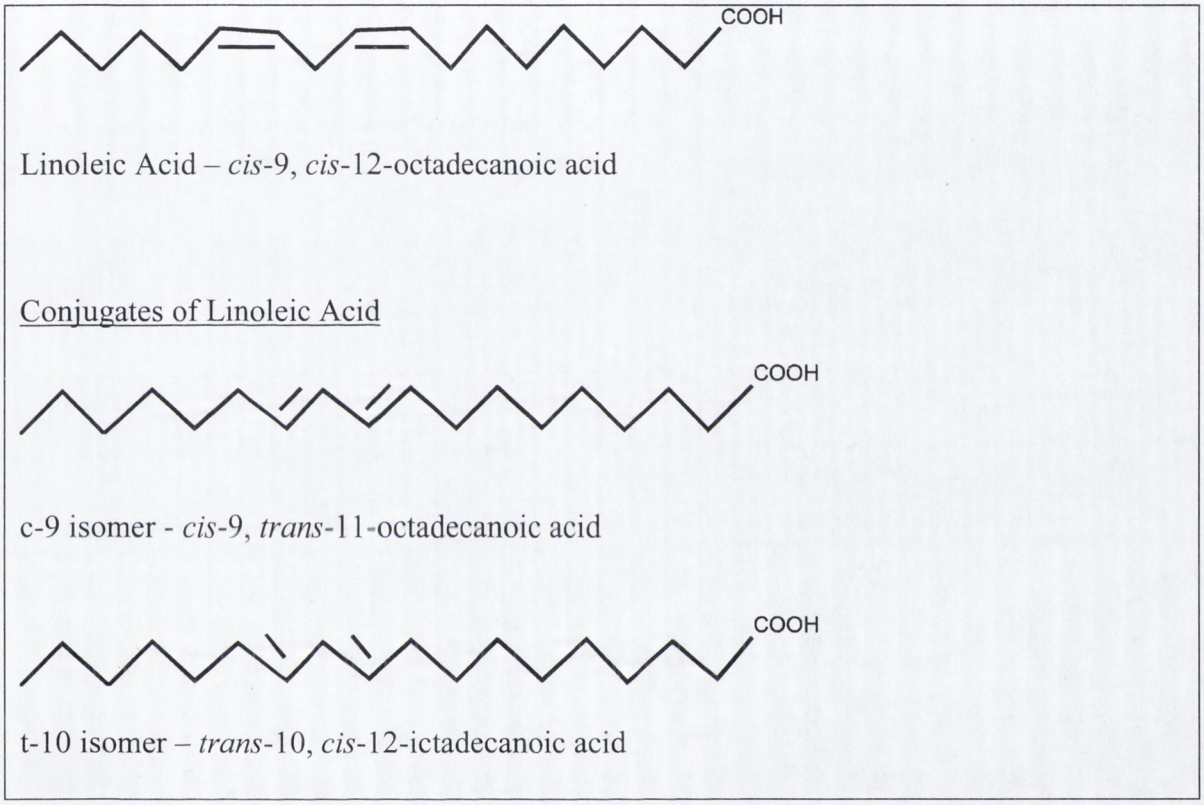


Figure 1.6.1 Molecular and geometric structure of linoleic acid and its conjugates, the c-9 and t-10 isomers (Belury 1995).

1.6.1 Body Composition Changes Due to CLA Intake

CLA intake has been associated with enhanced feeding efficiency and body compositional changes. Park *et al.* (1999) showed that mice given a diet containing 0.5% CLA (40.8-41.1% c-9 and 43.5-44.9% t-10), fed less frequently than control animals and manifested an increase in whole body protein and water and a decrease in body fat. The reduction in body fat is subsequent to the increase in whole body protein. The t-10 isomer is thought to be responsible for the body compositional changes in mice. The t-10 isomer was observed to inhibit LPL activity and enhance lipolysis in murine 3T3-L1 adipocytes (Pariza *et al.* 1999). Lee *et al.* (1994) found reduced TAG levels in rabbits fed CLA and hypothesised

that they may be due to increased carnitine palmitoyltransferase (CPT) activity or reduced TAG secretion from the intestine and/or liver. CTP is the rate limiting fatty acid β-oxidising enzyme; therefore increased CPT activity would reduce fatty acid levels in the organs where it is expressed. A study by Noone *et al.* (in press) also found that the t-10 isomer was the active component of CLA (3g/d of either 80:20 or 50:50 c-9 to t-10), which caused a reduction in fasting plasma TAG levels possibly leading to body compositional changes in humans. A human study on the effects of CLA supplementation over a 90 day period resulted in a 20% reduction in body fat (Brodie *et al.* 1999). Two additional studies investigated the effects of supplementing moderately obese individuals with CLA. The first found that high doses of CLA (3.6g/d or 7.2g/d) supplemented over a 12 week period could reduce body fat mass and percentage of body fat. In addition, the 7.2g/d dose could increase lean body mass (Gudmundsen *et al.* 1999). In the second study, Atkinson (1999) found that supplementation of 2.7g/d CLA over a 6 month period did not change body composition when the individuals were ingesting a calorie-restricted diet and taking regular exercise. Calculations on average diets consisting of meat and dairy products estimate that under normal circumstances humans ingest approximately 1g/day of CLA in the U.S. (Lee *et al.* 1994). Therefore the amounts of CLA ingested in the studies mentioned above are extremely high. The increased body protein due to CLA feeding suggests that CLA feeding may help in the prevention of cachexia (severe wasting in the later stages of carcinogenesis).

1.6.2 Effect of CLA Ingestion on Eicosanoid Synthesis

A diet high in n-6 PUFA is generally associated with increased risk of tumorigenesis. In contrast, n-3 PUFA is associated with inhibition of carcinogenesis (Belury 1995). Although CLA is an n-6 PUFA, it has been associated with decreased risk of tumorigenesis. CLA is believed to reduce the risk of atherosclerosis, cancer and cachexia following immune stimulation by activating the cytokine signal transduction mechanism. It is thought that the CLA isomers function by modifying eicosanoid intracellular signalling thus altering cellular responses to cytokines, particularly tumour necrosis factor α (TNF-α). This is also the mechanism by which n-3 PUFAs are thought to prevent cachexia following immune stimulation (Park *et al.* 1997).

The proportion of fatty acids present in murine adipocytes is altered by 10mg/l (0.0357mM) CLA treatment. The proportions of palmitic acid (16:0) and palmitoleic acid

(16:1) were increased by between 17-71%. In addition, the proportions of stearic acid (18:0) and oleic acid (18:1) were reduced by 30% and of arachidonic acid (20:4n-6) reduced by 50% (Satory & Smith 1999). This suggests that acetyl-CoA carboxylase and/or fatty acid synthetase may be stimulated but stearoyl-CoA desaturase activity and fatty acid elongase activity are not be stimulated. Chicken eggs, HepG2 cells and mammary cells, which were treated with CLA showed a reduction in MUFA due to a reduction in stearoyl-CoA desaturase activity (Lee *et al.* 1998, Loor & Herbein 1998). The decrease in stearoyl-CoA desaturase activity is thought to be due to suppression of *scdI* mRNA expression levels by the t-10 isomer. It has been suggested that CLA may inhibit mammary gland, skin, stomach and colon tumorigenesis in animals by altering the fatty acid composition of cells. CLA has been seen to suppress the growth of a human breast cancer cell line (MDA-MB-468), xenotransplanted into SCID mice. It is hypothesised that CLA may decrease the turnover of normal cells and inhibit clonal expansion of early transformed cells (Thompson *et al.* 1997). In particular, CLA may reduce the level of arachidonic acid, thus effecting eicosanoid synthesis (Liu & Belury 1998).

Eicosanoids are involved in cell proliferation and inflammation and are derived from arachidonate. Arachidonate-derived eicosanoids such as prostaglandin E_2, prostaglandin $F_{2\alpha}$ and prostaglandin D_2 have been shown to modulate carcinogenesis in the mammary gland and skin. Each eicosanoid exhibits a slightly different modulatory effect on tumorigenesis. For example prostaglandin E_2 is involved in enhancing tumour induction. When arachidonic acid is partially replaced by n-3 PUFA in the PL fraction of cells, there is a reduction in prostaglandin E_2 concentrations. Inhibition of prostaglandin E_2 synthesis in both mammary gland and skin has been associated with reduced tumorigenesis (Belury 1995).

1.6.3 Interaction between CLA and the Cytokine Signal Transduction Mechanism

It has been suggested that CLA can influence effects of the cytokine tumour necrosis factor α (TNF-α) on cells. TNF-α is known to induce the inflammatory response, and be a mediator in cachexia, atherosclerosis, carcinogenesis and obesity. An experiment was carried out which examined the effects of TNF-α on CLA supplemented mice. CLA fed mice were shown to experience less weight loss than control animals, suggesting that CLA may protect against TNF-α induced cachexia. CLA may possibly directly affect how TNF-α influences the expression of cell surface proteins associated with neoplastic progression, metastasis and atherosclerotic plaque formation (Pariza *et al.* 1999).

1.6.4 CLA as a Peroxisome Proliferator

Another potential mechanism by which CLA may modulate cancer, atherosclerosis and diabetes may be through its effect on lipid transport and metabolism. CLA has some features in common with peroxisome proliferators. For example, both reduce body weight and increase hepatic lipid concentration, which results in hyperlipidaemia due to increased hydrolysis of TAG by LPL. Peroxisome proliferator-activated receptor (PPAR) is a pivotal transcription factor of lipid metabolism. Of three types of PPAR (α, β, and γ), PPARα is found in liver, kidney and heart, PPARβ is found in all tissues and PPARγ is found in adipose tissue where it is involved in adipocyte differentiation. Several CLA isomers are high affinity ligands and activators of PPARα (Moya-Camarena *et al.* 1999). PPARα has been shown to be a regulator of α-peroxisomal β-oxidation, mitochondrial β-oxidation, lipid metabolising enzymes and plasma TAG clearance (Moya-Camarena & Belury 1999). One or more peroxisome proliferator-response element has been found upstream of the transcriptional start site of many genes involved in lipid metabolism (Moya-Camarena & Belury 1999). Brodie *et al.* (1999) showed that murine adipocyte differentiation is inhibited in a dose dependent manner by CLA. The increase in PPARγ, which results in cell differentiation, was not observed after adipocytes were treated with CLA. CLA also inhibited cellular proliferation of non-confluent cells (Brodie *et al.* 1999, Satory & Smith 1999). CLA has been shown to be chemoprotective in several tissues, but induces ornithine decarboxylase activity in liver. Ornithine decarboxylase activity is a marker of tumour promotion and cell proliferation, and is induced by peroxisome proliferators (Belury *et al.* 1997) and prostaglandin E_2 (Liu & Belury 1998). Therefore, unlike other n-6 PUFAs, CLA is chemoprotective in tissues such as stomach, mammary gland, skin, pancreas and colon but may be tumorigenic in the liver.

1.6.5 Summary

There is much interest in CLA as it is a *trans* fatty acid, which appears to have anti-atherogenic and anti-carcinogenic properties. CLA has been shown to alter fasting plasma TAG levels, which may be due to altered intestinal TAG metabolism, enhanced TAG clearance or increased fatty acid oxidation due to enhanced CPT activity. CLA can influence lipid metabolism by altering expression profiles of proteins involved in lipid metabolism. One way is through its action as a PPAR ligand. CLA has been shown to be

incorporated into the cell membrane and to alter eicosanoid synthesis thus effecting tumour promotion. Again its PPAR ligand function has been shown to reduce tumour promotion.

1.7 Aims

The aim of this doctorate has been to characterise some of the genetic components involved in modulating the postprandial response. In addition, the effects of diet on the postprandial response were studied, since the magnitude and duration of the postprandial response are modulated by both genetic and environmental factors.

To characterise possible genetic modulating factors of the postprandial response, various polymorphisms in some of the genes involved in lipid metabolism were examined for possible associations with postprandial response levels using statistical analyses. To study the effects of diet on the postprandial response, a model cell system of intestinal epithelial cells (Caco-2 cells) was subjected to various long- or short-term fatty acid treatments. Subsequently, expression and secretion levels of apoB100, apoB48, TAG and PLs were determined and compared to control cells using statistical analysis.

When this thesis was begun very few studies had investigated the relationship between population polymorphisms in genes involved in lipid metabolism and the postprandial response. However, many studies had been published on effects of polymorphisms on fasting plasma lipid levels. Therefore the first hypothesis, which was tested during this doctorate, was whether population polymorphisms affect the postprandial response in a similar manner to which they affect fasting plasma lipid levels. The degree of influence that postprandial TRLs have on individuals with two well documented polymorphisms in the coagulation *FVII* gene was investigated. It previously had been suggested that the different *FVII* variants stimulate FVII activation to different degrees during the postprandial response.

A third study, carried out during this doctorate, investigated effects of chronic feeding of fatty acids to Caco-2 cells on how they responded to an acute fatty acid dose. It was thought that the Caco-2 cell line might be an appropriate model system of the human intestine in which to assess affects of different fatty acids on TAG synthesis and secretion by the intestine. So far, the literature only has discussed the acute effects of fatty acid treatments on lipid and apolipoproteins synthesis and secretion levels in Caco-2 and

HepG2 cells. Therefore looking at effects of chronic low dose treatments of Caco-2 cells on lipid and apolipoprotein synthesis and secretion levels was a novel concept.

The fourth and final study discussed in this thesis involved CLA. Given that plasma TAG level is a central feature of this thesis and given the recent evidence that CLA lowers plasma TAG concentrations, CLA was included in the Caco-2 cells studies. The CLA study was carried out to determine whether the gut had any role in the reduction in TAG levels due to CLA. These four studies hopefully contribute toward clarifying the effects of genetics and fatty acids on lipid metabolism.

The experiments outlined in chapters 2 and 3 draw on a study of a cohort of subjects involved in a study of postprandial metabolism. A post-doctoral fellow and three graduate students, of which the author was one, managed the project as joint collaborators. In addition to the shared responsibility of managing the days on which postprandial responses were measured, each collaborator had specific responsibilities for different aspects of the analyses. The author had sole responsibility for all the genotyping and analysis of genotype effect on postprandial metabolism.

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2 Effect of Polymorphisms in Genes Involved in Lipid Metabolism on the Postprandial Lipid Response

2.1 Introduction

Plasma postprandial TAG concentrations are known to affect an individual's risk of coronary heart disease (CHD). The higher the postprandial plasma TAG concentrations are, the higher an individual's risk is of developing CHD. An elevated and prolonged postprandial response is linked to the formation of cholesterol ester- (CE) enriched chylomicron remnants, may stimulate the formation of the highly atherogenic small dense LDL particles and may decrease the pool of anti-atherogenic HDL_2 particles (Roche & Gibney 1999) (section 1.2.1.2). Ordovas & Schaefer (1999) suggested that reducing total fat intake, especially SFA and *trans*-fatty acids might be beneficial towards the prevention of CHD. In addition, they recommend increased intake of fruit, vegetables and fibre. However, as discussed in chapter 1 (section 1.3.3) a "one-size-fits-all" message of increasing one's health by dietary intake is simplistic as genetic polymorphisms in many of the genes involved in lipid metabolism can influence an individual's response to different fatty acid diets.

When commencing this doctorate most of the studies on the effect of polymorphisms on lipid metabolism measured fasting lipid, lipoprotein and apolipoprotein levels. Given that humans spend most of the day in a postprandial state (Williams 1998) it seemed reasonable to explore how such polymorphisms influence postprandial lipid metabolism. The following introduction summarises the relevant literature up to the date that the study outlined in this chapter was begun.

2.1.1 Effect of Five Proteins Involved in Lipid Metabolism

From the many genes involved in lipid metabolism discussed in section 1.3.2, five genes were examined for the effects their polymorphisms have on lipid metabolism. The first, *apoB,* is involved in transport of dietary and endogenous lipid around the body and is a ligand for the removal of large LDL particles by the liver (Rosseneu & Labeur 1995). ApoB is discussed in detail in section 1.3.2.4. The second, *lpl,* encodes the rate limiting protein involved in the hydrolysis of TAG into NEFA and monoacylglycerol. The NEFAs

are stored in adipose tissue during the postprandial lipid response (Ong & Kern 1989). LPL is discussed in more detail in section 1.3.2.9. The third and fourth genes investigated, *lcat* and *cetp,* encode major proteins involved in the reverse cholesterol transport pathway. LCAT esterifies cholesterol into CE, which migrates to the HDL core causing a gradient of cholesterol between cells and HDL particles (Qu *et al.* 1995) (section 1.3.2.10). CETP transfers CE from HDL to chylomicrons, VLDL and their remnants. In addition, it transports TAG from TRL particles to HDL particles (Kinoshita *et al.* 1993) (section 1.3.2.11). The fifth gene studied, *apoE,* encodes the apoE protein, which is transferred from HDL particles to triacylglycerol-rich lipoprotein (TRL) remnants. There, apoE acts as a ligand for the LDL receptor, and LDL-receptor related protein in the liver, causing the removal of these particles from the circulatory system (Rosseneu & Labeur 1995) (section 1.3.2.8). Therefore genes involved in all stages of lipid metabolism, that is lipid transport and removal, and the reverse cholesterol pathway, were examined in this study.

2.1.2 Linkage Disequilibrium

In the section below some of the polymorphisms will be described as being in linkage disequilibrium. This is a measure of how frequently polymorphisms at different sites occur together. The sites are not independently segregating and so are linked. This can be observed if particular haplotypes (alleles from different linked sites) occur more frequently than others in a population, although the alleles at each locus are in Hardy Weinberg equilibrium. Hardy Weinberg equilibrium assumes normal gene segregation, equal fertility of parents, equal fertilising capacity of gametes, a large population, random mating, equal gene frequencies in male and female parents and equal variability. When a two alleled polymorphism is in Hardy Weinberg equilibrium, $(p+q)^2=1$, where p and q are the frequencies of each of the two alleles. When there is more than one locus of interest on the same chromosome, it is necessary to calculate how likely it is for the different alleles to remain combined. If loci are far apart on a chromosome the likelihood of recombination during second division meiosis between the alleles is greater than if the loci are close to each other. An example of a calculation of linkage disequilibrium for two different loci (M and S) each with two alleles (M,m and S,s) is as follows.

Firstly the allele frequencies are calculated;

Allele	Hypothetical frequency	Symbol of frequency
M	0.525	p_1
m	0.475	p_2
S	0.308	q_1
s	0.692	q_2

Then the observed haplotype frequencies (D) are calculated, remembering each individual has two chromosomes.

Haplotype	Observed /Total chromosomes	Frequency	Symbol of frequency
MS	474/2000	0.237	P_{11}
Ms	611/2000	0.3055	P_{12}
mS	142/2000	0.071	P_{21}
ms	773/2000	0.3865	P_{22}

$D = P_{11}P_{22}-P_{12}P_{21}$
$= (0.237)(0.3865)-(0.3055)(0.071)$
$= 0.06991$

D_{max} is the lesser of p_1q_2 or p_2q_1 if D is positive, and the lesser of p_1q_1 or p_2q_2 if D is negative. In this hypothetical example D is positive therefore D_{max} is either p_1q_2 or p_2q_1.
$D_{max} = (0.525)(0.692)$ or $(0.475)(0.308)$
$= 0.3633$ or 0.1463
As p_2q_1 is less than p_1q_2, D_{max} is $p_2q_1 = 0.1463$

Linkage disequilibrium (D') can then be calculated as D/D_{max}.
$D' = 0.06991/0.1463$
$= 0.4779$

When D'=1 loci are in complete linkage disequilibrium, which suggests the loci are right beside each other on the chromosome. The loci are then said to be linked with zero recombination.

2.1.3 Polymorphisms of Five Proteins Involved in Lipid Metabolism

The biochemical and epidemiological effects of polymorphisms of the five genes mention in section 2.1.1 are summarised in Table 2.1.1. The signal peptide insertion/deletion polymorphism (-14 to –16, Leu Ala Leu), XbaI polymorphism (ACC2488ACT), MspI polymorphism (Arg3611Gln), EcoRI polymorphism (Gln4154Lys) and the TspRI polymorphism (Asn4311Ser) in the *apoB* gene were studied. The insertion/deletion polymorphism was expected to affect lipid transport while the XbaI, MspI, EcoRI and TspRI polymorphisms may be involved in remnant uptake by the liver (Renges *et al.* 1992). Individuals with the D (deletion) allele of the insertion/deletion polymorphism have been found to have lower fasting plasma TAG concentrations than II individuals

(individuals homozygous for the insertion allele) (Renges *et al.* 1992, Xu *et al.* 1990). The X+ allele of the XbaI polymorphism has been associated with increased total cholesterol, LDL-cholesterol and fasting plasma TAG levels. However, the X- allele is associated with increased risk of CAD (Juvonen *et al.* 1995, Renges *et al.* 1992). The MspI polymorphism has been found to be in linkage disequilibrium with the EcoRI polymorphism. The Gln allele has been associated with increased risk of premature CAD (Glisic *et al.* 1995). No biochemical affects on lipid metabolism have previously been associated with the EcoRI polymorphism (Renges *et al.* 1992, Glisic *et al.* 1995, Juvonen *et al.* 1995). Finally, individuals homozygous for the Ser allele of the TspRI polymorphism have been shown to have lower apoB, fasting plasma TAG and apoB/apoE lipoprotein levels and higher apoA-I levels than heterozygotes or individuals homozygous for the Asn allele (Renges *et al.* 1992).

The polymorphisms in the *apoE* gene were examined for their effect on postprandial lipid concentrations. Previous studies had found the ε2 allele to be associated with low total cholesterol, LDL-cholesterol and apoB levels and high apoE concentrations. The ε4 allele had been associated with high total cholesterol, LDL-cholesterol and apoB concentrations and low apoE levels. ε3ε3 individuals had intermediate plasma lipid levels (Ordovas *et al.* 1995).

The PvuII, HindIII and Ser447Ter polymorphisms in the *lpl* gene were examined for their effect on the postprandial response. Previously the P+P+ genotype had been associated with high fasting plasma TAG concentrations and low HDL-cholesterol concentrations (Ukkola *et al.* 1995). The H- allele had been associated with low fasting plasma TAG and in some studies-high HDL-cholesterol levels (Jemaa *et al.* 1995). The T allele of the Ser447Ter polymorphism also had been associated with low fasting plasma TAG and high HDL-cholesterol levels (Jemaa *et al.* 1995). Strong linkage disequilibrium had been found between the P-, H- and T alleles, suggesting that the P-H-T haplotype is anti-atherogenic (Jemaa *et al.* 1995).

No population polymorphisms associated with lipid metabolism, had been described in the *lcat* gene. However, two polymorphisms, which appeared to be interesting, had been described. The first was found in a Danish LCAT deficiency patient. This patient had two mutations, Ala93Thr and Arg158Cys. COS-1 monkey kidney cells were transformed with human *lcat* genes with each of these mutations and *in vitro* expression of the mutant genes showed that the Ala93Thr mutation caused the disease phenotype (Qu *et al.* 1995). Interestingly LCAT with the Arg158Cys mutation had 20% less specific activity than wild

type LCAT. It was suggested to be a polymorphism of LCAT, as 80% homology was found for this amino acid between human, pig, mouse and rat (Hill *et al.* 1993, Qu *et al.* 1995). The Arg158Cys mutation was deemed to be suitably interesting to be tested for its population frequency in the current study. The second polymorphism was observed in a Finnish family with LCAT deficiency. This family was found to have two mutations in the *lcat* gene. The first was an insertion in exon 1 that causes the formation of a premature stop codon, resulting in a truncated protein product, and the second was a C-T transition in exon 6, which caused a Cys399Arg mutation. When the Cys399Arg mutant *lcat* gene was transfected in COS-1 cells, the resulting LCAT protein was significantly less active than the wild type protein (Miettinen *et al.* 1995). A population of Finns with HDL levels >0.9 mmol/l was screened for this mutation and more than one individual was found to be heterozygous for the mutation (Miettinen *et al.* 1995). Therefore the frequency of this mutation was determined in the normolipidaemic study population.

Lastly the TaqIB polymorphism in the *cetp* gene was genotyped, and analysed for effects on postprandial lipaemia. In previous studies the B- allele was found to have a gene dosage effect on CETP levels, with B-B- individuals having the lowest CETP levels (Juvonen *et al.* 1995, Kuivenhoven *et al.* 1998). Kuivenhoven *et al.* (1998) also observed that the increased CETP concentrations were associated with decreased HDL-cholesterol levels.

Table 2.1.1 Table containing the published frequencies of the alleles of polymorphisms investigated in the five genes studied in this chapter. A summary of the biochemical and epidemiological factors associated with the alleles of these polymorphisms is given.

Gene	Polymorphism	Frequency	Effect on Lipid Metabolism
Apolipoprotein B (apoB)	Insertion/Deletion	I – 0.69, D – 0.31	D allele is associated with reduced fasting plasma TAG concentrations
	XbaI	X+ - 0.6, X- - 0.4	X+ allele associated with increased total cholesterol, LDL-cholesterol and fasting plasma TAG levels. X- allele associated with increased risk of CAD.
	MspI	Arg - 0.89, Gln - 0.11	Gln allele associated with increased risk of premature CAD.
	EcoRI	R+ - 0.8, R- - 0.2	No biochemical affects on lipid metabolism observed.
	TspRI	Asn – 0.8, Ser – 0.2	SerSer individuals lower apoB, TAG and apoB/apoE lipoprotein levels and higher apoA-I levels than AsnAsn and AsnSer individuals.
Apolipoprotein E (apoE)	ε2	ε2 – 0.08, ε3 – 0.77,	ε2 allele is associated with low total cholesterol, LDL-cholesterol and apoB levels and high apoE concentrations. The ε4 allele is associated with high cholesterol, LDL-cholesterol and apoB levels and low apoE concentrations. ε3ε3 individuals have intermediate plasma lipid levels.
	ε3	ε4 – 0.15	
	ε4		
Lipoprotein Lipase (lpl)	PvuII	P+ - 0.46, P- -0.54	P- allele is associated with low fasting plasma TAG and high HDL-cholesterol levels.
	HindIII	H+ - 0.71, H- - 0.29	H- allele is associated with low fasting plasma TAG concentrations.
	Ser447Ter	S – 0.89, T – 0.11	T allele is associated with low fasting plasma TAG and high HDL-cholesterol levels.
			These polymorphisms were found to be in linkage disequilibrium, with the P-H-T haplotype occurring more frequently than expected.
Cholesteryl ester transfer protein (cetp)	TaqIB	B+ - 0.6, B- - 0.4	B-B- individuals have lowest CETP concentrations, and due to inverse relationship between CETP concentration and HDL-cholesterol levels, they have the highest HDL-cholesterol concentrations.
Lecithin:cholesterol acyltransferase (lcat)	Arg158Cys		Mutant LCAT had 20% less specific activity than wild type LCAT expressed from COS-1 cells.
	Cys399Arg		Mutant LCAT had significantly less activity than wild type LCAT.

2.2 Materials and Methods

2.2.1 Study Design

The Ethics Committee of the Federated Dublin Voluntary Hospitals approved this study. Polymorphisms in the *apoB* gene (Insertion/Deletion, XbaI, EcoRI, MspI and TspRI), *apoE* gene (ε2, ε3 and ε4), *lpl* gene (PvuII, HindIII and Ser447Ter), *cetp* gene (TaqIB) and *lcat* gene (Arg158Cys, Cys399Arg) were genotyped in a non-smoking Caucasian population, consisting of 119 individuals. Of the 119 individuals screened for a normolipideamic profile, 82 individuals (48 male, 34 female) underwent a postprandial lipid test. These 82 individuals formed the Nutrifish study population.

2.2.2 DNA Isolation from Blood

Blood for DNA isolation was collected in 2ml EDTA vacutainers. The DNA was either isolated immediately from fresh blood or whole blood was frozen (-70°C) for subsequent DNA isolation. DNA was isolated, by adding 30µl of blood to 1ml of TE pH 7.5 buffer, which haemolysed the red blood corpuscles. The white blood cells were pelleted at 13,200rpm for 2 minutes in a microcentrifuge (Sigma), and the pellet was washed in TE buffer until the red pigment had been removed. 50µl of Lysis Buffer (15mM Mg^{2+} PCR buffer (see PCR amplification section 2.2.3), 0.5% TWEEN-20 and 100µg/ml Proteinase K (Sigma)) was added to the clean white blood cells, which were then incubated at 55°C for 45 minutes. The Proteinase K was then denatured for 10 minutes at 90°C, and the resulting product stored for subsequent use at -20°C.

2.2.3 Polymerase Chain Reaction Amplification

The polymerase chain reaction (PCR) was used to amplify small fragments of DNA containing each polymorphic site for genotyping. The primers for PCR amplification were synthesised on PCR-MATE (Applied Biosystems, 391 DNA synthesizer). PCR amplifications were carried out in 25µl reactions, containing the following:

1.25mM dATP
1.25mM dCTP
1.25mM dGTP
1.25mM dTTP

6% Mg^{2+} PCR buffer 500mM KCl
100mM Tris.Cl pH 8.5
10mM Triton-100
5, 7.5, 10, 12.5, 15, 20 or 25mM $MgCl_2$

50nmol of forward primer
50nmol of reverse primer
6 units Taq polymerase
1µl of DNA (in Lysis Buffer which contained 15mM PCR buffer and brings Mg^{2+} PCR buffer concentration to 10%).
All H_20 used in PCR reactions had been deionised, autoclaved, filtered and treated with ultraviolet radiation for 20 minutes.

Samples were amplified on either a Hyband (OmniGene) or PTC-100 (Genosys) PCR machine. Amplification programmes for each specific PCR amplification and primer sequences are given in Table 2.2.1.

2.2.4 Restriction Enzyme Digests of Polymerase Chain Reaction Amplification Products and their Visualisation

Specific enzymes used for distinguishing the different polymorphic alleles of the genes of interest are listed in Table 2.2.1. Generally, digests of PCR products were carried out according to the restriction enzyme manufacturers' instructions. In the cases of the insertion/deletion polymorphism in *apoB* and the Ser447Ter polymorphism in *lpl,* restriction digestions were not necessary to distinguish between the different alleles. The insertion/deletion polymorphism was genotyped as described in Boewinkle & Chan (1989) (Table 2.2.1) except that the resulting PCR amplified fragments were run on 4% agarose gels instead of 6% agarose gels, to separated the two PCR products (I allele 93bp, D allele 84bp). The apoE genotypes were distinguished as described in Hixson & Vernier (1990), except that the resulting digest products were separated on 20% polyacrylamide nondenaturing gels (20cm long) instead of 8% polyacrylamide nondenaturing gels (25cm long). Polyacrylamide gels were emerised in 0.005% ethidium bromide after elecrophorsis to stain the DNA bands, which were visualised under UV light. All other PCR products and digestion products were separated on either 2% or 3 % agarose gels containing 0.005% ethidium bromide. Separated products were visualised under a UV light. All

polymorphisms but the apoE polymorphism were sized using either the kilobase or 100bp DNA ladder (GibcoBRL). The apoE polymorphism was sized using the 10bp DNA ladder (GibcoBRL).

2.2.5 Postprandial Investigation; Isolation of Postprandial Components from Plasma

Postprandial tests were conducted in the Nutrition Laboratory at Trinity College Medical School, St James' Hospital, Dublin by 4 members of the group involved in the study. Each postprandial study began between 07:30 and 08:00 following a 12 hour overnight fast. All subjects abstained from alcohol and refrained from strenuous exercise for 24 hours before the postprandial investigation. A 21 gauge, 32mm venous catheter (Abbott) was inserted into the antecubital vein of the non-dominant forearm and a fasting blood sample was drawn. Subsequently, subjects were asked to consume a test meal composed of a milk shake, consisting of 40g sunflower oil (Flora) and 30g dried skimmed milk powder (Marvel) mixed with 150ml skimmed milk (Avonmore). Test meals were consumed under supervision, within a 20 minute period. During postprandial investigations, subjects abstained from food (including chewing gum) and drinks, with the exception of caffeine-free, low-calorie (<1kcal/ml) drinks. Postprandial blood samples were drawn every 2 hours over an 8 hour period. Blood for plasma lipid and apolipoprotein analyses was collected in heparinised vacutainers. Blood samples were centrifuged immediately at 2000g for 15 minutes at room temperature (RT) (carried out by all 4 members of the group). Heparinised plasma was harvested and divided into two portions. One portion of plasma was vortexed, aliquoted and frozen (-20°C) and the second portion was stored (<5°C) for 24 hours. The second portion of plasma was overlaid with 1.006g/ml NaCl solution and then ultracentrifuged to isolate the triacylglycerol-rich lipoprotein (TRL) and the triacylglycerol-poor lipoprotein (TPL) fractions (Roche *et al.* 1998). HDLs were isolated from the fresh plasma using the Quantolip precipitation kit (Allphar Services Ltd.), as previously described (Roche & Gibney, 1996) and the cholesterol concentrations of the HDL_2 and HDL_3 subfractions were measured (section 2.2.6).

2.2.6 Lipid and Protein Analyses

Analyses of plasma TAG (TAG Enzymatique PAP 1000, bioMerieux), plasma cholesterol (Cholesterol Enzymatique PAP 1200, bioMerieux), TRL-TAG, TPL-TAG (bioMerieux)

and plasma NEFA (NEFA, Randox Laboratories) concentrations were determined using enzymatic colorimetric assays on a Technicon RA-XT analyser (Bayer). Using external control plasma with known TAG, cholesterol (Lyotrol N, bioMerieux) and NEFA (Wako Control Serum II, Wake Chemicals GmbH) concentrations, the respective inter-assay coefficient of variation (CV) could be calculated. The TAG inter-assay CV for 1.31 mmol/l and 3.11 mmol/l were 1.45% and 1.67%, the cholesterol inter-assay CV for 4.02 mmol/l was 1.01% and the NEFA inter-assay CV for 1.04 mmol/l was 2.8%. Samples for apoB48 analysis were stored with a preservative, and the concentration of apoB48 in plasma was determined using a competitive enzyme-linked immunoabsorbent assay (ELISA), using a specific polyclonal anti-apoB48 antiserum (Lovegrove *et al.* 1996). The apoB48 inter-assay CV for 0.723 μg/ml was 13.13%. Plasma HDL and HDL_2 were precipitated out of the plasma using the Quantolip protocol (Immuno AG). The cholesterol fraction was measured on the Technicon RA-XT analyser using Lyotrol N (bioMerieux) and Immunolip control serum (IMMUNO art.) as controls. The HDL_3-cholesterol fraction was calculated from the HDL-cholesterol and HDL_2-cholesterol fractions as follows. $HDL_3 = HDL - HDL_2$. Plasma CETP mass was assayed using ELISA devised Clark *et al.* (1995), and activity using fluorescence kit (Wak-Chemie Medical GMBH).

2.2.7 Statistical Analysis of Genotype Frequencies

The frequencies of the polymorphic variants were calculated to assess whether the alleles were in Hardy Weinberg Equilibrium ($(p+q)^2=1$). Linkage disequilibrium between polymorphisms of the same gene was calculated using the Estimate of Haplotype Frequency programme (Xie & Ott, 1993), which calculates the observed and expected frequencies for each haplotype. Then linkage disequilibrium (D') was calculated for each of two polymorphisms (section 2.1.2).

$D' = D/D_{max}$

$D = P_{11}P_{22}-P_{12}P_{21}$ (observed frequencies)

D_{max} = lesser of p_1q_2 or p_2q_1 if D positive, and lesser of p_1q_1 or p_2q_2 if D negative (expected frequencies).

Statistical analysis was carried out using statistical packages, Data Desk 4.0 and Minitab 8.1. The postprandial data are presented in two ways. Firstly the area under the postprandial response curve (AUC) was calculated. The postprandial response curve is a

graph of time versus concentration. The AUC was calculated as AUC = 0hr +2(2hr + 4hr + 6hr) + 8hr (Matthews *et al.* 1990). Secondly the maximum postprandial TAG concentrations (max) and minimum postprandial NEFA concentrations (min) are presented. Before statistical analysis was carried out, the data were normalised: 1/square root(TAG-0hr), 1/square root(TAG-max), 1/square root(TAG-AUC), square root(TRL-TAG), $\log_{10}$(TRL-TAG-max), $\log_{10}$(TRL-TAG-AUC), $\log_{10}$(apoB48-0hr), $\log_{10}$(apoB48-4hr), square root(apoB48-8hr), Cholesterol-0hr, square root(cholesterol-AUC), 1/square root(HDL-cholesterol), square root(HDL_2-cholesterol), HDL_3-cholesterol, square root(NEFA-0hr), ln(NEFA-8hr), NEFA-AUC, $\log_{10}$(CETP mass-0hr) $\log_{10}$(CETP mass-6hr) square root(CETP activity-0hr) and $\log_{10}$(CETP activity-6hr). Then analysis of variance (ANOVA) and two-sample-t-tests were carried out on the normalised data. These two tests compare means of the data distribution with the null hypothesis that the means are the same. A two-sample-t-test compares the means of two groups, whereas the ANOVA compares the means of more than two groups. The simple linear regression model was used to investigate the possible relationship between genotype and the ability of fasting TAG levels to predict postprandial TAG levels. A plot was made of each individual's fasting TAG concentration (x-axis) and postprandial TAG-AUC concentration (y-axis), and then a line was plotted. How closely the points are to the regression line informs one of the relationship between the fasting TAG and postprandial TAG concentrations.

Table 2.2.1 Table of experimental conditions of PCR amplifications and restriction digests (where necessary) used to genotype polymorphisms. NEB = New England Biolabs. Boeh Mann = Boehringer Mannheim. u = unites. F = forward. R = reverse. Ins/Del = Insertion/Deletion polymorphism.

			PCR				Digestion		
Gene	Polymorphism	Primer	Machine	Programme	PCR Buffer	Size of Product	Restriction Enzyme	Incubation	Size of Products
apoB	Ins/Del M19808	F cag ctg gcg atg gac ccg ccg a R acc ggc cct ggc gcc cgc cag ca (Renges *et al.* 1992) (Boewinkle & Chan 1989)	OmniGene PTC-100	94°C 4mins 94°C 1min 64°C 1:30mins 35 cycles 94°C 4mins 94°C 1:30mins 64°C 2mins 35 cycles	15mM and 7% DMSO	I 93bp D 84bp			
	XbaI ACC2488ACT M14162	F gga gac tat tca gaa act aa R gaa gag cct gaa gac tga ct (Renges *et al.* 1992)	PTC-100	94°C 5mins 94°C 1:20mins 60°C 1:30mins 72°C 1:30mins 35 cycles 72°C 5mins	15mM	710bp	2 units/reaction NEB XbaI	10µl PCR product in 15µl reaction 37°C 4hours	433 & 277bp
	MspI Arg3611Gln M14162	F gga agt ggc cct gaa tgc taa cac R gct ctt gtc ata gac tgg tag g	OmniGene	94°C 7mins 94°C 1min 55°C 1min 72°C 1:12mins 35 cycles 72°C 5mins	15mM	195bp	1 unit/reaction NEB MspI	10µl PCR product in 15µl reaction 37°C 4hours	135 & 60bp
	EcoRI Gln4154Lys M14162	F ctg aga gaa gtg tct tcg aag R ctc gaa agg aag tgt aat cac (Renges *et al.* 1992)	OmniGene	94°C 7mins 94°C 1min 55°C 1min 72°C 1:12mins 35 cycles 72°C 5mins	15mM	480bp	1 unit/reaction Boeh Mann EcoRI	10µl PCR product in 15µl reaction 37°C 4hours	253 & 227bp
	TspRI Asn4311Ser M14162	F gac cta gtg att aca ctt cct ttc ga R gat gga tct gct gta act ctt gag	OmniGene	94°C 7mins 94°C 1min 55°C 1min 72°C 1:12mins 35 cycles 72°C 5mins	12.5mM	393bp	0.5 units/reaction NEB TspRI	10µl PCR product in 15µl reaction 65°C 5-6hours	271 & 122bp

Table 2.2.1 continued

			PCR				Digestion		
Gene	Polymorphism	Primer	Machine	Programme	PCR Buffer	Size of Product	Restriction Enzyme	Incubation	Size of Products
Ipl	PvuII X15736	F atg gca ccc atg tgt aag gtg R gtg aac ttc tga taa caa tct a (Peacock *et al.* 1992)	OmniGene	94°C 5mins 58°C 5mins 94°C 1min 58°C 5mins 35 cycles	15mM	430bp	1 unit/reaction NEB PvuII	10µl PCR product in 15µl reaction 37°C 4hours	320 & 110bp
	HindIII M76722 M76723	F ttt agg cct gaa gtt tcc ac R ctc cct aga aca gaa gct c (Ahn *et al.* 1993)	OmniGene	94°C 5mins 94°C 1 min 58°C 1min 72°C 1:12mins 35 cycles 72°C 5mins or 94°C 5mins 94°C 1 min 60°C 1min 72°C 1min 35 cycles 72°C 5mins	5mM or 10mM or 12.5mM or 20mM	1300bp	1 unit/reaction NEB HindIII	10µl PCR product in 15µl reaction 37°C 4hours	700 & 600bp
	Ser447Ter M76722 M76723	F tgt tct aca tgg cat att cac (Jemaa *et al.* 1995) S cca gaa tgc tca cca gcc ag T cca gaa tgc tca cca gcc ac	OmniGene	94°C 5mins 94°C 1min 60°C 1min 72°C 30secs 35 cycles 72°C 2mins	10mM	156bp			

Table 2.2.1 continued

			PCR				Digestion		
Gene	Polymorphism	Primer	Machine	Programme	PCR Buffer	Size of Product	Restriction Enzyme	Incubation	Size of Products
cetp	TaqIB M32992 J02898	F ggt cct agc tgc att gca aac R gat gga gcc tcc gtc acc tga	OmniGene	94°C 7mins 94°C 1min 55°C 1min 72°C 1:12mins 35 cycles 72°C 5mins	20mM	213bp	1 unit/reaction Boeh Mann TaqIB	5µl PCR product in 10µl reaction 65°C 4 hours	142 & 71bp
lcat	AciI Arg158Cys X04981	F cta tga ctg gcg gct gga R acc agc atg ggc ttg atg	OmniGene	94°C 5mins 94°C 1min 63°C 30secs 72°C 1min 40 cycles 72°C 5mins	7.5mM or 10mM	319bp	1 unit/reaction NEB *AciI*	10µl PCR product in 15µl reaction 37°C overnight	193, 126, 106, 64 & 23bp
	AciI Cys399Arg X04981	F cac agc ctg tgc acc tgc R gtg tgg gac tct agt gcc	OmniGene	94°C 5mins 94°C 1min 60°C 30secs 72°C 30secs 35 cycles 72°C 2mins	5mM	231bp	1 unit/reaction NEB AciI	15µl PCR product in 20µl reaction 37°C overnight	127, 104, 82 & 45bp
apoE	ε2, ε3, ε4	F taa gct tgg cac ggc tgt cca agg a R aca gaa ttc gcc ccg gcc tgg tac ac (Hixson & Vernier, 1990)	OmniGene	95°C 5mins 95°C 1min 60°C 1min 70°C 2min 35 cycles	15mM and 10% DMSO	244bp	5 units/reaction NEB HhaI	25µl PCR product in 30µl reaction 37°C 4 hours	ε2 91 & 83 bp ε3 91, 48 & 35bp ε4 72, 48, 35 & 19bp

2.3 Results

In the present study the effects of genotype on the phenotype of fasting and postprandial lipid, lipoprotein and apolipoprotein concentrations were investigated. PCR amplifications over polymorphic sites in 5 genes involved in lipid metabolism were carried out. Polymorphic variants were identified using restriction digestion and effects of different genotypes on fasting and postprandial TAG, TRL-TAG, cholesterol, NEFA, apoB48, HDL-cholesterol and CETP mass and activity were examined.

2.3.1 Genotype Frequencies and Haplotype Analysis of Genes Involved in Lipid Metabolism

Polymorphic sites in the *apoB*, *apoE*, *lpl, cetp* and *lcat* genes were genotyped in a population consisting of 119 Caucasians. Pictures of gels visualising the different genotypes found for these polymorphisms are shown in Figures 2.3.1-2.3.4. The two polymorphisms at amino acid positions 158 and 399 of the *lcat* gene were not detected in the 119 normolipidaemic individuals. Therefore, data are not shown. The observed allele frequencies of each of the 12 polymorphisms typed in the study population is contained in Table 2.3.1. The alleles are in Hardy Weinberg equilibrium and are not significantly different from frequencies reported in the literature (comparison with Table 2.1.1).

A total of 5 polymorphisms were typed in the *apoB* gene, and 3 in the *lpl* gene. Two tables (Table 2.3.2 and Table 2.3.3) show the linkage disequilibrium, which exists between the polymorphisms of these two genes. From Table 2.3.2 it becomes apparent that most recombination has occurred around the EcoRI polymorphic site. This causes the DX+AR+A, DX+GR+A, IX-AR+S and IX-AR-A haplotypes to be observed more frequently in the sample population, than would be expected from the allele frequencies (observed frequency – expected frequency; 0.227 – 0.108, 0.085 – 0.012, 0.163 – 0.037 and 0.182 – 0.047). In the *lpl* gene there is more recombination between the intronic mutations (PvuII and HindIII) than between either of the intronic mutations and the Ser447Ter polymorphism. The haplotypes P+H+S, P-H-S and P-H-T were found to be over-represented occurring more frequently than expected from allele frequencies (observed frequency – expected frequency; 0.507 – 0.405, 0.121 – 0.102 and 0.063 – 0.007).

Table 2.3.1 Allele frequencies of the polymorphisms that were genotyped in the study population.

Allele Frequencies for the Study Population

Gene	Polymorphism	Allele	Frequency
apoB	Ins/Del	I	0.664
		D	0.336
	XbaI	X+	0.538
		X-	0.462
	MspI	Arg (A)	0.903
		Gln (G)	0.097
	EcoRI	R+	0.794
		R-	0.206
	TspRI	Asn (A)	0.832
		Ser (S)	0.168
lpl	PvuII	P+	0.588
		P-	0.412
	HindIII	H+	0.735
		H-	0.265
	Ser447Ter	S	0.937
		T	0.063
apoE	ApoE	ε2	0.05
		ε3	0.75
		ε4	0.20
cetp	TaqIB	B+	0.622
		B-	0.378
lcat	Arg158Cys	M+	1.0
		M-	0.0
	Cys399Arg	M+	0.0
		M-	1.0

Table 2.3.2. Table of the linkage disequilibrium (D') between the *apoB* gene polymorphisms, when pairs of polymorphisms are examined.

Linkage Disequilibrium between Polymorphisms in *ApoB*

	XbaI	MspI	EcoRI	TspRI
Ins/Del	0.9667	0.9124	0.603	0.9997
XbaI		0.9998	0.741	0.99992
MspI			0.458	0.9993
EcoRI				0.9994

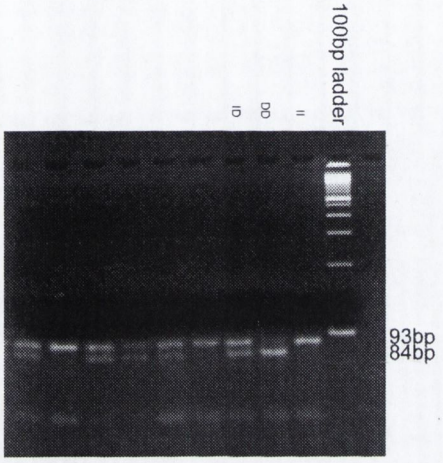


Figure 2.3.1a Photograph of an agarose gel showing the *apoB* insertion/deletion polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 9 DNA samples that were amplified and run on this gel, 3 are I homozygotes (II), 5 are heterozygotes (ID) and 1 is a D homozygote (DD).

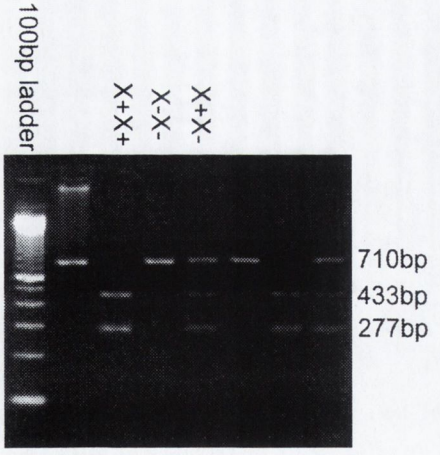


Figure 2.3.1b Photograph of an agarose gel showing the *apoB* XbaI polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 7 DNA samples that were amplified, digested and run on this gel, 2 are X+ homozygotes (X+X+), 2 are heterozygotes (X+X-) and 3 are X- homozygotes (X-X-).

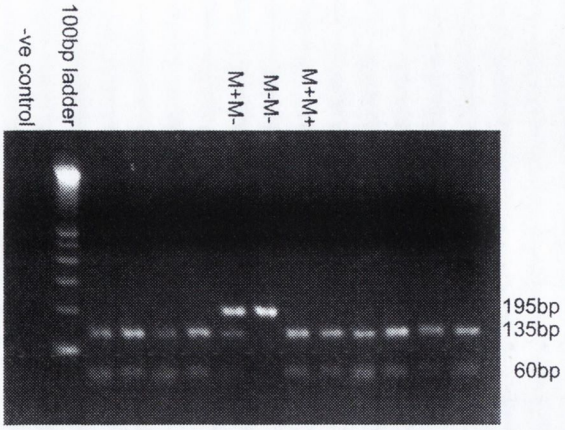


Figure 2.3.1c Photograph of an agarose gel showing the *apoB* MspI I polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 12 DNA samples that were amplified, digested and run on this gel, 10 are M+ homozygotes (M+M+), 1 is a heterozygote (M+M-) and 1 is a M- homozygote (M-M-).
-ve control = PCR and digest carried out with no DNA present.

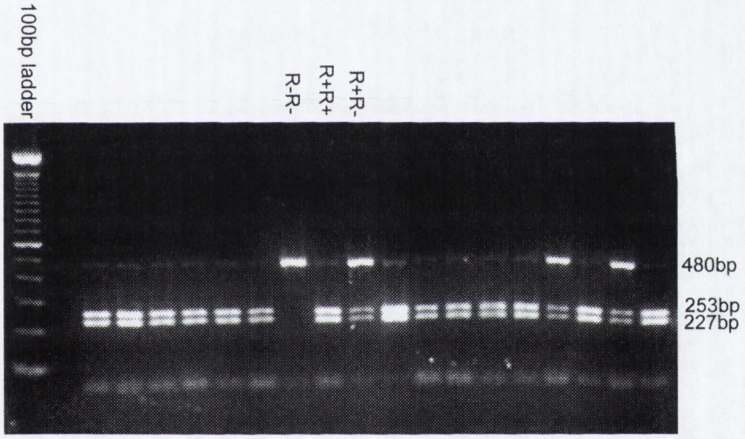


Figure 2.3.1d Photograph of an agarose gel showing the *apoB* EcoRI polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 18 DNA samples that were amplified, digested and run on this gel, 14 are R+ homozygotes (R+R+), 3 are heterozygotes (R+R-) and 1 is a R- homozygote (R-R-).

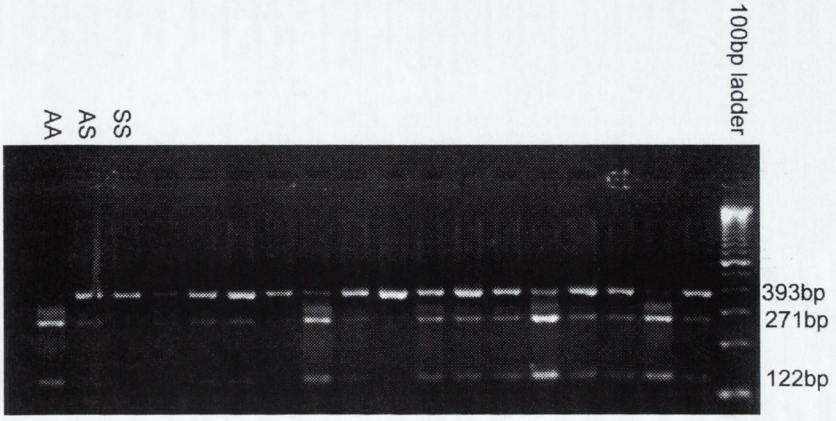


Figure 2.3.1e Photograph of an agarose gel showing the *apoB* TspRI polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 17 DNA samples that were amplified, digested and run on this gel, 4 are Asn homozygotes (AsnAsn), 10 are heterozygotes (AsnSer) and 3 are Ser homozygotes (SerSer).

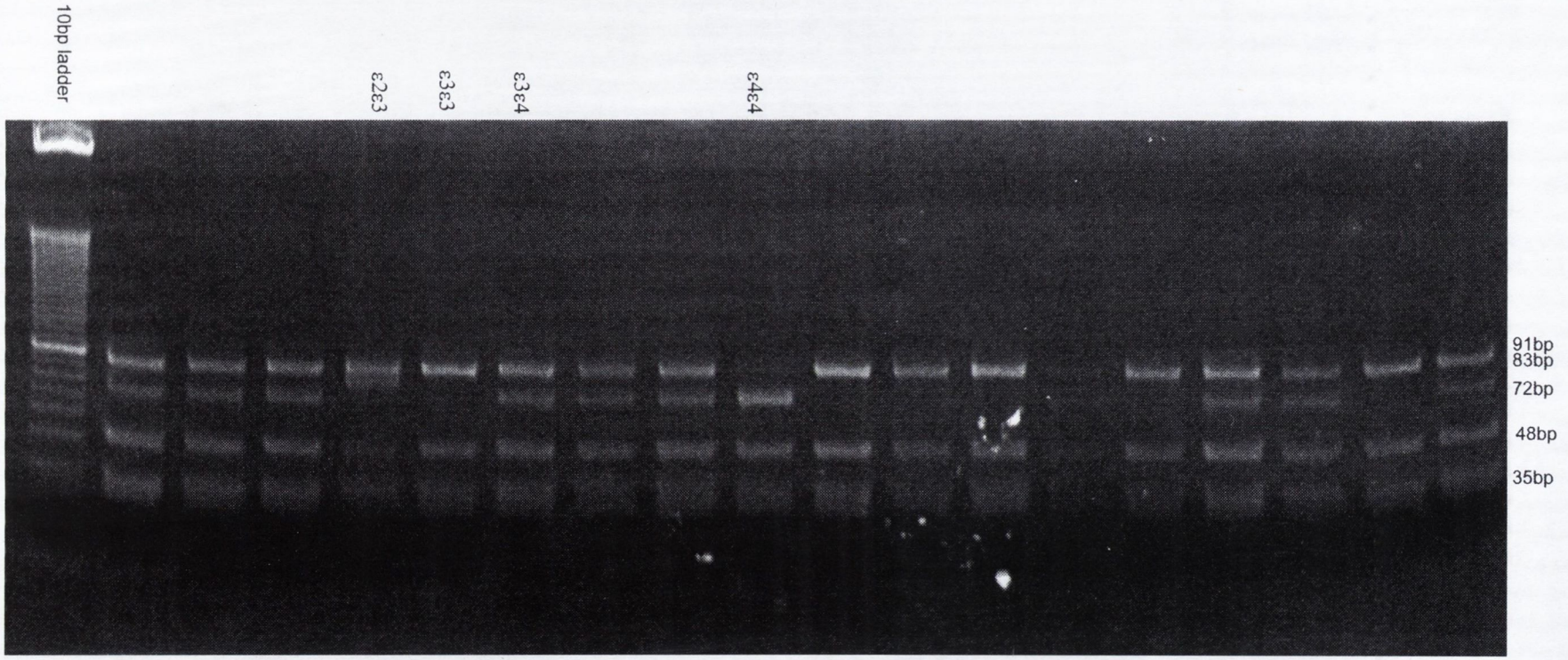


Figure 2.3.2 Photograph of an acrylamide gel showing the *apoE* polymorphism. The PCR amplification fragments were sized using the 10bp ladder (GibcoBRL). Of the 17 DNA samples that were amplified and run on this gel, 9 individuals are ε3ε4, 1 individual is ε2ε3, 6 individuals are ε3ε3 and 1 individual is ε4ε4. One individual's genotype can not be determined with certainty (6^{th} from the right).

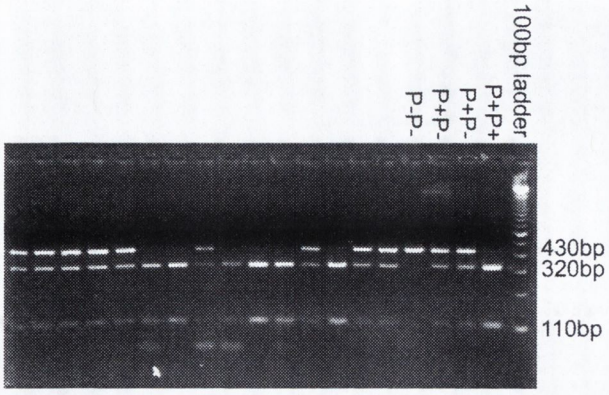


Figure 2.3.3a Photograph of an agarose gel showing the *lpl* PvuII polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 19 DNA samples that were amplified, digested and run on this gel, 7 are P+ homozygotes (P+P+), 11 are heterozygotes (P+P-) and 1 is a P- homozygote (P-P-).

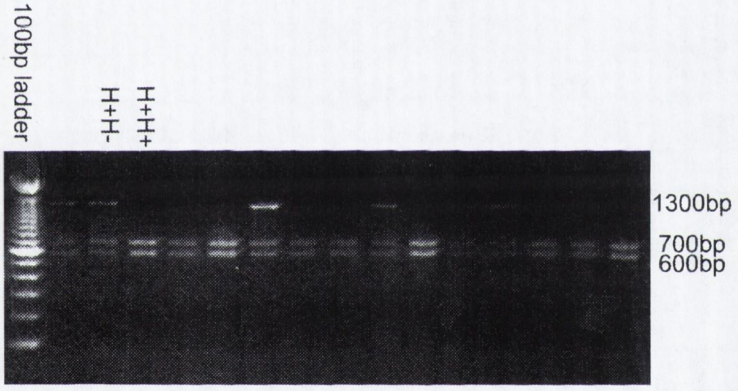


Figure 2.3.3b Photograph of an agarose gel showing the *lpl* HindIII polymorphism. The PCR amplification fragments were sized using the 100bp ladder (GibcoBRL). Of the 15 DNA samples that were amplified, digested and run on this gel, 9 are H+ homozygotes (H+H+) and 6 are heterozygotes (H+H-).

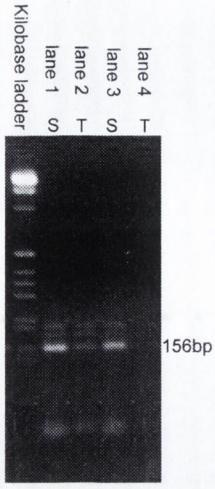


Figure 2.3.3c Photograph of an agarose gel showing the Ser447Ter polymorphism of the *lpl* gene. PCR amplification products were sized using the kilobase ladder (GibcoBRL). The Ser447Ter polymorphism was genotyped using primers specific for either the T or S alleles (Table 2.2.1). For each DNA sample two PCR reactions were carried out, one to amplify the S allele if present and the second to amplify the T allele if present. Lanes 1 and 3 represent PCR products of the S allele and lanes 2 and 4 represent amplification products of the T allele. One DNA sample is amplified in lanes 1 and 2 and this individual is ST. A second DNA sample is amplified in lanes 3 and 4 this individual is SS (no amplification occurs with the T primer).

Table 2.3.3 Table of the linkage disequilibrium (D') between the *lpl* gene polymorphisms, when pairs of polymorphisms are examined.

Linkage Disequilibrium Between Polymorphisms in *LPL*

	HindIII	**Ser447Ter**
PvuII	0.509	0.9998
HindIII		0.99998

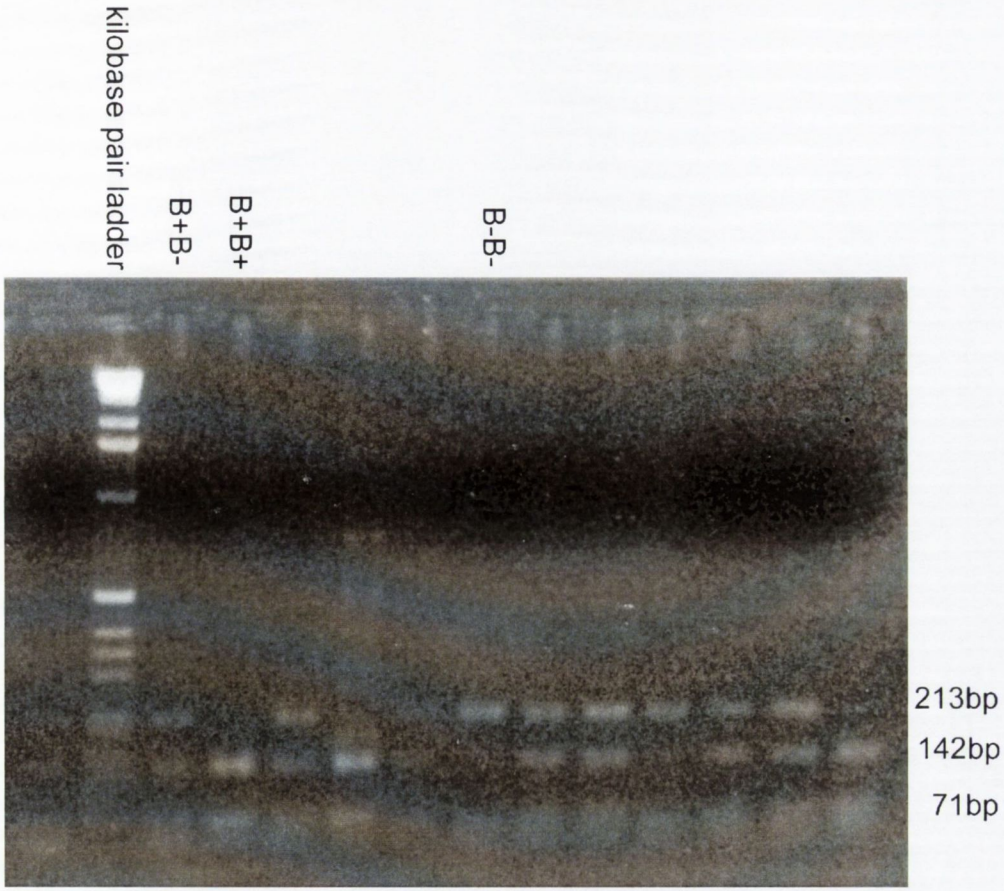


Figure 2.3.4 Photograph of an agarose gel showing the *cetp* TaqIB polymorphism. The PCR amplification fragments were sized using the kilobase ladder (GibcoBRL). Of the 12 DNA samples that were amplified, digested and run on this gel, 3 are B+ homozygotes (B+B+), 7 are heterozygotes (B+B-) and 2 are B- homozygotes (B-B-).

2.3.2 Effect of Genotype on Fasting and Postprandial Lipid Metabolism

The postprandial study was carried out on 82 individuals, subsequent to the genotype analysis. The fasting lipid, lipoprotein and glucose levels and genotype distribution of the normolipidaemic study population (82 individuals) are given in Table 2.3.4. The Arg3611Gln and Asn4311Ser polymorphisms in the *apoB* gene were not included in this study, due to low frequency of one of their alleles. In this population no polymorphisms were detected in the *lcat* gene. Therefore no *lcat* polymorphisms were included in this study.

The fasting and postprandial lipid, lipoprotein and apolipoprotein distributions measured in the 82 individuals were normalised (section 2.2.7), and categorised by genotype. The means of these measurements found in each category were then compared. The values for the genotypic effect on fasting and postprandial lipid and protein levels are reported in Table 2.3.5[1].

2.3.2.1 Apolipoprotein B

Associations were found between the insertion/deletion polymorphism in the *apoB* gene, and postprandial lipid and apolipoprotein levels. Carriers of the D allele had significantly lower body mass indexes (BMI) ($p=0.02$) and a trend towards lower postprandial plasma cholesterol levels ($p=0.09$) than II homozygotes. DD homozygotes had a trend towards having lower apoB48-4hr levels ($p=0.2$) and significantly higher HDL_2-cholesterol levels ($p=0.04$) than II and ID individuals (Table 2.3.5).

2.3.2.2 Apolipoprotein E

The apoE genotypes were divided into three groups consisting of ε2ε2 and ε2ε3 (ε2ε2/ε2ε3) individuals, ε3ε3 individuals and ε3ε4 and ε4ε4 (ε3ε4/ε4ε4) individuals. The two individuals with the ε2ε4 genotype were not included in the analysis. The apoE polymorphism was found to associate with plasma TAG distributions and CETP activity levels. ε3ε4/ε4ε4 individuals had a trend towards having higher fasting, maximum and postprandial (AUC) TAG levels ($p=0.25$, $p=0.1$ and $p=0.11$) than ε2ε2/ε2ε3 and ε3ε3 individuals (Table 2.3.5). The difference became more pronounced between the *apoE* genotypes during the postprandial response. ε2ε2/ε2ε3 individuals had a trend towards having lower CETP activity at fasting ($p=0.08$) than individuals with the other genotypes.

[1] The complete statistical analysis of genotypic effect on fasting and postprandial lipid metabolism can been found in appendix I.

However, no difference in CETP mass was distinguished between the three different groups. At 6 hours postprandial the genotypic effect on CETP activity disappeared (Table 2.3.5). The BMI was significantly lower in individuals with the ε2ε2/ε2ε3 genotype than with ε3ε3 or ε3ε4/ε4ε4 genotypes (p=0.05). ε3ε3 individuals have intermediate BMIs due to their having intermediate fasting and postprandial TAG concentrations.

2.3.2.3 Lipoprotein Lipase

Individuals with H+H-/H-H- (HindIII) and ST/TT (Ser447Ter) genotypes were found to have lower fasting plasma TAG (p=0.02 and p=0.2) and fasting TRL-TAG levels (p=0.03 and p=0.08) than H+H+ and SS individuals, and this difference was significant for the HindIII polymorphism (Table 2.3.5). This TAG effect is not so obvious during the postprandial response. Thus the LPL polymorphisms may only be associated with fasting plasma TAG levels. The associated increase in plasma HDL-cholesterol levels in individuals with low fasting plasma TAG levels was not observed in this population sample. Nor was any genotypic association found between this polymorphism and plasma apoB48 levels either at fasting or postprandially. The *lpl* gene polymorphisms, HindIII and Ser447Ter, are known to be in linkage disequilibrium and some work has been carried out on analysing plasma TAG distribution by haplotype (Humphries *et al.* 1998). When the population is divided into the 3 existing haplotypes (H+S, H-S and H-T) of the HindIII and Ser447Ter polymorphisms and the mean plasma TAG levels are calculated for each of these 3 groups, it seems that the fasting effect (lower plasma TAG levels) is linked to the HindIII polymorphism (Table 2.3.6a). Individuals with both H-S and H-T haplotypes have similar fasting plasma TAG levels, which tend to be lower than the H+S haplotype level (p=0.07 and p=0.06). The same data were then divided by gender and reanalysed. Notably, the male plasma TAG distribution seemed to be significantly linked to the H-T haplotype distribution at fasting (p=0.02) and postprandially (max p=0.01, AUC p=0.01). The female distribution however, was significantly linked only to the H-T haplotype at fasting (p=0.05) (Table 2.3.6b and 2.3.6c). The simple linear regression model (section 2.2.7) was used to test the effect of the HindIII polymorphism (H+H+ or H+H-/H-H-) and the H+S, H-S or H-T haplotypes (whole population and just males) on the relationship between fasting plasma TAG and postprandial plasma TAG levels. The HindIII polymorphism had no significant effect on the relationship between fasting TAG and postprandial TAG levels. However, H+H-/H-H- individuals had lower fasting plasma TAG levels and consistently maintained lower plasma TAG levels than H+H+ individuals throughout the postprandial response. No difference could be observed between the

different haplotypes when analysing the population as a whole. However, when a regression line was plotted depicting the relationship between the HindIII and Ser447Ter polymorphisms and fasting and postprandial plasma TAG levels in the male population with the H+S genotype, the slope of the regression line was observed to be smaller than for either of the other two haplotypes (H+S s=0.14, H-S s=0.27, H-T s=0.27). This implies that H+S males have higher fasting plasma TAG levels than men with other haplotypes, but do not have as great postprandial plasma TAG response. This is directly contradicts the idea that fasting plasma TAG levels are the best predictor of postprandial plasma TAG concentrations (section 1.2.1.3).

2.3.2.4 Cholesteryl Ester Transfer Protein

The intronic polymorphism (TaqIB) in the *cetp* gene was found to be associated with BMI, fasting and postprandial plasma cholesterol, HDL-cholesterol and HDL_3-cholesterol levels. B-B- homozygotes had significantly higher fasting (p=0.04) and a trend towards higher postprandial (p=0.08) plasma cholesterol levels than the B+B+ and B+B- individuals (Table 2.3.5). These individuals also had significantly higher HDL_3-cholesterol levels (p=0.05), which resulted in them having higher HDL-cholesterol levels than B+ homozygotes and heterozygotes. B- carriers had significantly higher BMI levels (p=0.02) than B+ homozygotes, which may be partly due to B-B- individuals having higher plasma cholesterol levels both at fasting and postprandially. A sub-population of 63 individuals had their CETP mass and activity measured. Differences in CETP mass and activity at fasting and postprandially were found, in the case of mass, partly to be associated with the TaqIB polymorphism. Individuals who are B- homozygotes had significantly lower CETP masses at fasting (p=0.04) than B+B+ and B+B- individuals. This effect however, is diminished at 6hrs after the test meal. No association between CETP activity and the TaqIB polymorphism was observed in this study population (Table 2.3.5).

Table 2.3.4 Summary of the study parameters. i.e. fasting plasma lipids and glucose levels of the 82 individuals who partook in the study. In addition, the genotypes of this study population, determined in section 2.3.1 are given.

Parameters of Study Population

	Median (Range)
Number of Males	46
Number of Females	36
Age years (n=82)	33 (19-63)
Body Mass Index kg/m^2 (n=82)	24.175 (20.33-31.17)
Fasting TAG mmol/l (n-81)	0.87 (0.395-3.08)
Fasting TRLTAG mmol/l (n=76)	0.216 (0.0386-0.84)
Fasting TPLTAG mmol/l (n=77)	0.547 (0.199-1.04)
Fasting Cholesterol mmol/l (n=82)	5.0 (2.93-8.05)
Fasting HDL-C mmol/l (n=80)	1.181 (0.691-2.69)
Fasting NEFA mmol/l (n=80)	0.44 (0.11-1.21)
Fasting Glucose mmol/l (n=82)	5.185 (3.77-7.365)
Apolipoprotein B (n=82)	Insertion Deletion Polymorphism
	II 36
	ID 36
	DD 10
	XbaI Polymorphism
	X-X- 18
	X+X- 38
	X+X+ 26
	EcoRI Polymorphism
	R+R+ 56
	R+R- 23
	R-R- 3
Apolipoprotein E (n=82)	ε2ε2 1
	ε2ε3 2
	ε2ε4 2
	ε3ε3 48
	ε3ε4 24
	ε4ε4 5
Lipoprotein Lipase (n=82)	PvuII Polymorphism
	P+P+ 26
	P+P- 45
	P-P- 11
	HindIII Polymorphism
	H+H+ 43
	H+H- 34
	H-H- 5
	Ser447Ter Polymorphism
	SS 71
	ST 10
	TT 1
Cholesteryl ester transfer protein (n=82)	TaqIB polymorphism
	B+B+ 30
	B+B- 42
	B-B- 10

Table 2.3.5 Results of effect of polymorphisms on fasting and postprandial lipid metabolism. The effects of note are reported in this table, all other analysis can be found in appendix[1]. The genotypes have been grouped together to show which genotype was responsible for raising or lowering postprandial lipid or lipoprotein concentrations. Either two-sample-t-tests or ANOVA were used to analyse the data. The probability of the genotypes having the same effect is also given.

Gene Polymorphism		**BMI kg/m^2**		**Fasting TAG mmol/l**		**Maximum TAG mmol/l**	
		Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
Apolipoprotein B							
Insertion/Deletion	II	36	25.05 (24.27-25.83)				
	ID/DD	46	23.77 (23.02-24.52)				
			p=0.0234				
	II/ID						
	DD						
Apolipoprotein E	ε2ε2/ε2ε3	3	21.2 (20.2-22.19)	3	0.9 (0.66-1.28)	3	1.0 (0.68-1.61)
	ε3ε3	48	24.55 (23.86-25.24)	45	0.82 (0.74-0.91)	45	1.14 (1.02-1.28)
	ε3ε4/ε4ε4	29	23.87 (22.96-24.78)	29	0.95 (0.81-1.14)	29	1.38 (1.19-1.61)
			p=0.054		p=0.248		p=0.102
Lipoprotein Lipase							
HindIII	H+H+			41	0.97 (0.87-1.09)		
	H+H-/H-H-			38	0.79 (0.7-0.9)		
					p=0.022		
Ser447Ter	SS			68	0.9 (0.81-0.99)		
	ST/TT			11	0.77 (0.64-0,94)		
					p=0.198		
Cholesteryl ester transfer protein							
TaqIB	B+B+	30	23.43 (22.66-24.19)				
	B+B-/B-B-	52	24.66 (23.96-25.36)				
			p=0.022				
	B+B+/B+B-						
	B-B-						

Table 2.3.5 continued

	Postprandial TAG mmol/l		Fasting TRL-TAG mmol/l		Fasting Cholesterol mmol/l		Postprandial Cholesterol mmol/l	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II							35	42.08 (39.06-44.86)
ID/DD							45	39.02 (36.87-41.22)
								p=0.087
II/ID								
DD								
ε2ε2/ε2ε3	3	6.56 (4.49-10.61)						
ε3ε3	45	7.37 (6.64-8.21)						
ε3ε4/ε4ε4	28	8.85 (7.63-10.41)						
		p=0.113						
H+H+			38	0.27 (0.22-0.33)				
H+H-/H-H-			38	0.2 (0.16-0.24)				
				p=0.034				
SS			65	0.25 (0.21-0.29)				
ST/TT			11	0.17 (0.11-0.24)				
				p=0.077				
B+B+								
B+B-/B-B-								
B+B+/B+B-					72	5.1 (4.87-5.32)	70	39.75 (37.99-41.55)
B-B-					10	5.81 (5.0-6.61)	10	44.6 (39.03-50.54)
						p=0.042		p=0.076

Table 2.3.5 continued

	HDL-C mmol/l		**HDL3-C mmol/l**		**HDL2-C mmol/l**		**ApoB48-4hr**	
	Number	Mean (95% CI)	Number	Median (Range)	Number	Mean (95% CI)	Number	Mean (95% CI)
II ID/DD								
II/ID DD					70 10	0.22 (0.17-0.27) 0.35 (0.24-0.46) p=0.037	69 9	0.51 (0.47-0.56) 0.41 (0.3-0.56) p=0.2
ε2ε2/ε2ε3 ε3ε3 ε3ε4/ε4ε4								
H+H+ H+H-/H-H-								
SS ST/TT								
B+B+ B+B-/B-B-								
B+B+/B+B- B-B-	71 9	1.17 (1.12-1.22) 1.41 (1.15-1.77) p=0.12	71 9	0.94 (0.35-2.62) 1.12 (0.76-1.51) p=0.052				

Table 2.3.5 continued

	CETPmass-0hr		CETPmass-6hr		CETPactivity-0hr		CETPactivity-6hr	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II ID/DD II/ID DD								
ε2ε2/ε2ε3 ε3ε3 ε3ε4/ε4ε4			3 37 22	1.51 (1.14-2.0) 2.23 (2.1-2.4) 2.12 (1.9-2.3) p=0.284	3 37 22	110 (107-113) 153 (148-158) 143.6 (135-152) p=0.078	3 37 22	175.4 (161-191) 180.7 (171-190) 196.3 (182-212) p=0.616
H+H+ H+H-/H-H-								
SS ST/TT								
B+B+ B+B-/B-B- B+B+/B+B- B-B-	 57 6	 2.02 (1.87-2.18) 1.22 (0.85-1.74) p=0.041						

Table 2.3.6a Effect of *lpl* haplotype on TAG metabolism in the study population of 82 individuals. Probabilities of interest are in bold.

POPULATION H+H+	SS		ST/TT	
n	41			
Fasting TAG	0.97 (0.87-1.09)			
Maximum-TAG	1.32 (0.98-1.66)			
Postprandial TAG	8.29 (7.42-9.35)			
H+H-/H-H-				
n	27		11	
Fasting TAG	0.81 (0.69-0.96)	**p=0.074**	0.77 (0.64-0.94)	**p=0.062**
Maximum-TAG	1.08 (0.89-1.34)	p=0.11	1.14 (0.95-1.38)	p=0.2
Postprandial TAG	7.5 (6.36-8.96)	p=0.36	7.17 (6.04-8.65)	p=0.21

Table 2.3.6b Effect of *lpl* haplotype on TAG metabolism in males. Probabilities of interest are in bold

MALE	SS		ST/TT	
H+H+				
n	26			
Fasting TAG	1.24 (1.0-1.49)			
Maximum-TAG	1.71 (1.46-2.04)			
Postprandial TAG	11.24 (9.53-13.42)			
H+H-/H-H-				
n	13		8	
Fasting TAG	1.06 (0.82-1.43)	p=0.36	0.85 (0.69-1.07)	**p=0.024**
Maximum-TAG	1.48 (1.19-1.87)	p=0.31	1.1 (0.91-1.36)	**p=0.006**
Postprandial TAG	9.75 (7.76-12.66)	p=0.36	7.17 (5.95-8.86)	**p=0.0055**

.Table 2.3.6c Effect of *lpl* haplotype on TAG metabolism in females. Probabilities of interest are in bold

FEMALE	SS		ST/TT	
H+H+				
n	17			
Fasting TAG	0.89 (0.75-1.07)			
Maximum-TAG	1.1 (0.93-1.33)			
Postprandial TAG	7.41 (6.37-8.96)			
H+H-/H-H-				
n	14		3	
Fasting TAG	0.74 (0.61-0.91)	p=0.19	0.7 (0.63-0.79)	**p=0.05**
Maximum-TAG	1.02 (1.0-1.72)	p=0.62	1.32 (1.03-1.75)	p=0.32
Postprandial TAG	6.54 (5.26-8.35)	p=0.38	7.94 (7.11-8.87)	p=0.54

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2.4 Discussion

When this research was initiated, very little work previously had been published on the effect of genetic polymorphisms on the postprandial lipid response. However, at the conclusion of the project, several papers in this area had been published. Some had used large population sizes (EARSI cases n=515 controls n=930 and EARSII cases n=332 controls n=342, Humphries *et al.* 1998) while others had studied smaller populations (n=57 men, Byrne *et al.* 1996) comparable to the population size studied during this doctorate (n=82). Because of the relatively small sample size studied in this chapter (n=82) only major effects of polymorphisms on postprandial lipid metabolism were expected to be significant. It was expected that minor effects caused by polymorphisms might appear as trends in the analysis. Studies on larger population sample sizes are more likely to significantly detect small effects on lipid metabolism. However, at the commencement of this study any information on how genotypic variation effected postprandial lipid metabolism would have been novel.

2.4.1.1 Apolipoprotein B

In the *apoB* gene, only the insertion/deletion polymorphism was associated with any variation in the fasting and postprandial lipid and apolipoprotein distributions. The D allele was associated with a lower body mass index (BMI) and postprandial cholesterol concentration, while DD homozygotes had decreased apoB48 levels at 4 hours (apoB48-4hr) after ingesting the test meal and increased fasting HDL_2-cholesterol levels. Other studies had found that the insertion/deletion polymorphism was associated with variation in plasma TAG levels at fasting and postprandially, but this association had been shown to be dependent on the fatty acid components of the test meal (Ordovas *et al.* 1995). When a meal that is high in SFA is administered to a population, individuals who are II tend to have the highest plasma TAG levels, highest concentration of chylomicrons and highest concentration of VLDL particles (Regis-Bailly *et al.* 1995, Peacock *et al.* 1995, Syvanne *et al.* 1997). The test meal used in this study was high in n-6 PUFA, which may have masked any effect the polymorphism may have had on the fasting and postprandial plasma TAG levels. In this study, DD individuals were observed to have a reduction in apoB48-4hr levels, although Peacock *et al.* (1995) found that only one D allele was necessary to reduce the plasma postprandial apoB48 concentration. Byrne *et al.* (1996) had found an association between II individuals, maximum free fatty acid and TRL-8hr levels. The TRL-8 hr concentration, was determined by the maximum free fatty acid concentration in I

homozygotes, but not in DD or ID individuals. This association was not observed in this study, which may possibly be due to Byrnes *et al.* (1996) having used a test meal that was high in SFA and the test meal given in this study was low in SFA. The D allele has been associated with an anti-atherogenic effect, and although in the current study no link was found between the plasma TAG levels and the insertion/deletion polymorphism, the decreased BMI, cholesterol, apoB48 and increased HDL_2-cholesterol levels, which were observed in this study, are all associated with an anti-atherogenic lipid and lipoprotein profile (Roche & Gibney 1995).

2.4.1.2 Apolipoprotein E

Some studies have shown a genotypic effect of the different apoE alleles on TAG levels. Dallongeville *et al.* (1992) concluded that high fasting plasma TAG levels were associated with both the ε2 and ε4 alleles. The Dallongeville paper describes a meta-analysis (analysis of data from different study populations) of 45 population samples, covering populations with obesity, diabetes, CHD and hyperlipidaemia. The EARS study, which uses a study population consisting of descendents of individuals with early-onset CAD, also showed that the ε2 and ε4 alleles were linked to elevated plasma TAG concentrations (Tiret *et al.* 1994). However, the present study found that only ε4 carriers had elevated plasma TAG levels at fasting and postprandially. The increase in postprandial plasma TAG concentration has also been observed in ε4 carrying infants, suggesting that from the beginning of life the ε4 allele may increase atherosclerosis risk (Tammi *et al.* 2000). However, the population sample in this current study only contained 3 subjects with either the ε2ε2 or ε2ε3 genotype. Thus, it may be difficult statistically to observed differences in plasma TAG levels between ε3ε3 individuals and ε2 carriers.

Martin *et al.* (1993) gave men diets containing high and low doses of cholesterol over a 35 day period and found that men with the ε2 allele had increased plasma CETP concentrations. The present study population (Table 2.3.4), consisting of approximately equal numbers of males and females who were regularly consuming normal Western diets, showed that the ε2 allele was not associated with low plasma CETP mass and postprandial activity. If one looks at the data from this study (Table 2.3.5), it is apparent that the ε2 allele appears to associate with lower postprandial CETP mass and activity levels. However, this decrease is not significant, which is probably due to the small sample size (n=63) used in this study. ε2 carriers may be prone to the effects of cholesterol intake than ε3ε3 individuals or ε4 carriers. This may be due to the apoE2 isoform having a reduced

affinity to hepatic apoE receptors (Larson *et al.* 2000). Carriers of the ε2 allele were found to have lower BMI values than ε3 homozygotes or ε4 carriers (Table 2.3.5). This effect is probably due to the fact that the apoE2 isoform is associated with low LDL-cholesterol levels (Larson *et al.* 2000).

2.4.1.3 Lipoprotein Lipase

A third gene that was investigated for its potential role in the postprandial lipid response was the *lpl* gene. When the HindIII and Ser447Ter polymorphisms were examined separately for their effect on fasting and postprandial plasma TAG concentrations, the HindIII polymorphism showed a significant effect. Individuals who were H-H- had lower fasting plasma TAG levels than H+H+ or H+H- individuals. However, this was not so apparent for the postprandial plasma TAG levels. Synvanne *et al.* (1997) previously had observed the link between the H-H- genotype and fasting plasma TAG levels but had not observed a link between the H-H- genotype and lower postprandial plasma TAG levels. The LPL haplotypes H+S, H-S and H-T were shown to be linked with variation in fasting and postprandial plasma TAG concentrations (Humphries *et al.* 1998). Fasting plasma TAG levels seemed to be influenced by the HindIII polymorphism; the H- allele being linked to reduced TAG concentrations. However, when the population was divided by gender only the H-T haplotype was linked to reduced fasting plasma TAG levels (Table 2.3.6a). Although females with the H-T haplotype had reduced fasting plasma TAG levels during the postprandial response no reduction in postprandial plasma TAG levels was observed (Table 2.3.6c). In contrast to females, males with the H-T haplotype maintained lower plasma TAG levels seen at fasting throughout the postprandial response (Table 2.3.6b). Using a simple linear regression model of the effect of fasting plasma TAG levels on postprandial TAG levels, it was demonstrated that males with the H+S haplotype had smaller postprandial responses. This suggests that the H-T haplotype may be required for maintaining low levels of fasting TAG, thus retarding the postprandial TAG response in males. Interestingly fasting plasma TAG levels are thought to be the best predictor of postprandial TAG concentrations (Roche & Gibney 1995), therefore the observation that individuals with the highest fasting plasma TAG concentrations had the smallest increase in plasma TAG concentrations may be an artefact of the study population.

A study by Humphries *et al.* (1998) also showed that the H-T haplotype was associated with reduced fasting plasma TAG levels in both genders. However, that study looked at the postprandial response only in males. Individuals with the H-T haplotype maintained 12% lower TAG levels at fasting and at 4 hours postprandially than individuals with either

the H+S or H-S haplotype. The present study suggests that in females, unlike in males, the H-T haplotype may not be linked to reduced postprandial plasma TAG concentrations. However, as the sample size was extremely small (n=3) this observation may be an artefact of the small sample size. A larger study on females would be necessary to assess if there is indeed a gender difference in how the H-T haplotype influences postprandial plasma TAG levels.

2.4.1.4 Cholesteryl Ester Transfer Protein

In this study possible effects of the TaqIB polymorphism in the *cetp* gene on fasting and 6 hour postprandial CETP mass and activity and HDL-cholesterol concentrations were evaluated. The effects of the TaqIB polymorphism observed in this study were in agreement with the Bernard's *et al.* (1998) study; individuals who were homozygous B-B- had lower fasting CETP mass and higher fasting HDL-cholesterol levels than individuals who were B+B+ or B+B-. Examination of the HDL_2- and HDL_3-cholesterol fractions revealed that the observed increase in HDL-cholesterol concentration in B-B- individuals was not due to increased levels of the anti-atherogenic HDL_2-cholesterol fraction but instead an increase in HDL_3-cholesterol levels. It was expected that the decrease in fasting CETP mass would result in lower CETP activity. Interestingly, this was not observed. Individuals with different TaqIB genotypes had the same CETP activity. Bernard *et al.* (1998) suggested the effects of the TaqIB polymorphism on CETP mass and HDL-cholesterol concentrations may be independent of each other. The TaqIB polymorphism may have both pro-atherogenic and anti-atherogenic effects. Its effect on HDL-cholesterol levels may be pro-atherogenic because the increase in HDL-cholesterol resulted from to an increase in the HDL_3 subfraction. Such an increase is associated with a reduction in the anti-atherogenic HDL_2 subfraction. However in this current study no significant decrease in the HDL_2 fraction was observed. The anti-atherogenic effect, which is linked to the TaqIB polymorphism, was the decrease in fasting CETP mass levels. If lower CETP mass was linked to lower CETP activity this would result in less transfer of TAG from TRL particles to HDL particles. Thus, the HDL particles would not have high TAG concentrations and be removed from the circulation by hepatic lipase. Thus, the concentration of anti-atherogenic HDL_2 particles would not be decreased (section 1.2.2.9).

2.4.2 Conclusion

The aim of this study was to investigate the effects of genetic polymorphisms in five genes involved in all stages of lipid metabolism on plasma lipid, lipoprotein and apolipoprotein concentrations at fasting and postprandially. Firstly 5 polymorphic sites in *apoB*, 3 polymorphic sites in *lpl*, *apoE* polymorphism, the TaqIB polymorphism in *cetp* and 2 possible polymorphic sites in *lcat* were genotyped in a Caucasian population consisting of 119 individuals. The possible polymorphic sites in *lcat*, proved not to be polymorphic in this normolipidaemic population. Linkage disequilibrium was found between the sites in *apoB*, with most recombination having occurred about the EcoRI site. The 3 polymorphisms in *lpl* were found to be very strongly linked, especially to the Ser447Ter site. Lipid, lipoprotein and apolipoprotein variables at fasting and during the postprandial response were measured after a test meal high in n-6 PUFA. The resulting variables were analysed to investigate whether genotypes were associated with the fasting and postprandial lipid and apolipoprotein concentrations in a normolipidaemic population of 82 individuals. The study concluded that the *apoB* signal peptide D allele may be associated with lower BMI, postprandial cholesterol levels, apoB48-4hr concentrations and higher HDL_2-cholesterol levels. The *apoE* ε4 allele was linked to higher fasting and postprandial TAG concentrations and the ε2 allele was linked to lower CETP activity at fasting. Individuals with the H-T haplotype in the *lpl* gene were associated with lower fasting and postprandial plasma TAG concentrations in males, but only fasting TAG concentrations in females. The B- allele of the TaqIB polymorphism in *cetp* was observed to cause an elevation in HDL-cholesterol concentration due to increased HDL_3-cholesterol levels, and was associated with lower fasting CETP mass.

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3 The Effect of Coagulation Factor VII Polymorphisms on the Relationship Between Postprandial Lipaemia and Coagulation Factor VII Activity

3.1 Introduction

3.1.1 Association Between Coagulation Factor VII Activity and Risk of Coronary Heart Disease

Coagulation factor VII (FVII) is a vitamin K-dependent plasma protein produced by the liver. It is the first enzyme involved in the extrinsic pathway of blood coagulation (section 1.4.1) (Nakagaki *et al.* 1991). The extrinsic pathway of the coagulation cascade is stimulated by vascular injury, which causes the release of cytokines such as interleukin-1, which stimulates damaged cells to produce tissue factor on their surfaces. The active form of FVII forms a complex with tissue factor. This complex cleaves factor IX and factor X converting them to their active derivatives and thereby initiating the coagulation pathway that ends with the formation of thrombin (section 1.4.1) (Miller 1998). In plasma the majority (450 ng/ml) of FVII is in the inactive zymogen form (Fair 1983), with only low levels (4 ng/ml) of the active dimer form (Wildgoose *et al.* 1992). The active dimer form is the primer that triggers blood coagulation (Morrissey *et al.* 1993). There are three measures of FVII levels. The first, FVII activity (FVIIc), is a measure of enzymatic activity of the activated form (FVIIa) of the protein and in addition some of the zymogen, which is inadvertently activated during the assay (Mennen *et al.* 1996). The second measure (FVIIa) is a measurement of the quantity of the active dimer form of FVII. The third measure, FVII antigen (FVIIag), is a measure of total FVII concentrations, zymogen and FVIIa (Mennen *et al.* 1996). Historically studies have measured FVIIc levels but more recent assays quantify FVIIa.

In the Northwick Park Heart Study, some of the risk factors involved in causing CHD in middle-aged men were studied. The study demonstrated that high plasma FVIIc appeared to be associated with an increased risk of CHD, especially CHD fatality (Meade *et al.* 1986; Ruddock & Meade, 1994). The apparent increase in FVIIc in CHD patients or risk

groups has been shown to result from an increase in both the FVIIag and FVIIa forms of the protein (Hoffman *et al.* 1988, Hoffman *et al.* 1989, Broadhurst *et al.* 1990, Suzuki *et al.* 1991). Elevated levels of FVII may therefore be a marker for a pro-thrombotic state, which may predispose to increased cardiovascular risk. Tissue factor is a component of atheromatous plaques (Miller 1998), therefore increased FVIIa levels could promote a thrombotic event.

3.1.2 Factor VII Activity and Postprandial Triacylglyceride Concentrations

FVIIc has been shown to be positively associated with total fat intake (Bladbjerg *et al.* 1994), and plasma triacylglyceride concentration (Mitropoulos *et al.* 1989). Therefore following intake of a fat rich meal, plasma TAG levels and FVIIc levels both increase (Miller *et al.* 1991). Individuals with conditions such as hypertriacylglyceridaemia, show a long-term increase in FVIIc rather than an increase in the FVII plasma protein (Scarabin *et al.* 1985). The regulatory mechanism of FVII protein levels during the postprandial response may be such that triacylglycerol-rich lipoproteins (TRL) bind to FVII zymogen and cause cleavage of the single-chain molecule into the double chain FVIIa form (Miller *et al.* 1991).

3.1.3 Variation in FVII Activity Associated with Polymorphisms in the *FVII* Gene

3.1.3.1 R353Q Polymorphism

Polymorphisms in the *FVII* gene have been found to be associated with risk of developing CHD. A substitution of a glutamine (Q) for an arginine (R) at amino acid 353 of the FVII protein has been found to be linked to variation in levels of the plasma FVIIc. The Q allele occurs at a frequency of approximately 0.1 in all European populations studied; therefore approximately 18% of individuals carry the Q allele. Heterozygotes have 20-25% lower FVIIc levels, and QQ homozygotes 40% lower FVIIc and 50% lower FVIIag levels than RR individuals (Green *et al.* 1991; Lane *et al.* 1992; Humphries *et al.* 1994). This implies that the Q allele may result in a reduced risk of developing CHD. Analysis of the postprandial response in hypertriacylglyceridaemic subjects showed that RR homozygotes have a mean increase in FVIIa of 1.76 ng/ml, 6 hours after the meal, and Q carriers have only an increase of 0.60 ng/ml (Silveira *et al.* 1994). Therefore the majority of the observed increase in FVIIa during postprandial lipidaemia is due to the increase found in

RR individuals. Another study looking at postprandial FVII levels found that although Q carriers have lower fasting FVIIc and FVIIa levels than RR individuals, the FVIIc and FVIIa levels increased postprandially irrespective of genotype (Sanders *et al.* 1999). The Q allele is thought to be associated with a decrease in hepatic processing of the protein, destabilisation of secreted protein or a reduction in cleavage of FVII zymogen by TRLs into the FVIIa form (Humphries *et al.* 1994).

3.1.3.2 Decanucleotide Insertion Polymorphism

Another known polymorphism of in *FVII* gene is a decanucleotide insertion (CCT ATA TCC T) in the promoter region (-323). The insertion (10bp) allele also has been shown to be associated with a reduction in FVIIc levels (Humphries *et al.* 1996, de Maat *et al.* 1997). The 0 base pair (0bp) allele has been shown to be in linkage disequilibrium with the R allele (96%), and the 10bp allele to be linked to the Q allele (90%) (Humphries *et al.* 1996). Humphries *et al.* (1996) showed that the 10bp allele was associated with a reduction in FVIIc levels of 16% and the Q allele with an additional 4%. Thus the 20% decrease in FVIIc levels seen in individuals who are RQ heterozygotes may be mostly due to the 10bp insertion polymorphism and not the R353Q polymorphism. When adjustments were made on statistical data for the effects of the Q allele, effects associated with the 10bp allele remained significant. However, after adjustments for the 10bp allele, effects associated with the Q allele were shown to be non-significant, suggesting that the strongest effect on FVIIc levels is due to the decanucleotide polymorphism. This supports the hypothesis that low FVIIc levels seen in Q carriers may be due to reduced production of the secreted protein. De Maat *et al.* (1997) investigated whether the association between the promoter and R353Q polymorphism and FVIIc levels was also present in other populations. An association between the polymorphisms and FVIIc was found in Europeans, Inuit and Gujarati Indians. But only the R353Q polymorphism was associated with FVIIc in an Afrocaribbean population. De Maat *et al.* (1997) also concluded that the promoter polymorphism had the greatest effect on plasma FVIIc.

Previously postprandial studies on FVII levels had been carried out on a hypertriglyceridaemic population and a population genotyped for only the R353Q polymorphism. These studies measured FVIIc levels, which measures FVII activity but some zymogen is activated during the assay and adds to the FVIIc levels measured. As the promoter polymorphism may be more responsible than the R353Q polymorphism for plasma FVII levels in Caucasians, individuals with either the RR0bp0bp or RQ0bp10bp haplotypes were included in this study. The present study therefore investigated the

postprandial increase in FVIIa and FVIIag levels in normotriacylglycerolaemic subjects with either RR0bp0bp or RQ0bp10bp haplotypes. The results from the study discussed below have been published as a short communication in the British Journal of Nutrition (Roche *et al.* 2000).

3.2 Materials and Methods

3.2.1 DNA Samples

DNA was isolation as described previously (section 2.2.2). A population of 80 individuals was genotyped for the decanucleotide insertion and R353Q polymorphisms in the *FVII* gene. The study population consisted of a subset of the population genotyped in chapter 2 (section 2.2.1).

3.2.2 Genotyping the R353Q Polymorphism

Primers used to amplify a 214bp fragment of exon 8 of the *FVII* gene, which included the R353Q polymorphism, were designed from the sequence published by O'Hara *et al.* (1987). The forward (5' gga gta cat gtt ctg tgc cgg 3') and reverse primers (5' gtg gct ctg aga gct 3') were synthesised on a PCR-MATE (Applied Biosystems, 391 DNA synthesizer) oligonucleotide synthesis machine. 25µl PCR reactions contained 1.25mM dATP, dCTP, dGTP, dTTP, 50-100ng of each primer, 6% Mg^{2+} PCR buffer (section 2.2.3), 6 units of Taq polymerase and 1µl of DNA (in Lysis Buffer which contained 15mM PCR buffer and brings Mg^{2+} PCR buffer concentration to 10%). PCR samples were denatured for 7 minute at 94°C. Subsequently, samples underwent 30 cycles of the following: 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute and 12 seconds. Then samples underwent an additional extension step for 5 minutes at 72°C (Hyband, OmniGene). Digestion of 10µl of the PCR product with MspI (New England Biolabs) for 4 hours at 37°C resulted in fragments of 125, 67 and 18 base pairs for the R allele and fragments of 194 and 18 base pairs for the Q allele. DNA fragments could be visualised on 2% agarose gels with 0.005% ethidium bromide using a UV lamp. The restriction fragments were sized using the 100bp DNA ladder (GibcoBRL).

3.2.3 Genotyping the Decanucleotide Insertion Polymorphism

The promoter polymorphism was genotyped as described in Marchetti *et al.* (1993), using primer 1 as the forward primer (5' agg ctc tct tca aat aat tca atc 3') and primer 3 as the reverse primer (5' cgg gct ggc tcc tgg att t 3'). Primers were synthesised by GibcoBRL. PCR reactions were carried out in 50µl volumes, with 20mM Mg^{2+} PCR buffer (section 2.2.3). PCR products were ethanol precipitated to increase the concentration of the PCR product. Each 50µl PCR reaction was added to 7µl 3M Ammonium Acetate and 250µl 100% ethanol (-20°C) and incubated at -20°C for 30 minutes. The PCR product was pelleted by centrifugation at 13,200rpm for 10 minutes and the supernatant was removed. The pellet was washed in 250µl 70% ethanol (-20°C), and centrifuged at 13,200rpm for 5 minutes. The supernatant was removed and pellet dried before being resuspended in 10µl deionised H_2O. PCR products were digested with 1 unit MboI (New England Biolabs) per reaction for 4 hours at 37°C. Digestion products were visualised using a UV lamp on a 3% agarose gel with 0.005% ethidium bromide. The 0bp allele was digested into fragments of 439 and 133bp, whereas the 10bp allele was digested into 439 and 143bp fragments. The fragments were sized using the 100bp DNA ladder (GibcoBRL).

3.2.4 Study Conditions

The Nutrifish study population (section 2.2.5) was used to find 11 heterozygous individuals for the R353Q and insertion polymorphisms, and 11 individuals who were homozygous RR0bp0bp who had equal molar fasting and postprandial plasma TAG levels. During the postprandial response after ingestion of a test meal high in n-6 PUFA, bloods for factor VII analysis were collected into monovettes containing 0.106M sodium citrate at fasting and every 2 hours for 8 hours throughout the response. Blood for lipid analysis was collected as described in section 2.2.5. Fresh blood was immediately centrifuged at 2000g for 15 minutes at room temperature (RT), to separate the plasma layer from the cells. Plasma was harvested, aliquoted into 1.5ml tubes (Starstead), snap frozen in liquid nitrogen and stored at -70°C until FVII analysis was preformed.

3.2.5 Lipid and Factor VII Analysis

Analyses of fasting and postprandial plasma TAG, cholesterol and NEFA concentrations were carried out as described previously (section 2.2.6). Dr. Helen Roche measured levels

of FVII active dimer from (FVIIa), and antigen levels (FVIIag, FVII zymogen and FVIIa) in the 22 individuals. Fasting, 4 hour and 8 hour FVIIa concentrations were analysed, using the Staclot VIIa-rTF kit (Stago Diagnostica) as described by Roche *et al.* (1998). The inter-assay coefficient of variation (CV) was calculated to be 5.4% for 37.5mU/ml FVIIa. The concentration of FVIIag of the fasting, 4 hour and 8 hour time points was determined by enzyme-linked immunoabsorbance assay (ELISA) using a specific rabbit, anti-human factor VII antibody (Stago Diagnostica) (Roche *et al.* 1998). The inter-assay CV was 6.6% for 77.6% FVIIag.

3.2.6 Statistical Analysis

Linkage disequilibrium was calculated between the insertion and R353Q polymorphism by the method described in section 2.2.7. The Data Desk 4.1 package for Apple Macintosh (Data Description Inc.) was used to analyse the data. Prior to analysis, carried out by Dr. Helen Roche, the natural log (ln) was take of the TAG and FVII plasma levels to normalise the data.. The area under the curve (AUC) was calculated as 0hr+2*(2hr+4hr+6hr)+8hr. The incremental area under the curve (IAUC) was calculated as AUC-8*(0hr). Repeated-measures-ANOVA was used to investigate the differences in postprandial FVII responses compared to TAG response in the haplotype groups. Pooled-t-tests were used to investigate the differences in TAG and FVII variables between the haplotype groups (RR0bp0bp and RQ10bp10bp). Repeated-measures-ANOVA is used to analyse data when data is related. For example, fasting TAG concentrations are related to postprandial TAG concentrations. Pooled-t-tests are t-tests were the variances and sample size of two groups are used to calculate the pooled variance and the means of the groups are compared using the pooled variance. This method has the greatest statistical power as the variance is smaller and differences between groups become more significant.

3.3 Results

The present study investigated the relationship between postprandial lipaemia and activation of coagulation FVII in two haplotype groups. In the present study, 11 individuals with the RR0bp0bp haplotype and 11 individuals with the RQ0bp10bp haplotype in the *FVII* gene were identified, who were matched for equal fasting and postprandial plasma TAG concentrations. After identification of the individuals, the

fasting and postprandial FVIIa and FVIIag concentrations were compared between the two haplotype groups.

Genotyping of the two polymorphisms was carried out by PCR amplification followed by restriction digest analysis of the resulting PCR products. Photos of agarose gels with the resulting digested DNA samples are shown in Figure 3.3.1 and Figure 3.3.2. An example of each of the different possible genotypes is depicted. In the 80 Caucasian individuals typed for the R353Q and insertion polymorphisms, the frequencies of the alleles were the following:

Polymorphism	Frequency of Allele 1	Frequency of Allele 2
R353Q	R – 0.894	Q – 0.106
Decanucleotide Insertion	0bp – 0.881	10bp – 0.119

In addition to measuring the allele frequencies of the two polymorphisms, linkage disequilibrium (D') between the polymorphic sites was calculated. D' was calculated to be 0.93 with the R0bp and Q10bp haplotypes occurring more frequently than expected from their individual allele frequencies (observed frequency – expected frequency: 0.875 – 0.788 and 0.1 – 0.013 respectively).

Eleven individuals who were heterozygous for both the Q and 10bp allele (RQ/0bp10bp; consisting of 7 males and 5 females) and eleven individuals who were homozygous for the R and 0bp allele (RR/0bp0bp; consisting of 9 males and 3 females) were matched against each other for equimolar fasting and postprandial TAG levels. Differences in fasting and postprandial plasma lipid concentrations and FVII (FVIIa and FVIIag) levels were assessed between the RR/0bp0bp and RQ/0bp10bp populations (Table 3.3.1). No significant difference in age, weight and plasma lipid concentrations was found between the groups, with the exception of plasma cholesterol and LDL-cholesterol concentrations which were significantly higher in the RR/0bp0bp population than in the RQ/0bp10bp population ($p=0.01$ and $p=0.02$ respectively). The two populations had previously been selected to exhibit similar plasma TAG and TRL-TAG concentrations. FVII concentrations were found to vary between the haplotype groups. RR/0bp0bp individuals had higher fasting concentrations of FVIIa and FVIIag than RQ/0bp10bp individuals ($p=0.0001$ and $p=0.0001$ respectively). In addition, the ratio of fasting plasma FVIIa to FVIIag (FVIIa:FVIIag) was significantly higher in the RR/0bp0bp group than in the RQ/0bp10bp group ($p=0.0001$). RR/0bp0bp individuals had larger postprandial FVIIa and FVIIag area under the curve (AUC) levels than RQ/0bp10bp individuals ($p=0.0001$ and

p=0.003 respectively). The plasma FVIIa and FVIIag incremental area under the curve (IAUC) tended to be larger in the RR/0bp0bp group than the other population (p=0.09 and p=0.06 respectively).

The levels of FVIIa and the ratio of FVIIa:FVIIag during the postprandial response are shown for both populations in Figure 3.3.3. The RR/0bp0bp group showed an increase in levels of both FVIIa and the ratio of FVIIa:FVIIag during the postprandial response. RQ/0bp10bp individuals' levels of FVIIag and FVIIa did not increase during the postprandial response (Figure 3.3.3). Repeat measures ANOVA was used to express the differences between groups comparing their plasma TAG, FVIIa and FVIIa:FVIIag levels. The FVIIa concentrations varied significantly between the two groups, and the genotype by time interaction approached significance (p=0.0678). Again the FVIIa:FVIIag ratio was significantly lower in the RQ/0bp10bp group than the RR/0bp0bp group (p=0.0003), and the genotype by time interaction also approached significance (p=0.0574).

The Pearson correlation coefficient, which is a summary variable attained for linearly related data, revealed a linear relationship between fasting and postprandial plasma TAG concentrations, and fasting and postprandial FVIIa concentrations (r=0.611, p=0.035 RR/0bp0bp fasting; r=0.587, p=0.045 RR/0bp0bp postprandial) in the RR/0bp0bp group only. Therefore, for RR/0bp0bp individuals fasting and postprandial plasma TAG and FVIIa levels are linearly related.

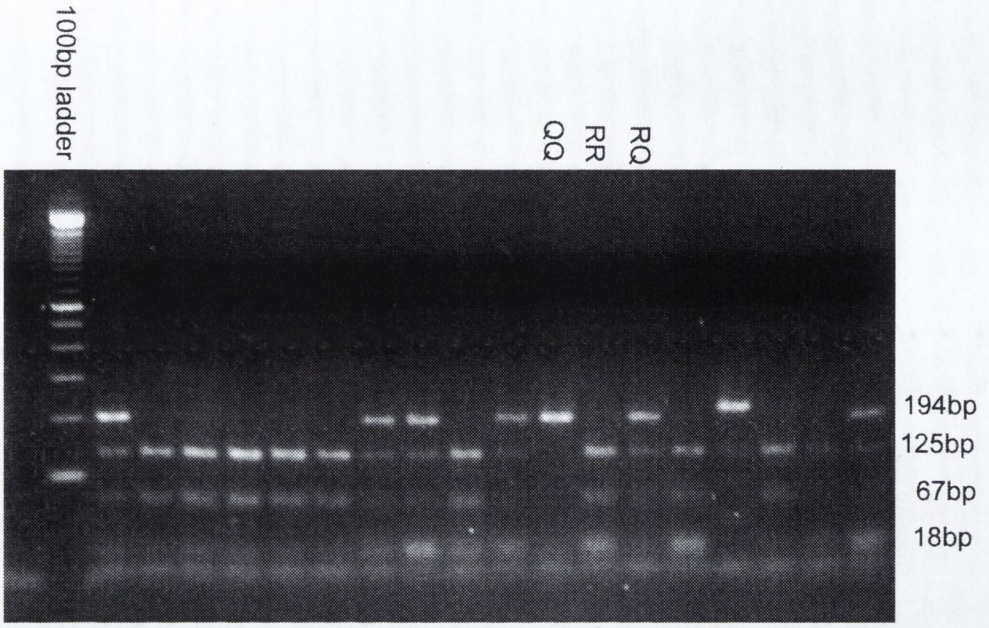


Figure 3.3.1 Photograph of 18 individuals from a total of 80 individuals genotyped for the R353Q polymorphism. 10 individuals are RR (R allele 125bp, 67bp & 18bp), 2 individuals are QQ (Q allele 194bp & 18bp) and 6 individuals are RQ.

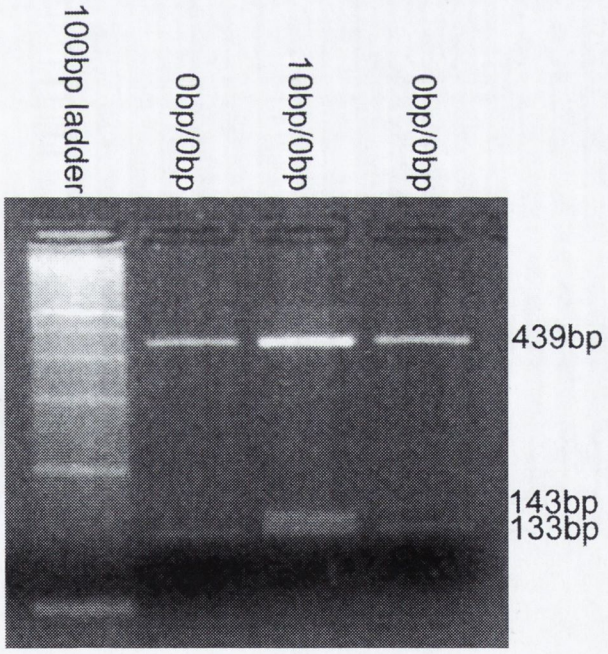


Figure 3.3.2 Photograph of 3 individuals from a total of 80 genotyped for the decanucleotide promoter polymorphism, 2 individuals are 0bp0bp (0bp allele 439bp & 133bp) and one is a heterozygote, 10bp0bp (10bp allele 439bp & 143bp).

Table 3.3.1 Mean and standard deviations (std) of age, body weight, fasting and postprandial lipid concentrations and factor VII levels in RR/0bp0bp and RQ/0bp10bp individuals (Roche *et al.* 2000). A comparison is made between the two haplotype groups in the last column. Std = standard deviation, NS = non-significant and mU = milliunits.

	RR/0bp0bp (n=11) Mean (std)	**RQ/0bp10bp** (n=11) Mean (std)	**Significance (P)**
Age (years)	35.25 (8.93)	29 (9.35)	NS
Weight (kg)	82.46 (17.12)	73.18 (11.47)	NS
Fasting variables			
Plasma TAG (mmol/l)	0.99 (0.41)	0.98 (0.41)	NS
TRL TAG (mmol/l)	0.6 (0.32)	0.56 (0.2)	NS
Plasma cholesterol (mmol/l)	5.7 (0.94)	4.69 (0.8)	P=0.01
LDL-cholesterol (mmol/l)	4.29 (0.85)	3.41 (0.81)	P=0.02
Plasma NEFA (mmol/l)	0.51 (0.2)	0.45 (0.16)	NS
FVIIa (mU/ml)	43 (4.8)	23.9 (6.5)	P=0.0001
FVIIag (%)	85.7 (5.4)	71.6 (7.7)	P=0.0001
FVIIa:FVIIag	0.5 (0.05)	0.33 (0.08)	P=0.0001
Postprandial variables			
TAG-max (mmol/l)	1.39 (0.51)	1.46 (0.55)	NS
TAG-AUC (mmol/l.8hr)	8.97 (3.43)	9.35 (3.68)	NS
TRL TAG-AUC (mmol/l.8hr)	5.785 (3.02)	6.31 (3.15)	NS
FVIIa-AUC (mU/ml.8hr)	396.4 (74.7)	194 (58.4)	P=0.0001
FVIIa-IAUC (mU/ml.8hr)	39.2 (57.89)	2.3 (41.89)	P=0.0877
FVIIag-AUC (%.8hr)	666.5 (35.89)	581.3 (79.2)	P=0.0026
FVIIag-IAUC (%.8hr)	-19.0 (28.9)	8.3 (38.8)	P=0.0637
FVIIa:FVIIag-AUC	2.04 (0.35)	1.2 (0.26)	P=0.0001

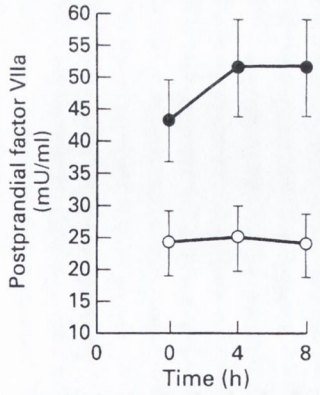


Figure 3.3.3a

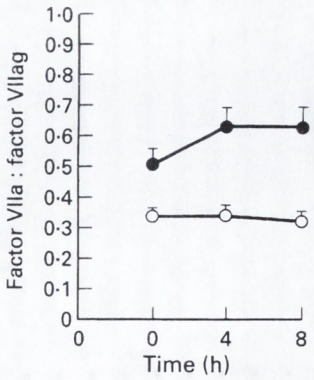


Figure 3.3.3b

Figure 3.3.3 "(a) Postprandial activated factor VII (FVIIa) concentrations and (b) the ratio of FVIIa to FVIIag in subjects with the Q/10bp (○) haplotype and R/0bp (●) haplotype following ingestion of an n-6 PUFA rich test meal. Values are untransformed group means for eleven subjects, with their standard errors represented by vertical bars." (Roche *et al.* 2000)

3.4 Discussion

This study investigated effects of two polymorphisms in the *FVII* gene, a decanucleotide insertion in the promoter region and R353Q polymorphism, on postprandial FVIIa and FVIIag levels. First, the allele frequencies of the polymorphisms were determined and the possibility of linkage disequilibrium (D') between the alleles was tested. Similar allele frequencies were found in this study population consisting of 80 Caucasians, to other published populations. In our study the frequencies of the Q and 10bp allele were 0.106 and 0.119 respectively. Bernardi *et al.* (1997) had found frequencies of 0.12 and 0.14 respectively in European populations (n=480). De Maat *et al.* (1997) found allele frequencies of 0.1 (Q) and 0.14 (10bp) also in a European population but the frequencies varied in other populations. An Inuit population had allele frequencies of 0.29 (Q) and 0.32 (10bp), an Afrocaribbean population had allele frequencies of 0.09 (Q) and 0.33 (10bp) and a Gujarati Indian population had allele frequencies of 0.26 (Q) and 0.24 (10bp) (de Maat *et al.* 1997). Previously Humphries *et al.* (1996) had found 3 major linkage groups in a study population consisting of approximately 760 British men; he found haplotypes R/0bp, R/10bp and Q/10bp at frequencies of 0.881, 0.014 and 0.103 respectively. In this study population the R/0bp and Q/10bp linkage groups were found a frequencies of 0.875 and 0.1 respectively.

This study showed variation in fasting plasma FVIIa and FVIIag levels between the haplotype groups. RQ/0bp10bp individuals had 44.3% lower plasma FVIIa concentrations and 16.4% lower plasma FVIIag concentrations than RR/0bp0bp individuals. Bernardi *et*

al. (1997) had demonstrated that Q/10bp individuals had 39% lower FVIIa and 4.9% lower FVIIag levels. Despite identical postprandial plasma TAG responses, postprandial plasma FVIIa levels and FVIIa:FVIIag ratios were significantly increased in RR/0bp0bp individuals but not in RQ/0bp10bp individuals. It has previously been shown that the decanucleotide polymorphism may have more of an effect on plasma FVIIc levels than the R353Q polymorphism (Humphries *et al.* 1996). However, the two polymorphisms are thought to cooperate in reducing fasting plasma FVIIc levels. When investigating the haplotype groups the decanucleotide polymorphism was found to account for a 16% reduction in FVIIc levels while the R353Q polymorphism accounted for a 4% reduction in FVIIc concentrations (Humphries *et al.* 1996). Therefore, in the RQ/0bp10bp group both the Q allele and 10bp allele may be cooperating in maintaining plasma FVIIa and FVIIa:FVIIag ratio levels at baseline during the postprandial TAG response. In European, Inuit and Gujarti Indian populations again the decanucleotide polymorphism was shown to have the greatest effect in determining plasma FVIIc levels. However, in an Afrocarribean population only the R353Q polymorphism was associated with a reduction in plasma FVIIc concentrations (de Maat *et al.* 1997).

FVIIag levels were lower in the RQ/0bp10bp group than the RR/0bp0bp group; both at fasting ($p=0.0001$) and postprandially ($p=0.0026$). One hypothesis that explains this result is that TRL may in some unknown manner enhance the rate of 0bp (but not 10bp) *FVII* transcription by interacting with the *FVII* 0bp promoter. This hypothesis has been partially substantiated by transactivation studies were *FVII* promoters with the 10bp insertion were shown to be 30% less active than 0bp promoters (Pollak *et al.* 1996). *In vitro* studies in COS-1 monkey kidney cells transiently transfected with *FVII* cDNA containing the R353Q polymorphism, have shown that the Q allele is linked to reduced secretion levels of FVIIag from these cells (Hunault *et al.* 1997). This reduced secretion of Q allele proteins may also occur in hepatocytes. Reduced transcription and secretion of FVII may account for the reduced FVIIag levels observed in Q/10bp individuals.

In this study the proportion of FVIIa to FVIIag was found to be lower in the RQ/0bp10bp group than the RR/0bp0bp group, suggesting that activation rate of the zymogen was decreased in the RQ/0bp10bp group. TRL possibly could stimulate the conversion of FVII zymogen to the active FVIIa form, probably due to hydrolysis of TAG by LPL (Marckmann *et al.* 1998). The Q polymorphic variant may affect the conformation and reactivity of the zymogen and thus reduce the ability of TRL to activate FVII. The concentration of TRL in plasma may also effect whether the FVII zymogen is activated. In

a hypertriacylglyerolaemic population given a high fat test meal (high TRL levels) the linear postprandial response of individuals with the Q allele was more than 50% smaller than the response of R homozygotes (Silveira *et al.* 1994). In the present study, normolipideamic RQ/0bp10bp individuals did not initiate the activation of FVII zymogen. Sanders *et al.* (1999) found that both RR and RQ individuals had increased FVIIa levels postprandially. However, the postprandial TAG concentrations in that study were greater than those found in this study. Thus, the Q zymogen may be less easily activated than the R zymogen, but this difference may be dependent on plasma TAG concentrations. Thus, the Q allele may cause decreased FVIIag secretion from hepatocytes, along with resisting the effect of TRL to convert FVIIag into FVIIa. With the 10bp allele also being associated with a reduction in *FVII* mRNA levels irrespective of TRL concentration. Therefore, individuals with the Q/10bp haplotype may have reduced risk of atherosclerosis.

3.4.1 Conclusion

The present study set out to investigate the apparent relationship between postprandial TAG concentrations and FVII activation in normolipidaemic individuals with either the RR/0bp0bp or RQ/0bp10bp haplotype. A population of 80 Caucasian individuals was genotyped for the decanucleotide insertion and R353Q polymorphisms and from the population 11 RR/0bp0bp and 11 RQ/0bp10bp individuals who had similar fasting and postprandial TAG levels were identified. The concentrations of FVIIag and FVIIa and the ratio of FVIIa:FVIIag were compared at fasting and postprandially between the two groups. The outcome showed that individuals with the RR/0bp0bp haplotype had higher levels of FVIIag and FVIIa at fasting, and these concentrations increased linearly during the postprandial response. However, RQ/0bp10bp individuals had 44.3% lower FVIIa and 16.4% lower FVIIag concentrations than RR/0bp0bp individuals at fasting and these levels did not increase during the postprandial response. It is hypothesised that the 10bp allele of the decanucleotide promoter polymorphism may be transcribed less efficiently than the 0bp allele, and the FVII protein with the Q allele of the R353Q polymorphism may be secreted less efficiently from hepatocytes than FVII proteins with the R allele. In addition, TRL may convert FVII zymogen with the Q allele into FVIIa less efficiently than the R zymogen.

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4 Characterisation of the Growth of Caco-2 Cells Under Chronic Fatty Acid Supplementation

4.1 Introduction

Postprandial lipaemia is affected by environmental conditions; therefore it was decided to use a cell line to investigate the molecular effects of fatty acid treatments on lipid (TAG) secretion. The colon carcinoma cell line (Caco-2) previously has been used as a cell model system for the study of acute fatty acid effects on lipid and lipoprotein synthesis and secretion (section 1.5.1). Nothing however was known about chronic fatty acid effects. An experiment was conceived to investigate effects of acute fatty acid treatment on Caco-2 cells that had been grown chronically in the presence of a range of fatty acid supplementations. This experiment will be discussed in chapter 5. In this chapter the ability of Caco-2 cells to grown and develop in various concentrations of fatty acid supplemented media was determined. Notably, it was also necessary to discover at what concentration of fatty acid supplementation the cell lipid component composition was altered. Therefore, in this chapter the growth and development of Caco-2 cells, treated chronically with various fatty acids was characterised.

4.1.1 Characteristics of the Colon Carcinoma Cell Line

The Caco-2 cell line was isolated from a colon adenocarcinoma in a 72-year-old male (Fogh *et al.* 1975). This cell line is used as a model of the human small intestine because it has been shown to spontaneously differentiate in cell culture and display some morphological and physiological characteristics of the small intestine. Caco-2 cells have 3 stages of differentiation: homogenously undifferentiated (pre-confluent), heterogeneously differentiated with cellular morphology and ultrastructural microvilli organisation (0-20 days post-confluent) and homogenously polarised and differentiated (21-30 days post-confluent) (Levy *et al.* 1995). The supports that Caco-2 cells are grown on have been found to affect cell differentiation. When the cells are grown on filters, which separate the media that the apical and basolateral surfaces of the cells are grown in, they differentiate more rapidly and produce a higher ratio of apoB48 to apoB100 than Caco-2 cells grown on

plastic (Levy *et al.* 1995). High cell passage number was found to affect the cell morphology of Caco-2 cells (Yu *et al.* 1997). Cells at passage 93-108 show a reduction in the expression of microvilli enzymes and transport proteins and less morphological heterogeneity than cells at passage 28-36. Therefore, confluent, low passage number Caco-2 cells that have been grown on filters are more similar to enterocytes than confluent, high passage number Caco-2 cells that have been grown on plastic.

Confluent Caco-2 cells express liver-fatty acid binding protein (L-FABP) for the uptake of apical dietary long-chain fatty acids (C>10:0). The long-chain fatty acids are synthesised into TAG by the glycerol 3-phosphate pathway (section 1.2.2.2) and assembled into VLDL, LDL and HDL density particles for secretion. The monoacylglycerol pathway (section 1.2.2.2) is not active in Caco-2 cells, although it is the dominant pathway in intestinal cells (Levy *et al.* 1995). In adults the intestine produces the apoB48 form of apoB, but Caco-2 cells have been found to produce both apoB100 and apoB48 in approximately equal quantities depending on the growth support (discussed above). Caco-2 cells appear to behave more like enterocytes of a 15 weeks gestational human foetus. The enterocytes of a 15 week gestational human foetus produce equal quantities of apoB100 and apoB48 (Pinto *et al.* 1983). Caco-2 cells have been shown to produce apoB100, apoB48, apoE, apoA-1, apoA-IV and apoC-III. ApoB100, apoB48, other major apolipoproteins, and 40% of the TAG are secreted in the 1.006g/ml TRL density fraction. ApoB100, apoE and 50% of the TAG are secreted in the 1.063g/ml (LDL) density fraction. The HDL density fraction (1.21g/ml) contains apoA-I and apoA-IV.

For the reasons of similarity of morphology, physiology and biochemistry Caco-2 cells were used as a cell model of the human intestine.

4.1.2 Characterisation of the Colon Carcinoma Cell Line

The aim of the study outlined in this chapter has been to characterise Caco-2 cells that were chronically supplemented with three different fatty acids and grown on filters (Transwells, Costar). The Caco-2 cells supplemented with the fatty acids were used as models of the human intestine after ingestion of diets high in different fatty acids. Much is known about the effect of acute (24hr) doses of fatty acid on lipoprotein and lipid synthesis and secretion by Caco-2 cells. However, the effects of low doses of fatty acids over a long-time have not previously been examined in Caco-2 cells. Long-term low dose

supplementation with fish oils in humans is known to reduce TAG concentrations comparable to ingestion of a fish oil test meal (Roche & Gibney 1996).

The fatty acids investigated in this study were palmitic acid (PA; C16:0) to simulate a diet high in animal fat, oleic acid (OA; C18:1) to stimulate a diet high in rapeseed or olive oil and eicosapentaenoic acid (EPA; C20:5n-3). EPA was added to Caco-2 cells to simulate a diet high in fish oil because it has been shown to be the active component of fish oils, which are known to have a hypotriglycerolaemic affect in animal models (Rambjor *et al.* 1996). Initial characterisation of the cells involved taking a number of measurements. Firstly, their transepithelial electrical resistance (TEER) and translocation of intra- and paracellular markers, which are both measures of cell confluency and tight junction formation, were measured (based on protocols in Claire Meaney's PhD. Thesis). Secondly, cell viability (based on protocols in Claire Meaney's PhD. Thesis) and proliferation were measured to ensure the health of the cells. Thirdly, cells were tested for their ability to differentiate. Caco-2 cells have been found to express sucrase-isomaltase, which is a well-characterised marker for functional differentiation of enterocytes (Green *et al.* 1987). A paper by van Beers *et al.* (1995) showed that in Caco-2 cells the biosynthesis and activity of lactase and sucrase-isomaltase are regulated at the mRNA level. There is a peak of lactase mRNA levels at day 8 post-seeding which subsequently falls dramatically, and a peak of sucrase-isomaltase mRNA from day 11 to 21.

The percentage of each fatty acid in TAG and PL isolated from cells which either had or had not been given fatty acid supplements was analysed. The fatty acid composition of TAG is known to be effected by the dietary fatty acid intake; that is the last feed given before measurement. The fatty acid composition of PL is known not to be effected by dietary fat intake. It is thought that the PL fatty acid composition may be effected by previous feeds of fatty acids (section 1.2.2.3) (Luchoomun & Hussain, 1999).

4.2 Materials & Methods

4.2.1 Caco-2 Cell Culturing

Adherent colon carcinoma cell line (Caco-2) was grown from passage 33 to 47. Cells were grown in media (consisting of Dulbecco's Modified Eagle Medium (DMEM; GibcoBRL) (High Glucose, with Glutimax and 4500mg/l D-Glucose), 10% foetal calf serum (FCS; GibcoBRL), 1X sodium pyruvate (GibcoBRL) and 0.1mg/ml gentimycin (GibcoBRL)).

Caco-2 cells were grown in $75cm^2$ or $125cm^2$ flasks (Falcon or Nunc) until confluence was attained. Cells were then harvested by aspirating off media and adding 1.5ml trypsin (GibcoBRL) (per $75cm^2$ flask) or 2.5ml trypsin (per $125cm^2$ flask). Cells with trypsin were incubated at 37°C for 5-10 minutes. Flasks were tapped gently to loosen cells and the cells were washed off with media to a volume of 10ml per flask. The cells were transferred to a 25ml Sterilin tube or 50ml Falcon tube. 10µl of cells and 10µl of trypan blue (GibcoBRL) were mixed and 10µl loaded onto a haemocytometer. The cells were counted under a light microscope. The tube containing the cells was centrifuged at 1000rpm for 5 minutes to pellet the cells. The supernatant was removed and the pelleted cells were resuspended in 1ml media per flask and added to seeding apical media. Cells that had been grown in the flasks were seeded in 24mm polycarbonate transwells (Costar) at a density of $3x10^5$ cells per well for experimentation unless otherwise stated. 1.5ml of cells was added to the apical compartment and 2.5ml of media to the basolateral compartment (Figure 4.2.1). The polycarbonate membrane had an area of $4.7cm^2$ and pore size of 0.4µm. Caco-2 cells are known to differentiate on this membrane (Levy *et al.* 1995). Cells were fed every 2 days with fresh media or media containing fatty acids until they were harvested.

4.2.2 Fatty Acid Supplementation

5,8,11,14,17-eicosapentaenoic acid (EPA), 9-octadecenoic acid (OA) and sodium hexadecanoic acid (Na^+PA) were stored at -20°C in ethanol at concentrations of 50mM, 500mM and 5mM respectively. Fatty acids were acquired from Sigma. Before use, EPA and OA had sodium ions added to them to make them soluble in media (van Greevenbroek *et al.* 1995) in the following way. EPA was added to 6 times the volume of 100mM NaOH (in ethanol), and OA was added to 60 times the volume of 100mM NaOH. An aliquot of Na^+PA comparable to the aliquots taken of EPA and OA was also taken. Fatty acids were then dried down (Savant, DNA Speed Vac). A 30mM fatty acid solution was made in deionised H_2O. Fatty acids were incubated in boiling water for 2 minutes, to solubilise them, before adding them to the apical cell culture media (37°C). Four concentrations of fatty acid in media were made: 0.5mM, 0.25mM, 0.1mM and 0.05mM. If media without FCS was used, bovine serum albumin (BSA; Sigma) was added to a concentration of 0.5% for the following reason. The serum long-chain fatty acid concentration appears to be equal to the serum fatty acid binding protein albumin in mammals (found in FCS), and fatty acid translocation into the cells may be dependent on a fatty acid albumin complex

being formed (McArthur *et al.* 1999). Therefore, it was assumed there was sufficient albumin in the FCS to bind the supplementary fatty acids, but when no FCS was added to the cells, BSA was included to bind the fatty acids and facilitate fatty acid uptake by the cell. The fatty acid supplement was delivered to the apical compartment of the transwells, while the basolateral compartment did not receive fatty acid supplementation (Figure 4.2.1). This was done to mimic the intestine, where dietary fats are found apically and processed fats are found basolaterally to the enterocytes.

4.2.3 Transepithelial Electrical Resistance

The transepithelial electrical resistance (TEER) is a measure of cell growth; younger cells have lower TEERs than older cells. This is because the TEER measures the resistance between cells. Cells that are confluent will have formed tight junctions and so have reached the TEER plateau. The TEER was measured using an EVOM epithelial voltohmmeter and STX2 electrode (World Precision Instruments). The electrode was sterilised for 20 minutes before use in 70% ethanol, and rinsed in media before measuring each transwell. A potential of 199mV was applied between the apical and basolateral compartments of the transwell and the resulting resistance (Ω) was measured (0-1999Ω). The electrode was placed over the 'pipette tip access port' (Figure 4.2.1). The TEER of the cells was measured every 2 days before the media was changed.

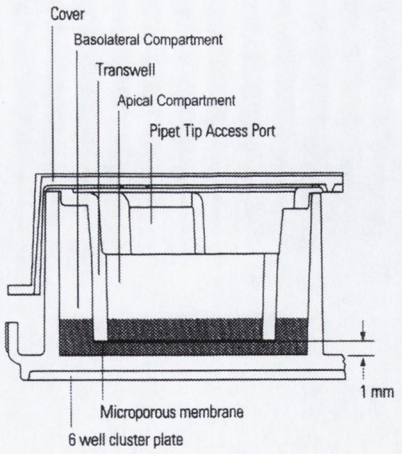


Figure 4.2.1 24mm Tranwell (Adapted from the Transwell Selection Guide, Costar)

4.2.4 Viability (MTT) Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide; thiazolyl blue (MTT, Sigma) is a tetrazolium salt cleaved by mitochondrial dehydrogenase to give a product, which can be measured with a dual filter on an ELISA plate reader at λ=490nm and λ=570nm.

An MTT solution of 5mg/ml in 1X PBS (GibcoBRL) was made and stored at 4°C. Cells were seeded at a density of $4x10^4$ cells/well, in a 96 well microtitre plate (Falcon) and grown for between 17-21 days. Each experiment was repeated eight times. To assess cell viability with long-term fatty acid supplementation (chronic treatment), cells were grown in media containing low levels of fatty acids (section 4.2.2). Cells given media without fatty acid supplementation were used as a control. Subsequently, high levels of fatty acids, in media without FCS, were given to cells grown in media alone over a 24 hour period to supply an acute fatty acid treatment. The media was changed every second day over the course of cell growth.

For experimentation 180µl of media or fatty acid treatment was added to cells and cells were incubated for 24 hours. Subsequently, 20µl of MTT solution was added to the media and the cells were incubated for an additional 4 hours at 37°C. 100µl of 10% SDS, 0.01M HCl was then added to the cells, which were then incubated overnight at 37°C before the absorbance was read at λ=490 and λ= 570nm (Elisa Plate Reader, Dynatech MR5000). By calculating means and standard deviations of results obtained from the 8 wells of cells given similar treatment (control, chronic or acute), and comparing cells given fatty acid treatment to those grown in media alone, the viability of the treated cells could be assessed. Two-sample-t-tests against the control were used to assess viability, with the null hypothesis that viability was not effected by supplementation. The null hypothesis was rejected if the viability of the cells was lower than 95% of the control.

4.2.5 Proliferation (MTS) Assay

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) was used to test the proliferation of cells under different fatty acid treatments. MTS is a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-3-(3-carbozymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), which is converted to formazan by

dehydrogenase enzymes found in metabolically active cells. The absorbance of formazan is read at λ=490nm.

20ml MTS and 1ml of an electron coupling agent, phenazine methosulfate (PMS) were mixed on arrival and stored at -20°C. Cells were seeded at 10^5 cells/well in 96 well microtitre plates (Falcon) and grown for 24 hours in control media or media supplemented with chronic fatty acids (0.1mM and 0.05mM). Each experiment was repeated eight times. To test the proliferation of the cells, 20µl of MTS/PMS solution was then added to each well and the cells were incubated for 90 minutes at 37°C. The absorbance at λ=490nm was read using an ELISA plate reader (Dynatech MR5000). Whether test cells had proliferated more than control cells grown in media alone was assessed using means and standard deviations. Two-sample-t-tests against the control were used to test for differences in the proliferation rate.

4.2.6 Translocation Assay

The permeability to macromolecules of the cells, on the polycarbonate membrane at 6 and 14 days post-seeding was assessed, in order to determine whether the cells had reached confluence and tight junctions had formed between cells. Caco-2 cells need to reach confluence before they can differentiate into enterocyte-like epithelial cells (Levy *et al.* 1995). Two radioactive compounds were used; the first was a lipophilic intracellular marker, ^{3}H-propranolol (15-30Ci/mmol, Amersham) and the second a paracellular marker, ^{14}C-mannitol (50-62mCi/mmol, Amersham).

Cells were seeded on 4.7cm^2 transwells (Costar) at a density of $3x10^5$ cells/well and grown for either 6 or 14 days with or without the addition of fatty acids. Two concentrations (either 0.1mM or 0.05mM) of EPA, OA or PA were used as treatments. At day 6 or 14 post-seeding the media was removed, and the cells washed in 1X PBS (GibcoBRL). Subsequently, cells were allowed to equilibrate in Hanks Balanced Salt Solution (HBSS; GibcoBRL) for 30 minutes at 37°C. The HBSS was removed and 1.5ml of HBSS, containing both ^{3}H-propranolol and ^{14}C-mannitol, each emitting 150,000 disintegrations per minute (DPM), was added to the apical compartment of the transwell. A number of six well plates (Falcon or Nunc) were set up containing 2.5ml HBSS in each well (one transwell per 6 well plate). The transwell was placed in the first well of a 6 well plate for 15 minute time point. Then the transwell was transferred to the second well for 15 minutes (30 minute time point) and so on until time points of 15, 30, 45, 60, 90 and 120 minutes

had been obtained. The plate was incubated at 37°C between time points. To assess the translocation of the intracellular and paracellular markers, 1ml of basolateral HBSS was removed from each well of the 6 well plates to vials containing 10ml of scintillation fluid (Lumagel safe, Lumac.LSC, Packard). Vials were counted for 10 minutes at radioactivity windows of 0.0 to 12.0 for ^{3}H and 12.0 to 156 for ^{14}C in a liquid scintillation counter (Packard, TriCarb 2100Tr, Liquid Scintillation Analyzer). The translocation assay was carried out approximately 5 times per treatment. The apparent permeability coefficient (P_{app}) was calculated from the following formula, were dQ/dt is the slope of the graph of the DPMs versus time in seconds, A is the area of the transwell (4.7cm^2) and C_o is the initial DPMs (150,000 DPM) added to the transwell.

$$P_{app}\ (cm.sec) = \frac{dQ/dt\ (sec)}{A(cm^2).C_o}$$

The P_{app} of the Caco-2 cells treated with each fatty acid (0.1mM EPA, 0.05mM EPA, 0.1mM OA, 0.05mM OA, 0.1mM PA and 0.05mM PA) was compared using means and standard deviations.

4.2.7 Cell Differentiation

The cells were assessed for differentiation at 3, 7 and 14 days post-seeding, using the Northern Blot technique with cDNA probes for human lactase-phlorizin hydrolase and sucrase-isomaltase. At day 3 and 7 post-seeding one expects to observe equal levels of lactase and sucrase-isomaltase mRNA, but at day 14 post-seeding one expects to find more sucrase-isomaltase than lactase mRNA present in the cells (section 4.1.2)

4.2.7.1 Isolation of mRNA

Cells were seeded at a density of 7.5x10^5 cells per 24mm transwell and grown for 7 days. To harvest the cells they were washed with 1X PBS (4°C). Then 200µl of 1X PBS (4°C) was added to each well and the cells, scraped from two wells, were pooled into one RNase-free eppendorf (Robbins Scientific). Cells were pelleted and the supernatant removed. 250µl of guanidinium thiocyanate solution (4M guanidinium thiocyanate, 5mM EDTA, 25mM Citrate, 0.5% Sarcosyl, in DEPC-H_2O) and 3.55µl of β-mercaptoethanol (Sigma) were added to the cells, which were vortexed and left overnight at 4°C. The next day 25µl

2M sodium acetate pH 4.0, 250µl DEPC-H_2O-saturated phenol (100g Phenol plus 100ml DEPC-H_2O) and 100µl chloroform:isoamyl alcohol (49:1) were added to the cells which were mixed gently. The cells were left on ice for 30 minutes and then centrifuged at 13,200rpm for 20 minutes. The supernatant was transferred to a new tube and 1µl of glycogen (GibcoBRL) and 500µl of isopropanol were added. The solutions were mixed and left at -20°C for 2 hours before being centrifuged at 13,200rpm for 30 minutes. The supernatant was discarded and pellet washed with 75% ethanol-DEPC-H_2O, and then centrifuged at 13,200rpm for 5 minutes. The supernatant was discarded and the pellet dried before being resuspended in 10µl DEPC-H_2O. Isolated total RNA was stored at -70°C.

4.2.7.2 Synthesis of cDNA Probe

The SuperScript Preamplification System for First Strand cDNA Synthesis (GibcoBRL) was used to synthesise first strand cDNA used in subsequent PCR reactions. An oligo-dT primer was used from the kit, because this hybridises to 3'polyA tails of all mRNAs. The single strand cDNA was amplified with the following primer pair to generate an 840bp fragment of lactase-phlorizin hydrolase gene spanning exons 7 and 8 (Mantei *et al.* 1988). In addition, an 800bp fragment of sucrase-isomaltase was amplified with the following primers ((from S12) Green *et al.* 1987). Primers were synthesised by Sigma-Genosys.

Lactase-phlorizin hydrolase (accession number: M61838)
Forward 5′ atc ctc ttg gat tcg tgt ggt g 3′
Reverse 5′ cca agc cat tga tca gcc tgt 3′
Sucrase-isomaltase (accession number: M22616)
Forward 5′ cgt tca aga cat gac acc 3′
Reverse 5′ atc agt gac ctg tgt atc 3′

PCR was carried out as described by the manufactures of the SuperScript Preamplification System for First Strand cDNA Synthesis kit, in 22.5µl reactions containing 12.5pmol of each primer and 20mM Mg^{2+} buffer. In the lactase-phlorizin hydrolase amplification reaction 6µl of single stranded cDNA was used, and in the sucrase-isomaltase amplification reaction 4µl of single stranded cDNA was used. PCR amplifications were carried out at 94°C for 3 minutes, and 33 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final extension at 72°C for 2 minutes (Biometra TRIO-thermoblock). Resulting PCR products were cleaned using two phenol:chloroform

extractions, one chloroform extraction, an ethanol precipitation and a 70% ethanol wash. 100µl of phenol:chloroform 1:1 was added to 100µl of cDNA (made up to 100µl with dH_2O), mixed and centrifuged for 20 seconds. The supernatant was transferred to a clean tube and the step repeated. Afterwards, 100µl of chloroform was added to the cDNA fraction, mixed and spun for 20 seconds. Again the supernatant was removed and 14µl 3M sodium acetate, 400µl 100% ethanol was added and left at -20°C for 30 minutes. The cleaned cDNA was pelleted at 13,200rpm for 10 minutes, washed with 70% ethanol, and spun for 5 minutes. DNA pellets were dried before being resuspended in 10µl of deionised H_2O. Resulting clean cDNA probes were stored at -20°C.

4.2.7.3 Setting-up Northern Blot

Cells were seeded at a density of $3x10^5$ cells/well and grown for 3, 7 or 14 days post-seeding, in the presence of 0.05mM EPA, 0.05mM OA or 0.05mM PA. Cells were harvested and total RNA isolated as above (section 4.2.7.1). The Hybond N (Amersham) protocol was used to generate northern blots. The mRNAs for lactase-phlorizin hydrolase and sucrase-isomaltase measure approximately 6kb each (Mantei *et al.* 1988, Green *et al.* 1987). 15µg (measured on a spectrophotometer (Cecil CE 3021, 3000 Series) of total RNA per well was loaded on 1% agarose gels. RNA samples were separated for 75 minutes at 100mV. After transfer of the total RNA to Hybond N membranes, these were baked at 80°C for 2 hours to crosslink the RNA to the membrane.

4.2.7.4 Northern Blot

Hybond N membranes were prehybridised in a solution of 5X SSC, 5X Denhardts, 0.05% Sodium Pyrophosphate and 0.5% SDS for 1 hour at 65°C. cDNA probes were labelled with ^{32}P α–dCTP (Amersham) using the Boehringer Mannheim High Prime kit, according to the manufacturer's protocol. Non-incorporated dNTPs were removed using a Quick Spin Column (Boehringer Mannheim). Probes were heated to 94°C for 5 minutes, cooled on ice and added to the prehybridisation solution and the blots. Blots were incubated overnight at 65°C. The next day the blots were washed once in 5X SSC, 1% SDS, once in 2X SSC, 1% SDS and once in 1X SSC, 1% SDS at 65°C for 30 minutes, per wash. A β-actin partial cDNA probe was used as a control for equal loading of the RNAs onto the gel. The β-actin cDNA probe of 400bp (Gift from Professor P. Humphries laboratory), was generated by PCR amplification using the following primers; Forward 5' cgt acc act ggc atc gtg 3' Reverse 5' gtt tcg tgg atg cca cag 3' (accession number: M10277). Northern

blots were exposed to autoradiograph films (Hyperfilm, Amersham) for approximately 7 days to visualise RNA.

4.2.8 Assay of Effect of Fatty Acid Supplementation on mRNA Levels of Microsomal Triglyceride Transfer Protein

The microsomal triglyceride transfer protein (MTP) is involved in the addition of TAG to apoB in the endoplasmic reticulum (section 1.2.2.3). Addition of TAG to apoB stabilises the protein against degradation. The expression profile of MTP mRNA during cell differentiation (day 3, 7 and 14 post-seeding) was investigated to test whether fatty acid supplementation affected MTP mRNA expression levels. A probe of 720bp for MTP was synthesised using PCR and the following primers (Sharp *et al.* 1993).

Microsomal triglyceride transfer protein (accession number: X59697)	
Forward	5′ gta tta cct cag ctg gtg 3′
Reverse	5′ gtg agc gac cat ttc ctt c 3′

The synthesis of the cDNA probe was as described in section 4.2.7.2, except 2µl of single stranded cDNA was added to the PCR reaction for amplification of the cDNA probe. The northern blots prepared in the above section (4.2.7.3) were used for investigation of MTP mRNA levels. MTP mRNA is approximately 3kb long (Sharp *et al.* 1993). The northern blots were probed with the partial cDNA probe for MTP using the protocol described in section 4.2.7.4.

4.2.9 Gas Chromatography

Gas chromatography was used to assay how chronic doses of fatty acids, EPA, OA and PA, administered to Caco-2 cells could change the lipid composition of their cellular and secreted TAG and PL fractions. Cells were grown for approximately 19 days with different doses of fatty acid treatment; 0.5mM, 0.25mM, 0.1mM and 0.05mM. Cells and basolateral media were harvested. Basolateral media (approximately 3ml) was transferred to a sterile 15ml tube (Falcon) and stored at -80°C before lipid isolation (section 4.2.9.1). Apical media was removed from cells, which were washed in 1X PBS. Subsequently, 1ml of 1X PBS was added to the apical compartment of the transwell and the cells were scraped off the membranes and transferred to 1.5ml tubes (Starstead). Cells were also

stored at - 80°C. To prepare the TAG and PL fractions for chromatography, firstly the lipids were isolated from the cell and basolateral media (section 4.2.9.1), and the TAG and PL fractions separated (section 4.2.9.2). Lastly, the lipid molecules were methylated (section 4.2.9.3) before loading on a gas chromatography column (section 4.2.9.4).

4.2.9.1 Lipid Isolation

The basolateral media was divided into three 16x100mm borosilicate glass test tubes (approximately 1ml/tube). In addition, the 1ml of cells was transferred to one 16x100mm borosilicate glass test tube. 2.5ml chloroform:methanol (2:1), containing 0.01% butylated hydroxy toluene (BHT) was added and the solution was vortexed for 60 seconds. The tubes were centrifuged at 4,500rpm for 15 minutes to separate the organic and aqueous phases. The organic phase was transferred to a 12x75mm borosilicate tube. The three organic phases from the 16x100mm borosilicate glass tubes containing the basolateral media were pooled into one 12x75mm borosilicate tube. 2ml of chloroform, containing 0.01% BHT was added to the aqueous phase and again the solution was vortexed and centrifuged. The organic phase was removed and added to the first isolate. Organic isolates were dried under a vacuum (AGB Scientific Ltd) and the isolated lipids stored at -80°C under nitrogen gas.

4.2.9.2 Separation of TAG and PL

Lipid entities were separated by thin layer chromatography (TLC). The Silica 60 LKD 19 Lane TLC plate (Whatman) was baked at 120°C of 1 hour to activate the plate. 30µl of chloroform was added to the 12x75mm borosilicate tubes containing the lipids, which were vortexed to solubilise the lipids. The lipids were transferred to the TLC plate in 5µl aliquots, drying between each addition, until all 30µl of lipid was added as one lipid spot. Then another 30µl of chloroform was added to the tube, which was vortexed and lipids aliquoted as before. In addition, 5µl of a TAG standard consisting of 100µl Flora in 1ml chloroform was added to the TLC plate. 20µl of a PL standard consisting of phosphatidyl choline in chloroform (2mg/ml) (Sigma) was also added to the TLC plate. The basolateral lipids could not be visualised on the TLC plate. For this reason cellular and basolateral lipid fractions isolated from each transwell were loaded beside each other on the TLC plate. This was done because it was observed that when Caco-2 cells were fed with different fatty acids, the TAG fractions isolated from these cells would run at different rates. The TLC plate was put into running solution consisting of 80ml petroleum spirit (boiling point 40-80°C), 20ml diethyl ether and 2ml formic acid (Gibney & Bolton-Smith

1988), which had been equilibrating for 1 hour. TLC plates were run for 33 minutes. Plates were removed, air dried for 30 minutes and sprayed with a vapour of 0.1% (w/v) 2,7-dichlorofluorsein, 95% methanol to visualise lipid fractions under UV light. After separation of TAG and PL into separate bands, lipid bands were scraped off TLC plates and the lipids dissolved out of the silicone powder of which the TLC plates consist. 3ml of chloroform:methanol (2:1) was added to TAG and/or PL fractions which were vortexed, and centrifuged for 5 minutes at 2,400rpm. The supernatant was removed and the extraction was repeated. The TAG and PL were dried down under a vacuum and stored under nitrogen gas at -80°C.

4.2.9.3 Methylation of TAG and PL

TAG and PL were methylated before being loaded onto the gas chromatograph column in order to be able to identify the fatty acid components of TAG and PL by gas chromatography (GC). 500µl of boron trifluoride methanol complex (BDH Chemicals) was added to either a TAG or PL fraction. Lipids were suspended by vortexing and incubated for 30 minutes at 75°C in tubes that were sealed with a marble to inhibit evaporation. Subsequently, the lipids were extracted with hexane as follows. 500µl of hexane was added, tubes were vortexed for 40 seconds and the supernatant removed. This was repeated twice more. Samples were dried under a vacuum and stored at -80°C under nitrogen gas before being analysed on the GC machine (Shimadzu GC-14A Series Gas Chromatograph).

4.2.9.4 Analysis of TAG and PL Fractions by Gas Liquid Chromatography

The GC column was a BP 21 polar aluminium coated silica column (Scientific Glass Engineering Pty. Ltd.). The analytical conditions were (taken from Anne-Marie Tully's PhD. Thesis 2000) as follows:

Make up gas	Oxygen free Nitrogen	1.0kg/cm^3
Column gas	Oxygen free Nitrogen	1.25kg/cm^3
	Hydrogen	0.6kg/cm^3
	Dry air	0.5kg/cm^3

Temperature programme	Column initial temperature	120°C
	4°C/min for 20 minutes to 200°C	
	200°C for 12 minutes	
	4°C/min for 5 minutes to 220°C	
	220°C for 12 minutes	
	Injection temperature	240°C
	Detection temperature	280°C

Data output appears as a series of peaks at different retention time points. The area under the peak represents the percentage of fatty acid present in the sample. The retention time is dependent on the type of fatty acid that is present. Longer chain fatty acids appear later than shorter chain fatty acids, and saturated fatty acids appear before unsaturated fatty acids. Fatty acid standards (Sigma) were used to identify retention times. The following fatty acids were present in the standard used; FAME 1 (8:0, 10:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2n-6, 18:3n-6, 18:3n-3, 18:4n-3, 20:1, 20:2, 20:3n-6, 20:4n-6, 20:3n-3, 20:5n-3, 22:4n-6 and 22:6n-3). The quantity of each fatty acid in the sample was calculated from the area of the peaks using a Shimadzu C-R6A chromatograph integrator (Mason Technologies).

Anne-Marie Tully loaded the samples onto the GC machine. Prior to loading samples each day, 2 hexane washes and the standard were run on the GC machine to standardise the machine. Cellular TAG samples were resuspended in 100µl hexane. 65µl was injected into the machine at a split ratio of 1/60 (dilution factor). Cellular PL samples were resuspended in 80µl hexane. 65µl was injected into the machine again at a split ratio of 1/60. For the basolateral samples, there was no split ratio. Basolateral TAG samples were resuspended in 80µl hexane and 80µl was injected into the machine. Basolateral PL samples were resuspended in 65µl hexane and 80µl was injected into the machine.

4.3 Results

In this chapter the influence of EPA, OA and PA, administered chronically to Caco-2 cells, on aspects of cell growth was studied. Firstly, TEER and translocation assays were carried out to measure cell growth. In addition, cell viability and proliferation were assayed, and potential effects of chronic fatty acid supplementation on cellular and basolateral lipids noted.

4.3.1 Transepithelial Electrical Resistance

The TEER is a measure of cell growth. Caco-2 cells were grown for 19-21 days in the presence of 0.5mM, 0.25mM, 0.1mM or 0.05mM EPA, OA or PA. For all treatments the TEER was measured so a comparison could be made between fatty acid supplementation and no fatty acid supplementation (control). The TEER throughout cell growth of control

cells and test cells was measured when all experiments were carried out. This gives a sample size of approximately 45 for the control cells, and between 15-30 for Caco-2 cells undergoing supplementation. The mean TEERs (in Ω) measured in the cells are given in Table 4.3.1. EPA and OA at doses of 0.5mM and 0.25mM seem to inhibit the growth of the cells to confluency compared to control cells, which reached confluency at day 7 post-seeding. Supplementation of Caco-2 cells with 0.1mM and/or 0.05mM EPA resulted in cells reaching confluency at approximately day 5 post-seeding. 0.1mM OA retarded growth to confluency until day 10 post-seeding, but cells supplemented with 0.05mM OA reach confluency at day 7 post-seeding. Supplementation with PA did not effect Caco-2 cells TEERs. The graph (Figure 4.3.1) shows how the supplementation of 0.05mM of each fatty acid to Caco-2 cells affects their TEER. At a concentration of 0.05mM fatty acid supplementation cell growth appears normal.

4.3.2 Viability (MTT) Assay

The viability of cells treated with fatty acid compared to control cells also was assessed. In living cells MTT is broken down into a blue product by the action of mitochondrial dehydrogenase. The production of this product can be measured spectrophotometrically at absorbances of λ=490nm and λ=570nm. From Table 4.3.2 one can see that cells treated with 0.1mM or 0.05mM OA or EPA have similar viability to control Caco-2 cells. However, 0.5mM and 0.25mM supplementation of OA and EPA severely hampered cell viability. All PA treatments result in full viability. Viability of cells was not affected by a 24 hour acute fatty acid treatment with 0.5mM fatty acid, 0.5% BSA in media without FCS.

4.3.3 Proliferation (MTS) Assay

The proliferation assay was used to assess if fatty acid treated cells proliferated at similar rates to control (media treated) cells. Cells were treated with either 0.1mM or 0.05mM EPA, OA or PA for one day. Cells were incubated in MTS for 90 minutes and formazan levels measured. The quantity of formazan (blue product), the product made by the action of dehydrogenase enzymes on MTS, was assessed at λ=490nm. From the Table 4.3.3, it is obvious that cells given either 0.1mM or 0.05mM fatty acid, proliferate at the same rate as the control cells.

4.3.4 Translocation Assay

This assay was used to assess the confluency of the cell monolayer at day 6 and 14 post seeding. Two markers were used; ^{3}H-propranolol which is a lipophilic intracellular marker and ^{14}C-mannitiol which is a paracellular marker. More translocation was expected to be detected using the paracellular marker at day 6 than day 14, due to confluency having been achieved by day 14. In addition, it was hypothesised that the apparent permeability coefficient (P_{app}) measured using the intracellular marker at day 6 and 14, may also be affected by the lack of confluency at day 6. The translocation experiment was carried out to determine whether fatty acid supplemented cells behave similarly to control cells.

The effects of 0.1mM and 0.05mM fatty acid supplementation, with EPA, OA or PA, on P_{app} levels were studied. Using both the paracellular ^{14}C-mannitiol and intracellular ^{3}H-propranolol markers P_{app} levels were assessed and found to be similar in control cells and fatty acid supplemented cells (Tables 4.3.4a-d). This suggests that fatty acid supplementation of 0.1mM or 0.05mM has no effect on the growth of Caco-2 cells.

4.3.5 Cell Differentiation

cDNA probes for lactase-phlorizin hydrolase, sucrase-isomaltase and microsomal triglyceride transfer protein were prepared (sections 4.2.7.2 and 4.2.8). In addition northern blots with total RNA from Caco-2 cells of 3, 7 and 14 days post-seeding were generated (section 4.2.7.3). However, for medical reasons it became no longer possible to carry out the probing of the northern blots with the ^{32}P labelled cDNA probes using the protocol outlined in section 4.2.7.4.

Table 4.3.1 The mean TEER (in Ω) measured in control cells and cells given different fatty acid supplementations. The mean TEER in control cells was calculated from all cells grown in media alone in both chapters 4 and 6.

Day	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Treatment																			
Control	126	144	191	260	250	364	329	401	394	364	363	384	379	372	417	431	438	439	410
0.5mM EPA		136			165		238		312			354		377		384			
0.25mM EPA		170		283		375			320		306		325			329		308	
0.1mM EPA		153		334	261	429	367		450		410	378	407	384		400		362	
0.05mM EPA		155		331	334	373	398	496	371		359	412	361	329		352		333	
0.5mM OA			138		126		132			157		202		317			441		388
0.25mM OA		143			132		347		476			487		389		416			454
0.1mM OA		414	138	189	223	244	213		311	160	310	278	355	344		408		403	421
0.05mM OA	122	143		288	238	384	400	427	479		376	468	359	402		436		565	465
0.5mM PA				154		235		358			425		342		326			343	
0.25mM PA			165		262		390			404		371		381			411		393
0.1mM PA		135		149	169	224	388	390	282		274	359	297		295	379		371	318
0.05mM PA		137	266	305	347	351	475	453	343	456	334	384	381	393	546	411	536	442	480

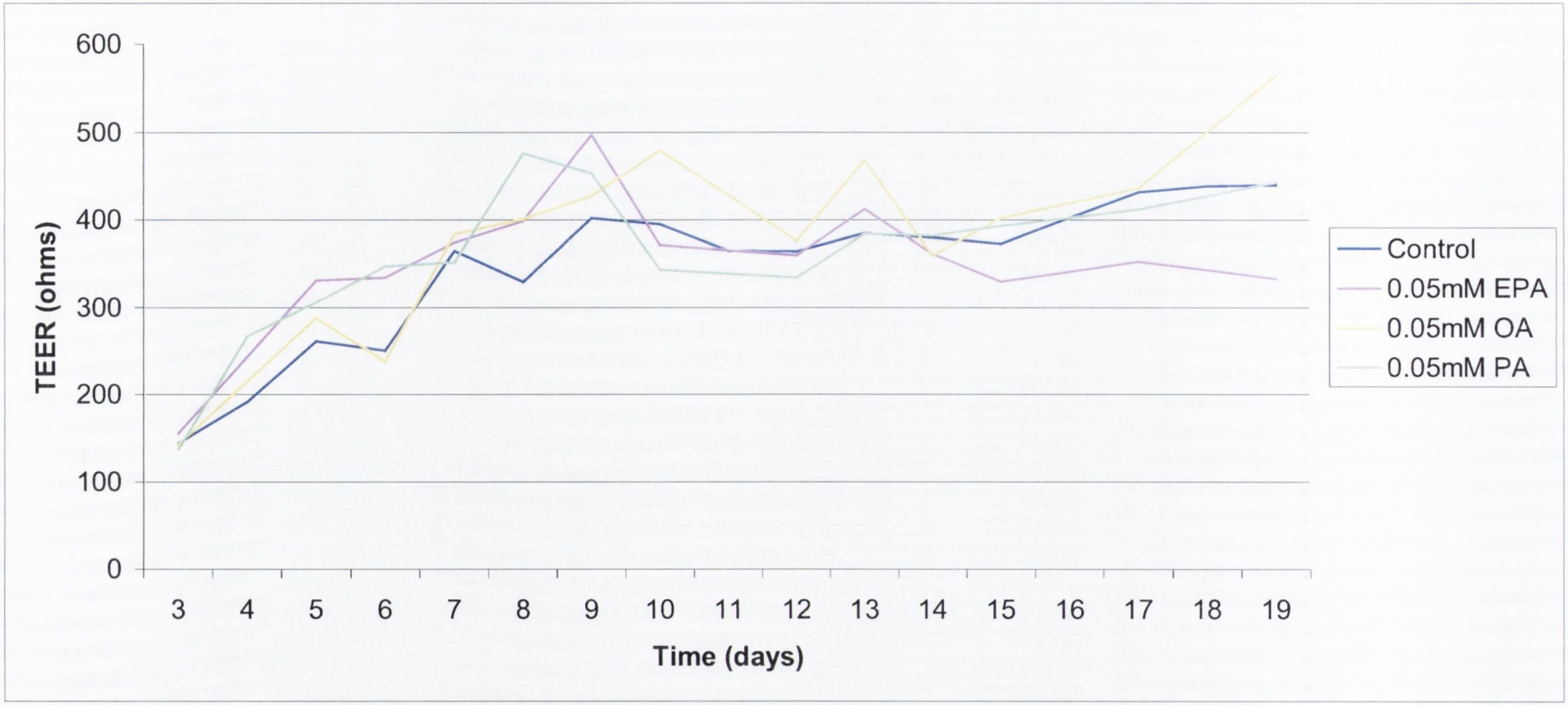


Figure 4.3.1 TEER measured in cells grown in the presence of 0.05mM fatty acid. The graph represents the measured TEER (in Ω) versus time (days). Growth rate does not appear to be different in fatty acid supplemented cells than in control cells. Where data were not present the mean between the previous and following mean TEER value was plotted.

Table 4.3.2 Viability (MTT) assay. Conditions, refers to the age of the cells post seeding and type of treatment given, either chronic or acute. The column entitled 'Mean λ' gives the mean absorbance measured at λ=490nm and λ=570nm in 8 different wells of a 96 well plate. The standard deviation of absorbance is given in the next column. Viability has 3 states, '*' viable, 'poor' low viability and 'dead' not viable.

Viability (MTT) Assay

Conditions	Treatment	Mean λ	Standard deviation λ	Viability
21 days	Media (Control)	0.611	0.145	
Chronic	0.5mM OA	0.013	0.004	Dead
Chronic	0.5mM OA, 0.5% BSA	0.273	0.055	Poor
Chronic	0.1mM OA	0.584	0.12	*
Chronic	0.1mM OA, 0.1% BSA	0.497	0.067	*
21 days	Media (Control)	0.495	0.063	
Chronic	0.5mM EPA	-0.007	0.001	Dead
Chronic	0.5mM EPA, 0.5% BSA	-0.007	0.003	Dead
Chronic	0.1mM EPA	0.494	0.062	*
Chronic	0.1mM EPA, .1% BSA	0.556	0.037	*
21 days	Media (Control)	0.626	0.081	
Chronic	0.25mM OA	0.311	0.067	Poor
Chronic	0.25mM OA, 0.25% BSA	0.366	0.043	Poor
Chronic	0.05mM OA	0.583	0.079	*
Chronic	0.05mM OA, 0.05% BSA	0.627	0.072	*
19 days	Media (Control)	0.477	0.081	
Chronic	0.25mM EPA	0.006	0.002	Dead
Chronic	0.25mM EPA, 0.25% BSA	0.158	0.108	Poor
Chronic	0.05mM EPA	0.512	0.139	*
Chronic	0.05mM EPA, 0.05% BSA	0.52	0.076	*
21 days	Media (Control)	0.594	0.069	
Chronic	0.5mM PA	0.562	0.135	*
Chronic	0.5mM PA, 0.5% BSA	0.537	0.187	*
Chronic	0.1mM PA	0.537	0.042	*
Chronic	0.1mM PA, 0.1% BSA	0.431	0.041	*
17 days	Media (Control)	0.509	0.184	
Acute	0.5% BSA	0.45	0.079	*
Acute	0.5mM EPA, 0.5% BSA	0.406	0.104	*
Acute	0.5mM OA, 0.5% BSA	0.315	0.058	*
Acute	0.5mM PA, 0.5% BSA	0.481	0.05	*
Chronic	0.25mM PA	0.335	0.155	*
Chronic	0.25mM PA, 0.25% BSA	0.255	0.07	*
Chronic	0.05mM PA	0.317	0.109	*
Chronic	0.05mM PA, 0.05% BSA	0.299	0.086	*

Table 4.3.3 Proliferation assay. Experimental conditions, refers to density of seeding, age of cells after seeding, and length of incubation of cells with MTS. Treatment refers to the fatty acid supplementation given to the cells. The mean is the mean absorbance (λ=490nm) of measurements taken from eight separate wells. The '*' signifies similar proliferation rate to control

Proliferation (MTS) Assay

Experimental Conditions	Treatment	Mean λ	Standard deviation λ	Proliferation
500 cells/μl 1 day	Media (Control)	0.507	0.107	
90 minute MTS	0.05mM EPA	0.349	0.043	*
	0.05mM OA	0.378	0.182	*
	0.05mM PA	0.32	0.043	*
	0.1mM EPA	0.376	0.031	*
	0.1mM OA	0.293	0.102	*
	0.1mM PA	0.433	0.087	*

Table 4.3.4a & b Translocation assay of mannitol from the apical compartment to the basolateral compartment of transwells with Caco-2 cells over a 2 hour period. The effects of fatty acid supplementation on mean P_{app} value, standard errors and the probability of P_{app} values of test cells being similar to the P_{app} values of control cells calculated by 2-sample-t-test (against control). NS = not significant

Table 4.3.4a ^{14}C-mannitol P_{app} values in cells grown for 6 days.

Treatment	**Mean P_{app} *10^{-6}**	**Standard Error**	**Probability**
Control (media)	3.92	1.43	
0.1mM EPA	7.61	2.61	NS
0.1mM OA	1.304	0.342	NS
0.1mM PA	2.38	0.613	NS
0.05mM EPA	1.205	0.666	NS
0.05mM OA	5.33	2.66	NS
0.05mM PA	12.66	4.69	NS

Table 4.3.4b ^{14}C-mannitol P_{app} values in cells grown for 14 days.

Treatment	**Mean P_{app} *10^{-6}**	**Standard Error**	**Probability**
Control (media)	0.692	0.153	
0.1mM EPA	0.662	0.085	NS
0.1mM OA	1.203	0.453	NS
0.1mM PA	0.365	0.044	NS
0.05mM EPA	0.988	0.2	NS
0.05mM OA	0.883	0.445	NS
0.05mM PA	0.557	0.094	NS

Table 4.3.4c & d Translocation assay of propranolol from the apical compartment to the basolateral compartment of transwells with Caco-2 cells over a 2 hour period. The effects of fatty acid supplementation on mean P_{app} value, standard errors and the probability of P_{app} values of test cells being similar to the P_{app} values of control cells calculated by 2-sample-t-test (against control). NS = not significant.

Table 4.3.4c ^{3}H-propranolol P_{app} values in cells grown for 6 days.

Treatment	Mean P_{app} $*10^{-5}$	Standard Error	Probability
Control (media)	1.73	0.096	
0.1mM EPA	1.998	0.175	NS
0.1mM OA	1.973	0.151	NS
0.1mM PA	1.908	0.227	NS
0.05mM EPA	1.513	0.129	NS
0.05mM OA	1.76	0.183	NS
0.05mM PA	1.682	0.116	NS

Table 4.3.4d ^{3}H-propranolol P_{app} values in cells grown for 14 days.

Treatment	Mean P_{app} $*10^{-5}$	Standard Error	Probability
Control (media)	1.413	0.04	
0.1mM EPA	1.6	0.072	NS
0.1mM OA	1.406	0.058	NS
0.1mM PA	1.49	0.054	NS
0.05mM EPA	1.448	0.065	NS
0.05mM OA	1.525	0.087	NS
0.05mM PA	1.41	0.084	NS

4.3.6 Gas Chromatography

Gas chromatography was used to assess how fatty acid treatments of Caco-2 cells affect fatty acid composition of cellular and secreted lipids (basolateral). The levels of 16:0 (PA), 16:1, 18:0, 18:1 (OA) and 20:5n-3 (EPA) present in the fatty acid supplemented cells and basolateral media were compared to the levels of these fatty acids found in control cells and their basolateral media. Fatty acids 16:0, 16:1, 18:0 and 18:1 occur at a high percentage in Caco-2 cells. Due to small sample size and large variance of measurements of fatty acid composition, significance was not attained in all experiments. No 20:5n-3 was detected in cellular TAG, basolateral TAG and basolateral PL fractions of control cells.

Feeding Caco-2 cells different fatty acids changed the cellular and secreted fatty acid composition of the cells. Analysis was not done on the fatty acid composition of TAG and PL fractions from the basolateral media of cells fed fatty acid concentrations of 0.5mM and 0.25mM, as these concentrations had proved to be fatal (Table 4.3.2). Statistical analysis of the difference in 20:5n-3 levels between fatty acid supplemented cells and control cells

could not be carried out, due to no 20:5n-3 being detected in the control cells. The results of the statistic analysis of differences between fatty acid supplemented cells and control cells can be found in Tables 4.3.5a-d. Bar graphs have been drawn of the percentage fatty acid detected in supplemented cells minus the percentage fatty acid detected in control cells versus the concentrations (0.5mM, 0.25mM, 0.1mM and 0.05mM) and supplemented fatty acid species (EPA, OA or PA) (Figures 4.3.2a-d).

All doses of EPA (0.5mM, 0.25mM, 0.1mM and 0.05mM) caused a reduction in the quantity of 16:0, 18:0 and 18:1 measured in both cellular and secreted TAG (Figures 4.3.2a, 4.3.2c). In the PL fractions a reduction in 16:1 and 18:1 levels were observed in cells treated with EPA (Figure 4.3.2b). However, the secreted PL fraction showed reduction in 18:0 and 18:1 levels (Figure 4.3.2d). This was observed to be dose dependent. The reduction of the above fatty acids due to EPA supplementation was accompanied by an increase in 20:5n-3, in all fractions except the basolateral TAG fraction of the 0.05mM supplemented cells. This is probably an artefact of this study due to small sample sizes (n=2). The additional 20:5n-3 appeared to be substituted for 18:1 in the lipid fractions.

Cells treated with OA showed a reduction in levels of 16:0 and 18:0. This reduction may result from the increase in 18:1 (OA) levels, found in the TAG fractions (Figures 4.3.2a, 4.3.2c). In addition a reduction in 16:1 and 18:0 and an increase in 18:1 levels was observed in the cellular PL fraction of cells treated with OA (Figure 4.3.2b). Interestingly, these changes were not observed in the basolateral PL fraction. Instead, there was a reduction in 18:1 in the basolateral PL fraction (Figure 4.3.2d). This suggests that the supplementation of Caco-2 cells with OA caused OA to be synthesised into TAG rather than PL for secretion.

Feeding Caco-2 cells different concentrations of PA increased the quantity of 16:0 in the TAG fractions (Figure 4.3.2a &c) and 16:0 and 16:1 in the PL fractions (Figures 4.3.2, 4.3.2d). A reduction in 18:1 was observed in the cellular TAG fraction (Figure 4.3.2a). In addition, reductions in 18:0 and 18:1 levels in the basolateral TAG fraction (Figure 4.3.2c) and in 18:1 in the basolateral PL fraction were observed (Figure 4.3.2d). These reductions appear to be necessary to facilitate the increase in 16:0 observed after PA supplementation. The increases in 16:0 and 16:1 observed in the cellular PL fraction did not seem to cause a reduction in any of the other fatty acids, which were measured (Figure 4.3.2c).

Thus, supplementing Caco-2 cells with EPA, OA or PA alters the percentage of fatty acids 16:0, 16:1, 18:0, 18:1 and 20:5n-3 in a fatty acid specific and a dose dependent manner.

Tables 4.3.5a-d Effects of fatty acid supplementation to Caco-2 cells on fatty acid composition of TAG and PL. All fatty acid compositions have been compared to composition of control cells (grown in media).

Tables 4.3.5a & b Significant differences in fatty acid composition found in cellular TAG and PL fractions respectively. NS = not significant, P = probability

Table 4.3.5a No EPA (20:5n3) could be detected in control cells grown in media and so no comparison could be made between fatty acid supplemented cells and control cells.

Cellular TAG

Treatment	16:0	16:1	18:0	18:1	20:5n-3
0.5mM EPA	P=0.0000	P=0.004	P=0.0000	P=0.0000	
0.25mM EPA	NS	P=0.0000	NS	P=0.0000	
0.1mM EPA	P=0.0000	NS	NS	P=0.0000	
0.05mM EPA	NS	NS	NS	P=0.008	
0.5mM OA	P=0.036	NS	P=0.0000	P=0.004	
0.25mM OA	NS	NS	NS	NS	
0.1mM OA	NS	P=0.004	P=0.04	NS	
0.05mM OA	P=0.006	NS	NS	NS	
0.5mM PA	NS	NS	NS	NS	
0.25mM PA	P=0.012	NS	NS	NS	
0.1mM PA	P=0.002	NS	NS	P=0.0000	
0.05mM PA	NS	NS	NS	NS	

Table 4.3. 5b

Cellular PL

Treatment	16:0	16:1	18:0	18:1	20:5n-3
0.5mM EPA	NS	NS	NS	NS	NS
0.25mM EPA	NS	P=0.006	NS	P=0.0000	P=0.02
0.1mM EPA	NS	P=0.0000	NS	P=0.0000	P=0.0000
0.05mM EPA	NS	P=0.0000	NS	P=0.0000	P=0.0000
0.5mM OA	NS	P=0.0000	P=0.0000	NS	
0.25mM OA	NS	NS	NS	NS	NS
0.1mM OA	NS	P=0.004	NS	P=0.002	NS
0.05mM OA	NS	NS	NS	NS	NS
0.5mM PA	NS	NS	P=0.006	NS	
0.25mM PA	NS	P=0.0000	NS	NS	
0.1mM PA	P=0.01	NS	NS	P=0.024	
0.05mM PA	P=0.01	P=0.034	NS	NS	NS

Table 4.3.5 continued

Tables 4.3.5c & d Significant differences in fatty acid composition found in basolateral TAG and PL fractions respectively. No EPA (20:5n3) could be detected in control cells grown in media and so no comparison could be made between fatty acid supplemented and control cells. NS = non significant. P = probability.

Table 4.3.5c

Basolateral TAG

Treatment	16:0	16:1	18:0	18:1	20:5n-3
0.1mM EPA	NS	NS	NS	NS	
0.05mM EPA	NS	NS	NS	NS	
0.1mM OA	NS	NS	NS	P=0.04	
0.05mM OA	NS	NS	NS	NS	
0.1mM PA	NS	NS	NS	NS	
0.05mM PA	NS	NS	NS	NS	

Table 4.3.5d

Basolateral PL

Treatment	16:0	16:1	18:0	18:1	20:5n-3
0.1mM EPA	NS	NS	NS	NS	
0.05mM EPA	P=0.028		NS	P=0.034	
0.1mM OA	NS		NS	NS	
0.05mM OA	NS	NS	NS	NS	
0.1mM PA	NS	NS	NS	NS	
0.05mM PA	NS	NS	NS	NS	

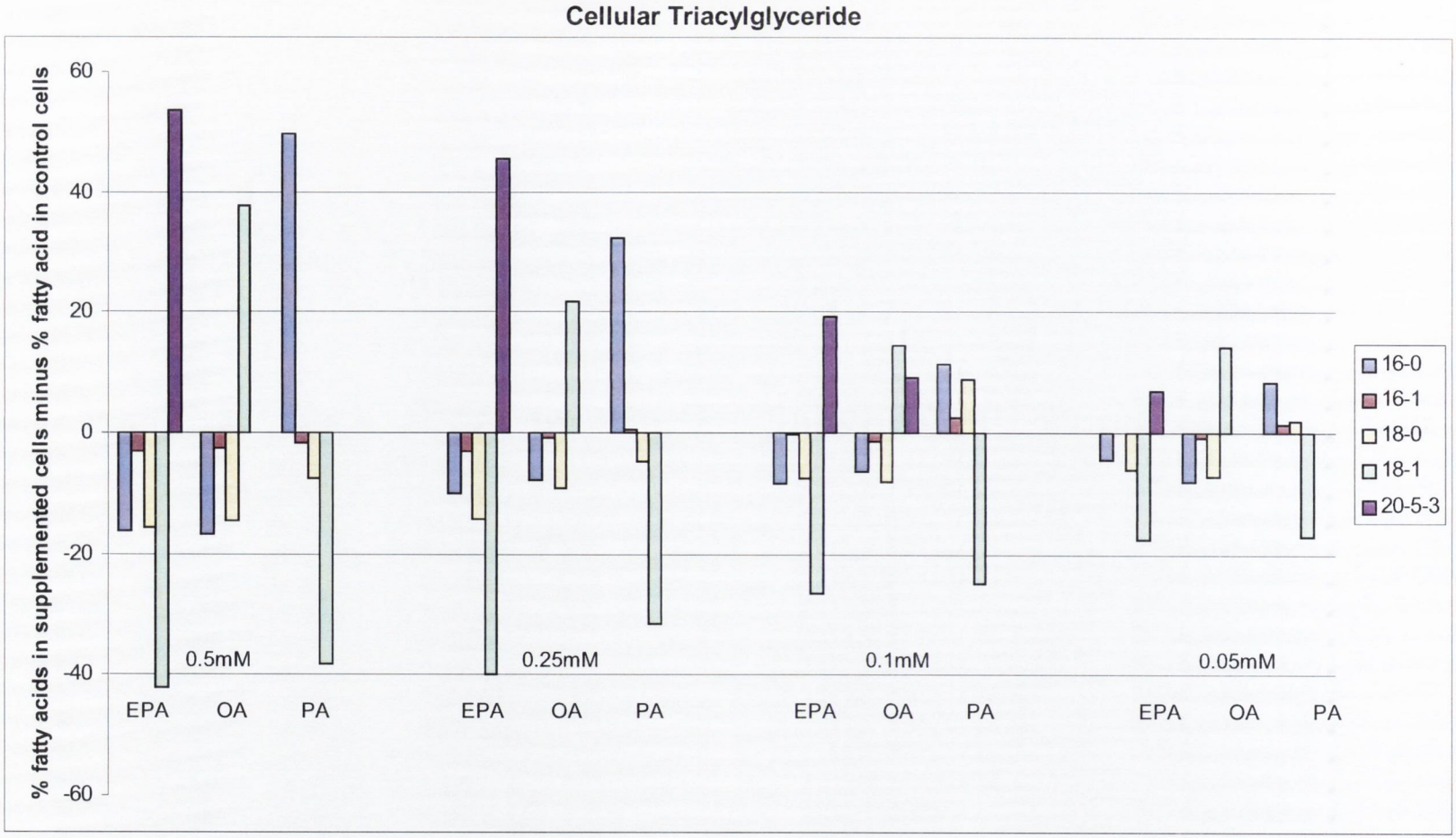


Figure 4.3.2a A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 20:5n-3) found in cellular TAG fractions of cells treated with various concentrations of EPA, OA or PA compared to control cells. The y-axis represents the percentage of fatty acids detected in supplemented cells minus the percentage of fatty acids detected in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1and 20-5-3 is 20:5n-3.

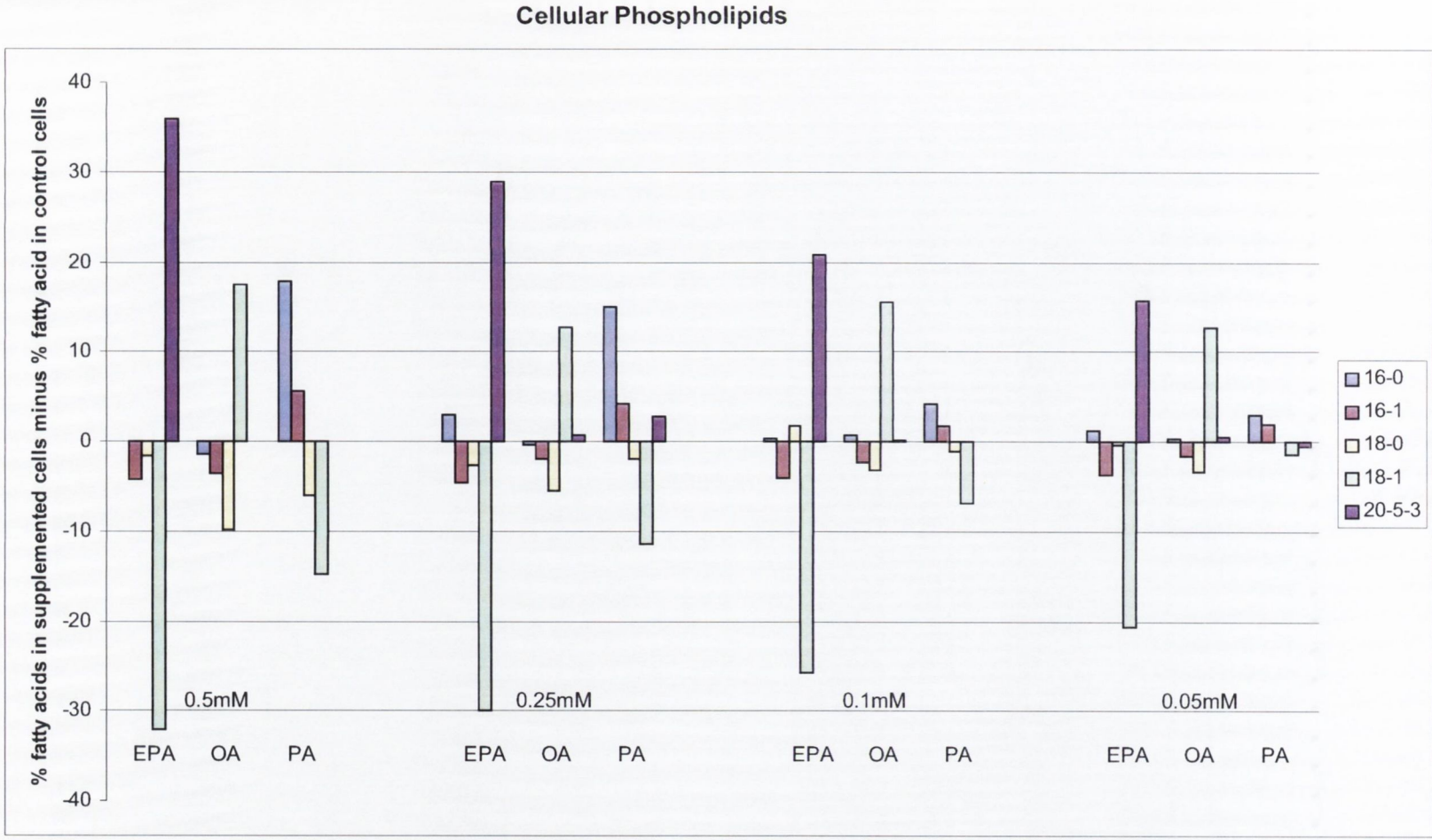


Figure 4.3.2b A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 20:5n-3) found in cellular PL fractions of cells treated with various concentrations of EPA, OA or PA compared to control cells. The y-axis represents the percentage of fatty acids detected in supplemented cells minus the percentage of fatty acids detected in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1and 20-5-3 is 20:5n-3.

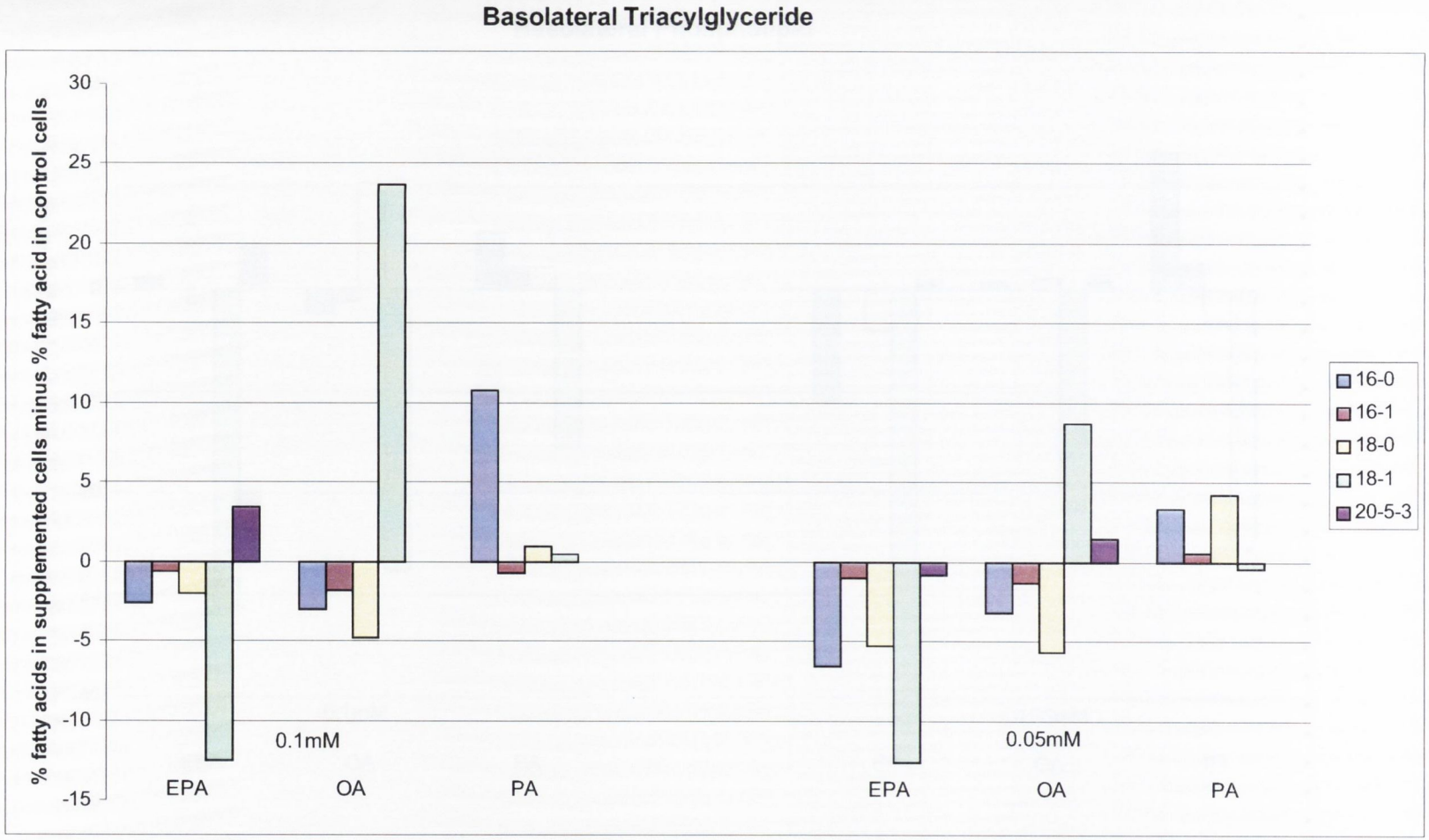


Figure 4.3.2c A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 20:5n-3) found in basolateral TAG fractions of cells treated with various concentrations of EPA, OA or PA compared to control cells. The y-axis represents the percentage of fatty acids detected in supplemented cells minus the percentage of fatty acids detected in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1and 20-5-3 is 20:5n-3..

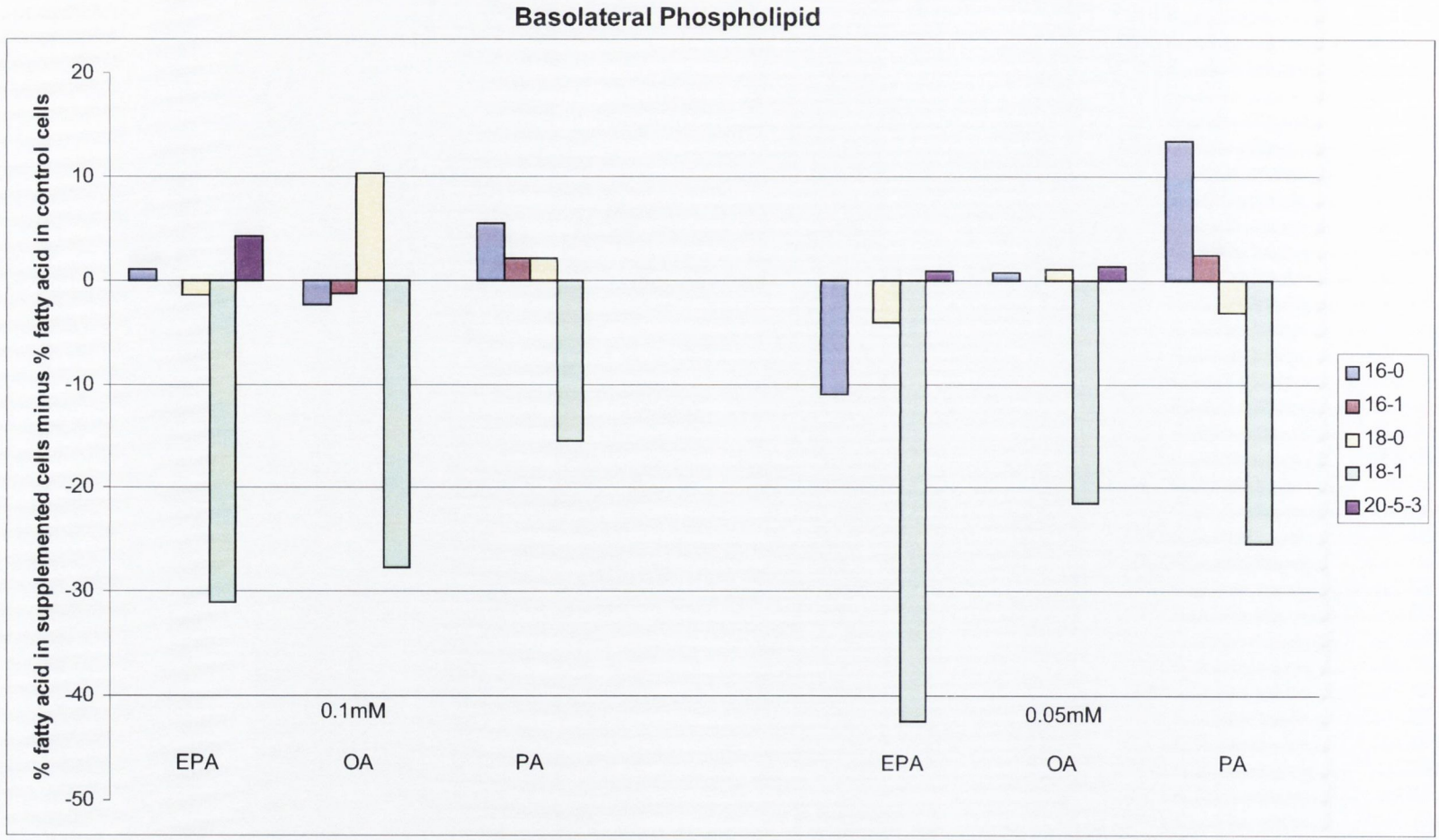


Figure 4.3.2d A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 20:5n-3) found in basolateral PL fractions of cells treated with various concentrations of EPA, OA or PA compared to control cells. The y-axis represents the percentage of fatty acids detected in supplemented cells minus the percentage of fatty acids detected in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1and 20-5-3 is 20:5n-3.

4.4 Discussion

4.4.1 Experimental Design

The aim of the experiments described in this chapter was to characterise a cell model system in which to study the effects of chronic low dose SFA (PA), MUFA (OA) or n-3-PUFA (EPA) diets on lipid metabolism. EPA was used instead of a mix of EPA and docosahexaemoic acid (DHA) or DHA alone, as EPA has been shown to be the hypotriglyceridemic component in fish oil (Rambjor *et al.* 1996). One of the ideas behind the experiments undertaken in this chapter was to mimic a human situation where a person is administered fatty acid supplements, which are ingested along with their normal diet. Therefore, fatty acids were administered to Caco-2 cells in media containing FCS, which contains all the fatty acids normally necessary for Caco-2 growth. Cells, which had been fed supplementary fatty acids, were characterised and compared to cells grown without supplementary fatty acids (control). It was expected that no difference in the cell's growth would be detected.

4.4.2 Characterisation of Fatty Acid Supplemented Caco-2 Cells

4.4.2.1 Cell Viability and Proliferation

MTT and MTS assays suggested that growing Caco-2 cells in the presence of supplementary fatty acid concentrations of 0.1mM or 0.05mM had no impact on either cell viability or proliferation.

4.4.2.2 Cell Growth

TEER, which measures the cell growth rate and indicates when cells attain confluency, plateaued approximately 7 days post-seeding in control cells. EPA supplementation of either 0.1mM or 0.05mM caused cells to reach the TEER plateau at about day 5 post-seeding, and in addition to have constantly higher TEERs than control cells. Higher TEERs were also observed by Jiang *et al.* (1998) in a vascular endothelial cell line ECV304 that had been grown in the presence of EPA supplements. Caco-2 cells that had been fed 0.1mM OA supplements did not reach confluency until day 10 post-seeding. However, cells grown in the presence of 0.05mM OA, like control cells, reached confluency at approximately day 7 post-seeding. Caco-2 cells grown in 0.1mM and/or

0.05mM PA, like control cells, reached the TEER plateau at about 7 days post-seeding. Caco-2 cells grown in concentrations of 0.5mM or 0.25mM EPA or OA were not viable over a 19 day growth period. Therefore, in subsequent cell permeability experiments, only fatty acid concentrations of 0.1mM and 0.05mM were studied.

The paracellular permeability of cells is a measure of tight junction formation. Both the paracellular and intracellular markers revealed no difference in cell permeability between Caco-2 cells fed with fatty acid supplements and control cells. This was an unexpected result in cells fed EPA supplements, as these cells manifested higher TEERs than control cells, especially at day 6 post-seeding, which could have resulted in these cells being less permeable to the paracellular marker than control cells. However, in this experiment, EPA treated cells showed no decrease in permeability to mannitol. Jiang *et al.* (1998) found that ECV304 cells fed EPA supplements showed both increased TEERs and decreased permeability to intracellular markers. However, unlike this study, the Jiang study gave a single fatty acid dose to confluent ECV304 cells. In addition, cell permeability was only measured at one time point.

A lipophilic intracellular marker was used to test if the increase in fatty acid concentration present in the media caused the cells to secrete fatty acids at a faster rate than control cells. This was not observed, suggesting that the fatty acid supplements were not passed through the Caco-2 cells and therefore, may have been incorporated into the cell structure. Overall, 0.1mM or 0.05mM fatty acid treatments did not affect cellular permeability, either through cells or between cells. Therefore fatty acid treated cells seem to develop similarly to control cells.

4.4.2.3 Cell Differentiation

The state of differentiation of the cells could not be assessed. However, as supplemented cells behaved similarly to control cells in all other characteristics measured, it was assumed that fatty acid supplementation did not affect cellular differentiation.

4.4.3 Incorporation of Supplementary Fatty Acids into Cellular and Secretion TAG and PL Fractions

4.4.3.1 Cellular Lipids

It was hypothesised that there may be a difference in the consistency of the fatty acids incorporated into cellular lipids between control cells and fatty acid treated cells. The

differences between lipid composition of fatty acid treated cells and control cells are represented in Figures 4.3.2a and 4.3.2b. As the concentration of fatty acid is reduced, the difference between the treated and untreated cells decreased. However, even when cells are grown in the presence of only 0.05mM fatty acid a difference in the fatty acid composition of the TAG and PL fractions is observed between treated and untreated cells. Cells grown in the presence of 0.05mM EPA showed an increase in EPA and a decrease in OA in their cellular TAG and PL fractions. Cells given 0.05mM OA supplements showed an increase in OA and a decrease in both PA and stearic acid (18:0) in their cellular TAG fractions. However, 0.05mM OA supplemented cells only manifest a decrease in only 18:0 in their cellular PL fractions. Finally, cells supplemented with 0.05mM PA had increased PA and decreased OA levels in both cellular lipid fractions.

4.4.3.2 Secreted Lipids

The basolateral lipid fractions contained very small quantities of lipid, which made it difficult to find significance, or to be sure that there were differences between basolateral lipid compositions of treated and untreated cells. However, from Figures 4.3.2c and 4.3.2d trends towards similar fatty acid composition of cellular TAG and PL fractions were seen in both the basolateral lipids and cellular lipids. Cells supplemented with 0.05mM EPA appeared to incorporate EPA preferentially into the PL layer of the cells rather than into the TAG fraction. This is unremarkable, as EPA treatment is hypothesised to inhibit diacylglycerol acyl transferase activity, thus inhibiting the final step in the synthesis of TAG (section 1.2.2.2) and reducing the competition for diacylglycerol to be biosynthesised into PL (Ranheim *et al.* 1992). Fish oil is known to prevent cachexia (severe wasting) and weight loss following immune stimulation. EPA has been found to replace arachidonic acid in the PL moiety of cells thus effecting eicosanoid synthesis. Eicosanoid derivatives are known to effect tumorigenesis (Belury 1995), which may also be linked to the effects of tumour necrosis factor-α (TNF-α) and interleuken-1 (IL-1) (Park *et al.* 1997) (sections 1.6.2 and 1.6.3). When cells are grown in the presence of 0.1mM and/or 0.05mM OA, OA is preferentially incorporated into the TAG fraction; this is especially visible in the basolateral TAG fraction. It has previously been shown that when cells are fed OA, it is synthesised into TAG, which causes more *de novo* TAG to be secreted from these cells, than from cells fed SFA or EPA (van Greevenbroek *et al.* 1995, Murthy *et al.* 1990). PA is found preferentially in the cellular TAG moiety. In the basolateral media the lack of significance and small sample size may be reasons why whether the PA is preferentially

incorporated in the secreted lipids could not be assessed. As PA is found in the cellular TAG fraction it may also be preferentially found in the basolateral TAG fraction.

4.4.4 Conclusion

A cellular model of chronic feeding of fatty acids was set up and characterised in the colon carcinoma cell line (Caco-2). It was hypothesised that chronic fatty acid feeding may effect how Caco-2 cells respond to acute fatty acid loads discussed in the literature (section 1.5.1.1, 1.5.1.2 and chapter 5). Caco-2 cells were supplemented for 19 days with 0.5mM, 0.25mM, 0.1mM or 0.05mM EPA, OA or PA. The cells were characterised by measuring TEERs, cell viability, cell proliferation and translocation of macromolecules. TEER and translocation assay measure cell growth and tight junction formation. Cell viability and proliferation rate measure the health of the cells. Fatty acid supplemented Caco-2 cells were compared to control (non supplemented) cells. The effects of fatty acid supplementation on fatty acid composition of the cellular and basolateral TAG and PL fractions were investigated. Supplementation with the various fatty acids had a dose dependent effect on fatty acid composition of cellular lipids. The investigation concluded that growing Caco-2 cells in 0.05mM EPA, OA or PA does not affect their growth rate or health and the supplemented fatty acids were shown to be utilised by the cells (TAG fraction), and to be synthesised into structural lipids (PL fraction). Therefore, in the experiments discussed in chapter 5, a chronic dose of fatty acid refers to cells incubated in 0.05mM fatty acid for approximately 19 days.

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5 Caco-2 Cell Model of Intestinal Lipoprotein Metabolism to Investigate the Effect of Dietary Fat Composition on Lipid and Apolipoprotein Synthesis and Secretion

5.1 Introduction

5.1.1 Effects of Diet in Humans on Postprandial Response

In humans, an individual's habitual or background fatty acid diet is known to affect the magnitude of the postprandial response, after ingestion of a lipid test meal (Williams 1998) (section 1.2.3.2). Zampelas *et al.* (1998) investigated the postprandial lipid response in Southern Europeans (Greeks) who consume a diet high in monounsaturated fatty acids (MUFA) and Northern Europeans (British and Irish) who consume a diet high in saturated fatty acids (SFA). The study demonstrated that during the postprandial response Southern Europeans show a rapid influx and removal of TAG and apoB48 from the circulation, while Northern Europeans had prolonged and elevated plasma TAG levels. This suggests that Southern Europeans metabolise dietary fat more efficiently than Northern Europeans. A concurrent study by Roche *et al.* (1998) investigated the effects of a background diet containing MUFA by isoenergically substituting an MUFA diet for an SFA diet in a study population for 8 weeks. Subsequently the study population was shown to have larger postprandial TAG and apoB48 peaks, which occurred earlier in the postprandial response and enhanced TAG and apoB48 clearance. It was hypothesised that this early peak was due to enhanced fatty acid absorption from the gut and faster TAG metabolism. Much interest has centred on omega-3 polyunsaturated fatty acids (n-3 PUFA) as they have been shown to attenuate the postprandial TAG response when supplemented at low concentrations (section 1.2.3.2). However, n-3 PUFAs appear to effect hepatic TAG production rather than intestinal TAG production (Roche & Gibney 1999). Although, Zampelas *et al.* (1994) investigated the effect of an n-3 PUFA test meal on postprandial plasma TAG levels, they hypothesised that an n-3 PUFA background diet may affect chylomicron-TAG concentrations. A recent study by this group demonstrated that individuals given even 0.9g of n-3 PUFA per day over a 16 week period showed a

significant attenuation of the postprandial TAG response after an n-6 PUFA test meal compared to controls given 0.9g per day of MUFA supplements (Tully *et al.* 2000).

5.1.2 Acute Fatty Acid Treatment of Caco-2 Cells

The colon carcinoma cell line is used as a model system of the human small intestine because it has similar properties to enterocytes (section 4.1.1). Previous studies using Caco-2 cells have investigated the affects of short-term (acute) doses of various fatty acids on the synthesis and secretion levels of TAG and apoB (Field *et al.* 1988, van Greevenbroek *et al.* 1995, van Greevenbroek *et al.* 1996, van Greevenbroek *et al.* 1998, van Greevenbroek *et al.* 2000, Moberly *et al.* 1990, Murthy *et al.* 1990 & Ranheim *et al.* 1992). An acute dose refers to one dose of fatty acid given to cells in serum free media for no longer than 24 hours. Most studies have investigated the effects of acute doses of oleic acid (OA), which is a MUFA, palmitic (PA) or stearic acids (SA), which are SFAs, linoleic acid (LA), which is an n-6 PUFA, and eicosapentaenoic acid (EPA) which is an n-3 PUFA. In Caco-2 cells, OA has been found to be the most efficient promoter of *de novo* TAG secretion followed by LA and PA. In addition, OA treatment induces a higher rate of TAG synthesis in Caco-2 cells than either LA or PA treatments, which induce similar TAG synthesis rates (section 1.5.1.1) (Field *et al.* 1988). In addition, 6 hours post-treatment, Caco-2 cells fed up to 0.25mM OA had the highest levels of *de novo* TAG in the TRL fraction (Field *et al.* 1988). The addition to Caco-2 cells of varying ratios of OA to PA (final fatty acid concentration of 0.5mM) has been shown to cause a change in the ratio of secreted TRL and IDL/LDL sized particles by the cells (van Greevenbroek *et al.* 2000). The higher the ratio is of OA to PA administered to Caco-2 cells, the more secreted lipoproteins are found in the TRL density fraction. Caco-2 cells given PA treatments have been shown to secrete lower levels of *de novo* TAG than cells given unsaturated fatty acid treatments (section 1.5.1.1) (van Greevenbroek *et al.* 1996). Fish oils attenuate the postprandial lipaemic response. This effect is primarily attributed to decreased VLDL production by the liver (Harris *et al.* 1990). Nevertheless, *in vitro* studies show that n-3 PUFAs also affect intestinal lipoprotein metabolism. Studies using Caco-2 cells found that an acute dose (0.25mM) of EPA reduced the synthesis and secretion levels of newly generated TAG but had no effect on secreted TAG mass (section 1.5.1.2) (Murthy *et al.* 1990). EPA was found to be more readily incorporated into the cellular PL moiety of Caco-2 cells fed EPA than cells fed OA (Ranheim *et al.* 1992, Murthy *et al.* 1990).

5.1.3 Aim of the Study

To date, all *in vitro* studies on intestinal lipoprotein metabolism have focused on the acute effect of fatty acids. The studies have been conducted in Caco-2 cells grown in media where the source of exogenous fatty acids has been foetal calf serum (FCS). Results from human studies have indicated that habitual dietary fat consumption also affects intestinal lipid metabolism. Therefore, in this PhD. thesis the effects of both chronic and acute fatty acid treatments on lipid and apolipoprotein secretion by the Caco-2 cells were investigated. It was hypothesised that chronic supplementation with 0.05mM EPA, OA or PA would effect how Caco-2 cells responded to acute fatty acid treatments consisting of 0.5mM EPA, OA or PA. In chapter 4 Caco-2 cells grown in the presence of 0.05mM fatty acid for 19 days were characterised. While this concentration of fatty acid was shown not to affect cell growth, the fatty acid composition of the cellular and secreted TAG and PL fractions had been altered. Therefore, in this chapter chronic fatty acid supplementation of Caco-2 cells refers to fatty acid doses of 0.05mM administered over a 19 day period as the cell develop.

5.2 Materials and Methods

5.2.1 Apolipoprotein B Isoform Separation

Before carrying out the chronic-acute experiment, separation of the two forms of apoB, apoB100 and apoB48, had to be achieved. Firstly, it was necessary to obtain apoB100 and apoB48 standards so they could be sized using protein ladders. An apoB100 standard was acquired (Sigma). ApoB100 was also isolated from plasma and plasma TRLs and an apoB48 standard was isolated from plasma TRLs. To acquire as much apoB48 as possible, it was isolated from 2 hour postprandial plasma, taken from a male who had consumed a meal high in n-6 PUFA. 10ml of postprandial blood was isolated and centrifuged for 10 minutes at 2,500rpm to separate the plasma from the cellular layer. 1.1ml of plasma was aliquoted into 3.2ml polycarbonate centrifuge tubes (Beckman) and overlaid with 1.1ml of saline at a density of 1.006g/ml (9.6g NaCl to 1l deionised water). TRL particles were isolated by ultracentrifugation at 100,000rpm for 22 minutes at 4°C in an Optima TLX Ultracentrifuge (Beckman). The cloudy TRL layer was removed using a fine tipped glass Pasteur pipette and stored at -20°C. ApoB100 and apoB48 proteins were isolated from

plasma and TRL fractions by immunoprecipitation (Boehringer Mannheim). For this purpose an anti-human apoB antibody synthesised in mouse was acquired from ICN, and Protein A-agarose from Boehringer Mannheim. 20µl of plasma and different volumes of the TRL fraction (10µl, 20µl, 40µl, 80µl and 160µl) were immunoprecipiated. The volume was made up to 200µl with wash 1 (Boehringer Mannheim) and protein inhibitors (Leupeptin 1µg/µl, Pepstatin 1µg/µl, Aprotinin 1µg/µl, PMSF 10µg/µl and Benzamidine 1µg/µl (Sigma)). Immunoprecipitations were further carried out as outlined in the Boehringer Mannheim protocol.

An SDS-PAGE gel was cast, which consisted of a 4% running gel (Total 20ml – 2ml 40% acrylamide:bis-acrylamide (38:2), 5ml 1.5M Tris.Cl pH 8.3, 12.6ml dH_2O, 200µl 10% SDS, 200µl 10% ammonium persulphate, 20µl TEMED), and a 2% stacking gel (Total 6ml – 320µl 40% acrylamide:bis-acrylamide (38:2), 750µl 1M Tris.Cl pH 6.8, 4.8ml dH_2O, 60µl 10% SDS, 60µl 10% ammonium persulphate, 6µl TEMED). Two protein ladders (Prestained Protein Large Molecular Weight Ladder (GibcoBRL) and Protein Marker Broad Range (New England Biolabs) (NEB)), apoB100 (Sigma) 20µl of plasma and 10µl, 20µl, 40µl, 80µl and 160µl of TRL were loaded on the gel. The gel was run in 1X running buffer (25mM Tris.Cl, 250mM Glycine, 0.1% SDS pH 8.3) for approximately 90 minutes at 100mV. The gel was then dismantled and stained with Coomassie Brilliant Blue (100ml – 0.25g Coomassie, 30ml methanol, 10ml acetic acid and 60ml dH_2O) and then destained (500ml – 50ml methanol, 50ml acetic acid and 400ml dH_2O).

Isolated apoB100 protein could be visualised in all plasma and TRL samples, but isolated apoB48 could only be observed in the 40µl, 80µl and 160µl TRL fraction samples (Figure 5.2.1). The apoB100 protein is approximately 500kDa and the apoB48 protein is approximately 230kDa (Figure 5.2.1).

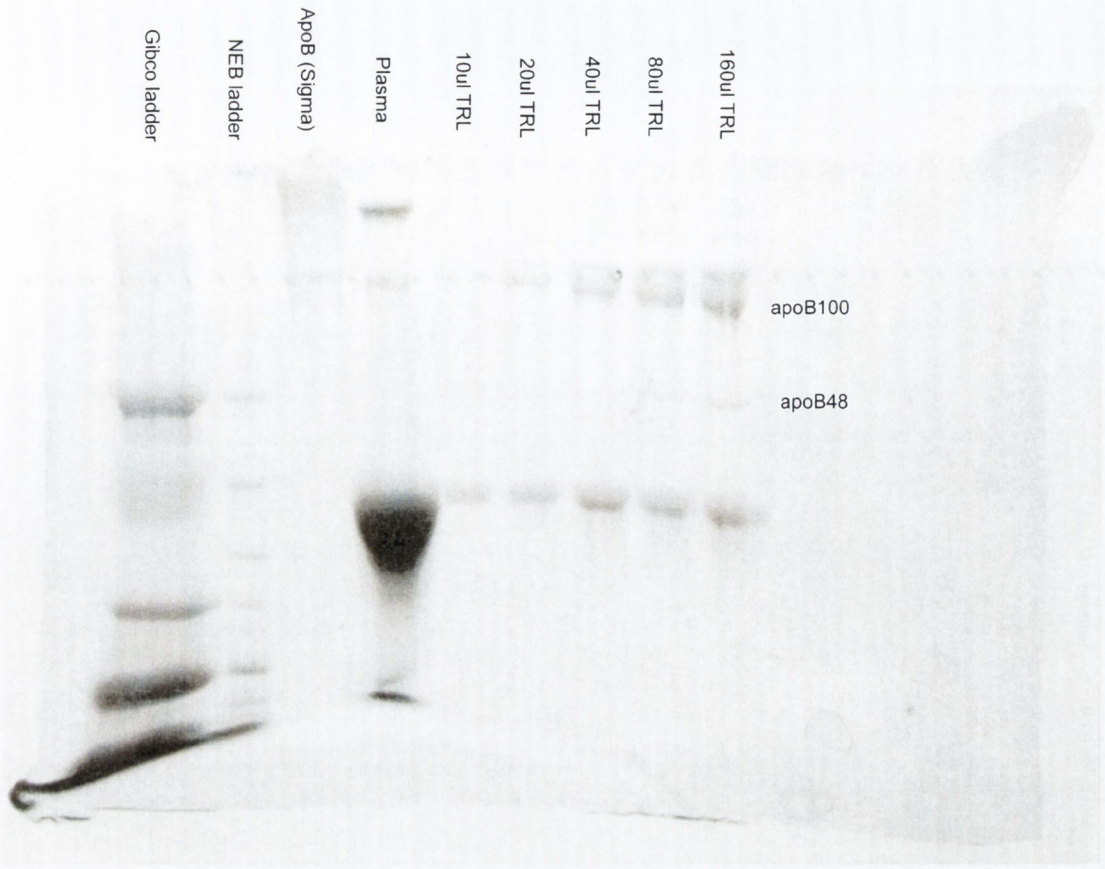


Figure 5.2.1 A 4% SDS-PAGE gel with 15µl of Prestained Protein Large Molecular Weight Ladder (GibcoBRL) (200, 97.4, 68, 43, 29, 18.4, and 14.3kDa), 10µl of Protein Marker Broad Range (New England Biolabs) (212, 158, 116, 97.2, 66.4, 55.6kDa), 15µl of apoB standard (Sigma), apoB isolated from 20µl of plasma, 10µl, 20µl, 40µl, 80µl and 160µl of TRL fractions respectively. ApoB100 can be seen in all lanes, whereas the apoB48 band can only be seen in the last 3 lanes. As can clearly be seen from the gel, apoB100 and apoB48 are approximately 500kDa and 230kDa respectively. The band at about 140kDa is probably partially degraded apoB protein

5.2.2 Acute Experiment Set-up

An experiment was conceived to decide how the acute part of the chronic-acute experiment would be carried out. Cells were seeded at a density of $3.3x10^5$ cells per 24mm transwell (Costar) and grown (section 4.2.1) for 19 days. On day 19 post-seeding the media was removed and the cells were washed with 1X PBS. 2.5ml of media without FCS was added to basolateral compartments and 1.5ml of media without FCS and containing 0.5mM OA and 0.5% BSA was added to apical compartments. Then 13µl (400ng) of ^{14}C-glycerol (1,443,000 DPM) (140-168mCi/mmol, Amersham) was added immediately to 2 wells (24 hour time point). Twelve hours later 13µl of ^{14}C-glycerol was added to another 2 wells (12 hour time point) and 8 hours later 13µl of ^{14}C-glycerol was added to another 2 wells (4

hour time point). Twenty-four hours after the OA had been administered, the basolateral media and cells were harvested. Basolateral media from each well was stored in 15ml tubes (Falcon) at -20°C for further analysis. Cells were washed in 1X PBS, and then scraped off the membranes into eppendorf tubes (Starstead). Cells were pelleted at 13,200rpm for 2 minutes, supernatant was removed and 1ml of 1X PBS added (section 4.2.9). Cells were stored at -20°C for further analysis.

The TRL fraction was isolated from the basolateral media on the day of harvesting. The media was made to a density of 1.006 g/ml with 1.34 g/ml density solution (153g NaCl + 354g KBr in 1l deionised H_2O) using the equation;

$$1.006 \text{ g/ml} = \frac{(\text{vol. media X den. media}) + (\text{vol. den. sol. X den. den. sol.})}{\text{vol. media} + \text{vol. den. sol.}}$$

vol. media = volume of media
den. media = density of media
vol. den. sol. = volume of density solution
den. den. sol. = density of density solution

The density of the basolateral media was assumed to be 1.000 g/ml. Then 10µl of human plasma was added to the media (Murthy *et al.* 1998). The media was transferred to 3.2ml polycarbonate ultracentrifugation tubes (Beckman) and ultracentrifuged for 22 minutes at 100,000rpm at 4°C in an Optima TLX Ultracentrifuge (Beckman). The top third of the ultracentifuged media was removed and stored at -20°C for further study.

Lipids were isolated from the media and cells as in chapter 4 (section 4.2.9.1) and run on thin layer chromatography (TLC) plates to separate TAG and PL fractions (section 4.2.9.2). TAG and PL fractions were then scraped off the TLC plate directly into 10ml scintillation fluid (Lumagel safe, Lumac.LSC, Packard) and counted for 10 minutes on a scintillation counter (Packard, TriCarb 2100Tr, Liquid Scintillation Analyzer).

Caco-2 cells that had received ^{14}C-glycerol for 24 hours had the least radioactivity in the apical compartment, and the most labelled cellular TAG, PL and basolateral TAG. Caco-2 cells that had received the 4 hour incubation showed the opposite with highest levels of radioactivity in the apical compartment and lowest levels of radioactive cellular TAG, PL and basolateral TAG. Cells that had been incubated with ^{14}C-glycerol for 12 hours showed intermediate apical radioactivity levels and labelled cellular TAG, cellular PL and basolateral TAG levels. Thus it was decided that the acute feed would consist of an

approximate 24 hour incubation of Caco-2 cells with 0.5mM fatty acid and 0.5% BSA and ^{14}C-glycerol.

The TRL fraction could not be separated by the above method and so it was decide to separate the TRL particles by increasing the density of the media to 1.063g/ml and layering it with 1.006g/ml saline solution prior to ultracentrifugation. Exact details can be found in section 5.2.3.2.

5.2.3 Chronic-Acute Experimental Set-up

5.2.3.1 Cell Culture

During this experiment Caco-2 cells were grown from passages 33-47, and *de novo* lipid production was measured using radiolabelled glycerol rather than radiolabelled fatty acids as in other studies (Field *et al.* 1988, van Greevenbroek *et al.* 1995, van Greevenbroek *et al.* 1996, van Greevenbroek *et al.* 1998, van Greevenbroek *et al.* 2000, Moberly *et al.* 1990 & Ranheim *et al.* 1992). The present Caco-2 cell line has been found to secreted less *de novo* TAG than reported in van Greevenbroek's papers (van Greevenbroek *et al.* 1995, van Greevenbroek *et al.* 1996, van Greevenbroek *et al.* 1998 & van Greevenbroek *et al.* 2000). When Caco-2 cells were grown at passages 70-90, which is the same as van Greevenbroek's experiments twice as much lipid was secreted from the cell but it was still lower than reported by van Greevenbroek *et al.* (Seeballuck & O'Driscoll personal communication). However, Caco-2 cells with passages of 93-108 have been found to behave less like enterocytes than cells with passages of 30-40 (Yu *et al.* 1997). Therefore the cells used at the present passages are more likely to produce results similar to what occurs in the human gut.

Radiolabelled glycerol has been used previously to measure lipid secretion from Caco-2 cells (Murthy *et al.* 1990). This study used ^{14}C-glycerol as the radioactive label of *de novo* lipid production because it was in aqueous solution and was therefore easier to dispose of than fatty acids that were in toluene. Although ^{14}C-oleic acid and ^{14}C-palmitic acid could be acquired no radiolabelled EPA could be bought and so to allow comparison between fatty acids treatments and the BSA control, ^{14}C-glycerol was used. ^{14}C-Glycerol may not be incorporated into all *de novo* TAG as endogenous glycerol may be present, while using the radiolabelled fatty acids directly measures all *de novo* lipid production as all other fatty acids have been removed. Therefore, using ^{14}C-glycerol may result in lower

concentrations of labelled *de novo* TAG because either an unlabelled or labelled glycerol molecule could be used to synthesis the TAG.

Caco-2 cells were seeded at a density of $3.3x10^5$ cells per 24mm transwell (Costar) and grown in the presence of 4 supplementations, media, 0.05mM EPA, OA or PA for approximately 19 days (section 4.2.1 and 4.2.2). Subsequently the cells were washed before addition of the one of 4 acute treatments consisting of 0.5% BSA, 0.5mM EPA and 0.5% BSA, 0.5mM OA and 0.5% BSA or 0.5mM PA and 0.5% BSA plus ^{14}C-glycerol as a marker of *de novo* lipid production (section 5.2.3.3) and ^{35}S-methionine as a marker of *de novo* protein production (section 5.2.3.3). Cells were incubated with the acute treatment at 37°C for approximately 22 hours. The 16 different combinations of chronic and acute fatty acid treatments and controls and the sample sizes of the study can be seen in Table 5.2.1. Six well transwell plates (Costar) were set up for each of the 16 combinations of chronic and acute treatments. The first two wells (1 and 2) were harvested to determine total *de novo* cellular and basolateral media lipid concentrations. The middle two wells (3 and 4) were harvested to determine total *de novo* cellular lipid concentrations and basolateral media *de novo* TRL lipid concentrations and the last 2 wells (5 and 6) were harvested to measure cellular and basolateral *de novo* apoB concentrations (Figure 5.2.2).

Well 1	**Well 3**	**Well 5**
Cellular *de novo* TAG & PL Basolateral *de novo* TAG & PL	Cellular *de novo* TAG & PL Basolateral *de novo* TRL-TAG and TRL-PL	Cellular *de novo* apoB100 and apoB48 Basolateral *de novo* apoB100 and apoB48
Well 2	**Well 4**	**Well 6**
Cellular *de novo* TAG & PL Basolateral *de novo* TAG & PL	Cellular *de novo* TAG & PL Basolateral *de novo* TRL-TAG and TRL-PL	Cellular *de novo* apoB100 and apoB48 Basolateral *de novo* apoB100 and apoB48

Figure 5.2.2 Diagram of fractions isolated from each well of the 6 well plate that was given one chronic and one acute treatment.

5.2.3.2 Lipid Analysis

The 4 transwells, in which either total or TRL lipid levels were determined, were washed in 1X PBS. Subsequently, new media with an acute fatty acid treatment was administered to these transwells in the following manner. 2.5ml of media without FCS was added to each basolateral compartment and 1.5ml of media without FCS but containing an acute fatty acid treatment (0.5mM EPA and 0.5% BSA, 0.5mM OA and 0.5% BSA or 0.5mM

PA and 0.5% BSA) was added to each apical compartment of the transwells. Subsequently, 13µl (400ng, 1,443,000 DPM) of ^{14}C-glycerol (140-168mCi/mmol, Amersham) was added to each apical compartment, and cells were incubated for 22 hours at 37°C. This quantity of ^{14}C-glycerol was used because, when the concentration of cellular TAG was measured, there appeared on average to be 400ng of total TAG. Thus, an excess of glycerol was added. The basolateral media for total lipid determination was harvested into falcon tubes and frozen immediately. The basolateral media to be used for TRL isolation was made to a density of 1.063 g/ml using the following equation (section 5.2.2).

$$1.063 \text{ g/ml} = \frac{(\text{vol. media X den. media}) + (\text{vol. den. sol. X den. den. sol.})}{\text{vol. media} + \text{vol. den. sol.}}$$

Subsequently, 15µl of FCS was added to each basolateral media fraction (Murthy *et al.* 1998). Duplicates each containing 1.6ml of media layered with 1.6ml of 1.006 g/ml density saline (section 5.2.2) were ultracentrifuged for 30 minutes at 100,000rpm at 4°C in polycarbonate ultracentrifugation tubes (Beckman). The top quarter was removed for further analysis.

The cells were harvested as described in the above section 5.2.2. TAG and PL levels were examined to determine how chronic and acute fatty acid treatments effect Caco-2 cell lipid production. Lipids were isolated from the cellular and basolateral fractions and separated by thin layer chromatography (TLC) (section 4.2.9.1, 4.2.9.2). TAG and PL fractions were scraped into liquid scintillation fluid (Lumagel safe, Lumac.LSC, Packard) and counted on a scintillation counter (Packard, TriCarb 2100Tr, Liquid Scintillation Analyzer). The TRL fractions were analysed to determine whether any of the fatty acid treatments either increased or decreased the production of TRL density lipoproteins compared to control (chronic treatment = media, acute treatment = 0.5% BSA).

5.2.3.3 Immunoprecipitation and Protein Analysis

The last 2 wells of the 6 well transwell plate (Costar) were washed with 1X PBS before addition of 2.5ml media without FCS and methionine (DMEM minus methionine, GibcoBRL), to the basolateral compartment. In addition, 1.5ml of media without FCS and methionine, but containing acute fatty acid treatment (0.5mM EPA and 0.5% BSA, 0.5mM

OA and 0.5% BSA or 0.5mM PA and 0.5% BSA), was added to the apical compartment. Subsequently, 10µl (2,220,0000 DPM) of ^{35}S-methionine (40-500mCi/mmol, Amersham) was added to each apical compartment and the cells were incubated for 22 hours at 37°C (Murthy *et al.* 1998). The basolateral media was harvested into falcon tubes and protease inhibitors were added (Leupeptin 1µg/µl, Pepstatin 1µg/µl, Aprotinin 1µg/µl, PMSF 10µg/µl and Benzamidine 1µg/µl (Sigma)).

The cells were harvested by first being washed in 1X PBS at 4°C. Then 200µl of Boehringer Mannheim wash 1 with protease inhibitors was added to the cells, which were scraped into eppendorf tubes (Starstead). Cells were centrifuged to remove cellular debris and the supernatant was transferred to a new tube.

Cellular and basolateral fractions were pre-cleared (removal of any other antibodies) using the Boehringer Mannheim protocol, and anti-human apoB antibody (ICN) was added. The immunoprecipitation of apoB48 and apoB100 was carried out using the Protein A-agarose (Boehringer Mannheim) protocol (section 5.2.1). The apoB isoforms were separated on 4% running, 2% stacking SDS-PAGE gels (section 5.2.1). The large molecular weight protein ladder (GibcoBRL) was used to assess how rapidly proteins were running through the gel, as it is a stained protein ladder, and subsequently used to identify the apoB isoforms in the gel. Gels were dried on a gel dryer before autoradiography film (Hyperfilm, Amersham) was put on the gels. Films were developed after about 2 days. A ruler was used to identify the apoB100 and apoB48 bands on the autoradiography film. Comparison between the large molecular weight protein ladder (GibcoBRL) and gel discussed in section 5.2.1 was fundamental in identification of the apoB isoforms. The quantity of both forms of apoB, apoB100 and apoB48, were measured using an instant imager (Packard). No apoB48 could be detected consistently in the basolateral media after experimentation; therefore no information could be gained about the quantity of secreted apoB48 (Figure 5.2.3).

In conclusion, the following 9 variables were examined in an attempt to characterise the possible effect that chronic-acute fatty acid treatment of Caco-2 cells may have on them: cellular apoB100, secreted apoB100, cellular apoB48, cellular TAG, secreted TAG, cellular PL, secreted PL, secreted TRL-TAG and secreted TRL-PL levels. .

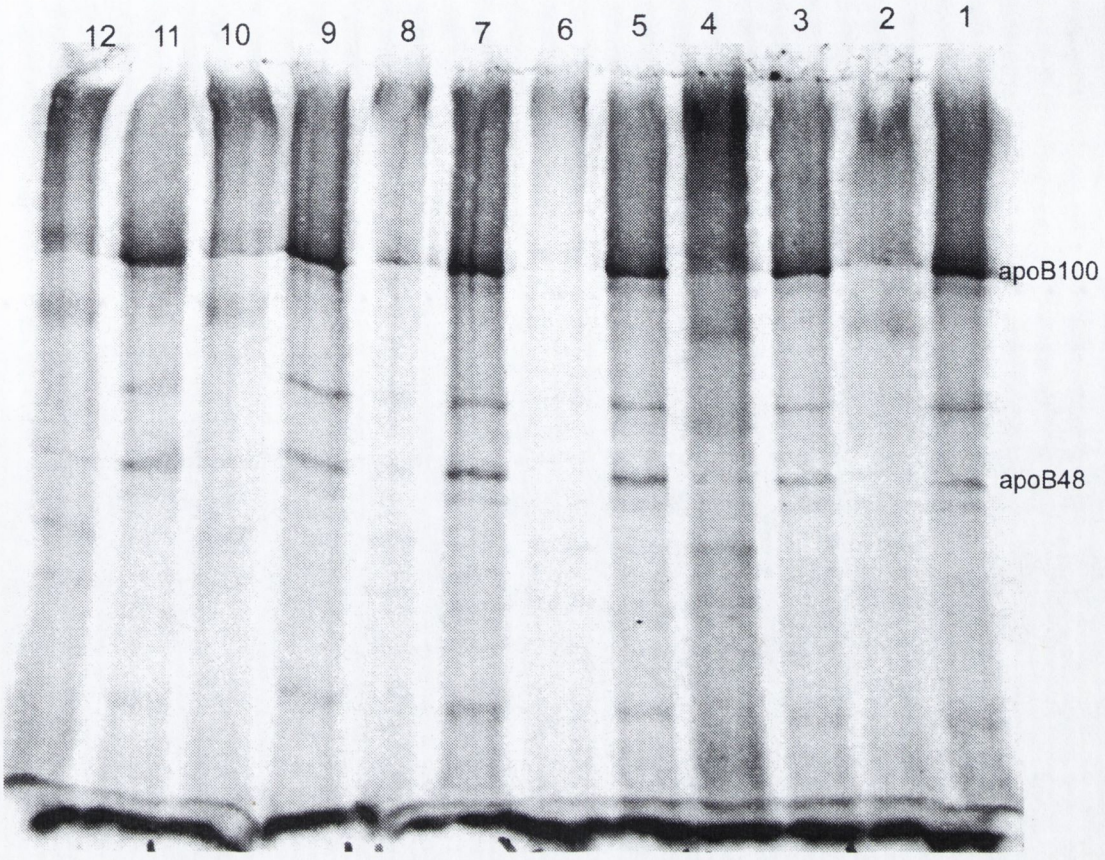


Figure 5.2.3 Autoradiograph of apoB separation on a 4% SDS-PAGE running gel. ApoB100 and apoB48 bands are indicated.

Lane No.	Isolated From	Chronic Treatment	Acute Treatment
1	Cellular	0.05mM PA	0.5mM PA, 0.5% BSA
2	Basolateral	0.05mM PA	0.5mM PA, 0.5% BSA
3	Cellular	Control	0.5% BSA
4	Basolateral	Control	0.5% BSA
5	Cellular	Control	0.5mM EPA, 0.5% BSA
6	Basolateral	Control	0.5mM EPA, 0.5% BSA
7	Cellular	0.05mM EPA	0.5mM EPA, 0.5% BSA
8	Basolateral	0.05mM EPA	0.5mM EPA, 0.5% BSA
9	Cellular	0.05mM PA	0.5mM PA, 0.5% BSA
10	Basolateral	0.05mM PA	0. 5mM PA, 0.5% BSA
11	Cellular	Control	0.5% BSA
12	Basolateral	Control	0.5% BSA

No information could be gained consistently on apoB48 secretion by Caco-2 cells, as no apoB48 band could be detected in samples from basolateral fractions. Other bands may be apoB degradation products.

5.2.4 Statistical Analysis

The means and standard deviations of the 9 variables measured were calculated using statistical package Minitab 8.1.

The primary purpose of the present study was to examine the acute by chronic effects of fatty acids on Caco-2 cell TAG synthesis and secretion levels. Thus of the many variables and combinations of variables that could be evaluated, the following were selected for their capacity to describe TAG and apoB (both apoB100 and apoB48) synthesis and secretion levels: percentage apoB100 secreted (bB100/(cB100 + bB100)*100), percentage of apoB48 present in the Caco-2 cell cellular fraction (cB48/(cB100 + cB48)*100), ratio of cellular TAG to basolateral TAG, ratio of cellular TAG to cellular PL, ratio of basolateral TAG to basolateral PL and ratio of basolateral TAG to basolateral TRL-TAG. Calculation of the ratio of basolateral TAG to basolateral TRL-TAG was carried out by first adding the DPMs in the two transwells from a plate, from which the basolateral TAG was isolated and secondly calculating the average then DPMs of the two transwells from the same plate, from which the basolateral TRL-TAG was isolated, thirdly the average of TAG was divided by the average of TRL-TAG to calculate the ratio (Figure 5.2.2). This leads to a sample size of approximately 2 per chronic-acute treatment.

$$\text{Ratio of Basolateral TAG to Basolateral TRL-TAG} = \frac{\frac{\text{Basolateral TAG Well 1} + \text{Basolateral TAG Well 2}}{2}}{\frac{\text{Basolateral TRL-TAG Well 3} + \text{Basolateral TRL-TAG Well 4}}{2}}$$

The effect of acute treatment alone on the variables above in Caco-2 cells was analysed. Results were compared to results published in the literature. Analysis of variance (ANOVA) (Data Desk 6.0) was used to identify significant effects caused by different acute treatments. In cases where significant acute effects were observed, least significance difference (LSD) tests was used to identify which fatty acids treatments had different effect from the others.

The data obtained from Caco-2 cells that had been given chronic supplementation followed by acute treatment were analysed by 2-way-ANOVA with interactions (Data Desk 6.0) where the main factors were the acute effect, the chronic effect and the acute by chronic interaction. Since the study involved 4 different acute and chronic treatments, a matrix of 16 variables was created. Where significantly acute by chronic, acute or chronic effects were observed, the means were compared by LSD tests to identify which fatty acids were responsible for the significant effect.

Table 5.2.1 Table of chronic-acute experimental set-up. Each combination of chronic treatment followed by acute treatment (16 in all), which was studied is indicated in the table. Sample sizes are given. Two plates yield 8 cellular lipid fractions, 4 basolateral lipid fractions, 4 basolateral TRL fractions, 4 cellular apoB fractions and 4 basolateral apoB fractions. Two and a half plates yield 10 cellular lipid fractions, 5 basolateral lipid fractions, 5 basolateral TRL fractions, 5 cellular apoB fractions and 5 basolateral apoB fractions.

	Acute Treatment			
Chronic Treatment	0.5% BSA (Control)	0.5mM EPA, 0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA
Media (Control)	2 plates	2 plates	2 plates	2 plates
0.05mM EPA	2 plates	2 ½ plates	2 ½ plates	2 ½ plates
0.05mM OA	2 ½ plates	2 ½ plates	2 ½ plates	2 ½ plates
0.05mM PA	2 ½ plates	2 ½ plates	2 ½ plates	2 ½ plates

5.3 Results

The aim of experiments carried out in this chapter was to determine whether long-term diet might affect how test meals are metabolised by the gut. Caco-2 cells a model system for the gut, were used to test the hypothesis that chronic fatty acid supplementation could effect how the cells respond to acute fatty acid treatments.

5.3.1 Chronic and Acute Effects of Fatty Acid Treatment

Since the aim of this study was to determine how fatty acids effect synthesis and secretion of lipids and apolipoproteins, the percentage of secreted apoB100, percentage of apoB48 present in total apoB in the cells, ratio of non-secreted to secreted TAG, ratio of non-secreted TAG to non-secreted PL, ratio of secreted TAG to secreted PL and ratio of secreted TAG to secreted TRL-TAG were examined. The following are the abbreviations used in this chapter and what variables the abbreviations refer to.

cB100	Non-secreted apoB100 (cellular)
bB100	Secreted apoB100 (basolateral)
cB48	Non-secreted apoB48 (cellular)
cTAG	Non-secreted TAG (cellular)
bTAG	Secreted TAG (basolateral)
cPL	Non-secreted PL (cellular)
bPL	Secreted PL (basolateral)
bTRL-TAG	Secreted TRL-TAG (basolateral d=1.006g/ml)
%bB100	Percentage secreted apoB100
%cB48	Percentage cellular apoB48
cTAG/bTAG	Ratio of non-secreted to secreted TAG
cTAG/cPL	Ratio of non-secreted TAG to PL
bTAG/bPL	Ratio of secreted TAG to PL
bTAG/bTRLTAG	Ratio of secreted TAG to TRL-TAG

Table 5.3.1 The mean and standard error (SE) of the measured disintegrations per minute (DPM) of protein and lipid variables. The first two columns refer to the treatments given. Control refers to cells fed media alone for 19 days before acute treatment was given. Abbreviations in the first row are explained in section 5.3.1. There are four spaces in this table as it appeared degradation had occurred of the apoB48 product.

Fraction Counted		cB100		bB100		cB48		cTAG		cPL	
Chronic Treatment	Acute Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	0.5% BSA	16,040	7,045	2,986	1,847	2,585	1,017	10,056	937	25,460	2,787
Control	0.5mM EPA 0.5% BSA	22,117	8,549	840	313	4,623	1,704	16,019	4,397	40,881	1,776
Control	0.5mM OA 0.5% BSA	11,485	2,965	522	224	2,650	651	20,436	551	39,460	4,607
Control	0.5mM PA, 0.5% BSA	3,686	641	1,802	1,579	1,887	755	45,829	4,087	47,161	7,553
0.05mM EPA	0.5% BSA	8,015	1,621	2,350	896	2,151	512	4,059	655	18,756	1,950
0.05mM EPA	0.5mM EPA 0.5% BSA	17,751	6,471	4,876	938	6,428	2,055	7,440	1,785	25,155	3,719
0.05mM EPA	0.5mM OA 0.5% BSA	8,688	2,094	3,624	3,234	1,276	533	15,795	1,687	24,702	1,241
0.05mM EPA	0.5mM PA, 0.5% BSA	6,072	1,404	4,431	2,298	1,281	249	24,886	1,416	38,677	1,610
0.05mM OA	0.5% BSA	7,093	2,059	2,511	1,567	2,428	1,641	9,992	882	20,949	2,251
0.05mM OA	0.5mM EPA 0.5% BSA	6,276	702	2,619	1,365	3,353	1,317	9,639	926	27,385	4,106
0.05mM OA	0.5mM OA 0.5% BSA	8,841	2,123	5,832	1,309	3,555	901	21,768	2,044	25,497	1,368
0.05mM OA	0.5mM PA, 0.5% BSA	9,220	3,366	8,271	3,498	3,646	1,660	38,687	2,732	28,799	3,247
0.05mM PA	0.5% BSA	9,244	3,383	775	321	2,428	608	9,603	772	19,355	1,379
0.05mM PA	0.5mM EPA 0.5% BSA	7,712	3,117	1,237	869			8,718	1,146	26,015	3,912
0.05mM PA	0.5mM OA 0.5% BSA	14,598	7,375	5,080	3,288			23,533	1,779	24,554	1,305
0.05mM PA	0.5mM PA, 0.5% BSA	37,034	7,482	7,424	3,792	8,334	744	34,871	3,882	19,973	3,826

Table 5.3.1 continued

Fraction Counted		bTAG		bPL		bTRL-TAG		bTRL-PL	
Chronic Treatment	**Acute Treatment**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	0.5% BSA	516	40	448	203	390	69	408	172
Control	0.5mM EPA 0.5% BSA	1,068	274	909	191	495	114	476	118
Control	0.5mM OA 0.5% BSA	6,417	1,226	889	324	1,683	402	539	177
Control	0.5mM PA, 0.5% BSA	4,857	883	830	301	1,337	369	509	171
0.05mM EPA	0.5% BSA	400	74	165	40	326	51	196	29
0.05mM EPA	0.5mM EPA 0.5% BSA	763	239	410	96	602	153	193	50
0.05mM EPA	0.5mM OA 0.5% BSA	2,589	151	302	7	806	282	318	101
0.05mM EPA	0.5mM PA, 0.5% BSA	1,609	177	404	82	960	236	362	112
0.05mM OA	0.5% BSA	778	53	143	14	1,442	823	266	45
0.05mM OA	0.5mM EPA 0.5% BSA	878	139	591	206	709	117	219	25
0.05mM OA	0.5mM OA 0.5% BSA	4,588	270	377	25	1,268	100	266	34
0.05mM OA	0.5mM PA, 0.5% BSA	3,137	465	455	70	1,094	135	285	20
0.05mM PA	0.5% BSA	842	118	180	26	714	142	232	30
0.05mM PA	0.5mM EPA 0.5% BSA	1,237	326	437	169	729	269	252	51
0.05mM PA	0.5mM OA 0.5% BSA	3,870	241	359	24	1,207	33	273	49
0.05mM PA	0.5mM PA, 0.5% BSA	2,555	291	379	94	842	144	258	42

5.3.1.1 Acute Effects of Fatty Acid Treatment

The effect of acute fatty acid treatment alone on the above mentioned ratios can be seen in Table 5.3.2. Acute fatty acid treatment alone refers to row one of Table 5.2.1 which is Caco-2 cells grown in control media (no supplementation of fatty acid) for approximately 19 days before the addition of 0.5% BSA, 0.5mM EPA and 0.5% BSA, 0.5mM OA and 0.5%BSA or 0.5mM PA and 0.5% BSA. The current study found a significant effect of acute OA treatment on the ratio of secreted TAG to secreted PL (bTAG/bPL). Acute OA treatment caused a significant increase in bTAG/bPL compared to acute doses of either 0.5% BSA (LSD p=0.005) or 0.5mM EPA and 0.5% BSA (LSD p=0.002). However, acute OA treatment was not significantly different from acute PA treatment (LSD p=0.369), and acute PA treatment was also shown to be significantly different from acute BSA (LSD p=0.02) and acute EPA (LSD p=0.01) treatments.

An acute EPA dose resulted in no significant difference in the variables measured, compared to control and other acute fatty acid treatments.

Acute PA treatment caused a significant accumulation of cellular *de novo* TAG compared to cellular *de novo* PL (cTAG/cPL p=0.0001). Acute PA treatment differed from all other acute treatments; 0.5% BSA (LSD p=0.000), 0.5mM EPA and 0.5% BSA (LSD p=0.000) and 0.5mM OA and 0.5% BSA (LSD p=0.000)

5.3.1.2 Chronic Effects and Acute on Chronic Effects of Fatty Acid Treatment

Six parameters were chosen to determine the main effects of fatty acid treatments on Caco-2 cell lipid and apolipoprotein synthesis and secretion levels. Five of these are shown in Tables 5.3.3a to 5.3.3e. The percentage of secreted *de novo* apoB100 (%bB100) is given in Table 5.3.3a. Two-way-ANOVA showed no acute effect (p=0.187) of fatty acid treatment on %bB100 levels. However, a significant chronic fatty acid supplementation effect (p=0.004) was observed for this variable. Supplementation of Caco-2 cells with OA increased %bB100 levels significantly compared to control cells (LSD p=0.001) and cells given chronic PA treatment (LSD p=0.008). However, this difference was not significant compared to cells given chronic EPA supplementation (LSD p=0.08). No significant acute by chronic interaction (p=0.442) was shown to influence %bB100 levels.

The percentage of cellular *de novo* apoB48 to apoB100 (%cB48) was investigated after chronic and acute fatty acid treatments and appeared not to be effected significantly (data not shown); acutely (p=0.968), chronically (p=0.219) or acute by chronically (p=0.607).

The ratios of non-secreted TAG to secreted TAG (cTAG/bTAG) are given in Table 5.3.3b. There was no significant chronic effect observed (p=0.468) on this ratio. However, a significant acute effect was observed (p=0.0001). Cells given acute OA treatment had a reduced ratio of cTAG/bTAG due to more *de novo* TAG secretion than cells given acute BSA (LSD p=0.000) and EPA treatments (LSD p=0.000). Acute PA treatment was also shown to have significantly different affects on cells than acute OA treatment (LSD p=0.000). An acute by chronic interaction was also observed for the ratio of cTAG/bTAG (p=0.024). As the aim of this study was to characterise in what manner chronic supplementation effects how the Caco-2 cells respond to acute fatty acid treatment, the most relevant way to describe the interaction is the following[2]:

- Caco-2 cells supplemented chronically with EPA and then treated with acute BSA were significantly different from cells, which were not supplemented with fatty acids and were then given acute BSA treatments (LSD p=0.004). Chronic EPA supplementation (8.29) resulted in an increase in *de novo* TAG secretion.
- Chronic EPA supplementation followed by acute EPA treatment had a significantly different affect on cells than no fatty acid supplementation followed by acute EPA treatment (LSD p=0.015). Cells supplemented with PA and subsequently treated with EPA were significantly different from cells, which had not had fatty acid supplementation (LSD p=0.016). Cells given chronic EPA (7.54) and PA (7.59) supplementation followed by acute EPA treatment manifested higher *de novo* TAG secretion levels compared to Caco-2 cells which had not been chronically supplemented with fatty acids and which had been treated acutely with EPA (14.21).
- Cells supplemented chronically with EPA and subsequently treated with acute doses of PA were significantly different to cells which had not been chronically supplemented with fatty acids and then had been fed acute doses of PA (LSD p=0.05). Caco-2 cells given chronic supplementation of EPA (14.25) had lower levels of *de novo* TAG secretion compared to cells which had not been supplemented with fatty acids and then had been given acute PA treatment (8.88).

Significant chronic (p=0.041) and acute (p=0.001) effects were found after fatty acid treatments on the ratio of non-secreted TAG to non-secreted PL (cTAG/cPL) (Table 5.3.3c). When LSD tests were carried out to analyse which of the chronic fatty acid treatments differed from each other, chronic EPA and PA supplementations appeared to

[2] The unabbreviated results of the least significance difference test can be found in appendix II.

differ most (LSD p=0.006). However, chronic PA treatment also appeared to differ significantly from cells not supplemented with fatty acids (LSD p=0.03). Cells given chronic EPA supplementation had the lowest ratio of cTAG/cPL (0.443), while cells given chronic PA supplementation had the highest ratio of cTAG/cPL (1.255). Control cells had a ratio of cTAG/cPL of 0.62. The significant acute effect was due to acute PA treatment being significantly different to all other fatty acid treatments: acute BSA doses (LSD p=0.001), acute EPA doses (LSD p=0.0008) and acute OA doses (LSD p=0.04). Acute PA treatment caused an increase the ratio of cTAG/cPL. No acute by chronic effect was observed for the ratio of cTAG/bTAG (p=0.127).

The ratios of secreted TAG to secreted PL (bTAG/bPL) are given in Table 5.3.3d. Both significant chronic (p=0.002) and acute effects (p=0.0001) were observed. Chronic OA and PA supplementation to cells stimulated TAG secretion compared to PL secretion significantly more than control (LSD p=0.02 and 0.03 respectively) or chronic EPA supplementation (LSD p=0.001 and 0.002 respectively). All acute fatty acid treatments altered *de novo* TAG secretion levels over PL secretion levels. Acute EPA treatment resulted in a decrease in the ratio of bTAG/bPL (LSD p=0.05). Acute OA and PA treatments both caused an increase in the ratio of bTAG/bPL (LSD p=0.000 and LSD p=0.0001 respectively). Acute fatty acid treatments also significantly differed from each other. Acute EPA doses resulted in an increase in PL secretion levels. However acute OA and PA treatments caused an increase in TAG secretion levels (LSD p=0.000 and LSD p=0.000 respectively). Acute OA treatments resulted in a greater increase in TAG secretion levels than acute PA treatments (LSD p=0.000). No acute by chronic interaction was observed for the ratio of bTAG/bPL (p=0.344).

The effects of fatty acid treatments on the ratios of secreted TAG to secreted TRL-TAG (bTAG/bTRL-TAG) are given in Table 5.3.3e. No chronic fatty acid supplementation effect was observed (p=0.0468). However, acute fatty acid treatment did cause a significant change in the ratio of bTAG/bTRL-TAG (p=0.004). Both acute OA and PA treatments caused a reduction in the amount of TAG associated with the TRL-TAG fraction. LSD tests showed that the effects of acute OA and PA doses were not significantly different from each other (p=0.254). However, acute OA and PA treatments were significantly different to acute BSA doses (LSD p=0.000 and p=0.002 respectively) or acute EPA doses (LSD p=0.000 and p=0.007 respectively). No significant acute by chronic interaction (p=0.962) was observed for the ratio of bTAG/bTRL-TAG.

Table 5.3.2 Table showing effects (mean and standard error (SE)) of acute fatty acid feeding on cells grown in media without chronic fatty acid enhancement. Each column represents the acute dose administered to the cells. The coloured superscripts are the notable values calculated from least significance difference (LSD) test, an EPA effect is green, an OA effect is red and a PA effect is blue. The ratio was calculated per well if possible and by plate for the ratio of bTAG/bTRL-TAG. For example, to calculate cTAG/bTAG, DPMs of cellular TAG were divided by DPMs of basolateral TAG. The mean ratios were then calculated. MSE = Mean Squared Error. NS = Non-significant

Acute fatty acids effects

Acute Fatty Acid Treatment	0.5% BSA (Control)	0.5mM EPA, 0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA	MSE	Significance
Measured Variables	Mean & (SE)	Mean & (SE)	Mean & (SE)	Mean & (SE)		
%bB100	15.33 (7.3)	6.57 (4.5)	3.5 (1.4)	17.8 (10.4)	248	NS
%cB48	20 (3.5)	22.1 (3.0)	20.4 (2.6)	26.7 (9.8)	87	NS
cTAG/bTAG	17.0 (2.68)	14.21 (5.44)	3.68 (0.66)	8.88 (0.85)	37.5	NS
cTAG/cPL	0.401 (0.026)[a]	0.391 (0.102)[a]	0.564 (0.059)[a]	1.1 (0.148)[b]	0.075	P=0.0001
bTAG/bPL	1.79 (0.83)[a]	1.5 (0.57)[a]	9.53 (2.15)[b]	7.58 (1.6)[b]	8.595	P=0.0059
bTAG/bTRL-TAG	1.79 (n=1)	2.14 (0.05)	3.89 (0.21)	3.92 (0.66)	0.327	NS

Table 5.3.3 Tables of the effects of chronic, acute and acute by chronic treatments of different fatty acids on Caco-2 cells.
A mean for each chronic-acute fatty acid treatment is calculated using the arithmetic mean of each treatment. The last row in each table represents the average acute effect of a fatty acid treatment and is calculated by taking the arithmetic mean of all acute treatments with the fatty acid. The last column in each table represents the average chronic effect of a fatty acid supplementation and is calculated by taking the arithmetic mean of all results obtained from cells given chronic doses of fatty acids. The acute, chronic and acute by chronic interaction were analysed using a 2-way-ANOVA with interactions. LSD tests were used to identify the fatty acid effects and superscripts are used to identify fatty acids which had similar and different effects. The important means are highlighted using different colours for the different fatty acids which caused the effect EPA, OA and PA. MSE = Mean squared error.

Table 5.3.3a Percentage secreted apoB100. In this table the percentage of apoB100 secretion by Caco-2 cells following various chronic and acute fatty acid treatments were compared. It is clear that chronic OA supplementation increases levels of apoB100 secretion irrespectively of the acute fatty acid treatment (32.64) compared to cells not supplemented (10.33) or supplemented with PA (15.19).

Percentage secreted apoB100 (%bB100)

Acute	0.5% BSA	0.5mM EPA, 0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA		Chronic Effect
Chronic	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)		Mean (SE)
Control	15.33 (7.3)	6.57 (4.5)	3.5 (1.4)	17.8 (10.4)		10.33 (3.23)[a]
0.05mM EPA	17.6 (4.9)	27.4 (12.8)	7.75 (3.9)	32.6 (18.0)		22.05 (5.81)[a b]
0.05mM OA	23.6 (11.9)	23.14 (10.7)	42.71 (6.7)	39.5 (11.5)	MSE = 423	32.64 (5.02)[b]
0.05mM PA	6.5 (1.7)	7.8 (2.4)	25.8 (8.0)	22.4 (6.8)	Acute by Chronic P=0.4418	15.19 (3.24)[a]
Acute Effect	15.32 (3.75)	16 (4.44)	21.82 (4.5)	28.62 (5.86)	Acute P=0.1868	Chronic P=0.0041

Table 5.3.3 continued

Table 5.3.3b Ratio of cTAG/bTAG. In this table the cTAG/bTAG levels were compared in cells given chronic followed by acute fatty acid treatments. Interestingly acute OA supplementation decreases cTAG/bTAG levels (4.76). An acute by chronic interaction was observed, where Caco-2 cells supplemented with EPA were significantly different to control cells after acute BSA (8.29 and 16.99), EPA (7.54 and 14.21) and PA (14.25 and 8.88) treatments. Caco-2 cells chronically supplemented with PA and then acutely treated with EPA (7.59) were also significantly different to control cells treated with acute doses of EPA (14.21).

Ratio of non-secreted TAG to secreted TAG (cTAG/bTAG)

Acute	0.5% BSA	0.5mM EPA, 0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA		Chronic Effect
Chronic	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)		Mean (SE)
Control	16.99 (2.68)[1]	14.21 (5.44)[1]	3.68 (0.66)	8.88 (0.85)[1]		10.54 (1.94)
0.05mM EPA	8.29 (1.06)[2]	7.54 (1.28)[2]	5.08 (0.44)	14.25 (1.38)[2]		8.79 (1.0)
0.05mM OA	12.01 (0.65)	11.12 (1.14)[12]	4.38 (0.56)	12.7 (0.67)	MSE = 14.05	10.05 (0.93)
0.05mM PA	11.76 (2.08)	7.59 (2.33)[2]	5.91 (0.93)	13.83 (1.65)	Acute by Chronic P=0.0236	9.77 (1.16)
Acute Effect	11.95 (1.08)[a]	10.11 (1.55)[a]	4.76 (0.37)[b]	12.41 (0.77)[a]	Acute P=0.0001	Chronic P=0.4678

Table 5.3.3c Ratio of cTAG/cPL. In this table the ratios of non-secreted TAG to PL are given, found in cells which have been fed chronic followed by acute doses of various fatty acids. The table shows that Caco-2 cells given chronic PA treatment (1.255) have a higher ratio of cTAG/cPL than cells given no chronic supplements (0.62) or chronic EPA supplements (0.443). In addition acute doses of PA (1.6) increase levels of cTAG/cPL in Caco-2 cells.

Ratio of cellular TAG to cellular PL (cTAG/cPL)

Acute	0.5% BSA	0.5mM EPA, 0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA		Chronic Effect
Chronic	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)		Mean (SE)
Control	0.401 (0.026)	0.391 (0.102)	0.564 (0.059)	1.099 (0.148)		0.62 (0.071)[a]
0.05mM EPA	0.21 (0.022)	0.283 (0.042)	0.634 (0.043)	0.645 (0.026)		0.443 (0.039)[a]
0.05mM OA	0.496 (0.042)	0.394 (0.052)	0.86 (0.086)	1.448 (0.16)	MSE = 1.369	0.799 (0.087)[ab]
0.05mM PA	0.495 (0.018)	0.363 (0.042)	0.961 (0.06)	3.201 (1.621)	Acute by Chronic P=0.1273	1.255 (0.437)[b]
Acute Effect	0.401 (0.03)[a]	0.358 (0.032)[a]	0.755 (0.042)[a]	1.6 (0.426)[b]	Acute P=0.0001	Chronic P=0.0406

Table 5.3.3 continued

Table 5.3.3d Ratio of bTAG/bPL. Basolateral media TAG and PL levels were measured in cells given chronic followed by acute doses of fatty acids. Clearly, both chronic and acute doses of OA (6.771 and 10.37) and PA (6.694 and 6.659) caused an increase in the ratio of bTAG/bPL, with OA having the greatest effect. However, an acute dose of EPA (2.221) significantly reduces the ratio of bTAG/bPL.

Ratio of secreted TAG to secreted PL (bTAG/bPL)

Acute / Chronic	0.5% BSA Mean (SE)	0.5mM EPA, 0.5% BSA Mean (SE)	0.5mM OA, 0.5% BSA Mean (SE)	0.5mM PA, 0.5% BSA Mean (SE)		Chronic Effect Mean (SE)
Control	1.787 (0.833)	1.498 (0.572)	9.525 (2.153)	7.582 (1.602)		5.319 (1.158)[a]
0.05mM EPA	2.595 (0.332)	1.905 (0.33)	8.575 (0.4)	4.435 (0.874)		4.377 (0.711)[a]
0.05mM OA	5.622 (0.663)	1.858 (0.367)	12.462 (1.472)	7.142 (0.839)	MSE = 3.959	6.771 (1.067)[b]
0.05mM PA	4.747 (0.315)	3.625 (0.724)	10.927 (0.92)	7.475 (1.254)	Acute by Chronic P=0.3436	6.694 (0.825)[b]
Acute Effect	3.815 (0.47)[a]	2.221 (0.317)[b]	10.37 (0.731)[c]	6.659 (0.626)[d]	Acute P=0.0001	Chronic P=0.0021

Table 5.3.3e Ratio of bTAG/bTRL-TAG. Ratios of basolateral TAG to basolateral TRL-TAG were measured in cells supplemented with chronic fatty acids and then given acute fatty acid treatment. The ratio was calculated using the formula: bTAG/bTRL-TAG = Plate 1: [(bTAG Well 1 + bTAG Well 2)/2]/[(bTRL-TAG Well 3 + bTRL-TAG Well 4)/2]. Acute doses of OA (3.687) and PA (3.137) caused a decrease in the quantity of TAG secreted in the form of TRL-TAG. This is an unexpected result in the case of OA (Moberly *et al.* 1990) but not in the case of PA (van Greevenbroek *et al.* 2000)

Ratio of secreted TAG to secreted TRL-TAG (bTAG/bTRLTAG)

Acute / Chronic	0.5% BSA Mean (SE)	0.5mM EPA, 0.5% BSA Mean (SE)	0.5mM OA, 0.5% BSA Mean (SE)	0.5mM PA, 0.5% BSA Mean (SE)		Chronic Effect Mean (SE)
Control	1.787	2.139 (0.053)	3.894	3.918 (0.665)		3.098 (0.413)
0.05mM EPA	1.208 (0.138)	1.256 (0.031)	4.002 (1.834)	2.544 (0.83)		2.252 (0.577)
0.05mM OA	0.871 (0.355)	1.258 (0.071)	3.646 (0.228)	3.053 (1.143)	MSE = 0.858	2.207 (0.498)
0.05mM PA	1.171 (0.049)	2.081 (0.613)	3.206 (0.072)	3.033 (0.011)	Acute by Chronic P=0.9623	2.373 (0.33)
Acute Effect	1.184 (0.14)[a]	1.684 (0.199)[a]	3.687 (0.37)[b]	3.137 (0.349)[b]	Acute P=0.0004	Chronic P=0.4677

5.4 Discussion

5.4.1 Acute by Chronic Interaction

The hypothesis that chronic fatty acid supplementation of Caco-2 cells effects how the cells respond to acute fatty acid treatment was tested in this chapter. A significant interaction between the two types of fatty acid treatment was observed for the ratio of non-secreted TAG to secreted TAG. Chronic EPA supplementation was significantly different from no fatty acid supplementation after acute treatments of BSA, EPA and PA. However, this effect was not consistent as chronic EPA treatment of cells increased TAG secretion levels after acute BSA and EPA treatments but decreased TAG secretion levels after acute PA treatments. In the literature EPA treatment is associated with decreased TAG secretion by both Caco-2 cells (Murthy *et al.* 1990) and human hepatocytes (Lin *et al.* 1995). Although one might expect that the extra fatty acids present after supplementation result in there being more TAG present for secretion, EPA treatment is usually associated with increased PL synthesis levels and reduced TAG synthesis levels (Ranheim *et al.* 1992). Also, as a significant increase in TAG secretion levels was not observed after either OA or PA supplementation, EPA supplementation is an unlikely reason for the increased TAG secretion. OA treatment is usually associated with increased TAG secretion levels (Field *et al.* 1988) and PA treatment with increased cellular TAG levels (Arrol *et al.* 2000). If the study were repeated with a larger sample size (eg. n=16 rather than n=8) possibly a consistent interaction would be observed. In cells the effect of background diet on how they respond to an acute test meal may be very small, making it difficult to identify this difference with significance without the use of larger samples sizes.

5.4.2 Acute and Chronic Effects of Fatty Acid Treatments on Caco-2 Cell Lipid Metabolism

It is apparent that the fatty acid treatments have the same biochemical effects on Caco-2 cells, whether given chronically or acutely. However, the treatments may not have significant effects on all the variables measured in this study. It appears that cells given chronic supplementation of fatty acids may behave in a similar fashion to cells given low doses of fatty acids and cells given acute treatments may behave in a similar fashion to

cells given high doses of fatty acids. However, the effects of high doses of fatty acids may be easier to detect than the effects of low doses. This suggests that Caco-2 cells may not be useful for measuring the effects of background diets. Future studies into background diets in humans should therefore most probably be carried out in humans.

5.4.2.1 Palmitic Acid

Caco-2 cells treated with either acute or chronic PA treatments showed increased cellular TAG concentrations (cTAG/bTAG and cTAG/cPL) compared to cells given other fatty acid treatments. Previous cell studies have demonstrated that acute PA treatments are associated with high cellular TAG levels (Arrol *et al.* 2000, van Greevenbroek *et al.* 1996). Arrol *et al.* (2000) found that HepG2 cells given acute PA treatments had the highest levels of cellular TAG followed by HepG2 cells treated with acute doses of OA or EPA. Van Greevenbroek *et al.* (1996) however, observed that Caco-2 cells treated with OA had higher cellular TAG concentrations than cells treated with PA, but acute OA treatment resulted in cells having a lower cTAG/bTAG ratio (5) than acute PA treatment (6.8). Therefore, in both van Greevenbroek *et al.* (1996) and the present study, PA treatment has been found to be associated with retarded TAG secretion compared to acute OA treatment. Van Greevenbroek *et al.* (2000) showed that in Caco-2 cells acute PA treatments caused secretion of IDL/LDL density lipoproteins, which are secreted after all TAG consisting of MUFAs has been secreted in chylomicron/VLDL density lipoproteins. However, cells given 0.1mM PA secreted fewer IDL/LDL lipoproteins than control cells (van Greevenbroek *et al.* 1998), which is hypothesised to be due to their having an increased ratio of unsaturated to saturated fatty acids. SFAs may be less good substrates for the microsomal triglyceride transfer protein (MTP) than unsaturated fatty acids. The present study found that cells given acute PA treatments had significantly less *de novo* TAG associated with TRL particles. This was not observed in cells given chronic doses of PA, which may be due to lower SFA to unsaturated fatty acid ratio in the chronic PA supplemented Caco-2 cells. Karpe *et al.* (1997) stated that large chylomicrons are cleared faster from the circulation than small VLDL-sized intestinal lipoproteins because they contain more unsaturated fatty acids with are better substrates for LPL hydrolysis than TAG comprised of SFAs. Therefore, as previously acknowledged, the TAG synthesised after an SFA meal is cleared more slowly from the circulation than TAG synthesised after an n-3 PUFA or n-6 PUFA meal (Zampelas *et al.* 1994).

5.4.2.2 Oleic Acid

In this study as in other studies OA treatment was found to be associated with increased TAG secretion levels (cTAG/bTAG and bTAG/bPL) (Field *et al.* 1988). In human studies MUFA test meals (Zampelas et al. 1998) and background diets (Roche *et al.* 1998) are known to cause a faster influx and clearance of TAG than SFA test meals or diets. Since Caco-2 cell culture studies have not found any difference in the absorbance of fatty acids by the cells (Ranheim *et al.* 1992, van Greevenbroek *et al.* 1995), OA treatment most likely effects TAG synthesis or secretion levels. Caco-2 cells synthesise TAG by the glycerol-3-phosphate pathway (Levy *et al.* 1995), while enterocytes synthesise TAG by the monoacylglycerol pathway (Gurr & Harwood 1991). Therefore, the same influx of TAG observed from Caco-2 cells given OA is also observed from enterocytes (postprandial response) after a MUFA meal. Thus the increased TAG secretion levels seen in Caco-2 cells after OA treatment is unlikely to be due to increased TAG synthesis. Although the promoter region of the *microsomal triglyceride transfer protein* (*MTP*) gene has a binding site for a transcription factor that is known to respond directly to fatty acid conditions (Hagan *et al.* 1994), mediators of lipoprotein secretion, such as OA, have been shown not to effect MTP gene expression, mass or activity (Mathur *et al.* 1997). Van Greevenbroek *et al.* (1998) hypothesised that TAG synthesised from MUFAs such as OA is a better substrate for the MTP complex than TAG synthesised from SFAs. In the endoplasmic reticulum the MTP complex folds apoB protein as it is being translated, forming a hydrophobic core. Subsequently, it transfers lipids to this core (Gordon 1997) (section 1.2.2.3), thus stabilising the apoB protein and protecting it from degradation. Acute OA treatment is usually associated with increased apoB secretion levels. However, this increase was not observed in this study, which may just be an artefact of the study. However, chronic OA supplementation may, like acute OA treatments described in the literature, increase apoB secretion levels by protecting apoB from degradation (Dashti *et al.* 1990, Dixon *et al.* 1991). OA treatment of cells is usually associated with increased concentrations of <1.006 g/ml density lipoproteins (van Greevenbroek *et al.* 2000). In the present study *de novo* TAG was not found to be associated with TRL particles. This is probably due to the fact that the secreted lipoproteins were left in contact with Caco-2 cells for 22 hours before they were harvested. Field *et al.* (1988) had previously shown that Caco-2 cells secrete all TRLs associated with acute OA treatment within 6 hours. They also found that by 24 hours after OA treatment 0.17% of the TRLs were degraded and 50% of the *de novo* TAG had been hydrolysed. HepG2 cells have been found to hydrolyse TAG from secreted lipoproteins reducing the size of the lipoproteins (Arrol *et al.* 2000).

This hydrolysis may also occur if secreted lipoproteins are left in contact with Caco-2 cells, causing the *de novo* TAG not to be associated with the TRL sized fraction. This may be the reason why in this study acute OA treatments were not observed to associate with increased levels of TRL sized particles. From tissue culture studies it appears more likely that the influx of postprandial TAG, observed in humans after a MUFA test meal or diet, is due to enhanced lipoprotein assembly as MUFAs are better MTP substrates than SFAs.

5.4.2.3 Eicosapentaenoic Acid

In the present study chronic and acute EPA treatments of Caco-2 cells were found to be associated with increased *de novo* PL levels over TAG levels (cTAG/cPL and bTAG/bPL), suggesting that EPA treatments induce PL synthesis. Caco-2 cells given acute EPA treatments previously have been shown to incorporate EPA into the PL fraction of cellular lipids (Ranheim *et al.* 1992, section 4.4.3). This is possibly achieved by a reduction of diacylglycerol acyl transferase activity (Ranheim *et al.* 1992). It is therefore less difficult for diacylglycerol to enter into the PL synthesis pathways due to less competition. Diacylglycerol, synthesised from phosphatidic acid by phosphatidate phosphohydrolase (glycerol-3-phosphate pathway section 1.2.2.2), is used for the synthesis of TAG, phosphatidylcholine and phosphatidylethanolamine (Gurr & Harwood 1991[a]). Phosphatidylcholine and phosphatidylethanolamine are highly abundant phospholipids. Phosphatidylcholine is the most abundant animal phospholipid and phosphatidylethanolamine is both abundant and widely distributed (Gurr & Harwood 1991[b]). However, although enterocytes do not synthesise TAG by the glycerol-3-phosphate pathway, diacylglycerol that is formed may be free to be synthesised into structural lipoproteins due to the inhibition of diacylglycerol acyl transferase activity by EPA. Although n-3 PUFA test meals and background diets are believed to attenuate postprandial lipaemia by inhibiting VLDL-TAG secretion by the liver (Roche *et al.* 1999), it appears that EPA treatment can also inhibit chylomicron-TAG secretion by the gut by retarding TAG synthesis. The microsomal triglyceride transfer protein (MTP) may be responsible for retarding lipoprotein secretion after EPA (Lin *et al.* 1995) or n-3 PUFA treatments, because non-polar lipids are the preferred substrate for MTP (Jamil *et al.* 1995). Therefore, TAG molecules are bound preferentially over PL molecules to apoB by MTP.

5.4.3 Conclusion

In the study outlined in this chapter the possibility was examined that the fatty acid constituents of cell culture media that Caco-2 cell develop in affect how the cells respond to acute fatty acid treatments. The results of the study showed that the combination of chronic and acute fatty acid treatments affects the ratio of cTAG/bTAG. However, the affect was inconsistent. Therefore, to determine unambiguously whether an acute by chronic interaction indeed exists the experiment needs to be repeated. Investigation of acute and chronic effects of fatty acid treatments on Caco-2 cells showed that acute PA treatment might increase TAG synthesis. An increase in TAG synthesis was also observed after chronic PA treatments (cTAG/cPL). Acute and chronic OA and PA treatments increased the ratio of bTAG/bPL secreted from cells. In addition, acute OA and PA treatments decreased the amount of *de novo* TAG associated with the TRL fraction. EPA (n-3 PUFA) caused less TAG to be synthesised by Caco-2 cells when administered chronically, thus decreasing the ratio of cTAG/cPL. Acute doses of EPA, however, significantly decreased the bTAG/bPL ratio. In addition, chronic OA supplementation seemed to enhance apoB secretion levels, which may be due to the protective effect of OA on apoB stability. In conclusion, chronic supplementation of fatty acids administered to Caco-2 cells appeared to have similar effects on lipid and apolipoprotein concentrations as acute fatty acid treatments. However, in Caco-2 cells chronic fatty acid supplementation affected the cells in a similar manner as a low-dose of fatty acid might and acute treatments affected the cells as a high-dose of fatty acid might.

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6 Effect of Conjugated Linoleic Acid on Growth and Lipid Metabolism of Caco-2 Cells

6.1 Introduction

Conjugated linoleic acid (CLA) is a term, which refers to a mixture of naturally occurring positional and geometric isomers of linoleic acid (LA). CLA is a *trans* n-6 PUFA that is naturally found in ruminant meat and diary products (Park & Pariza 1998). There are two main conjugates of linoleic acid; t-10 (*trans*-10, *cis*-12 isomer) and c-9 (*cis*-9, *trans*-11 isomer) (Figure 1.6.1). CLA in animal foodstuffs consists of approximately 80% c-9. However, in vegetable oils the isomers are found at a ratio of approximately 43% t-10:43% c-9 with other isomers accounting for the additional 14%, due to partial hydrogenation during the extraction process (Pariza *et al.* 1999).

There is much interest in CLA as its intake has been associated with reduced fasting plasma TAG levels in humans (Noone *et al.* in press) and reduced serum TAG levels in rabbits (Lee *et al.* 1994). Lee *et al.* (1994) hypothesised that the reduced TAG levels in rabbits may be due to increased carnitine palmitoyltransferase (CPT) activity or reduced TAG secretion from the intestine and/or liver. CTP is the rate limiting fatty acid β-oxidising enzyme; therefore increased CPT activity would reduce fatty acid levels in the organs where it is expressed. CLA also has been found to effect body composition and growth in humans and rodents (Belury *et al.* 1997, Brodie *et al.* 1999, Chin *et al.* 1994), by enhancing lean body mass and reducing body fat levels. In a study on mice, a CLA diet did not alter body weight, but did reduce body fat and enhance body protein levels. CPT activity was increased in fat pads and skeletal muscles of mice fed CLA but not in their livers, suggesting there is increased fatty acid oxidation after CLA ingestion. Supplementation of 0.1mM CLA to differentiated murine 3T3-L1 adipocytes reduced their LPL activity but enhanced lipolysis (Park *et al.* 1997, Brodie *et al.* 1999). Subsequently, the t-10 isomer was found to be responsible for reducing LPL activity and enhancing lipolysis in this cell line (Pariza *et al.* 1999). CLA has similar features to peroxisome proliferators, in that it reduces body weight and increases hepatic lipid levels. The peroxisome proliferator activated receptor α (PPARα) is found in the liver and peroxisome proliferator response elements have been found upstream of transcription

initiation sites of many genes which encode enzymes involved in lipid metabolism (Moya-Camarena *et al.* 1999, Moya-Camarena & Belury 1999). Therefore, CLA has the ability to effect gene expression and alter lipid metabolism (section 1.6.4).

Previous studies have shown that CLA effects TAG clearance. The present study set out to investigate whether CLA may effect plasma TAG concentrations at a gut secretory level. The effects of the two major CLA isomers, t-10 and c-9, on TAG secretion, using the Caco-2 cell line as a model system for intestinal lipid metabolism, were examined. How chronic supplementation of LA or its isomers effects how Caco-2 cells respond to acute fatty acid treatments of oleic or palmitic acid was investigated. However, since this cell line was originally derived form an adenocarcinoma and CLA is known to inhibit proliferation of tumors (Liu & Belury 1998) (section 1.6.2), caution was required when dealing with this cell line. Therefore cell proliferation was tested in both chronic and acute fatty acid treated cells. In addition to proliferation assays, whether CLA isomer supplementation, either acutely or chronically, effects cell development and whether the CLA is incorporated into lipid moieties was characterised.

6.2 Materials and Methods

Caco-2 cells were seeded and grown under the same conditions as reported in chapter 4 (section 4.2.1). However, in this chapter apical media was supplemented with either linoleic acid (LA) or its conjugates t-10 and c-9. LA refers to *cis*-9, *cis*-12 octadecadienoic acid (Sigma), t-10 refers to the *trans*-10, *cis*-12 isomer and c-9 refers to the *cis*-9, *trans*-11 isomer (Loders Croklaan BV, Wormerveer, The Netherlands) (Figure 1.6.1). Fatty acids were stored at concentrations of 50mM in ethanol at -20°C. Caco-2 cells were fed either 0.1mM or 0.05mM of one of the three LA isomers under the same conditions as described in chapter 4, section 4.2.2. To ensure that any effects on cell growth, fatty acid assimilation or lipid and apolipoprotein synthesis and secretion of supplemented Caco-2 cells were due to CLA and not n-6 PUFA, LA was used as a control of n-6 PUFA effects. Six assays were carried out on Caco-2 cells grown in the presence of 0.1mM and 0.05mM LA, t-10 or c-9: transepithelial electrical resistance (TEER) was measured, viability, proliferation, translocation assays were carried out, Caco-2 cells' ability to differentiate was tested and gas chromatography was carried out on cellular and basolateral TAG and PL fractions. In addition, the acute effects of LA and its two isomers on lipid and apolipoprotein synthesis and secretion were investigated. Concurrently, the chronic effects

of LA and its isomers after acute BSA, OA and PA treatments were tested. Cell TEERs were measured to assess cell growth, cell viability assay, the translocation assay and cellular differentiation assays were carried out as described in chapter 4 sections 4.2.3, 4.2.4, 4.2.6 and 4.2.7 respectively. Statistical analysis was calculated as described in previous chapters, in the corresponding sections.

6.2.1 Proliferation (MTS) Assay

The following protocol is based on the protocol described in section 4.2.5. To test the proliferation of the cells when fed LA or its conjugates, cells were either seeded at 10^5 cells/well in a 96 well plate (Falcon) and grown for 24 hours before the addition of MTS/PMS (3-(4,5-dimethylthiazol-2-yl)-3-(3-carbozymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and phenazine methosulfate (Promega)) or seeded at $4x10^4$ cells/well in a 96 well plate (Falcon) and grown for 6 days before the addition of MTS/PMS. Media was changed every second day in the later case. For the proliferation assay, 20µl of MTS/PMS solution was added to each well and the cells were incubated for 90 minutes or 4 hours at 37°C before reading the absorbance at λ=490nm on an ELISA plate reader (Dyntech MR5000).

6.2.2 Gas Chromatography

6.2.2.1 Preparation of CLA Isomer Standards

A new standard was made for analysis of the fatty acid composition of TAG and PL moieties. The fatty acid 18:4n-3 was removed from the standard described in section 4.2.9.4 and the two LA isomers CLAt-10 and CLAc-9 were added to the standard at the same concentration as LA (18:2n-6), i.e. 0.526mg. 7mg of t-10 and c-9 was dried under a vacuum before the addition of 1ml of boron trifluoride, methanol complex (BDH Chemicals). The isomers were then incubated at 70°C for 30 minutes so that the standard fatty acids got similar treatment to the experimental fatty acids resulting in the same ratio of artefacts being synthesised (section 4.2.9.3). Methylated isomers were isolated by addition of 1ml of hexane, vortexing and removal of the hexane layer. This procedure was repeated three times. Isomers were dried before addition of 1.235ml of hexane, (i.e. 0.526mg/50µl). Then 50µl of each isomer was added to the standard before being loaded on the GC machine (Shimadzu GC-14A Series Gas Chromatograph). The new standard

FAME2 contained the following fatty acids: 8:0, 10:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2n-6, 18:3n-6, 18:3n-3, CLAc-9, CLAt-10, 20:1, 20:2, 20:3n-6, 20:4n-6, 20:3n-3, 20:5n-3, 22:4n-6 and 22:6n-3.

6.2.2.2 Gas Chromatography

For the details of the methods involved in preparation of lipids for gas chromatography see section 4.2.9. The experimental samples were loaded after methylation on the GC machine (Shimadzu GC-14A Series Gas Chromatograph) using the same analytical method as described in section 4.2.9.4. However, in this case cellular samples were at a split ratio of 1/60, TAG samples were resuspended in 100µl of hexane and PL samples resuspended in 80µl of hexane. 30µl of each sample from both lipid fractions was injected into the machine. Basolateral TAG and PL fractions were also at a split ratio of 1/60 and reconstituted in 100µl of hexane. 40µl of these samples was injected onto the machine.

6.2.3 Effect of Linoleic Acid and its conjugates on lipid and apolipoprotein synthesis and secretion

To test whether cells fed with LA or its conjugates (t-10 or c-9) synthesised and secreted similar levels of lipid and apolipoproteins to control cells (fed media for the 19 day development period), the chronic-acute experiment described in chapter 5 was carried out (section 5.2.3). This experiment was carried out in conjunction with the experiments described in chapter 5. Therefore cells supplemented with no fatty acids and treated with 0.5% BSA, 0.5mM OA and 0.05% BSA and 0.5mM PA and 0.5% BSA are the same as those described in chapter 5. The results obtained from chronic CLA supplementation followed by acute BSA, OA and PA treatments are described separately because they differ from those observed in chapter 5.

To investigate the acute effects of LA, t-10 and c-9 on lipid and apolipoprotein synthesis and secretion, cells were grown for 19 days in media and subsequently treated with acute doses of 0.5mM LA and 0.5% BSA, 0.5mM t-10 and 0.5% BSA or 0.5mM c-9 and 0.5% BSA. Cellular (n=8) and basolateral (n=4) lipids were isolated as in section 5.2.3.2 and analysed as described in section 5.2.4. TRL density lipoproteins (n=4) were isolated as in section 5.2.3.2 and analysed as in section 5.2.4. In addition, cellular (n=4) and basolateral (n=4) apoB100 and apoB48 levels were measured (section 5.2.3.3) and analysed (section 5.2.4).

The possible chronic effects of t-10 and c-9 isomer supplementation on Caco-2 cells were also examined. Caco-2 cells were grown in the presence of chronic doses of 0.05mM LA, 0.05mM t-10 or 0.05mM c-9 over a 19 day period. Subsequently cells were either treated with acute doses of 0.5% BSA, 0.5mM OA and 0.5% BSA or 0.5mM PA and 0.5% BSA. Cellular (n=14) and basolateral (n=7) lipids and TRL density lipoproteins (n=7) were isolated and analysed (sections 5.2.3.2 and 5.2.4). In addition, cellular (n=7) and basolateral (n=7) apoB100 and apoB48 levels were determined (sections 5.2.3.3 and 5.2.4).

6.3 Results

Investigation into the effect of CLA isomers on intestinal lipid metabolism was carried out using the Caco-2 cell line. Characterisation of Caco-2 cells was necessary as the cells were supplemented with fatty acids during their development. The effect of chronic supplementation on the percentage fatty acid components in the TAG and PL fractions were also examined as a control on whether supplemented fatty acids had been incorporated into the cells' lipid fractions.

6.3.1 Transepithelial Electrical Resistance (TEER)

Caco-2 cells were grown in the presence of control, 0.1mM and 0.05mM LA or 0.1mM and 0.05mM of its conjugates, t-10 and c-9. The TEER, which is a measurement of cell growth, was taken approximately every 2 days (Table 6.3.1, Figure 6.3.1). Control cells and cells given LA or c-9 supplements all reached confluency at about 7-8 days post-seeding. Caco-2 cells supplemented with either 0.1mM or 0.05mM t-10, however, took 13 days to reach TEER values associated with confluency. Administration of the t-10 isomer caused the cells to have basal TEERs until about day 8 post-seeding.

6.3.2 Viability (MTT) Assay

Since t-10 clearly inhibited cells reaching confluency (Table 6.3.1, Figure 6.3.1), cell viability was assessed using the MTT assay in all fatty acid supplemented cells and readings of test cells at λ=490nm and λ=570nm were compared against control cells. Supplementation of cells with LA and its conjugates at concentrations of 0.1mM and 0.05mM over a 19 day period before addition of MTT, gave the following results (Table

6.3.2): all cells, except those grown in the presence of 0.1mM t-10, had full viability. The 0.1mM t-10 supplemented cells had poor viability. When the cells were grown for 19 days in control media and then given an acute (24 hour) dose of 0.5mM LA and 0.5% BSA, 0.5mM t-10 and 0.5% BSA or 0.5mM c-9 and 0.5% BSA in FCS-free media, the viability of cells was found not to be affected (Table 6.3.2).

6.3.3 Proliferation (MTS) Assay

MTS assays were carried out to compare the proliferation rates of control and fatty acid supplemented cells. MTS incubation times were either 90 minutes or 4 hours. The 4 hour incubation was used to confirm that the assay had reached completion. Caco-2 cells were plated at a density of 500 cells/µl in control media or media supplemented with 0.1mM or 0.05mM LA, t-10 or c-9 and incubated at 37°C for 24 hours before experimentation. Cell proliferation was not affected by a 24 hour fatty acid supplementation (Table 6.3.3). The proliferation assay was repeated on cells that had been seeded at a density of 200 cells/µl (comparable to transwell seeding density) and grown for 6 days in the presence of control media or media supplemented with 0.1mM or 0.05mM LA, t-10 or c-9. It is clear from the TEER results (Table 6.3.1) that cells grown in the presence of t-10 for 6 days will not have reached confluency, but in this experiment this was not found to be due to decreased proliferation rates of t -10 supplemented cells (Table 6.3.3).

6.3.4 Translocation Assay

Translocation assays were carried out to establish whether supplementation of Caco-2 cells with LA, t-10 or c-9 effected tight junction formation. Cells were grown in the presence of 0.1mM or 0.05mM LA, t-10 or c-9. Subsequently their apparent permeability coefficient (P_{app}) was measured (section 4.2.6). Paracellular (^{14}C-mannitol) and intracellular (^{3}H-propranolol) markers were studied. The paracellular marker assessed the confluency and tight junction formation of the cells at day 6 and 14 post-seeding, while the lipophilic intracellular marker was used to assess if the fatty acid supplements had changed the lipid metabolism of Caco-2 cells.

The P_{app} of the paracellular marker, ^{14}C-mannitol, was similar in control cells and Caco-2 cells grown in 0.1mM and 0.05mM LA and c-9 for 6 and 14 days post-seeding (Table 6.3.4a & b). However, cells grown for 6 days in either 0.1mM or 0.05mM t-10 had ten-

fold larger P_{app} values than control cells ($p=0.002$ and $p<0.000$ respectively) (Table 6.3.4a). In addition, Caco-2 cells grown for 14 days in the presence of 0.1mM t-10 had two-fold larger P_{app} values (not significant) and cells supplemented with 0.05mM t-10 showed a significant two-fold increase in P_{app} values ($p=0.023$) compared to control cells (Table 6.3.4b).

The P_{app} of the intracellular marker, ^{3}H-propranolol, was observed to be different in cells grown in media supplemented with 0.1mM t-10 for 6 days ($p=0.0004$) (Table 6.3.4c) and in cells grown for 14 days in 0.05mM c-9 supplemented media ($p=0.003$) compared to the relevant control cells (Table 6.3.4d). The effect of the c-9 treatment may be unique to this sample set, as no difference was detected at day 6. Another explanation is that c-9 treatment may cause more lipids to be transferred through Caco-2 cells. The high P_{app} value seen at day 6 post-seeding of cells grown in 0.1mM t-10 supplemented media may be due to paracellular passage of ^{3}H-propranolol, since Caco-2 cells have been shown to be less confluent than control cells at this time point using the ^{14}C-mannitol marker.

6.3.5 Cell Differentiation

As described in sections 4.2.7.2 and 4.2.8, cDNA probes for lactase-phlorizin hydrolase, sucrase-isomaltase and microsomal triglyceride transfer protein (MTP) were prepared. Northern blots with total RNA from 0.05mM LA, t-10 or c-9 supplemented Caco-2 cells of 3,7 and 14 days post-seeding were generated as outlined in section 4.2.7.3. However, for medical reasons it became no longer possible to carry out the probing of the northern blots with the ^{32}P labelled cDNA probes using the protocol outlined in section 4.2.7.4.

Table 6.3.1 The mean TEER (Ω) of cells fed control, LA and CLA supplemented media. Control values are the same as those reported in Table 4.3.1 (total number of controls measured) since the mean was calculated from a larger sample number giving a more accurate mean TEER. The table shows clearly that t-10 supplements inhibit cell growth at both 0.1mM and 0.5mM.

Day	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Control	126	144	191	260	250	364	329	401	394	364	363	384	379	372	417	431	438	439	410
0.1mM LA		132		138	270	267	400		439		385	406	379	372		475			
0.05mM LA		144	156		265	302	331		381	423		393		402		398			
0.1mM t-10		131		122	138	127	156		221		209	319	266	374		438			
0.05mM t-10		137	134		128	129	133		181	269	189	297	256	330		349			
0.1mM c-9		133		141	194	253	373		413		384	390	344	435		404			
0.05mM c-9		138	158		274	294	389		407	431	351	407	347	431		385			

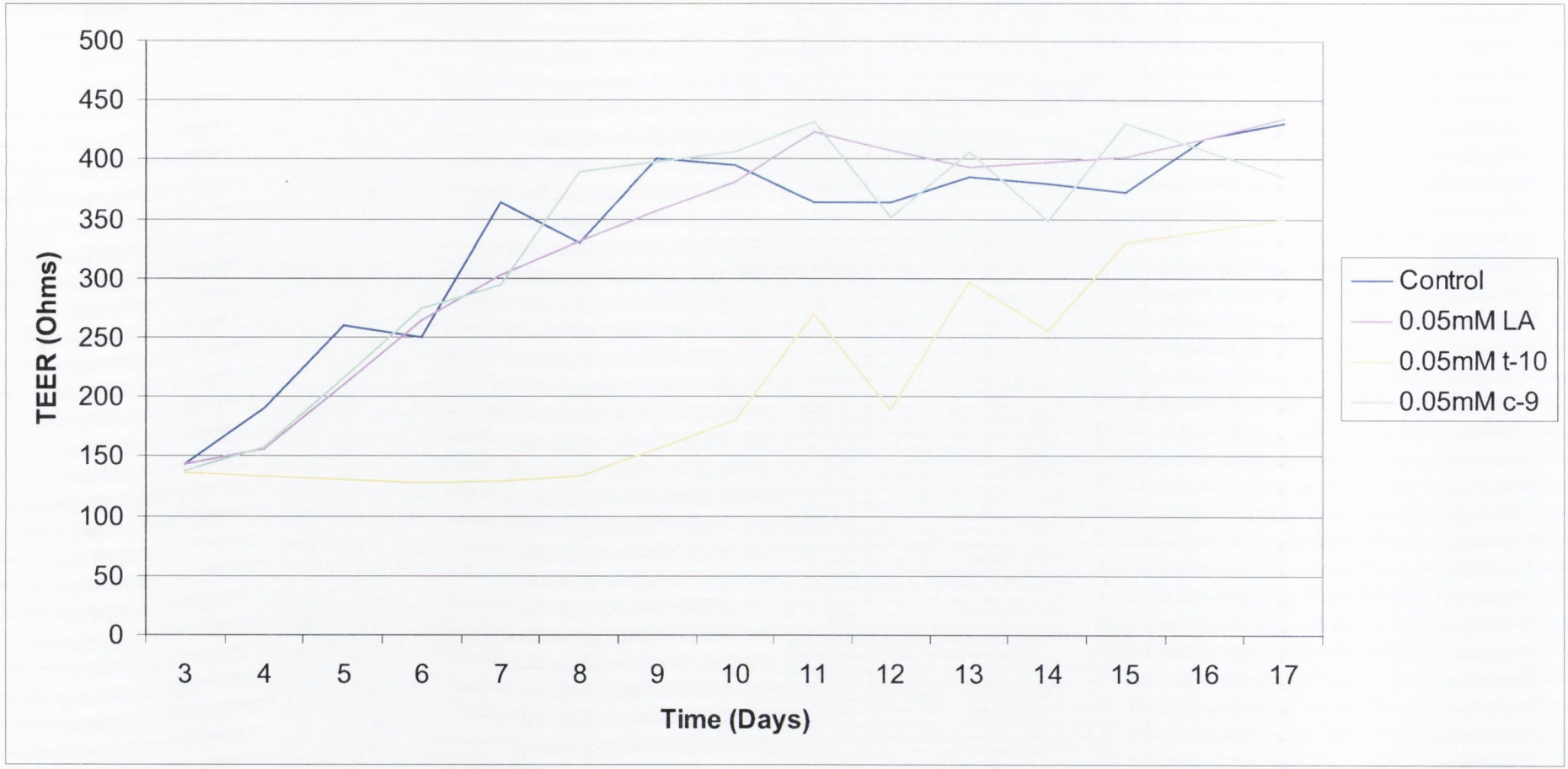


Figure 6.3.1 Graph of the TEER (Ω) versus time (days) of cells fed control, or 0.05mM LA, t-10 or c-9 supplemented media. The growth of Caco-2 cells supplemented with 0.05mM t-10 is retarded compared to control cells until day 11 post-seeding. Where data were not present the mean between the last and next TEER value was plotted.

Table 6.3.2 Viability (MTT) assay on cells given either chronic or acute dose of *cis* or *trans* n-6 PUFAs. Conditions, refers to the age of the cells post-seeding and type of treatment given, either chronic or acute. The column entitled 'Mean λ' gives the mean absorbance (λ=490nm and λ=570nm) measured in 8 wells of a 96 well plate. The standard deviation of absorbance is given in the next column. Viability had 2 states, '*' viable or 'poor' low viability. The table shows that cells treated chronically with 0.1mM t-10 had low cell viability compared to control cells. All other treatments resulted in fully viable cells.

Viability (MTT) Assay

Conditions	Treatment	Mean λ	Standard deviation λ	Viability
20 days, Acute	0.5% BSA	0.532	0.092	*
Acute	0.5mM LA, 0.5% BSA	0.657	0.091	*
Acute	0.5mM t-10, 0.5% BSA	0.747	0.181	*
Acute	0.5mM c-9, 0.5% BSA	0.672	0.137	*
17 days	Media (Control)	0.397	0.089	
Chronic	0.1mM LA	0.364	0.132	*
Chronic	0.1mM t-10	0.174	0.1	Poor
Chronic	0.1mM c-9	0.359	0.416	*
Chronic	0.05mM LA	0.383	0.144	*
Chronic	0.05mM t-10	0.297	0.095	*
Chronic	0.05mM c-9	0.306	0.078	*

Table 6.3.3 Proliferation assay on cells given chronic fatty acid supplements. Experimental conditions, refers to density of seeding, age of cells after seeding, and length of incubation of cells with MTS. Treatment refers to the fatty acid supplement given to the cells. The mean is the mean absorbance (λ=490nm) measured in eight separate wells. The '*' signifies similar proliferation to control cells. None of the fatty acid supplements effected cell proliferation. In addition, since cells seeded at the same cell density as those seeded on transwells (200 cells/µl) and grown for 6 days in the presence of 0.1mM or 0.05mM t-10 show no reduction in proliferation rate compared to control cells, the low TEER associated with t-10 supplemented cells can not be due to decreased proliferation.

Proliferation (MTS) Assay

Experimental Conditions	Treatment	Mean λ	Standard deviation λ	Proliferation
500 cells/µl, 1 day	Media (Control)	0.356	0.062	
90 minute MTS	0.05mM LA	0.368	0.038	*
	0.05mM t-10	0.367	0.089	*
	0.05mM c-9	0.366	0.059	*
500 cells/µl ,1 day	Media (Control)	0.507	0.107	
90 minute MTS	0.1mM LA	0.426	0.083	*
	0.1mM t-10	0.342	0.045	*
	0.1mM c-9	0.392	0.09	*
500 cells/µl, 1 day	Media (Control)	0.929	0.142	
4 hours MTS	0.1mM LA	0.852	0.126	*
	0.1mM t-10	0.816	0.207	*
	0.1mM c-9	0.718	0.163	*
	0.05mM LA	0.809	0.142	*
	0.05mM t-10	0.762	0.076	*
	0.05mM c-9	0.888	0.163	*
200 cells/µl, 6 days	Media (Control)	0.857	0.356	
4 hours MTS	0.1mM LA	1.184	0.181	*
	0.1mM t-10	0.841	0.202	*
	0.1mM c-9	1.055	0.117	*
	0.05mM LA	1.22	0.164	*
	0.05mM t-10	1.094	0.103	*
	0.05mM c-9	1.262	0.183	*

Table 6.3.4a & b The effects of fatty acid supplementation on mean P_{app} value and standard error are given. In addition, the probability that test cells have similar translocation rates of mannitol from the apical compartment to the basolateral compartment to control cells has been assessed using 2-sample-t-tests. NS = not significant

Cells grown in the presence of 0.1mM or 0.05mM t-10 showed a significant ten-fold increase in P_{app} values at day 6 post-seeding. However, by day 14 post-seeding the difference between P_{app} values was only two-fold. The increase associated with 0.1mM t-10 supplementation was not found to be significant.

Table 6.3.4a ^{14}C-mannitol P_{app} values in cells grown for 6 days.

Treatment	**Mean P_{app} $*10^{-6}$**	**Standard Error**	**Probability**
Control (media)	3.92	1.43	
0.1mM LA	5.5	1.27	NS
0.1mM t-10	31.32	3.96	p=0.002
0.1mM c-9	8.7	2.44	NS
0.05mM LA	1.22	0.4	NS
0.05mM t-10	29.82	1.25	p<0.000
0.05mM c-9	5.71	2.92	NS

Table 6.3.4b ^{14}C-mannitol P_{app} values in cells grown for 14 days.

Treatment	**Mean P_{app} $*10^{-6}$**	**Standard Error**	**Probability**
Control (media)	0.692	0.153	
0.1mM LA	0.623	0.155	NS
0.1mM t-10	1.138	0.209	NS
0.1mM c-9	0.536	0.142	NS
0.05mM LA	0.693	0.401	NS
0.05mM t-10	1.143	0.069	p=0.023
0.05mM c-9	0.975	0.301	NS

Table 6.3.4c & d The effects of fatty acid supplementation on mean P_{app} value and standard error are given. In addition, the probability that test cells have similar translocation rates of propranolol from the apical compartment to the basolateral compartment to control cells has been assessed using 2-sample-t-tests. NS = not significant
Cells grown for 6 days in the presence of 0.1mM t-10 show an approximate two-fold increase in P_{app} value compared to control cells. In addition, cells grown for 14 days with 0.05mM c-9 show a small, yet significant reduction in P_{app} value.

Table 6.3.4c ^{3}H-propranolol P_{app} values in cells grown for 6 days.

Treatment	Mean P_{app} *10^{-5}	Standard Error	Probability
Control (media)	1.73	0.096	
0.1mM LA	1.62	0.242	NS
0.1mM t-10	3.096	0.165	p=0.0004
0.1mM c-9	1.692	0.181	NS
0.05mM LA	1.723	0.038	NS
0.05mM t-10	1.79	0.17	NS
0.05mM c-9	1.575	0.115	NS

Table 6.3.4d ^{3}H-propranolol P_{app} values in cells grown for 14 days.

Treatment	Mean P_{app} *10^{-5}	Standard Error	Probability
Control (media)	1.413	0.04	
0.1mM LA	1.449	0.053	NS
0.1mM t-10	1.568	0.062	NS
0.1mM c-9	1.454	0.094	NS
0.05mM LA	1.357	0.037	NS
0.05mM t-10	1.453	0.154	NS
0.05mM c-9	1.226	0.052	p=0.003

6.3.6 Gas Chromatography

Gas chromatography (sections 4.2.9 and 6.2.2) was used as a check to investigate possible compositional changes in TAG and PL fractions of cells supplemented with LA, t-10 or c-9 compared to Caco-2 cells fed media without fatty acid supplementation (control) over a 19 day period. This procedure was carried out as part of the characterisation of the supplemented cells and not as an investigation into what lipid fractions the supplemented fatty acids were incorporated into. Therefore a sample size of two transwells was used, which meant no informative statistical analysis could be carried out, as the standard deviations would most likely have been too high to find significant incorporation.

The standard used to compare the composition of lipids from cells and basolateral media was FAME 2 (section 6.2.2), which contained methylated t-10 (CLAt-10) and c-9 (CLAc-9) isomers (section 6.2.2). The percentages of 16:0 (PA), 16:1, 18:0, 18:1 (OA), 18:2n-6 (LA), 18:3n-3, CLAt-10 (t-10), CLAc-9 (c-9) and 20:4n-6 (arachidonic acid; AA) in the

PL and TAG fractions were compared in control cells and in cells supplemented with 0.1mM or 0.05mM LA, t-10 and c-9. Control cells in this study, which were fed FCS without LA, t-10 or c-9 supplementation, were found to contain the t-10 and c-9 isomers of LA in both the TAG and PL fractions of the cellular lipid (Figure 6.3.2a & 6.3.2b). The c-9 isomer alone was found in secreted TAG fractions (Figure 6.3.2c), and both isomers were found in secreted PL fractions (Figure 6.3.2d).

Bar graphs show trends caused by the supplementation of different fatty acids. Caco-2 cells supplemented with LA (either 0.1mM or 0.05mM) showed and increase in 18:2n-6 levels in both the TAG and PL fractions. To facilitate this increase, a reduction in 18:1 was observed (Figure 6.3.3a, 6.3.3b, 6.3.3c & 6.3.3d).

Supplementation with t-10 caused an increase in the percentage of 16:0, CLAt-10 and CLAc-9 but a decrease in 18:1 in the TAG and cellular PL fractions (Figure 6.3.3a, 6.3.3b & 6.3.3c). The basolateral PL fraction of cells given 0.1mM t-10 (Figure 6.3.3d) had an increase in 16:0, CLAt-10 and CLAc-9 levels. However, in basolateral PL fraction of cells given 0.05mM t-10 showed no increase 16:0 and CLAt-10 levels but had a reduction in 18:1 levels.

Treatment of cells with 0.1mM or 0.05mM c-9 caused an increase in 16:0 and a reduction in 18:1 levels but neither of these differences were to the same extent as those caused by the analogous t-10 treatments. An increase in CLAc-9 levels was observed in all lipid fractions irrespective of the concentration of c-9 supplementation. In addition, an increase in CLAt-10 was observed in the cellular PL fractions of Caco-2 cells supplemented with c-9. Along with the increase in both CLAc-9 and CLAt-10 levels in cellular PL fractions due to c-9 supplementation, there was a decrease in the percentage of 20:4n-6. This decrease in 20:4n-6 level was not observed in the secreted PL fraction (Figure 6.3.3a, 6.3.3b, 6.3.3c & 6.3.3d).

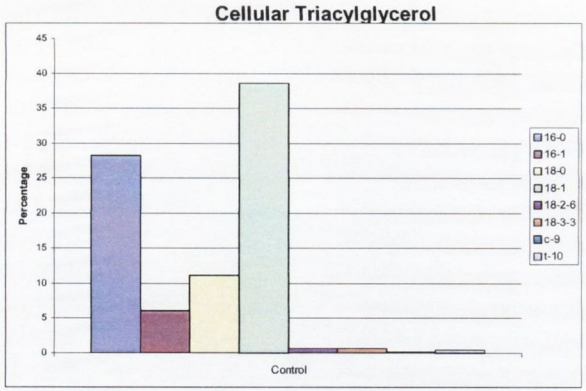


Figure 6.3.2a Fatty acid compostion of cellular TAG.

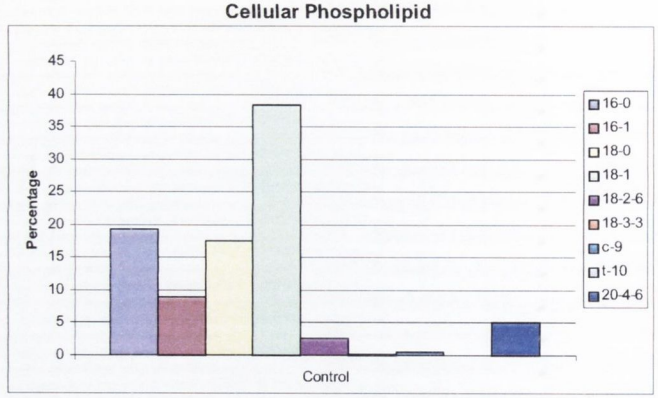


Figure 6.3.2b Fatty acid compostion of cellular PL.

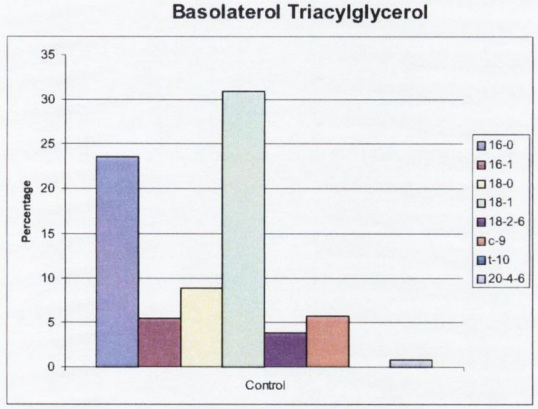


Figure 6.3.2c Fatty acid compostion of basolateral TAG.

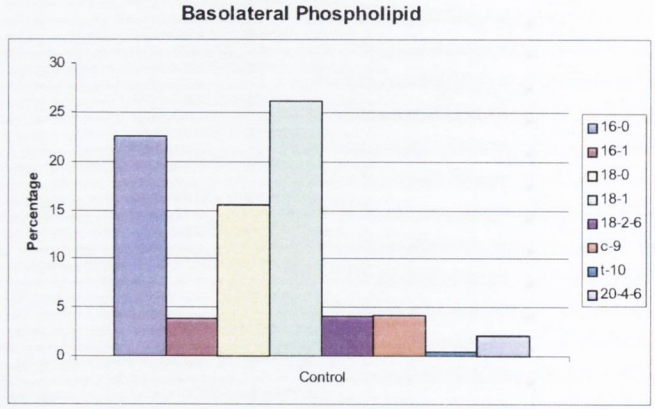


Figure 6.4.2d Fatty acid compostion of basolateral PL.

Figure 6.3.2 Fatty acid composition of cellular and basolateral TAG and PL fractions of control cells. In Figures 6.3.2a-d 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1, 18-2-6 is 18:2n-6, 18-3-3 is 18:3n-3 and 20-4-6 is 20:4n-6.

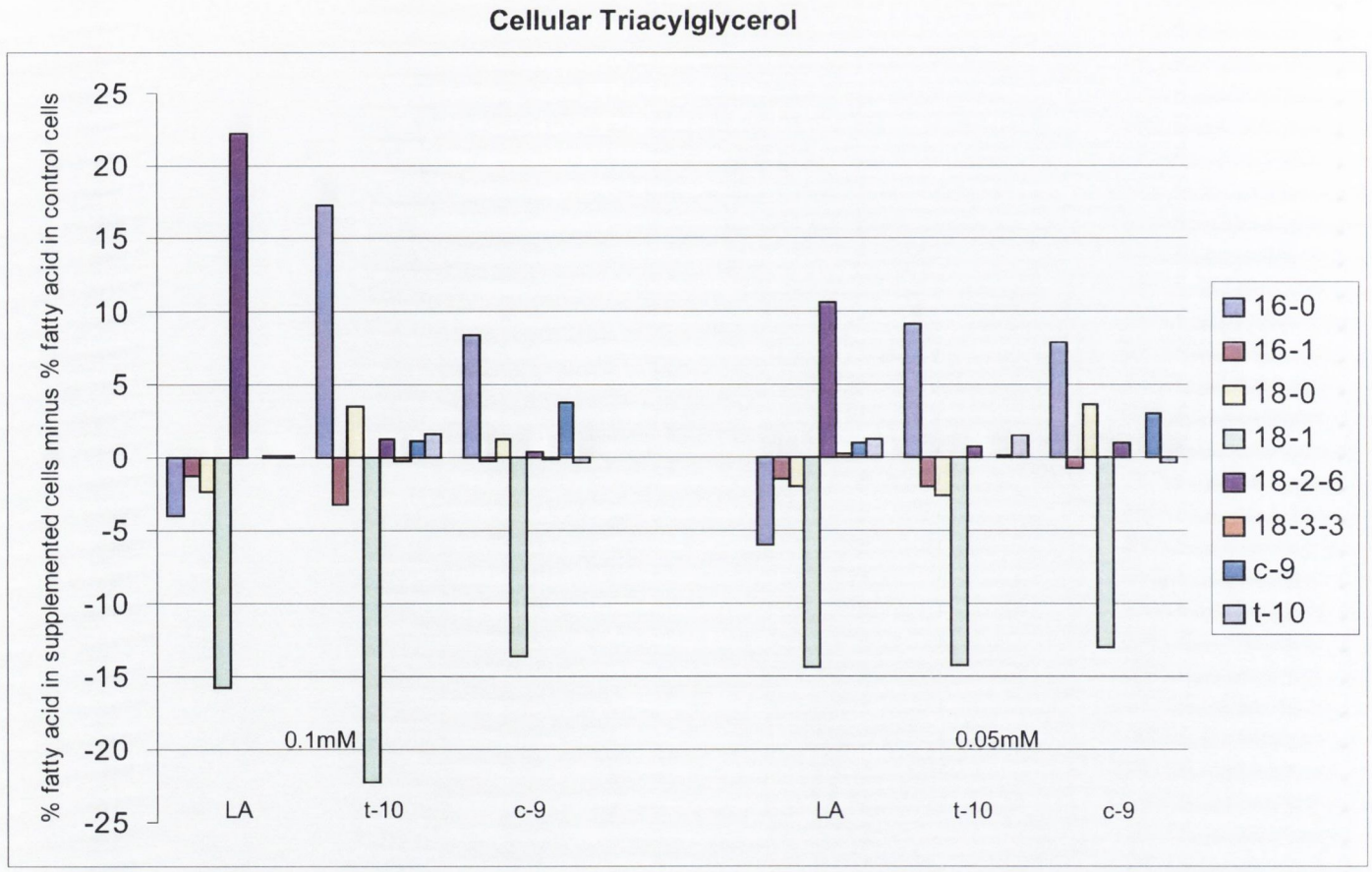


Figure 6.3.3a A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 18:2n-6, 18:3n-3, c-9 and t-10) found in cellular TAG fractions of cells treated with EPA, OA or PA compared to control levels. The vertical axis represents the percentage of each fatty acid in supplemented cells minus the percentage in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1, 18-2-6 is 18:2n-6 and 18-3-3 is 18:3n-3.

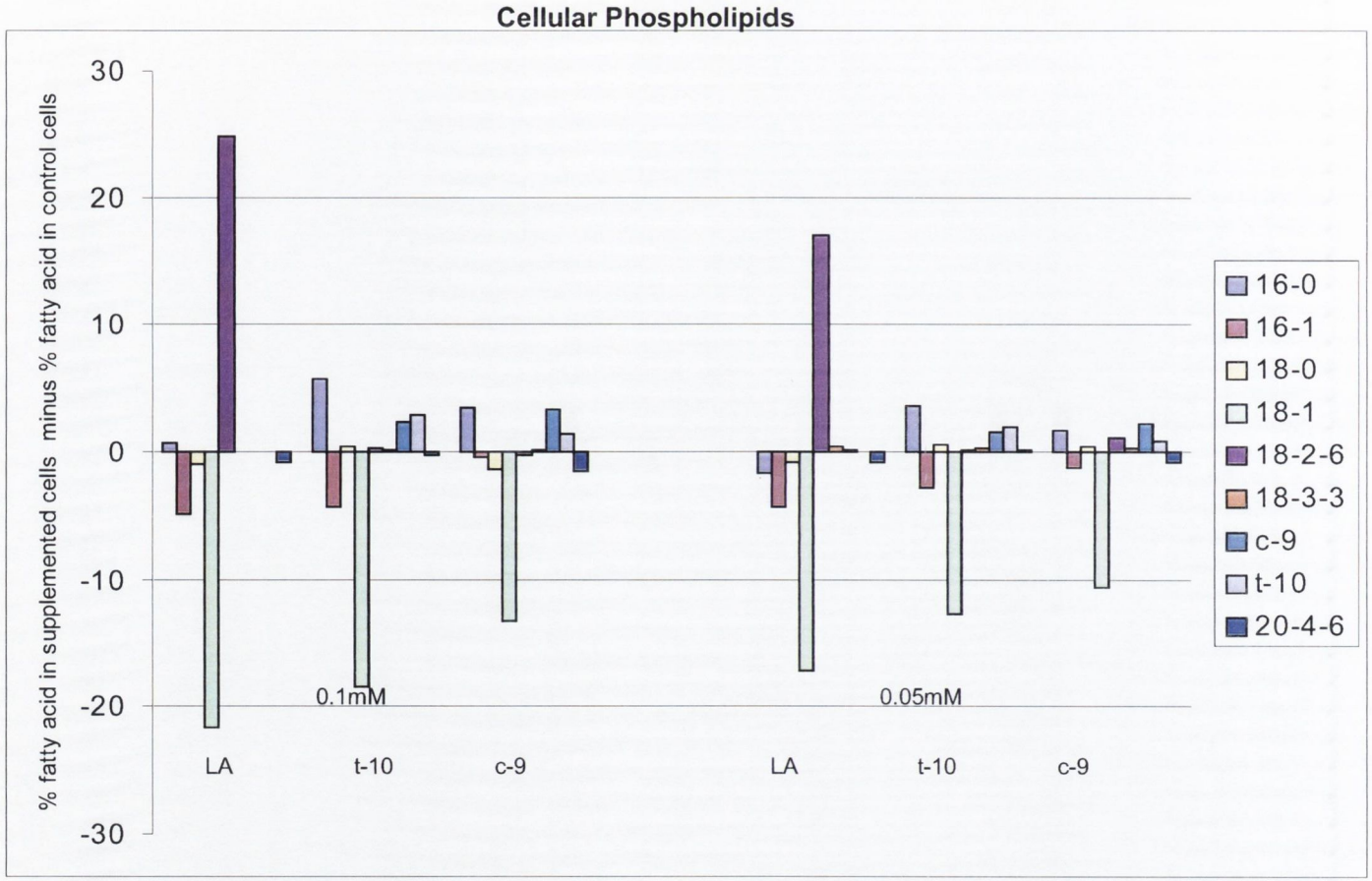


Figure 6.3.3b A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 18:2n-6, 18:3n-3, c-9, t-10 and 20:4n-6) found in cellular PL fractions of cells treated with EPA, OA or PA compared to control levels. The vertical axis represents the percentage of each fatty acid in supplemented cells minus the percentage in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1, 18-2-6 is 18:2n-6, 18-3-3 is 18:3n-3 and 20-4-6 is 20:4n-6.

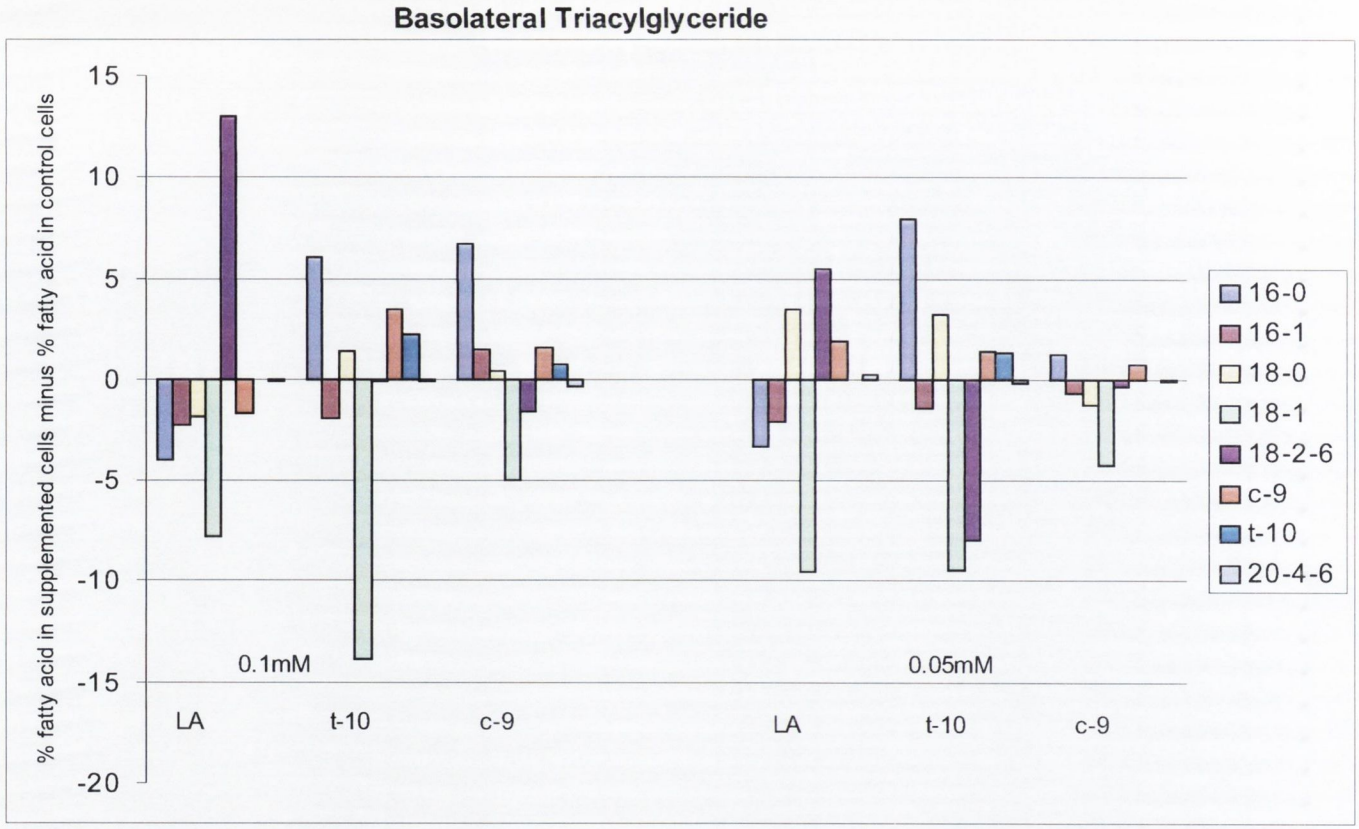


Figure 6.3.3c A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 18:2n-6, c-9, t-10 and 20:4n-6) found in basolateral TAG fractions of cells treated with EPA, OA or PA compared to control levels. The vertical axis represents the percentage of each fatty acid in supplemented cells minus the percentage in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1, 18-2-6 is 18:2n-6 and 20-4-6 is 20:4n-6.

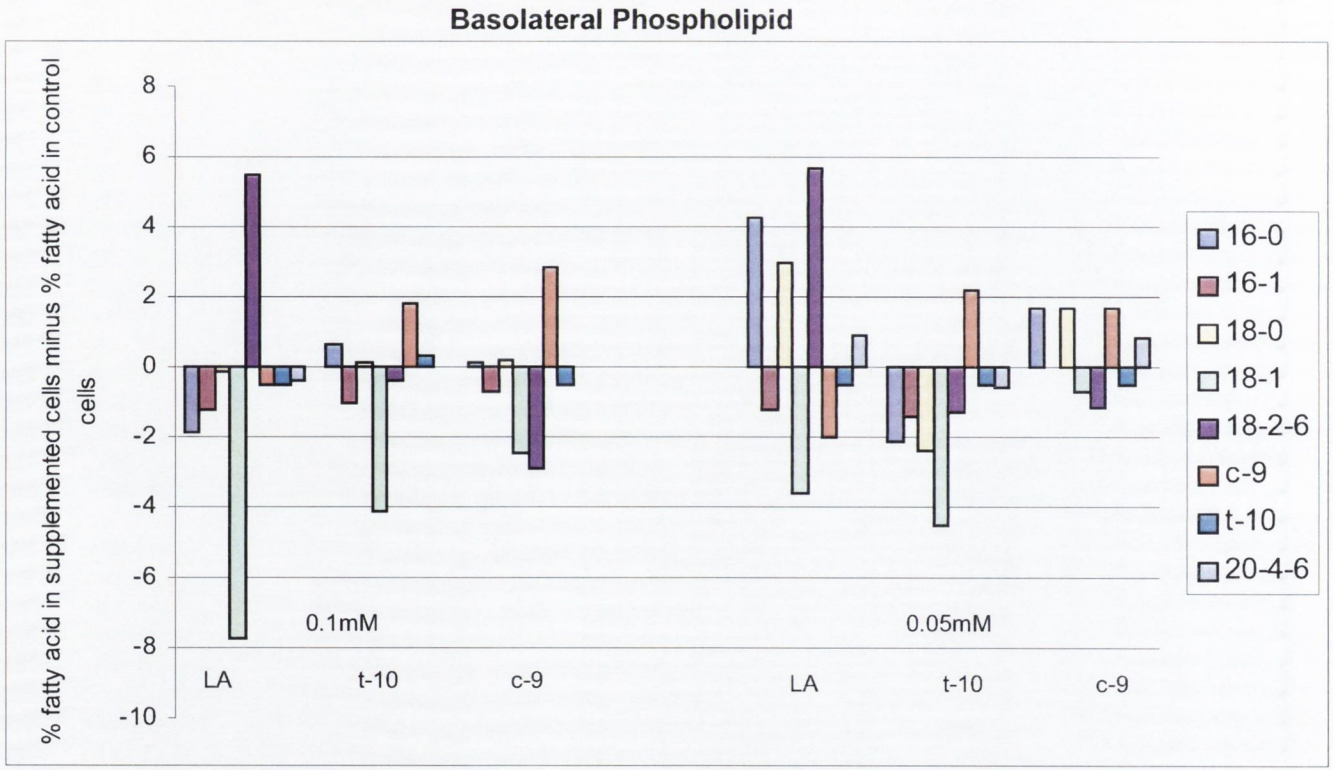


Figure 6.3.3d A bar graph representing levels of fatty acids (16:0, 16:1, 18:0, 18:1, 18:2n-6, c-9, t-10 and 20:4n-6) found in basolateral PL fractions of cells treated with EPA, OA or PA compared to control levels. The vertical axis represents the percentage of each fatty acid in supplemented cells minus the percentage in control cells. 16-0 is 16:0, 16-1 is 16:1, 18-0 is 18:0, 18-1 is 18:1, 18-2-6 is 18:2n-6 and 20-4-6 is 20:4n-6.

6.3.7 Acute Effect of Conjugated Linoleic Acid Isomers on Fatty Acid Synthesis and Secretion

Using the Caco-2 cell line as a model of the human intestine, the hypothesis was tested that LA or either of its conjugates, t-10 or c-9 may effect lipid and/or apolipoprotein synthesis and secretion by the gut. As most research on Caco-2 cells in the literature discusses the effects of acute doses of fatty acids on their metabolism, the effects of acute doses of t-10 and c-9 were investigated in this chapter. Caco-2 cells fed acute doses of LA and BSA were used as controls. An investigation was carried out to discover what affects a physiological (acute) dose (0.5mM) of LA or its conjugates has on lipid or apolipoprotein synthesis and secretion by Caco-2 cells. As before (section 5.2.3) cells and basolateral media were harvested and apoB100, apoB48, TAG and PL levels were determined. The following variables were analysed: percentage basolateral apoB100 (%bB100), percentage cellular apoB48 (%cB48), ratio of cellular TAG to basolateral TAG (cTAG/bTAG), ratio of cellular TAG to cellular PL (cTAG/cPL), ratio of basolateral TAG to basolateral PL (bTAG/bPL) and the ratio of basolateral TAG to basolateral TRL-TAG (bTAG/bTRL-TAG). The disintegrations per minute (DPM) values are reported in Table 6.3.5. The DPM values of the cells treated with 0.5% BSA are the same as those reported in chapter 5. As can be seen from Table 6.3.6, no acute fatty acid treatment had any significant effect on the %bB100, %cB48 and ratios of cTAG/bTAG, cTAG/cPL and bTAG/bTRL-TAG. However, acute fatty acid treatment did have a significant effect on the ratio of bTAG/bPL ($p=0.0151$). Least significance difference (LSD) tests were carried out to identify which acute treatment was different. Acute LA treatment appeared to increase TAG secretion levels compared to all other acute treatments, BSA (LSD $p=0.003$), t-10 (LSD $p=0.012$) and c-9 (LSD $p=0.05$).

6.3.8 Chronic Effect of Conjugated Linoleic Acid Isomers on Fatty Acid Synthesis and Secretion

Caco-2 cells were grown in the presence of chronic levels of LA or its conjugates and subsequently given acute doses of various fatty acids. As in chapter 5, the %bB100, %cB48, cTAG/bTAG, cTAG/cPL, bTAG/bPL and bTAG/bTRL-TAG levels were determined to gain information on possible long-term effects of diets high in *cis* n-6 PUFA and *trans* n-6 PUFAs on lipid metabolism. Controls for analysis were the same as reported in chapter 5. The controls were cells which had not been chronically supplemented with

fatty acids but which had been treated acutely with 0.5% BSA, 0.5mM OA and 0.5% BSA or 0.5mM PA and 0.5% BSA. The measured DPMs are reported in Table 6.3.5.

No significant acute (p=0.063), chronic (p=0.394) or acute by chronic effects (p=0.611) of chronic LA, t-10 and c-9 supplementations followed by acute BSA, OA or PA treatments on %bB100 levels were observed (Table 6.3.7a).

Acute by chronic effects on %cB48 were analysed, but no significant acute (p=0.732), chronic (p=0.422) or interaction (p=0.426) effects were observed (data not shown).

An acute effect (p=0.0001) was observed on the ratio of cTAG/bTAG (Table 6.3.7b). Caco-2 cells treated with OA had increased secretion levels of *de novo* TAG compared to those given other acute treatments, BSA (LSD p=0.000) and PA (LSD p=0.000). No chronic effect was observed on the ratio of cTAG/bTAG (p=0.354). However, an acute by chronic interaction was observed (p=0.019). Again, since the hypothesis to be tested was whether chronic supplementation affected the way Caco-2 cells responded to acute fatty acid treatment, the most informative way to analyse the data is to look for significant differences between cells fed different chronic treatments and the same acute treatment using LSD tests[3]. Caco-2 cells supplemented with t-10 and subsequently given acute PA treatment were significantly different from cells supplemented with no fatty acids (LSD p=0.000) or with LA (LSD p=0.003) followed by acute PA treatments. Cells chronically supplemented with c-9 followed by acute PA treatment were significantly different from control cells given acute PA treatment (0.03). All fatty acid (LA 12.25, t-10 23.75, c-9 17.1) supplementations decreased the level of *de novo* TAG secretion compared to no fatty acid supplementation (8.88), after acute PA treatments.

Chronic treatment of Caco-2 cells with LA, t-10 or c-9 resulted in their having higher cTAG/cPL ratios (Table 6.3.7c p=0.0001). LSD tests show that supplementations were different from each other and the control (LSD LA - control p=0.04, t-10 - control p=0.000, t-10 - LA p=0.005, c-9 - control p=0.000, c-9 - LA p=0.04 and c-9 - t-10 p=0.417). Cells given chronic fatty acid treatments had larger ratios of *de novo* cellular TAG to PL (LA 0.807, t-10 0.965 and c-9 0.92) than control cells (0.688). A significant effect was also observed due to acute treatments (p=0.0001). Both acute OA and PA treatments increased the quantity of cTAG/cPL compared to control treatments (LSD p=0.000 and p=0.000 respectively) and each other (LSD p=0.000). Acute PA treatment (1.152) increased TAG synthesis more than the other acute treatments, BSA (0.448) and OA (0.935). In addition, an acute by chronic interaction was observed (p=0.0002). Two

[3] Full LSD test of interaction can be found in appendix III.

types of significant interactions[4] were observed using LSD tests on cells chronically supplemented with different fatty acids but given the same acute treatments.

- Test cells given chronic supplementations with LA (LSD p=0.000), t-10 (LSD p=0.0.000) and c-9 (LSD p=0.000) followed by acute OA treatment were significantly different from control cells given acute OA treatment. Chronic LA (LSD p=0.001) and c-9 (LSD p=0.005) supplementations were also significantly different from t-10 supplementation followed by acute OA treatments. Chronic fatty acid supplements followed by acute OA treatments increased TAG synthesis over PL synthesis compared to control treatment (0.564), with chronic t-10 supplementation enhancing TAG synthesis the most (1.262), followed by c-9 (0.988) and LA (0.972).

- The second interaction that was observed was that chronic supplementation of cells with LA followed by acute PA treatment was different to chronic supplementation with c-9 followed by acute PA treatment (LSD p=0.007). Chronic c-9 supplementation followed by acute PA treatment appeared to increase TAG synthesis over PL synthesis (1.285), while this ratio (cTAG/cPL) calculated in cells given chronic LA supplementation followed by acute PA treatment (1.028) was slightly lower than that observed in cells not supplemented with fatty acids and given acute PA treatment (1.099).

An acute effect was observed on the ratio of bTAG/bPL (p=0.0001) (Table 6.3.7d). Acute OA treatment significantly increases the ratio of bTAG/bPL compared to all other acute treatments (BSA (LSD p=0.000) and PA (LSD p=0.001)). Acute PA treatment also increased the *de novo* TAG secretion of PL secretion compared to acute BSA treatment (LSD p=0.000). However, acute OA treatment (11.08) caused a larger increase in the ratio of bTAG/bPL than acute PA treatments (7.542). No effect of chronic supplementation (p=0.2776) or interaction (0.79) was observed.

No significant effects were found between the ratio of bTAG/bTRL-TAG and fatty acid treatments of Caco-2 cells (Table 6.3.7e), acute (p=0.242), chronic (p=0.574) or acute by chronic interaction (p=0.327).

[4] Full LSD test analysis of acute by chronic interaction can be found in appendix IV.

Table 6.3.5 The mean disintegrations per minute (DPM) and standard error (SE) of measured protein and lipid variables.

Fraction Counted		cB100		bB100		cB48		cTAG		cPL	
Chronic Treatment	**Acute Treatment**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	0.5% BSA	16,040	7,045	2,986	1,847	2,585	1,017	10,056	937	25,460	2,787
Control	0.5mM LA 0.5% BSA	8,641	2,502	1,715	964	2,374	371	14,855	2,189	33,855	4,434
Control	0.5mM t-10 0.5% BSA	8,352	2,124	1,491	501	2,156	559	23,544	1,685	40,758	4,747
Control	0.5mM c-9, 0.5% BSA	7,573	2,095	683	336	1,474	346	13,894	1,266	42,414	4,158
0.05mM LA	0.5% BSA	11,250	2,027	1,546	578	3,581	1,153	7,530	684	16,558	1,686
0.05mM LA	0.5mM OA 0.5% BSA	8,866	2,094	833	313	2,019	594	22,819	2,081	24,461	1,755
0.05mM LA	0.5mM PA, 0.5% BSA	7,711	1,714	1,785	1,191	1,350	490	30,433	2,983	29,640	2,764
0.05mM t-10	0.5% BSA	10,021	3,113	2,584	1,168	2,502	750	7,074	568	17,860	2,920
0.05mM t-10	0.5mM OA 0.5% BSA	8,260	1,650	901	509	1,734	340	22,517	2,254	19,608	2,989
0.05mM t-10	0.5mM PA, 0.5% BSA	7,258	2,153	2,069	1,368	2,223	768	31,667	3,729	27,487	3,636
0.05mM c-9	0.5% BSA	8,802	2,762	5,360	3,185	1,777	511	11,531	900	23,580	1,788
0.05mM c-9	0.5mM OA 0.5% BSA	7,347	1,546	1,088	352	2,016	451	28,061	1,891	28,528	1,975
0.05mM c-9	0.5mM PA, 0.5% BSA	6,720	1,823	564	240	1,780	689	38,196	2,680	30,610	1,978

Table 6.3.5 continued

Fraction Counted		bTAG		bPL		bTRL-TAG		bTRL-PL	
Chronic Treatment	**Acute Treatment**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	0.5% BSA	516	40	448	203	390	69	408	172
Control	0.5mM LA 0.5% BSA	4,208	1,426	496	107	1,570	325	333	46
Control	0.5mM t-10 0.5% BSA	1,922	663	573	159	1,322	192	500	118
Control	0.5mM c-9, 0.5% BSA	2,581	328	586	65	790	92	294	55
0.05mM LA	0.5% BSA	595	81	117	15	437	118	154	13
0.05mM LA	0.5mM OA 0.5% BSA	3,624	408	322	25	973	142	195	21
0.05mM LA	0.5mM PA, 0.5% BSA	2,576	386	347	36	498	116	228	19
0.05mM t-10	0.5% BSA	587	57	172	25	367	103	147	30
0.05mM t-10	0.5mM OA 0.5% BSA	5,319	926	452	55	804	187	227	61
0.05mM t-10	0.5mM PA, 0.5% BSA	1,640	417	665	407	1,022	398	467	195
0.05mM c-9	0.5% BSA	932	169	208	24	442	86	173	46
0.05mM c-9	0.5mM OA 0.5% BSA	4,719	774	408	67	1,195	258	262	32
0.05mM c-9	0.5mM PA, 0.5% BSA	2,391	356	303	84	1,122	254	236	50

Table 6.3.6 Table showing the effect of acute treatment with LA and its conjugates (t-10 or c-9) on apolipoprotein levels and cellular and secreted lipid ratios of cells grown for 19 days in control media. ANOVA was used to analyse the acute effects, and LSD tests were used to identify which fatty acid treatment caused significant changes. An effect caused by LA has a superscript coloured in dark green. NS = Not Significant. MSE = Mean Squared Error.

Acute fatty acids effects

	0.5% BSA Mean (SE)	0.5mM LA, 0.5% BSA Mean (SE)	0.5mM t-10, 0.5% BSA Mean (SE)	0.5mM c-9, 0.5% BSA Mean (SE)	MSE	Significance
%bB100	15.33 (7.3)	13.14 (5.58)	21.67 (9.91)	6.17 (2.54)	205	NS
%cB48	20 (3.5)	25.9 (3.4)	22.2 (3.0)	17.2 (3.1)	70.2	NS
cTAG/bTAG	17.0 (2.68)	8.84 (6.27)	20.1 (6.68)	4.82 (0.64)	95.9	NS
cTAG/cPL	0.401 (0.026)[a]	0.491 (0.091)	0.637 (0.1)	0.36 (0.056)	0.042	NS
bTAG/bPL	1.79 (0.83)[a]	7.47 (1.71)[b]	3.27 (0.45)[a]	4.41 (0.23)[a]	3.85	P=0.0151
bTAG/bTRL-TAG	1.79 (n=1)	2.48 (0.58)	2.58 (n=1)	3.27 (0.02)	0.34	NS

Table 6.3.7 Tables a-e show trends in lipid and apolipoprotein secretion and synthesis levels by Caco-2 cells which have been given chronic LA, t-10 or c-9 supplements followed by acute BSA, OA or PA treatment. Acute, chronic and acute by chronic effects are presented. The arithmetic mean of each chronic-acute fatty acid treatment was calculated. The last row in each table represents the average acute effect of a fatty acid treatment and is calculated by taking the arithmetic mean of the effects of all acute treatments with the fatty acid. The last column in each table represents the average chronic effect of a fatty acid supplementation and is calculated by taking the arithmetic mean of effects of all chronic treatments with the fatty acid. A 2-way-ANOVA with interactions was used to calculate whether any effects of fatty acid treatments were apparent in the measured variables and LSD tests were used to identify the fatty acid which causes the significant difference. Effects due to chronic supplementation of LA, t-10 and c-9 are coloured in dark green, dark red and dark blue respectively. Effects due to acute treatment of OA or PA are coloured in red or blue. MSE = Mean Squared Error.

Table 6.3.7a Percentage secreted apoB100. In this table the percentages of apoB100 secretion by Caco-2 cells following various chronic and acute fatty acid treatments were compared. No significant effects were observed.

Percentage secreted apoB100

Acute	0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA		Chronic Effect
Chronic	Mean (SE)	Mean (SE)	Mean (SE)		Mean
Control	15.33 (7.3)	3.5 (1.4)	17.8 (10.4)		11.88 (4.25)
0.05mM LA	11 (2.34)	7.8 (2.28)	17.4 (9.83)		12.07 (3.37)
0.05mM t-10	28.75 (15.32)	9 (3.24)	21.8 (11.28)	MSE = 284	19.21 (5.97)
0.05mM c-9	32.6 (6.42)	15.75 (4.84)	11.25 (4.61)	Acute by Chronic P=0.6108	20.85 (4.02)
Acute Effect	21.25 (4.28)	8.4 (1.64)	17.37 (4.7)	Acute P=0.063	Chronic P=0.3938

Table 6.3.7 continued

Table 6.3.7b Ratio of cTAG/bTAG. In this table the cTAG/bTAG levels were compared in cells given various chronic followed by acute fatty acid treatments. Acute doses of OA increased (4.88) TAG secretion levels in Caco-2 cells irrespective of chronic supplementation. An acute by chronic interaction was found. Supplementation with t-10 (23.75) and c-9 (17.1) affected how the cells responded to acute PA treatment. These cells were significantly different from the control cells (8.88). t-10 supplementation was also significantly different from chronic LA supplementation followed by acute PA treatment (12.25).

Ratio of non-secreted TAG to secreted TAG (cTAG/bTAG)

Acute	0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA		Chronic Effect
Chronic	Mean (SE)	Mean (SE)	Mean (SE)		Mean
Control	16.99 (2.68)	3.68 (0.66)	8.88 (0.85)[1]		9.85 (1.94)
0.05mM LA	11.24 (1.02)	5.89 (0.99)	12.25 (1.79)[12]		9.79 (1.03)
0.05mM t-10	11.27 (1.3)	4.05 (0.68)	23.75 (8.39)[3]	MSE = 29.56	13.02 (3.11)
0.05mM c-9	12.15 (1.29)	5.89 (0.46)	17.1 (2.89)[23]	Acute by Chronic P=0.0188	11.71 (1.67)
Acute Effect	12.91 (0.85)[a]	4.88 (0.41)[b]	15.5 (2.29)[a]	Acute P=0.0001	Chronic P=0.3542

Table 6.3.7c Ratio of cTAG/cPL. In this table the ratios found of non-secreted TAG to PL are given in cells which have been fed chronic followed by acute doses of various fatty acids. This table highlights three fatty acid effects. Firstly, all chronic treatments of the Caco-2 cells resulted in their having higher cTAG/cPL levels (LA 0.807, t-10 0.9651 and c-9 0.9203). Secondarily, cells give acute doses of OA (0.935) or PA (1.152) were significantly different from control cells (0.448) and each other. Thirdly, acute by chronic interactions were observed. Fatty acid supplemented cells given acute OA treatment (LA 0.972, t-10 1.2622 and c-9 0.988) were significantly different from control cells (0.564). Such cells given chronic LA and c-9 supplementation had similar ratios. In addition, chronic c-9 (1.285) followed by acute PA treatment was significantly different from chronic LA supplementation followed by acute PA treatment (1.028).

Ratio of cellular TAG to cellular PL (cTAG/cPL)

Acute	0.5% BSA	0.5mM OA, 0.5% BSA	0.5mM PA, 0.5% BSA		Chronic Effect
Chronic	Mean (SE)	Mean (SE)	Mean (SE)		Mean (SE)
Control	0.401 (0.026)	0.564 (0.059)[1]	1.099 (0.148)[i,ii]		0.688 (0.051)[a]
0.05mM LA	0.466 (0.02)	0.972 (0.039)[2]	1.028 (0.034)[i]		0.807 (0.049)[b]
0.05mM t-10	0.435 (0.035)	1.262 (0.119)[3]	1.198 (0.065)[i,ii]	MSE = 0.044	0.965 (0.083)[c]
0.05mM c-9	0.488 (0.016)	0.988 (0.033)[2]	1.285 (0.105)[ii]	Acute by Chronic P=0.0002	0.92 (0.072)[c]
Acute Effect	0.448 (0.013)[a]	0.935 (0.051)[b]	1.152 (0.047)[c]	Acute P=0.0001	Chronic P=0.0001

Table 6.3.7 continued

Table 6.3.7d Ratio of bTAG/bPL. Basolateral media TAG and PL levels were measured in cells given chronic followed by acute doses of fatty acids. Both OA and PA have an acute effect on bTAG/bPL ratio irrespective of chronic supplementation. However, acute OA treatment (11.08) causes a larger increase in secreted TAG levels versus secreted PL levels than acute PA treatment (7.542).

Ratio of secreted TAG to secreted PL (bTAG/bPL)

Acute / Chronic	0.5% BSA Mean (SE)	0.5mM OA, 0.5% BSA Mean (SE)	0.5mM PA, 0.5% BSA Mean (SE)		Chronic Effect Mean (SE)
Control	1.787 (0.833)	9.525 (2.153)	7.582 (1.063)		6.298 (0.757)
0.05mM LA	5.478 (1.124)	11.206 (0.809)	7.59 (1.207)		8.091 (0.849)
0.05mM t-10	3.734 (0.645)	11.702 (1.036)	6.233 (2.67)	MSE = 8.267	7.223 (1.241)
0.05mM c-9	4.88 (0.54)	11.878 (1.584)	8.764 (1.142)	Acute by Chronic P=0.7896	8.377 (1.023)
Acute Effect	3.872 (0.48)[a]	11.08 (0.673)[b]	7.542 (0.779)[c]	Acute P=0.0001	Chronic P=0.2776

Table 6.3.7e Ratio of bTAG/bTRL-TAG. Ratio of basolateral TAG to basolateral TRL-TAG was measured in cells supplemented with chronic fatty acids and then given acute fatty acid treatment. The ratio was calculated using the formula: bTAG/bTRL-TAG = Plate 1: [(bTAG Well 1 + bTAG Well 2)/2]/[(bTRL-TAG Well 3 + bTRL-TAG Well 4)/2]. Neither chronic, acute or acute by chronic fatty acid treatments of Caco-2 cells significantly affected the ratio of bTAG/bTRL-TAG.

Ratio of secreted TAG to secreted TRL-TAG (bTAG/bTRLTAG)

Acute / Chronic	0.5% BSA Mean (SE)	0.5mM OA, 0.5% BSA Mean (SE)	0.5mM PA, 0.5% BSA Mean (SE)		Chronic Effect Mean (SE)
Control	1.787 (n=1)	3.894 (0.213)	3.918 (0.665)		3.2 (0.285)
0.05mM LA	1.453 (0.394)	3.71 (0.373)	11.06 (7.018)		5.408 (2.496)
0.05mM t-10	1.703 (0.471)	6.445 (0.957)	2.85 (1.597)	MSE = 16.63	3.666 (0.925)
0.05mM c-9	2.26 (0.666)	3.883 (0.583)	2.242 (0.443)	Acute by Chronic P=0.3268	2.795 (0.395)
Acute Effect	1.805 (0.288)	4.483 (0.463)	5.018 (2.226)	Acute P=0.2421	Chronic P=0.5739

6.4 Discussion

6.4.1 Effects of LA, t-10 and c-9 on Lipid and Apolipoprotein Synthesis and Secretion by Caco-2 Cells

A study in which humans were supplemented with CLA over a 90 day period resulted in a 20% reduction in their body fat (Brodie *et al.* 1999). A study by Noone *et al.* (in press) suggested that humans given t-10:c-9 (50:50) supplements over an 8 week period had lower fasting plasma TAG levels than individuals given t-10:c-9 (20:80) supplements. Observations such as these beg the question whether the CLA plasma TAG reduction occurs at a gut level. The present study investigated long- and short-term effects of LA and its two conjugates, t-10 and c-9, on Caco-2 cell lipid metabolism as a model for intestinal lipid metabolism. The study in this chapter was carried out concurrently with the study discussed in chapter 5.

Investigation of short-term effects of LA, t-10 and c-9 showed that the LA isomer increased TAG secretion over PL secretion levels (bTAG/bPL) (Table 6.3.6). Previously acute LA treatment had been shown to increase TAG secretion levels by Caco-2 cells. This newly synthesised TAG was shown to be associated with TRL particles (van Greevenbroek *et al.* 1995). Although in the current study LA treatment did not increased TAG secretory levels compared to cellular TAG levels (cTAG/bTAG) the elevation in the ratio of TAG secretory levels compared to PL secretory levels (bTAG/bPL), suggests that LA treatment stimulates TAG secretion more than treatments with BSA, t-10 or c-9. It is possible that Caco-2 cells lipolyse TAG associated with TRL particles when in contact with the cells thus increasing lipoprotein density (Field *et al.* 1988). Therefore the newly synthesised TAG is not associated with TRL fractions. The CLA isomers did not significantly affect lipid and apolipoprotein synthesis or secretion compared to control treatment, LA or each other.

Interactions between long- and short-term fatty acid treatments showed that chronic t-10 and c-9 supplementation followed by acute OA or PA treatments appeared to decrease TAG secretion levels (cTAG/bTAG) (Table 6.3.7b) while increasing TAG synthesis levels (cTAG/cPL) (Table 6.3.7c). This observation is similar to results obtained after acute or chronic PA treatments (section 5.4.2.1). *Trans* fatty acids are considered to be

pseudosaturated fatty acids (Kritchevsky 1999) and have been observed in the present study to have the same effect on TAG homeostasis as PA, which is an SFA. A significant chronic effect was observed in this study, which is a generalisation of the interaction effects observed for the ratio of cTAG/cPL. The foetal calf serum (FCS) that the Caco-2 cells are grown in contains similar quantities of CLA isomers to dairy and beef fat (Park & Pariza 1998). Therefore FCS may strongly affect the secretion profile of lipids and lipoproteins after acute fatty acid treatments. It appears that the reduction in plasma TAG concentrations after CLA treatment is not due to a reduction in TAG secretion levels from the gut, since the present study found no reduction in TAG secretion levels. However, the reduction in plasma TAG levels may be due to enhanced lipolysis due to enhanced LPL activity. Noone *et al.* (in press) found that the t-10 isomer was associated with reduced fasting plasma TAG levels and Pariza *et al.* (1999) previously had shown that the t-10 isomer enhanced lipolysis in murine adipocytes.

The outcome of acute fatty acid treatments observed in the present study (Tables 6.3.7b-d) are comparable to those discussed in chapter 5 (sections 5.4.2.1 and 5.4.2.2). Thus, acute OA treatments cause increased TAG secretion levels (cTAG/bTAG and bTAG/bPL) and increased TAG synthesis over PL synthesis levels (cTAG/cPL). OA treatments have been associated with increased *de novo* TAG secretion (Field *et al.* 1988), which may possibly be due to OA being a good substrate for the microsomal triglyceride transfer protein (MTP) (van Greevenbroek *et al.* 1998). The increased TAG synthesis levels are probably due to the increased availability of fatty acids for the synthesis of TAG. Acute PA treatments were associated with increased cellular TAG levels (cTAG/cPL and bTAG/bPL). This may be because Caco-2 cells, which are fed SFA, first secrete MUFA containing TAG and subsequently secrete SFA containing TAG (van Greevenbroek *et al.* 2000).

6.4.2 Characterisation of Caco-2 Cells Supplemented with LA, t-10 or c-9

Characterisation of Caco-2 cells showed that cells supplemented with 0.1mM or 0.05mM of the t-10 isomer of CLA did not develop at the same rate as unsupplemented cells or cells supplemented with LA or the c-9 isomer of CLA. By day 19 post-seeding, however, t-10 supplemented cells did not have significantly different TEERs to control cells. Supplementation of Caco-2 cells with 0.1mM t-10 for 19 days lead to poor cell viability

but neither concentration (0.1mM or 0.05mM) of t-10 arrested cell proliferation. The paracellular apparent permeability coefficient (P_{app}) of the cell monolayer of cells grown in the presence of t-10 was higher than control P_{app} values at both day 6 and day 14 post-seeding, although by day 14 the P_{app} values were more similar to control values than at day 6 post-seeding. This suggests that the t-10 isomer may retard tight junction formation. In a subsequent study within this group, Roche *et al.* (2001) investigated the effects of the CLA isomers on adhesion molecules Occludin and ZO-1, which are integral structural components of tight junctions. The distribution of Occludin and ZO-1 was found to be altered by t-10 supplementation. Occludin and ZO-1 were not associated with the cell membrane but randomly spread within the cell (Roche *et al.* 2001). Previous studies had shown that at very low concentrations (0.5mg/l = 0.00178mM) CLA inhibits cellular proliferation of murine adipocytes (Satory & Smith 1999). In addition, CLA inhibits colon tumorgenesis in experimental animals (Liu & Belury 1998). The Caco-2 cell line was originally derived from an adenocarcinoma (Fogh *et al.* 1977) and so it was though that CLA might affect cell growth. From this study it appears that the active growth retarding isomer is the t-10 isomer.

6.4.3 Effect of LA and CLA Isomer Supplementation to Caco-2 Cells on the Fatty Acid Composition of their TAG and PL Fractions

Commercial FCS and newborn calf sera have been found to contain similar quantities of CLA to dairy and beef fat, although the levels of the t-10 isomer are higher than usually seen in ruminant products (Park & Pariza 1998). Control cells in this study, which were fed FCS without LA, t-10 or c-9 supplements were found to contain the t-10 and c-9 isomers of CLA in their lipid fractions. Satory & Smith (1999) had found a proportional increase in cellular 16:0, 16:1 and CLA levels and a decrease in 18:0 and 18:1 levels in 3T3-L1 preadipocytes fed 10mg/l CLA compared to control cells. In the present study a similar result was observed. Cells given LA, t-10 or c-9 supplements (particularly t-10) showed an increase in 16:0 levels and a decrease in 18:1 levels, compared to control cells. Cells that were given chronic LA supplementation were found to be directly exchanging 18:1 for 18:2n-6 (i.e. they had an increase in 18:2n-6 concentration and a decrease in 18:1 concentration). However, no increase in their 16:0 levels was observed compared to controls.

In the current study an increase in 16:0 and decrease in 18:1 was observed in Caco-2 cells given chronic doses of CLA (t-10 or c-9). The increase in 16:0 levels may be due to stimulation of acetyl CoA carboxylase and or fatty acid synthetase activity (Satory & Smith 1999). Similarly, the decrease in 18:1 levels is thought to be due to a reduction in stearoyl-CoA desaturase activity (Lee *et al.* 1998, Loor & Herbein 1998). The reduction in desaturase activity is believed to be due to a reduction in the levels of stearoyl-CoA desaturase 1 mRNA levels. This reduction has previously been found not to be due to treatment with the c-9 isomer (Lee *et al.* 1998) and in the present study supplementation with the t-10 isomer reduced 18:1 levels to a greater extent than supplementation with the c-9 isomer.

In the current study, supplementation of Caco-2 cells with t-10 caused an increase in CLAt-10 and CLAc-9 fatty acid levels found in cellular TAG fractions. However, c-9 supplementation did not result in an increase in the CLAt-10 levels in cellular TAG fractions (Figure 6.3.3a). In human tissue, CLA has been shown to be partly derived from diet and partly by the conversion of 18:1 *trans*-fatty acids (vaccenic acid), although in that study t-10 levels were too low for detection (Salminen *et al.* 1998). In this study, secreted TAG from the t-10 supplemented Caco-2 cells contained approximately 5-times as much c-9 as t-10 (Figure 6.3.3c). Therefore the t-10 isomer may be converted into the c-9 isomer by Caco-2 cells (human small intestine), causing higher levels of c-9 than t-10 to be found in human tissues. Previous studies had found the c-9 isomer to be present at twice the concentration of the t-10 isomer in the liver, fat pad and skeletal muscle of mice fed a diet containing equal quantities of both isomers (Park *et al.* 1999). When the mice were withdrawn from the diet, the level of t-10 decreased more rapidly in the skeletal muscle than in liver or fat pad, suggesting conversion of the t-10 isomer to the c-9 form.

CLA is known to inhibit tumorgenesis by reducing the quantity of arachidonic acid (AA) in the phosphatidyl choline fraction of PL (Liu & Belury 1998). AA is the substrate for the formation of eicosanoids, which are mediators of many biochemical processes and play critical roles in the coordination of physiological interactions between cells. It has been shown that dietary modifications, where some of the AA in the PL fraction has been replaced by n-3 PUFA, are positively correlated with a reduction of prostaglandin E_2. Prostaglandin E_2 is involved in tumour induction (Belury 1995). Therefore the anti-carcinogenic effect of CLA may occur through a similar mechanism as the n-3 PUFA anti-carcinogenic effect. In the present study supplementation of Caco-2 cells with the c-9 isomer caused a reduction in AA levels in the PL fraction of the cells (Figure 6.3.3b).

However, this reduction was not observed in the secreted PL fraction (Figure 6.3.3d). Supplementation of Caco-2 cells with the t-10 isomer did not result in a decreased level of AA (Figures 6.3.3a, 6.3.3b, 6.3.3c & 6.3.3d). Therefore, the anti-carcinogenic properties of CLA through the eicosanoid mechanism may be due to the c-9 and not the t-10 component of CLA.

6.4.4 Conclusion

The present study set out to investigate whether the reduction in plasma TAG concentrations associated with CLA occurs at the level of the small intestine. A model cell system of the small intestine, in which the effects of fatty acid supplementation on metabolism could be studied, was characterised. The effects of chronic supplementation of LA, t-10 and c-9 were studied in this Caco-2 cell model system. During characterisation the t-10 isomer was found to affect cell development. This has been shown to be due to disruption of tight junction formation (Roche *et al.* 2001).

CLA treatment was found to alter the fatty acid composition of cellular and basolateral lipid fractions. The t-10 isomer was found to be more potent at reducing 18:1 levels and increasing 16:0 levels than the c-9 isomer. In addition, it is thought that the t-10 isomer may also be converted into c-9 by Caco-2 cells, thus increasing the concentration of c-9 in t-10 supplemented cells. However, it was the c-9 isomer, which was found to decrease arachidonic acid levels in the cellular PL fraction, possibly interfering with eicosanoid synthesis.

The CLA isomers were found not to effect lipid and apolipoprotein synthesis and secretion when administered acutely. However, when Caco-2 cells were chronically supplemented with the isomers over a 19 day period the supplementation effected TAG synthesis and secretion levels (cTAG/cPL and cTAG/bTAG). Both CLA isomers were associated with reduced TAG secretion levels and increased TAG synthesis suggesting that the CLA isomers were behaving in the pseudosaturated fatty acid manner often associated with *trans* fatty acids. Ingestion of SFA is usually associated with increased plasma TAG levels (Williams 1999). Therefore the CLA associated reduction in TAG levels and body weight is most probably not regulated at the intestinal level. However, the results of investigations into fatty acid metabolism using cell models will be dependent on the quantity of CLA isomers present in the FCS given to the cells.

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7 Conclusion

7.1.1 Introduction

Postprandial lipaemia is a quantitative trait. Many genetic and environmental factors interact and modulate the response. Postprandial lipaemia response refers to the increase in plasma TAG concentration and associated decrease in NEFA and HDL-cholesterol levels after ingestion of a meal containing more than 15g of long-chain fatty acids. This thesis set out to explain some of the variation in individual's lipid and apolipoprotein concentrations observed during the postprandial response. Firstly, the influence of polymorphisms in various genes involved in lipid metabolism was studied (chapters 2 and 3). Secondly, the influence of fatty acid treatments both long- and short-term, on the postprandial response was analysed in Caco-2 cells, a model system of the human small intestine (chapter 5 and 6). Notably, certain genetic polymorphisms are known to have different effects on the postprandial response depending on whether the study populations are hyper-, hypo- or normotriglyceridaemic (Ordovas & Schaefer 1999). In addition the effects of genetics may vary depending on what the fatty acid constituents of the test meal are; saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), n-6 polyunsaturated fatty acid (PUFA) or n-3 PUFA (section 1.3.3).

7.1.2 Effects of Polymorphisms in Genes Involved in Lipid Metabolism on Fasting and Postprandial Lipid and Lipoprotein Levels

The study discussed in chapter two investigated the effects of 8 polymorphisms in four genes involved in lipid metabolism on postprandial lipid and apolipoprotein levels. The findings of this study, carried out on a normolipidaemic study population (n=82), were that the D allele of the insertion/deletion polymorphism in *apoB* and the ε2 allele in *apoE* were linked to a healthier lipid profile than II homozygotes and ε4 allele carriers. The H-T haplotype of the *lpl* gene was found to be significantly associated with lower fasting plasma TAG concentrations in both males and females, but only with lower postprandial TAG concentrations in males. At fasting individuals with the B- allele of *cetp* had higher

HDL-cholesterol levels due to increased concentration of HDL_3-cholesterol and lower CETP mass, than B+ homozygotes.

The outcome of the study highlights the need for using large population sizes when investigating effects of genetics on postprandial lipaemia, since genetics can have major and minor effects on the response. After identifying the genotypes of the study population and measuring their fasting and postprandial lipid and lipoprotein levels, data were trawled through for associations between the nature of the postprandial response and genetic polymorphisms. However, when effects of a large number of polymorphisms are analysed in a population of this size (n=82) using statistics, some effects are likely to appear to be significant by chance even after allowing for multiple testing. A better way in which to study the genetics of multifactorial diseases and/or traits may be to use single nucleotide polymorphism (SNP) maps, which are being generated by the SNP Consortium. These maps may be extremely valuable in the analysis of genetic contribution to quantitative genetic traits. Using SNP maps of genes involved in lipid metabolism, study populations could be chosen on the basis of their genetics. Subsequently, postprandial variables could be measured and compared between haplotype groups. The ability to associate health risk factors with individuals' haplotypes may lead to a more 'personalised' medical treatment for many complex disorders, where disease prevention strategies and treatments are tailored to an individual's genetic profile. However, before SNP maps of genes involved in lipid metabolism become available, a larger study with separate male and female populations chosen on the basis of their *lpl* haplotype, H+S, H-S or H-T could be carried out to investigate postprandial triacylglycerolaemia in the 6 groups.

7.1.3 Relationship Between Postprandial Triacylglycerolaemia and Activation of Coagulation Factor VII is Modulated by a Factor VII Haplotype

Chapter three discussed the relationship between the initiation of the extrinsic coagulation pathway and postprandial triacylglycerolaemia in individuals who had either RR/0bp0bp or RQ/0bp10bp haplotype of the R353Q and decanucleotide insertion polymorphisms in the *FVII* gene. The study found that individuals who were normolipidaemic with an RQ/0bp10bp haplotype did not have the increase in activated factor FVII levels (FVIIa) and the FVIIa to total FVII (FVIIag) ratio usually associated with postprandial plasma TAG levels. However, individuals with RR/0bp0bp haplotype did have a postprandial

increase in FVIIa levels and the ratio of FVIIa to FVIIag. Possibly a future larger study carried out on a normolipidaemic population in which FVII phenotypes associated the R0bp, R10bp, Q0bp and Q10bp haplotypes are analysised, could shed some light on the effect of each of the polymorphisms on fasting and postprandial FVII concentrations. The ability to investigate the effects of each of the haplotypes separately may show how the different alleles cooperate to cause the FVII phenotypes.

7.1.4 Effects of Chronic Fatty Acid Supplementation on the Response of Caco-2 Cells to Acute Fatty Acid Supplementation

The experiments in chapter four were carried out in order to set up a model system for studying effects of chronic fatty acid supplementation on postprandial response. The system utilised Caco-2 cells with passage numbers between 33 and 47. Fatty acid concentrations of 0.05mM EPA, OA or PA, did not affect the growth of Caco-2 cells. In addition, cells grown in the presence of 0.05mM fatty acid appeared to incorporate the fatty acids into their cellular and secreted TAG and PL fractions. These compositional changes in their lipid fractions are thought to alter how Caco-2 cells respond to acute fatty acid load (section 1.5.1).

Chapter five, discussed how chronically supplemented Caco-2 cells given acute doses of fatty acid synthesised and secreted varying levels of apoB100, TAG and PL depending on the type of chronic and acute fatty acid administered. The hypothesis tested in this doctoral thesis was whether chronic doses of fatty acids effect how cells respond to acute doses of fatty acids. Van Greevenbroek *et al.*, who have carried out much work in this area, experimented on Caco-2 cells with higher passage numbers (70-90). When the Caco-2 cells used in our studies reached passage numbers between 70-90, they were shown to secrete twice as many lipids as Caco-2 cells with passage numbers between 33-47. However, the levels observed were still lower than those reported by the van Greevenbroek group (Seeballuck & O'Driscoll personal communication). Possibly if this chronic-acute experiment had been carried out on Caco-2 cells with passage numbers of between 70-90 (same as van Greevenbroek) more secreted lipids would have been observed than were found in chapter five. However, Caco-2 cells with lower passage numbers (30-40) are known to resemble enterocytes more closely than Caco-2 cells with higher passage numbers (93-108) (Yu *et al.* 1997). Despite the different levels of secreted TAG observed

in this study and van Greevenbroek's studies, the same effects were observed in this study as in studies reported in the literature.

An acute by chronic interaction was observed to affect the ratio of non-secreted TAG to secreted TAG levels. However, the effect appeared to be inconsistent. Chronic and acute treatments with each of the fatty acids appeared to cause the same types of response in Caco-2 cells. EPA was associated with increased PL synthesis compared to TAG synthesis, which is believed to be due to inhibition of diacylglycerol acyl transferase activity. OA treatments were associated with increased TAG secretion levels. The fact that acute OA treatments did not appear to increase the levels of *de novo* TAG present in TRL particles may possibly be due to lipolysis of the secreted TAG molecules. PA treatments were associated with increased cellular TAG concentrations, which is believed to be due to TAG consisting of PA molecules not being as good a substrate for microsomal triglyceride transfer protein as TAG molecules consisting of OA.

To clarify whether an interaction between the two types of fatty acid treatment, chronic and acute, is indeed present, the experiment could be duplicated. Further investigations into chronic effects of fatty acids alone on lipid metabolism could also be carried out. This may help establish whether slight variations in the effects of acute fatty acid treatments and chronic fatty acid supplementations on cell lipid metabolism exist.

7.1.5 Effects of CLA Isomers (t-10 and c-9) on Caco-2 Cells: A Model of the Human Small Intestine

Recently there has been much interest in conjugate linoleic acid (CLA), as it has been shown, amongst others, to have anti-atherogenic and anti-carcinogenic properties. CLA has been shown to cause body compositional changes in animals and humans (section 1.6.1). It has also been shown to affect eicosanoid synthesis (section 1.6.2) and act as a ligand for the peroxisome proliferator response receptor, thus affecting expression of lipid regulating enzymes from many tissues (section 1.6.4).

The study discussed in chapter 6 on the effects of CLA on lipid synthesis and secretion by Caco-2 cells, was carried out to test whether CLA causes a reduction in plasma TAG levels due to changes in lipid metabolism at the intestinal level. CLA does not usually share characteristics with other *trans* fatty acids. However, this study found that chronic t-10 or c-9 isomer supplementation of Caco-2 cells increased their TAG synthesis levels and

decreased their TAG secretion levels. Therefore the isomers appeared to have a similar affect to SFA. The finding that chronic CLA supplementation affected how Caco-2 cells responded to physiological fatty acid doses is of considerable significance. Foetal calf serum contains CLA and therefore different brands or batches of FCS could contain different levels of CLA. Therefore, results of studies investigating lipid variables in cell lines, which use different brands or batch numbers of FCS, may not be comparable. The reduced plasma TAG levels, which have been associated with the t-10 isomer most probably occur at a TAG clearance level and may be due to increased carnitine palmitoyltransferase activity. Alternatively, the reduction may be due to the ability for CLA to act as a ligand for peroxisome proliferator activated receptors and thus regulate expression of lipid metabolising enzymes. Future investigation into the molecular mechanisms involved in enhanced TAG clearance after CLA ingestion in humans should be carried out in human hepatocytes, HepG2 cells and adipocytes.

Prior to the investigation of chronic effects of CLA on lipid synthesis and secretion by Caco-2 cells, comprehensive characterisation of the chronically supplemented Caco-2 cells was carried out. Results from the characterisation experiments showed that CLA isomers are likely to act independently to inhibit the development of cancerous cells. The t-10 isomer inhibits tight junction formation while the c-9 isomer appears to alter eicosanoid synthesis by reducing arachidonic acid levels. However, these results have been obtained from a pilot study. Therefore for full understanding of the anti-carcinogenic effects of both of the CLA isomers, larger and more in-depth investigations into the effects of each isomer on cancerous cell growth at all stages of cell development should be carried out.

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Appendix I

Complete analysis of genotypic effect on fasting and postprandial lipaemia

Gene Polymorphism		BMI kg/m^2		Fasting TAG mmol/l	
		Number	Mean (95% CI)	Number	Mean (95% CI)
Apolipoprotein B					
Insertion Deletion	II	36	24.77 (23.47-25.5)	34	0.88 (0.78-1.01)
	ID	36	23.7 (22.83-24.56)	35	0.78 (0.75-0.96)
	DD	10	24.04 (22.46-25.62)	10	1.0 (0.78-1.32)
			p=0.181		p=0.526
	II	36	25.05 (24.27-25.83)		
	ID/DD	46	23.77 (23.02-24.52)		
			p=0.0234		
	II/ID				
	DD				
EcoRI	R+R+	56	24.09 (23.42-24.76)	54	0.86 (0.78-0.96)
	R+R-	23	24.65 (23.67-25.63)	22	0.95 (0.81-1.13)
	R-R-	3	23.11 (22.22-24)	3	0.76 (0.62-0.94)
			p=0.493		p=0.485
	R+R+/R+R-				
	R-R-				
Apolipoprotein E	ε2ε2	1	20.34	1	0.83
	ε2ε3	2	21.63 (20.71-22.54)	2	0.93 (0.57-1.79)
	ε2ε4	2	25.53 (24.32-26.73)	2	1.44 (0.94-2.26)
	ε3ε3	48	24.55 (23.86-25.24)	45	0.82 (0.74-0.91)
	ε3ε4	24	23.68 (22.83-24.54)	24	0.94 (0.78-1.15)
	ε4ε4	5	24.74 (21.19-28.29)	5	1.04 (0.8-1.4)
			p=0.193		p=0.346
	ε2ε2/ε2ε3	3	21.2 (20.2-22.19)	3	0.9 (0.66-1.28)
	ε3ε3	48	24.55 (23.86-25.24)	45	0.82 (0.74-0.91)
	ε3ε4/ε4ε4	29	23.87 (22.96-24.78)	29	0.95 (0.81-1.14)
			p=0.054		p=0.248
Lipoprotein Lipase					
PvuII	P+P+	26	23.96 (23.03-24.89)	25	0.92 (0.78-1.05)
	P+P-	45	24.62 (23.92-25.32)	43	0.84 (0.76-0.93)
	P-P-	11	23.13 (21.51-24.76)	11	0.94 (0.74-1.25)
			p=0.169		p=0.515
HindIII	H+H+	43	24.28 (23.53-25.04)	41	0.97 (0.87-1.09)
	H+H-	34	23.97 (23.12-24.81)	33	0.8 (0.69-0.92)
	H-H-	5	25.23 (23.66-26.79)	5	0.77 (0.63-0.97)
			p=0.554		p=0.072
	H+H+			41	0.97 (0.87-1.09)
	H+H-/H-H-			38	0.79 (0.7-0.9)
					p=0.022
Ser447Ter	SS	71	24.19 (23.61-24.78)	68	0.9 (0.81-0.99)
	ST/TT	11	24.32 (22.9-25.74)	11	0.77 (0.64-0,94)
			p=0.87		p=0.198
Cholesteryl ester transfer protein					
TaqIB	B+B+	30	23.43 (22.66-24.19)	29	0.84 (0.72-1.06)
	B+B-	42	24.78 (24.1-25.7)	40	0.87 (0.79-0.98)
	B-B-	10	24.17 (23.47-24.88)	10	1.04 (0.85-1.31)
			p=0.073		p=0.363
	B+B+	30	23.43 (22.66-24.19)		
	B+B-/B-B-	52	24.66 (23.96-25.36)		
			p=0.022		
	B+B+/B+B-				
	B-B-				

	Maximum TAG mmol/l		Postprandial TAG mmol/l		Fasting TRL-TAG mmol/l	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II	34	1.22 (1.07-1.41)	33	7.87 (6.89-9.07)		
ID	35	1.21 (1.06-1.38)	35	7.77 (6.85-8.86)		
DD	10	1.3 (1.03-1.69)	10	7.47 (5.78-9.89)		
		p=0.883		p=0.923		
II						
ID/DD						
II/ID						
DD						
R+R+	54	1.2 (1.08-1.34)	53	7.45 (6.75-8.31)	51	0.23 (0.18-0.28)
R+R-	22	1.33 (1.13-1.6)	22	8.78 (7.55-10.82	22	0.26 (0.21-0.32)
R-R-	3	0.99 (0.83-1.2)	3	6.84 (5.78-8.21)	3	0.13 (0.11-0.15)
		p=0.364		p=0.231		p=0.285
R+R+/R+R-					73	0.24 (0.2-0.28)
R-R-					3	0.13 (0.11-0.15)
						p=0.0003
ε2ε2	1	0.77	1	5.1		
ε2ε3	2	1.15 (0.68-2.37)	2	7.55 (4.38-16.0)		
ε2ε4	2	1.57 (1.09-2.49)	2	5.83 (2.91-17.08)		
ε3ε3	45	1.14 (1.02-1.28)	45	7.37 (6.64-8.21)		
ε3ε4	24	1.32 (1.12-1.56)	23	8.49 (7.23-10.14)		
ε4ε4	5	1.73 (1.26-2.53)	5	10.78 (7.93-15.5)		
		p=0.172		p=0.183		
ε2ε2/ε2ε3	3	1.0 (0.68-1.61)	3	6.56 (4.49-10.61)		
ε3ε3	45	1.14 (1.02-1.28)	45	7.37 (6.64-8.21)		
ε3ε4/ε4ε4	29	1.38 (1.19-1.61)	28	8.85 (7.63-10.41)		
		p=0.102		p=0.113		
P+P+	25	1.25 (1.04-1.52)	24	7.63 (6.38-9.24)	22	0.3 (0.21-0.39)
P+P-	43	1.19 (1.08-1.34)	43	7.63 (6.85-8.55)	43	0.2 (0.16-0.24)
P-P-	11	1.29 (1.05-1.63)	11	8.71 (7.11-10.89)	11	0.25 (0.19-0.32)
		p=0.823		p=0.609		p=0.049
H+H+	41	1.32 (1.18-1.48)	40	8.29 (7.42-9.35)	38	0.27 (0.22-0.33)
H+H-	33	1.14 (0.98-1.33)	33	7.28 (6.34-8.45)	33	0.2 (0.15-0.24)
H-H-	5	1.11 (0.87-1.28)	5	7.21 (5.4-10.08)	5	0.21 (0.15-0.27)
		p=0.247		p=0.351		p=0.105
H+H+	41	1.32 (1.18-1.48)	40	8.29 (7.42-9.35)	38	0.27 (0.22-0.33)
H+H-/H-H-	38	1.13 (1.0-1.3)	38	7.28 (6.41-8.31)	38	0.2 (0.16-0.24)
		p=0.094		p=0.147		p=0.034
SS	68	1.24 (1.12-1.37)	67	7.87 (7.15-8.7)	65	0.25 (0.21-0.29)
ST/TT	11	1.14 (0.95-1.38)	11	7.17 (6.04-8.65)	11	0.17 (0.11-0.24)
		p=0.507		p=0.459		p=0.077
B+B+	29	1.12 (0.97-1.32)	29	7.43 (6.44-8.7)		
B+B-	40	1.29 (1.22-1.36)	39	8.05 (7.19-9.07)		
B-B-	10	1.3 (1.01-1.73)	10	7.7 (5.89-10.54)		
		p=0.336		p=0.708		
B+B+						
B+B-/B-B-						
B+B+/B+B-						
B-B-						

	Maximum TRL-TAG mmol/l		Postprandial TRL-TAG mmol/l		Fasting TPL-TAG mmol/l	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II ID DD II ID/DD II/ID DD						
R+R+ R+R- R-R- R+R+/R+R- R-R-	51 22 3	0.39 (0.33-0.47) 0.49 (0.39-0.61) 0.33 (0.22-0.5) p=0.329	44 22 3	2.11 (1.74-2.57) 2.76 (2.16-3.53) 2.0 (1.39-2.87) p=0.253	52 22 3	0.57 (0.52-0.63) 0.57 (0.49-0.66) 0.49 (0.44-0.56) p=0.758
ε2ε2 ε2ε3 ε2ε4 ε3ε3 ε3ε4 ε4ε4 ε2ε2/ε2ε3 ε3ε3 ε3ε4/ε4ε4						
P+P+ P+P- P-P-	22 43 11	0.46 (0.33-0.65) 0.4 (0.34-0.47) 0.39 (0.29-0.53) p=0.642	18 40 11	2.51 (1.73-3.64) 2.17 (1.8- 2.61) 2.44 (1.86-3.2) p=0.679		
H+H+ H+H- H-H- H+H+ H+H-/H-H-	38 33 5	0.45 (0.37-0.56) 0.37 (0.3-0.45) 0.43 (0.32-0.77) p=0.404	34 31 4	2.46 (1.98-3.07) 2.09 (1.67-2.62) 2.54 (1.58-4.08) p=0.561		
SS ST/TT	65 11	0.42 (0.36-0.5) 0.37 (0.28-0.5) p=0.47	59 10	2.37 (2.0-2.81) 1.88 (1.43-2.48) p=0.17		
B+B+ B+B- B-B- B+B+ B+B-/B-B- B+B+/B+B- B-B-					28 39 10	0.56 (0.49-0.64) 0.56 (0.51-0.61) 0.66 (0.53-0.8) p=0.339

	Maximum TPL-TAG mmol/l		Postprandial TPL-TAG mmol/l		Fasting Cholesterol mmol/l	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II					36	5.35 (5.0-5.69)
ID					36	5.1 (4.74-5.46)
DD					10	4.92 (4.54-5.31)
						p=0.417
II						
ID/DD						
II/ID						
DD						
R+R+	46	0.71 (0.63-0.8)	46	4.63 (4.28-5.0)	56	5.11 (4.85-5.37)
R+R-	22	0.68 (0.61-0.77)	22	9.32 (1.87-22.36)	23	5.34 (4.99-5.69)
R-R-	3	0.58 (0.54-0.62)	3	4.05 (3.78-4.34)	3	5.43 (3.23-7.63)
		p=0.611		p=0.292		p=0.612
R+R+/R+R-						
R-R-						
ε2ε2					1	4.13
ε2ε3					2	5.01 (3.74-6.27)
ε2ε4					2	4.25 (2.79-5.72)
ε3ε3					48	5.21 (4.91-5.5)
ε3ε4					24	5.26 (4.81-5.71)
ε4ε4					5	5.29 (4.66-5.92)
						p=0.728
ε2ε2/ε2ε3						
ε3ε3						
ε3ε4/ε4ε4						
P+P+	20	0.7 (0.6-0.82)			26	5.15 (4.76-5.54)
P+P-	40	0.67 (0.62-0.71)			45	5.16 (4.85-5.48)
P-P-	11	0.8 (0.54-1.28)			11	5.36 (4.8-5.92)
		p=0.364				p=0.833
H+H+					43	5.09 (4.79-5.38)
H+H-					34	5.3 (4.93-5.67)
H-H-					5	5.25 (4.14-6.35)
						p=0.66
H+H+						
H+H-/H-H-						
SS					71	5.24 (5.0-5.48)
ST/TT					11	4.86 (4.22-5.49)
						p=0.26
B+B+	25	0.64 (0.57-0.71)			30	5.23 (4.9-5.56)
B+B-	36	0.73 (0.64-0.84)			42	5.0 (4.69-5.31)
B-B-	10	0.73 (0.62-0.88)			10	5.81 (5.0-6.61)
		p=0.298				p=0.082
B+B+						
B+B-/B-B-						
B+B+/B+B-					72	5.1 (4.87-5.32)
B-B-					10	5.81 (5.0-6.61)
						p=0.042

	Postprandial Cholesterol mmol/l		HDL-C mmol/l		HDL3-C mmol/l	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Median (Range)
II	35	42.08 (39.06-44.86)				
ID	35	39.15 (36.57-41.82)				
DD	10	38.56 (35.928-42.15)				
		p=0.228				
II	35	42.08 (39.06-44.86)				
ID/DD	45	39.02 (36.87-41.22)				
		p=0.087				
II/ID						
DD						
R+R+	54	39.62 (37.66-41.63)				
R+R-	23	42.04 (38.61-45.62)				
R-R-	3	40.56 (27.67-55.86)				
		p=0.479				
R+R+/R+R-						
R-R-						
ε2ε2	1	33.27				
ε2ε3	2	38.5 (32.19-45.37)				
ε2ε4	2	32.91 (22.67-37.58)				
ε3ε3	47	40.77 (38.59-43.01)				
ε3ε4	23	40.48 (36.84-44.28)				
ε4ε4	5	41.09 (36.48-45.97)				
		p=0.693				
ε2ε2/ε2ε3						
ε3ε3						
ε3ε4/ε4ε4						
P+P+	35	39.83 (36.65-43.14)				
P+P-	44	40.27 (37.96-42.67)				
P-P-	11	41.8 (37.55-46.51)				
		p=0.793				
H+H+	42	39.81 (37.45-42.24)			42	1.0 (0.35-2.62)
H+H-	33	40.83 (38.22-43.53)			33	0.96 (0.6-1.51)
H-H-	5	41.62 (33.07-51.14)			5	0.8 (0.62-0.99)
		p=0.805				p=0.259
H+H+						
H+H-/H-H-						
SS	69	40.54 (38.69-42.43)			69	1.0 (0.36-2.62)
ST/TT	11	39.1 (34.54-43.94)			11	0.9 (0.64-1.21)
		p=0.574				p=0.295
B+B+	30	41.09 (38.37-43.9)	30	1.15 (1.06-1.27)	30	0.91 (0.35-1.61)
B+B-	40	38.76 (36.49-41.1)	41	1.18 (1.09-1.28)	41	0.98 (0.62-2.62)
B-B-	10	44.6 (39.03-50.54)	9	1.41 (1.15-1.77)	9	1.12 (0.76-1.51)
		p=0.097		p=0.15		p=0.145
B+B+						
B+B-/B-B-						
B+B+/B+B-	70	39.75 (37.99-41.55)	71	1.17 (1.12-1.22)	71	0.94 (0.35-2.62)
B-B-	10	44.6 (39.03-50.54)	9	1.41 (1.15-1.77)	9	1.12 (0.76-1.51)
		p=0.076		p=0.12		p=0.052

	HDL2-C mmol/l		Fasting NEFA mmol/l		Postprandial NEFA mmol/l	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II	36	0.21 (0.15-0.28)	36	0.46 (0.39-0.54)	35	3.96 (3.79-4.12)
ID	34	0.23 (0.16-0.31)	35	0.42 (0.37-0.47)	34	4.09 (3.72-4.46)
DD	10	0.35 (0.24-0.46)	9	0.56 (0.39-0.76)	9	4.34 (3.37-5.31)
		p=0.257		p=0.185		p=0.637
II						
ID/DD						
II/ID	70	0.22 (0.17-0.27)				
DD	10	0.35 (0.24-0.46)				
		p=0.037				
R+R+			55	0.45 (0.39-0.5)	53	3.98 (3.68-4.28)
R+R-			22	0.49 (0.4-0.58)	22	4.3 (3.85-4.75
R-R-			3	0.35 (0.25-0.47)	3	3.69 (3.35-4.03)
				p=0.456		p=0.432
R+R+/R+R-						
R-R-						
ε2ε2			1	0.82	1	5.46
ε2ε3			2	0.52 (0.45-0.6)	2	4.6 (2.6-6.6)
ε2ε4			2	0.38 (0.23-0.57)	2	3.5 (2.86-4.13)
ε3ε3			47	0.45 (0.39-0.51)	46	3.98 (3.66-4.29)
ε3ε4			24	0.46 (0.4-0.54)	23	4.33 (3.88-4.78)
ε4ε4			4	0.4 (0.19-0.69)	4	3.13 (2.59-3.67)
				p=0.647		p=0.203
ε2ε2/ε2ε3						
ε3ε3						
ε3ε4/ε4ε4						
P+P+			25	0.45 (0.39-0.52)	24	3.87 (3.49-4.25)
P+P-			44	0.45 (0.39-0.52)	45	4.05 (3.72-4.38)
P-P-			11	0.46 (0.36-0.56)	11	4.5 (4.34-4.66)
				p=0.996		p=0.288
H+H+			42	0.47 (0.41-0.54)	41	4.1 (3.78-4.41)
H+H-			33	0.43 (0.37--0.51)	32	4.09 (3.67-4.52)
H-H-			5	0.41 (0.35-0.47)	5	3.52 (3.02-4.02)
				p=0.597		p=0.525
H+H+						
H+H-/H-H-						
SS			69	0.45 (0.4-0.5)	67	4.08 (3.81-4.34)
ST/TT			11	0.5 (0.41-0.59)	11	3.95 (3.32-4.58)
				p=0.421		p=0.718
B+B+	30	0.25 (0.19-0.31)	30	0.5 (0.41-0.59)	30	4.34 (3.99-4.7)
B+B-	41	0.2 (0.15-0.26)	40	0.43 (0.38-0.48)	38	3.88 (3.57-4.2)
B-B-	9	0.32 (0.14-0.58)	10	0.42 (0.3-0.55)	10	3.87 (3.25-4.41)
		p=0.339		p=0.319		p=0.189
B+B+						
B+B-/B-B-						
B+B+/B+B-						
B-B-						

	Fasting ApoB48		ApoB48-4hr		Postprandial ApoB48	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II	34	0.37 (0.33-0.42)	33	0.5 (0.44-0.56)	31	1.83 (1.63-2.03)
ID	36	0.38 (0.33-0.43)	36	0.53 (0.47-0.61)	34	1.91 (1.68-2.14)
DD	9	0.4 (0.31-0.52)	9	0.41 (0.3-0.56)	9	1.63 (1.35-1.95)
		p=0.901		p-0.198		p=477
II						
ID/DD						
II/ID			69	0.51 (0.47-0.56)	65	1.87 (1.72-2.02)
DD			9	0.41 (0.3-0.56)	9	1.63 (1.35-1.95)
				p=0.2		p=0.21
R+R+						
R+R-						
R-R-						
R+R+/R+R-						
R-R-						
ε2ε2						
ε2ε3						
ε2ε4						
ε3ε3						
ε3ε4						
ε4ε4						
ε2ε2/ε2ε3						
ε3ε3						
ε3ε4/ε4ε4						
P+P+						
P+P-						
P-P-						
H+H+						
H+H-						
H-H-						
H+H+						
H+H-/H-H-						
SS						
ST/TT						
B+B+						
B+B-						
B-B-						
B+B+						
B+B-/B-B-						
B+B+/B+B-						
B-B-						

	CETPmass-0hr		CETPmass-6hr		CETPactivity-0hr		CETPactivity-6hr	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
II								
ID								
DD								
II								
ID/DD								
II/ID								
DD								
R+R+								
R+R-								
R-R-								
R+R+/R+R-								
R-R-								
ε2ε2								
ε2ε3								
ε2ε4								
ε3ε3								
ε3ε4								
ε4ε4								
ε2ε2/ε2ε3			3	1.51 (1.14-2.0)	3	110 (107-113)	3	175.4 (161-191)
ε3ε3			37	2.23 (2.1-2.4)	37	153 (148-158)	37	180.7 (171-190)
ε3ε4/ε4ε4			22	2.12 (1.9-2.3)	22	143.6 (135-152)	22	196.3 (182-212)
				p=0.284		p=0.078		p=0.616
P+P+								
P+P-								
P-P-								
H+H+								
H+H-								
H-H-								
H+H+								
H+H-/H-H-								
SS								
ST/TT								
B+B+	23	2.0 (1.74-2.29)	23	2.23 (1.87-2.66)	23	152 (134-171)	23	189 (166-216)
B+B-	34	2.03 (1.85-2.24)	34	2.2 (1.96-2.47)	34	148 (139-156)	34	183 (163-205)
B-B-	6	1.22 (0.85-1.74)	6	1.48 (0.93-2.34)	6	128 (101-160)	6	180 (141-231)
		p=0.002		p=0.074		p=0.327		p=0.904
B+B+								
B+B-/B-B-								
B+B+/B+B-	57	2.02 (1.87-2.18)	57	2.21 (2.0-2.44)				
B-B-	6	1.22 (0.85-1.74)	6	1.48 (0.93-2.34)				
		p=0.041		p=0.15				

Appendix II

Two-way-ANOVA with interactions for the ratio of cellular TAG to basolateral TAG (cTAG/bTAG). The least significance difference test was calculated to identify the differences between the fatty acid treatments. The '*' marks the treatments were supplementation of Caco-2 cells with fatty acid resulted in a difference in how the cells dealt with an acute fatty acid load. The chronic treatment is the number to the left of the comma and acute treatment in the number to the right of the comma. Chronic treatments are, 1 = media, 2 = EPA, 3 = OA and 4 = PA. Acute treatments are, 1 = BSA, 2 = EPA, 3 = OA and 4 = PA.

cTAG/bTAG

General Results

Analysis of Variance For cTAG/bTAG
No Selector
127 total cases of which 64 are missing

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	6019.2	6019.2	428.35 [2]	0.0001
Act	3	606.066	202.022	14.377 [2]	0.0001
Chc	3	36.3094	12.1031	0.86131	0.4678
Act*Chc	9	306.705	34.0783	2.4252	0.0236
Error	47	660.443	14.052		
Total	62	1581.89			

Expected Cell Means of: cTAG/bTAG on Act*Chc

Level of Act*Chc	Expected Cell Mean	Cell Count
1,1	16.99	3
1,2	14.21	4
1,3	3.683	4
1,4	8.878	4
2,1	8.293	4
2,2	7.538	4
2,3	5.08	4
2,4	14.25	4
3,1	12.01	4
3,2	11.12	4
3,3	4.385	4
3,4	12.7	4
4,1	11.76	4
4,2	7.585	4
4,3	5.905	4
4,4	13.83	4

LSD Post Hoc Tests

	Difference	std. err.	Prob
1,2 - 1,1	-2.78833	2.863	0.335088
1,3 - 1,1	-13.3108	2.863	0.0000272456
1,3 - 1,2	-10.5225	2.651	0.000245007
1,4 - 1,1	-8.11583	2.863	0.00674149
1,4 - 1,2	-5.3275	2.651	0.0502039
1,4 - 1,3	5.195	2.651	0.0559529
2,1 - 1,1	-8.70083	2.863	0.00386946*
2,1 - 1,2	-5.9125	2.651	0.0305205
2,1 - 1,3	4.61	2.651	0.0885476
2,1 - 1,4	-0.585	2.651	0.826282
2,2 - 1,1	-9.45583	2.863	0.00183537
2,2 - 1,2	-6.6675	2.651	0.0153661*
2,2 - 1,3	3.855	2.651	0.152494
2,2 - 1,4	-1.34	2.651	0.615546
2,2 - 2,1	-0.755	2.651	0.777022

	Difference	std. err.	Prob
2,3 - 1,1	-11.9133	2.863	0.000133682
2,3 - 1,2	-9.125	2.651	0.00122064
2,3 - 1,3	1.3975	2.651	0.600515
2,3 - 1,4	-3.7975	2.651	0.158572
2,3 - 2,1	-3.2125	2.651	0.231584
2,3 - 2,2	-2.4575	2.651	0.358597
2,4 - 1,1	-2.74833	2.863	0.342
2,4 - 1,2	0.04	2.651	0.988024
2,4 - 1,3	10.5625	2.651	0.00023367
2,4 - 1,4	5.3675	2.651	0.0485719*
2,4 - 2,1	5.9525	2.651	0.0294667
2,4 - 2,2	6.7075	2.651	0.014798
2,4 - 2,3	9.165	2.651	0.00116751
3,1 - 1,1	-4.98833	2.863	0.0879929
3,1 - 1,2	-2.2	2.651	0.410745
3,1 - 1,3	8.3225	2.651	0.00292084
3,1 - 1,4	3.1275	2.651	0.243979
3,1 - 2,1	3.7125	2.651	0.167903
3,1 - 2,2	4.4675	2.651	0.0985347
3,1 - 2,3	6.925	2.651	0.0120297
3,1 - 2,4	-2.24	2.651	0.402351
3,2 - 1,1	-5.87083	2.863	0.0459092
3,2 - 1,2	-3.0825	2.651	0.250733
3,2 - 1,3	7.44	2.651	0.00725941
3,2 - 1,4	2.245	2.651	0.40131
3,2 - 2,1	2.83	2.651	0.291127
3,2 - 2,2	3.585	2.651	0.182689
3,2 - 2,3	6.0425	2.651	0.0272128
3,2 - 2,4	-3.1225	2.651	0.244723
3,2 - 3,1	-0.8825	2.651	0.740663
3,3 - 1,1	-12.6083	2.863	0.0000610579
3,3 - 1,2	-9.82	2.651	0.000556189
3,3 - 1,3	0.7025	2.651	0.792146
3,3 - 1,4	-4.4925	2.651	0.0967183
3,3 - 2,1	-3.9075	2.651	0.147105
3,3 - 2,2	-3.1525	2.651	0.240285
3,3 - 2,3	-0.695	2.651	0.794314
3,3 - 2,4	-9.86	2.651	0.00053117
3,3 - 3,1	-7.62	2.651	0.00605614
3,3 - 3,2	-6.7375	2.651	0.0143844
3,4 - 1,1	-4.2908	2.863	0.140639
3,4 - 1,2	-1.5025	2.651	0.57352
3,4 - 1,3	9.02	2.651	0.00137131
3,4 - 1,4	3.825	2.651	0.155642
3,4 - 2,1	4.41	2.651	0.102819
3,4 - 2,2	5.165	2.651	0.0573305
3,4 - 2,3	7.6225	2.651	0.00604081
3,4 - 2,4	-1.5425	2.651	0.563394
3,4 - 3,1	0.6975	2.651	0.793591
3,4 - 3,2	1.58	2.651	0.553983
3,4 - 3,3	8.3175	2.651	0.00293638
4,1 - 1,1	-5.23833	2.863	0.0736505
4,1 - 1,2	-2.45	2.651	0.360052
4,1 - 1,3	8.0725	2.651	0.00380098
4,1 - 1,4	2.8775	2.651	0.283202
4,1 - 2,1	3.4625	2.651	0.197814
4,1 - 2,2	4.2175	2.651	0.118288
4,1 - 2,3	6.675	2.651	0.0152581
4,1 - 2,4	-2.49	2.651	0.352334

	Difference	std. err.	Prob
4,1 - 3,1	-0.25	2.651	0.925259
4,1 - 3,2	0.6325	2.651	0.812438
4,1 - 3,3	7.37	2.651	0.00778447
4,1 - 3,4	-0.9475	2.651	0.722349
4,2 - 1,1	-9.40833	2.863	0.00192532
4,2 - 1,2	-6.62	2.651	0.0160665*
4,2 - 1,3	3.9025	2.651	0.147611
4,2 - 1,4	-1.2925	2.651	0.62809
4,2 - 2,1	-0.7075	2.651	0.790702
4,2 - 2,2	0.0475	2.651	0.985778
4,2 - 2,3	2.505	2.651	0.349467
4,2 - 2,4	-6.66	2.651	0.0154748
4,2 - 3,1	-4.42	2.651	0.102063
4,2 - 3,2	-3.5375	2.651	0.188445
4,2 - 3,3	3.2	2.651	0.233377
4,2 - 3,4	-5.1175	2.651	0.0595712
4,2 - 4,1	-4.17	2.651	0.122382
4,3 - 1,1	-11.0883	2.863	0.000331493
4,3 - 1,2	-8.3	2.651	0.00299141
4,3 - 1,3	2.2225	2.651	0.40601
4,3 - 1,4	-2.9725	2.651	0.267806
4,3 - 2,1	-2.3875	2.651	0.37233
4,3 - 2,2	-1.6325	2.651	0.540942
4,3 - 2,3	0.825	2.651	0.756992
4,3 - 2,4	-8.34	2.651	0.00286703
4,3 - 3,1	-6.1	2.651	0.0258545
4,3 - 3,2	-5.2175	2.651	0.0549385
4,3 - 3,3	1.52	2.651	0.569079
4,3 - 3,4	-6.7975	2.651	0.0135888
4,3 - 4,1	-5.85	2.651	0.0322341
4,3 - 4,2	-1.68	2.651	0.529281
4,4 - 1,1	-3.16833	2.863	0.274084
4,4 - 1,2	-0.38	2.651	0.886618
4,4 - 1,3	10.1425	2.651	0.000382878
4,4 - 1,4	4.9475	2.651	0.0682147
4,4 - 2,1	5.5325	2.651	0.0423168
4,4 - 2,2	6.2875	2.651	0.0218359
4,4 - 2,3	8.745	2.651	0.00185426
4,4 - 2,4	-0.42	2.651	0.87478
4,4 - 3,1	1.82	2.651	0.495695
4,4 - 3,2	2.7025	2.651	0.313158
4,4 - 3,3	9.44	2.651	0.000857623
4,4 - 3,4	1.1225	2.651	0.673877
4,4 - 4,1	2.07	2.651	0.438755
4,4 - 4,2	6.24	2.651	0.0227971
4,4 - 4,3	7.92	2.651	0.0044542

Appendix III

Two-way-ANOVA with interactions for the ratio of cellular TAG to basolateral TAG (cTAG/bTAG). The least significance difference test was calculated to identify the differences between the fatty acid treatments. The '*' marks the treatments were supplementation of Caco-2 cells with fatty acid resulted in a difference in how the cells dealt with an acute fatty acid load. The chronic treatment is the number to the left of the comma and acute treatment in the number to the right of the comma. Chronic treatments are, 1 = media, 5 = LA, 6 = t-10 and 7 = c-9. Acute treatments are, 1 = BSA, 3 = OA and 4 = PA.

cTAG/bTAG

General Results

Analysis of Variance For cTAG/bTAG
No Selector
120 total cases of which 66 are missing

Source	df	Sums of Squares	Mean Square	F-ratio		Prob
Const	1	6300.29	6300.29	213.12	²	0.0001
Act	2	1123.46	561.73	19.002	²	0.0001
Chc	3	98.8045	32.9348	1.1141		0.3542
Act*Chc	6	513.579	85.5965	2.8955		0.0188
Error	42	1241.61	29.5622			
Total	53	2931.99				

Expected Cell Means of: cTAG/bTAG on Act*Chc

Level of Act*Chc	Expected Cell Mean	Cell Count
1,1	16.99	3
1,3	3.683	4
1,4	8.877	4
5,1	11.24	5
5,3	5.892	5
5,4	12.25	5
6,1	11.27	5
6,3	4.05	5
6,4	23.75	4
7,1	12.15	4
7,3	5.894	5
7,4	17.1	5

LSD Post Hoc Tests

	Difference	std. err.	Prob
1,3 - 1,1	-13.3108	4.153	0.00257799
1,4 - 1,1	-8.11583	4.153	0.0573368
1,4 - 1,3	5.195	3.845	0.183853
5,1 - 1,1	-5.75733	3.971	0.154499
5,1 - 1,3	7.5535	3.647	0.0445433
5,1 - 1,4	2.3585	3.647	0.521385
5,3 - 1,1	-11.1013	3.971	0.00777386
5,3 - 1,3	2.2095	3.647	0.547917
5,3 - 1,4	-2.9855	3.647	0.417666
5,3 - 5,1	-5.344	3.439	0.127674
5,4 - 1,1	-4.74133	3.971	0.239148
5,4 - 1,3	8.5695	3.647	0.0235738
5,4 - 1,4	3.3745	3.647	0.360149
5,4 - 5,1	1.016	3.439	0.7691
5,4 - 5,3	6.36	3.439	0.0714283
6,1 - 1,1	-5.72733	3.971	0.156607
6,1 - 1,3	7.5835	3.647	0.0437431
6,1 - 1,4	2.3885	3.647	0.516126
6,1 - 5,1	0.03	3.439	0.993081

	Difference	std. err.	Prob
6,1 - 5,3	5.374	3.439	0.125608
6,1 - 5,4	-0.986	3.439	0.775726
6,3 - 1,1	-12.9433	3.971	0.00221472
6,3 - 1,3	0.3675	3.647	0.920221
6,3 - 1,4	-4.8275	3.647	0.192805
6,3 - 5,1	-7.186	3.439	0.0427372
6,3 - 5,3	-1.842	3.439	0.595017
6,3 - 5,4	-8.202	3.439	0.0216596
6,3 - 6,1	-7.216	3.439	0.0419191
6,4 - 1,1	6.75917	4.153	0.111075
6,4 - 1,3	20.07	3.845	0.00000519254
6,4 - 1,4	14.875	3.845	0.000374381*
6,4 - 5,1	12.5165	3.647	0.00135893
6,4 - 5,3	17.8605	3.647	0.0000148691
6,4 - 5,4	11.5005	3.647	0.00297993*
6,4 - 6,1	12.4865	3.647	0.0013914
6,4 - 6,3	19.7025	3.647	0.00000286268
7,1 - 1,1	-4.84083	4.153	0.250306
7,1 - 1,3	8.47	3.845	0.033128
7,1 - 1,4	3.275	3.845	0.399134
7,1 - 5,1	0.9165	3.647	0.802823
7,1 - 5,3	6.2605	3.647	0.0934454
7,1 - 5,4	-0.0995	3.647	0.978365
7,1 - 6,1	0.8865	3.647	0.809146
7,1 - 6,3	8.1025	3.647	0.0317625
7,1 - 6,4	-11.6	3.845	0.00432083
7,3 - 1,1	-11.0993	3.971	0.00778405
7,3 - 1,3	2.2115	3.647	0.547556
7,3 - 1,4	-2.9835	3.647	0.417976
7,3 - 5,1	-5.342	3.439	0.127812
7,3 - 5,3	0.002	3.439	0.999539
7,3 - 5,4	-6.358	3.439	0.071514
7,3 - 6,1	-5.372	3.439	0.125744
7,3 - 6,3	1.844	3.439	0.594619
7,3 - 6,4	-17.8585	3.647	0.0000148955
7,3 - 7,1	-6.2585	3.647	0.0935467
7,4 - 1,1	0.104667	3.971	0.979095
7,4 - 1,3	13.4155	3.647	0.00066224
7,4 - 1,4	8.2205	3.647	0.029484*
7,4 - 5,1	5.862	3.439	0.0956386
7,4 - 5,3	11.206	3.439	0.00222061
7,4 - 5,4	4.846	3.439	0.166126
7,4 - 6,1	5.832	3.439	0.0972924
7,4 - 6,3	13.048	3.439	0.000468526
7,4 - 6,4	-6.6545	3.647	0.0751976
7,4 - 7,1	4.9455	3.647	0.182369
7,4 - 7,3	11.204	3.439	0.00222424

Appendix IV

Two-way-ANOVA with interactions for the ratio of cellular TAG to cellular PL (cTAG/cPL). The least significance difference test was calculated to identify the differences between the fatty acid treatments. The '*' marks the treatments were supplementation of Caco-2 cells with fatty acid resulted in a difference in how the cells dealt with an acute fatty acid load. The chronic treatment is the number to the left of the comma and acute treatment in the number to the right of the comma. Chronic treatments are, 1 = media, 5 = LA, 6 = t-10 and 7 = c-9. Acute treatments are, 1 = BSA, 3 = OA and 4 = PA.

cTAG/cPL

General Results

Analysis of Variance For cTAG/cPL
No Selector
120 total cases of which 9 are missing

Source	df	Sums of Squares	Mean Square	F-ratio		Prob
Const	1	81.529	81.529	865.6	²	0.0001
Act	2	9.41752	4.70876	107.75	²	0.0001
Chc	3	1.18158	0.39386	9.0124	²	0.0001
Act*Chc	6	1.31437	0.219062	5.0126		0.0002
Error	99	4.3265	0.043702			
Total	110	16.4569				

Expected Cell Means of: cTAG/cPL on Act*Chc

Level of Act*Chc	Expected Cell Mean	Cell Count
1,1	0.4014	7
1,3	0.5637	8
1,4	1.099	8
5,1	0.466	10
5,3	0.927	10
5,4	1.028	10
6,1	0.435	10
6,3	1.262	9
6,4	1.198	10
7,1	0.4878	9
7,3	0.988	10
7,4	1.285	10

LSD Post Hoc Tests

	Difference	std. err.	Prob
1,3 - 1,1	0.162321	0.1082	0.136723
1,4 - 1,1	0.697321	0.1082	0.00000000422575
1,4 - 1,3	0.535	0.1045	0.00000151569
5,1 - 1,1	0.0645714	0.103	0.532247
5,1 - 1,3	-0.09775	0.09916	0.32665
5,1 - 1,4	-0.63275	0.09916	0.00000000568725
5,3 - 1,1	0.525571	0.103	0.00000162608
5,3 - 1,3	0.36325	0.09916	0.000402261*
5,3 - 1,4	-0.17175	0.09916	0.0863833
5,3 - 5,1	0.461	0.09349	0.00000329798
5,4 - 1,1	0.626571	0.103	0.0000000223966
5,4 - 1,3	0.46425	0.09916	0.00000905077
5,4 - 1,4	-0.07075	0.09916	0.477225
5,4 - 5,1	0.562	0.09349	0.0000000308403
5,4 - 5,3	0.101	0.09349	0.282621
6,1 - 1,1	0.0335714	0.103	0.745211
6,1 - 1,3	-0.12875	0.09916	0.19717
6,1 - 1,4	-0.66375	0.09916	0.00000000132241

	Difference	std. err.	Prob
6,1 - 5,1	-0.031	0.09349	0.740903
6,1 - 5,3	-0.492	0.09349	0.000000824824
6,1 - 5,4	-0.593	0.09349	0.00000000678211
6,3 - 1,1	0.860794	0.1054	1.03283e-12
6,3 - 1,3	0.698472	0.1016	5.58387e-10*
6,3 - 1,4	0.163472	0.1016	0.110737
6,3 - 5,1	0.796222	0.09605	5.73243e-13
6,3 - 5,3	0.335222	0.09605	0.000722729*
6,3 - 5,4	0.234222	0.09605	0.0165303
6,3 - 6,1	0.827222	0.09605	1.15095e-13
6,4 - 1,1	0.796571	0.103	8.95498e-12
6,4 - 1,3	0.63425	0.09916	0.00000000530274
6,4 - 1,4	0.09925	0.09916	0.319318
6,4 - 5,1	0.732	0.09349	5.54915e-12
6,4 - 5,3	0.271	0.09349	0.00461483
6,4 - 5,4	0.17	0.09349	0.0720308
6,4 - 6,1	0.763	0.09349	1.082e-12
6,4 - 6,3	-0.0642222	0.09605	0.505294
7,1 - 1,1	0.0863492	0.1054	0.414396
7,1 - 1,3	-0.0759722	0.1016	0.456291
7,1 - 1,4	-0.610972	0.1016	0.0000000303771
7,1 - 5,1	0.0217778	0.09605	0.821102
7,1 - 5,3	-0.439222	0.09605	0.0000139455
7,1 - 5,4	-0.540222	0.09605	0.00000017292
7,1 - 6,1	0.0527778	0.09605	0.58392
7,1 - 6,3	-0.774444	0.09855	4.81456e-12
7,1 - 6,4	-0.710222	0.09605	4.64814e-11
7,3 - 1,1	0.586571	0.103	0.000000127401
7,3 - 1,3	0.42425	0.09916	0.0000434976*
7,3 - 1,4	-0.11075	0.09916	0.266754
7,3 - 5,1	0.522	0.09349	0.000000206748
7,3 - 5,3	0.061	0.09349	0.515607
7,3 - 5,4	-0.04	0.09349	0.669688
7,3 - 6,1	0.553	0.09349	0.0000000475728
7,3 - 6,3	-0.274222	0.09605	0.00524407*
7,3 - 6,4	-0.21	0.09349	0.0269115
7,3 - 7,1	0.500222	0.09605	0.00000104027
7,4 - 1,1	0.883571	0.103	1.37469e-13
7,4 - 1,3	0.72125	0.09916	8.32991e-11
7,4 - 1,4	0.18625	0.09916	0.0632892
7,4 - 5,1	0.819	0.09349	5.49667e-14
7,4 - 5,3	0.358	0.09349	0.000225471
7,4 - 5,4	0.257	0.09349	0.00710686*
7,4 - 6,1	0.85	0.09349	1.04559e-14
7,4 - 6,3	0.0227778	0.09605	0.813038
7,4 - 6,4	0.087	0.09349	0.354335
7,4 - 7,1	0.797222	0.09605	5.44384e-13
7,4 - 7,3	0.297	0.09349	0.00198629