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Dietary Fatty Acids and Diabetes Mellitus: effect on postprandial lipoproteins and apolipoproteins

by Claire Madigan B. Sc (Hons), M. Sc

Ph. D. thesis

Submitted to the faculty of Clinical Medicine, Trinity College, University of Dublin

Direction and Supervision
Professor Gerald Tomkin MD., F.R.C.P.I., F.R.C.P., F.A.C.P.

September 2001
Declaration

This thesis is submitted by the undersigned to the University of Dublin for examination for the degree of Doctor of Philosophy. The work herein is entirely my own and has not been submitted as an exercise for a degree to any other university. The library of Trinity College Dublin has my permission to lend or copy this thesis upon request.

Signed: Claire Madigan

Claire Madigan
This thesis explores the relative merits of dietary polyunsaturated and monounsaturated fat in reducing the postprandial dyslipidaemia which is associated with diabetes. Of particular interest are the apolipoproteins which control lipoprotein metabolism - apo B, apo E, apo C and apo A. The effect of diabetes on the production of intestinally-derived chylomicrons is also investigated. An increased risk of atherosclerosis exists in diabetes even in the absence of the classical lipid abnormalities of raised plasma triglycerides and reduced HDL cholesterol and increasing evidence shows that an elevation of postprandial particles may have an important role to play in this elevated risk. Diet is a cornerstone in the treatment of type 2 diabetes and this study attempts to establish whether the usually prescribed polyunsaturated diet is in fact prudent.

In a randomised, crossover study two diets high in either mono- or polyunsaturated fat using olive oil or sunflower oil were compared. Adherence of subjects to the assigned diet was ensured by measuring the oleic and linoleic acid content of subjects' LDL using Gas Chromatography. To assess the effect of diet on the postprandial response, a high fat test meal was given at the end of each dietary period, before switching subjects to the second diet. Plasma analysis showed that the oleic acid diet improved insulin resistance and reduced LDL cholesterol levels compared to the linoleic acid diet. Fasting and postprandial lipoproteins were isolated by ultracentrifugation. Apo B48 and apo B100 were measured by SDS-PAGE and densitometric scanning, reflecting the relative number of intestinally- and hepatically-derived particles respectively. The oleic acid diet normalised, to varying degrees, levels of both particles in the diabetic subjects.

In an attempt to identify a mechanism for this improvement, apo E, CII and CIII were measured by SDS-PAGE and isoelectric focusing (IEF) These are central to particle catabolism and clearance. The linoleic acid diet produced apo E deficient particles relative to non-diabetic subjects. This was normalised again by the oleic acid diet. The effect on apo C was more complex, but in general the smaller, more numerous linoleic acid particles were deficient in apo C compared to the non-diabetic subjects. The oleic acid diet
normalised this. The ratio of apo CII/E was higher in the VLDL fraction. Discounting an abnormal apo E genotype distribution (determined by PCR and restriction endonuclease fragment sizing) these results were indicative of reduced particle clearance in the linoleic acid diet due to either secretion of abnormal lipoproteins by the intestine or liver or reduced transfer of the apolipoproteins from HDL during the postprandial phase.

To investigate this, HDL apo E and C were measured by SDS-PAGE and IEF. The linoleic acid diet produced apo E deficient HDL which transferred less postprandially. The oleic acid diet normalised this. There was no change in apo C. Neither was there a difference in either apo AI or apo AI - apolipoproteins which indicate the relative atheroprotective nature of HDL particles. Enzymes central to reverse cholesterol transport, CETP and LCAT, were not altered between the diets. However fasting and postprandial HDL cholesterol was lowered by the linoleic diet - an independent risk factor for coronary heart disease. The oleic acid diet returned levels to those of the non-diabetic subjects.

The effect of diabetes on the intestinal production of chylomicron particles was investigated by isolating lymph chylomicrons from alloxan-diabetic and non-diabetic, cholesterol-fed rabbits - a model of TRL dyslipidaemia. Apo E was found to be deficient on diabetic lymph particles although the absolute amount in the fractions was not different. This may indicate that diabetes does not effect apo E secretion from the intestine but it does effect the apo E content on particles. When transported to the plasma compartment, apo E remains deficient on diabetic particles.

In conclusion, the production of intestinally-derived particles is abnormal in diabetes and a deficiency in apo E may reduce the rate of particle clearance. A diet rich in oleic acid normalised apo E and apo C levels in both the TRL and HDL. The oleic acid diet improved glycaemic control and reduced insulin resistance. Plasma LDL was lowered and fasting and postprandial HDL cholesterol increased relative to a linoleic acid rich diet. This evidence may suggest that monounsaturated fat may be more prudent in the diet of type 2 diabetic patients.
Dedicated To Mother and Pop
and to Ronan
It cannot be that axioms established by argumentation

can suffice for the discovery of new works,

since the subtlety of nature is greater many times over

than the subtlety of argument.

Francis Bacon
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I am particularly grateful to the patients and college staff who dealt so well with their daily oil and for giving selflessly for this study. I am also grateful to the laboratory personnel who suffered the fatty breakfast...4 days in a row! I did indeed feel your pain.

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PUBLICATIONS AND PRESENTTIONS

Published


Under review


Published Abstracts


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**Poster Presentations**


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<th>Definition</th>
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<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-coenzyme A: cholesterol acyltransferase</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AOC</td>
<td>area over curve</td>
</tr>
<tr>
<td>ATP</td>
<td>adenocine triphosphate</td>
</tr>
<tr>
<td>AUCf</td>
<td>area under curve from fasting</td>
</tr>
<tr>
<td>AUCz</td>
<td>area under curve from zero</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CERP</td>
<td>cholesterol-efflux regulatory protein</td>
</tr>
<tr>
<td>CET</td>
<td>cholesteryl ester transfer</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CLAS</td>
<td>Cholesterol Lowering Atherosclerosis Study</td>
</tr>
<tr>
<td>COX2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>copper sulphate</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>dl</td>
<td>decilitre</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl ester</td>
</tr>
<tr>
<td>FIS</td>
<td>flame ionisation detector</td>
</tr>
</tbody>
</table>
g  gram
G  guanine
GC  gas chromatography
HDL  high density lipoprotein
HL  hepatic lipase
HLA  human leukocyte associated antigens
HSPG  heparin sulphate proteoglycan
HMG-CoA  3-hydroxy 3-methylglutaryl coenzyme A
IDDM  insulin-dependent diabetes mellitus
IDL  intermediate density lipoprotein
IEF  isoelectric focusing
IHD  ischemic heart disease
kb  kilobase
kcal  kilocalorie
kDa  kiloDalton
l  litre
LCAT  lecithin-cholesterol acyltransferase
LDL  low density lipoprotein
LDLR  low density lipoprotein receptor
LPL  lipoprotein lipase
Lp (a)  lipoprotein (a)
LRP  low density lipoprotein receptor-related protein
LSR  lipolysis-stimulated receptor
M  molar
m  milli
μ  micro
mA  milliampers
MgCl  magnesium chloride
min  minute
mmol  millimole
MODY  maturity-onset diabetes of the young
mRNA  messenger ribonucleic acid
MTP  microsomal triglyceride transfer protein
NIDDM  non-insulin-dependent diabetes mellitus
nm  nonometre
NaOH  sodium hydroxide
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDGF  platelet-derived growth factor
PMSF  phenyl methyl sulphonyl fluoride
PON  paraoxonase
PPACK  D-phenylalanyl-L-prolyl-L-arginine
PPAR  peroxisome proliferator-activated receptor
PPRE  peroxisome proliferator response element
rpm  revolutions per minute
RXR  retinoic acid X receptor
SD  standard deviation
SDS-PAGE  Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM  standard error of mean
Sf  Swedberg floatation unit
SR-BI  scavenger receptor-BI
T  thymine
Taq  *Thermus aquaticus*
TNF  tumor necrosis factor
TRL  triglyceride rich lipoprotein
VCAM  vascular cell adhesion molecule
V  volts
VLDL  very low density lipoprotein
W  Watts
WHO  World Health Organisation
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4.2.2 Lipoprotein Isolation and Analysis

4.3 RESULTS
4.3.1 Apo E in the Chylomicron Fraction
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4.4 DISCUSSION

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5.1 INTRODUCTION

5.2 METHODS
5.2.1 Subjects and Study Design
5.2.2 Lipoprotein Isolation and Analysis

5.3 RESULTS
5.3.1 HDL Apo A
5.3.2 Postprandial HDL Cholesterol and Triglyceride
5.3.3 HDL Apo E
5.3.4 HDL Apo C
5.3.5 CETP and LCAT Activity

5.4 DISCUSSION
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6.2 METHODS

6.3 RESULTS
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6.4 DISCUSSION

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Introduction
Chapter 1

1.1 Diabetes Mellitus.

In 2001 diabetes mellitus affects almost 16 million Americans. It has become a major public health problem and the number one cause of adult blindness, end-stage renal disease and non-traumatic amputations. It markedly increases the risk for cardiovascular, cerebrovascular and peripheral disease. The resultant increased morbidity and mortality produces a cost from diabetes of almost $100 billion annually in the United States alone (Kazi and Blonde, 2001). The World Health Organisation (WHO) predicts that between 1995 and 2025, the worldwide prevalence of diabetes among persons aged 20 years and older will increase from 4.0% to 5.4% (de Vegt et al., 2001).

1.1.1 Definition.

Diabetes mellitus is a syndrome caused by the lack of and/or diminished effectiveness of endogenous insulin. It is characterised by hyperglycaemia both in the fasting and postprandial states. The American Diabetes Association (ADA) propose that diagnosis be made on the basis of a simple fasting blood glucose determination rather than the oral glucose tolerance test (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). The WHO has also adopted this approach (Alberti and Zimmer, 1998). Both propose diagnostic cut-off values of ≥7.0mmol/l for a fasting sample and a random plasma glucose of ≥11.1mmol/l.

1.1.2 History.

Aretaios, living between 81 and 138 AD, is popularly attributed with the first comprehensive description of symptoms of diabetes mellitus - "intolerable thirst, burning in the intestines, passage of large amounts of urine". He even went on to classify the disease in two stages - chronic and acutely fatal. But there is evidence suggesting that the early Egyptians suffered from diabetes. Reference to a condition with 'over abundant urine' was made as early as 1550 BC. The evidence lies in a volume of script called the Ebers papyrus which was uncovered in an Egyptian tomb. Perhaps the cause of the untimely death of a fabulously powerful Pharaoh? Later, 200 years BC, diabetes - a derivation of the
Greek word *diabeinein* (to go to excess) was used, reputedly by Demetrios of Apamaia. The next major leap in the characterisation of the disease was the discovery by Thomas Willis in 1674 that the urine of these patients was sweet describing a honey-like flavour. The word *mellitus*, as a result, was added in the 18th century by William Cullen to describe this form of diabetes.

The next discovery came in 1889 when von Mering and Minkowski reported severe diabetes in dogs which had undergone a complete pancreatectomy. Of course, 20 years earlier Paul Langerhans had described the anatomy and histology of pancreatic islet cells and this lead them to believe that the pancreas was secreting a substance which was essential in glucose metabolism. This hypothetical substance was later named insulin and in 1921 was isolated finally by Banting and Best and purified by John MacCleod. It was in January of the next year that Frederick Banting made the brave decision to treat a critically ill young diabetic patient with insulin. The result, happily, was a considerable improvement in the patient's condition and the Nobel prize for Medicine and Physiology being awarded to Banting and MacCleod.

Insulin was the first protein to be sequenced by the method of Frederick Sanger for which he was awarded the Nobel prize in 1960. A radioimmunoassay was developed by Berson and Yalow who demonstrated that serum insulin was raised in more than 80% of diabetic patients. This was the origin of the classification of the disease into insulin-dependent (Type 1) and non-insulin dependent (Type 2) diabetes mellitus.

### 1.1.3 Classification of Diabetes

Previously diabetes was classified as either primary or secondary, the later being due to destruction or removal of the pancreas, excessive concentrations of drugs (e.g. thiazide and loop diuretics) or hormones (e.g. glucagon, cortisol, adrenaline and growth hormone) which have insulin-antagonistic effects. Diabetes may also be associated with a number of complex genetic syndromes such as Freidrich's Ataxia. The classification of primary diabetes was made based on the extent to which the patient was dependent on insulin.
Insulin-dependent diabetes mellitus (IDDM), formally juvenile-onset diabetes, by definition required treatment with insulin for the short-term survival of the patient. In non-insulin-dependent diabetes mellitus (NIDDM), originally called maturity- or adult-onset diabetes, hyperglycaemia and ketosis can be tolerated for longer periods without the same risk of ketoacidosis or mortality. Gestational diabetes is usually a transient condition which becomes apparent during the second trimester of pregnancy. It is associated with an increase in perinatal mortality and if untreated infant mortality is 7% (O’Sullivan et al., 1973). Glycaemic control usually returns to normal after pregnancy.

Both the WHO (Alberti and Zimmer, 1998) and the American Diabetes Association (ADA) (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997) reported recommendations for the alteration of this classification which have subsequently been adopted by the ADA. The new classification is based on the pathogenesis of the disease rather than insulin dependence and each type is described below.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Includes immune-mediated and idiopathic forms of beta cell dysfunction which leads to absolute insulin deficiency</td>
</tr>
<tr>
<td>Type 2</td>
<td>Of adult onset, which may originate from insulin resistance and relative insulin deficiency or from a secretory defect</td>
</tr>
<tr>
<td>Type 3</td>
<td>Covers a wide range of specific types of diabetes including the various genetic defects in insulin action and diseases of the exocrine pancreas</td>
</tr>
<tr>
<td>Type 4</td>
<td>Gestational diabetes</td>
</tr>
</tbody>
</table>

It was also recommended that the diagnosis of diabetes should be established by a repeated raised fasting glucose on a subsequent day. A change was also proposed in the diagnostic
cut off point for fasting blood glucose concentration, reducing it from ≥7.8 to ≥7.0 mmol/l, with the random blood glucose cut-off concentration of ≥11.1 mmol/l remaining. For epidemiological purposes the American Diabetes Association criterion used is a fasting glucose concentration of ≥7.0 mmol/l, in contrast with the previous WHO criterion of a 2 hour glucose concentration of ≥11.0 mmol/l. It has been suggested that this change in diagnostic strategy will greatly increase the number of previously undiagnosed patients in Europe (DECODE study group on behalf of the European Diabetes Epidemiology Study Group, 1998).

1.1.4 Type 1 Diabetes Mellitus.

Otherwise classified as insulin-dependent, type 1 diabetes mellitus occurs under the age of thirty and most frequently in children between the age of 12 and 15 years. The clinical symptoms tend to appear abruptly. Patients presenting with the condition display symptoms of hyperglycaemia, thirst, polyuria and polydipsia. They complain of constant thirst and tiredness and are often suffering from weight loss. Between 5 and 10% of newly presenting patients may be ketoacidotic denoted usually by nausea, vomiting and drowsiness. Without an exogenous supply of insulin these patients would die. The overall mortality in type 1 diabetes is 4 to 7 fold higher than in the matched non-diabetic population (Stephenson et al., 1992). The main causes of premature death are renal failure and coronary heart disease (CHD).

Type 1 diabetes may be auto immune in nature, caused by the destruction of the insulin-secreting islets cells of the pancreas (Bottazzo et al., 1985) meaning that these patients are completely dependant on exogenous insulin for survival. Although the acute symptom of imbalance in glucose homeostasis occurs very suddenly, it is the final stage in a long silent process that has been destroying the pancreatic B cells over the proceeding four or five years (Eisenbarth et al., 1987). Autoantibodies to proinsulin and insulin receptors have been identified. Putative cell autoantigens that may incite immune damage include glutamic acid decarboxylase, islet cell antigen-2 (IA-2) and insulin. Circulating antibodies against these and other antigens are detectable in most newly presenting type 1 patients.
1.1.5 Genetic Factors in Type 1 Diabetes.

Genetic influences result in increased susceptibility of certain individuals to the disease, but are by no means exclusively responsible for the development of type 1 diabetes. A recent study has shown that monozygotic twin siblings of type 1 diabetic parents have a higher risk of progression to diabetes and of expressing islet cell antibodies than dizygotic twin and non-twin siblings (Redondo, 1999). Genetic susceptibility is associated with genes in the major histocompatibility complex region of the genome. Certain class II human leukocyte associated antigens (HLA) on chromosome 6, most notably HLA DR3 or HLA DR4 and the HLA DQ genes linked to these DR genes, are associated with type 1 diabetes (Michaelson and Lernmark, 1987). It is thought that these gene products associated with type 1 diabetes provide antigen presentations which initiate an immune response resulting in the destruction of the insulin-producing cells in the islets of Langerhans. Although 95% of type 1 patients have one or other of these genes, they are not specific for type 1 diabetes since 60% of the normal population also have them (Nerup et al., 1984). This evidence, along with the low concordance rate of less than 50% in twins, suggests that the disease must be, at least partially, non-genetically or environmentally determined.

It is estimated that HLA is responsible for only 60% of the genetic susceptibility to type 1 diabetes. Currently, more than 15 candidate loci have been identified including the insulin gene on chromosome 11 and the cytotoxic T-lymphocyte antigen (CTLA-4) gene on chromosome 2. This gene is critical to the apoptosis of T-cells and it has been suspected that a gene polymorphism involving an AT repeat at the C terminus at the 3' end of the gene effects the stability of the resulting messenger ribonucleic acid (mRNA) and ultimately results in a prolonged survival of killer T-cells (Lenmark, 1999).

1.1.6 Environmental Factors in Type 1 Diabetes.

A number of cytotoxins have been identified as being associated with a higher risk of development of type 1 diabetes. These include the mumps virus, Coxsackie B and Echo virus and the rubella virus. Recently it has been demonstrated that intrauterine exposure to
enterovirus is associated with increased risk of the offspring developing type 1 diabetes (Dahlquist et al., 1995).

1.1.7 Epidemiology.

It is estimated that among the current total number of about 100 million children in Europe aged between 0-14 years of age, approximately 10,000 will develop type 1 diabetes each year (DECODE Study Group, 1998). A major feature of the epidemiology of Nordic countries is the high incidence of the disease, especially in Finland, compared to the rest of Europe (Tuomilehto et al., 1992). Overall there is about a 10 fold difference in incidence throughout Europe, ranging from about 35 new cases in Finland (Tuomilehto et al., 1992) to two or three cases in Macedonia (Kocova et al., 1993) per 100,000 children up to 14 years of age annually. It remains to be established whether the wide variation in the risk of type 1 diabetes within Europe can be attributed to variation in genetic susceptibility, to a differential distribution of environmental factors or a combination of these. The considerable variation between genetically similar populations strongly suggests that environmental factors contribute substantially to the geographical variation.

The epidemiology of type 1 diabetes outside Europe is reviewed by Karvonen et al., (1993). The incidence in North America is comparable to that of the United Kingdom and central Europe, but rates among non-Caucasians are somewhat lower than those of Caucasians (Kostraba et al., 1992). A particularly high incidence has been reported in Prince Edward Island in Canada (Tan et al., 1981). In Australia and New Zealand the general incidence among the population of European ancestry is comparable to that in central Europe (Kelly et al., 1992). No information, as yet, has been published on the incidence amongst the native Aboriginal population of Australia.

1.1.8 Type 2 Diabetes Mellitus.

Type 2 diabetes mellitus is a heterogeneous state involving various degrees of beta cell dysfunction, impaired insulin secretion and insulin resistance. It is usually, but not always, preceded by a period of less severe hyperglycaemia referred to as impaired glucose
tolerance. Type 2 or non-insulin-dependent diabetes, by definition, identifies patients who do not require insulin for survival, but paradoxically about 20% of patients are treated with insulin to achieve and maintain good glycaemic control.

Type 2 accounts for approximately 90% of all cases of diabetes world-wide. The catch phrase - "over 40, over-weight and over-tired" has been used to describe people who may be suffering from or who are at risk of developing the disease. Unlike type 1 diabetes, the symptoms of type 2 tend to be less severe and diagnosis therefore can often be delayed. The peak age of onset is 60 years, with most subjects diagnosed after 40 years of age. Hyperglycaemia is just one of the metabolic disorders associated with the disease for which insulin deficiency or ineffectiveness is the cause. Insulin deficiency causes reduced insulin-mediated uptake from muscle, exaggerated glucose production in the liver and increased free fatty acid mobilisation from adipose tissue. As such there are a number of very serious disturbances in lipid metabolism. So-much-so that over the past number of years type 2 diabetes has come to be described as a disease of lipid abnormalities rather than one of disturbed carbohydrate metabolism. Type 2 diabetes is associated with an elevation in plasma triglyceride and low density lipoprotein (LDL) - cholesterol, along with a lowering of the protective high density lipoprotein (HDL) - cholesterol. Studies have shown clearly that postprandial lipids, in-other-words, the lipids produced after eating, are quite profoundly disturbed in type 2 diabetes (Karpe et al., 1994; Curtin et al., 1996; Tkac et al., 1997; Sakata et al., 1998; Kugiyama et al., 1999; Phillips et al., 2000). These patients have a high risk of developing cardiovascular disease (The Diabetes and Complications Trial Group, 1993; Hadden et al., 1997; UK Prospective Study Group, 1998) and it is this aspect of the disease with which the work of this thesis is concerned.

1.1.9 Genetic Factors in Type 2 Diabetes.

As with type 1 diabetes, there appears to be a significant genetic element in the aetiology of type 2 diabetes. There is virtually a 100% chance of both identical twins of parents with type 2 diabetes developing the disease (Barnett et al., 1981). The search for susceptibility genes and gene defects is complicated by non-Mendelian inheritance of the disease. Normal
insulin sensitive individuals can develop a type 2 diabetic phenotype due to a monogenic defect that impairs pancreatic β-cell function. This subtype of diabetes was originally called Maturity-Onset Diabetes of the Young (MODY) and has an autosomal dominant inheritance. Point mutations have been identified in six specific genes, such as the Glucokinase gene on chromosome 7 and the Insulin Promoter Factor 1 on chromosome 13. However, MODY accounts for <5% of diabetic patients. Type 2 patients with polygenic defects account for approximately 85% of all diabetic patients (Lebovitz, 1999). Studies have detailed gene polymorphisms which are strongly associated with the disease. For example, the frequency of properidin factor B and glyoxylase on chromosome 6 is decreased in Polynesian type 2 patients. The Rhesus blood group has been associated significantly with type 2 diabetes in Mexican-Americans (Stern et al., 1986) and much earlier in Norwegian (Berg et al., 1967) and German type 2 diabetic populations (Scholz et al., 1975). The extent to which any of these genetic defects and associations may cause or impact on the aetiology of the disease is an extremely interesting and hugely complicated area which has yet to be investigated.

1.1.10 Environmental Factors in Type 2 Diabetes.

Environmental factors can influence the clinical expression of both monogenic and polygenic disorders. A good example of this is seen in Pima Indians. Those living in Arizona have a lifestyle which includes a high calorie diet and minimal physical activity. They are quite obese and have an extremely high incidence of type 2 diabetes - 34% in women and 54% in men. A second Pima tribe living in the mountains of northern Mexico lead a highly physically active life and survive on a low calorie diet. Their body weight is close to ideal and their incidence of type 2 diabetes is approximately that of the rest of the Mexican population - 10.5% and 6.3% in women and men respectively (Ravussin et al., 1998)

1.1.11 Insulin Resistance and Obesity.

Goran et al., (2001) described how obesity has reached epidemic proportions in the United States population. The author went on to detail the enormous up-surge in the incidence and
the approaching epidemic in type 2 diabetes and how weight loss was associated with a reduction in the incidence of the disease in overweight men and women. Obesity is present in over 50% of men and 70% of women with type 2 diabetes and predisposes strongly to the disease. Central obesity with visceral fat deposition is independently correlated with insulin resistance and as a result is considered particularly important in inducing insulin resistance. It is associated with glucose intolerance, hypertension, dyslipidaemia and coronary artery disease (CAD), otherwise referred to as the metabolic syndrome or syndrome X. Insulin resistance is an impairment of that function of insulin that causes the normal glucose uptake by muscles and/or restraint in glucose production by the liver.

Normally when insulin secretion is stimulated by glucose ingestion, uptake of glucose by splanchnic (liver and gut) and peripheral (mainly muscle) tissue is promoted and hepatic glucose production is suppressed. Insulin resistance, however, is characterised by hyperinsulinaemia and there is considerable debate as to whether insulin resistance or hyperinsulinaemia comes first in type 2 diabetes. If the primary defect is the desensitisation of tissue by hyperglycaemia itself, then hyperinsulinaemia would be the compensatory response. But if hyperinsulinaemia is the primary defect then insulin resistance will be secondary in an attempt to maintain normal glucose levels. With insulin resistance in the peripheral tissues, plasma glucose rises and the pancreas responds by increasing insulin output. Down regulation of insulin receptors results, further exacerbating the situation.

Reaven, (1988) showed in a series of experiments that not only glucose but also free fatty acid concentrations are associated with insulin resistance. There is an inability to suppress plasma free fatty acids by insulin. It has been proposed that hyperinsulinaemia serves to drive hepatic lipogenesis and very low density lipoprotein (VLDL) secretion (Tobey et al., 1981; Gibbons, 1990) by enhancing the stability of nascent apo B100 (the major solubilising protein of VLDL) and reportedly doubling the mass of microsomal triglyceride transfer protein (MTP) (the enzyme central to the assembly of the particles) in hepatocytes isolated from fructose-fed hamsters - a model of insulin resistance.
(Taghibiglou et al., 2000). This results in an increased flux of triglycerides and free fatty acids to muscle and adipose tissue. As fat accumulates in muscle, insulin-stimulated glucose metabolism at the site becomes compromised. Boden et al., (1994) showed that this happens both at the level of glycogen production and to a lesser extent, in glucose oxidation. Gluconeogenesis is promoted and along with postprandial hyperglycaemia further stimulates insulin secretion fuelling the cycle and progressing glucose intolerance. As a consequence of developing insulin resistance in the adipocyte, re-esterification of free fatty acids becomes impaired and plasma free fatty acids continue to rise. All of these factors compound insulin resistance, further driving hepatic glucose production and placing even further demands on the β-cells. Eventually β-cell failure occurs and glucose intolerance can give way to overt type 2 diabetes.

Apart from specific genetic mutations in the insulin receptor (O’Rahilly et al., 1991) there is very little clear-cut evidence regarding the pathogenesis of insulin resistance and syndrome X. Dietary factors however, seem to play a role. Phospholipids, the major component of the plasma membrane of each cell in the body, can be compositionally altered by diet. The fatty acid components of phospholipids greatly influence the fluidity of the plasma membrane (Field et al., 1988; Tong et al., 1995), embedded in which of course, are all the various receptors and transport proteins, including the insulin receptor and the glucose transporter proteins. Changes in second-messenger function may also play a role (Falholt et al., 1988). Altered lipid composition of plasma membranes in type 2 diabetes have been shown in experimental animals and in humans. In the streptozotocin-diabetic rat platelet linoleic acid is increased and oleic acid is decreased (Dang et al., 1988), with the reverse being observed in adipocytes of diabetic rats (Field et al., 1988). This latter group also showed that feeding a high polyunsaturated fatty acid diet reduced insulin/receptor binding in non-diabetic animals. An increased cholesterol/phospholipid ratio is widely observed (Watala et al., 1989, 1990; Juhan-Vague et al., 1986), due to abnormalities in plasma cholesterol and cellular cholesterol metabolism in diabetes which will be discussed in section 1.6. This is known to reduce the fluidity of the membrane. Tong et al. (1995) showed that membranes of mononuclear leucocytes isolated from type 2
diabetic subjects resisted the normal increase in fluidity in the presence of insulin compared to normal cells. They suggested that this lack of enhanced fluidity prevented the aggregation of insulin receptors and as such reduced insulin sensitivity.

1.2 Atherosclerosis.

Atherosclerosis is the disease of the large arteries which causes heart disease, stroke and peripheral vascular disease, all of which are major causes of mortality in the western world. It involves a complex process of the accumulation of lipids and cells in the extracellular matrix in the intimal lining of the artery (Figure 1.1). It is a chronic, slowly progressive disease, the beginnings of which can be seen, astonishingly, in early childhood (Stary, 1989). The process in normal circumstances is a protective response to trauma or damage to the innermost cells - the endothelium, and the smooth muscle cells of the artery wall. This happens most at the points of bifurcation or branching that seem to bare the brunt of sheer and stress. So as with damage to any other tissue of the body an inflammatory response is initiated and best describes the formation of atheromatus plaque. In a disease situation the advanced plaque or lesion is a result of an excessive response to injury. The outcome may be that the artery is eventually occluded by the ever-expanding plaque or more likely by the "flaking-off" of the unstable fibrous cap which surrounds the lesion and the blockage of the vessel distally from the site of injury.

1.2.1 The Pathogenesis of Atherosclerosis.

"Fatty streaks" are considered to be the earliest identifiable lesion because they were found in the coronary arteries of 50% of children between the ages of 10 and 14 years on autopsy (McGill, 1984). These lesions are characteristically yellowish in colour, are slightly raised and are composed of an aggregation of lipid-rich macrophage called foam cells and T lymphocytes within the innermost layer of the artery wall - the intima. Several sources of injury have been identified which cause platelet aggregation, the result of which is the release of proliferative agents such as epidermal growth factor (EGF), platelet-derived
**Figure 1.1:** Atherosclerotic plaque at an arterial branch.

1. Endothelial cells.
2. Smooth muscle cells.
3. Arterial branch.
4. Local intima thickening with deposition of lipid.
growth factor (PDGF) and tumor necrosis factor (TNF), all of which enhance smooth muscle cell proliferation. These include smoking, obesity, hypertension, diabetes and hypercholesterolaemia. Smooth muscle cells accumulate lipid and stimulate the production of the extracellular support matrix which acts like a mesh trapping migrating lipid-laden cells. Chemotactic factors such as monocyte chemotactic protein-1, transforming growth factor-β and various interleukins are also released and mediate the migration of monocytes and lymphocytes to the point of inflammation where they are localised subendotheliially and migrate further into the intima of the wall as the process continues. Monocytes become macrophages and further accumulate lipid to become foam cells. During this process the cells stop expressing and displaying LDL receptors (LDLRs) and express what are known as scavenger receptors instead. LDL is the major cholesterol carrying lipoprotein in the bloodstream and enters cells along with other lipoproteins through this and other receptors. This will be discussed in more detail in section 1.5. As a result of this receptor change the cells now preferentially take up modified forms of LDL such as oxidised LDL. Modified LDL (Shaikh et al., 1988) and other lipoproteins (Mamo et al., 1998; Yu and Mamo, 2000) have been identified in atherosclerotic plaque. As the plaque advances a dense, fibrous cap of connective tissue develops, covering the core of lipid and necrotic debris. Thrombus formation may occur at the point of lesion erruption. This is the advanced lesion which can increase in size to impair blood flow, can rupture and cause haemorrhaging into the plaque again occluding the vessel and impairing blood flow or parts of the cap can become unstable and slough off, travel to a narrower part of the vessel where it can cause a blockage.

1.2.2 Risk Factors for Atherosclerosis.

The majority of the population are aware (whether we realise it or not) of the main risk factors leading to heart disease, thanks to public health awareness programs in the media. These include lifestyle factors such as diet, exercise, smoking and stress management and also factors such as age, hypertension, obesity, diabetes, lipoprotein abnormalities, hormonal changes and of course genetic factors. One of the most controversial and aggressively studied areas is that of lipid abnormality and the effect of diet, in particular dietary lipid.
There is strong evidence to show that elevated serum cholesterol, most of which is contained in LDL, increases the risk of mortality from heart disease (Martin et al., 1986; Expert Panel 1988). Normal cholesterol homeostasis may be maintained by regulating synthesis, excretion and cellular uptake via alteration in the expression of the LDLR. However cholesterol metabolism and therefore levels can be profoundly effected by dietary cholesterol (McNamara et al., 1987) and other dietary fats, comprehensive reviews of which have been made, most notably by Khosla and Sundram (1996).

Cholesterol carried by the HDL particle is considered to have an atheroprotective effect and low levels have been shown to be predictive of the development of cardiovascular disease (Gordon et al., 1986; Stampfer et al., 1991). A number of mechanisms have been elucidated to explain the antiatherogenic effect of HDL. These include its role in reverse cholesterol transport, the mechanism by which cholesterol is removed from peripheral tissues, including the artery, and transported either directly in HDL or in apo B-containing lipoproteins to the liver where it can be excreted in bile (Glomset, 1968). HDL has also been shown to prevent oxidation of LDL by its associated enzyme paraoxanase (PON) (Mackness et al., 1993), an enzyme which has itself been reported to be a genetic risk factor for cardiovascular disease (Serrato and Marian, 1995). HDL has also been reported to suppress endothelium dependent vasorelaxation inhibition by oxidised LDL (Matsuda et al., 1993) and to interfere with cell adhesion by reducing the expression of vascular cell adhesion molecule (VCAM) (Cockerill et al., 1995). Along with smoking (Mero et al., 1998) and the reduction in plasma triglycerides (Schaefer et al., 1978), dietary lipids have been shown to influence plasma HDL levels (Mensink and Katan 1990 and Rudel et al., 1995).

As mentioned previously, patients with low levels of HDL frequently have elevated plasma triglyceride levels (Avogaro et al., 1992) for reasons which seem to be associated with the role of apo B-containing lipoproteins in reverse cholesterol transport. These lipoproteins are chylomicrons, which carry dietary lipids from the intestine mainly to the liver and VLDL which are secreted by the liver and are the main triglyceride transporting
lipoprotein. Indeed a negative correlation has been reported between HDL - cholesterol and apo B-48 and apo B-100, the solubilising proteins of chylomicrons and VLDL respectively (Karpe et al., 1993). According to data from the Framingham Heart Study, (Castelli, 1986) an increased risk of CHD in hypertriglyceridaemic patients was largely limited to those with low HDL cholesterol, prompting the suggestion of a "back seat" role for triglycerides in the risk of the disease. Only recently, despite a number of studies showing a very high correlation between fasting serum triglycerides and CHD (Hokanson and Austin, 1996), has the view emerged that triglyceride and triglyceride rich lipoproteins (TRL) are frequently crucial in atherogenesis. This argument is put very convincingly in a review by Durrington, (1998). As with HDL, triglyceride levels can be manipulated by diet. For example, high carbohydrate diets tend to raise triglycerides in diabetic patients (Coulston et al., 1987; Garg et al., 1988), while the polyunsaturated fats in fish oils have a well established hypotriglyceridaemic effect (Parks et al., 1990). Indeed, there has been speculation that the mechanism of lowering triglycerides by fatty acids is similar to that of a class of hypotriglyceridaemic drugs called fibrates (Auwerx et al., 1996). These are thought to act by altering gene expression of enzymes and apolipoproteins which are important in the catabolism of TRL through steroid hormone-like receptors called peroxisome proliferater-activated receptors (PPARs) (Reddy et al., 1982).

1.2.3 Diabetes Mellitus and Vascular Disease.

Much of the morbidity and mortality associated with diabetes is attributable to chronic complications of the disease - microvascular disease including diabetic retinopathy, nephropathy, neuropathy and premature macrovascular disease or atherosclerosis. Diabetes is responsible for 12% of all new cases of blindness, 25% of all cases of end-stage renal disease and 40% of all non-traumatic foot and leg amputation in the USA. These three pathologies have the common aetiology of stenosis of the small, non-arterial blood vessels. It occurs in types 1 and 2 diabetes but is most common in type 2 and has been shown to be related to the duration of the disease (Watkins et al., 1987).
Atherosclerosis, however, is by far the most common complication in diabetes, being the cause of death in three-quarters of the diabetic population (Steiner, 1994). Diabetic patients have been shown to have between a 2- and 5-fold higher incidence of cardiovascular disease than the non-diabetic population (Panzram, 1987; Anderson et al., 1987) which cannot be explained by an increase in the presence of standard risk factors such as hypertension, hyperlipidaemia, smoking or gender. While non-diabetic women have a lower incidence of cardiovascular disease, in diabetes there is very little difference between the sexes (Krowleski et al., 1987). Disease-related abnormalities such as hyperglycaemia, hyperinsulinaemia, insulin resistance, the classical definitive serum lipid abnormalities of elevated triglycerides, low HDL - and raised LDL - cholesterol and modification of lipoproteins by oxidation or glycosylation certainly have a role to play in enhancing atherosclerosis, but evidence is mounting to show that abnormalities in postprandial lipoproteins in diabetes may also play a key role (Karpe et al., 1994; Tkac et al., 1997; Sakata et al., 1998; Kugiyama et al., 1999). This topic is discussed in more detail in section 1.4 as it is the basis for the experimental investigation detailed in this thesis.

1.3 LIPOPROTEIN METABOLISM.

Due to their inherent hydrophobicity, lipids present a problem when it comes to transportation around the body. All the major fluid media of the body, including the cytosol of the cell, interstitial fluid and of course the blood are aqueous in nature and therefore somewhat incompatible with extremely hydrophobic lipids such as cholesterol and triglyceride. The problem is overcome in quite an ingenious way with the help of phospholipids, molecules containing both a hydrophobic fatty acid "tail" and a hydrophilic or charged "head". Phospholipids, when linearly aligned, form a barrier between hydrophobic and hydrophilic phases. Lipoproteins are spherical arrangements of phospholipid arranged with the non-polar, fatty acid chains facing inward and the polar phosphate groups facing outward and packed inside in the resulting hydrophobic environment are the lipids - cholesterol ester and triglyceride (Figure 1.2). The polar
Figure 1.2: Generalised structure of a human plasma lipoprotein.
phosphate groups are hydrophilic and allow the lipoprotein ease of movement through aqueous lymph and blood. The protein moiety of the lipoprotein may be a single peptide or multiple, varying peptides called apolipoproteins or apolipoproteins, having a variety of functions such as solubilisation of the particle, receptor ligands or enzyme co-factors, all of which play a role in the metabolism of the particular lipoprotein.

Lipoproteins vary widely in size and composition and plasma lipoproteins are constantly being remodelled, by exchanging core lipids and apolipoproteins and by the hydrolysis of core triglyceride, but the basic components shown in Figure 1.2 are common to all.

1.3.1 Fatty Acids.
Fatty acids consist of chains of carbon atoms which are hydrophobic and a carboxylic acid group, which is hydrophilic and readily forms esters and amides. Fatty acids differ primarily in chain length and in the number and position of double bonds between the carbons (Figure 1.3). Saturated fatty acids contain no double bonds, monounsaturated have one and polyunsaturated have more than one. There are two types of double bond, cis and trans, depending on the position of the hydrogen atoms on the carbons involved in the bond. Intake of trans fatty acids such as the hydrogenated fats present in hard margarines has an unfavorable effect on plasma lipoproteins. It tends to raise LDL cholesterol and lower HDL cholesterol - an effect similar to that of saturated fat (Zock et al., 1997). This is discussed further in section 1.5.

Most fatty acids can be synthesised in the human body but certain polyunsaturated fatty acids, such as linoleic acid, cannot and have to be taken in the diet. These are called essential fatty acids. Figures 1.4 and 1.5 show a summary of the synthesis of fatty acids by the desaturation and elongation pathways. Four desaturase enzymes, called delta (Δ) desaturases have been identified in humans. They are given numbers denoting the specific carbon (numbered from the carboxy terminal) after which it has the ability to form a double bond. The enzymes are Δ⁴, Δ⁵, Δ⁶ and Δ⁹. As a result of the absolute specificity of the desaturases, double bonds can only be formed up to carbon 9 and fatty acids containing
Figure 1.3: Fatty acid structural formulas. Examples of saturated, monounsaturated and polyunsaturated fatty acids all in the cis configuration.
Figure 1.4: The principle fatty acids of the n-6 series, including linoleic acid (9,12,-C18:2).
Figure 1.5: The principle n-7 and n-9 fatty acids, including oleic acid (9, C18:1).
double bonds beyond this must be obtained from dietary sources. The elongation of fatty acids involves the addition of two carbons and as a result all fatty acids naturally occurring in humans are even-numbered. Fatty acids are grouped into series depending on double bond furthest from the carboxy terminus and which are denoted by the letter n and the number of the first carbon in the bond counting from the methyl terminus. Since both linoleic and oleic acid are the focus of the main studies in this dissertation, examples of the main members of the series to which they belong are shown in Figures 1.4 and 1.5.

Fatty acids, being hydrophobic are transported in the plasma as free fatty acids bound to the hydrophobic blood protein albumin or as esterified fatty acids in lipoproteins. The main fatty acid-containing molecules are phospholipid, triacylglycerol and cholesterol ester. Phospholipids are derived from the three carbon compound glycerol. Two fatty acids are attached at two of the carbons. At the third carbon a charged phosphate group is linked and attached to that is a hydrophilic base such as choline or ethanolamine for example, producing phosatidylcholine and phosphatidylethanolamine respectively (Figure 1.6).

Triacylglycerols or triglycerides consist again of a glycerol backbone to which three fatty acid chains are linked via ester bonds (Figure 1.7). These molecules are the form in which fatty acids are stored in adipocytes mainly and constitute the body's main store of readily accessible energy. Triglycerides may be produced in the intestine from dietary fatty acids and monoacylglycerol or they can be synthesised de novo in the fasting gut and fasting and fed liver by the phosphatidic pathway.

1.3.2 Cholesterol Synthesis.

Cholesterol is a twenty-seven carbon steroid with a hydroxyl group at position 3 and a double bond between carbon 5 and 6 (Figure 1.8 (a)). It occurs in two forms in the body, as this free sterol which is highly hydrophobic and exists mainly interspersed between phospholipid in plasma membranes and also on the surface of lipoproteins. The cholesterol/phospholipid ratio determines the viscosity of the membrane. The ringed structure of cholesterol makes it very rigid increasing the viscosity of the membrane. It also
**Figure 1.6:** (a) Generalised structure of phospholipid. (b) Phosphatidylcholine (lecithin).
Figure 1.7: (a) Glycerol. (b) Triacylglycerol.
Chapter 1

exists in an esterified form, where a long-chain fatty acid is attached through the oxygen of the alcohol group (Figure 1.8 (b)). Two enzymes are responsible for esterification of cholesterol. Cholesterol esterase, a pancreatic enzyme, is absorbed into the intestinal mucosal cell, its site of action. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) is a microsomal enzyme and catalyses intracellular esterification. The majority of cholesterol exists in this form and it comprises the bulk of core lipoprotein cholesterol. Apart from its structural function, cholesterol is also required for the synthesis of steroid hormones, bile salts and as a precursor of vitamin D. It is synthesised de novo in the liver mainly and also in the distal region of the small intestine, but it is also obtained from the diet.

Figure 1.9 outlines the synthetic pathway of cholesterol. It occurs mainly in the liver but also in the distal region of the intestine. All the carbons in the molecule are derived from acetate. Briefly, it involves the reaction of three molecules of acetyl CoA to form 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA), catalysed by the enzymes acetyl CoA-acyl transferase and HMG CoA synthase. The conversion of HMG-CoA to mevalonate is the rate-limiting step catalysed by HMG CoA reductase. The reaction is regulated by negative feedback of the end product - cholesterol. Mevalonate then undergoes a number of enzymatic isomeric transformations to produce farnesyl pyrophosphate, an isoprenoid. Six of these polymerise to form squalene by the action of squalene synthtase. After oxidation by epoxidase, squalene oxide is cyclised to form cholesterol by the enzyme oxidosqualene-lanosterol cyclase.

1.3.3 Absorption of Dietary Lipid.

Dietary lipids, including cholesterol, may be absorbed from the gastrointestinal tract. They are broken down into their constituent fatty acids, monoacylglycerols and some diacylglycerols, lysophospholipids and free cholesterol by various salivary, pancreatic and intestinal digestive enzymes. Micelles much like lipoproteins are formed with bile acids and diffuse at the microvillus surface of the enterocyte. The scavanger receptor-BI (SR-BI) was the first receptor identified to be involved in the absorption of cholesterol from the intestine (Hauser et al., 1998). The glycerides and about half of the cholesterol are re-
Figure 1.8: (a) Cholesterol. (b) A cholesteryl ester - cholesteryl stearate.
Figure 1.9: The synthetic pathway of cholesterol in mammalian cells.
esterified in the enterocyte (Dietschy and Wilson, 1970) and the lysophospholipid is reacylated to form phospholipid, before being packaged into the chylomicron, the largest of the lipoproteins. This particle is secreted into the mesenteric lymphatics and from there enters the bloodstream via the thoracic duct, in the subclavian vein. The chylomicron will be described in substantially more detail later in this section.

1.3.4 Cholesterol Homeostasis.

Cholesterol homeostasis is vital from the point of view of maintaining normal cellular function and hormone production, but also because of its potential for atherogenesis.

Dietary cholesterol is delivered to each cell mainly by LDL and is taken up either by receptor-mediated endocytosis involving the LDL- or B/E receptor (Goldstein and Brown, 1977) or by the putative non-receptor pathway, of bulk-phase endocytosis. Over-accumulation of cholesterol in the cell results from an upset in the balance of dietary cholesterol uptake, cholesterol synthesis and the removal of cholesterol from the cell mainly by HDL. Synthesis is controlled by product inhibition of HMG CoA reductase. Uptake of dietary cholesterol is also regulated by the alteration the gene expression of the LDLR by cellular cholesterol (Goldstein and Brown, 1977).

The liver has a central role to play in regulating plasma lipoprotein levels. It is the major site of cholesterol and triglycerol synthesis in the body. It is responsible for the removal of dietary lipid (in the form of chylomicron remnants) from the blood (Redgrave, 1970) and the turnover of VLDL and LDL. It is the site of assembly of VLDL, production of which is controlled by free fatty acids, dietary lipid and insulin (Gibbons, 1990; Sparks and Sparks 1994; Fungwe et al., 1992; Cartwright and Higgins, 1999). It is also responsible for mediating the excretion of cholesterol from the body. This includes cholesterol removed from the peripheral tissues by HDL (Webb et al., 1998).

1.3.5 The Lipoproteins.

The description of lipoprotein components, so far in this section, has concentrated on the lipid moiety of the particles. Whilst each will be described more thoroughly throughout this
section, an understanding of the lipoproteins requires a brief familiarisation with the proteins involved - the apolipoproteins (Table 1.2).

### Table 1.2: The apolipoproteins, their site of synthesis, localisation and function.

<table>
<thead>
<tr>
<th>Apo</th>
<th>Site of synthesis</th>
<th>Lipoprotein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>Intestine</td>
<td>HDL, chylomicron</td>
<td>Structural protein of HDL, LCAT activator, receptor ligand</td>
</tr>
<tr>
<td>AII</td>
<td>Liver</td>
<td>HDL₂, chylomicron</td>
<td>Structural protein of HDL, LCAT inhibitor</td>
</tr>
<tr>
<td>AIV</td>
<td>Intestine</td>
<td>HDL, chylomicron</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>B48</td>
<td>Intestine</td>
<td>Chylomicron</td>
<td>Structural protein of chylomicron, receptor ligand</td>
</tr>
<tr>
<td>B100</td>
<td>Liver</td>
<td>VLDL, IDL, LDL</td>
<td>Structural protein of VLDL,IDL,LDL, receptor ligand</td>
</tr>
<tr>
<td>CI</td>
<td>Liver mainly, intestine</td>
<td>VLDL, HDL, chylomicron</td>
<td>LCAT activator, inhibitor of apo E/receptor binding</td>
</tr>
<tr>
<td>CII</td>
<td>Liver mainly, intestine</td>
<td>VLDL, HDL, chylomicron</td>
<td>Cofactor of LPL, inhibitor of apo E/receptor binding</td>
</tr>
<tr>
<td>CHI</td>
<td>Liver mainly, intestine</td>
<td>VLDL, HDL, chylomicron</td>
<td>Inhibitor of LPL, inhibitor of apo E/receptor binding</td>
</tr>
<tr>
<td>D</td>
<td>Liver</td>
<td>HDL</td>
<td>LCAT activator (?), part of CET complex</td>
</tr>
<tr>
<td>E</td>
<td>Liver mainly, intestine</td>
<td>VLDL, HDL, chylomicron</td>
<td>Receptor ligand</td>
</tr>
<tr>
<td>J</td>
<td>Liver</td>
<td>HDL</td>
<td>PON modulator</td>
</tr>
</tbody>
</table>

LCAT (lecithin-cholesterol acyltransferase); CET (Cholesteryl ester transfer); PON (paraoxanase)

There are five major classifications of plasma lipoproteins - chylomicron, VLDL, intermediate density lipoprotein (IDL), LDL and HDL, in descending size and increasing density. They were originally classified and identified according to their electrophoretic mobility, but now are separated in groups depending on their density as determined by
their ultracentrifugal properties which largely reflects their size and also depending on their constitutive components. The TRL, namely the chylomicron and VLDL, will be described first. While they are distinctly different in their site of origin and function, they have a similar method of assembly and catabolism and as such will be discussed together with comparison and contrast highlighted. A full description of all the apolipoproteins will be given in the context of the lipoproteins to which they are associated and the function they perform. Figures 1.10 (a) and (b) show an overview of the lipoproteins and their functions. This may be divided into two. Dietary lipid is carried from the intestine to the peripheral cells or the liver and this is called the exogenous pathway and it involves only the chylomicron. Lipid delivered by the chylomicron to the liver and newly synthesised lipid is carried either to the periphery or from the periphery in a complicated pathway called the endogenous pathway and all the other lipoproteins are involved.

1.3.6 Lipoprotein of the Intestine - The Chylomicron.

Chylomicrons (Greek: chylo - chyle; mikron - any minute thing) are triglyceride-rich lipoproteins, synthesised within cells of the jejunal part of the small intestine, which provide the means to introduce newly absorbed dietary fat into the body. This has traditionally been referred to as the exogenous lipid transport pathway. Chylomicrons are large, spherical particles ranging in size between 75 and 450nm in diameter and after ingestion of dietary fat give a milky appearance to lymph and a cloudy appearance to plasma. They have a density of <0.95g/ml. Their Svegberg flotation rate (Sf) varies from Sf > 400 to Sf > 1000. Larger particles are less dense and contain more triacylglycerol. Their constituents are triacylglycerols (85-92%), phospholipids (6-12%), cholesterol (1-3%) and proteins (1-2%). The apolipoproteins found associated with newly synthesised chylomicrons are apo B48, apo AI, apo AIV, a small amount of various apo Cs and a small amount of apo E. Only one molecule of apo B48 is present on the particle but multiple and varying numbers of all the other smaller apolipoproteins are present (Chan, 1992). Only apo B48 is required for the assembly of the particle.
Figure 1.10: (b) The endogenous lipid pathway. Both dietary cholesterol and triglyceride and de novo synthesised lipid is secreted from the liver in the form of the apo B100-containing VLDL particle. Apo E and apo Cs received from HDL allowing the action of LPL to release fatty acids to the peripheral tissues and reduce the size of the particles to IDL and LDL, loosing apoproteins back to HDL in the process. Triglyceride is exchange for cholesterol from HDL by LCAT and CETP. Both VLDL and IDL may be taken up by the liver or peripheral tissues via apo E. LDL is cleared via apo B100 and the LDLR. HDL effluxes cholesterol from peripheral tissues which is transferred to apo B-containing particles.
1.3.7 Lipoprotein of the Liver - Very Low Density Lipoprotein.

These particles are somewhat smaller than the chylomicrons, although there is overlap in their sizes and therefore their density. VLDL range in size between 30 and 100nm in diameter and have a density of <1.006g/ml and an Sf of 20-400. They contain less triglyceride and more cholesteryl ester per particle than chylomicrons, but because of their relatively longer residence time in the plasma and their apparent greater abundance, they are considered the major triglyceride carrying particle in the bloodstream. They are produced in the liver and like the chylomicron have apo B as their main structural protein. In contrast however, VLDL contains the apo B100 form, a massive hydrophobic protein with a molecular weight in excess of half a million Daltons.(Da) It is one of the largest naturally-occurring monomeric proteins. The function of these particles is to deliver fatty acids to the cells of the periphery where they can by oxidised as a fuel source upon depletion of glycogen stores or it can be stored as triglyceride in the adipocytes. The process of hydrolysis which facilitates this is described in section 1.3.10. The cholesterol of the VLDL and some triglyceride remains with the particle and is delivered to peripheral cells or back to the liver by uptake of this or smaller remnants of the particle, IDL and LDL. This process is referred to as the endogenous lipid transport pathway. Apart from apo B100, of which there is only one copy per particle (Elovson et al., 1988), VLDL contains apo E and apo C1, CII and CIII, all of which are crucial in the catabolism and cellular uptake of the particles.

1.3.8 Chylomicron and VLDL Assembly.

Apo B48, with a molecular weight of 256,000Da is approximately 48% that of apo B100. Both are translational products of the APOB gene. This gene is 43kb long and consists of 29 exons and 28 introns. The gene has been localised to chromosome 2 in humans (Knott et al., 1985). It is mainly expressed in the intestine and the liver in humans but is also expressed in small amounts in the kidney, colon and stomach (Teng et al., 1990). It is tissue-specifically transcribed producing apo B48 in the intestine and the full length apo B100 specifically in the liver. In the enterocyte the gene is transcribed, edited and polyadenylated in the nucleus resulting in the production of two mRNAs of 14 and 7kb.
The post-transcriptional editing results in the change of cytosine at nucleotide number 6666 to a uracil by the enzyme apob mRNA editing enzyme, catalytic polypeptide # 1 (apobec-1) (Teng et al., 1993) thereby changing codon 2153 from glycine (CAA) to a stop codon (UAA). The edited mRNAs comprise 48% of the 5' end of the gene and on translation in the cytosol produces apo B48. In contrast to the intestine, the 7kb mRNA is not synthesised in the hepatocyte. The vast majority of APOB gene product in the intestine is apo B48, but a small amount of apo B100 has been shown to be expressed and produced in human intestinal biopsy organ cultures (Hoeg et al., 1990; Levy et al., 1990).

Assembly of the chylomicron particle has much in common with assembly of the hepatically derived, apo B100-containing VLDL particle. The APOB gene is transcribed continuously and lipid addition takes place at the membrane of the endoplasmic reticulum. If there is insufficient lipid present, translocation of either protein across the membrane is interrupted and the protein is degraded (Davis et al., 1990). Addition of lipid to the nascent apo B is initiated by the MTP complex (White et al., 1998). The rate of triglyceride synthesis and therefore dietary fatty acids have been shown to play a role in regulating the assembly and secretion of particles (Benoist and Grand-Perret 1996), but not by directly effecting the expression of apo B (Davidson et al., 1987). Phospholipid availability is also thought to play a role (Mathur et al., 1996).

1.3.9 Chylomicron and VLDL Clearance.

The mechanism of removal or clearance of these particles from the circulation occurs in two steps (Redgrave, 1970). The first involves the hydrolysis of the triglyceride in the particle core to release fatty acids to the periphery by the enzyme lipoprotein lipase (LPL). Triglyceride is also removed from the particles by the action of the cholesteryl ester transfer protein (CETP) complex and donated to HDL in exchange for cholesterol in the process of reverse cholesterol transport. This will be described in the context of HDL in section 1.3.17. The resulting cholesterol-enriched particle is called a remnant. The second step is the receptor- or non-receptor mediated uptake of the remnant particles into cells,
mainly the hepatocytes but also peripheral tissues. These processes are, for the most part, common to both intestinally-derived and hepatically-derived particles.

When the particles enter the bloodstream they immediately lose apo A (AI from the chylomicron and AII from VLDL). This is the beginning of a complex process of apolipoprotein exchange which occurs between the TRL and HDL (Havel et al., 1973a). It is enormously important in the metabolism of both of these types of lipoproteins from the point of view of modulation of enzyme activity, ligand/receptor interaction and the modulation of that interaction. Apo A will be discussed later in the context of HDL (Section 1.3.18). Apo Cs and apo E are donated by HDL to the TRL (Havel et al., 1973). It has been well established that apo CII is the essential cofactor for LPL (Havel et al., 1970) and once present on the particle allows hydrolysis of as much as 85% of the triglyceride core of the particle. Apo C111 inhibits the hydrolysis by LPL (Krauss et al., 1973). The apo Cs have a dual role, however, in that they inhibit binding of lipoproteins to cell surface receptors which mediate the uptake of the particles (Windler et al. 1980; Clavey et al., 1995). Apo E is the ligand for a number of these receptors in the liver and on extrahepatic tissue. Apo B100, but not apo B48, is the ligand for the LDL or B/E receptor and this is a fundamental difference in the clearance of VLDL and chylomicrons (Mahley and Ji, 1999). Apo B48 is the ligand for newly discovered receptors on the surface of monocyte/macrophages (Gianturco et al., 1998).

1.3.10 Lipoprotein Lipase.

The importance of LPL in the clearance of TRL is demonstrated in type 1 hyperlipoproteinaemia where there is a congenital defect in the enzyme. Patients show massive accumulation of chylomicrons and large VLDL. There is no build-up of remnant particles because their formation is limited without the hydrolytic activity of LPL. This has been observed again recently in a mink animal model homozygous for a mutation in the enzyme (Savonen et al., 1999). It is present, attached by electrostatic interactions, to glycosaminoglycans on the endothelial cells in the lumen of the microvasculature (Saxena and Goldberg, 1990). Apo CII is its essential co-factor (Olivercrona and Beisiegel, 1997).
and apo CIII and apo CII when in excess inhibit the activity of LPL (Havel et al., 1973; Goldberg et al., 1990; Lambert et al., 1996). The free fatty acids which it releases are taken up by the surrounding tissue. A number of groups have shown that chylomicrons and VLDL compete for binding to the enzyme (Karpe et al., 1993; Bjorkegren et al., 1996). It has been shown that chylomicron triglyceride clearance is up to 10-fold faster than that of VLDL triglyceride and that this may be due to an higher affinity of apo B48-containing lipoproteins for LPL (van Beek et al., 1998; Xiang et al., 1999). Martins et al., (1996) demonstrated that rather than size, the number of particles present in the system may be more important in determining clearance, suggesting that LPL hydrolysis is a saturable process, although this is disputed (Goldberg, 1996). The role of apo E in LPL activity is controversial. It has been suggested that it facilitates LPL hydrolysis of TRL by anchoring them to the endothelial surface next to the enzyme. A recent study in apo E-deficient mice showed that they were resistant to heparin-induced lipolysis and that they accumulated both chylomicron and VLDL particles. When levels of these particles were reduced to the level of wild-type mice, however, lipolysis returned to normal (Zsigmond et al., 1998). They attributed the reduction in lipolysis to saturation of the pathway rather than an apo E effect.

1.3.11 Receptors Mediating Uptake of TRL.

Although endocytosis of LDL particles has been established since the early 1980s (Handley et al., 1983), the understanding of remnant particle clearance began with the elucidation of the LDLR pathway for the clearance of LDL through apo B100 (Brown and Goldstein, 1986). It was later discovered that the LDLR had a higher affinity for particles containing several molecules of apo E rather than for a single molecule of apo B100 (Innerarity and Mahley, 1978), implicating the LDLR in TRL clearance. Studies in fibroblasts (Floren et al., 1981), macrophages (Ellsworth et al., 1987) and knockout animals (Ishibashi et al., 1994) confirmed this. It was also observed in these experiments that LDLR-knockout mice did not accumulate TRL in their plasma when fed a low-fat diet. This prompted the hypothesis that there was another receptor involved in remnant clearance. It was discovered to be a protein with significant homology to the LDLR. It was
identified by Herz et al., (1988) and was called the LDLR-related protein (LRP). The LRP binds both apo E and apo B100. The importance of the LRP in the clearance of remnant particles was demonstrated in LDLR-knockout mice infected with an adenovirus expressing receptor associated protein, a protein which blocks all the binding site of the LRP. These animals display elevated levels of remnant lipoproteins (Ishibashi et al., 1994). A third member of the LDLR family is the VLDL receptor. It is expressed on non-hepatic tissue and has been shown also to bind chylomicron remnants which are rich in apo E (Niemeier et al., 1996).

A fourth receptor was characterised by Bihain and Yen, (1992) and was proposed to be a chylomicron remnant receptor. It was activated by free fatty acids and was named the lipolysis-stimulated receptor (LSR). It is not related to the LDLR family. It binds both apo B and apo E and unlike the LDLR who's expression is down-regulated by cellular cholesterol, the LSR is constitutively expressed and unaffected by cholesterol levels. The most compelling evidence so far for the role of the LSR in chylomicron clearance is that a strong negative correlation has been observed between the number of LSR expressed in rat liver and plasma triglyceride levels (Mann et al., 1995). The relative importance of each of these receptors in the clearance of both chylomicrons and VLDL is not fully understood.

Finally, a receptor was recently discovered on the surface of monocytes/macrophages which has apo B48 as a ligand (Gianturco et al., 1998). This has significant implications for the involvement of intestinally-derived particles in the atherosclerotic process. The discovery of this receptor further substantiates the theory that chylomicron remnants contribute to the cholesterol build-up in cells which mediate plaque formation.

1.3.12 Hepatic Uptake of Chylomicron and VLDL Remnants.

Approximately 80% of chylomicron cholesterol is rapidly taken up by the liver, making it the major site of chylomicron remnant clearance. Cooper, (1997) proposed a model describing the interplay of these various receptors and other molecules in this process (Figure 1.11). When triglyceride hydrolysis has been carried out, the particle has lost apo
Figure 1.11: Proposed mechanism for the uptake of chylomicron remnants and VLDL remnants (IDL and LDL) in the Space of Disse in the liver. Cooper A. D (1997) Journal of Lipid research. 38: 2173-3192.

HSPG - heparin sulphate proteoglycan.
HL - hepatic lipase.
C, allowing further acceptance of apo E and the particle achieves a size that allows it to traverse the fenestrae in the endothelium, it enters the space of Disse. This is the area between the endothelium and the hepatocyte. Once inside, there are a number of pathways open to the particle. It can bind directly to the LDLR or the LRP and be taken up directly. Further accumulation of apo E on the particle is thought to be required to facilitate binding to the LRP. On saturation or insufficient numbers of these receptors particles are "sequestered" in the space of Disse. This involves the attachment of the particles to heparin sulphate proteoglycans (HSPGs) via apo E or to hepatic lipase (HL) via apo B.

There is also evidence to show that LPL, which comes away from the vascular endothelium and becomes attached to partially hydrolysed chylomicron remnants, acts as a docking protein here also (Felts et al., 1975). The enzyme HL hydrolyses triglyceride and phospholipids mainly in the liver. It also has a role in remodelling of HDL. Like LPL, HL is attached to the endothelium by HSPGs. During sequestration particles may undergo further triglyceride hydrolysis by both LPL and HL and may also receive more apo E which is produced in the hepatocytes and is secreted into the space of Disse. Finally particles are transferred to receptors and internalised. Exactly how this transfer occurs is unknown.

This model does not, however, take the LSR into account. It has been argued that the LSR plays a significant role in chylomicron remnant clearance as its expression is not downregulated by cholesterol as is that of the LDLR and its affinity is greatest for triglyceride-depleted, remnant particles rather than TRL, which is the opposite for the LDLR (Bihain and Yen, 1992). This group propose that the LSR is responsible for 50% of the clearance of intestinally-derived remnant particles and studies with apo CIII in cultured rat hepatocytes suggest that the LSR represents a physiological rate-limiting step in the removal of remnants from the circulation (Mann et al., 1997).

The removal of the larger, apo E-rich VLDL particles is reported to be identical to that of chylomicron remnants (Cooper et al., 1982). However, as VLDL is reduced further in size, producing IDL and eventually LDL, apo C and also apo E is gradually lost affecting its
affinity for various receptors until, with only apo B100 remaining, LDL can only be
removed by the LDLR.

Remnant removal has been shown to be decreased by cholesterol feeding in vivo (Redgrave
and Snibson, 1977). This may be due to the expansion in the lipoprotein pool size rather
than a reduction in the removal capacity of the liver (Kris-Etherton and Cooper, 1980).
Insulin has been shown to increase hepatic uptake in vitro. Insulin is known to stimulate
LDLR activity (Jensen et al., 1988). However, diabetes has been shown to greatly reduce
the rate of remnant clearance in vivo (Redgrave and Snibson, 1977; Phillips et al.,
submitted). This will be discussed further in section 1.4.1. The fatty acyl chain component
of triacylglycerols in chylomicron emulsions has been shown to effect their clearance in
vivo (Mortimer et al., 1994). They propose that there are acyl preferences in the steric
interactions between particles and enzymes and apolipoproteins or alterations in the
physio-chemical properties (such as fluidity) of the lipids in the particles.

1.3.13 Apolipoprotein C and the PPAR.
There are four subclasses of apo C, named apo CI - IV. The genes encoding apo CI, apo CII
and apo CIV are located in a 48kb gene cluster on chromosome 19 along with that of apo E.
That of apo CIII is located in a gene cluster along with apo AI and Apo AIV on the long
arm of chromosome 11 (Table 1.3).

Isoelectric focusing separates apo CIII into three different isoforms that differ in their
degree of □-linked sialylation at the threonine residue in position 74 (Brown et al., 1970).
Apo CIII0 is unsialylated, CIII1 has one sialic acid residue and CIII2 has two. The protein
is secreted in the sialylated forms, suggesting that apo CIII2 must be desialylated after
secretion into the plasma to the monosialo and asialo forms (Roghani and Zannis, 1988).
Once secreted from the hepatocyte, apo Cs rapidly associates with HDL and TRL due to
their high affinity for lipid. In the fasting state apo Cs are mainly associated with HDL and
redistribute preferentially to chylomicrons and VLDL postprandially (Havel et al., 1973a;
Mahley et al., 1984; Klein et al., 1992; Björkegren et al., 1997, 1998). Apo Cs are also
synthesised in the intestine, albeit to a lesser extent, the relative importance of this newly synthesised apo C, relative to that transferred from HDL, on the apo C levels on chylomicron particles has not been elucidated.

Table 1.3: Properties of human APOC genes and proteins.

<table>
<thead>
<tr>
<th>Property</th>
<th>APOCI</th>
<th>APOCII</th>
<th>APOCIII</th>
<th>APOCIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal location</td>
<td>19q13.2</td>
<td>19q13.2</td>
<td>11q23-qter</td>
<td>19q13.2</td>
</tr>
<tr>
<td>Size of gene, kb</td>
<td>4.7</td>
<td>3.4</td>
<td>3.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Tissue expression</td>
<td>Liver, lung, skin, testis, spleen</td>
<td>Liver, intestine</td>
<td>Liver, intestine</td>
<td>Low amounts in liver</td>
</tr>
<tr>
<td>No. of amino acids</td>
<td>57</td>
<td>79</td>
<td>79</td>
<td>102</td>
</tr>
<tr>
<td>Molecular mass, kDa</td>
<td>6.6</td>
<td>8.8</td>
<td>8.75</td>
<td>Not reported</td>
</tr>
<tr>
<td>Plasma concentration, mg/dl</td>
<td>6</td>
<td>4</td>
<td>12</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

Numerous in vitro studies have demonstrated the inhibitory effect of apo CII on LPL-mediated hydrolysis of triglyceride-rich lipoproteins (Havel et al., 1970; LaRosa et al., 1970; Goldberg et al., 1990). It is essential for the activation of the enzyme. The exact mechanism by which activation is induced is not yet clear. It was suggested in the early eighties from experiments with synthetic apo CII peptides that direct protein-protein interaction was required (Smith et al., 1980). Recently it has been shown that the lipid binding domain of apo CII is essential for the activation of LPL (Olivecrona and Beisiegel, 1997). High concentrations of apo CII have, however, been shown to inhibit the enzyme (Havel et al., 1973). This group also showed that apo CI and apo CIII inhibited LPL activity in vitro. On the other hand, transgenic mouse models over-expressing apo CI did not have an alteration in the lipolysis of VLDL in vivo (Jong et al., 1996; Shachter et al.,
Chapter 1

The ratio, therefore of apo CII to CIII and the overall level of each of the apolipoproteins is important in the regulation of LPL activity (Lambert et al., 1996).

Another effect of apo CIII which is extremely important in the metabolism of chylomicrons and VLDL is its ability to inhibit the uptake of these particles in the liver. Normally this occurs to enable the triglyceride core of the particles to be hydrolysed, releasing fatty acids to the peripheral tissues and permitting the reduction in particle size which is required for entrance to the space of Disse in the liver. The inhibitory effect was shown in the rat model twenty years ago (Windler et al., 1980). It was later discovered that the apo E-mediated uptake of triglyceride emulsions was inhibited in cultured hepatocytes by both apo CI and apo CIII (Quarfordt et al., 1982). Each of the apo Cs have been shown to inhibit apo E-mediated binding to the LDLR (Sehayek and Eisenberg, 1991), the LRP (Kowal et al., 1990) and the LSR (Mann et al., 1997). This latter group also investigated the inhibitory effect of the different apo CIII isoforms, showing that despite a two-fold greater binding of apo CIIIb to VLDL, it was a less efficient inhibitor of the binding of VLDL to the LSR than apo CIII1 and apo CIII0. Inhibition of the apo B-dependent uptake of VLDL by the LDLR by both apo CII and apo CIII has also been demonstrated in HeLa cells (Clavey et al., 1995). Overexpression of human apo CIII in mice results in impaired clearance of VLDL triglycerides (Aalto-Setälä et al., 1992) and also apo B48-containing chylomicron remnants (de Silva et al., 1994). This defect was neutralised by cross-breeding with mice overexpressing apo E and the authors proposed that the ratio of apo CIII to E was a key determining factor in the uptake of these particles. This has since been questioned, and it seems that the absolute amount of each apolipoprotein is more important in determining clearance (Attia et al., 2000). In the face of all this evidence to say that apo Cs have an inhibitory effect on the binding of TRL to receptors Chung and Dashti (2000) have recently published a paper with a contradictory observation. They studied the effect of HDL on VLDL remnant formation and uptake in cultured hepatocytes. They have shown that in the presence of increasing levels of HDL, the apo C/apo E ratio decreases on remnant formation, due to greater acceptance of apo C in surface vesicles (phospholipid, free cholesterol, apo C and apo E) by HDL. The resulting particles with the lowest apo
C/E ratio had a similar affinity for hepatic receptors and a similar rate of internalisation and catabolism to those which contained a large apo C/E ratio.

Due to the extremely low expression and non-detectable levels of apo CIV in human plasma, it is thought not to function in lipoprotein metabolism. It is synthesised to a more substantial level in rabbit and is associated with VLDL and HDL (Zhang et al., 1996). Transgenic mice expressing human apo CIV are hypertriglyceridaemic, suggesting a role in TRL clearance in the mouse but not in humans (Allan and Taylor, 1996).

Apo CI has been shown to activate the enzyme lecithin-cholesterol acyltransferase (LCAT), which is responsible for the esterification of both cholesterol and lysophosphatidylcholine in reverse cholesterol transport (section 1.5.18). Apo CII and CIII have been reported to inhibit LCAT activity by replacing activating apolipoproteins such as apo AI and apo CI on the surface of HDL particles (Nishida et al., 1986). Synthetic peptide corresponding to the N-terminal of apo CI have been shown to inhibit CETP activity in vitro (Kushwaha et al., 1993).

Hypertriglyceridaemia is often treated with fibric derivatives— a group of drugs known as the fibrates (Reddy et al., 1994). These drugs act primarily on the liver by modifying the expression of key genes involved in lipoprotein metabolism. These genes include LPL and apo CIII and the transcription factors common to their expression are called the PPARs (Auwerx et al., 1996). The PPARs belong to the steroid hormone-like nuclear receptor gene super-family. They are members of the class II receptors - those which dimerise with 9-cis retinoic acid X receptors (RXR). There are four forms characterised to date - α, β, γ and δ which have been shown to be activated by a diverse group of compounds including fatty acids (Schmidt et al., 1992), thus fuelling the argument that fatty acids act as hormone-like messengers orchestrating lipid metabolism between the liver, the adipocytes, muscle tissue and other peripheral cells. The functions of the β form are largely unknown, but fatty acids act as ligands for it. The α form has fatty acids and fibrates as ligands and plays a role in β-oxidation of fatty acids, regulation of the expression of LPL, apo CIII, apo AI and apo All
genes. In experiments in PPARα-deficient mice, fibrates show no effect on \textit{APOCIII} expression levels compared to a marked depression of expression in the wild-type mice (Peters \textit{et al.}, 1997). It also has a role in inflammation by inhibiting cyclooxygenase 2 (COX2) (Lehmann \textit{et al.}, 1997) and may be involved in apoptosis. PPARα is expressed in cardiac muscle cells, liver and intestine. PPARγ has no natural ligands except oxidised metabolites of linoleic acid in the adipocyte. The thiazolidinedione class of anti-diabetic drugs (the "glitazones") are high affinity binders (Grossman and Lessem 1997). PPARγ is involved in the differentiation of cells (Wright \textit{et al.}, 2000), the induction of glucose transporters and the uptake of oxidised LDL. There is, as yet, very little known about PPARδ.

Fibrates and fatty acids enhance LPL expression in the liver and the adipocyte and reversibly reduce expression of the \textit{APOCIII} gene in the liver (Staels \textit{et al.}, 1995). Figure 1.12 details this regulation diagramatically. On ligand binding, PPAR is activated by dephosphorylation mediated by heat shock proteins and heterodimerises with the retinoic acid-bound, activated RXR. This complex enters the nucleus of the cell and binds to peroxisome proliferation response elements (PPREs) adjacent to the target gene, which induces transcription of the LPL gene and represses expression of the \textit{APOCIII} gene.

Treatment of rats with fibrates has been shown to reduce \textit{APOCII} gene expression, but not \textit{APOCI} expression in the liver and not in the intestine (Andersson \textit{et al.}, 1999). This group showed that \textit{APOCII} gene expression was not altered by a synthetic PPARγ ligand and suggested that its regulation is through PPARα. It has also been shown that the expression of apo CIII is mediated by insulin via a negative insulin response element in the gene promoter (Dammernan \textit{et al.}, 1993). This has been established in diabetic mice (Chen \textit{et al.}, 1994). The consequence of hyperinsulinaemia on this regulatory mechanism is not well understood.
1.3.14 Apolipoprotein E.

Apo E is a 299 amino acid glycoprotein with a molecular weight of 34kDa. It has a wide tissue distribution, but is mainly expressed in the liver, with lesser expression in the brain, intestine, spleen, lung, adrenal glands, ovary, kidney, muscle (Mahley and Innerarity, 1988) and macrophages (Mazzone, 1989). Apo E is present on chylomicrons and VLDL and on HDL. The function of apo E on HDL will be discussed in section 1.3.17. During the postprandial state an exchangeable pool of apo E is donated by HDL to the postprandial lipoproteins (Björkegren et al., 1997, 1998; Blum 1982). Apo E is also secreted in to the space of Disse where in can be incorporated into sequestered remnant particles and aid their uptake (Cooper, 1997). Several approaches have shown that apo E is a critical ligand for the clearance of remnant lipoproteins. Intravenous injection of apo E into cholesterol-fed rabbits reduced the characteristically high levels of remnant lipoproteins (Mahley et al., 1989). This was shown more recently in apo E knockout mice which displayed severe hypercholesterolaemia and a marked elevation in remnants (Mortimer et al., 1995 and Zhang et al., 1992). There is also an accumulation of LDL in plasma of transgenic rabbits overexpressing human apo E (Fan et al., 1998). The reciprocal effects of apo E and apo C on binding of chylomicron and VLDL remnants to cell surface receptors has been described in section 1.3.9

Apo E is also expressed by and secreted onto the surface of macrophages (Wang-Ivenson et al., 1985). This observation had significant implications in both the accumulation of cholesterol esters in these cells (Werb and Cohn, 1972) and its contribution to the development of atherosclerotic plaques. Apo E on macrophages also plays a role in the efflux of cholesterol from cells of the normal artery (Campbell et al., 1990) and also the efflux of cholesterol from the macrophage itself into HDL (Mazzone and Reardon 1994, Lin et al., 1998, Lin et al., 1999) for removal to the liver directly or via the apo B-containing lipoproteins. The expression of apo E by cultured mouse peritoneal macrophages has been shown to be regulated by cholesterol in the cells - increased cholesterol accumulation enhanced apo E expression and secretion on the surface of the
macrophages. Interestingly, expression returned to normal after cholesterol efflux from the cell by HDL (Mazzone et al., 1989).

The actions of apo E in lipid metabolism are primarily a consequence of its ability to bind to the LDLR (Innerarity and Mahley, 1978), the LRP (Herz et al., 1988), the LSR (Bihain and Yen, 1992), HSPGs (Felts et al., 1975) and plasma lipases (Saxena et al., 1993). Residues 136-150 in human apo E constitutes the domain responsible for these binding activities. This region is also the site which is affected by naturally occurring polymorphisms in the APOE gene (Utermann et al., 1977). The APOE gene is part of the 48kb APOE/C1/C2 gene cluster on chromosome 19. It consists of four exons, with most of the protein-coding sequence in exon four (Das et al., 1985). Its expression is regulated by the hepatic control region (HCR), an element located 17kb downstream of the gene (Shachter et al., 1993). More recently however a 185bp region located in this same region was reported to shown to increase by 10-fold luciferase activity in cells transfected with a PPARg expression plasmid. Sequence analysis revealed a high levels of analogy to the consensus PPRE. Apo E expression in macrophages was doubled on treatment with ciglitazone, a PPARg agonist (Galetto et al., 2001). The expression of apo E therefore may be effected by other PPARg ligands such as fatty acids, although this has not yet been demonstrated.

In humans, the APOE gene has three alleles denoted by e2, e3 and e4. The e3 allele is thought to be the original form being the most frequently occurring in the general population - 77%, with 7% for e2 and 15% for e4. This is slightly different in Ireland where the frequency of both e2 and e4 is elevated - 66% for e3, 12% for e2 and 22% for e4 (Sheehan et al., 2000). There are, as a result of this polymorphism and co-dominant expression of the alleles, three homozygous phenotypes denoted by E2/2, E3/3 and E4/4 and three heterozygous phenotypes - E3/2, E4/3 and E4/2. In the general population the distribution of these phenotypes are 1% for E2/2, 11% for E2/3, 2% for E2/4, 60% for E3/3, 23% for E3/4 and 3% for E4/4 (Ordovas et al., 1987).
The resultant alteration of the apo E peptide involves a substitution of amino acids 112 and 158 (Figure 1.13). The E3 peptide has a cysteine and arginine, respectively, at these points. The E4 peptide has an arginine at both sites and the E2 peptide a cysteine at both points.

(a)

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<tr>
<td>E3</td>
<td>Cysteine</td>
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<td>E4</td>
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(b)

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Figure 1.13: The apo E polymorphism at the level of the peptide (a) and the gene (b).

Various population-based studies have suggested that the different APOE alleles have distinct influences on lipid metabolism in humans. Possession of at least one copy of the e2 allele has been associated with higher plasma apo E (Boerwinkle and Utermann, 1988), lower plasma cholesterol, LDL cholesterol and apo B levels (Davignon et al., 1988) relative to e3 homozygotes. The e2 allele is also associated with lower risk of CAD (Wilson et al., 1996), except in 5-10% of e2 homozygotes who develop type III hyperlipoproteinaemia and premature atherosclerosis (Brewer et al., 1983). The E2 protein has a greatly reduced
affinity for the LDLR compared to both E3 and E4 resulting in reduced competition with LDL apo B100 for the receptor and a more efficient LDL clearance (Woolett et al., 1995). It does however have normal binding to HSPGs and it is thought that remnants particles may still be sequestered in the liver and plasma remnants do not accumulate (Mahley, 1996). On the other hand, at least one e4 allele is associated with lower plasma apo E (Boerwinkle and Utermann, 1988) and increased plasma cholesterol, LDL cholesterol and apo B levels (Davignon et al., 1988) and a greater risk of CAD (Wilson et al., 1996), when compared to e3 homozygotes. It has been demonstrated in mice expressing the E4 isoform only, that the E4 peptide competes with LDL apo B for binding to the LDLR at a higher rate than the E3 peptide (Knouff et al., 1999). This enhanced uptake of remnant particles, including intestinally-derived particles (Weintraub et al., 1987), competitively reduces the binding of LDL to the LDLR causing its accumulation in the plasma. This is further exacerbated by down regulation of the LDLR as a result of the heightened uptake of remnant cholesterol. The presence of 1 allele for E4 has been shown to increase the conversion of VLDL to LDL and to lower the fractional catabolic rate of LDL apo B100 (Welty et al., 2000). These plasma cholesterol observations were corroborated in a meta analysis carried out in 1992 (Dallongeville et al., 1992) which included studies of subjects from 17 different countries. This study also showed a consistent elevation of plasma triglycerides with both the e2 and e4 alleles compared to the e3 allele. There was also lower HDL cholesterol associated with the e4 allele, both of these possibly accounting for the increased risk of cardiovascular disease associated with the e4 allele.

As well as effecting remnant clearance, apo E polymorphism is responsible for alterations in cholesterol absorption. It has been demonstrated that the sum of the subscripts of apo E alleles (e.g. e2/2 = 4, e3/4 = 7 etc.) was positively correlated with cholesterol absorption and negatively correlated with endogenous cholesterol synthesis (Kesaniemi et al., 1987) - the 4 allele being that associated with the greatest absorption efficiency. The effect of diet on the apo E phenotypes is contradictory. A study by Tikkanen et al. (1990) showed that in e4 allele-carriers the reduction in plasma cholesterol associated with a low cholesterol, low fat diet was greater than in the other genotypes. This was also observed by other
groups (Miettinen, 1991). In an alternative study however, no difference was found in plasma cholesterol reduction between the apo E genotypes (Boerwinkle et al., 1991).

The relative frequencies of the apo E alleles between diabetic (Siegel et al., 1996; Inamdar et al., 2000) or insulin resistant (Meigs et al., 2000) and normoglycaemic populations does not differ. One exception to this observation seems to be the Hispanic population in the San Luis Valley, Colorado where the difference in distribution was not observed between diabetic and non-diabetic groups of the non-Hispanic population (Kamboh et al., 1995). However, the level of hypertriglyceridaemia which is normally associated with hyperinsulinaemic conditions may be associated with apo E genotype (Despres et al., 1993 and Inamdar et al., 2000). Hypertriglyceridaemia is absent in hyperinsulinaemic women carrying the e4 allele. It has also been suggested that apo E genotype may be associated with changes in insulin output during the oral glucose tolerance test. In a study of non-diabetic, non-obese men, the 3/4 genotype frequency was higher in those with high insulin output, regardless of blood pressure (Dembinska-Kiec et al., 1998).

### 1.3.15 Intermediate Density Lipoprotein.

Like the other classes of lipoproteins, IDL is a heterogeneous group of particles ranging in size between approximately 25 and 30nm and falling within the density range of 1.006-1.025g/ml. This lipoprotein contains the original apo B100 molecule from VLDL, some of the apo E and a high concentration of cholesterol and are considered as VLDL remnants produced by the lipolysis of VLDL. They are removed from the circulation by interaction with the LDLR in the liver via apo B or the small amount of remaining apo E (Friedman et al., 1990). Alternatively, IDL can be converted to LDL by the action of HL (Gibson and Brown, 1988).

### 1.3.16 Low Density Lipoprotein.

These are the major cholesterol carriers in the bloodstream and as such are considered the most potentially atherogenic. The particles are derived from VLDL and are thought to contain only one molecule of apo B100 and no other apolipoproteins, although there are
reports that as much as 11% of total plasma apo CIII may be associated with LDL and as much as 62% in hyperlipidaemic subjects (Bury and Rosseneu, 1985). Particles range in size between approximately 19 and 25 nm and a density between 1.025 and 1.063 g/ml. Three different classes of LDL have been described based on their density which have varying atherogenicities. These three classes are 1) large buoyant particles termed LDL-I (density: 1.025-1.034 g/ml); 2) intermediate sized particles termed LDL-II (density: 1.034-1.044 g/ml and 3) small dense LDL termed LDL-III (density: 1.044-1.063 g/ml) (Krauss and Burke, 1982). There is a strong positive correlation between plasma triglyceride level and small dense LDL-III abundance, suggesting that plasma triglyceride concentrations may influence LDL subclass distribution (McNamara et al., 1987; Kondo et al., 2001). This latter group showed that smaller LDL particles are more atherogenic. The predominance of large, triglyceride-rich VLDL particles has recently been shown to be associated with the production of small, dense LDL particles (Packard et al., 2000). The preponderance of small, dense LDL has been specifically linked to an increased risk of myocardial infarction and the presence of heart disease (Austin et al., 1988; Campos et al., 1992; Griffin et al., 1994).

Lipoprotein (a) (Lp(a)) is a particle which is essentially a modified LDL. It is slightly larger than LDL and contains apolipoprotein (a), a large glycoprotein, which is attached by a disulphide bond to the B100 molecule on LDL. There is wide variation in the concentration of Lp(a) in the plasma (0-200 mg/dl). Apo (a) has considerable amino acid sequence similarity to plasminogen. The association between Lp(a) and risk of ischaemic heart disease is not clear. Elevated Lp (a) has been shown to increase the delivery of cholesterol to the artery wall (Rath et al., 1989) and to increase smooth muscle cell proliferation (Grainger et al., 1993). An independent association between high Lp (a) and CAD was identified in the mid-eighties (Dahlen et al., 1986), but since then neither the Helsinki Heart Study (Jauhiainem et al., 1991) nor the Quebec Cardiovascular Study (Cantin et al., 1998) made the same association.
1.3.17 High Density Lipoproteins and Reverse Cholesterol Transport.

The smallest of the lipoproteins is HDL (density: 1.063-1.21 g/ml). It is the second major cholesterol carrier in the body and is considered to be antiatherogenic or protect against the development of atherosclerosis. A reduced HDL cholesterol level in plasma is a recognised risk factor for CAD (Gordon and Rifkind, 1989). Its function as a carrier and donor of apo E and apo C has been discussed in sections 1.3.13 and 1.3.14. A second function of HDL is in the removal or efflux of cholesterol from peripheral cells and delivery of it, either directly, to the liver and steroidogenic tissue such as the adrenal glands, or indirectly by transfer of cholesterol to apo B-containing particles (mainly VLDL and LDL) where it can be taken up by the receptor-mediated endocytosis already described for these particles (section 1.3.11). From here, the cholesterol can be excreted from the body or recycled in the endogenous lipid transport pathway. This process is called reverse cholesterol transport (Figure 1.13).

There are three classes of HDL, grouped according to their size. The smallest is nascent or pre-\( \beta \) HDL. There are two forms. Pre-\( \beta 1 \) HDL is lipid-poor apo AI, cannot be isolated by ultracentrifugation and apart from plasma is found in large vessel lymph (Fielding and Fielding, 1995). The second is discoidal or pre-\( \beta 2 \) HDL. These are flattened, non-spherical particles consisting of phospholipid and apo AI mainly. These particles are also present in interstitial fluid (Sloop et al., 1987) and have been shown to play an important role in the efflux of cholesterol from peripheral cells (Oram, 1986). They are produced either by the liver, the intestine or from the catabolism of triglyceride-rich lipoproteins in the form of phospholipid and apolipoprotein (apo C and apo E) surface remnants. They function in cholesterol efflux, which is the removal of cholesterol from the cells of the body, including those of the arterial wall and macrophages (Bilicki et al., 1999). Apo E produced by macrophages has been shown to promote HDL-mediated cholesterol efflux from the macrophage (Mazzone and Reardon, 1994). The mechanism by which cholesterol is released from the cell has just recently been elucidated (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). It is mediated by a protein called cholesterol-efflux regulatory protein (CERP) which is the product of the adenosine triphosphate (ATP)-
Figure 13: Reverse cholesterol transport. Cholesterol is effluxed from the peripheral tissues mediated by the cholesterol-efflux regulatory protein (CERP) and is transferred to HDL₃ which is present in the surrounding interstitial fluid and binds to the Scavenger Receptors BI and BII (SR-BI and BII). Cholesterol and triglyceride exchange occurs with chylomicrons and other apo B-containing lipoproteins in lymph and also in the plasma, by the action of LCAT and CETP. Effluxed cholesterol may then be taken up by the liver via apo b- or apo E-mediated clearance of TRL or directly from HDL bound through the SR-BI/II receptors. Cholesterol is excreted from the body in bile.
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binding transporter 1 (ABCI) gene. Mutations in this gene are present in patients with Tangier disease and people with familial HDL deficiency. These conditions are characterised by a severe or moderate decrease in HDL levels respectively and premature coronary artery disease. But HDL is not the only lipoprotein involved in cholesterol efflux. A paper published three years ago (Chung et al., 1998) reported in vitro efflux of cholesterol from red blood cells by all the lipoprotein classes in fasting plasma. Interestingly, they also observed a 356% increase in the cholesterol content of chylomicrons in postprandial plasma. Isolated chylomicrons supplemented into fasting plasma were 9 times more potent than HDL at boosting cholesterol efflux. With the rapid removal of chylomicrons from the circulation this evidence suggests an important role for chylomicrons in reverse cholesterol transport. However, there is no attempt to discriminate between purely cholesterol efflux effects and that of cholesterol transfer by CETP and incubations were carried out for 18h periods, a situation which does not exist in vivo, as clearance of chylomicrons occurs in a matter of minutes.

The effluxed cholesterol is esterified by the enzyme lecithin-cholesterol acyltransferase (LCAT) and as the pre-β HDL accumulate cholesterol ester they increase in size and are referred to as HDL3. These particles have a density of between 1.125 and 1.21g/ml. They participate also in cholesterol efflux and are also acceptors of surface remnants. As they increase in size they are referred to as HDL2, the largest HDL particle (density 1.063-1.125g/ml). Both HDL 2 and HDL 3 contain apo Al (two to four copies per particle) and apo All (one or two copies per particle) (Cheung et al., 1987). Some particles of these classes may however contain apo Al only. These particles are rich in both apo E and apo C and constitute the major apo E and apo C pool in the circulation. It is from this apolipoprotein pool that both apo C and apo E are donated from HDL to the TRL postprandially and also in the reverse direction once TRL lipolysis occurs. Kinetic studies in vitro with radiolabelled apo CIII have shown that this transfer is not dependant on the number of donor or receptor particles present and that transfer can occur across a 50kDa molecular mass cutoff semipermeable membrane suggesting that transfer of apo CIII at least is achieved by an aqueous diffusion mechanism (Boyle et al., 1999). Rich in cholesterol
ester, HDL\textsubscript{2} particles participate in the exchange of this cholesterol for triglyceride from the apo B-containing particles by the action of the large protein complex - cholesteryl ester transfer protein (CETP) (Hesler et al., 1987). The effluxed cholesterol may then be taken up by the liver via apo B- or apo E-mediated clearance of TRL or directly from HDL by binding through the SR-BI and SR-BII (Webb et al., 1997. Just this year a 95kDa protein with strong HDL binding ability was discovered in fetal hepatocytes which has subsequently been shown to be widely expressed in human cells and cell lines (Bocharov et al., 2001). Authors claim it to be new HDL receptor candidate which may participate in HDL particle or cholesterol uptake. From here the cholesterol may be excreted from the body, completing reverse cholesterol transport.

**1.3.18 Apolipoproteins Al, AII and AIV.**

Apo AI is a 243 amino acid protein, the gene for which is located on chromosome 11q and is part of a 15kb gene cluster with apo CIII and apo AIV (Naganawa et al., 1997). It is secreted by both the small intestine and the liver as pro-apo AI, which undergoes extra cellular cleavage of a hexapeptide by a circulating peptidase to yield the mature apolipoprotein. It has a molecular weight of 28,330Da and has a concentration of approximately 1g/ml in normal plasma, making it one of the most abundant proteins in human plasma. About 8% of this occurs free in the plasma (Neary and Gowland, 1987), the majority is associated with chylomicrons and HDL. Low plasma apo AI has been identified as an independent risk factor for CAD, even in subjects which would otherwise be classed as being low-risk (Francis and Frohlich, 2001). It is rapidly transferred from chylomicrons as a result of the action of LPL to become part of HDL. It has been demonstrated that free apo AI is more efficient than plasma HDL at promoting cholesterol efflux from macrophage-foam cells, indicating that it represents a major metabolic pathway of cellular cholesterol efflux (Yancey et al., 1995). Apo AI activates LCAT and is thought to be a ligand for the SR-BI (Acton et al., 1996). It has been shown recently however that apo AI knockout mice do not accumulate cholesterol ester in the adrenal glands even though HDL particles do bind normally to the SR-BI, suggesting a role for apo AI in cholestrol transfer beyond a receptor binding function (Williams et al., 2000). Knockout mice which
do not express the SR-BI receptor have a significantly increased cholesterol level and decreased adrenal gland cholesterol content - one of the major tissues responsible for the uptake of HDL cholesterol (Rigotti et al., 1997). Recently, a second receptor SR-BII has been described as having HDL cholesterol uptake activity and has been shown to be a splice variant of the same gene (Webb et al., 1998).

In comparison to apo AI, there is relatively little known about apo AII. It exists as a 17,400Da disulphide-linked homodimer in humans and higher primates and as a 8,600Da monomer in lower animals. Also, of both of these apolipoproteins, only the plasma concentration of apo AI is inversely correlated with the development of atherosclerosis (Kwiterovic et al., 1992). It has also been shown in transgenic mice that the presence of apo AII on HDL diminishes the anti-atherogenic effect of apo AI (Schultz et al., 1993). This is consistent with the observation previously that HDL particles containing only apo AI were atheroprotective and those containing both apo AI and apo AII were not (Pichois et al., 1987). Apo AII is present on HDL in plasma and has also been identified in human peripheral lymph (Reichl et al., 1989). The role of apo AII on HDL is not well defined. It has been shown to modulate the activity of LCAT by decreasing its binding activity to reconstituted HDL particles (Durbin and Jonas, 1997). It has also been suggested that there is a role for apo AII on native HDL in stimulating hepatic lipase activity (Mowri et al., 1996). It has also been suggested that apo AII exerts its effect on HDL metabolism by modifying apo AI structure and function, making apo AI more easily exchangeable from the HDL particle (Durbin and Jonas 1997). Recently a more positive role for apo AII has been suggested. Studies in vitro using reconstituted HDL showed that particles containing both apo AI and apo AII had a 4-5-fold greater cholesterol uptake ability into SR-BI-transfected Chinese hamster ovary cells, despite a reduction in binding (de Beer et al., 2001). Clearly the role of apo AII is not fully defined.

Apo AIV is a 46,000Da protein produced in the intestine and is found free in interstitial fluid and plasma or present on chylomicrons and HDL. The actual function of apo AIV is not defined but it has been shown to be an activator of LCAT (Steinmetz and Uterman,
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1985) and may influence LPL activity (Goldberg et al., 1990). It has also been shown to mediate cholesterol efflux from adipocytes in vitro (Steinmetz et al., 1990).

1.3.19 Lecithin-Cholesterol Acyltransferase.

Central to the metabolism of plasma lipoproteins, LCAT is responsible for the esterification of cholesterol in lipoproteins and controlling the levels of free and esterified cholesterol in the various cells of the body. It is a 416 amino acid peptide secreted by the liver and has a molecular weight of 47,090Da. It is a serine hydrolase and it catalyses the transfer of an acyl chain from the sn-2 position of phosphatidylcholine (previously called lecithin) to cholesterol, producing lysophosphatidylcholine and cholesterol ester, or in the absence of cholesterol it functions as a phospholipase, producing free fatty acids. It is also responsible for the re-distribution of fatty acyl groups between position 1 and 2 on phosphatidylecholine (Czarnecka and Yokoyama, 1993). Cholesterol ester is a more hydrophobic molecule than free cholesterol and may be carried in the core of the particle. This reaction takes place on the surface of HDL and is mediated, as described in section 1.5.17, by both apo AI and AII. As such, LCAT is responsible for the maturation of HDL, the removal of excess cholesterol from the inner leaf of the cell membranes and from lipoproteins such as LDL, VLDL and chylomicrons. The most effective substrate of LCAT in nascent, discoidal HDL which contains only apo AI (Hamilton et al., 1976). A region of apo AI spanning amino acids 143 to 165 has been shown to be essential for the activation of LCAT (Sorci-Thomas et al., 1993).

In patients with LCAT deficiency, free cholesterol levels in the plasma are raised, there are high levels of small spherical and discoidal nascent HDL particles and lamellar structures of LDL density (Jonas, 1991). These deficiencies may be in both HDL and apo AI, such as Tangier disease and Fish-eye disease, where the level of apo AI in the plasma is 50% and 15%, respectively, of normal controls (Chen and Albers, 1985; Funke et al., 1991). Here the mass and activity of LCAT is reduced to between 20 and 60% of controls, but the specific activity is equal to controls, indicating that the enzyme itself is fully functional but not under these conditions of reduced apo AI. Familial LCAT deficiency may cause a
reduced (50% in heterozygotes) or absent (in homozygotes) LCAT activity, along with a reduction in apo AI mass (Frohlich et al., 1988). Observations suggest that HDL components are reduced in the plasma of these patients due to a rapid catabolism of HDL (Teremoto et al., 1986). It should also be mentioned that smoking has a profound effect on LCAT activity, amongst other things (De Parscau and Fielding, 1986; Moriguchi et al., 1990; Dullart et al., 1994). Alterations in CETP and phospholipid transfer protein (PLTP) activities along with a reduction in the level of apo E associated with HDL have been proposed as the mechanism for the high levels of apo B48-containing, postprandial particles and low levels of HDL which are seen in smokers (Mero et al., 1998) and may help to explain the higher incidence of atherosclerosis in smokers (Gottlieb et al., 1994).

1.3.20 Cholesteryl ester transfer protein.

This hydrophobic glycoprotein appears as a broad band between 66,000 and 74,000Da on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Hesler et al., 1987). Cloning experiments have shown human plasma CETP to be a single polypeptide species of 476 amino acids, 44% of which are hydrophobic residues, giving it an unusually high hydrophobicity compared even to apolipoproteins (Drayna et al., 1987). The CETP gene consists of 16 exons separated over about 25kb located on the long arm of chromosome 16, near the LCAT locus (Lusis et al., 1987). In humans, it is expressed mainly in the liver and adipose tissue, with lower levels of expression in the small intestine, adrenal gland, kidney and heart (Drayna et al., 1987). Cholesteryl ester transfer activity has been found in cerebrospinal fluid (Albers et al., 1992). The protein is heavily sialylated and glycosylated post-transcriptionally. In human plasma it exists primarily, attached to HDL (80%) through a neutral lipid binding site in the C-terminal domain of the peptide (Swenson et al., 1989) and also via hydrophobic interactions between positively charged residues and the negatively charged head groups of HDL phospholipid (Pattnaik and Zilversmit, 1979).

Plasma CETP mediates the transfer or exchange of neutral lipids between lipoproteins. Unlike an enzyme, CETP is energy-independent, and must work down a pre-existing
concentration gradient. This may mean an equimolar transfer of cholesterol ester from HDL to the apo B-containing lipoproteins and triglyceride in the opposite direction (Yen et al., 1989). But CETP can also transfer cholesterol ester from HDL without the back-transfer of triglyceride (Barter et al., 1990).

There are three proposed mechanisms for the transfer of lipids via CETP (Tall, 1993). First, that CETP can act as a lipid carrier between donor and acceptor lipoproteins. Evidence for this exists in the fact that CETP can bind free neutral lipids and these protein/lipid complexes have been isolated from plasma (Swenson et al., 1988). This mechanism relies on the random collision of HDL with the various lipoproteins. The second mechanism proposed is the formation of a ternary complex of donor and acceptor lipoproteins with CETP. The CETP binds first to the donor and while still attached, travels through the plasma and binds to the acceptor and transfer of lipid occurs. In this way CETP acts as a hydrophobic "bridge" across which lipids can traverse in both directions. This mechanism suggests an alternative and distinct binding site for donor and acceptor lipoproteins on CETP. The third proposed mechanism involves the formation of a similar ternary structure, except CETP exists as a dimer. This would mean that both donor and acceptor could bind without the existence of a second binding site. There is no evidence, however to show that dimer formation is necessary for lipid transfer.

The net transport of cholesteryl ester by CETP depends mainly on the availability of suitable TRL acceptor particles, not on the CETP concentration (Mann et al., 1991). Transfer activity of CETP is enhanced by the lipolysis of VLDL in vitro. This has been shown to be due to the build-up of free fatty acids of the surface of the VLDL which enhances binding of CETP (Sammett and Tall, 1985). There is also evidence to show that the length and unsaturation of these free fatty acids is a factor in this enhanced transfer activity (Lagrost and Barter, 1991). The enrichment of lipoproteins with unesterified cholesterol may also enhance transfer of cholesteryl ester from HDL to the TRL (Morton, 1988). Both of these situations exist in type 2 diabetes (Reaven, 1988; Watala et al., 1989;
1990; Juhan-Vague et al., 1986) and may explain in part the observed increase in CETP activity in these patients.

Studies in trans- and normogenic animals overexpressing CETP show a reduction in HDL cholesterol by 20-30%, an increase in VLDL and LDL cholesterol and an increase in plasma apo B (Marotti et al., 1992 and Groener et al., 1989). A strong positive correlation between CETP levels and LDL cholesterol and apo B levels has been demonstrated in animals fed an atherogenic, high cholesterol diet (Quinet et al., 1990). Point mutations causing splicing defects in the CETP gene were described about ten years ago, which are responsible for CETP deficiency in humans (Brown et al., 1989). Homozygotes for the mutation have a complete absence of the protein and very high levels of HDL in the plasma. They also tended to have lower LDL cholesterol and apo B levels. Both advantageous and detrimental effects may be seen on elevation and diminution of CETP activity. On one hand, a reduction results in an elevation of HDL cholesterol and its associated benefits, along with lower LDL cholesterol and lower apo B. On the other hand, an increase in CETP activity may improve efflux of cholesterol from cells and its eventual removal from the body. As with many biological systems, the appropriate level may depend on many other factors, which in concert perform a fine balancing act.

1.3.21 Antioxidant properties of HDL.

The oxidative modification of LDL is central in the pathogenesis of atherosclerosis. Over recent years it has been elucidated that HDL acts to prevent this occurring by interruption of the lipid-peroxidation cascade by the enzyme paraoxonase 1 (PON 1) (Mackness et al., 1991). This enzyme is carried on HDL and its activity is decreased in subjects who have suffered a myocardial infarction (McElveen et al., 1986). A number of polymorphisms of the PON 1 gene which reduce its capacity to protect HDL from oxidation such as those at amino acid 55 and 192, and have been associated with the presence of CHD. The activity and concentration of the enzyme have also shown to be important in determining the presence of the disease (Mackness et al., 2001). Low serum PON 1 activity has been demonstrated in Type 2 diabetic patients with retinopathy (Mackness et al., 2000).
However PON 1 activity does not seem to contribute to the greater risk of CHD in Type I diabetes (Valabhji et al., 2001). Interestingly, a study carried out in Type II diabetic patients showed that thermally stressed dietary olive oil increased paraoxonase activity postprandially compared to safflower oil (polyunsaturated) (Wallace et al., 2001). The authors suggest that an olive oil diet may be preferential to a polyunsaturated fat diet for Type II diabetic patients.

### 1.4 Diabetic Dyslipidaemia.

As mentioned in section 1.2.3, the dyslipidaemia evident in diabetic patients plays a part in their higher incidence of vascular disease, along with hypertension, hyperglycaemia, hyperinsulinaemia and insulin resistance. Although not always strictly the case, the classical lipid abnormalities are hypertriglyceridaemia, due largely to increased levels of VLDL and also chylomicrons and a decrease in HDL cholesterol. Both of these have been shown to be potent predictors of coronary artery disease morbidity and mortality (Laakso et al., 1993; Hanefeld et al., 1996). There is also evidence that LDL (Feingold et al., 1992; Reaven et al., 1993) and HDL (Lane et al., 1991; Taskinen, 1996) particle density is altered in diabetes to the more atherogenic subclasses. The concept of atherosclerosis being a postprandial phenomenon was introduced by Zilversmit (1979) over twenty years ago and since then has been confirmed by others (Patsch et al., 1992; Karpe et al., 1994; Tkac et al., 1997). Levels of intestinally-derived postprandial particles are significantly raised in diabetes (Curtin et al., 1994) and their role in the development of atherosclerosis is a matter of continuous investigation and debate.

#### 1.4.1 Intestinally-Derived Postprandial Particles.

Elevated levels of apo B48 have been demonstrated in type 2 diabetes fasting and postprandially (Curtin et al., 1994). There is increasing evidence to suggest that chylomicrons and their remnant particles are atherogenic, including the discovery of a receptor on the surface of macrophages which binds apo B48 (Gianturco et al., 1998).
More recently this group have identified this receptor on a human endothelial cell line (Bradley et al., 1999). It was a number of years ago that chylomicron remnants were shown to be able to gain access to human smooth muscle cells, following endothelial injury and contribute to cholesterol accumulation (Floren et al., 1981). More recently, visual evidence of this has been demonstrated using fluorescent-labelled chylomicron remnants (Proctor and Mamo, 1998). Investigators were able to see that chylomicron remnants rapidly penetrated arterial tissue and that chylomicron remnant cholesterol accumulates in the subendothelial space. This evidence is consistent with the idea of atherogenesis being a postprandial phenomenon and may go some way to explaining the positive correlation which has been demonstrated by Karpe and colleagues (1994) between apo B48 in the remnant fraction and the progression of atherosclerosis in non-diabetic men which was unrelated to triglyceride levels. These investigators published results recently showing that mean postprandial remnant lipoprotein cholesterol is strongly correlated with common artery intima-media thickness (thickening being an early stage in atherogenesis) in healthy middle aged men (Karpe et al., 2001). They also showed that the postprandial increase in remnant cholesterol was strongly related to the generation of TRL apo B48 and large TRL apo B100. It has been suggested that because of the extremely rapid clearance of chylomicrons and their remnants normally, that they do not contribute significantly to the atherosclerotic process (Nordestgaard and Nilson, 1994). But chylomicron metabolism in diabetes seems to be anything but normal, with significantly elevated numbers in the plasma postprandially and a significantly higher number present in fasting plasma, the indication is that in the diabetic patient chylomicron remnants have a extended residence time in the blood, making them more likely to contribute to plaque formation.

There seem to be a number of abnormalities in diabetes which might explain the elevation of intestinally-derived particles. A study was carried out in type 2 diabetic patients, using a double isotope method to trace ingested cholesterol and showed that absorption of cholesterol from the intestine was significantly lower in comparison to controls but cholesterol synthesis was significantly higher (Gylling and Meittinen, 1997). This corroborates evidence that the expression, (Feingold et al., 1994) and activity (Feingold et
of HMG CoA reductase, the rate-limiting enzyme in cholesterol synthesis, is increased in diabetic animal models. Somewhat contradictory to this, the enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT) which is the rate-limiting step in cholesterol absorption in the intestine has been shown to be increased in diabetic animals fed cholesterol (O'Meara et al., 1991; Maechler et al., 1992). An increase in particle size in lymph chylomicrons isolated from diabetic rats (Gleeson et al., 1999) and an increase in the number of chylomicron particles in lymph isolated from alloxan-treated diabetic rabbits (Phillips et al., submitted) has been observed. A 4-fold increase in MTP expression has been demonstrated in the intestine of diabetic rats (Gleeson et al., 1999) and a 2-fold increase in the intestine of diabetic, cholesterol-fed rabbits compared to non-diabetic cholesterol-fed animals (Phillips et al., 2001 submitted 2).

This former study in rabbits also demonstrated a reduced rate of clearance of chylomicron particles in diabetes and that both the particle isolated from diabetic animals and the diabetic state itself was important in this effect. Particles isolated from lymph of diabetic animals was significantly depleted of apo E and may be contributing to the reduced clearance. Interestingly, the overexpression of apo E in transgenic, streptozotocin-induced diabetic mice was shown to prevent hyperlipidaemia compared to diabetic wild-type mice and that the reduction in plasma cholesterol and triglyceride levels was due to the disappearance of apo B-containing lipoproteins (Yamamoto et al., 1995). There is, however, no differentiation made between hepatically- and intestinally-derived particles. Transcription of the APOCIII gene has been shown to be increased and is related to an increase in plasma triglyceride levels in streptozotocin-induced diabetic mice (Chen et al., 1994). This suggests a higher level of LPL inhibition and a reduced rate of particle clearance in diabetes. An interesting study published recently used streptozotocin-induced mice, engineered to produce only apo B100 or only apo B48 to show that a reduction in the hepatic heparin sulphate proteoglycan core protein, called perlecan, may be responsible for the reduced rate of clearance of apo B48-containing lipoproteins (Ebara et al., 2000). A reduced rate of clearance of chylomicrons has ramifications for the metabolism of hepatically-derived particles as they share a common method of lipolysis and uptake.
1.4.2 Hepatically-Derived Postprandial Particles.

Postprandial lipaemia or fat intolerance is an inherent feature of diabetic dyslipidaemia (Chen et al., 1993; DeMann et al., 1996) and much of this is due to disturbance in the regulation of VLDL metabolism in the liver. Normally the insulin secreted following the ingestion of a meal acts to reduce VLDL secretion in vivo in humans (Pietri et al., 1983) and in cultured rat hepatocytes (Patsch et al., 1986; Sparks et al., 1989) and HEP-G2 cells (Dashti and Wolfbauer, 1987), even though it has been shown to enhance triacylglycerol production in the liver (Durrington et al., 1982; Björnsson et al., 1992). This is achieved by the reduced translation of apo B mRNA (Theriault et al., 1992) and increased apo B degradation (Yao and Vance, 1988; Sparks and Sparks, 1990). Insulin also suppresses fatty acid release from adipose tissue (Lewis et al., 1995), possibly through hormone sensitive lipase, reducing substrate for the production of VLDL in the liver. This is disturbed in insulin resistance (Gibbons, 1990). In states of prolonged hyperinsulinaemia, such as that seen in insulin resistance and type 2 diabetes, this regulation is disturbed and VLDL secretion is enhanced. This has been shown in rat hepatocyte cultures exposed to insulin for longer than 24h (Björnsson et al., 1992) and is evident also in diabetic patients (Tobey et al., 1981; Malmström et al., 1997). Recently, it has been shown in a fructose-fed hamster model of insulin resistance that there is an increased stability of apo B and enhanced expression of MTP, facilitating enhanced assembly and secretion of VLDL (Taghibiglou et al., 2000).

There is also evidence for a reduced rate of clearance of VLDL particles. This may be due to saturation of LPL by increased numbers of postprandial particles (Poapst et al., 1985; Martins et al., 1996). This may be considerably exacerbated by the presence of higher than normal number of apo B48-containing particles (Curtin et al., 1994; Steiner et al., 1998). Martins and co-workers (1996) have shown that chylomicrons are the preferred substrate for LPL. Insulin has been shown to increase LPL levels in adipose tissue and diabetes has been associated with diminished tissue LPL activity (Taskinen et al., 1981). The observation of increased APOCIII gene expression in diabetic mice (Chen et al., 1994) may also be of significance in reducing the lipolysis of VLDL particles as well as chylomicrons.
A recent study of VLDL subfractions in type 2 diabetic patients showed that both the largest and the smallest subfractions were more susceptible to oxidation in the patients compared to control VLDL and also that diabetic subfractions contained significantly less monounsaturated fatty acids compared to control VLDL (McEneny et al., 2000). This may indicate a more atherogenic VLDL particle in type 2 diabetes, contributing to their higher incidence of cardiovascular disease.

1.4.3 Diabetic LDL.

Elevated LDL cholesterol has been reported in type 2 diabetes (Reaven, 1987). Stinson and colleagues (1993) showed that normal postprandial suppression of cholesterol synthesis is disrupted in type 2 diabetes and that hyperinsulinaemia was associated with the stimulation of cholesterol synthesis in both type 1 and type 2 diabetes (Stinson et al., 1993). However, results from the Framingham Offspring Study showed that diabetes was not associated with an increase in LDL cholesterol (Siegel et al., 1996). It did, on the other hand show that diabetic patients are more likely to have small dense LDL particles compared to non-diabetic subjects. Decreased LDL particle size has been associated with premature coronary artery disease (Austin et al., 1988; Griffin et al., 1994) and indeed has been reported to be an independent predictor of the disease (Campos et al., 1992). The small LDL subclass has been associated with hypertriglyceridaemia (James and Pometta, 1991), with reduced clearance of TRL (Feingold et al., 1992) and with insulin resistance (Reaven et al., 1993). It has also been shown that postprandially, chylomicron remnant clearance is a major determinant of the preponderance of small dense LDL in type 2 diabetic patients (Tan et al., 1995). Recently it has been suggested through compartmental modelling, that large, triglyceride-rich VLDL particles which occur in hypertriglyceridaemic states including diabetes, have been shown, through differential lipolysis, to be the precursors of small dense LDL species (Packard et al., 2000).

Modification of lipoproteins is important in the development of atherosclerosis and the oxidation of LDL has long been thought to be the major modification which promotes plaque formation (Steinberg et al., 1989). Diabetic LDL is enriched in linoleic acid and has
been shown to be more easily oxidised than oleic acid-rich LDL isolated from diabetic patients (Dimitriadis et al., 1995; 1996). Oxidised LDL is known to be avidly taken up by macrophage-derived foam cells through scavenger receptors (deVries et al., 1999) and oxidatively modified apo B-containing lipoproteins have recently been shown to induce monocyte chemotaxis and adhesion to the endothelium (Lee et al., 1999), all processes in the progression of plaque formation. Chronic hyperglycaemia also induces an increase in non-enzymatic glycation of circulating and indeed structural proteins along with a glucose-generated oxidative and carbonyl stress, known as glycoxidation. Glycoxidation endproducts play a role in the formation of superoxidative products leading to lipoperoxidation of lipoproteins. This is an irreversible process and has grave implications for diabetic patients as it is related to mean blood glucose. LDL particles modified in this way have been shown to contribute to the development of atheroma in diabetic rats (Meng et al., 1998).

1.4.4 Diabetic HDL and Reverse Cholesterol Transport.

Reduced plasma HDL cholesterol is a typical feature of type 2 diabetes (Howard, 1987; Siegel et al., 1996). These patients have reduced levels of HDL particles containing both apo Al and apo AII (Syävnne et al., 1995). This group later demonstrated that these particles are positively related to cholesterol efflux (Syävnne et al., 1996). Particles containing apo Al only are also reported to be reduced in type 2 diabetic men (Cavallero et al., 1995). The singular atheroprotective importance of these particles has been reported (Fruchart et al., 1994). The effect of diabetes on HDL particle size has been investigated and a number of studies have shown a preponderance of small particles, low in cholesterol (Singh et al., 1995; Taskinen, 1996) which is also indicative of defective reverse cholesterol transport. Recently the reduction in the ratio of LPL to HL activity in type 2 diabetes has been implicated in increasing the triglyceride content of HDL and the fractional catabolic rate of apo Al and reducing HDL (Frenais et al., 2001).

There is considerable disagreement in the activity of the enzymes involved in reverse cholesterol transport. The activity of LCAT is reported to be elevated in type 2 diabetes
(Jones et al., 1996; Riemens et al., 1998). Cholesteryl ester transfer activity is not so well defined. There are studies reporting raised CETP activity in type 2 diabetes (Jones et al., 1996, Durlach et al., 1996; Riemens et al., 1998) related to the elevated TRL levels in these patients. Others have related the higher free cholesterol content in TRL which is evident in type 2 diabetes to an elevation (Bhatnagar et al., 1995) and a reduction in CET (Fielding et al., 1984). Recently, increased glycation of lipoproteins in poorly-controlled diabetic patients has been reported to be responsible for increased CET activity (Passarelli et al., 1997). There is yet another report of no difference in either fasting or postprandial CETP activity between diabetic and non-diabetic subjects (Lottenberg et al., 1996). There is also varying opinions on the part increased CETP activity has to play on the increased incidence of vascular disease in diabetes. Increased transfer would suggest a more efficient reverse cholesterol transfer mechanism, but on the other hand it may also lead to a reduction in HDL cholesterol. Compelling evidence for a detrimental effect of elevated CETP activity is its association with angiographically-confirmed coronary artery disease, again related to the enrichment of TRL with free cholesterol (Bhatnagar et al., 1993).

1.4.5 The cholesterol-fed diabetic rabbit- a model of postprandial dyslipidaemia.

The development of diabetes in rabbits by the production of cytotoxic reactive oxygen species on administration of alloxan and the accompanying severe hypertriglyceridaemia on cholesterol feeding has been utilized as an animal model of diabetic dyslipidaemia for over fifty years (Duff and McMillan, 1949). Within days of starting a cholesterol diet the serum cholesterol of normal New Zealand White rabbits raises from a low of approximately 1.5mmol/l by more than 10-fold (Bitman et al., 1976). This occurs because rabbits are unable to increase sterol excretion, resulting in enhanced secretion of hepatic particles which in turn causes saturation of the LDLR clearance pathway. Repression of LDLR expression results from the negative feedback of cholesterol, further exacerbating the situation. Early studies showed that the hypertriglyceridaemia produced in diabetic, cholesterol-fed rabbits resulted from a prevalence of partially metabolized intestinally-derived particles also (Van Sickle et al., 1985), making them a good model of diabetic
dyslipidaemia, as intestinal lipoprotein metabolism is known to be disturbed in diabetes (Curtin et al., 1995, 1996).

1.5 Dietary Fatty Acids.

1.5.1 Fatty Acids and Serum Lipid Levels.

The ability of dietary fats to influence total plasma cholesterol has been studied since the 1950s. It was also at this time that the fatty acid classes of saturated, polyunsaturated and monounsaturated were investigated for their cholesterolaemic effects (Keys, 1957). These investigators showed that saturated fatty acid was twice as effective in raising plasma cholesterol as polyunsaturated fatty acids were at lowering it. Monounsaturated fatty acids were considered neutral. Epidemiological studies revealed a positive association between saturated fat intake and risk of coronary heart disease (Kato et al., 1973; Kromhout and de Lezenne Coulander, 1984; Kushi et al., 1985; McGee et al., 1985). As such, dietary guidelines for the general public were made, to increase polyunsaturated fat at the expense of saturated fat. This is where clarity and general agreement ends in the evaluation of dietary fatty acids. There has been relentless investigation in this field and the literature is enormously divergent, with very few undisputed conclusions. Within the saturated fats, for example, there seems now to be differential effects with varying chain length. So those with 12, 14 carbon chains (lauric and myristic) increased total plasma cholesterol and the 18 carbon stearic acid was either neutral (Hegsted et al., 1965) or as effective as oleic acid in reducing plasma cholesterol (Bonanome and Grundy, 1988). There is also some contention as to the effect of 16 carbon palmitic acid. Hypercholesterolaemic (Zock et al., 1994) or similar (Tholstrup et al., 1994) effects relative to myristate (14:0) have been observed. Palmitoleic acid (16:1), a minor monounsaturated fatty acid which has commanded very little investigative attention, is now considered to be more like a saturated fat (Nestel et al., 1994).
In the past 15 years or so, studies have challenged the idea of monounsaturated fatty acids being "neutral". Mattson and Grundy (1985) were the first to show that oleic acid (18:1), the main monounsaturated fatty acid in plasma, was as effective in decreasing LDL cholesterol as linoleic acid, a polyunsaturated fatty acid. This has been confirmed by others (Zock and Katan, 1992; Gustafsson et al., 1992; Kris-Etherton et al., 1993). A more recent study demonstrated the reduction of both plasma cholesterol and triglyceride with a monounsaturated fatty acid diet compared to saturated fat (Kris-Etherton et al., 1999). A study by Howard et al. (1995) substituted monounsaturated fat for polyunsaturated fat incrementally in the diet and showed that while at low levels polyunsaturated fat was slightly better at reducing plasma cholesterol than monounsaturated fat, at higher levels polyunsaturated fat also reduced HDL cholesterol. This did not occur with the monounsaturated fat diet. As with most observations in this area, there are exceptions. These are detailed in a meta-analysis by Gardener and Kraemer (1995). This observation with HDL had been seen previously and the effect on HDL was shown to be similar to that of saturated fat (Mata et al., 1992). An interesting study in hamsters suggested a number of mechanisms to try to explain the differential effects of linoleic acid and oleic on plasma cholesterol levels in animals fed a moderately high cholesterol diet (Kurushima et al., 1995). Both fatty acids prevented hepatic LDLR suppression which would normally occur with an increase in cholesterol. Only oleic acid, however, increased hepatic cholesterol 7a-hydroxylase activity, the rate limiting enzyme in the conversion of cholesterol to bile acids, suggesting an enhanced rate of cholesterol excretion. Oleic acid also prevented the raise in CETP activity which was evident in animals fed cholesterol alone or cholesterol and linoleic acid, possibly impacting HDL levels. Studies in cultured human skin fibroblasts, J774 macrophages and HepG2 cells have shown that oleic acid enhances ACAT activity, as oleate is a precursor of co-enzyme A, an integral component of the ACAT enzyme (Rumsey et al., 1995). They suggest that this activation of ACAT shifts the balance of esterified and free cholesterol in the cell, to reduce the free cholesterol and increase LDLR activity. It is thought that this pool of free cholesterol regulates gene expression of the LDLR.
Epidemiological studies in Greenland Eskimos (Kromann and Green, 1980) showed that despite a diet high in fat, Eskimos had an incidence of ischaemic heart disease which was about 8% that of the comparable Danish population. Eskimos consume large quantities of n-3 fatty acids - about 7g/day, which is over 100 times higher than in most western diets, from which they obtained from marine sources. This fuelled intense investigation into the atheroprotective properties of fish oils rich in n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (C20:5) and docosahexaenoic acis (22:6), which are reviewed by Harris (1989). In this, it is concluded that n-3 fatty acids lower plasma triglycerides, especially in hypertriglyceridaemic patients, increase HDL cholesterol slightly (5-10%) and show very little effect on plasma and LDL cholesterol levels. The consumption of fish was related to arterial wall pathology in a study in Australia. They concluded that as little as one or two fish dishes per week may prevent CAD by improving arterial wall characteristics (Walqvist et al., 1989). This did not extend to the type 2 diabetic patients in this study group.

With the widespread acceptance that dietary saturated fat is detrimental to health, the consumption of butter has been greatly reduced and replaced by margarines and spreads made from various different fat sources. To obtain a texture similar to that of butter the fats used, mainly vegetable oils, are hydrogenated. This increases the unsaturation of the fatty acids, producing an artificially high incidence of trans (geometric isomers) rather than the more normal cis double bonds. There are, however, several reports of adverse effects of these trans fatty acids on plasma lipids, the first of these being made by Mensink and Katan (1990). They showed that dietary trans fatty acids actually raised LDL cholesterol and lowered HDL cholesterol in humans. Epidemiological studies also indicate that high intakes of trans fatty acids increase the risk of CHD (Ascherio et al., 1996), although there are again contradictory publications (Aro et al., 1995).

1.5.2 Dietary Fatty Acids and Postprandial Particles.

The majority of the studies investigating fatty acids with regard to postprandial lipoproteins involve the reduction of VLDL by n-3 fatty acids as its hypotriglyceridaemic
effect has been known for some time. It is generally believed that this effect is caused, for
the most part, by a suppression in hepatic VLDL triglyceride and apo B secretion, which
has been demonstrated both in isolated rat hepatocytes (Lang and Davis, 1990) and in
humans in vivo (Nestel et al., 1984). The former group also suggest that the observed
reduction in VLDL secretion is not related to an alteration in triglyceride synthesis. Studies
in primary rat hepatocytes showed that eicosapentaenoic acid, when added to culture
medium, increased the rate of both apo B48 (the main form of hepatic apo B in rat) and apo
B100 degradation and showed no effect on the rate of synthesis of either (Wang et al.,
1993). Disturbance in VLDL particle assembly and the targeting of apo B for degradation
has been shown in hepatocytes of hamsters fed fish oil (Kendrick and Higgins, 1999).
Brown and co-workers (1999), however, report divergent results depending on whether the
fatty acids are added to culture medium of whether hepatocytes are isolated following a
diet rich in the n-3 fatty acids. In the latter situation they do report an inhibition of cellular
triglyceride synthesis and only apo B48 VLDL was inhibited and not apo B100 VLDL.
Synthesis of apo B48 was also decreased in this situation. The authors suggest that
differential results may be due to genetic modulation through PPARs, for which
polyunsaturated fatty acids have been shown to be ligands (Rustan et al., 1992) and which
may only be facilitated by prolonged exposure to n-3 fatty acids in the diet. Evidence for
the effect of oleic acid on hepatic particle secretion is mixed, depending on the cell type
used. There is no effect in primary rat hepatocytes (Patsch et al., 1982), increased secretion
of apo B48, triglyceride and cholesterol in HepG2 cells (Dixon et al., 1991) and a decrease
in apo B48 secretion in McArdle RH-7777 rat hepatoma cells (Sparks et al., 1997). A
recent study in patients with severe hypertriglyceridaemic men showed that n-3 fatty acids
were effective in reducing levels of large chylomicrons and VLDL, but had little effect on
chylomicron remnants (Westphal et al., 2000).

There are relatively few studies reported on the effect of dietary fatty acid on the intestine
and on chylomicron formation and secretion. A recent study in enterocytes isolated from
rabbits fed a high fat diet for a period of 2 weeks showed an increase in the secretion of apo
B48, triacylglycerol and cholesterol ester (Cartwright and Higgins, 1999). The response to
sunflower oil (linoleic acid) was greater than western diet lipid (saturated), which in turn was greater than fish oil. The authors suggest that the enterocytes adapt in the presence of different fatty acids and are "turned-on" to lipid absorption and that the extent of this adaptation is dependant on the type of dietary fatty acid. Oleic acid was reported to be the most potent stimulator of lipoprotein secretion by Caco-2 cells, followed in descending order by linoleic acid, linolenic acid and myristic and palmitic acids. A more recent study however, showed that relative to saturated fatty acid, oleic acid caused the production of larger, triglyceride rich chylomicrons by Caco-2 cells (van Greevenbroek et al., 2000). This may aid the clearance of chylomicron triglyceride as fewer, larger particles are known to be lipolysed more efficiently by LPL than a larger number of smaller particles (Martins et al., 1996). Chylomicrons produced in rats fed polyunsaturated fatty acid have been shown to be cleared more rapidly compared to n-3 fatty acids and oleic acid when injected into recipient animals (Bravo et al., 1995). This same group also studied the kinetics of LPL using as substrate, chylomicrons derived from rats fed various fatty acids. They showed that highest V\text{max} of LPL occurred with chylomicrons derived from olive oil and corn oil (linoleic acid), compared to fish oil and palm oil (palmitic acid). They also showed that olive oil-derived chylomicrons produced the highest K\text{m} for the enzyme, meaning that these actually had the lowest affinity for the enzyme. Clearly, this and the many other aspects of clearance need further investigation.

1.5.3 Oleic Acid, Linoleic Acid and Cardiovascular Disease

An epidemiological study, known as the Seven Countries Study (Keys et al., 1986), showed that death rates from CHD were relatively low in countries such as Greece and southern Italy, where the traditional diet was high in olive oil, which is rich in oleic acid. While there are other aspects to the Mediterranean-style diet which may contribute to this observation such as, a higher proportion of legumes, fruit, vegetables and grains, a slightly higher consumption of fish and a lower consumption of red meat, there is increasing evidence to show that oleic acid plays a very important role. Not least of which is the improvement in the LDL/HDL cholesterol ratio mentioned above. The benefit of this on the development of atherosclerosis is well established. Linoleic acid reduces both LDL and
HDL cholesterol, sometimes increasing the LDL/HDL cholesterol ratio (Howard et al., 1995). Linoleic acid was also implicated in the development of coronary artery disease following the observation that linoleic acid levels in adipocyte and platelet membranes were positively related to angiographically-assessed disease of the arteries (Hodgson et al., 1993). The membrane lipids of both these tissues are known to reflect dietary lipid intake (Garg et al., 1988; Seidelin, 1995). The intake of linoleic acid was also reported to increase the risk of new atherosclerotic lesions in human arteries (Blankenhorn et al., 1990). There is, however, directly contradictory evidence to this (Wood et al., 1984). A study in African Green Monkeys, where they were fed specific fatty acid-enriched, atherogenic diets for a period of 5 years, showed that a polyunsaturated diet was the best at preventing atherosclerosis, as measured by intimal area and that monounsaturated fatty acids were similar to saturated fatty acids in this regard, despite showing that the monounsaturated diet produced the lowest LDL/ HDL cholesterol ratio (Rudel et al., 1995).

The final report of the Lyon Diet Heart Study showed that the atheroprotective nature of a Mediterranean-type diet was maintained up to 4 years after the occurrence of a first myocardial infarction, reducing second infarcts and other cardiovascular complications when compared to a control group consuming a prudent Western-style diet (de Lorgeril et al., 1999). This study does not investigate the role of postprandial lipoproteins in the observed benefits, however an interesting aspect of their results was that there was no difference in either plasma lipids, blood pressure or glycated haemoglobin between the groups, suggesting the involvement of alternative effects in the atheroprotection of the Mediterranean-type diet. One such effect may be the ability of oleic acid to dramatically reduce the susceptibility of LDL to oxidation. This was first shown in rabbits fed diets rich in either linoleic acid or oleic acid (Parthasarathy et al., 1990). The fatty acid composition of LDL can be altered by diet and Bononome et al., (1992) showed that a diet rich in monounsaturated fatty acids greatly reduced the oxidisibility of LDL isolated from men, compared directly to that isolated after a polyunsaturated fatty acid-rich diet and that this was independent of the antioxidant content of the LDL. Other studies have confirmed this comparison (Abbey et al., 1993; Aviram and Elias 1993). This latter group also
demonstrated that LDL isolated after 1 and 2 weeks on an olive oil diet reduced cholesterol accumulation in macrophages. A study published last year showed that certain subfraction of diabetic VLDL contained significantly less monounsaturated fatty acids compared to controls. all subfraction of diabetic VLDL had significantly higher levels of preformed peroxides and that the largest and smallest subfractions were significantly more susceptible to in vitro oxidation compared to control VLDL. (McEneny et al., 2000). The authors suggest that this atherogenic lipoprotein characteristic may be contributing to the elevated incidence of heart disease in diabetes.

An interesting study published two years ago showed a 53% reduction in monocyte chemotaxis and a 77% reduction in monocyte adhesion due to minimally oxidised LDL isolated from American subjects on a oleic acid-rich liquid diet compared to a similar linoleic acid diet (Tsimikas et al., 1999). These processes are central to atherosclerotic plaque formation. This paper detailed results of a second study carried out in Greeks, Greek-Americans and Americans. The Greek group, living in north-west Greece and consuming a diet typical of the area, mirrored the results obtained in the American subjects consuming a liquid oleic acid diet in the previous study - lower chemotaxis and lower cell adhesion, when compared to Greek-Americans and Americans, living in America and consuming the typical American diet. The LDL vitamin A and E content was similar in all groups. The interesting point about this study is that it shows that by simply altering the oleic acid content of the diet, LDL properties similar to those of native Greeks and which are central in reducing the development of atherosclerosis, can be obtained. This study highlights the specific importance of oleic acid in the Mediterranean diet.

1.5.4 Dietary Fatty Acids and Diabetes Mellitus.

Diet has been described as the "cornerstone" in the management of type 2 diabetic patients, from the point of view of achieving and maintaining good metabolic control, normalising dyslipidaemia and maintaining normal blood pressure. In the last decade, the dietary recommendation for diabetic patients have changed somewhat. The ADA, while in agreement that saturated fat should be avoided, recommended instead of the low fat/high
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complex carbohydrate diet which was suggested in 1986, that more of the percentage of the calories from fat or carbohydrate should be varied to suit the individual patients needs (ADA Position Statement, 1994). In most cases this involves an increase in fat intake and the debate arose as to the best type of fat to include in the diet. There are studies to recommend both monounsaturated and polyunsaturated (n-6 and n-3) fats.

In a ten-year follow up study, the risk of developing type 2 diabetes was related to the fatty acid content of serum cholesteryl esters, which are known to reflect diet over the proceeding weeks. They showed that those who went on to develop the disease had a significantly higher saturated fatty acid level and a reduced level of linoleic acid compared to those who did not (Vessby et al., 1994). There are studies outlining the benefits of a monounsaturated fat diet relative to a high carbohydrate diet. These show improvements in glycaemic control at fasting and especially postprandially (Garg et al., 1988; Lerman-Garber et al., 1994) on the monounsaturated diet. Dietary monounsaturated fatty acids have been shown to potentiate the positive effects on glycaemia produced by weight loss in patients (Low et al., 1996). Even moderate weight loss has been shown to result in marked improvements in glycaemic control as well as an improvement in lipid profile (Henry et al., 1991). Fish oils have also been shown to enhance insulin sensitivity in the miniature pig model (Behme, 1996), but there are reports of fish-oil supplementation raising LDL cholesterol levels in diabetic patients (Mori et al., 1990). Fish oil has also been shown to adversely effect glycaemic control in diabetic patients (Borkman et al., 1989). This may help explain the lack of association between fish oil intake and beneficial artery wall characteristics which has been shown in non-diabetic subjects but not in the type 2 diabetic group in the same study (Wahlqvist et al., 1989). A polyunsaturated fatty acid diet has been shown to improve insulin binding in adipocytes of control rats but not diabetic rats (Field et al., 1988). However, in a study of type 2 diabetic patients glucose transport was shown to be enhanced in isolated adipocytes after a diet rich in olive oil (oleic acid) compared to a diet rich in sunflower oil (linoleic acid) (Ryan et al., 1999). This was accompanied by an enrichment of adipocyte membranes with oleic acid. The lipid composition of cell membranes of diabetic people has been shown to have altered but there
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is very little agreement as to exactly what the alteration is (Watala, 1993). The importance of this with regard to insulin resistance has been discussed in section 1.1.11.

An important feature of dietary fatty acids is that they become incorporated into lipoproteins - not just the postprandial lipoproteins but LDL and HDL also. As discussed in section 1.4.3, different fatty acids have very different oxidation potential, linoleic acid having a very high potential for oxidation (Esterbaur et al., 1987). This has very important implications for the atherogenicity of the lipoproteins in which these fatty acids are contained. It has been shown that LDL from diabetic patients has an elevated level of linoleic acid (Dimitriadis et al., 1995), which may be due to dietary treatment or the diminished rates of microsomal fatty acid 6- and 9-desaturase which has been demonstrated in experimental models of diabetes (Faas and Carter, 1980). It has been demonstrated, however, in type 2 diabetic subjects that a diet rich in oleic acid marked reduces the oxidisibility of isolated LDL when compared to a diet rich in linoleic and this has been related to a higher amounts of oleic acid in the LDL (Dimitriadis et al., 1996).

While dietary mono- and polyunsaturated fatty acids seem, for the most part, to have similar effects on levels of fasting lipids, except HDL which has a tendency to be higher on monounsaturated fatty acid diets in non-diabetic subjects, there is very little information on the postprandial responses of diabetic patients after either diet and even less on the role the intestine may have to play in responding to changes in diet. With increasing evidence of vascular disease in diabetes being a postprandial lipid phenomenon, evaluation of various dietary fatty acids may lead to a practical, treatment option. This thesis endeavours to explore these issues paying particular attention to the complex role played by the apolipoproteins.
Chapter 2

Materials and Methods
2.1 MATERIALS.

All chemicals used were ACS grade and from Sigma/Aldrich, PO Box 14508, St Louis, Missouri 63178-9916, USA, unless otherwise stated.

2.1.1 Specialised Biochemicals.

Apolipoprotein standards Apo E, CI, CII, CIII, AI and AII. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone di-hydrochloride, Aprotinin.

Pharmacia
Clean gels for IEF, Gel Pool, Pre-mixed Ampholine pH 3.5-9.5, Pre-mixed ampholine pH 4.5-6.5.

Promega Corporation, 2800 Woods Hollow Rd., Madison, WI53711 - 5399 USA. Taq DNA polymerase, Hha I restriction enzyme, 10bp DNA step ladder.

Gentra Systems Inc., 13355 10th Ave. North, Suite 120, Minneapolis, MN 55441 USA.
Puregene® DNA isolation kit.

2.1.2 Specialised Apparatus.

Beckman Coulter Inc., Fullerton, CA 92834-3100
L7 Ultracentrifuge, Fixed angle rotor

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, Ca. 94547, USA.
Mini-Protean II electrophoresis cell, Pre-cast, Tris-HCl polyacrylamide gradient gels (4-15%, 10-20% and 15%)

Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan.
Gas Chromatograph model 14A with Flame Ionisation Detector and C-R6A chromatopac integrator.

Supelco Inc., Supelci Park, Bellefonte, PA 16823-0048, USA.
Supelcowax™ 10 capillary, fused silica cap column for Gas Chromatography 30m (length) x 0.25m (internal diameter) x 0.25μm (film).

Unicam Ltd., York St., Cambridge, UK.
2.2 SUBJECTS AND ETHICS

Type 2 diabetic subjects were recruited from patients attending the Adelaide Hospital Diabetic Clinic, Dublin. Non-diabetic control subjects were drawn from hospital orthopaedic wards and college and laboratory personnel. Subjects were male and female and between the ages of 25 and 70 years. All control subjects were within the normal lipid profile ranges.

All subjects who volunteered to take part in the studies involved in this thesis gave informed consent and all studies were approved by the ethics committee of the Federated Dublin Voluntary Hospitals.

2.3 BLOOD SAMPLING.

All venesections were carried out using the vacuutainer system. Blood for isolation of lipoproteins and determination of plasma lipids were taken into lithium heparin glass tubes. Fluoride oxalate tubes were used for blood from which glucose determination was made and tubes coated with ethylenediaminetetra-acetic acid (EDTA) for the determination of glycosylated haemoglobin.
2.4 SERUM ANALYSIS

2.4.1 Total Cholesterol.
Total serum cholesterol was estimated using a commercially available diagnostic kit from Roche Diagnostics Ltd., Sussex, England. This involved the colorimetric assay described by Roschlau et al., (1974). Commercially available cholesterol standards (0-80μg) and samples were measured in duplicate. Following addition of assay reagent, standards and samples were incubated at 37°C for 15 min. and absorbances measured at 500nm. A standard curve was constructed from which sample cholesterol content was determined.

2.4.2 Triglyceride.
Serum triglyceride content was measured using a commercially available diagnostic kit also from Roche Diagnostics Ltd., Sussex, England. This involved the colorimetric assay described by Wahlefeld, (1974), where standards (0-31μg) were used to construct a standard curve. Duplicate samples and standards were incubated at 37°C for 15 min and the absorbance measured at 500nm.

2.4.3 Glucose.
This was performed using a Beckman 2 glucose analyser using glucose oxidase reagent and an oxygen electrode. The machine was calibrated daily using the following standards (2.2, 8.3, 15.5 and 20.2mmol/l glucose). All samples were refrigerated immediately after being drawn and analysed on that day. Intra-assay variation was 1.73% and inter-assay variation was 1.82%.

2.4.4 Insulin.
This was performed using a microparticle enzyme immunoassay kit from Abbott Laboratories, Illinois, USA. Results are expressed in μU/ml. Intra-assay variation was 3.2% and inter-assay variation was 4%. The assay did not cross-react with pro-insulin.
2.4.5 Glycosylated Haemoglobin.

Blood HbA1c was determined by the enzyme immunoassay kit from Novo Nordisk Diagnostics Ltd., Cambridge, England, containing monoclonal antibody specific for HbA1c. The normal value is < 4.9%.

2.5 Design of Polyunsaturated and Monounsaturated Fat Diets and Fat-loading Meal.

2.5.1 Diets.

The two diets used in the human study are detailed below in Table 2.1. They were designed to alter the ratio of polyunsaturated fatty acids to monounsaturated fatty acids in the diet without altering the saturated fat content or the daily total caloric intake of the subject. This was achieved by the daily consumption of 30ml of either sunflower oil (Flora) which is rich in the polyunsaturated fatty acid - linoleic acid or 30ml of extra virgin olive oil (Don Giovanni) which is rich in the monounsaturated fatty acid - oleic acid. The subject’s usual dairy spread was replaced with the appropriate spread -Flora or Golden Olive for the duration of the diet. Diets were assigned in random order and for a period of 2 weeks each.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Linoleic acid-rich diet</th>
<th>Oleic acid-rich diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g)</td>
<td>97.6</td>
<td>97.6</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>150.19</td>
<td>150.19</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>105.24</td>
<td>93.9</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>36.8</td>
<td>25.6</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>31.3</td>
<td>57.6</td>
</tr>
<tr>
<td>Saturated</td>
<td>30.7</td>
<td>27.0</td>
</tr>
<tr>
<td>Total kcal</td>
<td>1899</td>
<td>1897</td>
</tr>
</tbody>
</table>
2.5.2 Test Meal

Following an overnight fast, patients were given a high fat test meal containing 50g of glucose which was consumed over 30 minutes. The caloric content was 1172 kcal in which 53% of calories were derived from fat and 28% from carbohydrates. The meal consisted of:

- 200ml orange juice
- 30ml sunflower or olive oil
- 30g breakfast cereal
- 150ml full fat milk
- 2 fried eggs
- 2 slices (60g) of bread fried in either sunflower or olive oil
- 2 fried tomatoes
- A cup of tea (200ml)

Blood samples were collected from each individual while fasting, prior to the test meal and at 2 hour time intervals after the meal for a total of 8 hours. Tables 2.2 and 2.3 give the specific constituents of the test meal and a breakdown of the various fat types.

The fat used in the preparation of the test meal was either sunflower oil or olive oil depending on the dietary period directly preceding the test. Table 2.3 details the amount of each fatty acid type in both test meals.
Table 2.2: Detailed constitutive analysis of test meal giving quantity, protein, fat and carbohydrate content and calorific value of each.

<table>
<thead>
<tr>
<th>Food</th>
<th>Quantity (g)</th>
<th>Protein (g)</th>
<th>Carbohydrate (g)</th>
<th>Fat (g)</th>
<th>Energy (Kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>150</td>
<td>9.9</td>
<td>14.1</td>
<td>5.7</td>
<td>97.5</td>
</tr>
<tr>
<td>Corn flakes</td>
<td>30</td>
<td>2.9</td>
<td>23.6</td>
<td>0.5</td>
<td>110</td>
</tr>
<tr>
<td>Sugar</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>White bread</td>
<td>40</td>
<td>4.1</td>
<td>8.1</td>
<td>14.9</td>
<td>223</td>
</tr>
<tr>
<td>Fried eggs</td>
<td>100</td>
<td>12.3</td>
<td>1.3</td>
<td>19.5</td>
<td>232</td>
</tr>
<tr>
<td>Oil</td>
<td>30</td>
<td>0</td>
<td>0.1</td>
<td>30</td>
<td>269</td>
</tr>
<tr>
<td>Fried tomatoes</td>
<td>40</td>
<td>1.3</td>
<td>0.5</td>
<td>2.7</td>
<td>27.5</td>
</tr>
<tr>
<td>Orange juice</td>
<td>200</td>
<td>1.1</td>
<td>26.1</td>
<td>0.2</td>
<td>132</td>
</tr>
<tr>
<td>TOTAL</td>
<td>610</td>
<td>31.6</td>
<td>93.8</td>
<td>73.5</td>
<td>1170</td>
</tr>
</tbody>
</table>

This meal contained approximately 580mg of cholesterol.

Table 2.3: Fat composition of the linoleic acid-rich and oleic acid-rich breakfasts in grams and, in parenthesis, as a percentage of total fat.

<table>
<thead>
<tr>
<th>Fat Composition of Breakfasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat type</td>
</tr>
<tr>
<td>Saturated</td>
</tr>
<tr>
<td>Polunsaturated</td>
</tr>
<tr>
<td>Monounaturated</td>
</tr>
</tbody>
</table>

These meals were designed to maximise lipoprotein changes without altering the patients' metabolic control. Participating subjects were encouraged to drink water throughout the study time period. No food of any kind was taken.
2.6 Lipoprotein Isolation.

Blood was collected into heparinised tubes by venepuncture and centrifuged within 1 hour to separate plasma and cells. One tube (10ml) was frozen at -20°C for deoxyribonucleic acid (DNA) isolation and apo E genotyping as described in section 2.12. The following preservatives and protease inhibitors were added to the plasma to prevent degradation of apo B: D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone di-hydrochloride (PPACK) (1mmol/l), phenyl methyl sulphonyl fluoride (PMSF) (0.1mmol/l), aprotinin (0.005%(V/V)), sodium azide (0.02%(V/V)) and EDTA (0.1mg/ml). A chylomicron-rich fraction was isolated as previously described (Curtin et al., 1996) by layering the plasma under an equal volume of saline (density 1.006g/ml) containing the above preservatives and centrifuging for 30 minutes at 20,000 rpm at 4°C in a Beckman L7-55 ultracentrifuge using a fixed angle rotor. Very low density lipoprotein (VLDL, density < 1.006g/ml) was isolated from the infranate by ultracentrifugation at 40,000rpm for 24h at 4°C. The density of the sample infranate was then raised to 1.025g/ml and centrifuged for 24h to remove intermediate density lipoprotein (IDL). Low density lipoprotein (LDL, density 1.025-1.063g/ml) was removed and fasting samples retained. High density lipoprotein (HDL, density 1.063-1.21g/ml) was isolated from samples by ultracentrifugation of the remaining infranate (Havel et al., 1955). Chylomicron and VLDL apo B48 and apo B100 were analysed within 2 days. Aliquotes of chylomicron, VLDL, LDL and HDL samples were stored either at 4°C and compositional analysis carried out within 1 week or at -20°C for analysis of apo C, apo A and apo E in HDL. Aliquotes of LDL were frozen for fatty acid analysis.

2.7 Particle Composition Analysis.

2.7.1 Lipids.

Lipoprotein cholesterol and triglyceride were measured using the commercially available kits described in sections 2.4.1 and 2.4.2. Phospholipid determination was made using a
colorimetric assay kit from BioMérieux. Charbonaires les bains, France. As with all other colorimetric assays, phospholipid standards (0-62μg) and samples were prepared in duplicate. Tubes were incubated at 37°C for 15 min and absorbances measured at 505nm.

2.7.2 Protein.

All protein quantification was carried out using a modification (Markwell et al., 1978) of the method of Lowry et al., (1951). The following solutions were used:

Solution A : 2% (W/V) Na2CO3, 0.2% (W/V) sodium/potassium tartrate in 0.1M NaOH
Solution B : 1% (W/V) CuSO4.5H2O
Solution C : 100ml Solution A and 1ml Solution B
Solution D : Folin Ciocalteau's phenol reagent diluted 1:1 with distilled water.

Solutions C and D were prepared fresh immediately before use. Standards (0-100μg) of human serum albumin were used to construct a standard curve. Solution C (1ml) was added to duplicate samples and standards and incubated at 37°C for 15 min. Solution D (100μl) was then added, tubes were immediately vortexed and incubated again at 37°C for 15 min. Absorbancy was measured at 660nm and sample protein determination made with reference to the standard curve. Cloudy, triglyceride-rich samples were cleared with Triton.

2.7.3 LDL Fatty Acid Analysis using Gas Chromatography.

To assess the adherence of both the diabetic and non-diabetic subjects to their assigned diets, fasting LDL fatty acids were analysed at the end of each 2 week intervention period. Having a half-life of approximately 4 days, LDL reflects well the dietary fatty acid intake over a period of time.

Gas chromatography was used, which allows for both qualitative and quantitative analysis. Separation is achieved on the basis of relative solubility of the volatile methyl ester (ME) form of the fatty acid in a hydrophobic liquid which lines the inner wall of a capillary column. Hydrophobicity increases with fatty acid chain length and degree of saturation. So for example, palmitic acid, having a 16 carbon chain and no double bonds is eluted first and
arachidonic acid, having a 20 carbon chain and four double bonds elutes much later. The length of time spent on the column is called the retention time and this is constant for a given fatty acid under specific conditions. This allows for qualitative identification of fatty acids of interest relative to previously identified standards. These are called fatty acid methyl ester (FAME) standards and are usually chromatographed at the beginning of each day, in order to accurately determine of the retention times of the fatty acids to be measured.

Quantitative analysis is made relative to an internal standard. As fatty acids elute from the column they are picked up by the detector, in this case a flame ionisation detector (FIS) and the change in absorption is reflected in the movement of the printer pen from left to right, to produce a chromatogram. The area of the peak is proportional to the amount of fatty acid eluting. So by comparison to the peak area of a known standard, the concentration of each fatty acid may be determined. The standard in this case was heptadecanoic acid (17:0), a fatty acid with an uneven number of carbon atoms, which does not occur in humans and can therefore be easily identified without interfering with the fatty acids of interest. A known amount is added to samples at the beginning of sample preparation therefore reflecting any loss of sample which might occur. It is called an internal standard and is also included in the FAME standards for identification.

FAME standards were dissolved in 20ml iso-octane to give final concentrations as follows

- Palmitic Acid ME (16:0) 1mg/ml
- Palmitoleic Acid ME (16:1) 1mg/ml
- Heptadecanoic Acid ME (17:0) 1mg/ml
- Stearic Acid ME (18:0) 1mg/ml
- Oleic Acid ME (18:1) 1mg/ml
- Linoleic Acid ME (18:2) 1mg/ml
- Linolenic Acid ME (18:3) 1mg/ml
- Arachidonic Acid ME (20:4) 0.5mg/ml

Aliquotes of 0.25ml were stored under nitrogen in crimper-sealed glass tubes wrapped in aluminium foil at -20°C.
The method of fatty acid extraction involves a procedure first described by Folch (1957). All organic solvents used were of the highest purity grade available. All laboratory-ware was glass.

Protein determination was made of LDL isolated from fasting plasma by the modified Lowry method (section 2.7.2) and 1mg LDL protein placed in 10ml glass, screw cap tubes and volume made up to 1ml with phosphate buffered saline (PBS). An 100µl aliquot of freshly prepared heptadecanoic acid in methanol (1mg/ml) was added to each tube as internal standard. Negative control was 1ml PBS and 100µl methanol. Positive control was 1ml PBS and 100µl internal standard. To each tube was added 2ml of a 2:1 chloroform/methanol mix. Tubes were vortex-mixed for 30 seconds and centrifuged at 3,000rpm for 15min, at room temperature. The bottom chloroform phase was removed and filtered through Whatmann # 1 filter paper into fresh 10ml glass tubes. A further 2ml chloroform/methanol was added and the extraction repeated another 3 times and filtrates pooled. To this 2ml 0.88% potassium chloride was added, tubes inverted a few times and centrifuged at 3,000rpm for 10min. The aqueous upper phase was discarded and 2ml 50% methanol in water added. Tubes were again inverted to mix and centrifuged as before for 10min after which the upper phase was again discarded. The remaining water was removed by adding a spatula tip-full (approx. 0.5g) of anhydrous sodium sulphate and allowed to stand for 30min. Extracts were then transferred quantitatively to 10ml pyrex culture tubes with teflon screw-on lids. Extracts were dried under vacuum at room temperature.

The method of fatty acid transmethylation is a modification of the transmethylation procedure of Dimitriadis et al., (1995).

Extract solids were redissolved by swirling in 0.5ml toluene. An aliquot of 1ml boron trifluoride (14% in methanol) was than added and mixed. Tubes were placed in an oven between 100-110°C with the lids sitting on loosely. After 5min the lids were screwed down tightly and left for another 85min. Tubes were cooled and 1.5ml water and 2ml n-hexane added. Tubes were vortexed for 30 seconds and centrifuged for 15min. Methylated
fatty acids in the upper hexane phase were removed to clean 10ml glass tubes. This was repeated a further 2 times and the pooled hexane extract evaporated to dryness. Samples were stored at -20°C for analysis. Before injection tubes were brought to room temperature, 40µl iso-octane added to carefully redissolve sample and 0.5µl loaded onto the column.

The conditions for gas chromatography were as follows.

The carrier and make-up gas was GC grade nitrogen set at pressures of 1.25kg/cm³ and 0.5kg/cm³ respectively. The gases for flame ionisation detection were GC grade air and hydrogen set at 0.5kg/cm³ and 0.5kg/cm³ respectively. The column temperature program was as follows

- 120°C, ramping at 8°C/min for 10min and holding at 200°C for 12min.
- 200°C, ramping at 4°C/min for 5min and holding for 25min.

The injection port temperature was 250°C and the detector temperature was 260°C. The total chromatographing time was 52min. An iso-octane solvent run was carried out at the start of each day, followed by the FAME standards (0.5µl), to accurately determine the retention time of each of the fatty acids to be quantified. Fatty acids were quantified from peak areas, with reference to the internal standard peak area. A FAME standard chromatogram and an example of sample LDL chromatogram are shown in Figure 2.1 and 2.2 respectively.
Figure 2.1: An example gas chromatogram of the FAME standards, showing retention times of 18.12min for palmitic acid, 19.12min for palmitoleic acid, 21.88min for heptadecanoic acid (Internal standard), 25.93min for stearic acid, 26.75min for oleic acid, 28.56min for linoleic acid, 31.38min for linolenic acid and 42.32min for arachidonic acid. The iso-octane solvent peak appears at 2.74min.
Figure 2.2: Example of LDL fatty acid methyl ester gas chromatogram with heptadecanoic acid added as internal standard.
Chapter 2

2.8 Quantification of Chylomicron and VLDL Apolipoproteins.

Quantification of the various apolipoproteins in the three lipoprotein fractions - chylomicron, VLDL and HDL, was made by separation of the particular protein by gel electrophoresis, staining with Coomassie Brilliant Blue 250 and by densitometric scanning to determine the concentration relative to known standards. There were two basic types of gel electrophoresis used.

1. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE). Separation is made on the basis of size, the smaller peptides travelling furthest down the gel. Gradient gels were used to optimise separation of proteins in a very wide molecular weight range - from 500,000Da for apo B100 to 8,800Da for apo C.

2. Isoelectric focusing (IEF). Separation is made on the basis of net pH of the denatured proteins. Two pH ranges, broad and narrow, were used depending on the resolution required. This is an extremely powerful technique that allows separation of proteins of equal molecular weight but with a difference of a single net charge.

2.8.1 Standard Proteins for Quantification of Sample Apolipoproteins.

All purified, standard proteins were obtained commercially (see section 2.1.1), except apo B100, which was partially delipidated LDL isolated from human plasma described below. Apo Al from human HDL was used for the identification and quantification of sample Al, apo All from human HDL for sample All and apo CII from human VLDL for quantification of both sample CII and CIII. Apo CIII and apo E from human VLDL were used for the identification of sample CII and E respectively. Concentrations of each was verified by colorimetric protein assay (Lowry) and all were >95% pure, as determined by the manufacturer.

Apo B100 was isolated from human LDL using the method of Kotite, (1995). Fasting blood was drawn from a healthy, normolipidaemic volunteer into heparinised tubes. Plasma was isolated within 1 hour by ultracentrifugation at 4°C. Preservatives and protease
inhibitors were added as described in section 2.6 to prevent scission of apo B100. An LDL fraction within the density range of 1.025g/ml and 1.063g/ml was isolated by sequential ultracentrifugation (as above) and re-isolated at the upper density limit. The LDL was dialysed overnight at 4°C against 0.15M NaCl, 0.05% EDTA and 0.02% Na3Na pH 7.4 and partially delipidated by 4 washes of cold diethylether. Protein determination was made in triplicate by the Lowry method described above, using five separate standard curves.

Purification and integrity of the protein was confirmed by SDS-PAGE (see section 2.8.2). Two dilutions of protein were made (0.1mg/ml and 1.5mg/ml) in sample buffer (2.0% (v/v) B-mercaptoethanol, 4% (w/v) SDS, 0.01% (v/v) bromophenol blue, 0.1mmol/l Tris-HCl, 20% (v/v) glycerol, pH 6.8) and stored at -20°C for use throughout the study.

2.8.2 Determination of Apos B100, B48 and E in Chylomicron and VLDL Fractions.

Chylomicron and VLDL apo B100, apo B48 and apo E were separated by SDS-PAGE using 4-15% gradient gels as previously described (Curtin et al., 1996; Taggart et al., 1997). Non-delipidated lipoprotein samples (28µl) were reduced in SDS sample buffer (2.0% (v/v) B-mercaptoethanol, 4% (w/v) SDS, 0.01% (v/v) bromophenol blue, 0.1mmol/l Tris-HCl, 20% (v/v) glycerol, pH 6.8) using a 1:1 ratio of sample buffer for 4 minutes at 97°C. Samples were loaded onto the gel and electrophoresed at 60mA constant current in 0.019mol/l Tris/0.192mol/l glycine until the tracker dye reached the bottom of the gel. Gels were stained for 1h with Coomassie Brilliant Blue staining solution and destained in water: acetic acid: methanol (5:1:4) with several changes of solvent, until gel was clear. The chromogenicity of apo B48 has been shown to be similar to that of apo B100 (Karpe et al., 1994), and that of apo E has been shown to be twice that of apo B100 (Kotite et al., 1995). An apo B100 protein standard prepared from the LDL of a single individual was stored at -20°C and used throughout the study for the quantification of these three apolipoproteins. Four concentrations of LDL apo B100, depending on the expected apolipoprotein concentration were applied to each gel. High range molecular weight marker from Bio-Rad Laboratories (see section 2.1.2) were loaded into the last lane of each gel for identification of apolipoproteins. The bands were quantified by densitometry as described in section 2.8.7. Apo E values were divided by a factor of two. An example of a chylomicron gel is
Figure 2.3: Example of a 4-15% SDS-Polyacrylamide gradient gel for the quantification of apo B100, apo B48 and apo E in Postprandial lipoprotein fractions.

Lanes 1-4: LDL apo B100 standard curve.
Lanes 5-9: Fasting and postprandial samples of chylomicron fraction.
Lane 10: Commercial apo E standard.
shown in Figure 2.3. Levels of apolipoproteins were in agreement with Kotite et al., (1995), Karpe et al., (1993, 1994), Curtin et al., (1995, 1996) and Bjorkegren et al., (1997, 1998).

2.8.3 Linearity of Staining and Assay Variation of Apo B100 and Apo E.

Kotite et al., (1995) reported apo B100 staining to be linear within the range 0.1-20\(\mu\)g protein, facilitating the measurement of both apo B48 and apo B100 both in the same sample. Apo E was reported to be linear between 0.1 and at least 8\(\mu\)g. This was verified by electrophoresis of eight concentrations of standard between 0.1 and 30\(\mu\)g apo B100 and 0.1 and 5\(\mu\)g apo E on 4-15% SDS polyacrylamide gradient gels stained with Coomassie Blue (Figure 2.4). To confirm Kotite's observed two-fold higher chromogenicity of apo E compared to B100 two standard curves of between 0.2 and 2.5\(\mu\)g B100 and between 0.1 and 1.5\(\mu\)g E were electrophoresed along with duplicate samples of VLDL (Figure 2.5). Standard curves were constructed and VLDL apo E quantified using both curves.

Table 2.4: Estimation of VLDL apo E using both apo B100 and apo E standard curves to determine the difference in chromogenicity between the two apolipoproteins.

<table>
<thead>
<tr>
<th>Estimation of VLDL apo E ((\mu)g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B100 curve</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>1.</td>
</tr>
<tr>
<td>2.</td>
</tr>
</tbody>
</table>

Mean ratio = 2.075
Figure 2.4: Chromogenicities of (a) apo B100 (0 - 30μg) and (b) apo E (0 - 5μg) extrapolated to 20μg for comparison, on 4-15% SDS gels stained with Coomassie Blue.
Figure 2.5: Standard curves for apo B100 and apo E to assess chromogenicity.

Lanes 1-7: 0.2 to 2.5μg apo B100 and 1.5 to 0.1μg apo E.

Lanes V: VLDL sample for identification of apo B100 and apo E.
Intra-assay variation was determined using 4 hour postprandial VLDL and chylomicron samples from a non-diabetic subject applied to six lanes (3 chylomicron and 3 VLDL) of an SDS-PAGE gel containing an apo B100 standard curve. The gel was densitometrically scanned three times, constructing a new standard curve each time. The inter-assay variation was estimated by application of both these samples to three separate gels, each containing an apo B100 standard curve. Again samples were scanned in triplicate and the mean value taken.

Table 2.5: Mean coefficient of variation for determination of chylomicron and VLDL apolipoproteins, separated on 4-15% SDS-PAGE gels, stained with Coomassie Blue and quantified by densitometric scanning relative to purified standards.

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>B100</td>
<td>3.1</td>
<td>4.5</td>
</tr>
<tr>
<td>B48</td>
<td>3.9</td>
<td>6.0</td>
</tr>
<tr>
<td>E</td>
<td>1.8</td>
<td>3.6</td>
</tr>
</tbody>
</table>

2.8.4 Determination of Apo CII and Apo CIII in the Chylomicron Fraction.

Apo CII and CIII in the chylomicron fraction were separated using the method described by Shachter et al., (1996). The method is similar to that employed to quantify apo B and apo E, but given the considerably smaller size of the apo C peptides (approx. 8.8kDa) and the very small difference in molecular weight between apo CII and apo CIII, higher percentage gradient gels of 10-20% were found to give the best separation. Gels were also found to electrophorese more satisfactorily at a lower constant current of 30mA. The equivalent of between 28 and 60μl chylomicron fraction was loaded. The more dilute samples were concentrated using Microcon™ centrifugal filter devices with a 3,000 Da
molecular weight cut-off. Standard apo CII was used to quantify both apolipoproteins. Four standards between 0.1 and 1µg were loaded on each gel. The chromogenicity of apo CII is 1.25 times that of apo CIII (Catapano et al., 1978, Bugugnani et al., 1984) and as such apo CIII values were multiplied by a factor of 1.25. A VLDL sample or an apo CIII standard was loaded in the last lane of each chylomicron gel for the positive identification of apo CIII. An example of an apo C chylomicron gel is shown in Figure 2.6. Levels of apo CII and CII in the chylomicron fraction were in agreement with Karpe et al., (1993) and Attia et al., (2000).

2.8.5 Identification of Apolipoproteins in VLDL and Chylomicrons.

Contained on VLDL are well defined and easily identifiable apo CII, CIII and apo E. As such, a VLDL sample was chromatographed along-side samples on chylomicron and HDL SDS-PAGE gels (see section 2.8.4 and 2.9.1 respectively) to identify apo E and/or apo CIII by comparing their exact positions on the gel. In order to do this, a positive identification was made of the VLDL apolipoproteins of interest and also those of the chylomicron sample. Samples of postprandial VLDL and chylomicron were electrophoresed on a 15% gel along with purified apo B100 from LDL, apo CII, apo CIII, apo CI, apo E, apo AI and apo AII obtained from a commercial source. Apo E has a molecular weight of 34,200Da and chromatographs as the only band in the centre of the VLDL sample. Apo CIII has a molecular weight of 8,750Da and chromatographs as the top, major two bands in a group of three at the bottom of the gel. Apo CII has a molecular weight of 8,800Da and chromatographs below apo CIII as the bottom band of the triplet. This apparent reversal of molecular weights and the appearance two bands in apo CIII is due to the presence of a varying number of sialic acid residues on the different isoforms of apo CIII. Apo CI is the smallest apo C peptide at 6,613Da and could not be identified on this gel. Apo AI has a molecular weight of 28,330Da and chromatographs just below apo E and is visible in the chylomicron sample. Apo AII has a molecular weight of 17,380Da but exists as a homodimer and chromatographs at the very bottom of the gel. There is no apo AII visible in either chylomicrons or VLDL isolated from human subjects.
Figure 2.6: Example of SDS 10-20% gradient gel for the quantification of apo CII and apo CIII in postprandial lipoprotein fractions.

Lanes 1-4: Apo CII standard curve using commercial apo CII.
Lanes 5-9: Fasting and postprandial samples of the cylomicron fraction.
Lane 10: Commercial apo CIII standard.
2.8.6 Linearity of Staining and Assay Variation of Apo CII and CIII.

Catapano et al., (1978) reported linearity of staining of apo CII and apo CIII up to 40μg. A range of seven concentrations of both apo CII and CIII between 0.2 and 8μg were electrophoresed on a 10-20% gradient gel. In order to confirm Catapano’s observed 1.25 fold higher chromogenicity of apo CII compared to apo CIII three samples of postprandial VLDL were also loaded onto the gel. Following Coomassie Blue staining, as described previously, both standard curves were plotted and the VLDL apo CIII estimated using both curves.

Table 2.6: Estimation of VLDL apo CIII using both apo CII and apo CIII standard curves to determine the difference in chromogenicity between the two apolipoproteins.

<table>
<thead>
<tr>
<th>Estimation of VLDL apo CIII (μg)</th>
<th>Apo CII curve</th>
<th>Apo CIII curve</th>
<th>Ratio CIII/CII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.526</td>
<td>0.679</td>
<td>1.29</td>
</tr>
<tr>
<td>2.</td>
<td>0.558</td>
<td>0.670</td>
<td>1.20</td>
</tr>
<tr>
<td>3.</td>
<td>0.540</td>
<td>0.661</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Mean ratio = 1.24

Figure 2.7: Chromogenicities of apo CII and CIII (0 - 8μg) on 10-20% SDS gels stained with Coomassie Blue.
Figure 2.8: IEF gel pH 3.5 - 9.5 for separation and quantification of apo CII and CIII in the VLDL fraction.

Lanes 1-3: Apo CII standard curve.
Lanes 4-6: VLDL samples showing apo CII and apo CIII isoforms.
Assay variation was determined in a similar manner (n=4 for intra- and inter assay variation) to that for apo B and apo E (see section 2.8.3), using an apo CII standard curve.

**Table 2.7**: Assay variation for VLDL apolipoproteins, separated on 5% polyacrylamide IEF gels, stained with Coomassie Blue and quantified by densitometric scanning relative to purified standards.

<table>
<thead>
<tr>
<th>Chylomicron Apolipoprotein</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CII</td>
<td>7.3</td>
<td>8.1</td>
</tr>
<tr>
<td>CIII</td>
<td>5.4</td>
<td>6.4</td>
</tr>
</tbody>
</table>

**2.8.7 Quantitative Apolipoprotein Determination by Densitometric Scanning.**

Sample apolipoproteins on Coomassie Blue-stained gels were quantified using the Viber Lourmat gel documentation system by comparison of sample to known standards. A video image of each gel was generated and imported into Bio 1D version 6.32 software for analysis (See section 2.1.2). Density values were assigned to the purified standard bands and a standard curve constructed. Values were recalculated by linear regression and only curves with a correlation coefficient of >0.95 were accepted. The concentration of sample apolipoproteins was then determined from this standard curve. To maximise reproducibility camera settings were standardised to those which allowed for the largest gel image. Each sample band was scanned three times, redrawing the standard curve each time and an average taken.
2.8.8 Determination of Apo CII and CIII in the VLDL fraction.

The higher levels of apo C in VLDL facilitated the measurement of the separate isoforms of apo CIII, which have different affinities for the VLDL particle and also have varying abilities to inhibit binding of apo E to hepatic receptors. Therefore, the analysis was carried out on IEF gels, which separated the three isoforms - CIII0, CIII1 and CIII2 by difference in pH. The method was a modification of that described by Catapano et al., (1978).

Apo CII and CIII isoforms in the VLDL fraction were separated on 5% polyacrylamide, pre-cast gels (245 x 110 x 0.43 mm thick). Gels, supplied in dry form, were rehydrated with 10.4 ml de-gassed buffer containing 6M urea (purity >99.8%), 0.01M Tris-HCl pH 8.5 and 3% (V/V) pre-blended ampholites (0.4 mg/ml) pH 3.5-9.5. Gels were prefocused at 700V, 12mA, 8W for 20 min. Samples of VLDL (between 50 and 150 µg protein) were partially delipidated by addition of cold diethyl ether (4 volumes), vortex-mixed for 30 sec and centrifuged to separate the two phases for 15 min at 12,000 rpm at 4°C. Diethyl ether and the dissolved lipid, being less dense than the aqueous sample and practically immiscible, forms the upper phase and can be removed. This was repeated a further three times, samples dried under vacuum and redissolved in 20 µl freshly prepared sample buffer (6M urea, 0.01 M Tris-HCl pH 8.5, 1% SDS, 20% sucrose). Samples and apo CII standards (0-8 µg) were placed on to 1 cm² pieces of filter paper placed approx. 1.5 cm from the negative electrode. Sample entrance was carried out at 500V, 8mA, 8W for 20 min, isoelectric focusing at 2000V, 14mA, 14W for 90 min and band sharpening at 2500V, 14mA, 18W for 10 min. Gels were stained with Coomassie Blue and protein quantification made by densitometric scanning, as described in section 2.8.7. An example of a VLDL apo C IEF gel is shown in Figure 2.8. Levels were in approximate agreement with Fredenrich, (1998), Venkatesan et al., (1995), Bjorkegren et al., (1997, 1998) and Attia et al., (2000). Ratio of apo CII / CIII is in agreement with that measured by Bugugnani et al., (1984), absolute amounts were not reported in this paper.
2.8.9 Assay variation for determination of apo C in VLDL

Assay variation was determined in a similar manner (n=4 for intra- and inter assay variation) to that for apo B and apo E (see section 2.8.3), using an apo CII standard curve.

Table 2.8: Assay variation for VLDL apolipoproteins, separated on 5% polyacrylamide IEF gels, stained with Coomassie Blue and quantified by densitometric scanning relative to purified standards.

<table>
<thead>
<tr>
<th>VLDL Apolipoprotein</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CII</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>CIII_0</td>
<td>2.6</td>
<td>3.7</td>
</tr>
<tr>
<td>CIII_1</td>
<td>2.8</td>
<td>3.9</td>
</tr>
<tr>
<td>CIII_2</td>
<td>2.6</td>
<td>4.2</td>
</tr>
</tbody>
</table>

2.8.10 Recovery of apo B-containing particles.

To assess the recovery of the apo B-containing particles two methods were used following their isolation from plasma by the method of sequential ultracentrifugation described in section 2.6. The first involved the separation of all the various fractions - triglyceride-rich lipoproteins (TRL, density>1.006g/ml), intermediate density lipoprotein (IDL, density 1.006g/ml - 1.025g/ml), LDL (density 1.025 - 1.063g/ml), HDL (density 1.063 - 1.21g/ml) by the method of sequential ultracentrifugation described in section 2.6. Fasting (n=3) and postprandial (n=4) blood was obtained from normolipidaemic volunteers. Total apo B was determined in duplicate for plasma and each fraction on 4-15% SDS-PAGE gels as described in section 2.8.2. Plasma, LDL and HDL samples were partially delipidated with cold ether.

The second method used a total apo B enzyme-linked immunosorbant assay (ELISA) kit from AlerCHEK, inc., Portland, ME 04101-2548 USA. Briefly, the kit involved incubation of diluted plasma and lipoprotein samples in microwells coated with goat affinity-purified
antibody specific for human apo B. Both apo B100 and apo B48 bind to this antibody. Dilutions of lipoprotein fractions were made with supplied diluent to levels similar to that of plasma, according to gel analysis. Two dilutions were assayed in duplicate. An horseradish peroxidase-conjugated affinity-purified goat antibody specific for apo B and peroxide substrate was used for colour development proportional to the amount of bound conjugate, which in turn is proportional to the amount of apo B in the sample. Optical densities were read at 450nm using a microwell reader, using the sample diluent as control and to zero the machine. An apo B100 standard was supplied in the kit and a standard curve was constructed by serial dilution and duplicate assay of each, as per manufacturer's instructions. Unknown samples were determined manually, relative to the resultant standard curve. Figure 2.9 shows this hyperbolic standard curve and table 2.9 gives the calculated recovery of apo B-containing particles using the SDS-PAGE method and the ELISA method.

![Apo B standard curve](image)

**Figure 2.9**: Apo B standard curve constructed using the total apo B ELISA kit and standards between 0 and 200mg/dl.
Table 2.9: Recovery of apo B-containing lipoproteins in fasting (n=3) and postprandial (n=4) plasma samples. Results are given as mean of total fractions /plasma apo B x 100%.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Fasting</th>
<th>Postprandial</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE</td>
<td>62</td>
<td>59</td>
</tr>
<tr>
<td>ELISA</td>
<td>68</td>
<td>56</td>
</tr>
</tbody>
</table>

To assess our variation for this ELISA kit and to assess the variation between the SDS-PAGE method and the ELISA method, chylomicron and VLDL samples from 8 diabetic patients were assayed by both methods, one of these samples being applied 5 times.

The intra-assay coefficient of variation for the ELISA method was 4.6%. The mean variation between the two assays is given in Table 2.10 below.

Table 2.10: Variation in the determination of apo B between the SDS-PAGE and ELISA methods. Results given as the mean percentage difference (n=8) for chylomicron and VLDL fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td>5.8</td>
</tr>
<tr>
<td>VLDL</td>
<td>7.6</td>
</tr>
</tbody>
</table>


2.9 QUANTIFICATION OF HDL APOLIPOPROTEINS.

2.9.1 Determination of Apo E, Apo CIII and Apo AII in HDL.

While the levels of apo C in HDL permitted separation of apo CIII isoforms by IEF, apo AII co-chromatographed with apo CIII\(_0\), therefore only apo CII was measured by IEF. All other apolipoproteins were separated by SDS-PAGE using the method of Shachter et al. (1996). Aliquots of 50\(\mu\)l HDL were partially delipidated as described in section 2.8.8 using 6 washes of 12 volumes diethyl ether and were made up to 200\(\mu\)l with phosphate buffered saline (PBS) pH 7.4. Total protein was measured before and after delipidation and there was found to be virtually no loss due to this method of delipidation. Aliquots of between 30 and 60\(\mu\)g protein were dissolved in denaturing buffer and applied to 10-20% polyacrylamide gradient gels and electrophoresed as described in section 2.8.4. Again a VLDL sample was loaded in the last lane for positive identification of apo E and apo CIII.

Quantification of apolipoproteins was facilitated by the application of three standard curves (see section 2.7.1). Standard apo AII between the concentrations of 0.5 and 10\(\mu\)g, standard apo CII between 0.1 and 1\(\mu\)g for determination of apo CIII and standard apo B100 between 0.1 and 2\(\mu\)g for determination of apo E were loaded on each gel. An example of how all three standard curves may be electrophoresed in the same lanes is shown in Figure 2.10. HDL apolipoprotein levels are in agreement with Stocks et al., (1998), Fredenrich, (1997), Mero et al., (1998) and Breyer et al., 1999).

2.9.2 Linearity of staining of apo AII and assay variation of HDL apolipoproteins.

A range of eight standards (0-10\(\mu\)g) were loaded and chromatographed on 10-20% SDS-PAGE gels. Gels were stained, as described, by Coomassie Blue and scanned densitometrically (section 2.8.7). The staining of apo AII was found to be linear up to 10\(\mu\)g. This gel is shown in Figure 2.11 and the scanned densitometric results plotted as shown in Figure 2.12.
Figure 2.10: Standard curves of apo B100, apo CII and apo AII for the quantification of Apo E (apo B100 curve), apo CII and apo CIII (apo CII curve) and apo AII (apo AII curve) in HDL lipoprotein fractions.
Figure 2.11: Apo AII standard curve on 10-20% SDS-PAGE gel to assess chromogenicity with Coomassie Brilliant Blue stain.

Lanes 2-9: Apo AII standard curve using commercial apo AII (0.5-10μg).
Lane 10: Molecular weight maker (200-6.5kDa).
Figure 2.12: Linearity of staining of apo All (0 - 10μg) on 10-20% SDS gels stained with Coomassie blue.

Assay variation was determined in a similar manner (n=4 for intra- and inter assay variation) to that for apo B and apo E (see section 2.8.3).

Table 2.11: Assay variation for HDL apolipoproteins, separated on 10-20% SDS-PAGE gels, stained with Coomassie Blue and quantified by densitometric scanning relative to purified standards.

<table>
<thead>
<tr>
<th>HDL Apolipoprotein</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>2.9</td>
<td>5.3</td>
</tr>
<tr>
<td>CIII</td>
<td>3.8</td>
<td>6.2</td>
</tr>
<tr>
<td>All</td>
<td>6.4</td>
<td>8.2</td>
</tr>
</tbody>
</table>
2.9.3 Determination of HDL apo AI.

Total protein determination was made by the Lowry method of a 1/4 dilution of partially delipidated HDL, as in section 2.9.1. A further 1/5 dilution was made in PBS and a volume equivalent to 3μg protein was reduced in an equal volume of 2x denaturing buffer and separated on a 10-20% gel. Apo AI was found to stain linearly only to 4μg necessitating this dilution. Four concentrations of standard apo AI between 0.5 and 3μg were used for identification and quantification of samples. An typical SDS-PAGE gel for apo AI determination in HDL is shown in Figure 2.13. Levels are in agreement with Stocks et al., (1998).

2.9.4 Linearity of Staining and Assay Variation of Apo AI.

A range of seven purified standards between 1 and 20μg were loaded and chromatographed on 10-20% SDS-PAGE gels (Figure 2.14 (a)). Gels were stained, as described, by Coomassie Blue and scanned densitometrically (section 2.8.7). The staining of apo AI was found to be linear only to approximately. 4μg. To further assess this a second standard curve was set up loading samples between 0.5 and 4μg (Figure 2.14 (b)).

Assay variation was determined in a similar manner (n=4 for intra- and inter assay variation) to that for apo B and apo E (see section 2.8.3).

<table>
<thead>
<tr>
<th>HDL Apolipoprotein</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-Assay</td>
</tr>
<tr>
<td>AI</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 2.13: Example of an SDS-PAGE gel for the quantification of apo AI in HDL. Lanes 1-4: Apo AI standard curve (1-4μg). Lanes 5-9: Fasting and postprandial HDL (3μg). Lane 10: Molecular weight marker.
Figure 2.14: Linearity of staining of apo Al (a) 0 - 10 μg and (b) 0 - 4 μg on 10-20% SDS gels stained with Coomassie Blue.
2.9.5 Determination of Apo CII in HDL.

The method of IEF was found to be the most reliable for the determination of apo CII in HDL. This was done again by the method of Catapano et al., (1978) as described in section 2.8.8, using a pH gradient between 4.5 and 6.5 in order to maximise resolution of apo CII and apo AII.

Aliquots of 150 µl HDL were partially delipidated as described in section 2.8.8. Samples were then de-salted by centrifugation through mini-filters of 3,000 Da pore size from Millipore Inc. (section 2.1.2) at 14,000g for 50 min at 4°C. Retentate was redissolved in 30 µl sample buffer (6 M urea, 0.01 M Tris - HCl pH 8.5, 1% SDS, 20% sucrose) and 20 µl (equivalent of 100 µl HDL) loaded onto 5% polyacrylamide gels which had been rehydrated in 10.4 ml buffer (6 M urea, 0.01 M Tris pH 8.5 and 3% (v/v) preblended ampholites pH 4.5 - 6.5). Electrophoresis was carried out as described in section 2.8.8, extending the focusing time to 4 hours. Apo CII standards between 0.1 and 3.75 µg were used for quantification of samples.

2.9.6 Assay variation of apo CII in HDL.

Assay variation was determined in a similar manner (n = 4 for intra- and inter assay variation) to that for apo B and apo E (see section 2.8.3).

Table 2.13: Assay variation for HDL apo CII, separated on 5% IEF gels, between pH 4.5 and 6.5, stained with Coomassie Blue and quantified by densitometric scanning relative to purified standards.

<table>
<thead>
<tr>
<th>HDL Apolipoprotein</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CII</td>
<td>5.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

92
In a study of this design, where, due to the volume of analysis to be carried out, it was necessary for samples to be frozen and analysed at a later date. The decision to measure apo B100, B48 and apo E fresh was made because of the very large size of apo B and the possibility that it may be more susceptible to denaturation on freezing. Apo E, being measured relative to an apo B100 standard, was also measured in the fresh chylomicron and VLDL fractions. Once this was decided, a study was set up to assess the stability of the other apolipoproteins over time at -20°C. These were apo CII, apo CIII, apo AI, apo AII and apo E in HDL. Every precaution was taken in the preparation of the samples to maintain the integrity of the apolipoproteins including the use of protease inhibitors and preservatives, the immediate aliquoting and freezing of samples following isolation and the manipulation of samples, as far as was possible, at 4°C before freezing, during and after thawing. Thawed samples were used immediately.

Blood was drawn from 3 healthy normolipidaemic subjects into heparinised tubes. Plasma was isolated and preservatives and protease inhibitors added. TRL (density <1.006g/ml), IDL (1.006 - 1.025g/ml), LDL (1.025 - 1.63g/ml) and HDL (density <1.21g/ml) were isolated as described in section 2.6 and multiple aliquots of each frozen. Apolipoproteins were measured immediately and after 2 months and 6 months of freezing. Apo CII and CIII were measured in TRL on 10-20% SDS-PAGE gels, as described in section 2.8.4, apo AI in HDL by the method described in section 2.9.3 and apo AII and E by the method in section 2.9.1. Table 2.15 shows the measured amount expressed as a percentage of that measured at time zero.
Table 2.15: Results of stability study of apolipoproteins frozen at -20°C and measured before freezing and following 2 and 6 months of freezing. Results are expressed as a percentage of the measurement before freezing for n=3.

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>2 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>CII</td>
<td>95.9</td>
<td>93.4</td>
</tr>
<tr>
<td>CIII</td>
<td>101.2</td>
<td>97.2</td>
</tr>
<tr>
<td>AI</td>
<td>99.6</td>
<td>100.7</td>
</tr>
<tr>
<td>All</td>
<td>104.3</td>
<td>92.1</td>
</tr>
<tr>
<td>E</td>
<td>101.1</td>
<td>97.8</td>
</tr>
</tbody>
</table>

While there is a tendency to be lower at 6 months (except for apo AI) the differences from the initial measurement approximate the inter assay variations determined for the apolipoproteins. These apolipoproteins were taken to be stable until at least 6 months.

2.11 Determination of Cholesteryl Ester Transfer Protein (CETP) and Lecithin Cholesteryl Acyl Transferase (LCAT) Activity.

Cholesterol ester transfer to apo B-containing lipoproteins was determined using a modification of the method described by Channon et al. (1990). The LCAT activity was determined using a modification of the method of Stokke and Norum, (1971).

A cholesterol/albunin emulsion was prepared freshly by dissolving 40μl [1α, 2α(n)-3H] cholesterol (1 Ci/l in toluene) in 300μl acetone. This was added dropwise into 3ml of a constantly stirring solution of bovine serum albumin (BSA, 50mg/ml) in phosphate buffered saline pH 7.4. Acetone was evaporated under a stream of nitrogen gas. Of this emulsion 200μl was added to 2ml plasma, vortexed and incubated for 1 hour at 4°C to allow equilibration of the radiolabelled cholesterol with endogenous free cholesterol.
Samples were then transferred to a 37°C incubator for a further 3 hours, after which time transfer and esterification activities were stopped by removal of tubes to ice. Total radioactivity in 3 x 50μl aliquots of the incubation mixture was determined directly by liquid scintillation counting. A 200μl aliquot was assayed for free cholesterol using a kit from Roche Diagnostics Ltd., Lewes, East Sussex, England and the remaining mixture was divided into 3 x 300μl aliquots for CETP activity determination and 3 x 150μl for LCAT activity determination.

For CETP activity determination apo B-containing lipoproteins were precipitated from the incubation mixture by addition of 60μl of 4%(w/v) sodium phosphotungstate in 0.5M MgCl₂ solution. After centrifugation at 2,000 rpm for 20 min at 4°C, supernatant was removed and 100μl taken for scintillation counting. The pellet was washed twice with 3ml of a 1/10 dilution of the phosphotungstate/MgCl₂ solution, lipids extracted by the method of Folch et al (1957), extract evaporated to dryness and redissolved in 250μl of a 2:1 mixture of chloroform and methanol. Lipids were extracted from LCAT tubes by the same method. A 50μl volume of each was loaded onto glass-backed, silica 60, thin layer chromatography plates. Free and esterified cholesterol were separated and visualised by iodine staining relative to loaded commercially obtained standards. Both bands were removed for liquid scintillation counting. The activity of CETP was calculated as the fraction of radioactivity precipitated as cholesterol ester per hour and was expressed as nmol cholesterol transferred/h/ml plasma. This was corrected for recovery. The activity of LCAT was calculated as the fraction of initial free cholesterol esterified per h per ml plasma. Activity was expressed as nmol cholesterol esterified/h/ml plasma.

2.11.1 CETP and LCAT Activity Assay Variation.

Plasma from a fasting healthy, normolipidaemic volunteer was used for the assessment of intra-assay variation. The sample was split into six and the assays carried out as described in section 2.9. The day-to-day variation was determined at fasting and at 2 hours postprandially in five healthy, normolipidaemic volunteers and was carried out over a period of four days. Fasting samples were taken at 9.30 am each day. The fat-loading meal
detailed in section 2.5.2 was eaten over a period of 30min and postprandial samples taken 2 hours later.

Table 2.14: Assay variation for CETP and LCAT activity. Intra-assay variation (n=6) carried out at fasting, day-to-day variation (n=5) carried out at fasting and 2 hours postprandially.

<table>
<thead>
<tr>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer Assay</td>
</tr>
<tr>
<td>Fasting</td>
</tr>
<tr>
<td>CETP activity</td>
</tr>
<tr>
<td>LCAT activity</td>
</tr>
</tbody>
</table>

2.12 APO E GENOTYPING.

Apo E genotyping was carried out using a one-step polymerase chain reaction (PCR) method. It involves the use of specifically designed primers which flank a 292bp region of the \(\text{APOE} \) gene which includes both polymorphic sites at amino acid 112 and 158. This region is amplified by PCR and digested with the restriction enzyme HhaI. This enzyme cuts DNA specifically at the four base recognition site GCGC, the last three bases of which code for arginine. The point mutations change this to GTGC, the last three bases now coding for cysteine and making the site unrecognisable to the enzyme. Depending on the alleles present a number of restriction fragments are produced, each genotype producing a different fragment pattern. These can be resolved on a polyacrylamide gel and the genotype determined according to the pattern and relative to known standards.
2.12.1 Isolation of Genomic DNA.

The isolation of genomic DNA was done using the Puregene® DNA isolation kit from Gentra Systems Inc., 13355 10th Ave. North, Suite 120, Minneapolis, MN 55441 USA, and was carried out as per manufacturer's instructions. Briefly, 10ml of heparinised blood, drawn from 11 diabetic patients and 6 non-diabetic subjects was mixed with 30ml red blood cell lysis solution. Following incubation at room temperature for 10min, the remaining intact, white cells were pelleted by centrifugation at 4,600rpm for 10min. All but approximately 200μl supernatent was discarded. Pellets were broken up by vigorous vortex-mixing and 10ml cell lysis solution added. Samples were incubated at 37°C, usually for 10-20min, until suspensions were homogenous. Samples were cooled to room temperature and protein precipitated by adding 33.3ml precipitation solution to the cell lysate. Tubes were vortex-mixed for 20 seconds and then centrifuged at 4,600rpm for 10min to precipitate a dark brown protein pellet. Supernatent was decanted carefully to a fresh tube and DNA was precipitated by the addition of 10ml 100% isopropanol and gently inverting the tube until threads of DNA appeared. The DNA was precipitated as a white pellet by centrifuging at 4,600rpm for 3min and supernatent carefully decanted. The DNA pellet was washed in 10ml 70% ethanol, re-pelleted and allowed to air dry for 15min. Rehydration solution (1ml) was added to the DNA, incubated for 1h at 65°C and at room temperature overnight and stored at -20°C for apo E genotyping.

2.12.2 DNA Amplification.

Amplification of the APOE gene fragment was carried out by PCR using an automated thermocycler (Perkin-Elmer Cetus). Reactions, in a total volume of 200μl, were carried out in 0.5ml microcentrifuge tubes. Components of the PCR mixture were 6μl (200-500nmol) phenol-extracted DNA, 12μl each oligonucleotide primer at 1.2μM each, 8μl of 1mM Mg Cl₂, 20μl 10x Promega assay buffer, and 20μl of a deoxy nucleotide triphosphate (dNTP) mixture with each at 0.2mM. The base sequence of the primers was as follows:

upstream primer \[\text{5'}(AACAACTGACCCCGGTGCCG)3']\]
downstream primer \[\text{5'}(ATGGCGCTGAGGCCGCTC)3']\]
Oligonucleotides were made by the Oligonucleotide Synthesis Unit, Queen's University, Belfast, NI. Tubes were overlaid with four drops of mineral oil. A "hot-start" was used where DNA was denatured at 94°C for 12min, after which time 2 units (20μl) Taq DNA polymerase (Promega) was added under the mineral oil. The amplification temperature cycle was as follows: denaturation at 94°C for 1min, annealing at 65°C for 1min and extension at 72°C for 1min for 33 cycles, with a final extension period at 72°C for 5min. Negative controls of sterile water were analysed with each batch of samples. Positive control of phenol-extracted DNA which had previously been successfully amplified was used. PCR products were visualised on 8% polyacrylamide gels and sized at approximately 292bp by comparison to a DNA ladder produced by the digestion of the plasmid PBS by Hae III.

2.12.3 Restriction Endonuclease Digestion.

PCR product DNA was concentrated from 100μl reaction mixture by precipitation with 100μl 4M ammonium acetate and 400μl cold ethanol. Tubes were incubated at -20°C for 30min and centrifuged at 12,000g for 10min at 4°C. The supernatant was removed carefully and 600μl 70% ethanol added, mixed and centrifuged under the above conditions. This step was repeated. Pellet was redissolved in 20μl distilled, sterile water. An aliquote of 10μl from each sample was digested with 2μl 10x enzyme buffer C (supplied with restriction enzyme), 0.2μl acetylated BSA and 6.8μl water. After mixing, 1μl Hha I was added and mixture incubated at 37°C for 2 - 3 hours. Restriction fragments were seperated on 12% polyacrylamide and sized by comparison to a 10bp DNA step ladder (Promega). Genotype was determined on the basis of the fragment pattern as described in section 1.3.14.
2.13 ANIMAL STUDY.

2.13.1 Animals.
Male New Zealand White rabbits were used (n=18). These were 9-10 weeks old at the beginning of the study and weighed approximately 1.5Kg. Rabbits were housed individually in a reverse light cycle room (1am to 1pm light, 1pm to 1am dark).

2.13.2 Cholesterol Feeding and Induction of Diabetes.
Following acclimatisation of the animals for one week, with free access to standard chow and water, animals were fed a 0.5% cholesterol diet obtained commercially from Special Diet Services, UK, ad libitum for 6 weeks. Diabetes was induced in 9 of the rabbits at the beginning of the fifth week by intravenous infusion of a 10% (w/v) solution of alloxan monohydrate (150mg/kg) in physiological saline through a catheter inserted via a marginal ear vein. To counteract hypoglycaemia caused by the release of insulin from necrotic beta cells, blood glucose was determined at 2 hourly intervals for the first 16 hours using a drop of blood sampled by pricking the ear. Glucose was measured by glucose strip and glucometer. A 50% suspension of glucose was given intragastrically when required. Rabbits were also provided with a 20% (w/v) solution of glucose in their cages for the first 24h period. Diabetes were confirmed 48h later with blood glucose >22mmol/l. Urine ketones were monitored with assay dip sticks. Rabbits were not ketotic. Blood glucose and food intake of each rabbit was monitored daily. Diabetic rabbits were kept in moderate control by daily subcutaneous injection of long-acting insulin. Animals were diabetic for at least 8 days prior to experiment.

2.13.3 Collection of Lymph and Blood Samples.
A 5ml blood sample was taken from the ear artery for CETP and LCAT analysis 24 hours prior to lymph collection. Sample was placed on ice until plasma could be isolated which was for less than 1 hour. Lipid transfer assays were carried out as described in section 2.10.
To stimulate lymph production rabbits were given a lipid emulsion by gavage containing sunflower oil (75% (V/V)), water (25% (V/V)) and phosphatidylcholine (5% (W/V)). Rabbits were returned to their cages with access to water only. After a period of 5h rabbits were sedated by intramuscular injection of Hypnorm (0.3ml/kg) and anaesthetised 20-30 min later by intravenous injection of Hypnovel (2mg/kg). Rabbits were intubated, attached to a anaesthesia machine and administered a mixture of oxygen and diethylether. Rabbits received 50ml / hour warm saline intravenously. A laparotomy was performed and the left kidney tied off and removed. The mesenteric lymph duct, which lies ventrally above the abdominal aorta, was tied off and cannulated by insertion of a 2mm portex tube. Tubing was exteriorised through a stab wound in the left side and positioned to facilitate lymph flow. Lymph was collected for 4 hours into tubes containing EDTA (1mg/ml) and protease inhibitors (as described in section 2.6). When lymph collection ended, blood was sampled into tubes containing heparin, by cardiac puncture. Both lymph and blood samples were kept at 4°C for lipoprotein isolation. Rabbits were sacrificed with an overdose of Euthane.

2.13.4 Preparation of Lymph and Plasma Chylomicrons.

Plasma was isolated by centrifugation at 2,000rpm for 10 min at 4°C and preservatives and protease inhibitors added as described in section 2.6. Plasma and lymph chylomicrons were isolated by layering under 1.006g/ml density solution and centrifuging at 20,000rpm for 30 min at 10°C. The higher temperature was necessitated by the large quantity of lipid-rich chylomicrons and prevented solidification of the lipoproteins which occurred at 4°C. Aliquots of samples were immediately frozen at -70°C for analysis. Aliquots of chylomicrons were partially delipidated using the cold ether method described in section 2.9, evaporated to dryness and then frozen.

2.13.5 Compositional Analysis of Lymph, Plasma and Chylomicrons.

Lipids and total protein were measured by the methods described in section 2.7. Free cholesterol in plasma samples was measured using a colorimetric kit and HDL cholesterol was measured using an HDL-c reagent, both from Roche Diagnostics Ltd., Lewes, East Sussex, England.
2.13.6 Analysis of Plasma and Lymph Chylomicron Apolipoproteins.

Duplicate plasma and lymph chylomicron samples (100μl) were partially delipidated and concentrated by centrifugation through mini-filters of 3,000Da pore size from Millipore Inc., at 14,000g for 50min at 4°C. Retentate was made up to 20μl with PBS. Samples were reduced in sample buffer as described in section 2.8.2. All of the plasma chylomicron sample (equivalent to 100μl HDL) and half of the lymph chylomicron sample were loaded (equivalent to 50μl HDL) onto 10-20% gels. Two standard curves were applied to each gel - apo B100 between 0.1 and 1.2μg and apo AI between 0.25 and 2μg. Using these apo B100, apo B48, apo E and apo AI were quantified in both lymph and plasma chylomicrons from diabetic and control rabbits. A sample gel is shown in Figure 2.15.

2.14 ANIMAL LICENSING.

Animals were housed under licence from the Department of Health and experiments carried out according to Irish law as administered by the Department of Health.
Figure 2.15: Example of an SDS-PAGE gel for quantification of apo B100, apo B48, apo E and apo AI in rabbit plasma and lymph chylomicrons.

Lanes 1-4: Apo B100 and apo AI standard curves.

Lane 5: Plasma chylomicron sample.

Lanes 6 and 7: Lymph chylomicron samples.
2.15 Statistical Analysis.

All results were analysed using the software package Prism 2.0 for Macintosh (Graphpad Software Inc., San Diego, CA USA). For normally distributed data, groups were compared using the paired or unpaired student t-test and results expressed as the mean ± standard deviation (SD) in tables, unless otherwise stated. Graphs show mean and standard error of the mean (SEM). Non-parametric data were compared using either the Wilcoxon matched pairs test or the Mann-Whitney U test for unpaired data and results expressed as the median and the range. Area under curve was calculated either from fasting (AUCf) or from zero (AUCz) using the above program. Areas over curve (AOC) were calculated by subtracting postprandial points from the fasting value and obtaining the area under curve from zero as above. A p value of < 0.05 was regarded as statistically significant.
Chapter 3

The Effect of dietary linoleic and oleic acid on postprandial lipaemia in diabetes
3.1 INTRODUCTION

Postprandial particles are thought to be particularly atherogenic (Karpe, et al., 1994; Tkac et al., 1997; Sakata et al., 1998; Kugiyama et al., 1999). The major postprandial lipoprotein is the chylomicron, which has apolipoprotein B48 as its structural protein and is produced in the intestine. Recently apo B-specific binding sites, which bind to apo B48, have been discovered on the human monocyte/macrophage, which suggests a mechanism that may contribute to the atherogenicity of these particles (Gianturco et al., 1998). The second postprandial particle is the apo B100-containing VLDL, which is produced by the liver. Considerable disturbance in both the amount and size of these particles has been shown to occur in diabetes (Curtin et al., 1996; DeMann et al., 1996; Taggart et al., 1997; Phillips et al., 2000). It has been demonstrated in the experimental animal that clearance and synthesis of these particles accounts for the abnormalities found in diabetes (O'Meara et al., 1990; Gleson et al., 1998). Understanding of the regulation of hepatically-derived VLDL has advanced with the discovery of the PPARs and their effect on gene regulation (Rodriguez et al., 1994). This has provided an explanation of how the fibrates reduce VLDL and lower serum triglyceride (Schoonjans et al., 1995). Many studies have examined the effect of change from a carbohydrate-rich to a fat-rich diet in diabetes (Garg, 1998; Gumbiner et al., 1998; Lerman-Garber et al., 1994) or a saturated to an unsaturated fat diet in both diabetic and non-diabetic subjects. Comparison of a polyunsaturated to monounsaturated fat diet is of considerable interest in view of the continued interest in the beneficial effects of a Mediterranean-type diet (de Lorgeril et al., 1999). Van Greevenbroek et al., 2000) have shown different effects of polyunsaturated as compared to monounsaturated fat in the ability to stimulate the secretion of apo B48 in Caco-2 cells. However, there is very little information on the effect of a polyunsaturated diet compared to a monounsaturated fat diet on postprandial lipoproteins in diabetes and, in particular, on the intestinally-derived apo B48-containing chylomicron.

This section of the human study examines the effect of a polyunsaturated fat diet compared to an isocaloric monounsaturated fat diet on the levels of these postprandial lipoproteins.
lipoproteins, using apo B48 as a marker for chylomicrons and apo B100 as a marker for the liver-derived VLDL particles.

3.2 METHODS

3.2.1 Subjects.
Eleven well-controlled Type 2 diabetic, male patients with HbA1c lower than 6% were randomly recruited from those regularly attending the diabetic clinic. The mean age was 56.5 ± 2.5 years. Patients with renal or hepatic dysfunction were excluded as were those with triglycerides >4mmol/l and those on lipid lowering agents or other drugs which would interfere with lipid metabolism. Patients were euthyroid and their diabetes and weight had been stable for at least 3 months. One patient was treated with diet alone and ten with diet and sulphonylurea. Six healthy, normolipaemic, non-diabetic control men (mean age of 46.7 ± 4.8 years) were recruited from hospital and laboratory personnel. Subjects did not undergo cardiovascular screening prior to participation in the study.

3.2.2 Study Design.
A randomised, cross-over study was performed. Both diabetic and non-diabetic subjects were interviewed by a dietitian and randomly instructed on an isocaloric diet high in either monounsaturated fat, including 30ml olive oil per day, or high in polyunsaturated fat, including 30ml sunflower oil per day for a period of two weeks each. Details of diets are given in section 2.5.1. At the end of each two week dietary period fasting blood was drawn and subjects were given the appropriate high fat test meal detailed in section 2.4.2, which they consumed over 30 minutes. Patients on oral hypoglycaemic agents took their medication as usual. Blood was sampled 2-hourly over the next 8 hours.

3.2.3 Serum and Lipoprotein Isolation and Analysis.
Fasting lipid profiles, fasting glucose and insulin and HbA1c were determined as described in section 2.4. Chylomicron, VLDL, and LDL fractions were isolated from plasma by
sequential ultracentrifugation as described in section 2.6. Compositional analysis (total protein, triglyceride, cholesterol and phospholipid) of each fraction was carried out as described in section 2.7. Fatty acid analysis was carried out on fasting LDL samples by the method detailed in section 2.7.3. Apo B48 and apo B100 were measured in both the chylomicron fraction and VLDL fraction (see section 2.8.2) by separation on 4-15% SDS-gradient gels, staining with Coomassie Brilliant Blue and quantification by densitometric scanning relative to an apo B100 standard purified from LDL (section 2.8.1).

3.3 RESULTS.

To fully investigate any possible effect of the linoleic and oleic acid-rich diets in diabetic subjects, levels of the various lipids and apolipoproteins were firstly compared between the diets and then between diabetic and non-diabetic subjects on each of the diets. This format will be employed in all subsequent chapters detailing results of the human study.

3.3.1 LDL Fatty Acid Composition.

In order to assess adherence of subjects to their assigned diets, the fatty acid content of fasting LDL samples was determined at the end of each two-week period. The six main fatty acids were quantified and results for both diabetic and non-diabetic subjects given in Table 3.1. The percentage of oleic acid in the LDL was significantly higher on the oleic acid diet (p<0.05). The percentage of linoleic acid was higher on the linoleic acid diet but did not quite reach statistical significance (p=0.08). Linoleic acid was however, significantly higher on the linoleic acid diet when expressed as μg/mg LDL protein (p<0.01). The ratio of linoleic acid to oleic acid was significantly higher on the linoleic acid diet (p<0.02).

It was found that there was a significantly higher percentage of linoleic acid on the linoleic acid diet (p<0.02) and a significantly higher percentage of oleic acid on the oleic acid diet (p<0.02). The ratio of linoleic acid to oleic acid was also significantly higher on the linoleic acid diet (p<0.02).
3.3.2 Characteristics of Subjects.

Characteristics of the diabetic subjects on the two diets are given in Table 3.2. Diabetic patients' Body Mass Index (BMI) did not change between the diets. Fasting blood glucose was significantly higher on the linoleic acid-rich diet (p<0.01), as was fasting insulin (p<0.002). Insulin resistance, measured using the homeostasis model reported in the Framingham Offspring Study (Meigs et al., 2000) was significantly lower on the oleic acid diet (p<0.001). Also significantly higher on the linoleic acid-rich diet was plasma cholesterol (p<0.01) and LDL cholesterol (p<0.01). There was no change in either plasma triglyceride or HDL cholesterol between the diets.

Comparison of the characteristics of the diabetic and non-diabetic subjects is also detailed in Table 3.2. The only two differences in the non-diabetic subjects between the diets, among all the measurements made in the human study are also given in this table. These were a significantly lower fasting blood glucose (p<0.01) and fasting plasma cholesterol (p<0.01). However, fasting glucose was still significantly higher in the diabetic subjects in both the linoleic acid (p<0.0001) and the oleic acid (p<0.0001) diets, defining their diabetic state. Fasting insulin was also higher in diabetic subjects on both diets (p<0.02 for both). There was no difference in insulin resistance in the non-diabetic group and the diabetic subjects remained more insulin resistant on both the linoleic acid diet (p<0.0005) and the oleic acid diet (p<0.0001). There was no difference in the BMI of the two groups of subjects. Neither was there a significant difference in any of the fasting lipids between the two groups on either of the diets, although there was a tendency toward higher triglyceride, higher LDL cholesterol and lower HDL cholesterol in the diabetic group on both diets.

3.3.3 Fasting Chylomicron and VLDL.

Fasting chylomicron and VLDL apo B and lipids are shown in Table 3.3. All fasting components of the chylomicron fraction were higher on the linoleic acid-rich diet, although only apo B48 (p<0.05) and apo B100 (p>0.02) were statistically significant. Apo B48 and apo B100 in the VLDL fraction were also higher on the linoleic acid-rich diet, but not
significantly. Phospholipid in the VLDL fraction however, was significantly higher on the linoleic acid-rich diet (p<0.02). The other lipid components in this fraction were similar.

Differences in fasting chylomicron and VLDL composition between diabetic and non-diabetic subjects are also detailed in Table 3.3. Fasting apo B48 and apo B100 in the chylomicron fraction were approximately three-fold higher in the diabetic subjects on the linoleic acid diet (p<0.01 and p<0.02 respectively). These were not different on the oleic acid diet. There was no difference in chylomicron lipids on either of the diets. There was no difference in any of the fasting VLDL components on either of the diets.

### 3.3.4 Postprandial Chylomicron and VLDL.

Postprandial levels of the various fraction components were assessed in two ways. The first was to compare the area under curve measured from the fasting value (AUC\(_f\)). This gave a measurement of the postprandial response following the test meal, effectively disregarding the fasting levels. The second assessment was to compare the area under curve from zero (AUC\(_z\)). This gave a measurement of the total amount of the component present over the postprandial time period, including the fasting level. These results are given in Table 3.4 and Table 3.5 respectively.

Chylomicron apo B48 and apo B100 AUC\(_f\) were almost twice as high on the linoleic acid-rich diet compared to the oleic acid-rich diet (p<0.05). This difference is accentuated when the fasting value is taken into account (AUC\(_z\)), with p<0.001 for both apo B48 and apo B100. These curves are shown in Figure 3.1. There was no significant difference in the cholesterol, triglyceride or phospholipid content of the chylomicron fraction postprandially.

Postprandial curves for the VLDL fraction are shown in Figure 3.2. There was an approximate two-fold increase in the AUC\(_f\) of apo B48 (p<0.01) and apo B100 (p<0.05) in the VLDL fraction. With no difference in the fasting values, the AUC\(_z\) showed a similar magnitude of difference (p<0.05). There was no difference in either triglyceride or
cholesterol in the VLDL fraction, but phospholipid was significantly higher on the linoleic acid-rich diet from fasting and from zero (p<0.05)

Comparison of postprandial chylomicron and VLDL components between diabetic and non-diabetic subjects is made in Table 3.4 (AUC_f) and Table 3.5 (AUC_z) also. It may be easier to visualise the differences by examining the postprandial curves in Figures 3.3 - 3.6. AUC_f will be considered firstly, followed by the AUC_z.

Diabetic subjects had approximately six-fold higher apo B48 in the chylomicron fraction on both diets (p<0.002 for both diets). Apo B100 in the chylomicron fraction was also significantly higher in the diabetic subjects on the linoleic acid diet (p<0.01) and the oleic acid diet (p<0.05). Chylomicron triglyceride was lower in the diabetic subjects on the linoleic acid diet (p<0.05) but not on the oleic acid diet. Postprandial apo B48 and apo B100 in the VLDL fraction were significantly higher in diabetic subjects only on the linoleic acid diet (p<0.05). There was no difference in apo B100, cholesterol, triglyceride or phospholipid in this fraction between diabetic and control groups.

AUC_z analysis mirrors that for AUC_f - strengthening, for the most part, the p-values on the linoleic acid diet and showing less of a difference in apo B48 and apo B100 on the oleic acid diet. Triglyceride levels in the chylomicron fraction on the linoleic acid diet were similar between the groups when the AUC is taken from zero.

3.3.5 Composition of Fasting LDL.

Fasting LDL composition in the diabetic subjects differed between the diets (Table 3.6) with a higher level of phospholipid (p<0.01) and an increase in total fatty acids (p<0.05) on the linoleic acid diet compared to the oleic acid diet. Diabetic and non-diabetic subjects are compared in Table 3.6. There was no significant difference in the percentage composition of any of the constituents on either diet.
Table 3.1: Composition of the major fatty acids in fasting LDL from diabetic (n=11) and non-diabetic (n=6) subjects, following the linoleic and oleic acid-rich dietary periods. Results are expressed as a percentage of the total weight of the 6 major fatty acids measured (% W/w) and as μg fatty acid/mg LDL protein and given as mean ± SD.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Diabetic</th>
<th></th>
<th>Non-diabetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic</td>
<td>Oleic</td>
<td>Linoleic</td>
<td>Oleic</td>
</tr>
<tr>
<td>% major fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>24.5 ±3.4</td>
<td>25.4 ± 2.0</td>
<td>23.3 ± 0.3</td>
<td>24.6 ± 0.5</td>
</tr>
<tr>
<td>Palmitoleic (16:1)</td>
<td>2.6 ± 1.3</td>
<td>3.0 ± 1.6</td>
<td>18.6 ± 5.5</td>
<td>24.2 ± 8.3</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>7.0 ± 0.7</td>
<td>7.0 ± 0.8</td>
<td>81.4 ± 17.3</td>
<td>84.1 ± 8.5</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>20.5 ± 3.1 a</td>
<td>23.6 ± 3.6</td>
<td>22.8 ± 4.9</td>
<td>27.6 ± 8.7</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>39.0 ± 5.5</td>
<td>35.7 ± 4.2</td>
<td>54.8 ± 11.9</td>
<td>51.5 ± 13.5</td>
</tr>
<tr>
<td>Arachidonic (20:4)</td>
<td>6.0 ± 1.8</td>
<td>5.5 ± 1.9</td>
<td>79.9 ± 16.2</td>
<td>82.4 ± 29.3</td>
</tr>
</tbody>
</table>

μg/mg LDL protein

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Diabetic</th>
<th></th>
<th>Non-diabetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic</td>
<td>Oleic</td>
<td>Linoleic</td>
<td>Oleic</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>320.1 ± 80.2</td>
<td>285.2 ± 74.7</td>
<td>23.3 ± 0.7</td>
<td>24.6 ± 1.2</td>
</tr>
<tr>
<td>Palmitoleic (16:1)</td>
<td>34.7 ± 20.3</td>
<td>33.5 ± 19.2</td>
<td>1.6 ± 0.6</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>90.1 ± 17.3</td>
<td>80.4 ± 22.7</td>
<td>6.4 ± 0.3</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>259.7 ± 68.9</td>
<td>270.0 ± 82.2</td>
<td>18.3 ± 1.4 b</td>
<td>21.1 ± 2.1</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>512.3 ± 72.7 c</td>
<td>382.7 ± 124.0</td>
<td>43.9 ± 2.5 b</td>
<td>39.7 ± 2.2</td>
</tr>
<tr>
<td>Arachidonic (20:4)</td>
<td>76.9 ± 26.8</td>
<td>65.5 ± 29.4</td>
<td>6.5 ± 0.4</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>Ratio 18:2/18:1</td>
<td>2.09 ± 0.6 b</td>
<td>1.51 ± 0.3</td>
<td>2.4 ± 0.3 b</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

a p<0.05  b p<0.02 different from oleic acid diet in same group.
Table 3.2: Characteristics of diabetic (n=11) and non-diabetic (n=6) subjects following the linoleic and oleic acid-rich dietary periods. Comparison is made between the diets in both groups and between diabetic and non-diabetic groups on each diet. Results are expressed in mmol/l, with BMI in kg/m² and given as mean ± standard deviation (SD) and median and range for triglyceride. Insulin resistance was calculated using the homeostasis model: Fasting insulin x fasting glucose/22.5 (Meigs et al., 2000).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Diabetic</th>
<th>Non-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic</td>
<td>Oleic</td>
</tr>
<tr>
<td>BMI</td>
<td>27.7 ± 2.6</td>
<td>27.8 ± 2.3</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>8.5 ± 0.9</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>13.4 ± 2.9</td>
<td>11.8 ± 2.3</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>5.18 ± 1.4</td>
<td>3.97 ± 0.8</td>
</tr>
<tr>
<td>Plasma cholesterol</td>
<td>5.3 ± 0.6</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>Plasma triglyceride</td>
<td>1.6 (0.9-3.6)</td>
<td>1.5 (1.0-2.5)</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>3.2 ± 0.5</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>1.34 ± 0.1</td>
<td>1.31 ± 0.2</td>
</tr>
<tr>
<td>Postprandial</td>
<td>1.38 ± 0.2</td>
<td>1.37 ± 0.1</td>
</tr>
</tbody>
</table>

a p<0.01  b p<0.002  c p<0.001 different from oleic acid diet in same group (paired t-test).

† p<0.02  § p<0.0005  ¶ p<0.0001 different from non-diabetic on same diet (unpaired t-test).
Fasting apo B48, apo B100 and lipid in the chylomicron and VLDL fractions from diabetic (n=11) and non-diabetic (n=6) subjects following the linoleic and oleic acid-rich dietary periods. Comparison is made between the diets in both groups and between diabetic and non-diabetic groups on each diet. Results are expressed as μg/ml plasma and given as mean ± SD.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic Linoleic</th>
<th>Diabetic Oleic</th>
<th>Non-diabetic Linoleic</th>
<th>Non-diabetic Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>3.1 ± 1.6 a§</td>
<td>1.6 ± 1.7</td>
<td>1.0 ± 0.7</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Apo B100</td>
<td>6.3 ± 4.1 b†</td>
<td>2.6 ± 2.2</td>
<td>1.6 ± 0.9</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>14.0 ± 13.2</td>
<td>9.6 ± 8.1</td>
<td>16.6 ± 21.2</td>
<td>7.3 ± 5.9</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>61.2 (6.3-169)</td>
<td>34.1 (3.6-81)</td>
<td>41.8 (2.5-149)</td>
<td>28.3 (4.0-74)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>15.5 ± 13.7</td>
<td>9.5 ± 2.5</td>
<td>14.3 ± 13.2</td>
<td>13.1 ± 9.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic Linoleic</th>
<th>Diabetic Oleic</th>
<th>Non-diabetic Linoleic</th>
<th>Non-diabetic Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>1.7 ± 1.6</td>
<td>1.3 ± 0.8</td>
<td>1.1 ± 0.5</td>
<td>1.4 ± 1.1</td>
</tr>
<tr>
<td>Apo B100</td>
<td>21.0 ± 13.5</td>
<td>15.3 ± 9.1</td>
<td>13.6 ± 9.1</td>
<td>10.8 ± 8.4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>66.0 ± 37.2</td>
<td>78.1 ± 88.4</td>
<td>84.3 ± 80.1</td>
<td>81.3 ± 59.3</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>388 (24-882)</td>
<td>294 (41-563)</td>
<td>184 (20-641)</td>
<td>323 (23-602)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>104.7 ± 55.7 b</td>
<td>64.2 ± 30.7</td>
<td>243.9 ± 349</td>
<td>128.5 ± 89.3</td>
</tr>
</tbody>
</table>

\( a \ p < 0.05 \quad b \ p < 0.02 \) different from oleic acid diet in same group (paired t-test).

\( ^\dagger \ p < 0.02 \quad ^\S \ p < 0.01 \) different from non-diabetic on same diet (unpaired t-test).
Table 3.4: Postprandial apo B48, apo B100 and lipid in the chylomicron and VLDL fractions from diabetic (n=11) and non-diabetic (n=6) subjects following the linoleic and oleic acid-rich dietary periods. Comparison is made between the diets in both groups and between diabetic and non-diabetic groups on each diet. Results are expressed as AUCf and given as mean ± SD.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic Linoleic</th>
<th>Diabetic Oleic</th>
<th>Non-diabetic Linoleic</th>
<th>Non-diabetic Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chylomicron</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>38.1 ± 20.7 a¶</td>
<td>21.7 ± 10.3 q</td>
<td>5.9 ± 2.9</td>
<td>3.7 ± 4.6</td>
</tr>
<tr>
<td>Apo B100</td>
<td>69.4 ± 41.9 a§</td>
<td>34.2 ± 18.8 †</td>
<td>13.1 ± 12.2</td>
<td>11.5 ± 7.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>176.5 ± 99.9</td>
<td>140.1 ± 136.0</td>
<td>175.2 ± 185.5</td>
<td>109.2 ± 107.2</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>530 (179-3308) †</td>
<td>945 (165-6433)</td>
<td>2180 (405-4757)</td>
<td>1015 (298-2750)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>222.8 ± 171.0</td>
<td>274.7 ± 280.3</td>
<td>456.5 ± 297.8</td>
<td>268.3 ± 221.4</td>
</tr>
</tbody>
</table>

| **VLDL**        |                   |               |                       |                    |
| Apo B48         | 11.3 ± 9.2 b†     | 4.0 ± 2.0     | 2.9 ± 1.7             | 1.8 ± 2.4          |
| Apo B100        | 73.6 ± 57.7 a     | 42.1 ± 37.9   | 22.8 ± 25.7           | 22.2 ± 23.7        |
| Cholesterol     | 355.1 ± 301.5     | 465.4 ± 668.0| 223.5 ± 269.1         | 481.8 ± 388.7      |
| Triglyceride    | 2036 (362-4441)   | 1048 (387-4064)| 644 (246-3848)      | 1525 (341-3017)    |
| Phospholipid    | 380.2 ± 251.1 a   | 208.7 ± 166.2| 365.5 ± 326.0         | 297.0 ± 285.1      |

*a p<0.05 † p<0.01 different from oleic acid diet in same group (paired t-test).
‡ p<0.05  § p<0.01 ¶ p<0.002 different from non-diabetic on same diet (unpaired t-test). Triglyceride used a paired, non-parametric test (Wilcoxon matched pairs test) between diets and an unpaired, non-parametric test (Mann-Whitney U test) between groups.
Table 3.5: Postprandial apo B48, apo B100 and lipid in the chylomicron and VLDL fractions from diabetic (n=11) and non-diabetic (n=6) subjects following the linoleic and oleic acid-rich dietary periods. Comparison is made between the diets in both groups and between diabetic and non-diabetic groups on each diet. Results are expressed as AUC$_Z$ and given as mean ± SD.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic Linoleic</th>
<th>Diabetic Oleic</th>
<th>Non-diabetic Linoleic</th>
<th>Non-diabetic Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>61.8 ± 23.2 c,f</td>
<td>34.1 ± 18.8 §</td>
<td>13.6 ± 7.1</td>
<td>8.5 ± 4.2</td>
</tr>
<tr>
<td>Apo B100</td>
<td>119.8 ± 58.5 c§</td>
<td>53.9 ± 26.4 †</td>
<td>25.4 ± 16.0</td>
<td>21.5 ± 11.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>293.5 ± 198.1</td>
<td>253.5 ± 211.3</td>
<td>294.3 ± 312.5</td>
<td>153.9 ± 127.5</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>902 (420-4462)</td>
<td>1174 (194-7079)</td>
<td>2512 (426-5588)</td>
<td>1242 (349-3115)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>575.2 ± 705.9</td>
<td>346.5 ± 327.5</td>
<td>566.7 ± 355.0</td>
<td>365.8 ± 276.2</td>
</tr>
<tr>
<td>VLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>24.8 ± 20.3 a†</td>
<td>12.8 ± 6.2</td>
<td>11.8 ± 3.7</td>
<td>8.5 ± 4.2</td>
</tr>
<tr>
<td>Apo B100</td>
<td>234.1 ± 132.0 b</td>
<td>145.7 ± 72.9</td>
<td>123.6 ± 74.0</td>
<td>99.7 ± 47.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>583.3 ± 328.3</td>
<td>546.1 ± 330.2</td>
<td>748.3 ± 605.5</td>
<td>656.4 ± 321.1</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>3331 (1095-9837)</td>
<td>1810 (559-7443)</td>
<td>2744 (211-4671)</td>
<td>3248 (369-4390)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>970.2 ± 436.0 a</td>
<td>652.4 ± 388.9</td>
<td>2186 ± 3115</td>
<td>1166 ± 573.6</td>
</tr>
</tbody>
</table>

* p<0.05  † p<0.01  ‡ p<0.001 different from oleic diet in same group (paired t-test).
§ p<0.001 different from non-diabetic on same diet (unpaired t-test).
Table 3.6: Composition of fasting LDL from diabetic (n=11) and non-diabetic (n=6) subjects following the linoleic and oleic acid-rich dietary periods. Comparison is made between the diets in both groups and between diabetic and non-diabetic groups on each diet. Results are expressed as a percentage of the total weight (%W/W), with total fatty acid as μg/mg LDL protein and given as mean ± SD.

<table>
<thead>
<tr>
<th>LDL Component</th>
<th>Diabetic Linoleic</th>
<th>Diabetic Oleic</th>
<th>Non-diabetic Linoleic</th>
<th>Non-diabetic Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>39.4 ± 6.3</td>
<td>46.3 ± 18.8</td>
<td>42.0 ± 5.1</td>
<td>40.8 ± 10.9</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>8.3 (4.0-53)</td>
<td>8.6 (1.4-33)</td>
<td>3.4 (2.3-27)</td>
<td>3.8 (2.7-14)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>26.2 ± 5.2</td>
<td>20.8 ± 7.7</td>
<td>23.6 ± 5.0</td>
<td>18.5 ± 13.5</td>
</tr>
<tr>
<td>Protein</td>
<td>25.5 ± 8.2</td>
<td>24.8 ± 11.4</td>
<td>26.7 ± 4.5</td>
<td>29.6 ± 7.1</td>
</tr>
<tr>
<td>Total fatty acids</td>
<td>1294 ± 223.2</td>
<td>1117 ± 309.5</td>
<td>1235 ± 268.0</td>
<td>1301 ± 349.2</td>
</tr>
</tbody>
</table>

a p<0.05  b p<0.01 different from oleic acid diet in same group (paired t-test).
Figure 3.1: Apoproteins B48 (a) and B100 (b) in the chylomicron fraction at fasting and postprandially from Type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 3.2: Apoproteins B48 (a) and B100 (b) in the VLDL fraction at fasting and postprandially from Type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 3.3: Apo B48 in the chylomicron fraction at fasting and postprandially from Type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods.
Figure 3.4: Apo B100 in the chylomicron fraction at fasting and postprandially from Type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods.
Figure 3.5: Apo B100 in the VLDL fraction at fasting and postprandially from Type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods. (NS = not significantly different).
Figure 3.6: Apo B48 in the VLDL fraction at fasting and postprandially from Type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods.
3.4 DISCUSSION.

This section of the study details, primarily, changes in the levels of intestinally derived, apo B48-containing and hepatically-derived, apo B100-containing lipoproteins at fasting and postprandially when diabetic subjects are switched between monounsaturated and polyunsaturated fatty acid-rich diets. Even though postprandial lipoprotein fatty acid composition largely reflects the most recent meal, a two week dietary period was chosen to allow time for the observation of changes which may occur in gene expression through the PPAR system or alterations in cell membrane composition which may influence ligand-receptor interaction involved in particle clearance. A washout period was not included in the study. This would serve to prevent any possible overlap of effects from the first diet to the second. However, Jump et al., (1996) review considerable evidence which shows that the effect of polyunsaturated fatty acids, at least, on lipogenic gene expression is rapid and occurs within hours of changing the diet and also that the effects persist only as long as polyunsaturated fatty acid remains in the diet. Starting diets were also assigned randomly to subjects, reducing the statistical impact of any possible overlap in effect. This was verified by the observation of similar statistical trends when groups were divided into those who had the linoleic diet first and those who had the oleic diet first (results not shown).

This chapter also deals with lipid and other biochemical and physical characteristics of the diabetic patients, how they change from one diet to the other and also how they differ from non-diabetic subjects on the two diets. Changes in the composition of both the postprandial fraction particles and also fasting LDL particles are also shown.

The adherence of subjects to their assigned diets was verified by an increase in the ratio of linoleic acid to oleic acid in fasting LDL after the linoleic acid diet compared to that of the oleic acid diet. Both groups achieved this. There was no change in the mean BMI of either group of subjects between the diets, indicating that they neither lost nor gained weight on one diet compared to the other. There was no difference in the mean BMI between diabetic and non-diabetic groups. There were quite a number of changes in the lipid parameters and the parameters of metabolic control in the diabetic subjects. Fasting blood glucose levels improved on the oleic acid diet, but remained significantly higher than the non-diabetic
group on both diets. This was accompanied by a decrease in fasting insulin. Insulin resistance was significantly improved by the oleic acid diet in the diabetic subjects but it still remained significantly higher than the non-diabetic group. This observation is consistent with the finding that Type 2 diabetic patients on a similar olive oil diet have enhanced glucose transport in their adipocytes, relative to a sunflower oil diet (Ryan et al., 2000). This was associated with an accompanying alteration in the oleic/linoleic acid ratio in the adipocytes.

It was originally thought that the polyunsaturated fat diet was more effective in lowering cholesterol than a monounsaturated fat diet (Keys et al., 1957, Hegsted et al., 1993), but more recent studies have suggested that there may be no difference between the diets in their effect on LDL cholesterol levels (Gardner et al., 1995), at least in non-diabetic subjects. This current study shows a significant reduction in both total and LDL cholesterol on the oleic acid diet. With regard to the postprandial effect, there is little information on changing between linoleic and oleic acids in non-diabetic subjects, yet it has been shown experimentally that the rate of lipolysis of chylomicrons may depend on the fatty acid composition of the particle (Botham et al., 1997). It has been shown that not only fasting, but postprandial chylomicrons also are elevated in diabetes and that a considerably larger proportion are smaller in size, falling within the density range of VLDL and therefore are isolated ultracentrifugally along with VLDL (Curtin et al., 1996; Taggart et al., 1997).

Diabetic patients on a diet rich in linoleic acid have significantly more large chylomicron particles present in their blood while fasting than those present after a diet rich in oleic acid. The oleic acid diet levels resemble that of the non-diabetic group and may therefore be considered more normal. This normalisation may be indicative of an improvement in the lipolysis of these large particles on the oleic acid diet or a reduction in the number produced by the intestine. Postprandially, diabetic patients have significantly more of these large chylomicron particles present after a diet rich in linoleic acid. Indeed, the postprandial curve shows little reduction even 8 hours after the test meal. This difference is
more pronounced when AUC values are calculated from zero, without taking the fasting levels into account. An oleic acid-rich diet improves the levels of postprandial, large chylomicron particles in diabetic patients, but regardless of the diet, levels are significantly higher than those present in non-diabetic subjects. Again, this suggests that lipolysis of these larger particles, the preferred substrate of LPL, may be impaired in diabetes and that a diet rich in oleic acid may improve this to some degree. Alternatively, production of these larger particles postprandially and while fasting may be elevated in diabetes.

This study shows that diabetic patients have significantly higher levels of chylomicron remnants postprandially, when their diet is rich in linoleic acid. A diet rich in oleic acid normalises levels of chylomicron remnants. Levels at fasting show no difference between diabetic patients and non-diabetic subjects on either diet, suggesting that this may be mainly a postprandial disturbance and one which is of particular importance to diabetic patients, given the well-established elevation in remnants postprandially (DeMann et al., 1996; Curtin et al., 1996; Taggart et al., 1997; Phillips et al., 2000). Simons et al. (1987) have shown that the relative enrichment of apo B48 compared to apo B100 in the sf 60 fraction, 4 hours after a fat meal, was significantly greater in patients with CAD than in controls. It has also been shown that the postprandial concentration of apo B48 in the sf 20-60 fraction (chylomicron remnants) correlates with the progression of coronary atherosclerosis (Karpe et al., 1994). The importance of intestinally derived lipoproteins in the development of atherosclerosis was given further credence with the discovery of a receptor specific for apo B48 on the surface of monocyte/macrophages (Gianturco et al., 1998). Several studies have shown that chylomicron remnants penetrate arterial tissue rapidly, efflux poorly and in vitro serve as a lipid substrate for macrophages (Mamo and Wheeler, 1994; Mamo et al. 1996; Fujioka et al., 1998). Yu and Mamo, (2000) showed that chylomicron remnants prepared in vivo could induce foam cell formation in human monocyte-derived macrophages and that these particles were also cytotoxic to rabbit aorta smooth muscle cells. Mechanistically, chylomicrons may contribute to cholesterol and lipid accumulation in macrophages thereby contributing to plaque formation. It may be of particular importance therefore that a diet rich in oleic acid appears to normalise levels of remnants in diabetic patients.
With regard to apo B100-containing, liver derived particles, diabetic patients have significantly higher levels of the larger VLDL particles (isolated in the chylomicron fraction) at fasting and postprandially after a diet rich in linoleic acid. The predominance of large, triglyceride-rich VLDL particles have been shown to be associated with the production of small, dense LDL particles (Packard et al., 2000). This phenotype is two-fold more common in even normolipaemic Type 2 diabetic patients than age-matched control subjects of similar lipid levels (Feingold et al., 1992). The predominance of these particles is associated with an increased risk of CAD (Austin et al., 1988; Griffin et al., 1994). It is interesting that on the linoleic acid-rich diet the diabetic patients have elevated levels of these large VLDL particles and also significantly more phospholipid in the LDL fraction as a percentage of the total weight, suggesting the presence of smaller, more numerous particles with a greater combined surface area.

Apo B100-containing lipoproteins of the VLDL density fraction are the major carrier of triglyceride in the bloodstream. At fasting, the number of these particles present is similar between the diets in the diabetic patients and between groups. Postprandially, an oleic acid-rich diet reduces VLDL numbers in diabetic patients, but while numerically three times higher, it was not significantly different from control levels on either diet.

Tkac et al. (1997) investigated a possible relationship between the severity of CAD and the concentration of the major classes of lipoproteins. They found that the number of particles, reflected by the apo B levels of the TRL, were greater in those with moderate and severe disease than in those with mild disease. Analysis of triglyceride only did not show a significant correlation with CAD. The results of this part of the study indicate that both apo B48- and apo B100-containing particle levels are elevated for the most part in diabetes. It is interesting to note that particles of the chylomicron fraction produced after the linoleic acid-rich diet contain significantly less triglyceride compared to non-diabetic particles, suggesting a smaller particle size and this disturbance may be normalised or at least alleviated with the consumption of a diet rich in olive oil. The same trend does exist however on the oleic acid diet. The opposite trend is observed in the VLDL particles with diabetic particles being more
enriched with triglyceride on both diets. This may be due to an increased production and secretion of triglyceride from the diabetic liver, resulting in an increased number of VLDL particles which tend to be enriched in triglyceride. It may also represent a difference in particle assembly involving microsomal triglyceride transfer protein which has been shown to be increased in diabetic rabbits (Phillips et al., submitted) in the liver of the diabetic patients.

Subsequent chapters investigate possible differences in apolipoprotein composition of these particles in an attempt to begin to elucidate a mechanism, perhaps of a more efficient lipolysis or enhanced clearance, to explain the observed effects of the oleic acid diet.
Chapter 4

The effect of dietary linoleic and oleic acids on apolipoproteins involved in postprandial lipoprotein metabolism in diabetes
4.1 INTRODUCTION.

In the previous chapter the data show a significant reduction in the level of both intestinally- and liver-derived particles following an olive oil-rich diet in type II diabetic subjects compared to one rich in sunflower oil, bringing the levels more in line with non-diabetic subjects. This chapter investigates particle apolipoprotein composition in an attempt to explain the observed improvement in the diabetic dyslipidaemia.

Postprandial particle metabolism is complex. Both apo E and apo C have key roles to play. There is general agreement that both chylomicrons and VLDL share the same lipolytic pathway (Bjorkegren et al., 1996) involving the hydrolysis of the core triglyceride of the particles by the action of LPL to produce remnant particles and this process has been shown to be regulated for the most part by apo C - apo CII being its essential cofactor and apo CIII inhibiting its LPL activity. Over expression of apo CIII in transgenic mice was shown to lead to accumulation of VLDL (Aalto-Setälä et al., 1992) and also apo B48-containing chylomicron remnants (de Silva et al., 1994). But mice over expressing apo CII also developed hypertriglyceridaemia (Shachter et al., 1994). This was attributed to involvement of apo CII in the second step of the process - the receptor-mediated endocytosis of the particles into hepatocytes.

Particle uptake is mediated by apo E and involves the sequestration of particles in the Space of Disse in the liver through binding to HSPG, HL and indeed LPL (Cooper, 1997 and Mahley and Ji, 1999) and the subsequent internalisation by binding to the various receptors though apo E. There is controversy as to whether hepatically localised apo E plays a role in this process but recent evidence from experiments in perfused livers of apo E- and apo E/LDLR-knockout mice suggest it is the apo E present on the lipoprotein itself rather than hepatically localised apo E which is important in particle uptake (Yu et al., 2000). In a study with cholesterol-fed rabbits (used as a model of elevated triglyceride-rich lipoproteins), transgenic for and over expressing human apo E, investigators observed a 35% reduction in triglyceride due to a decrease in VLDL, showed that isolated VLDL had a
higher affinity for binding to the LDLR and that injected chylomicrons were cleared more rapidly in the transgenic animals compared to their non-transgenic litter mates (Fan et al., 1998). These animals also showed an increase in LDL, compelling evidence for the role of apo E as the primary ligand for such receptors as the LDLR, in the clearance of postprandial lipoproteins. A further demonstration of the crucial role of apo E in lipoprotein metabolism was presented in a study in LDLR-knockout mice over expressing rat apo E. These animals showed a consistent reduction in plasma LDL, even when fed a cholesterol-rich, atherogenic diet due to the enhanced removal of triglyceride-rich lipoproteins and also the suppression of fatty streak lesions in the aortic sinus compared to LDLR-transgenic animals with normal levels of apo E (Osuga et al., 1998). The authors go as far as to suggest stimulation of hepatic apo E as a promising adjunctive therapy of homozygous familial hypercholesterolaemia. The importance of apo E is also demonstrated by the differences in the removal rate of chylomicron remnants in normolipidaemic individuals with different apo E phenotypes. The $E_2$ isoform is significantly functionally less active (Brewer et al., 1983) while compared to the normal $E_3$ form the $E_4$ isoform has a higher affinity for VLDL (Bioletto et al., 1998) but homozygous, transgenic mice ($E_4/E_4$) clear VLDL at only half the rate of $E_3/E_3$ animals (Knouff et al., 1999).

Apo CIII has a duel role in postprandial lipoprotein metabolism. It inhibits the apo E-mediated uptake of the particles (Karpe et al., 1993), the exact mechanism for which is still unclear. Indeed it is now clear and has been shown that both apo CIII and apo CII have been shown to inhibit apo E-mediated binding of particles differentially to various receptors (Jong et al., 1999). Mann et al., (1997) also described differential effects on receptor binding inhibition between the various apo CIII isoforms, with nascent, sialylated forms having greater affinity for VLDL but a lesser ability to inhibit binding than the desialylated forms. Over expression of apo CIII in mice resulted in an accumulation of TRLs (Aalto-Setälä et al., 1992). Later the accumulation of apo B48 remnants in these transgenic mice was shown to be corrected by over expression of apo E, without altering particle-affinity for LPL (de Silva et al., 1994). This led to the suggestion that the ratio of apo CIII to apo E was the major determinant in the rate at which lipoproteins were cleared.
Insulin plays an important role in regulating the expression of LPL and apo CIII. In insulin deficient mice hepatic apo CIII mRNA levels were increased up to 1.5-fold and were reduced in a dose-dependant manner on the administration of insulin (Chen et al., 1994). In diabetic patients plasma apo CIII levels differ depending on the type and glycaemic control of diabetes. In Type I patients apo CIII levels were largely increased and returned to normal with achievement of good glycaemic control. Type II patients had marginally increased levels despite their associated hypertriglyceridaemia (Alaupovic et al., 1988). Elevations in plasma apo E have also been associated with Type II diabetes in the presence of CHD (Sylvänne et al., 1994).

Apo CIII and also apo CII gene expression has been shown to be regulated through PPARα (Peters et al., 1997) and expression of both can be reduced by the action of fibrates- known PPARα ligands (Andersson et al., 1999). Dietary polyunsaturated and monounsaturated fatty acids have also been shown to be ligands of PPARα (Auwerx et al., 1996; Jump et al., 1996), having a rapid effect (hours) on gene expression, particularly in the liver, which lasts only as long as the fatty acid remains in the diet (Clarke et al., 1997).

There is relatively little known about the effect of altering dietary fatty acids on apolipoprotein content in the postprandial lipoproteins, particularly with reference to the diabetic state. In this study a diet rich in linoleic acid has been shown to significantly increase postprandial lipoprotein levels and exacerbate hyperglycaemia relative to a diet rich in oleic acid (Chapter 3). The purpose of this part of the study was to investigate the effect of dietary fatty acids on the apolipoprotein composition of postprandial particles which may determine their metabolic fate.
Chapter 4

4.2 METHODS.

4.2.1 Subjects and Study Design.
This chapter details further analysis and results of lipoproteins isolated from the eleven type 2 diabetic and six non-diabetic subjects described in chapter 3. In summary, subjects were instructed on a diet rich in monounsaturated or polyunsaturated fatty acids (see section 2.4.1) for a period of two weeks, after which time subjects were swapped to the second diet for the same time period. Blood samples were taken following a test meal at fasting and every 2 hours, for 8 hours postprandially (see section 2.4.2).

4.2.2 Lipoprotein Isolation and Analysis.
Chylomycin and VLDL fractions were isolated from fasting and postprandial blood samples by ultracentrifugation as described in section 2.5. Apo E was measured in fresh, non-frozen samples of both fractions within 48 hours of isolation (section 2.7.2) by electrophoresis on 4-15% gradient gels, staining with Coomassie Blue and quantification by densitometric scanning relative to an apo B100 standard isolated from LDL (section 2.7.1). Apo C11 and apo C111 were measured in frozen chylomicron samples on 10-20% gradient gels as described in section 2.7.4, using an apo C11 standard (section 2.7.1). Apo C11 and isoforms of apo C111 in frozen samples of the VLDL fraction were measured on 5% polyacrylamide isoelectric focusing gels, pH 3.5-9.5, as described in section 2.7.8 using an apo C11 standard.

4.3 RESULTS.
The postprandial curves of the apolipoproteins analysed are complex and vary widely. As such, only AUCs from zero (AUCz) were used for analysis. Each individual time point, including fasting, has been analysed and results are displayed in the figures rather than tables.
4.3.1 Apo E in the Chylomicron Fraction.

When expressed in μg/ml plasma, the postprandial curves for apo E were remarkably similar in diabetic subjects between the diets. The AUC was higher in diabetic subjects particularly in the later postprandial phase compared to the non-diabetic subjects but do not quite reach statistical significance (p=0.07 and p=0.12 for linoleic and oleic acid diets respectively) (Figures 4.1 and 4.2). However, when expressed in μg/mg apo B both the AUCz (p<0.002) and individual postprandial time points were significantly lower in diabetic subjects on the linoleic acid-rich diet compared to the oleic acid-rich diet. The fasting value was lower, but not significantly (Figure 4.3). Apo E was significantly lower in the diabetic subjects on the linoleic acid-rich diet (AUCz p<0.01) and even on the oleic acid-rich diet (AUCz p<0.05) compared to non-diabetic subjects on the equivalent diets. Both fasting and the majority of the postprandial points were significantly lower on both diets (Figure 4.4). Numerical values for AUCz are given in Tables 4.1 and 4.2.

4.3.2 Apo C in the Chylomicron Fraction.

The postprandial curves for both apo CII and apo CIII in the chylomicron fraction when expressed in μg/ml plasma were also remarkably similar between diets in the diabetic subjects and also between diabetic and non-diabetic subjects on both diets (Figures 4.5 - 4.8). When these data were expressed in μg/mg apo B, however, apo CII (AUCz p<0.05) was significantly lower on the linoleic acid-rich diet. It was significantly lower at fasting and at most of the postprandial points (Figure 4.9). Apo CII was significantly lower in diabetic subjects compared to controls only on the linoleic acid-rich diet (AUCz p<0.002), with the latter time points being the most significantly different (Figure 4.10 (a)). The oleic acid curves differed only at the 2 hour time point (Figure 4.10 (b)). The AUCz for apo CIII was also lower on the linoleic acid diet (AUCz p<0.02) but none of the individual points reached statistical significance (Figure 4.11). Apo CIII was significantly lower in diabetic subjects compared to non-diabetic only after the linoleic acid-rich diet (AUCz p<0.001). All the postprandial time points were significantly lower in the diabetic subjects on the linoleic acid-rich diet (Figure 4.12 (a)) while only the 8 hour time point was significantly lower between subjects on the oleic acid-rich diet (Figure 4.12 (b)).
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Total apo C (numerical addition of apo CII and apo CIII) in the chylomicron fraction reflects these results. When expressed in μg/ml plasma there is no difference in the diabetic subjects between the diets or between diabetic and non-diabetic subjects on either diet (Table 4.1). When expressed in μg/mg apo B total apo C was significantly lower in diabetic subjects on the linoleic acid-rich diet (AUCz p<0.05) and this was significantly lower than non-diabetic subjects on the linoleic acid-rich diet (AUCz p<0.002). There was no difference between diabetic and non-diabetic subjects on the oleic acid-rich diet (Table 4.2).

The ratio of apo CIII / E is similar in the diabetic subjects between the diets and also between diabetic and non-diabetic subjects on both diets. There was also no difference in the ratio of total apo C / E nor apo CIII / CII (Table 4.3).

4.3.3 Apo E in the VLDL Fraction.

As in the chylomicron fraction apo E in the VLDL fraction, when expressed in μg/ml plasma, was similar after both diets in the diabetic group (Figure 4.13). But unlike the chylomicron fraction, apo E in the VLDL fraction was significantly lower in the diabetic subjects compared to the non-diabetic after the linoleic (AUCz p<0.02) and the oleic acid-rich (AUCz p<0.05) diets. The differences are quite profound with all of the postprandial time points being significantly lower in the diabetic group on the linoleic acid-rich diet (Figure 4.14 (a)) compared to the non-diabetic group with the 2 and 4 hour time points significantly lower in diabetic compared to non-diabetic subjects after the oleic acid-rich diet (Figure 4.14 (b)). Numerical values for the AUC data are shown in Table 4.1.

These differences in apo E are accentuated when the data are expressed in μg/mg apo B. The AUCz for diabetic subjects on the linoleic acid-rich diet (Table 4.2) was almost half that of the AUCz after the oleic acid-rich diet (p<0.01). Each postprandial time point was significantly lower on the linoleic diet (Figure 4.15). When compared to non-diabetic subjects, apo E expressed per mg apo B was very significantly reduced in diabetic subjects on the linoleic acid-rich diet (AUCz p<0.0001). There was also a significantly lower level in diabetic compared to non-diabetic subjects on the oleic acid-rich diet (AUCz p<0.01).
Both the fasting and all the postprandial time points were reduced in diabetic subjects on the linoleic acid-rich diet (Figure 4.16 (a)) compared to the non-diabetic group, whilst there were differences only at the later time points of the oleic curves (Figure 4.16 (b)).

4.3.4 Apo CII in the VLDL Fraction.

Unlike the chylomicron fraction, apo CII in the VLDL fraction when expressed in μg/ml plasma showed significantly higher AUCz in the diabetic subjects on the linoleic acid-rich diet (p<0.05) compared to the oleic acid-rich diet (Table 4.1). Most of the postprandial time points were higher on the linoleic acid-rich diet (Figure 4.17) when expressed in this way. There was however no significant difference in VLDL apo CII between diabetic and non-diabetic subjects on either diet (Table 4.1). Indeed the postprandial curves were quite similar between the groups on the two diets (Figure 4.18).

Expressing the results per mg apo B, the AUCz for apo CII was similar between the diets in the diabetic subjects (Table 4.2). The shape of the postprandial curves differ quite considerably however, with all the postprandial time points being higher on the oleic acid-rich diet (Figure 4.19). It reaches statistical significance at the 6 hour time point (P<0.05). Comparing diabetic with non-diabetic subjects, the AUCz was significantly higher in the non-diabetic group only after the linoleic acid-rich diet (p<0.05) with the greatest difference at 4, 6 and 8 hours postprandially (Figure 4.20 (a)). Curves for both groups were similar on the oleic acid-rich diet (Figure 4.20 (b)).

4.3.5 Apo CIII in the VLDL Fraction.

Similar to apo CII, apo CIII in the VLDL fraction, when expressed per ml plasma was significantly higher in diabetic subjects on the linoleic acid-rich diet (AUCz p<0.02) compared to the oleic acid-rich diet (Table 4.1). The 2, 6 and 8 hour time points showed the greatest difference (Figure 4.21 (a)). This difference was a result of all three apo CIII isoforms being significantly higher on the linoleic acid-rich diet (Figure 4.21 (b)). As with apo CII, there was no difference in apo CIII between diabetic and non-diabetic subjects on either diet (Table 4.1) and postprandial curves were quite similar (Figure 4.22). Neither
was there any difference in the individual apo CIII isoforms between diabetic and non-diabetic subjects on either diet (Figure 4.23) when expressed as μg/ml plasma.

When expressed per mg apo B, the AUCz for apo CIII was similar in the diabetic subjects between the diets (Table 4.1). There were no significantly different postprandial points (Figure 4.24 (a)). There was however, a significantly lower level of apo CIII1 in diabetic subjects on the linoleic acid-rich diet (p<0.05) compared to the oleic acid-rich diet (Figure 4.24 (b)). Comparing diabetic and non-diabetic subjects, apo CIII was significantly lower in the diabetic group (AUCz p<0.05) compared to the non-diabetic group. The greatest differences were in the later postprandial time points (Figure 4.25 (a)). There was no difference between the groups on the oleic acid-rich diet (Figure 4.25 (b)). All three isoforms of apo CIII were significantly lower in the diabetic group on the linoleic acid-rich diet compared to the non-diabetic group (Figure 4.26 (a)), but there only apo CIII2 was significantly lower in the diabetic group on the oleic acid-rich diet (Figure 4.26 (b)). AUCz values are given in Table 4.2 for apo CIII isoforms.

4.3.6 Apo E Genotyping.

As can be seen in figures 4.16 (a) and (b), one of the diabetic subjects had the 3/4, all the others having the 3/3 genotype. All the non-diabetic subjects were shown to have the 3/3 genotype.
Table 4.1: Postprandial apo E, apo C11 and apo C111 in the chylomicron and VLDL fractions from diabetic subjects (n=11), following the linoleic and oleic acid-rich dietary periods. Results are expressed as AUCz for the μg/ml plasma curve and given as mean ± SD.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic Linoleic</th>
<th>Diabetic Oleic</th>
<th>Non-diabetic Linoleic</th>
<th>Non-diabetic Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16.5 ± 8.8</td>
<td>12.6 ± 8.3</td>
<td>10.0 ± 4</td>
<td>8.9 ± 3</td>
</tr>
<tr>
<td><strong>Apo E</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Apo C11</strong></td>
<td>10.8 ± 7.4</td>
<td>13.8 ± 9.6</td>
<td>8.6 ± 6</td>
<td>6.8 ± 7</td>
</tr>
<tr>
<td><strong>Apo C111</strong></td>
<td>33.0 ± 19.1</td>
<td>36.7 ± 33.2</td>
<td>27.0 ± 25</td>
<td>18.7 ± 13</td>
</tr>
<tr>
<td><strong>Total apo C</strong></td>
<td>43.8 ± 23.7</td>
<td>50.5 ± 41.8</td>
<td>35.6 ± 31.5</td>
<td>25.6 ± 20.0</td>
</tr>
<tr>
<td>Chylomicron</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VLDL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Apo E</strong></td>
<td>20.2 ± 16.8 §</td>
<td>16.5 ± 10.5 †</td>
<td>47.9 ± 23.1</td>
<td>34.8 ± 25.9</td>
</tr>
<tr>
<td><strong>Apo C11</strong></td>
<td>22.4 ± 14.6 a</td>
<td>16.2 ± 12.0</td>
<td>28.9 ± 18.5</td>
<td>24.5 ± 16.8</td>
</tr>
<tr>
<td><strong>Total apo C111</strong></td>
<td>127.0 ± 67.0 b</td>
<td>93.2 ± 44.6</td>
<td>135.8 ± 94.9</td>
<td>129.9 ± 83.7</td>
</tr>
<tr>
<td><strong>C1110</strong></td>
<td>26.5 ± 20.3 a</td>
<td>20.1 ± 15.4</td>
<td>29.2 ± 19.4</td>
<td>30.5 ± 19.8</td>
</tr>
<tr>
<td><strong>C1111</strong></td>
<td>50.4 ± 26.4 c</td>
<td>34.6 ± 20.2</td>
<td>53.7 ± 38.6</td>
<td>49.2 ± 33.5</td>
</tr>
<tr>
<td><strong>C1112</strong></td>
<td>50.2 ± 24.4 a</td>
<td>37.4 ± 14.6</td>
<td>53.0 ± 37.6</td>
<td>49.6 ± 31.4</td>
</tr>
</tbody>
</table>

a p<0.05  b p<0.02  c p<0.01 different from oleic diet in same group (paired t-test)
† p<0.05 § p<0.02 different from non-diabetic on same diet (unpaired t-test)
Table 4.2: Postprandial apo E, apo C11 and apo C111 in the chylomicron and VLDL fractions from diabetic subjects (n=11), following the linoleic and oleic acid-rich dietary periods. Results are expressed as AUCz for the μg/mg apo B curve and given as mean ± SD.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic</td>
<td>Oleic</td>
</tr>
<tr>
<td><strong>Chylomicron</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo E</td>
<td>730.0 ± 339 c†</td>
<td>1349 ± 502 †</td>
</tr>
<tr>
<td>Apo CII</td>
<td>600.2 ± 545 a¶</td>
<td>1589 ± 1263</td>
</tr>
<tr>
<td>Apo CIII</td>
<td>1224 ± 719 b¶</td>
<td>3448 ± 2518</td>
</tr>
<tr>
<td>Total apo C</td>
<td>1824 ± 1404 a†</td>
<td>5037 ± 354</td>
</tr>
<tr>
<td><strong>VLDL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo E</td>
<td>495.8 ± 338.1 b¥</td>
<td>945.8 ± 673 §</td>
</tr>
<tr>
<td>Apo CII</td>
<td>755.5 ± 505 †</td>
<td>1088 ± 1005</td>
</tr>
<tr>
<td>Total apo CIII</td>
<td>4473 ± 2446 †</td>
<td>5984 ± 3406</td>
</tr>
<tr>
<td>CIII0</td>
<td>887.3 ± 720 †</td>
<td>1208 ± 936</td>
</tr>
<tr>
<td>CIII1</td>
<td>1807 ± 1095 a †</td>
<td>2507 ± 1607</td>
</tr>
<tr>
<td>CIII2</td>
<td>1779 ± 801 §</td>
<td>2269 ± 1068 †</td>
</tr>
</tbody>
</table>

a p<0.05  b p<0.02  c p<0.002 different from oleic diet in same group (paired t-test)
† p<0.05  § p<0.01  ¶ p<0.002  ¥ p<0.0001 different from non-diabetic on same diet (unpaired t-test).
Table 4.3: Apolipoprotein ratios in the chylomicron and VLDL fractions from diabetic (n=11) and non-diabetic subjects (n=6), following the linoleic and oleic acid-rich dietary periods. Results were calculated from the mean apolipoprotein content (µg/mg apo B) over the whole test period including and are given as mean ± SD.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic</td>
<td>Oleic</td>
</tr>
<tr>
<td>Chylomicron</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total apo C / E</td>
<td>4.87 ± 1.4</td>
<td>3.79 ± 1.2</td>
</tr>
<tr>
<td>Apo CIII / E</td>
<td>3.28 ± 0.9</td>
<td>2.40 ± 0.7</td>
</tr>
<tr>
<td>Apo CIII / CII</td>
<td>2.16 ± 1.0</td>
<td>2.08 ± 0.9</td>
</tr>
<tr>
<td>VLDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total apo C / E</td>
<td>13.1 ± 8.7</td>
<td>9.1 ± 5.2</td>
</tr>
<tr>
<td>Apo CIII / E</td>
<td>9.2 ± 1.6 a¥</td>
<td>6.0 ± 1.8 §</td>
</tr>
<tr>
<td>Apo CIII / CII</td>
<td>8.2 ± 8.9</td>
<td>7.5 ± 4.7</td>
</tr>
</tbody>
</table>

a p<0.02 different from oleic diet in same group (paired t-test)
¥ p<0.0001 different from non-diabetic on same diet (unpaired t-test).
Figure 4.1: Apo E in the chylomicron fraction (μg/ml plasma) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 4.2: Apo E in the chylomicron fraction (µg/ml plasma) at fasting and postprandially from type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods.
Figure 4.3: Apo E in the chylomicron fraction (µg/mg apo B) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.

* p<0.02  ** p<0.01  *** p<0.002 different from oleic acid-rich diet at same postprandial time point.
Figure 4.4: Apo E in the chylomicron fraction (μg/mg apo B) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05  ** p<0.01  *** p<0.005 different from non-diabetic at same postprandial time point.
Figure 4.5: Apo CII in the chylomicron fraction (μg/ml plasma) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 4.6: Apo CII in the chylomicron fraction (µg/ml plasma) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05 different from non-diabetic at same postprandial time point.
Figure 4.7: Apo CIII in the chylomicron fraction (µg/ml plasma) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 4.8: Apo CIII in the chylomicron fraction (µg/ml plasma) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods.
Figure 4.9: Apo CII in the chylomicron fraction (μg/mg apo B) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. * p<0.05  ** p<0.005 different from oleic acid-rich diet at same postprandial time point.
Figure 4.10: Apo E in the chylomicron fraction (μg/mg apo B) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.02  ** p<0.005  *** p<0.0001 different from non-diabetic at same postprandial time point.
Figure 4.11: Apo CIII in the chylomicron fraction (µg/mg apo B) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 4.12: Apo CIII in the chylomicron fraction (μg/mg apo B) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05  ** p<0.002  *** p<0.0001 different from non-diabetic at same postprandial time point.
Figure 4.13: Apo E in the VLDL fraction (μg/ml plasma) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 4.14: Apo E in the VLDL fraction (μg/ml plasma) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05  ** p<0.02  *** p<0.01 different from non-diabetic at same postprandial time point.
Figure 4.15: Apo E in the VLDL fraction (μg/mg apo B) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.

* p<0.05  ** p<0.005  *** p<0.0005 different from oleic acid-rich diet at the same postprandial time point.
Figure 4.16: Apo E in the VLDL fraction (µg/mg apo B) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.01  ** p<0.001  *** p<0.0001 different from non-diabetic at same postprandial time.

(a) LINOLEIC ACID

(b) OLEIC ACID
Figure 4.17: Apo CII in the VLDL fraction (µg/ml plasma) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. * p<0.05  ** p<0.01 different from oleic acid-rich diet at the same postprandial time point.
Figure 4.18: Apo CII in the VLDL fraction (μg/ml plasma) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods.
Figure 4.19: Apo CII in the VLDL fraction (μg/mg apo B) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. 

* p<0.05 different from oleic acid-rich diet at the same postprandial time point.
Figure 4.20: Apo E in the VLDL fraction (µg/mg apo B) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05 ** p<0.02 *** p<0.01 different from non-diabetic at same postprandial time point.
Figure 4.21: Apo CIII in the VLDL fraction (μg/ml plasma). (a) Total apo CIII postprandial curve and (b) apo CIII isoforms (AUCz) from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. * p<0.05   ** p<0.02   *** p<0.01 different from oleic acid-rich diet.
Figure 4.22: Apo CIII in the VLDL fraction (µg/ml plasma) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods.
Figure 4.23: Apo CIII isoforms in the VLDL fraction (μg/ml plasma AUCz) from diabetic and non-diabetic subjects following the linoleic (a) and oleic (b) acid-rich dietary periods.
Figure 4.24: Apo CIII in the VLDL fraction (μg/mg apo B). (a) Total apo CIII postprandial curve and (b) apo CIII isoforms (AUCz) from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. * p<0.05
Figure 4.25: Apo CIII in the VLDL fraction (μg/mg apo B) from type 2 diabetic and non-diabetic subjects at fasting and postprandially following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.02  ** p<0.01 different from non-diabetic at same postprandial time point.
Figure 4.26: Apo CIII isoforms in the VLDL fraction (µg/mg apo B AUCz) from diabetic and non-diabetic subjects following the linoleic (a) and oleic (b) acid-rich dietary periods. * $p<0.05$ ** $p<0.02$ different from non-diabetic subject on same diet.
Figure 4.27: Apo E genotyping.

Lanes 1, 13 and 14 - 10bp DNA ladder.
Lanes 2-12 - restriction fragment pattern for diabetic subjects.
Lane 10 - genotype 3/4. All others 3/3.
4.4 Discussion.

The first point of interest from the results is the apparent disparity in the effect of alteration in diet between the chylomicron and VLDL fractions, even though each contained a certain amount of both liver-derived and intestinally-derived particles. This was not entirely unexpected as chylomicrons and VLDL, while sharing the same catabolic and clearance mechanism, behave fundamentally differently. Chylomicrons have up to 50-fold higher affinity for LPL and the rate of lipolysis is faster for chylomicrons compared to VLDL even when the particles are of equal size (Xiang et al., 1999). Investigators in this same study also showed that large chylomicrons have a higher affinity for LPL than smaller chylomicrons, but no such difference has been shown for VLDL. An inverse relationship has been demonstrated between fasting triglyceride and the rate of chylomicron clearance (Brunzell et al., 1973). Postprandially, VLDL has a triglyceride response which may be up to 20-fold higher than that of chylomicrons yet VLDL still seems to inhibit chylomicron clearance (Xiang et al., 1999). This group suggest that given the larger numbers of VLDL but higher binding affinity of chylomicrons that there is approximately an even chance of either particle binding and being lipolysed giving the impression that chylomicrons are cleared at a much faster rate than VLDL. However these studies did not take differential apolipoprotein content into account.

In the chylomicron fraction there was no difference in the absolute amount of the apolipoproteins measured in the diabetic subjects between the diets. Diabetic apo E levels tended to be higher than non-diabetic levels, but not significantly. The difference was in the amount of each apolipoprotein with respect to apo B. This gave an indication of the relative amount of apolipoprotein per particle. In the diabetic subjects all three apolipoproteins were significantly decreased on the linoleic acid diet with apo CII and CIII most resembling normality on the oleic acid diet. Apo E remained abnormally low (compared to non-diabetic levels) even on the oleic acid-rich diet. This concomitant reduction in all three apolipoproteins resulted in an unchanged ratio of apo CIII/E, total apo C/E and also apo CIII/CII on the particles.
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In the VLDL fraction however the story was somewhat different. The absolute amount of apo E in the fraction was consistently lower in the diabetic subjects compared to the non-diabetic subjects and the oleic acid-rich diet did not seem to improve this. The absolute amount of both apo CII and CIII was significantly lower in diabetic subjects on the linoleic acid diet compared to the oleic acid diet and this included all three isoforms of apo CIII. Since the majority of particles (approximately 90%) in the VLDL fraction consist of apo B100-containing, hepatically-derived particles this may be indicative of an increased expression of both apo CII and apo CIII in the liver of diabetic subjects on the linoleic acid diet. Indeed since neither diet produced levels significantly different from non-diabetic subjects on the same diet it may also indicate an inhibition in the expression of apo C in the liver in diabetic patients on the oleic acid diet.

On the particle however, apo E was lower in diabetic subjects on the linoleic acid diet at every postprandial time point. The particle remained apo E-deficient compared to non-diabetic subjects even on the oleic acid-rich diet. Apo CII was enriched on particles at the 6 hour time point in diabetic subjects on the oleic diet, the triglyceride peak in diabetic patients postprandially (Curtin et al., 1995). The diabetic particles produced after the linoleic acid diet remained deficient in apo CII in the later postprandial time points compared to non-diabetic subjects and resembled levels on the non-diabetic particles after the oleic acid diet. While the levels over time of the total apo CIII postprandially are quite different between the diets in the diabetic subjects, there is no significant difference in the amounts on the particles.

Interestingly there was a difference in the apo CIII isoform profile on the diabetic particles between the diets. There was a tendency towards a more desialylated apo CIII on linoleic particles, with apo CIII1 being significantly lower. Experiments using radiolabelled apo CIII isoforms showed that the desialylated isoforms had a lower affinity but were more effective in inhibiting apo E-mediated binding of VLDL to the LSR (Mann et al., 1997). This may have contributed to some degree in the diminished clearance of VLDL in diabetic subjects on the linoleic acid-rich diet. Diabetic particles produced in the later postprandial
hours on the linoleic acid diet were deficient in all the apo CIII isoforms compared to non-diabetic particles, whilst only the CIII2 isoform was deficient in diabetic particles on the oleic acid-rich diet. Unlike the chylomicron fraction the VLDL fraction showed a significantly higher mean ratio of apo CIII/E on the particles produced by diabetic subjects on the linoleic acid diet. This remained higher than the non-diabetic particle ratio, even after the oleic acid diet. There was no difference in the ratios of total apo C/E or apo CIII/CII.

When these results are taken into account with regard to the levels of both apo B48 and apo B100 the linoleic acid-rich diet produced more particles in both fractions. An equal amount of triglyceride was carried in the particles produced on each diet and whilst it cannot be shown statistically, it does suggest that the linoleic acid-rich diet tends to produce smaller, but numerically more particles in each fraction. This may have been elucidated using gradient ultracentrifugation, which unfortunately was not used in the isolation of the lipoproteins. It has been shown that both particle number and size are determinants of the rate of plasma clearance of triglyceride-rich lipoproteins and that the process, which is common to both apo B48- and apo B100-containing particles, is saturable (Martins et al., 1996). This group showed that, given an equal amount of triglyceride, an emulsion containing fewer particles was cleared more rapidly than one containing a large number of small particles. This apparent size difference may be contributing to a more rapid clearance rate in particles produced on the oleic acid-rich diet.

Size is also an important factor in the process of apolipoprotein exchange from HDL to the triglyceride-rich lipoproteins. It has been proposed that apo CIII reduces particle uptake by displacing apo E from the surface of the particle. In hepatic perfusion studies, clearance of VLDL is markedly reduced when apo CIII is added to the lipoproteins in vitro (Windler et al., 1980). Both apo E and apo C are known to exchange between HDL and VLDL and there is evidence from in vivo, radioactive tracer studies to suggest the existence of two pools of apo E and apo C on the particle surface - the exchangeable (loose binding) pool and the non-exchangeable (tight binding) pool (Bukberg et al., 1985). It has also been shown that larger particles contain a larger non-exchangeable pool of apo E which is only
reduced on the lipolysis of the particles (Breyer et al., 1999). The same group observed no effect of apo E phenotype on pool size. Particles of the chylomicron fraction of this current study produced by diabetic patients after a diet of linoleic acid contained less of all the apolipoproteins measured. The smaller particles may have less non-exchangeable apolipoprotein pools resulting in a general apolipoprotein deficiency which is normalised by a diet rich in olive oil. While the evidence is strong to support the idea of an improvement in postprandial particle clearance with an increase in the ratio of apo E/CIII and apo CII/CIII there is a growing belief that the absolute amount of the apolipoproteins on the particle may be of equal importance in determining both LPL activity (Lambert et al., 1996; Andersson et al., 1999) and hepatic particle clearance, particularly in type II diabetes (Attia et al., 2000). This latter group showed that in type II diabetic patients apo CIII was transferred to chylomicrons in proportion to the triglyceride content and the ratio of apo CIII to E did not change after treatment with bezafibrate despite a reduction in plasma triglyceride.

The VLDL fraction did however show an increase in the ratio of apo CIII/E in the diabetic group on the linoleic acid diet which was also significantly higher than the non-diabetic group on the same diet. This was due to a deficiency in apo E on the particles with no change in apo CIII. This is more difficult to explain and may simply reflect reduced hepatic secretion of apo E. It does again highlight the divergent responses of both lipoprotein fractions to the change in diet.

Enhanced levels of apo E on the particles may also be as a result of greater enrichment intracellularly, before the secretion of the particle. Apo E has been shown to associate more readily with VLDL during rapid hepatic synthesis of the particles and is secreted with small, HDL-like particles during times of low VLDL synthesis (Fazio and Yao, 1995). A recent kinetic study in men using deuterated leucine to trace apo E in the TRL has revealed a strong correlation between the rate of production of apo E and the levels of apo E on TRL particles (Millar et al., 2001). Recently also a PPAR response element has been identified in the apo E/apo CI intergenic region (the control region of the apo E gene) which
may be involved in the regulation of apo E expression (Galetto et al., 2001). This raises the possibility that apo E expression could be directly effected by altering dietary fatty acid composition which in turn may effect the amount carried by each particle produced. It has been shown in Caco-2 cells that oleic acid is a more potent stimulator of lipoprotein secretion than linoleic acid (Field et al., 1988) and indeed oleic acid has been shown to be preferentially esterified into triglyceride in the enterocyte (Dashti et al., 1990) suggesting a more rapid absorption of oleic acid from the gut. However, the particles produced from oleic acid tend to be larger and richer in triglyceride (van Greevenbroek et al., 2000). There is further evidence to show that following a meal rich in oleic acid, particularly in subjects habituated to an oleic acid-rich diet, the rate of production of chylomicrons is very rapid during the early postprandial hours but returns quickly to fasting levels (Zampelas et al., 1998). As has been mentioned above, apo E associates more readily with VLDL during rapid production of the particles (Fazio and Yao, 1995). This may be particularly important in diabetic patients where the postprandial apo B and triglyceride peak is later, at 6 hours rather than at 4 hours as it is in non-diabetic subjects. A diet rich in oleic acid may normalise the dynamics of the diabetic postprandial response and may also explain the lack of effect in non-diabetic subjects in this study. However, with a smaller number in the non-diabetic group it may be more difficult to obtain statistically significant differences.

With such profound changes in apo E in both the chylomicron and the VLDL fractions in the diabetic subjects apo E genotype was determined to confirm that both patient and control groups were representative, to some degree, of the Irish population and that the effects observed were not due to the coincidental choice of a very abnormal genotype distribution. Apo E genotype has been shown to have an effect on both affinity of the resulting peptide for lipoprotein particles (E4 having the highest affinity) (Bioletto et al., 1998) and also the affinity of the peptide for hepatic receptors (E2 being functionally inactive) (Brewer et al., 1983). A recent comprehensive study of healthy Irish subjects showed that the E3/3 genotype was the most frequent at 66% and the E3/4 next at 20% (Sheehan et al., 2000). Whilst evidence of apo E genotype distribution in the Irish type II
diabetic population is lacking, results from the Framingham Offspring Study show that there is no association between apo E polymorphism and insulin resistance (Meigs et al., 2000). It has been shown that any effect of abnormal apo E polymorphism on the risk of cardiovascular disease in non-diabetic subjects may be completely overwhelmed by diabetes itself in those with the disease (Vauhkonen et al., 1997). Nevertheless, the genotype distribution displayed in both groups of this study did in fact resemble that of the Irish population, confirming the results observed were not due to an aberrant group of diabetic subjects.

In conclusion, the linoleic acid diet caused the production of apo E-deficient chylomicron and VLDL particles postprandially and a reduced total amount of apo E in the VLDL fraction. There was also a deficiency in apo CII and apo CIII in linoleic diet chylomicrons. The ratio of apo CIII/E in the VLDL fraction was higher on this diet and remained higher than non-diabetic VLDL on the oleic acid diet. These deficiencies and changes in ratio of apolipoproteins may greatly effect clearance of the particles and may account for elevated levels of particles in patients on the linoleic acid diet.
Chapter 5

The effect of dietary fatty acid on HDL and reverse cholesterol transport in Type 2 diabetes
The smallest of the lipoproteins is HDL. Its main protein components are apo AI and a lesser amount of apo AII. The HDL core contains mainly cholesteryl ester and the particle is central to the process of reverse cholesterol transport - the removal of cholesterol from peripheral tissues and its transport back to the liver from where it is excreted in the form of bile acids. An inverse relationship between HDL cholesterol and the risk of coronary heart disease has been well established (Gordon and Rifkind, 1989). A negative relationship has also been noted between VLDL triglyceride and HDL cholesterol concentrations (Schaefer et al., 1978), the classical lipid abnormality in Type 2 diabetes. Both apo E and apo C are components of HDL. Data from the Cholesterol Lowering Atherosclerosis Study (CLAS) have shown that reduced levels of HDL-linked apo CIII were the predominant risk factor for the progression of coronary artery lesions in hyperlipidaemic patients (Blankenhorn et al., 1990). Postprandial lipoprotein metabolism is disturbed in diabetes and it has not yet been elucidated what role the transfer of apo E, apo C and also cholesteryl ester from HDL to the TRL plays in this disturbance. Dietary polyunsaturated and monounsaturated fatty acids are thought to have a similar effect on plasma cholesterol levels (Mattson and Grundy, 1985; Zock and Katan, 1992; Gustafsson et al., 1992; Kris-Etherton et al., 1993), but unlike oleic acid, dietary linoleic acid has been shown to reduce HDL cholesterol as well as LDL cholesterol (Howard et al. 1995). As yet there is little information on the effect of these diets on the apolipoprotein components of HDL and how this may effect HDL cholesterol levels and postprandial lipoprotein metabolism - lipid abnormalities which may be central to the increased incidence of atherosclerosis in diabetes (Sakata et al., 1998; Takeichi et al., 1999).

As discussed in section 1.3.17, HDL has two major functions. The first is the efflux of cholesterol from peripheral tissues which is mediated by CERP (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999) and putatively the SR-BII (Webb et al., 1998), and in its transferral either through the triglyceride-rich lipoproteins to the liver or by direct removal of HDL to the liver, mediated by SR-BI (Webb et al., 1997). This
process is called reverse cholesterol transport. Apo AI is the main activator of the LCAT reaction which esterifies cholesterol in order to facilitate efflux and transfer of the resulting cholesteryl esters (Fielding and Fielding, 1995). It is also thought to be the most likely ligand for the scavenger receptor during HDL cholesterol uptake (Fielding and Fielding, 1995). Epidemiologically, low apo AI in the HDL fraction has been associated with atherogenesis and CAD (Miller, 1994) and for these reasons apo AI is considered to be anti-atherogenic. Efflux capacity has been shown to be reduced in Type 2 diabetic patients, particularly in the presence of CAD (Sylvan et al., 1996) and it has recently been shown that in Type 2 diabetes low serum apo AI is related to increased carotid intima-media thickness and early atherosclerotic lesions (Tkac et al., 2001).

The role of apo AII is less well defined. It has been shown to have a higher affinity for lipids than apo AI and therefore has the ability to displace apo AI on the HDL particle thus effectively inhibiting the LCAT reaction (Durbin and Jonas, 1997). Apo AII has been shown to inhibit CETP activity also (Lagrost et al., 1994). This is the protein which mediates transfer of lipid between HDL and the TRL. However, in some epidemiological studies levels of apo AII are negatively correlated with CAD susceptibility (Miller, 1987). In a more recent study using apo AII knockout mice, a dramatic decrease in HDL cholesterol, increased remnant clearance and insulin hypersensitivity were observed suggesting a complex role for apo AII in atherosclerosis (Weng and Breslow, 1996). There is disagreement as to the effect of diabetes on HDL apo AII (O'Brien et al., 1996; Contiero et al., 1998). There is also disagreement in the literature regarding the rate of cholesteryl ester transfer in diabetic subjects relative to non-diabetic subjects. It has been shown to be raised in Type 2 diabetic patients who also have raised triglyceride levels (Jones et al., 1996; Riemans et al., 1998) and unchanged in normotriglyceridaemic Type 2 diabetic patients (Lottenberg et al., 1996). There is however contradictory evidence (Bhatnagar et al., 1995). There is similar disagreement with regard to LCAT activity in diabetes (Misra et al., 1974; Reimens et al., 1998).
Apo E also plays a role in HDL metabolism. It has been proposed that apo E may mediate HDL particle clearance through hepatic apo E receptors and HSPGs (Ji et al., 1997). Studies in apo E knock-out mice suggest that apo E is central to the selective clearance of HDL cholesteryl ester by the liver (Arai et al., 1999). The authors propose that apo E facilitates the presentation of HDL to the SR-BI at the cell surface. Macrophages and macrophage-foam cells synthesise and secrete apo E and cholesterol efflux from these cells by nascent HDL has been shown to be enhanced by secretion of apo E into culture medium (Mazzone and Reardon, 1994; Bielicki et al., 1999). This more recently has been shown in studies with HDL isolated from patients with congenitally deficient apo E (Kinoshita et al., 2000). A recent study in cultured endothelial cells showed that apo E-containing HDL particles can stimulate HSPG production, molecules which are central to lipoprotein clearance, monocyte adhesion and smooth muscle cell proliferation (Paka et al., 1999). The authors suggested a strong anti-atherogenic role for HDL-apo E.

The second major function of HDL is that large HDL particles, enriched in apo E and apo C act as their major pool in plasma (Havel et al., 1973; Mahley et al., 1984; Klein et al., 1992; Björkegren et al., 1997, 1998). It is from HDL that both apo CII and CIII and of course apo E are donated to the TRL postprandially (Björkegren et al., 1997, 1998; Blum 1982) to drive and control both lipolysis of the particles by LPL and clearance of remnants via hepatic receptors using apo E as the ligand (Cooper, 1997 and Mahley and Ji, 1999). Apo C is returned almost immediately on the commencement of lipolysis and with the reduction in particle surface area, a certain amount of apo E is also returned to HDL before remnant uptake. Therefore HDL apolipoprotein may reflect an absence on the TRL.

Apo CIII and also apo CII gene expression has been shown to be regulated through PPARα (Peters et al., 1997) and expression of both can be reduced by the action of fibrates- known PPARα ligands (Andersson et al., 1999). Dietary polyunsaturated and monounsaturated fatty acids have also been shown to be ligands of PPARα (Auwerx et al., 1996; Jump et al., 1996). Recently a PPAR response element has been identified in the apo E/apo C intergenic region (the apo E gene control region) which may be involved in the
regulation of apo E expression (Galetto et al., 2001). The liver is the major site of apo E and apo C production (Mahley and Innerarity, 1988; Fredenrich, 1998) and it has been shown that both apolipoproteins associate preferentially with HDL during fasting conditions (Havel et al., 1973; Mahley et al., 1984; Klein et al., 1992; Björkegren et al., 1997, 1998). Differential expression of these genes may therefore be reflected in the apolipoprotein levels in the HDL fraction.

The purpose of this part of the study was three-fold. Firstly, non-esterified fatty acids have been shown to effect the rate of transfer of cholesteryl esters from HDL to LDL and the TRL and this was shown to be dependant on both the length and degree of unsaturation of the fatty acid (Lagrost and Barter, 1991). The activity of LCAT is influenced by the nature of fatty acid tails on phospholipids in the cell or lipoprotein surface (Jonas, 1991). Fasting and postprandial measurements of LCAT and CETP activity were made in conjunction with HDL cholesterol, apo AI and apo AII levels to investigate a possible effect of dietary fatty acid on both postprandial HDL cholesterol levels and TRL particles. Secondly, previous chapters have detailed an improvement in diabetic postprandial dyslipidaemia with consumption of a diet rich in oleic acid. This was accompanied by enrichment of the particles in apo E and also in apo C in particles of the chylomicron fraction. This study aimed to determine the effect of dietary mono- and polyunsaturated fatty acids on levels of apo E, apo CII and apo CIII on both fasting and postprandial HDL in diabetic patients to identify differential apolipoprotein transfer as a possible mechanism for the observed changes in apo E and C in the TRL. Thirdly, a non-diabetic subject group was included in the study and similar analysis carried out so comparison could be made with subjects having a normal postprandial lipoprotein metabolism.
Chapter 5

5.2 METHODS

5.2.1 Subjects and study design.
This chapter details the analysis and results of HDL isolated from a subgroup of seven of the type 2 diabetic subjects and the six non-diabetic subjects described in chapter 3. In summary, subjects were instructed on a diet rich in monounsaturated or polyunsaturated fatty acids (see section 2.4.1) for a period of two weeks, after which time subjects were swapped to the second diet for the same time period. Blood samples were taken following a test meal at fasting and every 2 hours, for 8 hours postprandially (see section 2.4.2).

5.2.2 Lipoprotein isolation and analysis.
Assays for CETP and LCAT activity were carried out on the fasting and 2 hour postprandial blood samples. Plasma was separated within 30 minutes at 4°C and the assays carried out immediately as described in section 2.9. Lipoproteins were isolated in the density range 1.063 - 1.21g/ml from fasting and postprandial blood as described in section 2.5 and constituted the HDL fraction. Cholesterol and triglyceride composition of the fraction was determined within 1 week as described in section 2.3. Apos E, CIII and AII were determined from partially delipidated, frozen HDL samples by separation on 10-20% polyacrylamide gradient gels and quantification with reference to known standards as described in section 2.8.1. Apo AII staining was shown to be linear to at least 10μg (section 2.8.2). Having determined that staining of apo AI was linear only to approximately 4μg (section 2.8.4), a second 10-20% gel was used to quantify apo AI, loading only 3μg total HDL protein as described in section 2.8.3. Apo CII was determined most easily by isoelectric focusing between pH 4.5 and 6.5 as detailed in section 2.8.5.

5.3 RESULTS

Data in this chapter are presented in two ways, as was the case in previous chapters. Tables of AUC are shown. As postprandial curves for the HDL components analysed
were shaped irregularly and as with apo E for example, the postprandial values were sometimes lower than the fasting value, AUC was calculated from zero only. As before, curves are represented diagramatically and individual time points compared in an attempt to identify differences in the shape of the curves. Unlike the chapters dealing with triglyceride lipoproteins in which results could be expressed both as an absolute amount (µg/ml plasma) and relative to the number of particles present (µg/mg apo B) there is no apolipoprotein on HDL which is present as a single copy and as such who's levels do not alter postprandially. Therefore all the results of this chapter have been expressed as an absolute amount and also as a ratio of apo AI - by far the most abundant HDL apolipoprotein.

5.3.1 HDL apo A.
All AUC results for HDL constituents are given in Table 5.1. There was no difference in the AUC for apo AI in the diabetic subjects between the linoleic and oleic acid diet and no difference in either the fasting or any postprandial time point (Figure 5.1). These AUC levels were numerically 2-fold lower in diabetic subjects on the linoleic acid diet compared to non-diabetic subjects but does not quite reach statistical significance (p=0.07). Levels of apo AI in diabetic subjects more resembled non-diabetic levels while on the oleic acid-rich diet. Apo AII was also remarkably similar between the diets in the diabetic subjects and also between diabetic and non-diabetic subjects regardless of the diet. This latter result means that the ratio of apo AII/AI is not different in any of the groups.

5.3.2 Postprandial HDL cholesterol and triglyceride.
There was no statistical difference between the absolute amount of HDL cholesterol between diets in the diabetic subjects (Table 5.1). At 4 hours postprandially, however, there was significantly less cholesterol carried in the HDL produced by diabetic subjects on the linoleic acid-rich diet compared to the oleic acid diet (Figure 5.2). Diabetic subjects had HDL cholesterol levels which were less than half that of the non-diabetic subjects on the linoleic acid-rich diet (p<0.002). Every time point, including fasting, was lower (Figure 5.3 (a)). The diabetic cholesterol AUC approached normality on the oleic acid diet, with only
the later time points being significantly lower than non-diabetic subjects on the oleic acid diet (Figure 5.3 (b)). The same pattern of results was shown when cholesterol was expressed per mg apo A1 (Figures 5.4 and 5.5).

Changing diet had no effect on levels of HDL triglyceride (Table 5.1) in the diabetic subjects with no difference at any time point (Figure 5.6). Interestingly there was significantly higher levels of HDL triglyceride in diabetic compared to non-diabetic subjects on both the linoleic acid (p<0.05) and the oleic acid diet (p<0.01). The 4 hour time point was most different on the linoleic acid diet (Figure 5.7 (a)), while the later time points were most different between diabetic and non-diabetic subjects on the oleic acid diet (Figure 5.7 (b)).

5.3.3 HDL apo E.
The absolute amount of HDL apo E in diabetic subjects on a linoleic acid diet was almost half that of subjects on the diet rich in oleic acid (p<0.05) with both the fasting and 4 hour time point lowest (Figure 5.8). Levels of apo E were significantly lower in diabetic subjects on the linoleic acid diet compared to non-diabetic subjects (p<0.05). Practically all time points were lower including fasting (Figure 5.9 (a)). The AOC from fasting (Table 5.1) was over 5 times lower in the diabetic subjects on the linoleic acid diet compared to the oleic acid diet (p<0.02) and compared to non-diabetic subjects on the same diet (p<0.005). There was no difference between diabetic and non-diabetic subjects on the oleic acid diet.
There was a similar pattern when apo E results were expressed per mg apo A1 (Figures 5.10 and 5.11). A strongly significant correlation (p<0.0005, r=-0.804) was observed between postprandial apo E and the postprandial total apo B which was measured in these seven patients and reported in chapter 3 (Figure 5.12).

5.3.4 HDL apo C.
Levels of both apo CII and apo CIII were remarkably similar between the diets in the diabetic subjects and also between diabetic and non-diabetic subjects on the same diet (Table 5.1).
5.3.5 CETP and LCAT activity.

Both CETP and LCAT activities were similar between the diets in the diabetic subjects and also between diabetic and non-diabetic subjects in fasting and postprandial plasma (Table 5.2).
Table 5.1: Lipid and apolipoprotein composition of HDL fraction from diabetic (n=7) and non-diabetic (n=6) subjects following the linoleic and oleic acid-rich dietary periods. Results are expressed as AUC₂₀ or Area Over the Curve (AOC) for both the μg/ml plasma (μg/dl plasma for apo E) and μg/mg apo AI curves and given as mean ± SD and the median and range for triglyceride.

<table>
<thead>
<tr>
<th>HDL component</th>
<th>Diabetic</th>
<th></th>
<th>Non-diabetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic</td>
<td>Oleic</td>
<td>Linoleic</td>
<td>Oleic</td>
</tr>
<tr>
<td>μg/vol plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo AI</td>
<td>3838 ± 1964</td>
<td>4153 ± 1332</td>
<td>6248 ± 2592</td>
<td>5224 ± 1568</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1193 ± 554</td>
<td>1640 ± 437</td>
<td>2772 ± 869</td>
<td>2263 ± 908</td>
</tr>
<tr>
<td>Apo E (AUC)</td>
<td>1240 ± 896</td>
<td>2104 ± 1335</td>
<td>3065 ± 1694</td>
<td>2800 ± 1257</td>
</tr>
<tr>
<td>Apo E (AOC)</td>
<td>268 ± 280</td>
<td>1528 ± 1072</td>
<td>1231 ± 637</td>
<td>948 ± 732</td>
</tr>
<tr>
<td>μg/mg apo AI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2784 ± 961 b§</td>
<td>3853 ± 1532</td>
<td>4859 ± 1476</td>
<td>3514 ± 1180</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1054 (494-2943) †</td>
<td>963 (499-1454) §</td>
<td>474 (117-1430)</td>
<td>512 (170-802)</td>
</tr>
<tr>
<td>Apo E</td>
<td>27.2 ± 17 a†</td>
<td>43.0 ± 10</td>
<td>42.5 ± 17</td>
<td>43.6 ± 12</td>
</tr>
<tr>
<td>Apo AII</td>
<td>2419 ± 875</td>
<td>2514 ± 1087</td>
<td>2579 ± 923</td>
<td>2753 ± 1091</td>
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<tr>
<td>Apo CII</td>
<td>24.5 ± 11</td>
<td>27.4 ± 9</td>
<td>34.9 ± 10</td>
<td>25.1 ± 8</td>
</tr>
<tr>
<td>Apo CIII</td>
<td>219 ± 120</td>
<td>231 ± 115</td>
<td>199 ± 110</td>
<td>195 ± 132</td>
</tr>
</tbody>
</table>

a p<0.05  b p<0.02 different from oleic diet (paired t-test. Triglyceride used paired, non-parametric test (Wilcoxon matched pairs test)).

† p<0.05  § p<0.01  ¶ p<0.005  ¶ p<0.002 different from non-diabetic on same diet (unpaired t-test. Triglyceride used unpaired, non-parametric test (Mann-Whitney U test))
Table 5.2: Activity of CETP and LCAT in fasting and postprandial plasma in diabetic (n=7) and non-diabetic (n=6) subjects. Results are expressed as nmol cholesterol transferred/hour/ml plasma and nmol cholesterol esterified/hour/ml plasma respectively and given as median and range.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Linoleic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CETP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>31.2 (25-52)</td>
<td>45.9 (28-50)</td>
<td>35.1 (18-46)</td>
<td>29.2 (16-50)</td>
</tr>
<tr>
<td>Postprandial</td>
<td>48.9 (23-65)</td>
<td>48.7 (23-58)</td>
<td>51.1 (17-63)</td>
<td>39.0 (17-52)</td>
</tr>
<tr>
<td>LCAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>45.1 (33-65)</td>
<td>59.1 (33-65)</td>
<td>39.6 (33-53)</td>
<td>44.4 (25-63)</td>
</tr>
<tr>
<td>Postprandial</td>
<td>53.4 (17-71)</td>
<td>63.8 (36-87)</td>
<td>43.0 (17-72)</td>
<td>51.1 (32-64)</td>
</tr>
</tbody>
</table>
Figure 5.1: Apo Al in the HDL fraction (μg/mg apo B) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.
Figure 5.2: Cholesterol in the HDL fraction (μg/ml plasma) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. * p<0.05 different from oleic diet in same group (paired t-test).
Figure 5.3: Cholesterol in the HDL fraction (µg/ml plasma) at fasting and postprandially from type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05  ** p<0.005  *** p<0.001 different from non-diabetic subjects on same diet. (unpaired t-test)
Figure 5.4: Cholesterol in the HDL fraction (μg/mg apo AI) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods.

* p<0.05 different from oleic diet in same group (paired t-test).
Figure 5.5: Cholesterol in the HDL fraction (μg/mg apo AI) at fasting and postprandially from type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05 different from non-diabetic subjects on same diet. (unpaired t-test)
Figure 5.6: Triglyceride in the HDL fraction (µg/mg apo AI) at fasting and postprandially from type 2 diabetic subjects following the linoleic and oleic acid-rich dietary periods. (Paired, non-parametric test (Wilcoxon matched pairs test)).
Figure 5.7: Triglyceride in the HDL fraction (µg/mg apo Al) at fasting and postprandially from type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05 different from non-diabetic subjects on same diet. (unpaired, non-parametric test (Mann-Whitney U test))
Figure 5.8: Apo E in the HDL fraction (μg/dl plasma) at fasting and postprandially from type 2 diabetic following the linoleic and oleic acid-rich dietary periods. AOC=Area over curve. * p<0.05  ** p<0.005 different from non-diabetic subjects on same diet. (paired t-test)
Figure 5.9: Apo E in the HDL fraction (μg/dl plasma) at fasting and postprandially from type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.05  ** p<0.02  *** p<0.01 different from non-diabetic subjects on same diet. (unpaired t-test)
Figure 5.10: Apo E in the HDL fraction (μg/mg apo AI) at fasting and postprandially from type 2 diabetic following the linoleic and oleic acid-rich dietary periods. 

* p<0.05  ** p<0.005 different from non-diabetic subjects on same diet. (paired t-test)
Figure 5.11: Apo E in the HDL fraction (μg/mg apo Al) at fasting and postprandially from type 2 diabetic and non-diabetic subjects following the linoleic (a) and oleic acid-rich (b) dietary periods. * p<0.02 different from non-diabetic subjects on same diet (unpaired t-test)
Figure 5.12: Correlation between HDL apo E (µg/dl plasma AUC) and TRL total apo B in Type 2 diabetic patients.
There is relatively little information in the literature about the effect of changing dietary fatty acid on postprandial HDL, particularly in Type 2 diabetic subjects. Interestingly this chapter answers some of the questions which were raised in the previous chapter. Diabetic subjects consuming a diet rich in linoleic acid have dramatically lower HDL cholesterol both at fasting and postprandially compared to non-diabetic subjects. Levels were more normal in diabetic subjects following the oleic acid diet. These particles also contained triglyceride levels which most resembled non-diabetic HDL. Since most of the day is spent in the postprandial state this substantial improvement in HDL cholesterol may represent a reduced risk of atherosclerosis in diabetic subjects consuming olive oil rather than sunflower oil since there is a negative correlation between HDL cholesterol and CAD (Gordon and Rifkind, 1989). This finding is in agreement with the observation that linoleic acid reduces plasma cholesterol by reducing both HDL and LDL cholesterol (Howard et al. 1995). It may also reflect the previously observed reduction in postprandial TRL in this group, since there is an inverse relationship between VLDL triglyceride and HDL cholesterol (Schaefer et al., 1978). An inverse relationship is known to exist between the clearance of postprandial chylomicrons and HDL cholesterol levels (Patsch et al., 1982). Diabetic subjects on the linoleic acid-rich diet did indeed have lower levels of HDL cholesterol along with higher levels of postprandial lipoproteins.

Apo AII levels were remarkably similar between the groups and diet did not seem to alter these. Apo AI was lower in diabetes on the linoleic acid diet, and was very close to reaching significance. This reduction in apo AI may be contributing to a reduction in cholesterol efflux which has been observed in diabetes (Syvänne et al., 1996) and the resulting reduction in HDL cholesterol levels. The similarity in CETP activity and a trend towards higher LCAT activity in diabetic subjects compared to non-diabetic subjects may reflect the somewhat lower levels of apo AI as it activates LCAT. These results suggest however that cholesterol transfer may not be responsible for the significant decrease in HDL cholesterol in diabetic patients on the linoleic acid diet. However the measurement of
cholesterol ester in each fraction may have given a more clear insight into its transfer. Unfortunately only total cholesterol was measured. The similarity in LCAT and CETP activity between the diets and between diabetic and non-diabetic groups is not entirely unexpected as the amount of triglyceride carried in the TRL is similar in all groups and it has been observed that elevated activity may only occur in the presence of hypertriglyceridaemia (Jones et al., 1996; Riemens et al., 1998; Lottenberg et al., 1996). However it may have been more insightful to have measured the postprandial activity at the 6 hour time point - the triglyceride peak for diabetic subjects, in order to assess the effect of the abnormally high postprandial response in the diabetic subjects.

Another interesting observation in this study was that the diet rich in linoleic acid produced HDL which was noticeably deficient in apo E at fasting and postprandially. This may represent a reduction in hepatic apo E gene expression by linoleic acid as newly synthesised apo E binds preferentially with HDL during periods of fasting (Havel et al., 1973; Mahley et al., 1984; Klein et al., 1992; Björkegren et al., 1997, 1998). As described above, apo E on HDL is important in the clearance of HDL particles through hepatic apo E receptors and HSPG (Ji et al., 1997), the final step in reverse cholesterol transport. This process may therefore be disturbed in diabetic patients on the linoleic acid diet, even though HDL cholesterol levels remained low. Apo E is important in cholesterol efflux from peripheral tissues (Oram and Yokoyama, 1994; von Eckardstein et al., 1995). It is not unreasonable then to suggest that a reduced efflux of peripheral cholesterol may be contributing to the reduction in HDL cholesterol in diabetic patients on the linoleic acid diet. It has also been shown that apo E-containing HDL stimulate HSPG production in cultured endothelial cells (Paka et al., 1999). These molecules are present in the Space of Disse in the liver and are central to the clearance of postprandial lipoproteins (Cooper, 1997 and Mahley and Ji, 1999). The normalisation of HDL apo E in diabetes by dietary oleic acid may therefore be important in the reduced postprandial particle levels also observed on this diet.
The reduction in HDL apo E levels on the linoleic diet may effect TRL clearance by a second method. The area over the HDL apo E postprandial curve (AOC) was less than 20% that of the diabetic subjects on the oleic acid diet and also that of the non-diabetic subjects. Apo E in the HDL fraction reduces postprandially as it is donated to newly secreted TRL particles and rises toward the end of the postprandial phase as the TRL are lipolysed and apo E is returned to HDL. The AOC measurement represents the loss, over time of apo E from HDL to the TRL. This may be the reason for the observed deficiency in apo E in diabetic TRL, and the subsequent elevation of postprandial particles, especially after a diet rich in linoleic acid as described in chapters 3 and 4. This relationship between HDL apo E and the TRL is further substantiated by the strong negative relationship existing between postprandial HDL apo E and postprandial TRL apo B in diabetic subjects. The linoleic acid diet caused a deficiency in HDL apo E along with an elevation in the number of postprandial chylomicron and VLDL particles in diabetic subjects.

Levels of apo CII and CIII were remarkably similar between diets at fasting and postprandially in the diabetic subjects and also between diabetic and non-diabetic subjects. This may indicate a lack of effect on hepatic gene expression and also that a differential transfer of these apolipoproteins from HDL to the TRL may not be responsible for the reduced levels on the postprandial lipoproteins seen in diabetic patients on the linoleic acid diet (Chapter 4).

In conclusion, a diet rich in linoleic acid reduces both fasting and postprandial HDL cholesterol levels in Type 2 diabetic subjects, whereas an oleic acid diet normalises HDL levels. This may be due to a slight reduction in apo AI and LCAT activity, effecting reverse cholesterol transport and a deficiency in apo E effecting cholesterol efflux. Reduced levels of apo E on HDL may limit transfer of the apolipoprotein to the TRL, reducing postprandial particle uptake. Diet seemed not to effect HDL apo C levels. A linoleic acid diet may contribute to a more atherogenic lipid profile in Type 2 diabetic patients.
Chapter 6

The effect of alloxan-diabetes on lymph chylomicron apo E in the cholesterol-fed rabbit
6.1 INTRODUCTION.

Postprandial lipoprotein metabolism is disturbed in diabetes (Curtin et al., 1996; DeMann et al., 1996; Taggart et al., 1997; Phillips et al., 2000) and similar findings have indeed been detailed in the human study of this thesis. Hyperglycaemia has recently been confirmed as a risk factor for mortality (Shaw et al., 1999) and improving hyperglycaemia results in an improvement in postprandial dyslipidaemia in diabetes (Phillips et al., 2000). It has been found that levels of both intestinally-derived (apo B48-containing) and hepatically-derived (apo B100-containing) particles are abnormally raised in diabetes (Curtin et al., 1994, 1996). The disturbance in these particles, particularly remnant particles, may be important in the 3-5-fold increase in the risk of atherosclerosis in diabetes (Sakata et al., 1998; Takeichi et al., 1999). Abnormal particle clearance and/or production may be involved in this disturbance but the relative contribution of each has not yet been resolved.

Abnormalities have been elucidated in aspects of particle production in diabetes. A study carried out in humans using a double isotope method to trace ingested cholesterol showed that absorption of cholesterol from the intestine was significantly lower in comparison to controls but cholesterol synthesis was significantly higher (Gylling and Meittinen, 1997). This corroborates evidence that the expression, (Feingold et al., 1994) and activity (Feingold et al., 1982; O'Meara et al., 1990; Gleeson et al., 2000) of HMG CoA reductase, the rate-limiting enzyme in cholesterol synthesis, is increased in diabetic animal models. The enzyme ACAT which is the rate-limiting step in cholesterol absorption in the intestine has been shown to be increased in diabetic animals fed cholesterol (O'Meara et al., 1991; Maechler et al., 1992). An increase in particle size in lymph chylomicrons isolated from diabetic rats has been observed along with a 4-fold increase in MTP expression in the intestine of diabetic rats (Gleeson et al., 1999). These data would suggest a role for both abnormal particle synthesis and abnormal particle clearance in diabetic dyslipidaemia. The issue remains unclear because human experiments do not allow the study of these processes separately. For this, chylomicrons produced by the intestine which are secreted into lymph need to be collected before entering the bloodstream where they are
immediately compositionally altered. Experiments in humans are therefore extremely
difficult, but experimental animals facilitate sufficient sample collection.

Apo E is central to the process of particle clearance. It is the ligand for hepatic receptors
which mediate uptake of the TRL (Cooper, 1997; Mahley and Ji, 1999). Apo E is
produced in the intestine at low levels (Mahley and Innerarity, 1988) and nascent particles
do contain small amounts of the apolipoprotein. It is also transferred from HDL to the
TRL during alimentary lipaemia for the purpose of particle clearance to the liver
(Björkegren et al., 1997, 1998; Blum 1982). The importance of apo E has been
demonstrated in animal models. Mahley et al., (1989) showed that the addition of apo E to
thoracic duct lymph chylomicrons resulted in the accelerated clearance of the chylomicrons
in rabbits. They also showed a 20-40% reduction in plasma cholesterol levels by
intravenous infusion of apo E in cholesterol-fed rabbits and suggested that apo E
availability is rate limiting in normal clearance of diet-induced remnant lipoproteins. More
recently experiments in transgenic animals have shown enhanced removal of TRL and
suppression of fatty streak lesions in the aortic sinus of LDLR-knockout mice
overexpressing apo E compared to LDLR-transgenic animals with normal levels of apo E
(Osuga et al., 1998). Overexpression of apo E in diabetic transgenic mice has also been
shown to prevent diabetic dyslipidaemia (Yamamoto et al., 1995).

Studies in humans have also demonstrated the central role of apo E in postprandial
lipoprotein metabolism and the development of atherosclerosis. It is known that
possession of at least one E4 allele (a polymorphism of the apo E gene) is associated with
lower plasma apo E (Boerwinkle and Utermann, 1988) and increased plasma cholesterol,
LDL cholesterol and apo B levels (Davignon et al., 1988) and a greater risk of coronary
artery disease (Wilson et al., 1996), when compared to E3 homozygotes (the wild-type
isoform of the apo E gene). A Japanese study in diabetic patients with and without IHD
showed that the ratio of VLDL apo E/VLDL triglyceride was significantly reduced in
patients with IHD compared to those without (Murakama et al., 1997). Recently kinetic
studies in American men using dueterated leucine to trace apo E and apo B100 showed that
the concentration of apo E in TRL was determined by the production rate of apo E and that those producing TRL with a low apo E content had the highest LDL apo B100 production rates (Millar et al., 2001). It is well accepted that elevated serum cholesterol, most of which is carried in LDL increases the risk of mortality from heart disease (Martin et al., 1986; Expert Panel, 1988).

Results from the human study which are detailed in chapter 4 show that particles of the chylomicron fraction in diabetic patients are depleted of apo E compared to non-diabetic particles, regardless of diet. The amount of apo E donated from HDL during the postprandial phase was also shown to be reduced in diabetic patients on the linoleic acid-rich diet compared to the oleic acid-rich diet and non-diabetic subjects (Chapter 5). This was accompanied by a significantly elevated fasting glucose (Chapter 3) and may suggest abnormal apo E transfer in diabetes. This current chapter investigates the effect of diabetes on intestinal particle production in an animal model of postprandial dyslipidaemia - the cholesterol-fed rabbit. Of particular interest are levels of intestinally-produced apo E on lymph chylomicrons which would give an indication of their ability to be cleared. Apo E was also measured in the plasma chylomicron fraction to assess apolipoprotein transfer from HDL. The production and incorporation of apo E into nascent chylomicron particles may be important in their metabolism and as yet there is little information about how this may be effected by diabetes.

6.2 METHODS.

Eighteen New Zealand White rabbits were fed an atherogenic diet of 0.5% cholesterol for a period of five weeks. At the beginning of the fifth week nine were made diabetic with alloxan as described in section 2.13.2 and moderate glycaemic control maintained with daily insulin injection. Blood was collected 24 hours before lymph collection for analysis of LCAT and CETP activities (section 2.13.3). Lymph and blood samples were collected from each animal following gavage of a lipid emulsion. Plasma was isolated and plasma and
lymph chylomicrons were isolated as described in section 2.13.4. Lipid analysis was made of plasma and lymph (section 2.13.5). Lipid and apolipoprotein analysis was made of plasma and lymph chylomicrons as described in section 2.13.6.

6.3 RESULTS.

6.3.1 Animal Characteristics and Plasma Measurements.

Animal characteristics are shown in Table 6.1 and show that diabetic rabbits were slightly, but not significantly lighter on the day of experiment and there was no difference in the daily food intake between the groups. Diabetic animals had a mean blood glucose of 18.2 mmol/l. Examination of plasma lipids showed significantly higher cholesterol in diabetic rabbits (p<0.05). Free cholesterol was also over 2-fold higher in diabetics (p<0.02) compared to non-diabetics. Triglyceride was significantly higher and HDL cholesterol significantly lower in diabetic animals. Both cholesterol esterification (p<0.05) and cholesteryl ester transfer (p<0.02) activities were significantly higher in diabetic rabbits.

6.3.2 Composition of Lymph and Plasma Chylomicron Fractions.

Absolute amounts (μg/ml plasma or lymph) of components are given in Table 6.2 and shows that the absolute amount of both apo B48 (p<0.001 and p<0.01) and apo B100 (p<0.005 and p<0.0001) were significantly higher in both lymph and plasma chylomicron fractions. Levels of apo E and apo A1 were similar in both fractions in the two groups. The amount of cholesterol carried in this fraction in the both lymph and plasma was numerically higher but not statistically different between the groups. The triglyceride content of the lymph chylomicron fraction was significantly higher in the diabetic animals compared to non-diabetic (p<0.01).

Lipid and apolipoprotein composition expressed in mg/mg apo B, representing the composition of the particles in the fractions are given in Table 6.3. Diabetic rabbits produced both lymph (p<0.005) and plasma (p<0.01) particles which contained
approximately half the amount of apo E that diabetic particles contained. Apo AI was significantly lower on the lymph particles of diabetic rabbits (p<0.005). The amount contained on the plasma particles was lower also in diabetic animals but did not reach statistical significance. Particles of the lymph fraction had a similar cholesterol content in both diabetic and non-diabetic animals but had a significantly lower triglyceride content in the diabetic group (p<0.01). Plasma particles carried similar amounts of cholesterol and triglyceride.

Table 6.1: Characteristics, plasma lipids and CETP and LCAT activities of diabetic (n=9) and non-diabetic (n=9) rabbits. Results are given as mean ± SD. Triglyceride expressed as median and range.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (Kg)</td>
<td>2.8 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>140 ± 38</td>
<td>131 ± 27</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>18.2 ± 2.2</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>23.9 ± 10.6</td>
<td>8.4 ± 4.2</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>9.1 (0.5-24.3)</td>
<td>0.9 (0.3 - 1.8)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Free cholesterol (mmol/l)</td>
<td>10.34 ± 5.3</td>
<td>4.03 ± 2.7</td>
</tr>
<tr>
<td>CETP activity (nmol/ml/h)</td>
<td>36.6 ± 13</td>
<td>21.1 ± 5.9</td>
</tr>
<tr>
<td>LCAT activity (nmol/ml/h)</td>
<td>41.5 ± 17</td>
<td>26.3 ± 5.0</td>
</tr>
</tbody>
</table>

† p<0.05  § p<0.02  ¶ p<0.01 different from non-diabetic rabbits. (unpaired t-test).

Triglyceride used non-parametric, unpaired test (Mann-Whitney U test).
Table 6.2: Apolipoprotein composition of plasma and lymph chylomicron fractions. Results expressed in μg/ml plasma or lymph and given as mean ± SD Triglyceride expressed as median and range.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymph chylomicron fraction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>9.06 ± 3.4</td>
<td>4.32 ± 1.1</td>
</tr>
<tr>
<td>Apo B100</td>
<td>9.23 ± 4.5</td>
<td>3.64 ± 2.5</td>
</tr>
<tr>
<td>Apo E</td>
<td>6.09 ± 2.3</td>
<td>5.80 ± 3.1</td>
</tr>
<tr>
<td>Apo A1</td>
<td>15.17 ± 8.1</td>
<td>18.93 ± 7.7</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>804 ± 516</td>
<td>390 ± 208</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>4793 (3140-13440)†</td>
<td>3478 (580-10690)</td>
</tr>
</tbody>
</table>

| **Plasma chylomicron fraction** |                |              |
| Apo B48                    | 4.50 ± 2.7     | 1.44 ± 1.2   |
| Apo B100                   | 15.47 ± 5.3    | 4.24 ± 3.1   |
| Apo E                      | 11.21 ± 5.8    | 6.64 ± 3.1   |
| Apo A1                     | 5.25 ± 3.6     | 3.14 ± 1.6   |
| Cholesterol                | 1474 ± 1074    | 475 ± 405    |
| Triglyceride               | 324 (137-4651) | 138 (76-861) |

† p<0.01  § p<0.005  ¶ p<0.001  ¥ p<0.0001 different from non-diabetic rabbits. (unpaired t-test). Triglyceride used non-parametric, unpaired test (Mann-Whitney U test).
Table 6.3: Lipid and apolipoprotein composition of chylomicrons from plasma and lymph. Results expressed in μg/mg apo B and given as mean ± SD. Triglyceride given as median and range.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymph chylomicron fraction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo E</td>
<td>0.35 ± 0.09 §</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>Apo AI</td>
<td>1.10 ± 571 §</td>
<td>2.45 ± 0.99</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>43.92 ± 32.14</td>
<td>49.07 ± 37.02</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>262 (157-905) †</td>
<td>486 (139-1678)</td>
</tr>
<tr>
<td><strong>Plasma chylomicron fraction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo E</td>
<td>0.63 ± 0.21 †</td>
<td>1.37 ± 0.44</td>
</tr>
<tr>
<td>Apo AI</td>
<td>0.37 ± 0.12</td>
<td>0.53 ± 0.31</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>73.8 ± 46.3</td>
<td>83.6 ± 59.4</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>15.8 (5.2-106)</td>
<td>24.8 (5.1-134)</td>
</tr>
</tbody>
</table>

† p<0.01  § p<0.005  ¶ p<0.001 different from non-diabetic rabbits.

(unpaired t-test). Triglyceride used a non-parametric, unpaired test (Mann-Whitney U test).
Chapter 6

6.4 Discussion.

Atherosclerosis is one of the major complications associated with diabetes and there is increasing evidence to show that the disturbance in postprandial lipoprotein metabolism, which has become a feature of diabetes, may be an important contributor to the development of heart disease. Elevated levels of apo B48-containing particles has been demonstrated in diabetic subjects (Curtin et al., 1994; 1996) and that an improvement in glycaemic control can improve this condition (Phillips et al., 1999). Feeding cholesterol to rabbits produces marked hypertriglyceridaemia (Minnich and Zilversmit, 1989) and hypercholesterolaemia (Bitman et al., 1976). This hyperlipidaemia has been shown to be due to an accumulation of both VLDL and chylomicron remnant particles (Fainaru et al., 1982; Kane et al., 1983; Hussain et al., 1995) and it was for this reason that the cholesterol-fed rabbit was chosen as a model of postprandial dyslipidaemia. While there is evidence to show that cholesterol absorption and synthesis are altered in diabetic animal models (O'Meara et al., 1990; 1991; Gleeson et al., 1999; 2000), there is little information on intestinal apolipoprotein and nascent chylomicron particle apolipoprotein composition and how it may be affected by diabetes. This study was undertaken to investigate the effect of diabetes on intestinal chylomicron production, particularly with regard to apo E levels on the particles, as it is the ligand responsible for the normally rapid uptake of chylomicron remnants from the circulation via hepatic receptors such as the LDL receptor (Innerarity and Mahley, 1978), the LRP (Herz et al., 1988), the LSR (Bihain and Yen, 1992). Alteration of the chylomicron particles on entering the plasma was also investigated by again assessing particle composition and measuring the activity of transfer proteins (CETP and LCAT) which effect cholesterol and triglyceride composition of the apo B-containing particles in the plasma.

Results from this study revealed that lymph from diabetic animals contained significantly more apo B48 and apo B100 particles isolated in the chylomicron density fraction compared to non-diabetic animals. While diabetic animals produced more triglyceride in this fraction, the ratio of triglyceride to apo B however was significantly higher in particles from non-diabetic animals. This showed that diabetic animals were producing significantly
more particles which were smaller in size (more lipid-depleted) compared to non-diabetic animals. This was reflected in plasma, where over 3 times more particles entered the bloodstream of diabetic animals and these particles tended to be smaller in size compared to the non-diabetic particles. It has been shown previously that both particle number and size are determinants of TRL clearance and that the process, which is common to both apo B48- and apo B100-containing particles, is saturable (Martins et al., 1996). This group showed that, given an equal amount of triglyceride, an emulsion containing fewer particles was cleared more rapidly than one containing a large number of small particles. Indeed in a study running concurrently with the one being reported in this chapter, lymph chylomicrons from a subgroup of these same animals showed a significantly slower clearance rate of both particle triglyceride (reflecting LPL activity) and cholesterol (reflecting particle uptake) when diabetic particles were injected into similarly fed rabbits, regardless of diabetic status (Phillips et al., submitted). It was therefore reasonable to suggest that the exacerbated accumulation of particles in diabetic animals was due, at least in part, to reduced particle clearance.

The process of chylomicron clearance is mediated primarily by apo E (Cooper, 1997 and Mahley and Ji, 1999). Results in previous chapters of this thesis show diabetic chylomicron particles to be depleted of apo E and a reduction in the amount of apo E donated from HDL to the TRL in diabetic patients compared to diabetic patients with very good glycaemic control and non-diabetic patients. These studies gave no information about the effect of diabetes on apo E levels on newly secreted particles i.e., those present in lymph and possessing only the apo E produced by the intestine. The rabbit model facilitated this.

Apo E composition was investigated in both lymph and plasma chylomicron fractions. The absolute amount of apo E carried in the chylomicron fraction of lymph and plasma was not altered by diabetes. This would suggest that intestinal production of apo E is not altered in diabetes. This supports the finding of Lennich et al., (1991) who showed that intestinal apo E mRNA levels were not altered on the induction of alloxan-diabetes in
rabbits fed a 0.5% cholesterol diet. They did however demonstrate a reduction in apo E mRNA in the diabetic liver. On the particle however, diabetes caused a dramatic reduction in apo E on both lymph and plasma particles. As discussed in chapter 4, particle size is important in determining its apolipoprotein content and larger particles contain more apo E which is only lost on reduction in particle size due to lipolysis (Breyer et al., 1999). This observation was made in plasma lipoproteins but may also be relevant to lymph lipoproteins. The presence of HDL and other plasma-like lipoproteins in lymph and interstitial fluid has been described in animals (Laplaud et al., 1990; Sloop et al., 1993; Wong et al., 1995) and humans (Reichl et al., 1989; Luc et al., 1996). Indeed it has been shown that a sizeable portion of plasma HDL enters intestinal lymphatics probably intact and is responsible for approximately 58% of lymph cholesterol (Oliveira et al., 1993). This group also showed using injected, duel-radiolabelled plasma HDL that cholesteryl esters and a small amount of apolipoprotein is transferred from HDL to the larger TRL particles in lymph. This may be an alternative method by which apo E is incorporated into lymph chylomicrons and the process may indeed be altered in diabetes.

In plasma, chylomicron particles of diabetic rabbits had less than half the amount of apo E that non-diabetic chylomicrons. This may be as a result of the diabetic lymph particles being produced with less apo E, but may also be due to reduced transfer of apo E from HDL in the plasma compartment, as has been demonstrated in the human study of chapter 5. With an inadequate complement of apo E it is reasonable to assume that reduced clearance of plasma particles is responsible to some extent for their further accumulation in diabetic, cholesterol-fed rabbits. In a study with cholesterol-fed rabbits (used as a model of elevated TRLs), transgenic for and over expressing human apo E, investigators observed a 35% reduction in triglyceride due to a decrease in VLDL and that injected chylomicrons were cleared more rapidly in the transgenic animals compared to their non-transgenic litter mates (Fan et al., 1998). These animals also showed an increase in LDL cholesterol. It has been shown that both apo B48- and apo B100-containing particles compete for hepatic receptor binding for clearance of the particles (Bjorkegren et al., 1996) and an accumulation
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TRL may be partly responsible for the greatly elevated plasma cholesterol levels observed in the diabetic animals.

The LCAT and CETP activity was significantly higher in the plasma of diabetic rabbits, giving a possible explanation for the reduced HDL cholesterol levels and suggesting a pro-atherogenic role for elevated transfer activity in this situation. Diabetic rabbits had elevated plasma free cholesterol levels. The enrichment of lipoproteins with unesterified cholesterol is thought to enhance transfer of cholesterol ester from HDL to the TRL (Morton, 1988). This increased cholesterol transfer may also have contributed to cholesterol loading of the chylomicron particles in plasma. The role of CETP in the development or prevention of atherosclerosis in humans is unclear and the literature is conflicting. However a recent article reports the attenuation of atherosclerosis in rabbits by a CETP inhibitor (Okamoto et al., 2000). Treatment with the inhibitor reduced chylomicron, VLDL and LDL cholesterol and increased HDL cholesterol, strongly suggesting a pro-atherogenic role for CETP. The absolute amount of apo AI produced by the intestine was not different between diabetic and non-diabetic rabbits. The amount carried per particle was reduced in diabetes. Intestinally-derived apo AI is immediately transferred to HDL once the chylomicron reaches the plasma and becomes reduced in size by the action of LPL. The amount present in the plasma fraction was also similar in both groups. These results suggest that intestinal apo AI may have no part to play in the reduced HDL cholesterol levels observed in the diabetic rabbits.

The accumulation of postprandial lipoprotein remnants has been shown to be pro-atherogenic (Tkac et al., 1997; Sakata et al., 1998; Kugiyama et al., 1999) and a positive correlation has been demonstrated between plasma apo B48 levels and the progression of coronary atherosclerosis (Karpe et al., 1994). Diabetic patients have elevated levels of postprandial TRL and results from this study would suggest that while the diabetic rabbit intestine does not produce less apo E, particles produced by the diabetic intestine are deficient in apo E and this may contribute to postprandial diabetic dyslipidaemia. An elevated cholesterol ester transfer activity in the diabetic animals may have contributed to
cholesterol loading in the chylomicron fraction in plasma and suggests a pro-atherogenic role for CETP in diabetes.
Chapter 7

Discussion and conclusions
Atherosclerosis is by far the most common complication in diabetes, the incidence of which is between 2- and 5-fold higher than that of the non-diabetic population (Panzram, 1987; Anderson et al., 1987). Beyond disease-related abnormalities such as hyperglycaemia, hyperinsulinaemia, insulin resistance and the classical lipid abnormalities of raised plasma triglycerides and depressed HDL cholesterol, some responsibility for this startling statistic has lately been aimed at abnormalities in postprandial lipoprotein metabolism (Curtin et al., 1995; 1996). Remnants of postprandial particles are thought to be particularly atherogenic (Karpe et al., 1994; Tkac et al., 1997; Sakata et al., 1998; Kugiyama et al., 1999) and the elevated level which is evident in diabetes has been shown to be exacerbated by poor glycaemic control (Phillips et al., 2000).

Studies in postprandial lipoprotein metabolism have advanced since the development of a method to separate and measure apo B48 and apo B100 (Kotite et al., 1995) - the marker apolipoproteins for intestinally-produced chylomicrons and hepatically-produced VLDL particles respectively. Each particle contains only one molecule of either protein (Elovson et al., 1988; Chan, 1992) so measuring the levels of these proteins gives an indication of the relative number of particles present. It has been shown that the number of particles present and also the size of the particle is important in determining the clearance rate of the triglyceride and cholesterol carried in the particles (Martins et al., 1996). Several studies have shown that chylomicron remnants penetrate arterial tissue rapidly, serve as a lipid substrate for macrophages (Mamo and Wheeler, 1994; Mamo et al., 1996; Fujioka et al., 1998) and induce foam cell formation in human monocyte-derived macrophages (Mamo et al., 2000). Remnants of VLDL particles (LDL) are the major carriers of cholesterol in the bloodstream and when oxidised, contribute considerably to cholesterol accumulation in atherosclerotic plaques (Shaikh et al., 1988). The predominance of large, triglyceride-rich VLDL particles which is evident in diabetic patients (Feingold et al., 1992) has been shown to be associated with the production of small, dense LDL particles (Packard et al., 2000). This phenotype is associated with increased risk of coronary artery disease (Austin et al., 1988; Griffin et al., 1994). It is now becoming more apparent that this diabetic
postprandial lipoprotein phenomenon is contributing to the increased incidence of heart
disease which is evident in these patients even in the presence of normal plasma lipids.

Diet is considered to be the cornerstone in the management of type 2 diabetes, the aim
being to maintain good glycaemic control, reduce or maintain body weight, control blood
pressure and normalise dyslipidaemia. Over recent years there has been much debate
regarding the attributes of various diets with respect to these criteria. In fact, a meta-
analysis of studies on the effects of dietary monounsaturated fatty acids in diabetic
patients concluded that this diet should be advised for diabetic patients because it reduced
plasma triglycerides, increased HDL cholesterol without increasing LDL cholesterol and
also improved glycaemic control (Garg, 1998). Dietary guidelines have been slow to change
but in the past the general concensus has been for a diet of low total fat, limiting saturated
fat

Studies have shown however, that monounsaturated fat improved glycaemic control
relative to a high complex carbohydrate diet (Garg et al., 1988; Lerman-Garber et al., 1994)
and recently an improved glucose transport has been demonstrated in adipocytes isolated
from Type 2 diabetic patients following an olive oil diet compared to the same patients on
a sunflower oil diet (Ryan et al., 2000). Also, LDL from diabetic patients following an
olive oil diet displays a marked reduction in oxidisibility when compared to LDL from the
same patients following a sunflower oil-rich diet (Dimitriadis et al., 1996). An oleic acid
diet has recently been shown to dramatically reduce both monocyte chemotaxis and
adhesion due to minimally oxidised LDL isolated from American subjects on a oleic acid-
rich liquid diet compared to a similar linoleic acid diet (Tsimikas et al., 1999). The final
report of the Lyon Diet Heart Study aimed to test whether a Mediterranean-type diet (the
main lipid component of which is olive oil) may reduce recurrence of myocardial infarction,
when compared to a prudent Western style diet (the main lipid component being
polyunsaturated fat) (Lorgeril et al., 1999). Investigators found that the protective effect of
the Mediterranean-type diet was maintained up to 4 years after the first infarction.
Interestingly in this study there was no difference in plasma lipids, blood pressure or
glycated haemoglobin between those on the diet and a control group even though the traditional risk factors were still identified in the diet-treated group. This may suggest the importance of alternative effects other than improving plasma cholesterol levels in the atheroprotective nature of the Mediterranean-style diet. A review published recently discussed the merits of different diets with regard to the metabolic syndrome and concluded that a diet which improves body weight is important, saturated fat should be limited and that monounsaturated fat rather than other fat should be permitted as it does not induce detrimental metabolic effects (Riccardi and Rivellese, 2000). So even though dietary mono- and polyunsaturated fatty acid are thought to have a similar effect in lowering LDL cholesterol (Mattson and Grundy, 1985; Zock and Katan, 1992; Gustafsson et al., 1992; Kris-Etherton et al., 1993) questions arise as to the suitability of polyunsaturated fat in the diet of Type 2 diabetic patients.

There is little information on the effect of poly- or monounsaturated fat diets on postprandial lipoproteins in diabetes. This project therefore endeavoured to investigate the relative merits of each in the context of diabetes mellitus, with respect to plasma lipids, glycaemic control, levels of postprandial particles, paying particular attention to the apolipoproteins which normally work in concert to mediate both intestinally- and hepatically-derived postprandial particles. The effect of diabetes on postprandial chylomicron production was also investigated, in an animal model.

This study turned up some interesting and also some quite controversial findings, not least in the plasma lipid levels. The linoleic acid diet produced higher fasting plasma cholesterol and fasting glucose levels in both groups. This is contrary to the studies mentioned above which have shown that linoleic acid and oleic acid had similar effects on LDL cholesterol levels (Gardener et al., 1995) and that linoleic acid may have a tendency to reduce HDL cholesterol also (Howard et al., 1995) - at least in non-diabetic subjects. There was no change in HDL cholesterol or triglyceride levels between the diets. This elevated LDL would indicate an increased risk of heart disease. The linoleic acid diet seemed to exacerbate insulin resistance in the diabetic group which is reflected in the higher fasting glucose and
insulin levels on this diet. This is in agreement with a previous study in diabetic patients which also demonstrated reduced glucose transport in patients' adipocytes after a linoleic acid diet (Ryan et al., 2000).

A central finding of this thesis was that a diet rich in linoleic acid resulted in significantly higher numbers of both intestinally- and hepatically-derived lipoproteins of chylomicron and VLDL density postprandially, whereas the oleic acid diet tended to normalise levels. Fasting apo B48 and apo B100 were elevated in both fractions but most significantly in the chylomicron fraction. Levels were completely normalised by the oleic acid diet. This may indicate either impaired clearance or enhanced secretion of postprandial particles in subjects on the linoleic acid diet, a condition which is rectified by the oleic acid diet. It has recently been reported that there is a strong positive relationship between fasting plasma apo B48 levels and the postprandial apo B48 response (Smith et al., 1999). The authors suggest that a single measurement of fasting plasma apo B48 levels could predict the postprandial levels. This relationship seems to hold for the postprandial apo B results of the current study.

The greatest improvement was in both apo B48- and apo B100-containing particles in the VLDL fraction. These include chylomicron remnants and the majority of newly secreted VLDL particles. The linoleic acid diet was most detrimental in elevating numbers of newly secreted, large chylomicrons and large hepatically-derived particles of the chylomicron fraction. Dietary oleic acid may therefore be very important in reducing risk of atherosclerosis in diabetic patients given the increasing evidence that chylomicron remnant particles are proatherogenic (Karpe et al., 1994; Mamo and Wheeler, 1994; Mamo et al. 1996; Fujioka et al., 1998; Proctor and Mamo, 1998; Yu and Mamo, 2000) and that the preponderance of large triglyceride-rich VLDL particles, a feature of diabetic dyslipidaemia (Feingold et al., 1992), is accompanied by the predominance of small, dense LDL (Packard et al., 2000) - particles which are easily oxidised (Siegel et al., 1996) and are associated with premature coronary artery disease (Austin et al., 1988; Griffin et al., 1994). The phospholipid content of the fasting LDL fraction was significantly higher in the diabetic
patients after the linoleic acid diet suggesting that the LDL particle size may indeed be smaller in this group.

Interestingly, a paper published earlier this year reported quite contradictory results. This study was in Japanese, Type 2 diabetic patients and compared fasting lipid levels and postprandial cholesterol in remnant-like particles following oleic and linoleic acid diets. They found no difference in fasting lipid profiles between the diets. They did however find that postprandial apo B48-containing remnant cholesterol was significantly higher in patients after the oleic acid diet. This was not changed in either fraction measured in this current study. But both apo B48- and apo B100-containing particles are present and measured in these fractions. The patients chosen in the Japanese study were poorly controlled metabolically and the authors suggest that plasma lipid results may have been different had well-controlled patients been used. Also the test meal given at the end of each dietary period was of cow's milk and cream and therefore contained mainly saturated fat and does not therefore accurately reflect the normal postprandial response whilst on the diets. It was also reported that LDL oxidisibility was higher following the oleic acid diet which is directly contradictory to many studies in animals (Parthasarathy et al., 1990) and in normoglycaemic (Bononome et al., 1992; Abbey et al., 1993; Aviram and Elias 1993) and diabetic humans (Dimitriadis et al., 1995, 1996). Even though levels of oleic acid and linoleic acid changed significantly in plasma of the Japanese subjects, the amount of fatty acid in the low fat diet (20%) may not have been sufficient to elicit differential lipid and lipoprotein changes.

The second most significant finding of this study was the deficiency of apo E on postprandial particles produced by patients after the linoleic acid diet. Apo E plays a key role in particle uptake (Mahley and Ji, 1999) and it was therefore suggested that the elevated levels of chylomicrons and VLDL in patients on the linoleic acid diet could be as a result of reduced clearance of the particles. A study in diabetic and non-diabetic subjects with and without CAD showed that apo E was highest in lipoprotein fractions from diabetic subjects with CAD. Their results did however show a reduced amount of apo E in
chylomicron and VLDL fractions in diabetic patients without CAD compared to healthy subjects (Syvänne et al., 1994). They also showed that TRL particles from diabetic patients were enriched in apo E relative to the triglyceride content of the particle and when expressed in this way particles from patients on the linoleic acid diet do have elevated apo E in the VLDL fraction compared to non-diabetic subjects (p<0.05, results not shown). Values are normalised on the oleic acid diet. This way of expressing the result does not reflect particle number and may therefore not be an accurate estimation of the apo E content on the particle. Expressing apo E as a ratio of apo B does this and unfortunately this was not done in the above publication.

There may be a number of different reasons for the observed deficiency in apo E follow the linoleic acid diet. Particle size has been shown to be a determinant of apolipoprotein content (Bukberg et al., 1985; Breyer et al., 1999) with larger particles having more non-exchangeable apolipoproteins. The linoleic acid diet produced more particles which were smaller in size, and contained less apo E. Interestingly the absolute amount of apo E present in the VLDL fraction of diabetic patients was significantly lower than in non-diabetic patients while the diets seemed to have little effect. Apo E genotyping showed that the allele distribution of the diabetic group was similar to that of the non-diabetic group (mainly 3/3) and was also consistent with that observed in the Irish population (Sheehan et al., 2000). We can therefore say that these effects are not due to the selection of an aberrant diabetic group. This would suggest that diabetes itself is contributing to either a reduction in the secretion of apo E or a reduction in the amount of apo E transferred from HDL. Indeed analysis of the HDL fraction showed significantly lower apo E in HDL following the linoleic acid diet. This was again normalised with the oleic acid diet. The amount transferred postprandially from linoleic HDL was only about one sixth that of the oleic HDL and the non-diabetic HDL. This would suggest that deficiency of apo E in the TRL could well be as a result of both lower levels on, and lower transfer of the apolipoprotein from HDL in diabetic subjects on the linoleic acid diet. It has been shown that apo E associates preferentially with HDL during fasting conditions and with TRL during alimentary lipaemia (Havel et al., 1973; Mahley et al., 1984; Klein et al., 1992;
Björkegren *et al.*, 1997, 1998). The reduced levels of HDL apo E in diabetic subjects on the
linoleic acid diet may also reflect the more elevated postprandial lipaemia on this diet. It
may also reflect a reduction in hepatic apo E expression by linoleic acid through the newly
identified PPAR response element (Galetto *et al.*, 2001).

The animal study revealed that apo E production by the intestine is not altered by
diabetes, confirming the finding of Lennich *et al.*, (1991) who showed that intestinal apo E
mRNA levels were not altered on the induction of alloxan-diabetes in rabbits fed a 0.5%
cholesterol diet. But the apportioning of apo E on the particles was different and diabetes
did indeed cause the production of apo E-deficient lymph chylomicrons, which remained
deficient in the circulation. Again this deficiency may be a result of particle size as diabetic
particles were more numerous (Apo B) and lipid-depleted - a similar finding to that of the
human study. Transfer of apo E from HDL present in lymph (Reichl *et al.*, 1989; Luc *et
al.*, 1996) cannot however be ruled out, although this has never been documented. It would
make for interesting investigation.

Apo C is also central to the metabolism of postprandial lipoproteins. Apo CII is the
essential co-factor of LPL (Olivecrona and Beisiegel, 1997), but both apo CII and apo CIII
when in excess can also inhibit the activity of the enzyme (Havel *et al.*, 1973; Goldberg *et
al.*, 1990; Lambert *et al.*, 1996). Both apo CII and apo CIII can also prevent apo E-
mediated uptake of particles by the liver, when in excess. So TRL lipolysis and clearance
seems to involve a fine balancing act between these apolipoproteins. It seemed reasonable
to predict that changing fatty acid diet may cause changes in apo C expression as this is
mediated through the PPARs and fatty acids are ligands to these steroid hormone-like

There was an increase in the absolute amount of apo C present in the VLDL fraction in
diabetic subjects on the linoleic acid diet but neither level was different from that of non-
diabetic subjects. This may suggest an effect on hepatic expression of the apo C genes. It
has also been shown that the expression of apo CIII is mediated by insulin via a negative
insulin response element in the gene promoter (Dammerman *et al.*, 1993; Chen *et al.*, 1994)
and the more severe insulin resistance displayed in diabetic patients on the linoleic acid diet may have a role to play here. However, the particle content was not different between diets in the VLDL fraction but was significantly lower than non-diabetic particles on the inoleic acid diet. In the chylomicron fraction however, the linoleic acid diet produced particles deplete of both apo CII and apo CIII, whereas the oleic acid diet seemed to normalise levels. This may again be a function of particle size as analysis of HDL showed no difference in apo CII or apo CIII whatever, ruling out differential transfer of the apolipoprotein.

Both the ratios of apo CIII/CII and apo CIII/E and also the levels of the apolipoproteins have been shown to be important in the clearance of TRL (Lambert et al., 1996; Andersson et al., 2000). A study recently in Type 2 diabetic patients showed that apo CIII transferred to chylomicrons in proportion to the triglyceride content (Attia et al., 2000). This is in agreement with the current study where the smaller particles of the linoleic acid diet contain less apo C. This group also showed that the ratio of apo CIII/E did not change after treatment with bezafibrate despite a reduction in plasma triglyceride. The ratios were not changed in the chylomicron fraction, but there seemed to be an overall deficiency in apo C which may have had a detrimental effect on particle lipolysis and uptake. The VLDL fraction however showed a significantly higher ratio of apo CIII/E in both diabetic groups which seems more as a result of the dramatic deficiency in apo E on the particle. There was some improvement on the oleic acid diet and this may have contributed to enhanced uptake of the oleic particles.

One of the more unexpected findings of the current study was that postprandially diabetic patients on the linoleic acid diet had a dramatic reduction in HDL cholesterol. Indeed contrary to the plasma analysis, fasting HDL cholesterol was also significantly lower in diabetic subjects on the linoleic acid diet compared to non-diabetic subjects when measured in the ultracentrifugally isolated fraction. It may be that this method is more sensitive, especially in samples containing elevated TRL. One reported difference between polyunsaturated and monounsaturated diets is that while both seem to lower plasma

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cholesterol, polyunsaturated fat also tends to lower HDL cholesterol (Howard et al., 1995). This indeed seems to be the case at least in diabetic patients in the study. Since most of the day is spent in the postprandial state the measurement of HDL cholesterol postprandially may be a better indicator of HDL cholesterol status as there was no difference between the diets in fasting samples. A reduced HDL cholesterol level in plasma is a well established risk factor for coronary artery disease (Gordon and Rifkind, 1989; Fruchart et al., 1994). This is a defining lipid abnormality of Type 2 diabetes and would strongly suggest the unsuitability of a linoleic acid-rich diet for diabetic patients.

Further analysis of the HDL fraction may give an insight into a possible cause for the cholesterol effect. As mentioned earlier HDL apo E was significantly reduced in diabetic HDL produced following the linoleic acid diet. Apo E is important in the process of cholesterol efflux from cells (Oram and Yokoyama, 1996; von Eckardstein et al., 1995; Kinoshita et al., 2000), the first step of reverse cholesterol transport. There tended to be lower apo AI in diabetic patients on the linoleic acid diet. Apo AI is also important in cholesterol efflux (Fielding and Fielding, 1995). It is the main activator of LCAT and the process of cholesterol efflux may indeed be impeded on the linoleic diet resulting in cholesterol-depleted HDL. A recent study in African green monkeys showed that plasma concentrations of large, cholesterol-rich HDL are determined by the production rate of apo AI in the fraction (Colvin et al., 1998). A low serum apo AI in Type 2 diabetic patients has been related to increased intima-media thickness and early atherosclerotic lesions (Tkac et al., 2001), indicating again the unsuitability of a linoleic acid-rich diet for diabetic patients.

HDL particles from diabetic patients tend to be smaller and deplete of cholesterol and it has been proposed that this does indeed reflect defective reverse cholesterol transport (Singh et al., 1995; Taskinen, 1996). There was however no difference in the activity of either LCAT or CETP between diets or between groups in this current study, suggesting that cholesterol transfer to the TRL was not responsible for the reduced HDL cholesterol levels following the linoleic acid diet. This was not entirely unexpected as the amount of
triglyceride carried in the TRL fractions and plasma triglyceride levels were similar between the groups and it has been observed in diabetes that elevation of CETP and LCAT activity is associated with hypertriglyceridaemia (Jones et al., 1996; Reimens et al., 1998; Lottenberg et al., 1996; Guerin et al., 2001).

The animal study did show increased levels of both LCAT and CETP activity in diabetes. The diabetic rabbits had only moderately controlled glycaemia and displayed the more classical lipid abnormalities of elevated plasma triglycerides and reduced HDL cholesterol. The rabbit model did allow us to assess apo Al production by the intestine and showed that diabetes had no effect in the alloxin-diabetic rabbit. Apo Al is transferred from the chylomicron to HDL once the particle reached the bloodstream and this result would suggest that intestinally produced apo Al may not influence HDL cholesterol levels in diabetes.

In conclusion, the fundamental problems of insulin resistance, glycaemic control, elevated postprandial particles and reduced HDL cholesterol seem to be exacerbated in Type 2 diabetic patients by a polyunsaturated fatty acid diet and normalised to a certain extent by a monounsaturated fatty acid diet. This seems to occur by an improvement in insulin sensitivity, reducing postprandial particles by normalising both apo E and apo C - apolipoproteins which are central to particle clearance. This is accomplished at least in part by increasing to more normal levels the amount of apo E transferred from HDL. LDL cholesterol was decreased and HDL cholesterol increased during fasting and postprandially. LDL cholesterol was lower possibly as a result of enhanced TRL clearance and HDL cholesterol was raised at fasting and postprandially possibly due to the enrichment of the particles in apo E and apo AI - apolipoproteins which mediate cholesterol efflux from the periphery. This lipid and lipoprotein profile is indicative of a reduced risk of atherosclerosis. Results of this study show also that benefits of the Mediterranean-style diet can be achieved by changing only the dietary fatty acid content of the diet to that of olive oil and that for diabetic patients at least, this may indeed be the most prudent diet.
The work of this thesis raises a very fundamental question regarding the treatment of diabetes. Should olive oil be the dietary fat of choice for diabetic patients? This may become particularly relevant in the coming years as diabetes edges ever closer to reaching epidemic proportions. Can the metabolic problems which have such a devastating effect on the vascular and general health of these patients be markedly improved by simply switching the oil in their diet? These studies show that the effect of doing so is quite dramatic and should certainly be considered as a simple, inexpensive and effective treatment to alleviate the profound metabolic disorder that is diabetes mellitus.
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