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Intestinal microsomal triglyceride transfer protein, promoter region polymorphisms and postprandial lipoproteins in Type 2 Diabetes

By Karen Mullan MB.BS., M.R.C.P.

M.D thesis

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

Direction and Supervision

Professor Gerald Tomkin M.D., F.R.C.P.I., F.R.C.P., F.A.C.P.

Dr Daphne Owens Ph.D.

2007
Declaration

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Signed: [Signature]

Karen Mullan
To my parents Seamus and Mary and my husband Gavin
Summary

Microsomal triglyceride transfer protein is an ancient lipid transfer protein responsible for the assembly of chylomicrons in the intestine and very low density lipoproteins in the liver.

Common MTP gene promoter region polymorphisms have been described that do not affect lipoproteins and cardiovascular risk in non-diabetic subjects. Their effect in diabetes has not been described in a Caucasian population.

Intestinal MTP gene expression and protein activity are increased in animal models of diabetes while human intestinal MTP expression has not previously been reported.

Eighty two diabetic subjects had MTP genotyping by PCR-RFLP and were characterized for evidence of cardiovascular disease by means of chart review, exercise stress tests and carotid artery intima-media thickness measurements. Twenty -seven diabetic and 24 non-diabetic subjects had duodenal biopsies taken during endoscopy and MTPmRNA was measured by a ribonuclease protection assay. Postprandial lipoprotein composition was determined in all subjects after a high fat meal.

Subjects heterozygous for the -493 G/T substitution had lower LDL cholesterol. They also had higher apo B48, lower cholesterol and lower cholesterol/apo B in the postprandial VLDL fraction. This suggests that the T allele effect is magnified in diabetes and that this common polymorphism may be protective from risk of atherosclerosis via its effect on LDL and postprandial lipoproteins. The -400 A/T substitution gave very similar lipoprotein results but there was significant linkage disequilibrium between the
two polymorphisms. No association was found between the -164 T/C polymorphism and either plasma lipids or postprandial lipoproteins. ApoE genotype did not influence the above results.

The few subjects with the rare TT -493 polymorphism had a high level of atherosclerotic disease, but no differences were found between the GG and GT genotypes. This is in keeping with other authors who have found that the TT polymorphism, rather than being protective against atherosclerosis, may paradoxically be a risk factor.

Diabetic subjects were found to have significantly higher intestinal MTP mRNA than non-diabetic subjects. There were positive correlations between intestinal MTPmRNA and chylomicron particle composition in diabetic subjects, consistent with the previously shown lipidation effect of MTP on apoB.

Intestinal MTP mRNA was higher in the statin - treated diabetic group compared to the statin- treated non-diabetic group. Within the non-diabetic group, statin therapy was associated with lower intestinal MTP mRNA. In the group as a whole there was a positive correlation between intestinal MTPmRNA and postprandial chylomicron cholesterol/apoB48. These results confirm the suggestion that the insulin and sterol response elements of the MTP gene are important regulators of MTP transcription.
Acknowledgements

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I would very much like to thank Dr Catherine Phillip for allowing me to work alongside her at the bench during the analysis of the intestinal MTPmRNA samples, the apoB and lipid isolations. I am also grateful for her analysis of the MTP polymorphisms which have made this MD possible.

I would like to acknowledge Professor Paddy Collins, Department of Biochemistry, The Royal College of Surgeons, for his help and support; also to the laboratory staff who kept me on track in the laboratory.

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**Irish Angiology Society Nov 2002**

"Relationship between intestinal microsomal triglyceride transfer protein and postprandial lipoproteins in type 2 diabetes"

**17 th Annual Medical and Scientific Meeting Heart UK Manchester 2003**

"Human intestinal microsomal triglyceride transfer protein: the effect of HMG CoA reductase inhibition"

**European Association for the study of Diabetes, Paris August 2003**

"Intestinal microsomal triglyceride transfer protein is raised in diabetes: A mechanism for the production of atherogenic postprandial lipoproteins?"

**Irish Endocrine Society November 2003**

"Increased intestinal microsomal triglyceride transfer protein in diabetes is related to increased cholesterol content but not the triglyceride content of the intestinally-derived particles"

**Association of Physicians of Great Britain and Ireland 2004**

"Microsomal triglyceride transfer protein and triglyceride rich lipoprotein metabolism in type 2 diabetes"
POSTER PRESENTATION

European Association of Diabetes, Budapest, Hungary September 2002

"Relationship between intestinal microsomal triglyceride transfer protein and postprandial lipoproteins in type 2 diabetic patients"

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Irish Angiology Society Nov 2002

"Relationship between intestinal microsomal triglyceride transfer protein and postprandial lipoproteins in type 2 diabetes"

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"Human intestinal microsomal triglyceride transfer protein: the effect of HMG CoA reductase inhibition"
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Irish Endocrine Society November 2003

Irish Journal of Medical Science vol 174:4: Suppl 1; 38

“Increased intestinal microsomal triglyceride transfer protein in diabetes is related to increased cholesterol content but not the triglyceride content of the intestinally-derived particles”
<table>
<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>Apo</td>
<td>apoprotein/apolipoprotein</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-coenzyme A:cholesterol acyltransferase</td>
</tr>
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<td>ADA</td>
<td>American Diabetes Association</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>CET</td>
<td>cholesterol ester transfer</td>
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<tr>
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<td>cholesterol ester transfer protein</td>
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<td>dl</td>
<td>decilitre</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
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<td>gram</td>
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<td>G</td>
<td>guanine</td>
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<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
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<td>hepatic lipase</td>
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<tr>
<td>HLA</td>
<td>human leukocyte associated antigens</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy 3-methylglutaryl coenzyme A</td>
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<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
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<tr>
<td>IDL</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kCal</td>
<td>kilocalorie</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>l</td>
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<tr>
<td>LCAT</td>
<td>lecithin-cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>lipoprotein(a)</td>
</tr>
<tr>
<td>LRP</td>
<td>low density lipoprotein receptor-related protein</td>
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<tr>
<td>M</td>
<td>molar</td>
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<td>m</td>
<td>milli</td>
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<tr>
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<td>micro</td>
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<tr>
<td>mA</td>
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<td>MgCl</td>
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<td>Min</td>
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</tr>
<tr>
<td>Mmol</td>
<td>millimole</td>
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<tr>
<td>MODY</td>
<td>maturity-onset diabetes of the young</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes</td>
</tr>
<tr>
<td>Nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>Rpm</td>
<td>revs per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>RXR</td>
<td>retinoic acid X receptor</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Sf</td>
<td>Swedberg floatation unit</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRL</td>
<td>triglyceride rich lipoprotein</td>
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<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoproteins</td>
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<td>watts</td>
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<td>World Health Organisation</td>
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Chapter 1

Introduction
1.1 Diabetes Mellitus

1.1.1 Definition

Diabetes mellitus is defined by the American Diabetes Association Expert Committee in their 1997 recommendations as “a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, action or both. The chronic hyperglycaemia is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidney, nerves, heart and blood vessels.”

1.1.2 Diagnosis

Guidelines were published by the World Health Organization (2002) and by the American Diabetes Association which was last updated in 2005. They are based on studies on the prevalence of diabetes-specific microvascular disease (e.g. retinopathy and nephropathy).

In the American Diabetes Association (ADA) criteria at least one of the following three criteria must apply and one of the criteria must be observed on a subsequent day:

(i) Symptoms of diabetes (polyuria, polydipsia, unexplained weight loss) as well as a casual plasma glucose concentration of ≥ 11.1 mmol/l (200 mg/dl).

(ii) Fasting plasma glucose of ≥ 7.0 mmol/l (126 mg/dl) with no caloric intake for at least 8 hours.
(iii) 2-hour plasma glucose of ≥11.1 mmol/l (200 mg/dl) during an oral glucose
tolerance test (OGTT), with the glucose load containing 75 g of anhydrous glucose in
water.

Two intermediate groups at risk of diabetes were also defined:

Impaired fasting glucose (IFG) was defined as a fasting plasma glucose between 5.6
and 6.9 mmol/l (100-125 mg/dl). Before 2003 their values had been 6.1-6.9 mmol/l.

Impaired glucose tolerance (IGT) was defined as a 2-hour glucose between 7.8 and
11.1 mmol/l (140-200 mg/dl).

The World Health Organization’s criteria is identical to those given by the ADA but
states that ideally both the 2-hour and fasting value should be used. It also states that
the OGTT should only be used where the casual blood glucose lies in the range 5.5-
11.1 mmol/l (100-200 mg/dl). It defines impaired fasting glucose as between 6.1-6.9
mmol/l.

1.1.3 Classification

Diabetes is classified both in stages (normoglycaemia, IGT/IFG, diabetes) and in
aetiological types of diabetes mellitus. The WHO and ADA have identical
recommendations with the exception of gestational diabetes which the WHO
classifies as a fasting glucose ≥7.0 mmol/l and/or 2-hour glucose ≥11.1 mmol/l, while
the ADA classifies the cut-off values as 5.3 fasting and 8.6 mmol/l at 2 hours post a
75 g glucose load.

The main classes are Type 1 diabetes (due to β-cell destruction) and Type 2 diabetes
(due to insulin resistance and a β-cell insulin secretory defect in varying proportions),
other specific types of diabetes and gestational diabetes.
Specific types of diabetes include genetic defects of β-cell function and insulin action, diseases of the exocrine pancreas, endocrinopathies, drug / chemical / infection induced, other genetic syndromes associated with diabetes and uncommon forms of immune-mediated diabetes.

Latent autoimmune diabetes of adulthood (LADA) is an autoimmune disease similar to Type 1 diabetes. However like patients with Type 2 diabetes, they do not require insulin initially and may have insulin resistance.

Mature onset diabetes of the young (MODY) usually develops before the age of 25 years. Currently six types have been identified which are autosomal dominant and caused by loss-of-function mutations in six different genes.

1.1.4 History

The first description of a polyuric state resembling diabetes dates to 1500 BC in Egypt. The term “diabetes” from the Greek word for siphon was first used in the 2nd century AD. John Rollo (d.1809) added the term “mellitus” from the Greek and Latin term for honey.

Minkowski and von Mering induced diabetes in a dog by pancreatectomy in 1889 thereby demonstrating the central role of the pancreas in this condition. Banting and Best (Toronto University) first discovered insulin in 1921 and the first patient received insulin in 1922 (review Pickup and Williams 3rd Edition). Oral hypoglycaemics followed in the 1950s.

More recently, the Diabetes Control and Complications Trial (DCCT 1993) and the United Kingdom Prospective Diabetes Study (UKPDS 33) gave conclusive evidence for the value of improved glycaemic control in delaying and preventing microvascular complications of Type 1 and Type 2 diabetes respectively.
1.1.5 Type 1 Diabetes Epidemiology

The incidence of Type 1 Diabetes has been increasing among most populations worldwide at a rate of about 3% per year (Karvonen et al 2000). There is a wide geographical variation with, for instance, an overall age adjusted incidence of 36 per 100,000/year in those <14 years old in Finland compared to 0.1 in Caracus, Venezuela.

In Europe, three out of the four countries with the highest incidences are in the northern regions (Finland, Sweden and Scotland). However, Sardinia in southern Europe has the second highest incidence. The European countries range from 1.3-48.5 per 100,000/year. The incidence in Eastern Europe and Asia countries tends to be low (1-11 per 100,000/year). Data on African incidence is sparse but data in Northern Africa give an intermediate incidence (10-20 per 100,000/year). Northern American incidence is comparable to those in Europe.

The global incidence of Type 1 diabetes in children and adolescents is increasing. In Europe the estimated annual rates of increases were 6.3% for children aged 0–4 years, 3.1% for 5–9 years, and 2.4% for 10–14 years (EURODIAB ACE Study Group 2000).

1.1.6 Genetic Factors in Type 1 Diabetes

Most cases are sporadic but siblings of children with Type 1 diabetes are 10 times more likely to develop diabetes before the age of 15 than those with no affected sibling.
Regions of the human genome that have been linked to Type 1 diabetes have been called the IDDM loci and range from 1 to over 20 (review Permutt 2005).

The major histocompatibility complex on chromosome 6 is part of the IDDM 1 locus and encodes cell surface glycoproteins called human leucocyte antigens (HLA). The HLA antigens (class I and II) have the ability to present autoantigens to T lymphocytes, and induce an autoimmune response. Some HLA antigens such as HLA-DR3 and HLA-DR4 are thought to present pancreatic β cell antigens as foreign and trigger autoimmune destruction of β cells, while some polymorphisms for example HLA-DQ6 appear protective against diabetes. Some authors find the effect of the HLA-linked locus on familiality to be minor. Risch (1987) found that HLA linkage accounts for a 3.42-fold increased risk in sibs of patients with Type 1 diabetes, while the observed value was 15. However other authors suggest that HLA accounts for about half of the genetic susceptibility for Type 1 diabetes (review Kim et al 2005).

Late onset Autoimmune Diabetes (LADA) is thought to be a hybrid of Type 1 and Type 2 diabetes in patients over 40 years old, who have the antibodies typically seen in early Type 1 Diabetes. LADA patients with multiple types of islet antibodies develop beta-cell failure within 5 years, whereas those with only GAD antibodies or islet cell antibodies tend to develop beta-cell failure later. Even though it may take up to 12 years for beta-cell failure to occur, impairments in the beta-cell response to intravenous glucose can be detected at diagnosis of diabetes (review Stenstrom 2005).
1.1.7 Environmental Factors in Type 1 Diabetes

As the incidence of Type 1 Diabetes is on the increase, this suggests that environmental factors play some role in its aetiology. Further argument for this comes from studies among monozygotic twins which report concordance rates of only 30-50%.

However there has been great difficulty in pinpointing these environmental factors. Viruses and diet have been the most explored factors. Most recently childhood vaccinations (measles, mumps and rubella) have been proposed as environmental factors in Type 1 diabetes but Hviid et al (2004) found no evidence for this in a cohort of all Danish children born between 1990 and 2000. The "fertile field hypothesis" proposes that viral infections alone do not induce disease in the absence of other inflammatory factors, while the "hygiene hypothesis" proposes that viruses and vaccinations can play a role in preventing rather than enhancing type 1 diabetes development (review Filippi et al 2005).

The "overload hypothesis" proposes that overload of the beta cell sensitizes it to immune damage and apoptosis. Environmental risks would include insulin resistance due to dietary excesses and fat cell accumulation, and increased insulin requirements due to a high growth rate, also dietary driven (Dahlquist 2006).

1.1.8 Type 2 Diabetes

Type 2 diabetes is a heterogeneous syndrome of insulin resistance and β-cell failure. It can be asymptomatic, at least initially, and therefore macro-vascular and micro-vascular complications are often already present at the time of diagnosis.
1.1.9 Epidemiology

Type 2 diabetes accounts for 85% of cases of diabetes in Caucasian populations and more in some other ethnic groups. Those particularly at risk are the Native Americans, Mexicans, and South Asians. The Pima Indians in USA have a prevalence of 50%.

There are estimates of 150 million people worldwide with diabetes. The total number is expected to double by 2025 (King et al 1998). There has been a 5-fold increase in India in the last 30 years and a 2-fold increase in Australia in the last 20 years (Shaw et al 2003).

Type 2 Diabetes is presenting in younger age groups, accounting for 80% of new cases of diabetes in children and adolescents in Japan, and 30% of new cases of adolescent diabetes in parts of USA. However, UK children still have a low prevalence of Type 2 diabetes (Ehtisham et al 2004). Risk factors for childhood type 2 diabetes includes obesity, positive family history and age >9 years.

1.1.10 Genetic factors

Genetic factors accounts for about 40-80% of susceptibility but is not Mendelian in nature. Type 2 diabetes is highly concordant in monozygotic twins (up to 90%) but concordance in dizygotic twins is only 17-37% (Poulsen et al 1999). No single major locus has been found to be linked to diabetes. Genetic defects in β-cell function have been identified on chromosomes 7, 12, and 20 in maturity onset diabetes of the young (MODY) but these account for less than 5% of cases. A locus on the short arm of chromosome 2 encoding caplain-10 has been identified as being responsible for 30%
of the risk in Mexican-American sibling pairs (Horikawa et al 2000). This proteolytic enzyme is involved in both insulin secretion and action.

1.1.11 Environmental factors

In the last number of years the idea of "fetal programming" leading to a "thrifty phenotype" has been proposed to explain the aetiology of Type 2 diabetes. Transient nutritional stimuli occurring at critical stages in development is thought to have lasting influence on the expression of various genes by interacting with mechanisms involved in transcription factor accessibility (review Gallou-Kabani 2005).

The “thrifty phenotype” term has been used to propose an evolutionary adaptation of the human gene pool. In previously food depleted environments it is thought that such adaptations would have resulted in more efficient storage of adipose fats which would give a survival advantage. The effects of such adaptations in an environment of food abundance would partly explain the insulin resistance and diabetes found in many of today’s populations.

1.1.12 Insulin resistance, Obesity, and the metabolic syndrome

The concept of insulin resistance emerged when it was noticed that patients following myocardial infarction had raised levels of insulin. Reaven (1988) showed, in a series of experiments set out in his Banting lecture of that year that resistance to insulin-stimulated uptake of glucose was present in the majority of patients with Type 2 diabetes and impaired glucose tolerance. However he also found evidence for this in 25% of non-obese subjects with normal glucose tolerance. He proposed that free fatty acid concentrations linked insulin resistance, hyperinsulinemia and glucose tolerance. Prospective studies with the Pima Indians provided evidence that insulin resistance
was a precursor for Type 2 diabetes (Lillioja et al 1993). The European Group for the
study of Insulin Resistance (Ferrannini et al 1997) has shown that obesity triples the
risk of insulin resistance, although many obese individuals were not insulin resistant.
They also showed that hyperinsulinaemia and insulin resistance could be mutually
exclusive in some patients. Those with insulin resistance but no fasting
hyperinsulinemia had raised triglyceride, free fatty acids (FFAs), and hepatic glucose
production, indicating hepatic insulin resistance and insulin resistance of lipolysis.
Those with fasting hyperinsulinemia but no insulin resistance had low waist hip ratio,
low FFAs, low glucose production and low insulin clearance, suggesting that insulin
level itself is only partially useful in characterizing insulin resistance syndrome.

The causes of insulin resistance have been explored including circulating insulin
antagonists such as growth hormone, corticosteroids, catecholamines, and glucagon;
antibodies to islet cells, and insulin receptors; resistin; cytokines (TNF-α, interleukin-
6) and elevated free fatty acids. Other rare causes include abnormal β-cell secretory
product such as an abnormal insulin molecule and incomplete conversion of pro-
insulin to insulin. Target tissue defects such as insulin receptor defects and post-
receptor defects may also cause insulin resistance. O’Rahilly et al (1991) have
described rare specific genetic mutations in the insulin receptor. The same authors
have also described mutations in human PPAR gamma (peroxisome proliferator-
activated receptor gamma) in association with severe insulin resistance, early onset
type 2 diabetes and hypertension (Barroso et al 1999).

Intracellular processing of the signal provided by insulin has also been examined.
Insulin receptor substrate (IRS)-2, the protein kinase B (PKB)-beta isoform and the
forkhead transcription factor Foxo1a (FKHR) are of interest as recent data have suggested that dysfunction of these proteins results in insulin resistance in-vivo (Previs at al 2000; Brozinick et al 2003; Hirota et al 2003).

Raised body mass index (BMI) as measured by weight/height$^2$ is a strong risk factor for Type 2 diabetes. Insulin resistance has been widely described in obesity; there is a rightward shift in dose response curve for insulin-stimulated glucose disposal.

There is a continuum of insulin resistance in obesity. Over a 10 year period the risk of developing diabetes is 40-80 fold higher if BMI is $>40$ kg/m$^2$ than if BMI is $<21$ kg/m$^2$ (Chan 1994). However when BMI is $>27$ further increases in total fat mass adds little to insulin resistance and the distribution of fat assumes greater importance.

Those with central adiposity are more insulin resistant than those with peripheral obesity, independent of the degree of obesity. Factors such as non-esterified fatty acids, TNF-α, IL-6, resistin and omentin (Yang et al 2006) secreted from this central fat tissue have been proposed to induce insulin resistance. Visceral fat is thought to be subject to more lipolysis with the release of these factors to the liver where they are postulated to impair insulin receptor function.

In recent years the hypothesis that the metabolic syndrome can be explained as “Cushing’s of the omentum” has been debated. Cortisol is generated from 11-keto cortisone in adipose tissue by 11β-hydroxysteroid dehydrogenase type 1 (11β HSD-1). Studies in mice in which 11βHSD-1 is over-expressed in adipose tissue show elevated intra-adipose and portal vein glucocorticoid levels in such animals, and also a tendency to obesity, hyperglycaemia, dyslipidaemia and hypertension. Conversely,
knockout mice deficient in 11\(\beta\) HSD-1 have lower levels of glucocorticoid in tissues where 11\(\beta\)HSD-1 is expressed, are resistant to dietary obesity, and are protected from dyslipidaemia and hyperglycaemia. In diet-induced obese mice inhibition of 11\(\beta\) HSD-1 reduces food intake and weight gain but maintains energy expenditure (Wang et al 2006).

Peterson et al (2004) looked at healthy lean insulin resistant offspring of patients with Type 2 diabetes. They found that the insulin-stimulated rate of glucose uptake by muscle was 60% lower in the insulin-resistant subjects than the insulin-sensitive controls and was associated with an 80% increase in intramyocellular lipid content, and a 30% reduction in mitochondrial phosphorylation. They postulated an association of the insulin resistance with dysregulated intramyocellular fatty acid metabolism, possibly due to an inherited defect in mitochondrial oxidative phosphorylation.

The effect of diet on peripheral insulin resistance has been studied: phospholipids can be altered by the fatty acid composition of the diet, and this can affect the rigidity of the plasma membrane of all the cells of the body (Field et al 1988; Tong et al 1995). It is possible that a change in the fluidity of the plasma membrane may affect the ability of the insulin receptors to aggregate and therefore affect glucose uptake into the cell.

**Metabolic syndrome**

Insulin resistance, as well as being a fundamental aspect of the aetiology of Type 2 diabetes is also closely linked to hypertension, hypertriglyceridaemia, low levels of HDL, all components (along with abdominal obesity) of Syndrome X or the metabolic
syndrome. Other components of the metabolic syndrome include polycystic ovarian syndrome, endothelial dysfunction, atherosclerosis, microalbuminuria, uricaemia, hypercoagulability and raised markers of inflammation. This cluster of abnormalities reflects the diverse actions of insulin.

Weiss et al (2004) found that the prevalence of the metabolic syndrome is high among obese children and adolescents, reaching 50% in severely obese youngsters.

Nuclear receptor modulation is thought by some to be central to the pathogenesis of the metabolic syndrome (Gurnell et al 2003 review). The retinoid X receptor (RXR) is a member of the nuclear-receptor superfamily which forms functional complexes with other nuclear-receptors. These heterodimers bind a variety of ligands derived from cholesterol, fatty acids, and fat-soluble vitamins. Animals treated with RXR agonists such as rexinoids have been shown to exhibit marked changes in cholesterol balance, including inhibition of cholesterol absorption (Repa et al 2000).

The metabolic syndrome is defined by the WHO, the International Diabetes Federation (IDF) and also by the National Cholesterol Education Program (NCEP). The latter requires at least 3 of the following to be present: abdominal obesity, hypertriglyceridaemia, low HDL cholesterol levels, hypertension and elevated fasting glucose (Diabetes and Cardiovascular Disease Review ATP-III). However the American Diabetes Association and the European Association for the Study of Diabetes (EASD) have concluded that at present there is a lack of certainty regarding the pathogenesis of the metabolic syndrome and that at present it does not warrant the title "syndrome" (Kahn et al 2005).
1.2 Atherosclerosis

1.2.1 Pathogenesis

1.2.2 Risk factors for atherosclerosis

1.2.3 Diabetes and atherosclerosis

1.2.4 Diabetic dyslipidaemia and atherosclerosis

1.2.5 Diabetic postprandial lipoproteins and plaque formation

1.2.1 Pathogenesis

Atherosclerosis is a disease of large and middle sized arteries where lesions develop causing coronary artery disease, cerebro-vascular and peripheral vascular disease. The lesions occur as a response to injury and predominate at vulnerable sites such as artery bifurcation points where blood flow turbulence is maximal (Stary et al 1992). They are graded by their histology and a temporal sequence has been found on examination of vessels of different ages in both animal models and human autopsies. Type 1 lesions are not grossly visible; they can be found in children as well as adults and comprise areas of increased numbers of intimal macrophages filled with lipid droplets called foam cells (Stary 1994). Type 2 lesions are visible fatty streaks and can also be found in children. They are characterized by layers of foam cells and layers of lipid droplets within intimal smooth muscle cells as well as droplets of extra cellular lipid. Type 3 lesions are generally only found in adults and are characterized by pools of
extra cellular lipid as well as the features of Type 2 lesions. Advanced lesions-Type 4 or “atheroma”, are thought to progress from these and are characterized by larger amounts of extra cellular lipid. Thick layers of fibrous connective tissue characterize Type 5 lesions, and in some cases are highly calcified. Large lesions can impair blood flow and cause ischaemic symptoms. When lesions become unstable and fissure, thrombus formation supervenes (Type 6), causing acute obstruction at the site or downstream if part of the thrombus sloughs off. In clinical practice those lesions found on arteriography to be causing most stenosis are not necessarily those lesions likely to progress to Type 6 lesions.

The pathogenesis of atherosclerosis lies in abnormalities in endothelial and smooth muscle cell function as well as a prothrombotic milieu. The abnormalities are thought to be the chronic and excessive inflammatory response to injury (reviews Ross 1999). The finding that the inflammatory marker C reactive peptide (CRP) is a marker for atherosclerotic disease would support this hypothesis.

Endothelial cells synthesize nitric oxide (NO) which causes vasodilatation in the subjacent vascular smooth muscle cells by activating guanylyl cyclase. This maintains adequate blood flow and nutrition to tissues distally. NO is synthesized by endothelial NO synthase (e NOS) and is degraded by oxygen-derived free radicals. Loss of endothelium derived NO permits an increase in the activity of nuclear factor kappa B (NF-kB), which is a pro-inflammatory transcription factor (Zeiher et al 1995). This leads to expression of leukocyte adhesion molecules and release of cytokines and chemokines. Procoagulant changes on the endothelial surface promote adhesion of macrophages and platelets.
Platelet aggregation results in release of proliferative agents such as epidermal growth factor, platelet-derived growth factor and tumour necrosis factor, all of which enhance smooth muscle cell proliferation. Smooth muscle cells accumulate lipid and stimulate production of extracellular support matrix. Monocytes and lymphocytes migrate to the sub endothelium by the action of chemotactic factors such as transforming growth factor-β and various interleukins, and the monocytes transform to foam cells. The cells change receptor expression from LDL receptors to scavenger receptors which preferentially accumulate oxidized LDL.

1.2.2 Risk factors

Epidemiological studies have identified smoking, hypertension, diabetes, hyperlipidaemia, obesity, the metabolic syndrome, genetic factors, diet, exercise and hormonal changes as chief underlying causes of this sequence of events.

The Framingham Study provided prospective evidence of the effect of smoking on development of atherosclerotic disease (Sparrow et al 1978). The mechanism of action of smoking continues to be investigated. Animal studies indicate that the intimal thickening with which smoking is associated may be due to increased expression of inducible nitric oxide synthase or iNOS which counteracts the effect of eNOS (Anazawa et al 2004).

The relationship between blood pressure and cardiovascular disease mortality is strong and predictive. A meta-analysis of observational studies involving 1 million individuals with no history of cardiovascular disease demonstrated that death from coronary heart disease and stroke increases linearly from BP levels of 115 mm Hg
systolic and 75 mm Hg diastolic (Lewington et al 2002). Among diabetic patients the UKPDS (no.72-2005) has calculated that the incremental cost per quality-adjusted life years gained for blood pressure control was £369 while that for intensive blood glucose control was £6,028.

Obesity has been recognized as an independent cardiovascular risk factor (Barrett-Conner 1985, review Sowers 2003) and predisposes to Type 2 Diabetes. Lifestyle changes such as physical inactivity, high fat and energy diets leading to obesity have been closely linked to increases in the appearance of Type 2 Diabetes.

A review of genetic markers by Gibbons et al (2004) outlines recent progress in identifying high risk genotypes aided by The Human Genome Project. The rare familial hyperlipidaemias are well described in the literature and are associated with premature atherosclerosis. More recently common polymorphisms of various genes have been examined as risk factors for atherosclerosis. These have included variants of the Cholesterol Ester Transfer Protein gene (Kuivenhoven et al 1998) and the Microsomal triglyceride transfer protein (MTP) which I will discuss in later chapters.

Prospective epidemiological studies have shown a causal relationship between incidence of coronary artery disease and levels of physical activity. Subjects who are most physically active have coronary artery disease rates 50% of those with sedentary lifestyles (review Thompson et al 2003). Moderate-intensity aerobic exercise has been shown to augment endothelium-dependent vasodilatation in man through increased production of nitric oxide (Goto et al 2003).
Pre-menopausal women have reduced atherosclerotic risk compared to age matched men, but the risk after the menopause increases to that of age-matched men. In diabetes this gender protection is lost. The benefits of postmenopausal oestrogen hormone replacement was extolled after observational studies indicated protection from atherosclerosis (Grady et al 1992). This view has now been reversed after publication of prospective studies, most notably the HERS study, showing the opposite effect (Hulley et al 1998). However the debate continues as a recent study has shown an association between hormone replacement therapy as combined oestrogen plus progestin and a lower incidence of diabetes (Margolis et al 2004).

Other hormonal states are associated with atherosclerosis, including hypercortisolaemic states, acromegaly and phaeochromocytoma. The effect is likely due in part to increased insulin resistance and hypertension.

Epidemiological studies in nondiabetic populations have found that dyslipidaemia is a strong and independent risk factor for cardiovascular disease. Low density lipoproteins, especially those that are small and dense, have been found to be pro-atherogenic while HDL has been found to be anti atherogenic. Hypertriglyceridaemia has been found to be an independent risk factor for atherosclerosis.
1.2.3 Diabetes and Atherosclerosis

Diabetes is associated with up to a 5 fold increase in atherosclerosis (reviews: Fisher 2004; Creager 2003; Luscher 2003). It is an independent cardiovascular risk factor and has been found to be comparable to the risk in non-diabetic subjects who have already had a myocardial infarct or stroke (Haffner et al 1998). The prognosis in those diabetic subjects who have had a cardiovascular event is worse than in those who have also had an event with normoglycaemia (Jacoby et al 1992).

Diabetes and insulin resistant states are closely associated with the other classical atherosclerotic risk factors. Hypertension is found in up to 50% of those with type 2 diabetes (Turner et al 1998). The reason for this association is not known. The hypertriglycerideremia and easily oxidisable small dense LDL found in insulin resistance may cause vasoconstriction by affecting nitrous oxide production by the endothelium.

In diabetes atherosclerotic plaques are found not just discretely at the points of stress such as the bifurcations of arteries, but also distally and confluenlly. The first changes of this process can be seen in the intimal thickening found by high resolution Doppler scanning of the carotids, and has been found in young adult diabetics(Yamasaki et al 2000). Studies have shown the intima in diabetes to have increased permeability, cell adhesiveness and impaired relaxation and fibrinolysis. The medial layers have been found in diabetes to undergo premature hyaline change and calcification and have impaired compliance.
The mechanisms by which hyperglycemia may have these effects have not been fully elucidated. One proposal is that advanced glycation end-products (AGEs) are formed in hyperglycaemic states which attach to a receptor (RAGE) and cause abnormal cross-linking of vessel-wall proteins. This causes a disturbance in the permeability of the vasculature, release of reactive oxygen species and increased expression of cell vascular cell adhesion molecules (VCAM1) (Chappey et al 1997).

Hyperglycaemia appears to induce a series of reactions which reduce NO production (review Creager et al 2003), and also increase reactive oxygen species which inactivate NO. Intra arterial infusions of the anti-oxidant ascorbic acid in diabetics restore endothelium dependent vasodilation (Timimi et al 1998).

The question of whether insulin is responsible for some of the atherosclerotic burden in diabetes has been debated. Insulin has potentially positive and negative effects on the vasculature. For example it induces vasodilatory endothelial nitric oxide via activation of Akt kinase and phosphatidylinositol-3 kinase. (Baron et al 1996) but in vitro, stimulates vascular smooth muscle proliferation (Jung et al 2000), enhances hepatic production of procoagulant plasminogen activator inhibitor PAI-1(Kooistra et al 1989), and retains sodium and water at the renal tubule.

In diabetes there is both a propensity for platelet activation and aggregation, and also a tendency for coagulation. Pro coagulant fibrinogen, thrombin, factor VII, and Von Willebrand factors are raised in diabetes while anti-fibrinolytic protein PAI-1 is also raised. Insulin and triglycerides, and TNF-α have been shown in vitro studies to enhance PAI-1 production in the liver, endothelium and adipocytes (Schneider et al 1996, Gottschling-Zeller et al 2000). Diabetes is associated with decreased levels of
anticoagulants protein C and thrombomodulin. This has the potential to increase the risk of thrombosis following plaque rupture.

Endothelial vasoconstrictors prostanoids and endothelin levels are increased in diabetes. Endothelin promotes inflammation and causes vascular smooth muscle growth and contraction. Insulin administration increases plasma endothelin-1 in healthy subjects and diabetics. Diabetes increases migration of vascular smooth muscle cells into atherosclerotic lesions where they replicate and produce extra cellular matrix. In diabetes these muscle cells also undergo apoptosis at an increased rate and matrix metallo-proteinases in the plaque are increased, both of which increase the chance of plaque rupture (review Moreno 2004).

1.2.4 Postprandial lipoproteins in diabetes and plaque formation

The metabolic state in the postprandial period is particularly disturbed in diabetes (Sniderman et al 2001; Tomkin et al 2001). Postprandial hypertriglyceridaemia is marked in diabetes. The postprandial lipoproteins are very low density lipoproteins (VLDL) and chylomicrons. The turnover of these lipoproteins is much faster than LDL, so that in fasting blood samples, their levels may disguise the true impact that they have on the vasculature in the postprandial period. There is evidence that in diabetes the time spent in the postprandial phase is prolonged as clearance of postprandial particles is impaired (Phillips et al 2002) (Howard et al 1987). Most of the epidemiological studies look at fasting lipid samples and may therefore, in diabetes, underestimate the effect of the postprandial lipidaemia on the vasculature. Karpe et al have demonstrated an association between apoB48 (the lipoprotein intrinsic to each chylomicron particle) and carotid artery atherosclerosis progression.
in non-diabetic subjects (1994). Mero et al (2000) have found an association between postprandial apoB48 levels in the chylomicron fraction and the severity of coronary artery stenosis found at angiography in diabetic subjects. Receptors for apoB48 have been found on the macrophage surface which may be one mechanism for entry of chylomicrons into the atherosclerotic plaque (Brown et al 2000). Animal models of diabetes have shown that arterial smooth muscle and fibroblasts take up postprandial remnant particles (which comprise apoB48 and 100 containing particles) with greater affinity than LDL (Proctor et al 2000). The same authors also demonstrated efficient retention of fluorescent labeled chylomicron (apoB48) remnants within the intima of the arterial wall (Proctor 1998).
1.3 Lipoprotein Metabolism

1.3.1 Introduction
1.3.2 Fatty acids
1.3.3 Cholesterol
1.3.4 Triglycerides
1.3.5 Phospholipid
1.3.6 Apoproteins A
1.3.7 Apoprotein C
1.3.8 Apoprotein E
1.3.9 Apoprotein B100
1.3.10 Apoprotein B48
1.3.11 Chylomicron
1.3.12 Very low density lipoprotein
1.3.13 Intermediate density lipoprotein
1.3.14 Low density lipoprotein
1.3.15 Lipoprotein (a)
1.3.16 High density lipoprotein

1.3.1 Introduction

Triglycerides and cholesterol are hydrophobic entities. In order for their transport in the aqueous milieu they are arranged in a hydrophobic core surrounded by a hydrophilic surface of phospholipids. Phospholipids have a hydrophobic fatty acid tail which points inwards and a phosphate hydrophilic head which prevents emulsification in the body's media. The resultant particles also contain protein molecules or apolipoproteins which serve to improve solubility and act as receptor ligands and as enzyme cofactors.

These lipoproteins can be classified according to their size and density and the particular apoproteins (or apolipoproteins) which they carry. The lipoproteins are not fixed in their composition and a dynamic process exists whereby apoproteins and core
lipids are exchanged between particles. There are 5 major classifications – chylomicron (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Chylomicrons are involved in the exogenous pathway of lipid transport from the intestine to the liver and peripheral cells, while the other 4 lipoproteins are involved in the endogenous pathway which transports endogenously synthesized lipids.

1.3.2 Fatty acids

Fatty acids consist of chains of carbon atoms (hydrophobic) and a carboxylic acid (hydrophilic) which forms esters and amides. They are constituents of triglycerides, cholesterol esters and phospholipids. They also exist free in the circulation, bound to albumin and other proteins.

Those fatty acids that are not made by the body and need to be consumed in the diet are termed “essential fatty acids”. These polyunsaturated fatty acids are termed Omega -3 and 6 essential fatty acids. They include linoleic acid (Omega-6), and linolenic acid.

Fatty acids are classified according to the number of double bonds between the carbons and include monounsaturated (1), polyunsaturated (>1) and saturated fatty acids (no double bonds). The type of double bond (cis or trans) depends on the position of attached hydrogen atoms.

Free fatty acids are elevated in insulin resistant states including diabetes. These acids activate Protein kinase C (PKC) which have a myriad of effects including activation of membrane-associated NAD (P) H dependent oxidases which produce super oxide anions. This enhances the ability of LDL to be oxidized and taken up by scavenger receptors in the atherosclerotic plaque (review Rask Madsen et al 2005).
High-fat oral or iv feeding which raise circulating free fatty acid levels has been shown to be associated with a decreased insulin-stimulated glucose uptake through impairing insulin activation of intracellular signaling in skeletal muscle (review McGarry 2002). Increased intracellular fatty acids downregulates the insulin driven Glut 4 mediated glucose uptake into cell, causing insulin resistance.

\[ \text{Serine/threonine phosphorylation} \]
\[ \text{P13Kinase} \]
\[ \text{Fatty acid} \]
\[ \text{CoA} \]
\[ \text{Insulin R} \]

Boden G et al EurJ Clin Invest 2002; 32(3) Skeletal muscle cell

In the pancreatic β cell, NEFA are known to be insulinotropic and this protects against ketosis in the fasting state when the glucose is not available for the stimulation of insulin secretion. However when there is a large inflow of free fatty acids and an accumulation of triglycerides in beta cells these cells have been shown to deteriorate and apoptose (review Shafrir et al 2003).
1.3.3 Cholesterol

Cholesterol is a steroid, and exists either as free sterol, or as cholesterol ester where cholesterol is linked to a fatty acid. For example cholesterol stearate is cholesterol bonded to stearic acid. Sources include that synthesized in the liver and intestine and also that absorbed from the diet.

![Cholesterol structure diagram]

The rate limiting enzyme for cholesterol synthesis is HMG CoA-acetyl reductase and this is regulated by negative feedback from cholesterol itself. HMGCoA reductase inhibitors (statins) are used in clinical practice to lower plasma cholesterol levels in human subjects. The efficacy of statins to reduce major vascular events have been reiterated in a prospective meta-analysis of over 90,000 participants in 14 randomized trials (Baigent et al 2005).

Dietary cholesterol is acted on by digestive enzymes; micelles which act like lipoproteins are then formed with bile acids. Niemann-pick C1 like protein and adenosine triphosphate-binding cassette protein ABC G5 and G8 are involved in its transport across the enterocyte. ABC-G5 and ABC-G8 regulate, in the liver, excretion...
into the bile, and in the intestine, efflux of cholesterol from the enterocyte into the intestinal lumen. Niemann-pick C 1 like 1 (NPC1LI) was found in jejunal enterocytes (Davis et al 2004) through studies designed to discover the mechanism of action of ezetimibe, a compound that has recently been shown to dramatically reduce intestinal absorption of cholesterol at the enterocyte brush border (Sudhop et al 2002).

Intestinal transport of cholesterol

Recently the transport of intestinal cholesterol has been shown in cell and animal models to involve apoB-independent pathways as well as apoB-dependent pathways (review Hussein 2005). Although both pathways transport free cholesterol, cholesterol esters are secreted only via the apoB-dependent pathway. ApoB-independent cholesterol transport involves adenosine triphosphate-binding cassette protein A1
(ABCA1) with apoA I also playing a role in its transport with HDL. If this pathway is deficient, for example in an ApoA I deficient state, the apoB-dependent pathways has been shown to compensate for this deficiency. Conversely increased levels of ABCA1 mRNA levels have been detected in ACAT2−/− mice which are incapable of esterifying free cholesterol. Temel et al (2005) measured cholesterol absorption in mice deficient in both ABCA1 and ACAT2. Compared with wild-type and single knock out mice, these double knock out mice displayed the lowest level of cholesterol absorption. They conclude that ABCA1, especially in the absence of ACAT2, can have a significant effect on cholesterol absorption. This apoB independent pathway may provide therapeutic targets in the future.

Cholesterol plays a role in cell membranes where it provides rigidity via its ringed structure. It is also a precursor for steroid hormones, vitamin D and bile salts. Serum plant sterols reflect cholesterol absorption and have been used to assess cholesterol absorption in diabetes, while cholesterol precursors have been used to assess endogenous cholesterol synthesis.

Animal models of diabetes have shown that intestinal synthesis but not absorption of cholesterol is increased (Gleeson et al 2000; Feingold et al 1994). Similar results have been shown in humans with Type 2 diabetes (Gylling et al 1997; Simonen 2002; Sutherland 1992). The mechanism appears to involve disruption of the action of ABC-G5 and ABC-G8. However in Type 1 diabetes Gylling et al (2004) has suggested that high absorption and low synthesis characterizes Type 1 diabetes. These findings could be related to low expression of ABC G5 and G8 genes. There is some emerging
evidence of the efficacy of ezetimibe in the setting of Type 2 diabetes (Gaudiani et al 2005, Stroup et al 2003).

**Reverse cholesterol transport**

This is the process whereby cholesterol is transported from peripheral tissues to the liver and excreted in bile. This is an anti-atherogenic process and pivotal to this is HDL transport of cholesterol. Low levels of HDL are an independent risk factor for atherosclerosis. Below is a pictorial summary of the process:

Key: SRA=scavenger receptor A; SRBl=scavenger receptor B1; ce=cholesterol ester; fc=free cholesterol; mØ=macrophage; ABCA 1= ATP-binding cassette transporter A1; CETP=cholesterol ester transport protein; LCAT=
Initial lipidation of apoA-I by hepatic ATP-binding cassette transporter AI (ABCA1) with phospholipid and free cholesterol is an important first step (Van Eck 2005). LCAT esterifies free cholesterol from various sources, aided by, among other factors, apo AI, A IV and C I.

Cholesterol is more efficiently cleared to faeces via bile in mice over expressing hepatic scavenger receptor class B type I (SR-BI), whereas the opposite is true in knockout mice (van der Velde et al 2005). Efflux of cholesterol from macrophages also occurs and, although representing only a small fraction of overall cellular cholesterol efflux, it is important with regard to atherosclerosis (review Lewis et al 2005).

Inhibition of cholesterol ester transfer protein (CETP) in the hypercholesterolemic rabbit increased HDL cholesterol by >50% but this effect is anti-atherosclerotic only in moderately severe hyperlipidaemia but not in severe hypertriglyceridemia. It may be that the anti-atherosclerotic effect varies with the metabolic milieu or the degree of CETP inhibition (review Forrester 2005). In humans, mutations resulting in CETP inhibition have been associated with both reduced and increased risk of atherosclerosis (Perez-Mendez O 2000; Brewer 2004).
1.3.4 Triglycerides

Triglycerides (or triacylglycerols) consist of 3 fatty acids linked to 1 glycerol via ester bonds.

\[
\begin{align*}
\text{H} & \quad \text{O} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} \\
\text{H} & \quad \text{C} & \quad \text{O} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad (\text{CH}_2)_n & \quad \text{C} & \quad \text{H} \\
\text{H} & \quad \text{C} & \quad \text{OCO} & \quad (\text{CH}_2)_n & \quad \text{CH}_3 \\
\text{H} & \quad \text{C} & \quad \text{OCO} & \quad (\text{CH}_2)_n & \quad \text{CH}_3 \\
\text{H}
\end{align*}
\]

Triglycerides are synthesized in the intestine and liver and are stored in adipose tissue. Dietary triglycerides are initially broken into its constitutive glycerol and fatty acids before being reesterified within the enterocyte and packaged into the chylomicron.

Triglycerides are classically elevated in Type 2 diabetes and insulin resistant states. They are mostly found in the intestinally derived chylomicron and the hepatically derived VLDL. Hypertriglyceridaemia has been found to be a predictor of coronary artery disease morbidity and mortality (Laakso et al 1993; Hanefeld et al 1996).
1.3.5 Phospholipids

Phospholipids consist of 1 glycerol attached to 2 fatty acids and a phosphate, which is itself attached to a hydrophilic (charged) group with differing chemical properties.

\[
\begin{align*}
&\text{H} \\
&\text{H—C — phosphate group} \\
&\text{H—C — fatty acid} \\
&\text{H—C — fatty acid} \\
&\text{H}
\end{align*}
\]

The plasma phospholipids are derived endogenously only. Dietary phospholipids are not absorbed but are hydrolyzed by phospholipases in the intestine. Phospholipid transfer protein is important in HDL metabolism and higher levels and activity have been demonstrated in diabetes (Tu et al 2001).

Apoproteins

Apolipoproteins or apoproteins are found in the lipid particles where they help to solubilize the hydrophobic core of cholesterol, cholesterol esters and fatty acids. They also serve as ligands for binding to sites in the periphery for uptake and transfer between particles.

1.3.5 ApoA –I

Apo A1 is one of the most abundant proteins in human plasma with a concentration in normal plasma of ~1 g/ml. Ninety two per cent is bound to HDL and chylomicrons. It
is secreted by the small intestine and the liver as pro-ApoA-1. A hexapeptide is then cleaved by a circulating peptidase. It has a molecular weight of 28,339 Da. Free ApoA1 has been shown to be superior to plasma HDL in reverse cholesterol transport from macrophages (Yancey et al 1995). Apo A1 is a ligand for scavenger receptor B1.

The gene for ApoA 1 is part of a gene cluster with apo CIII and apoA IV (Naganawa et al 1997). Lipoprotein lipase rapidly transfers ApoA 1 from chylomicrons to HDL. ApoA-1 activates LCAT which esterifies cholesterol in reverse cholesterol transport. A low level of apoA 1 is an independent risk factor for CAD (Francis et al 2001). Fibrates have the effect of increasing ApoA 1 and ApoA 11 expression. Navab et al (2004) have reviewed studies looking at the use of Apo A1 and apoA 1 mimetic peptides in the treatment of atherosclerosis. They concluded that the quality of HDL may be more important in cardio-protection than HDL-cholesterol levels and that these peptides may have therapeutic potential.

In work with mice expressing human apoA-I transgene against the Abca1 knockout background Lee et al (2005) noted that the hypercatabolism of normal HDL particles in knockout mice is attributable to a selective catabolism of HDL apoA-I by the kidney.

**ApoA-II**

Relatively little is known about this lipoprotein. It exists as a 17,400 Da homodimer in humans. It is present on HDL (Reichl et al 1989) and has been shown to be pro-atherogenic in transgenic mice (Schultz 1993) by inhibiting reverse cholesterol transport. It modulates the activity of LCAT by decreasing its binding to HDL (Durbin et al 1997) and also stimulates hepatic lipase activity (Mowri et al 1996). The effects of HDL oxidation (review Ferretti et al (2006)) include the formation of
heterodimers of ApoAI and ApoA II, which may play a role in the pathogenesis of atherosclerosis.

**ApoA-IV**

This is a 46,000Da protein produced in the intestine and is found on chylomicrons and HDL. The gene is found clustered with the genes for apo AI and CIII. It activates LCAT (Steinmetz et al 1985) and mediates cholesterol efflux from adipocytes in vitro (Steinmetz 1990) and may influence LPL activity (Goldberg et al 1990). More recent studies have demonstrated that human apoA-IV exerts an inhibitory effect against Cu(2+)-induced lipid peroxidation of VLDL (Ferretti et al 2002). Linkage and association of the apoAI-CIII-AIV gene region to familial combined hyperlipidaemia has been reported but is complex in its contribution, conferring both susceptibility and protection (Groenendijk et al 2001).

Apo A-IV/- mice have been used to demonstrate that Apo A IV is involved in the activation of the vagal afferent pathway which inhibits gastric emptying and acid secretion in response to dietary lipid (Whited 2005).

In the intestine Apo A-IV is thought to facilitate intestinal lipid secretion by aiding the expansion of nascent CM by means of maintaining the elasticity of the particle (review Hussain 2001).

**ApoA-V**

Recently, a new gene coding for apolipoprotein A-V was identified in the vicinity of Apo AI-CIII-AIV cluster on human chromosome 11q23. Studies in knock-out and transgenic mice revealed that its expression pattern correlates negatively with
triglyceride levels. Mice injected with endotoxin were found to have elevated levels of apoA IV and V in their serum (Khovidhunkit et al 2004). Recently a family with inherited apolipoprotein A-V deficiency and severe hypertriglyceridaemia has been described (Oliva et al 2005).

1.3.7 Apo C
There are 4 subclasses of apo C. Genes encoding Cl, CII, and CIV are located on a 48 kb gene cluster on chromosome 19 (alongside apoE). CIII is located on chromosome 11 in a gene cluster with apo Al and ApoA IV as described above. They are secreted by the liver and in the intestine to a lesser extent. In the fasting state, the apo Cs are mainly associated with HDL. Postprandially they redistribute to chylomicrons and VLDL (Bjorkegren 1997, 1998). Each of the apo Cs has been shown to inhibit apoE-mediated binding to the LDL receptor, the LRP and LSR.

Apolipoprotein C-I
Apolipoprotein C-I has been shown to activate LCAT responsible for cholesterol esterification in reverse cholesterol transport. However it also inhibits VLDL clearance. Apo Cl transgenic mice have been found to have elevated levels of free fatty acids but are not insulin resistant. In the ob/ob mice, over expression of apoC I improved insulin sensitivity. Cross breeding the apoC I transgenic mouse with ob/ob mouse actually protects from obesity (Jong et al 2001). Bjorkegren et al (2000) showed that human subjects with coronary artery disease but with normolipaemia have postprandial VLDL which is apolipoprotein C I and cholesterol rich.

Apolipoprotein C-II
Apolipoprotein C-II is a cofactor for LPL, and agents which increase these levels may have therapeutic value in the future. Apolipoprotein CII deficiency is rare but has
recently been described in an infant with massive chylomicronaemia and severe encephalopathy due to marked lipid deposition in the brain (Wilson et al 2003).

**Apo C-III**

Apo C-III is an important apoprotein which inhibits delipidation by LPL and uptake of chylomicrons and VLDL by the liver. Over expression of apo CIII in mice increases triglyceride levels by decreasing clearance of VLDL triglycerides (Aalto-Setala et al 1992) and apoB48 containing lipoproteins (de Silva et al 1994). This effect is counteracted by crossbreeding with transgenic apoE mice. Apo C III knock out mice have increased VLDL turnover due to increased LPL hydrolysis (Jong 2001).

Fibric derivatives-the “fibrates” are used to treat hypertriglyceridaemia. These act primarily on the liver by modifying gene expression for LPL and apo C-III. The transcription factors common to their expression are called the peroxisome proliferator-activated receptor or PPARs (α, β, δ, γ). The α-form plays a role in β-oxidation of fatty acids, and regulation of expression of LPL, apo C-III, apo A-I and apoA-II genes. It also inhibits cyclo-oxygenase 2 (COX 2) and therefore may also play a role in the inflammatory response. Ligands that act on PPAR –α also include fatty acids as well as the fibrates. The effect is an increase in LPL expression and reduction in apo C-III gene expression (Auwerx et al 1996; Haubenwallner et al 1995). Insulin acts at a negative insulin response element in the gene promoter for apo C-III (Dammerman et al 1993).

PPAR- γ is involved in adipocyte differentiation, glucose transporters, and uptake of oxidized LDL and in insulin sensitization. PPAR- γ agonists include the glitazones which have the effect of improving insulin sensitivity in insulin resistant states
(review Yki-Jarvinen 2004). PPAR α and -γ agonists have also been shown to affect inflammation, vascular function, and vascular remodeling (review Staels B et al 2005).

1.3.8 Apo E

This apoprotein is important in clearance of remnants of chylomicrons and VLDL (triglyceride rich lipoprotein particles exposed to lipoprotein lipase). It is found on chylomicrons, VLDL and HDL. In the postprandial state apoE is donated by HDL, and also secreted into the space of Disse in the liver to aid uptake of sequestered remnants. ApoE is also expressed by macrophages. ApoE binds to the LDL receptor, the LRP and LSR, and plasma lipases. In humans the APOE gene has 3 alleles affecting the binding domain- E-2, E-3, E-4. The E-2 protein has reduced affinity for the LDL receptor. This allows more efficient LDL clearance because of reduced competition of TRLs with LDL apoB100. E-2 allele is associated with lower levels of plasma cholesterol, LDL cholesterol and apoB levels and coronary artery disease.

In diabetic animals apoE on chylomicrons is decreased (Gleeson et al 1999). In diabetic subjects also the TRLs have reduced apoE/particle (Madigan et al 2000). Type III hyperlipidaemia is a genetic disorder of premature atherosclerosis and accumulation of remnant lipoproteins in the plasma. Receptor binding defects in apoE are the main causes of this disorder (Mahley et al 1999). However most individuals who are homozygous for the E2 allele do not have the disorder, therefore additional genetic or environmental interactions are also required to produce hyperlipidaemia.
ApoE E4 is associated with earlier onset of Alzheimer's disease. Cholesterol exits the brain through the blood-brain barrier via ApoE. Cholesterol has been shown to modulate the conversion of amyloid precursor protein to amyloid beta peptide, the protein which, if accumulates, is key to the development of Alzheimer's disease (review Reiss 2005).

1.3.9 Apolipoprotein B100

ApoB100 is an important structural apoprotein of VLDL, IDL, and LDL. It also acts as a ligand for receptor mediated endocytosis of LDL. Essentially all apoB is associated with lipoproteins and unlike other apoproteins, cannot exchange among lipoprotein particles.

There is 1 APOB gene on chromosome 2, size 43kb in man which is transcribed into a single mRNA of 15 kb. From here it is either translated into a single polypeptide of 4536 amino acids (apoB100) in the liver or post-transcriptional editing occurs in the intestine to produce a truncated product (apoB48). ApoB 48 is identical to the amino-terminal 48% of apoB100.

ApoB100 is required for VLDL production in the liver. It is synthesized in the rough endoplasmic reticulum. It translocates across the endoplasmic reticulum while being translated. Transfer of lipids to nascent apoB occurs during translation, a process aided by microsomal triglyceride transfer protein.

ApoB is synthesized in excess of its requirements for lipid transport, the excess (about 50%) being degraded within the cell before secretion. Degradation can occur in two ways. Firstly the ubiquitin-proteasome reaction occurs when the initial lipidation of apoB by MTP fails because of insufficient lipid levels. Inadequate lipid levels leads to incorrect folding of the protein and therefore susceptibility to conjugation by
ubiquitin. Secondly the LDL receptor can prevent secretion of dense apoB100-containing lipoproteins from the liver (Larsson et al 2004).

Apolipoprotein B100 consists of five structural domains: \( \beta a_1, \beta_1, \alpha_2, \beta_2, \alpha_3 \). It is the N-terminal \( \beta a_1 \) domain that is thought to interact with microsomal triglyceride transfer protein (MTP) at multiple sites during apoB-lipoprotein assembly (Hussain et al 2003 review). The LDL receptor binding and heparin binding sites are in the \( \beta_2 \) domain. The role of \( \alpha_2 \) and \( \alpha_3 \) are not yet known.

The atherogenicity of apoB containing LDLs is linked to their affinity for artery wall proteoglycans. The region of the apoB which binds to the LDL receptor has also been found to bind to proteoglycans (Boren et al 1998).

Mutations in the APOB gene occurring naturally have helped in delineating the role of apoB. Familial hypobetalipoproteinaemia is a condition in which mutations in the APOB gene cause a truncated molecule. Homozygote patients have hypocholesterolaemia, and low levels of apoB. Clinical manifestations include neuromuscular disorders, retinitis pigmentosa, and deficiencies of fat soluble vitamins. Fatty liver is also reported as VLDL assembly is impaired and lipids accumulate in hepatocytes. The features are similar to abetalipoproteinaemia which is due to a mutation in the MTP gene. Heterozygotes for familial hypobetalipoproteinaemia have low levels of cholesterol and apoB but are usually asymptomatic.

Familial defective Apolipoprotein B100 is a relatively common disorder caused by a mutation in apoB100 that impairs its ability to bind to the LDL receptor. A single mutation accounts for almost all cases to date unlike in familial hypercholesterolaemia (Innerarity et al 1990). Clearance of LDL is reduced by about
50%. Phenotypically the condition in the heterozygous form is indistinguishable from heterozygous familial hypercholesterolemia though the features may be less severe.

1.3.10 ApoB48

ApoB48 is an isoform of apoB100. The editing of apoB100 mRNA occurs post-transcriptionally in the intestine to result in a protein which is 48% the size of apoB100. It is a polypeptide of 2152 amino acids and does not contain the $\alpha_2$, $\beta_2$, $\alpha_1$ domain. ApoB48 is only synthesized in the intestine in man. Chylomicrons are taken up by the liver via LRP receptors and by endocytosis in the space of Disse after the action of hepatic lipase. ApoB48 has also been found recently to be a ligand for receptors on the surface of the monocyte/macrophage cells (Gianturco et al 1998) which may be important in atherosclerotic plaque development. Like apoB100, apoB48 also has a proteoglycan binding domain (Flood 2002).

1.3.11 Chylomicrons

The chylomicron particles are triglyceride rich lipoproteins, synthesized within cells of the jejunum. The name is derived from the Greek word “chyle” meaning milk, which was noted to be the colour of the lymph draining from the intestine. The sizes of the particles are variable depending on time from a meal, with larger less dense particles found in the postprandial state and smaller particles in the fasting state. They are the largest lipoprotein particles with a diameter range of 75-450 nm. In the fasting state there is some overlap in size with VLDL. The density of chylomicrons are less
than 0.95 g/ml and the Svägberg flotation rate (Sf) varies from >400-1000. These particles are triglyceride rich (85-92%), the rest of the particles made up of cholesterol (1-3%), proteins (1-2%) and phospholipids (6-12%). There is 1 apoB48 per chylomicron particle and it is the apoB48 which defines it as a chylomicron. This 1:1 ratio allows chylomicron particles to be quantified as there are negligible amounts of apoB48 free in the circulation. Varying amounts of the other apoproteins are present and include AI, AIV, various Cs, and E (Chan 1992).

Assembly of chylomicron

The apoB gene is transcribed continuously. If there is insufficient lipid available then the translocation of apoB across the endoplasmic reticulum membrane is interrupted and this triggers degradation of the apoB protein (Davis et al 1990). Chylomicron assembly is thought to involve three independent steps: the assembly of primordial lipoproteins, the formation of lipid droplets, and core expansion.

Homozygotes for the condition hypo lipoproteinaemia in which there is a molecular defect in apoB suffer from severe fat malabsorption, resembling patients with defective MTP or a(1)lipoproteinaemia. MTP is involved in the lipidation process and there is evidence that the transcription of MTP is controlled by the sterol response element, HNF 1 and 4, dietary fats, ethanol, and the insulin response element. Once in the circulation, HDL donates apo Cs and apoE to the chylomicron and apoA 1 is lost to HDL.

Three different mechanisms have been shown to modulate the secretion of intestinal lipoproteins (review Hussain 2005). First TGF-β studies in caco-2 cells and hepG2 cell models suggest that the transcriptional regulation of the apob gene plays a modest role in controlling intestinal lipoprotein assembly and secretion. Second, recent
studies have shown that fat feeding induces apoA IV synthesis, and apoA IV in turn facilitates intestinal lipid secretion levels by helping in the expansion of nascent chylomicrons into large chylomicrons by maintaining the elasticity of these particles. Third, in diabetes and insulin resistance, enhanced lipid synthesis has been shown to augment intestinal lipoprotein secretion.

**Clearance of Chylomicrons**

Clearance of VLDL and CM bear some resemblances though this section will concentrate on chylomicron clearance. Chylomicrons are cleared after release of a proportion of the triglyceride from its core, leaving “remnants”. This occurs in two ways: first by hydrolysis of triglyceride by lipoprotein lipase (LPL) which is found in the circulation and attaches to the particle. LPL requires the cofactor apo CII, transferred from HDL, and once present, allows hydrolysis of up to 85% of the triglyceride core. LPL is also involved in the transfer of ApoA I from the chylomicron almost immediately to HDL after it enters the bloodstream. The importance of LPL is demonstrated in Type 1 hyper-lipoproteinaemia where there is a congenital defect in LPL. Patients accumulate high levels of chylomicrons and large VLDL but not remnants.

Secondly cholesterol ester transfer protein complex (CETP) transfers triglyceride to HDL in exchange for cholesterol (reverse cholesterol transport). The resultant cholesterol -enriched particle is called a remnant and is then taken up into hepatic cells mostly.

ApoE acts as a ligand for the LDL receptor, LDL related protein (LRP) receptor and the lipolysis-stimulated receptor (LSR) in the liver and other peripheral tissues. ApoE is donated by HDL in the postprandial state and it is also secreted by the hepatocytes
into the space of Disse where it incorporates into sequestered remnants to aid uptake. The importance of apoE in this process is appreciated through the development of apoE knock out mice which displayed a marked elevation of remnants (Mortimer et al 1995).

Sequestration into the space of Disse is thought to occur when the remnant reduces in size sufficiently to enter the space between the hepatocytes and the endothelium. The particles attach to one of three structures: heparin sulphate proteoglycans via apoE; hepatic lipase via apoB; or possibly lipoprotein lipase (LPL). During sequestration further triglyceride hydrolysis by LPL and hepatic lipase are thought to occur. The LSR, unlike the LDL receptor is not down regulated by cellular cholesterol. Mann et al (1997) propose from work with cultured rat hepatocytes that this receptor represents a physiological rate-limiting step in clearing intestinally derived remnants from the circulation.

Unlike apoB100, apoB48 is not a ligand for the LDL receptor without apoE (Mahley et al 1999). Clearance of VLDL and chylomicron remnants has been shown to be reduced in diabetes. Phillips et al (2002) treated 5 diabetic rabbits and 5 control rabbits with [14C] linoleic acid and [3H] cholesterol by lavage. The lymphatic duct was then cannulated and the lymph drained. Radiolabelled chylomicrons were then isolated by ultracentrifugation. Diabetic donors had lipid and apoE deficient chylomicrons. These isolates were injected into paired diabetic and control animals. Diabetic recipients cleared both control and diabetic chylomicron triglyceride more slowly than control recipients. Not only was the diabetic cholesterol slow to clear when injected into in the control recipient but also there was a delay of clearance of chylomicron particles (cholesterol and triglycerides) of normal recipients when injected into the diabetic animals. Therefore the reduced clearance seen in diabetes is
likely multifactorial. The fatty acid chains of triglyceride in chylomicron emulsions may affect clearance in vivo (Mortimer et al 1994). There is also increasing evidence that the chylomicrons and their remnants are atherogenic. In 1981 Floren et al showed that chylomicron remnants were able to gain access to human smooth muscle cells, following endothelial injury and contribute to cholesterol accumulation. Fluorescein-labelled chylomicron remnants have been used to demonstrate chylomicron remnant penetration of arterial tissue and chylomicron remnant cholesterol accumulation in the subendothelial space (Proctor et al 1998). Around the same time it was demonstrated that macrophages have a receptor which binds apoB48 (Gianturco et al 1998). This group went on to demonstrate this receptor on a human endothelial cell line (Bradley et al 1999).

Clinical evidence that intestinally derived particles may play an important role in atherosclerosis is also growing. Karpe et al (1994) demonstrated that a positive correlation between apoB48 in the remnant fraction in non-diabetic men which was unrelated to triglyceride levels. These investigators more recently (2001) demonstrated a strongly positive correlation between mean postprandial remnant lipoprotein cholesterol and common carotid intimal thickening in middle aged non-diabetic men.

1.3.12 Very low density lipoproteins

These lipoproteins are derived from the liver and are involved in the “endogenous lipid transport pathway”. VLDL is made up of a core of cholesterol, cholesterol ester, triglyceride and a surface of phospholipids. It also contains apoproteins AII, B100, CI, CII, CIII and E. Although there is an overlap with chylomicrons, they tend to be smaller and denser than these intestinally derived triglyceride rich lipoproteins. The
diameter of the particles range from 30-100 nm and the density is less than 1.006g/ml; the Sf is 20-400. The VLDL delivers fatty acids to the periphery for storage or as a fuel source. The particles may be altered in the circulation to become IDL and LDL, or be taken up again by the peripheral cells and the liver. As with the chylomicron, VLDL possesses only one apoB protein (apoB100), which aids in their quantification after ultracentrifugation.

The assembly of the VLDL particle is similar in some respects to the chylomicron particle. The APOB gene is continuously transcribed and the gene product is translocated across the endoplasmic reticulum where lipids are added. Hepatic MTP is involved in the lipidation of apoB100 and the more lipid available the larger the VLDL particle. If insufficient lipid is available however, translocation is interrupted and the apoB100 is degraded.

On entering the bloodstream apo AII is lost to HDL, while apo Cs and apoE are gained. As with chylomicrons, the apo CII is a cofactor for LPL while apo CIII inhibits LPL; both inhibit binding of lipoproteins to cell surface receptors. Chylomicrons and VLDL are thought to compete for binding to LPL which is found bound to the endothelium in the microvasculature. Because chylomicron triglyceride is cleared more quickly than VLDL triglyceride, it is postulated that there is a greater affinity of LPL for apoB48 containing lipoproteins (Xiang et al 1999).

ApoB100 in VLDL is a ligand for the LDL receptor (unlike apoB48) as well as LRP, and possibly the LSR receptor. In the liver they are taken up in a similar way to the chylomicron. LPL and HL hydrolyze the particles in the space of Disse, which gain apoE and eventually become internalized. However in the circulation the VLDL particles are gradually transformed into IDL and LDL as the triglyceride content is hydrolyzed, and apoE and C are lost. As this happens there is less scope for receptor
binding, so that LDL, containing only apoB100 has the capacity to bind only to the LDL receptor.

1.3.13 Intermediate density lipoproteins

These are considered to be remnants of VLDL hydrolysis. They have a diameter range of 25-30nm and density of 1.006-1.025 g/ml. They contain apoB100 (the original from VLDL), a higher concentration of cholesterol than VLDL, but less triglyceride and apoE, and no apo C. They may be cleared by the liver as IDL either by the LDL receptor via apoB, or by the action of apoE on LSR and LRP (Friedman et al 1990). Alternatively HL may convert IDL into LDL (Gibson et al 1988).

1.3.14 Low density lipoproteins

LDL is the end product of VLDL conversion in the circulation. It has a particle diameter range of 19-25 nm and a density of 1.025-1.063 g/ml. No apo E or apoA is found in the particle and low levels of apo C III may be found in some cases of hyperlipidaemic patients (Bury et al 1985). Apoprotein apo B100 alone however characterizes this particle (1 per particle), along with the high cholesterol concentration.

LDL is the major transporter of cholesterol in the circulation.

There are 3 classes of LDL: LDL I, II and III. LDL- I has a density of 1.025-1.034 g/ml and is the largest and most buoyant of the LDLs. It is thought to be the class with least atherogenic potential. LDL II has a density of 1.034-1.044 g/ml. LDL III or "small dense LDL" has a density of 1.044-1.063 g/ml and has the most atherogenic potential. (Austin et al 1988; Campos et al 1992; Griffin et al 1994). There is a strong
positive correlation between small dense LDL, high levels of plasma triglycerides and large triglyceride rich VLDL (Kondo et al 2001; Packard 2000).

1.3.15 Lipoprotein (a)
This is a modified LDL particle in which the large apoprotein (a) is attached to the apoB100 molecule. Apo (a) is believed to be derived from plasminogen. The association between Lipoprotein (a) and risk of ischaemic heart disease is controversial, after initial reports linking the two (Mbewu et al 1990) was refuted by a prospective study by Ridker et al (1993). More recently a prospective study linked increased levels of lipoprotein (a) and risk of coronary heart disease among women with Type 2 diabetes (Shai 2005).

1.3.16 High density lipoproteins
These particles are the smallest and densest of the lipoproteins (1.063-1.21 g/ml). The particles contain apoA, apoE and apo C. HDL is involved in the reverse transport system of cholesterol. It transfers cholesterol from the peripheral cells and circulation back to the liver via the SRB1 receptor, where it may be stored, released back into the circulation as VLDL or excreted via the biliary system. It also transfers cholesterol via cholesterol ester transfer protein (CETP) to apoB containing particles (mainly VLDL and LDL) where it can be taken up by receptor mediated endocytosis. It is a carrier and donor of apoE and apo C postprandially and a recipient of these once TRL lipolysis has occurred. Low levels of HDL have been shown to be a risk factor for coronary artery disease (Gordon et al 1989).

There are 3 classes of HDL: nascent or pre β HDL, HDL₂ and HDL₃.
Nascent HDL is produced by the liver and gut. Excess surface apoproteins released from chylomicrons and VLDL during the process of triglyceride hydrolysis also contribute to HDL synthesis. They function in cholesterol efflux, including from macrophages and the arterial wall. Cholesterol release from cells is mediated by cholesterol-efflux regulatory protein (CERP) which is the product of the (ATP)-binding transporter 1 (ABC A1) gene. Mutations in this gene cause Tangier disease and familial HDL deficiency. In these conditions HDL levels are low and premature coronary artery disease occurs.

Nascent HDL is converted to HDL₃ and then HDL₂ by the addition of free cholesterol which is then esterified. This is catalyzed by the action of lecithin cholesterol ester transferase (LCAT) which is itself activated by apoA-1 on the HDL particle.

Apart from reverse cholesterol transport, HDL also acts as an antioxidant. The enzyme paraoxonase is an enzyme found in the HDL particle tightly associated with apoA 1. This enzyme hydrolyzes oxidized phospholipids and thereby protects LDL from oxidation (Navab et al 1996). ApoA-II, also found in HDL is pro-atherogenic unlike apo Al. It inhibits reverse cholesterol transport, has no antioxidant function and may play a role in insulin resistance. The gene locus has been postulated as a candidate gene for diabetes.

Structural modifications of HDL have been discovered to occur by oxidation, glycation, homocysteinylation and enzymatic degradation. These modifications may play a role in the pathogenesis of atherosclerosis (review Ferreti et al 2006).
1.4 Disorders of lipid metabolism

1.4.1 Introduction
1.4.2 Familial hypercholesterolemia
1.4.3 Familial combined hyperlipidaemia
1.4.4 Type III Hyperlipoproteinaemia
1.4.5 Lipoprotein lipase deficiency
1.4.6 Apolipoprotein CII deficiency
1.4.7 Familial hypertriglyceridemia
1.4.8 Primary disorders of HDL metabolism
1.4.9 Hypolipoproteinaemia
1.4.10 Chylomicron retention disease
1.4.11 Secondary causes of hyperlipidaemia

1.4.1 Introduction

Plasma lipid levels vary among individuals of different populations depending on genetic and dietary factors. However hyperlipidaemia has been defined as the upper 5-10% of values from population studies. For western men the mean plasma cholesterol is 5.4 mmol/l. Cholesterol concentrations greater then 6.2 mmol/l or triglyceride concentrations greater than 2.3 mmol/l are defined as significant hyperlipidaemia (NECP 2001).

The hyperlipidaemias have been classified in various ways. Initially plasma electrophoresis was used as the basis for classification.
<table>
<thead>
<tr>
<th>Type</th>
<th>Predominant elevated lipoprotein</th>
<th>Predominant Elevated lipid</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chylomicrons</td>
<td>Triglycerides</td>
<td>LPL deficiency</td>
</tr>
<tr>
<td>IIa</td>
<td>LDL</td>
<td>Cholesterol</td>
<td>Familial hypercholesterolaemia</td>
</tr>
<tr>
<td>IIb</td>
<td>VLDL+ LDL</td>
<td>Triglycerides+ Cholesterol</td>
<td>Familial combined hyperlipidaemia</td>
</tr>
<tr>
<td>III</td>
<td>Remnants(βVLDL)</td>
<td>Triglycerides+ Cholesterol</td>
<td>Type III hyperlipoproteinaemia</td>
</tr>
<tr>
<td>IV</td>
<td>VLDL</td>
<td>Triglycerides</td>
<td>Familial hypertriglyceridaemia</td>
</tr>
<tr>
<td>V</td>
<td>Chylomicrons+VLDL</td>
<td>Triglycerides+ Cholesterol</td>
<td>Apo-CII deficiency</td>
</tr>
</tbody>
</table>

However this has been overtaken by other classifications, as firstly lipoprotein phenotype by ultracentrifugation may change in the same patient over time; and secondly, different electrophoretic patterns may occur in different family members with the same condition.

Genetic abnormalities are used where possible to define primary disorders of hyperlipidaemia, although in some inherited conditions, for example, familial combined hyperlipidaemia, the genetic cause is unclear and is likely due to polygenic inheritance.

In the following paragraphs I will briefly outline the more common primary disorders of hyperlipidaemia with special emphasis on postprandial changes in chylomicron and VLDL.
1.4.2 Familial hypercholesterolaemia (FH)

FH is a relatively common autosomal dominant disorder (heterozygotes 1 in 500) and is caused by a mutation in the LDL receptor gene. This leads to elevated plasma LDL and total cholesterol concentrations in plasma.

Heterozygotes have plasma cholesterol levels at 2-3 fold above normal. Premature coronary artery disease is common (average age 45 years in men; 55 years in women) and they often have tendon xanthomas, xanthelasma and arcus cornea. Homozygotes have a 3-6 fold increase in plasma cholesterol and usually die of coronary disease by age 20.

The lack of LDL receptors impairs clearance of LDL which binds by means of the apoB100, and also impairs clearance of IDL which binds by means of apoE. It is thought that scavenger receptors on macrophages in the artery wall take up the excess LDL by a mechanism which does not saturate.

Despite aggressive therapy with LDL apheresis and statin therapy outcomes are presently poor (Masaki et al 2005).

Postprandial studies in FH

Postprandial studies have been done on animal models of FH (WHHL rabbits) and human subjects (Watts review 2000). It has been postulated that postprandial dyslipidaemia may partly explain the heterogeneity of atherosclerosis risk found in patients with FH. However results have been conflicting. This may be partly due to differences in methodology, as some workers have used retinol labeling to measure chylomicron remnants. This technique has been shown to be suboptimal, as retinol is able to transfer to the other lipoproteins including LDL (Castro-Cabezas et al 1998). Weintraub et al (1987) and Eriksson et al (1991) have not shown reduced clearance of
chylomicrons in heterozygote FH patients. In vitro studies suggest that co-existent defects in the LRP receptor are required for postprandial accumulation of chylomicrons in this condition (Linton 1998).

1.4.3 Familial combined hyperlipidaemia

This is a common disorder inherited in an autosomal dominant fashion. Its genetic cause is unclear and is likely due to polygenic defects. Elevated levels of cholesterol, triglycerides, or both are found corresponding to plasma electrophoresis types IIa, IIb, or IV. HDL levels are often low. Chylomicron remnant clearance is impaired and there are high levels of small dense LDL. Premature coronary artery disease is common but xanthomas and xanthelasma are not found. Obesity, insulin resistance, and hyperuricaemia make this condition similar in phenotype to Type 2 diabetes.

Postprandial studies in Familial combined hyperlipidaemia (FCHL)

These have been reviewed by Castro Cabezas (2003). The metabolic basis for the condition is overproduction of VLDL by the liver. The underlying mechanism for this has yet to be elucidated. Non-esterified or free fatty acids are elevated in the postprandial state (Meijssen et al 2000) and uptake of these by fibroblasts and adipocytes are impaired (Sniderman et al 1998). It is proposed that more free fatty acids are handled by the liver resulting in raised VLDL postprandially. As chylomicrons and VLDL share the same catabolic pathway with LPL hydrolysis, high levels of VLDL may lead to impaired clearance of both chylomicron and VLDL remnants. It is thought that this contributes to uptake of triglyceride rich lipoproteins by the vasculature and subsequent atherosclerosis.
1.4.4 Type III Hyperlipoproteinaemia (Familial Dysbetalipoproteinaemia)

This is an uncommon (usually autosomal recessive) disorder characterized by moderate-severe hypertriglyceridemia (3.4-4.5 mmol/l) and hypercholesterolemia (7.8-10.3 mmol/l). Palmer xanthomas are virtually pathognomonic of the disorder and premature vascular disease is common. Tuberose xanthomas are also common. The cause is a defect in apoE that results in defective clearance of remnants of chylomicron, VLDL, and IDL (Mahley et al 1999). It is associated with the apo-E2 isoform. The remnants have lost much of their triglyceride by ongoing LPL hydrolysis and therefore are cholesterol rich. Electrophoresis gives a β migration pattern rather than the usual VLDL pre β pattern. In most cases a secondary exacerbating factor is required for expression of the phenotype. These would include conditions in which overproduction of lipoproteins occur such as in diabetes.

1.4.5 Lipoprotein lipase deficiency

This is a rare autosomal recessive disorder resulting from mutations in the LPL gene. It gives rise to a chylomicronemia syndrome identified in childhood. Pancreatitis is a common complication as well as eruptive xanthomas and lipaemia retinalis. Chylomicrons can be assumed to be present if the fasting triglycerides are greater than 1.13 mmol/l. The diagnosis is established if no lipase activity occurs after heparin administration which would be expected to displace LPL from its binding sites on HSPG. The condition should be differentiated from apo-CII deficiency (another cause of chylomicronemia) as apo C-II is a cofactor for LPL. Treatment includes a fat free diet initially followed by a diet very low in fat. The type of triglycerides recommended are those with medium chain length as these are absorbed directly into
the portal circulation rather than packaged into chylomicrons. However this treatment may be associated with hepatic toxicity. Recently the use of diacylcerol cooking oils has been investigated and found to be of benefit (Yamamoto et al 2005).

1.4.6 Apolipoprotein CII deficiency

As mentioned above Apolipoprotein CII deficiency also causes a chylomicronemia syndrome similar to LPL deficiency. It is very rare (1 per million) and is autosomal recessive in inheritance. More than 10 mutations in this LPL cofactor have been identified. It has been shown that although LPL will bind to chylomicrons in these subjects it does so in a nonproductive manner and requires lipid binding of apo-CII (Olivecrona et al 1997).

1.4.7 Familial hypertriglyceridemia

Familial hypertriglyceridemia is not usually detected till after puberty and may be difficult to distinguish from FCHL. Triglycerides are raised (2.3-5.6 mmol/l) and LDL-C levels are usually normal. HDL-C is often low. The underlying metabolic disorder appears to be large triglyceride rich VLDL in the presence of near normal levels of apoB. LPL activity in adipose tissue has been found to be low both in the fasting and postprandial phases in this condition (Goldberg et al 1980). A hypolipidaemic diet has been shown to reduce fasting triglycerides but the postprandial response is poorly modified by a dietary regimen (Zoppo et al 1999).
1.4.8 **Primary disorders of HDL metabolism**

These conditions are summarized below. All but the last condition have low levels of HDL-C while CETP deficiency usually have levels >2.6 mol/l. Familial hypoalphalipoproteinaemia is the most common, and is an autosomal dominant condition with HDL-C levels are usually in the order of 0.5-0.8 mmol/l. The association with premature vascular disease is presumably related to defective reverse cholesterol transport or other protective effects of HDL (Ng review 2004). Mutations at the apoAI-CIII-AIV locus result in low HDL levels

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Mutant gene</th>
<th>Inheritance</th>
<th>Population frequency</th>
<th>Premature vascular disease</th>
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<td>1/400</td>
<td>yes</td>
</tr>
<tr>
<td>Familial apo-AI and apo-CIII deficiency</td>
<td>apoAI or apoAI/apo-CIII</td>
<td>AR</td>
<td>rare</td>
<td>yes</td>
</tr>
<tr>
<td>LCAT deficiency</td>
<td>LCAT</td>
<td>AR</td>
<td>rare</td>
<td>yes</td>
</tr>
<tr>
<td>Fish eye disease</td>
<td>LCAT</td>
<td>AR</td>
<td>rare</td>
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<tr>
<td>Tangier disease</td>
<td>ABCA1</td>
<td>AR</td>
<td>rare</td>
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</tr>
<tr>
<td>CETP deficiency</td>
<td>CETP</td>
<td>AR</td>
<td>rare</td>
<td>no</td>
</tr>
</tbody>
</table>

*Adapted from Uptodate website 2006*

LCAT deficiency results in decreased esterification of cholesterol on HDL particles. This impairs reverse cholesterol transport and most of the cholesterol found in the plasma is free. Free cholesterol accumulates on the surface of lipoproteins and accumulates in the vasculature leading to premature vascular disease and in the renal beds leading to renal failure. A less severe form called fish eye disease is also
described with corneal opacities (as in LCAT deficiency) and low HDL levels as the only clinical manifestations.

Tangier disease is caused by mutations in the ABCA1 transporter which aides cholesterol efflux from macrophages. HDL catabolism is enhanced and cholesterol esters accumulate in the reticulo-endothelial system (causing orange tonsils). The risk of coronary artery disease is variable. Postprandial studies in Tangiers disease are scanty but there has been some work indicating that postprandial hypertriglyceridemia may be a feature in the homozygous form (Kolovou et al 2003).

CETP deficiency causes reduced transport of cholesterol esters from HDL to apoB containing lipoproteins. Protection against atherosclerosis is not clear as transgenic mice that express CETP have increased plasma LDL-C, decreased HDL-C and increased atherosclerosis (Marotti et al 1993).

### 1.4.9 Hypolipoproteinaemia

This is an autosomal dominant disorder arising from molecular defects in apoB (review Schonlen 2003). The condition is usually associated with only mild symptoms, if any, of diarrhoea. It is defined as <5th percentile in plasma total cholesterol or LDL cholesterol, or total apolipoprotein B. Secondary causes of hypolipoproteinaemia include strict vegetarianism, intestinal fat malabsorption syndromes, severe liver disease, malnutrition and hyperthyroidism.

Most patients with familial hypobetalipoproteinaemia (FHBL) are asymptomatic heterozygotes with very low LDL cholesterol and apoB levels. However more recently an association with non alcoholic fatty liver has been described (Schonfeld et al 2003). This is due to defective export of lipid via VLDL. Homozygotes suffer from severe fat malabsorption, resembling patients with abetalipoproteinaemia.
In most cases the genetic causes of familial HBL are unknown. However 40 different abnormal truncations of the protein transcribed from the gene on chromosome 2 have been described.

1.4.10 Chylomicron retention disease

This is an autosomal recessive disorder first described by Anderson et al in 1961.

There is a specific defect in intestinal lipoprotein secretion (Patel 1994).

Clinical features include steatorrhea and low plasma LDL cholesterol levels. The liver is capable of VLDL assembly and secretion, but apoB100 plasma levels are reduced. Intestinal biopsies show lipid filled enterocytes.

The apoB gene and apoB mRNA editing activity are intact. MTP levels appear normal in the intestine. ApoB48 is synthesized in the enterocytes but is absent in plasma. Berriot-Varoqueaux et al (2001) investigated the intracellular transport of apolipoprotein B48 and found that apolipoprotein B48 is completely transported into the endoplasmic reticulum and to Golgi apparatus, suggesting that this is a post-Golgi secretion defect.

1.4.11 Secondary causes of hyperlipidaemia

Lipid levels are affected by a number of metabolic conditions. They can be split into non-endocrine and endocrine conditions. Non endocrine conditions include alcohol excess, renal, biliary and liver disease, and connective tissue disorders.

Endocrine disease includes hypothyroidism, lipodystrophy and oestrogen treatment, glucocorticoid and growth hormone excess syndromes. The dyslipidaemia associated with the latter three conditions can be linked to the opposing actions of these
hormones to insulin. Treatment of the dyslipidaemia is usually the treatment of the underlying disorder.

I will now outline the dyslipidaemia associated with another secondary disorder of lipid metabolism-Type 2 diabetes. There has been debate in recent years about whether diabetes is a secondary cause of hyperlipidaemia or whether the primary defect is the hyperlipidaemic state causing "diabetic lipidus" (Shafrir et al 2003).
1.5: Diabetic Dyslipidaemia

1.5.1 Introduction

1.5.2 Intestinally derived postprandial particles in diabetes

1.5.3 Hepatically derived postprandial particles in diabetes

1.5.4 Diabetic LDL

1.5.5 Diabetic HDL

1.5.1 Introduction

Both type 1 and type 2 diabetes are associated with multiple abnormalities of lipid and lipoprotein abnormalities. These abnormalities involve both the exogenous pathway of lipid metabolism which deals with diet derived lipids, and the endogenous pathway, which begins with the hepatic production of VLDL. Dyslipidaemia is a major cardiovascular risk factor in diabetes –especially in Type 2 diabetes where coronary artery disease is the commonest cause of death. The UKPDS trial has demonstrated that improved glycaemic control in Type 2 diabetes was not associated with significant reduction in macrovasular endpoints. However various studies have demonstrated the benefit of reduction of hyperlipidaemia to reduce cardiovascular endpoints in both diabetic and nondiabetic subjects.

Type 1 diabetes, if poorly controlled leads to increased lipolysis and overproduction of nonesterified or free fatty acids (NEFAs). Lipoprotein lipase activity decreases and this leads to increased levels of triglycerides in plasma.

Type 2 diabetes causes a characteristic dyslipidaemia which is also seen in the metabolic syndrome. This comprises of: raised triglycerides; low levels of HDL;
normal or slightly raised total cholesterol and LDL cholesterol; increased levels of small dense LDL; and increased levels of small dense HDL (HDL₃). VLDL production by the liver is increased and decreased clearance of triglycerides by LPL contributes to this pattern. These abnormalities are not completely reversed by tight diabetic control.

The concept of atherosclerosis as a postprandial phenomenon was introduced by Zilversmit (1979) over 25 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 continuing investigation and debate.

1.5.2 Intestinally derived postprandial particles in diabetes

Elevated levels of apoB48 have been demonstrated in Type 2 diabetes in both the fasting and postprandial state (Curtin et al 1994). The rapid clearance of chylomicrons from the plasma has led to the argument that they do not contribute significantly to the atherosclerotic process (Nordestgaard et al 1994). However in diabetes not only are the number of intestinally derived particles produced increased but their clearance is also delayed, so that in Type 2 diabetes more time is spent in the postprandial phase (Phillips et al 2002). Postprandial particles isolated from the lymph of diabetic animals were found to be depleted of apoE which may partly explain the reduced clearance. A reduced rate of clearance of chylomicrons has a knock on effect on the metabolism of hepatically derived particles as they share a common method of lipolysis and uptake.
In Type 2 diabetes, cholesterol synthesis has been shown to be increased in diabetes although intestinal absorption is reduced (Gylling et al 1997). HMGCoA reductase, the rate limiting enzyme in cholesterol synthesis has been found to have increased expression and activity in animal models of diabetes (Feingold et al 1994; O'Meara et al 1990).

1.5.3 Hepatically derived postprandial particles in diabetes

Normally the insulin secreted postprandially reduces VLDL secretion in humans (Pietri et al 1993). This is achieved by the reduced translation of apoB mRNA (Theriault et al 1992) and increased apoB degradation (Sparks et al 1990). Insulin also suppresses fatty acid release from adipocytes (Lewis et al 1995), possibly through hormone sensitive lipase, reducing substrate for the production on VLDL in the liver.

In insulin resistance and Type 2 diabetes, VLDL secretion is enhanced. There is also evidence of a reduced rate of clearance of VLDL particles. Lipoprotein lipase (LPL) levels in adipose tissue are increased by insulin and LPL activity is decreased in diabetes (Taskinen et al 1981). Saturation of LPL may result from increased numbers of postprandial particles (Martins et al 1996). Chylomicrons are increased in number in diabetes (Curtin et al 1994; Steiner et al 1998) and have been shown to be the preferred substrate for LPL (Martins et al 1996). Large VLDL 1 particles are increased in diabetes and these are associated with the generation of atherogenic remnants, small dense LDL and small dense HDL particles (Taskinen review 2003). The mechanism behind the increased level of large VLDL 1 in diabetes are incompletely understood but are thought to include: (i) low insulin signaling via PI-3 kinase pathway that enhances lipid accumulation into nascent VLDL particles; (ii)
upregulation of SREBP-1C that stimulates de novo lipogenesis and (ii) excess availability of polar molecules in hepatocytes that stabilize apoB100.

1.5.4 Diabetic LDL

Small dense LDL in which the cholesterol: triglyceride ratio is decreased, is a characteristic feature of diabetic dyslipidaemia even in the setting of normal or subnormal LDL cholesterol concentrations (Gray et al 1997; Sniderman et al 2001). LDL size and number are important determinants of CHD risk (St-Pierre et al 2001). Because small dense LDL binds poorly to the LDL receptor there is more time for intimal penetration into the atherosclerotic plaque. It is thought that small particle size favors LDL penetration into the arterial intima as small dense LDL particles have a higher binding affinity to intimal proteoglycans than large LDL particles (Caleano et al 1998). The specific segments of apoB100 which bind to proteoglycans are more exposed on the surface of small dense LDL which may contribute to their greater atherogenic potential (Skalen et al 2002). They also bind to the intima via bridging molecules for example lipoprotein lipase and decorin (Chait 2000). Once retained in the intimal space small dense LDL, especially if glycated, shows an increased susceptibility to oxidation (Onorato et al 2000). Free fatty acid composition of LDL particles in diabetes is important to its oxidisability (Dimitradis et al 1996). Oxidized LDL in the intima is ultimately the trigger that initiates foam cell generation and atherosclerotic plaque formation. LDL size is a strong determinant of endothelial function in Type 2 diabetes as well as in nondiabetic subjects (Makimatila et al 1999).
1.5.5 Diabetic HDL

HDL particles are the most malleable of the lipoproteins and undergo continuous remodeling in the circulation via LPL, HL, CETP, and phospholipid transfer protein. Patients with diabetes lack large HDL 2 particles while small dense HDL 3 particles are increased (Syvanne et al 1995; Pascot et al 2001). Some HDL particles contain apoproteins A-I but not A-II. In one cross-sectional study Type 2 diabetes patients had a decrease in HDL particles containing both A-I and A-II apoproteins thus making reverse cholesterol transport less efficient (Syvanne et al 1996). A low level of HDL is a dominant feature of diabetic dyslipidaemia. The activity of LCAT in Type 2 diabetes has been shown to be increased (Jones et al 1996). Levels of CETP activity in diabetes are disputed. Increased transfer would suggest a reduction in HDL cholesterol but also a more efficient reverse cholesterol transfer to the liver and apoB containing lipoproteins. The overall turnover of cholesterol may be the important factor with high turnover being the cardio-protective factor rather than the overall level of HDL.
1.6 Microsomal Triglyceride Transfer Protein overview

1.6.1 Introduction

1.6.2 Structure of MTP

1.6.3 Functional domains of MTP

1.6.4 Interactions between apoB and MTP

1.6.5 Model of apolipoprotein synthesis and secretion

1.6.6 Determinants of MTP levels and activity

1.6.7 MTP in models of diabetes

1.6.8 Promoter region polymorphisms

1.6.9 Aplipoproteinaemia

1.6.10 MTP inhibitors

1.6.11 Aims of the thesis

1.6.1 Introduction

Microsomal triglyceride transfer protein was first isolated from the microsomal fraction of bovine liver (Wetterau 1984). It was found to accelerate the transport of triglyceride, cholesterol ester and phosphatidyl choline between synthetic membranes. Later it was also found in the microsomes of the intestine (Wetterau 1986). MTP is essential for the secretion of chylomicrons in the intestine and very low density lipoproteins in the liver.
It has recently emerged that MTP is an ancient protein which is widely distributed throughout invertebrate and vertebrate species, its functions of intracellular lipid transport are likely to be even more widespread than thus far appreciated.

MTP has recently been discovered in adipocytes (Swift et al 2005). MTP fluorescence studies showed that MTP was particularly marked around lipid droplets. Its role in this tissue is as yet uncertain.

Nielson et al (1998) have demonstrated that the myocardium in both mice and man express the genes for apoB and MTP and have suggested that the secretion of lipoproteins by the heart may represent a pathway by which cardiac myocytes can unload surplus fatty acids during times of hypoxic stress. An increase of triglycerides in obese rat hearts has been shown to be associated with apoptosis of cardiac myocytes and contractile dysfunction (Zhou 2000). Nielson et al (2002) has shown ventricular MTP and apoB mRNA levels to be higher in CABG patients compared with valve replacement patients. In CABG patients, ventricular MTPmRNA levels were negatively associated with the triglyceride content in ventricular myocytes and with mRNA levels of sterol regulatory element binding protein-1.

MTP has also recently been found to be involved in the modulation of the immune system. It regulates the ability of CD1d (a lipid antigen presenting molecule) to activate invariant natural killer cells (Brozovic et al 2004). This may have implications for the use in clinical practice of MTP inhibitors for the amelioration of postprandial dyslipidaemia. Mice deficient in liver MTP have been found to be protected from the hepatocyte injury associated with CD1d-restricted invariant natural killer cell activity.
induced by injections of α-galactosy ceramide. Mice deficient in intestinal MTP have also been found to be protected from colitis and systemic sequelae associated with CD1d-restricted invariant natural killer cell activity induced by injections of oxazolone.

1.6.2 Structure

MTP exists as a hetero-dimer of 2 proteins, with SDS polyacrilamide gel electrophoresis mobilities consistent with molecular weights 55000 Da and 97000 Da. It has similarities to lipovitellin, the predominant lipoprotein found in the yolk of egg-laying animals which is involved in lipid storage.

The smaller subunit called the P subunit (55 kDa) has been characterized as protein disulfide isomerase (PDI), an enzyme resident in the lumen of the endoplasmic reticulum. This is a ubiquitous protein with multiple functions including chaperone and protein binding activities. If the small subunit and large subunit are separated by denaturants, the large subunit is insoluble. Ricci et al (1995) used insect Sf 9 cells to show that PDI is essential to maintain the structure and activity of MTP. When these were infected with viruses expressing only the large subunit of MTP, there was no detectable MTP activity and the expressed protein was found by Western blot analysis to be an insoluble aggregate. However the PDI subunit appears not to be involved in lipid transfer activity (Lamberg 1996).

The large subunit is called the M subunit and is 97 kDa in humans. It is a unique polypeptide, highly conserved between species, and is essential for the lipid transfer activity of the MTP.
Based on homology with lipovitellin, there are thought to be 3 structural domains: N-terminal β-barrel, central α-helical domain, and C-terminal lipid-binding cavity. These are thought to correspond to the three functional domains of MTP M subunit: membrane association domain, lipid transfer domain and apoB binding domain.

**1.6.3 Functional domains**

Evidence for the postulation that the three activities of MTP occur at different sites comes from inhibition studies. For example Bakillah et al (2000) showed that lipid transfer activity inhibitors do not inhibit apoB-MTP binding. Conversely they showed that inhibitors of apoB-MTP binding have no effect on lipid transfer activity of MTP. Bakillah et al (2001) also showed that the association of MTP with lipids resulted in increased binding to apoB, indicating that the apoB binding domain is different from the lipid transfer and membrane associating domains in MTP.

**1.6.4 Interactions between apoB and MTP**

The interactions between apoB and MTP were first investigated in 1996. Wu et al (1996) immuno-precipitated MTP from HepG2 cells and found that ~10% of the nascent apoB associated with MTP. Hussain et al (1997) immobilized lipoproteins to microtitre plates and incubated them with labeled heterodimeric MTP showing higher binding rates with LDL and VLDL than with HDL. Patel et al (1996) showed that washes of high salt content prevents co-immuno-precipitation of apoB-MTP complexes, but once made, these complexes are resistant
to this ionic milieu. From this it was concluded that only the initial interaction is ionic. There is evidence that positively charged amino acid residues interact with negatively charged residues in MTP but there appears to be no overlap between the MTP binding site and the heparin and LDL receptor binding sites of apoB (Bakilla et al 1998). The binding of apoB to MTP decreases with increasing length of apoB polypeptide. The point of binding has been localized by Mann et al and Bradbury et al (both 1999) to be at two sites on the N-terminal βα1, globular domain of apoB.

An inhibitor of apoB-MTP binding (AGI-S17) which has no effect on lipid transfer activity was found to inhibit secretion of apoB by ~70% (Bakilla 2000). This study underlines the importance of MTP-apoB binding in lipoprotein assembly and secretion. ApoB binds to several other proteins in the ER. However it is unclear whether these proteins play a role in lipoprotein assembly.

**MTP binding to lipids**

Increases in the concentration of negatively charged phospholipids can slow transport between membranes (Aztel 1993) in vitro. It is possible that an increase in free fatty acids into the hepatocytes changes the free fatty acid concentration in the ER membrane, which increases the production of lipoprotein particles by altering the affinity of the MTP for the ER membrane.

**Lipid transfer**

MTP has been found to accelerate transport of lipid molecules between synthetic small unilamellar vesicles (SUV). It is by measuring the rate of transfer of radiolabelled triglyceride from donor SUV to acceptor SUV, that MTP activity is
routinely determined. The transfer activity is expressed as % of the donor lipid transferred per unit of time (Wetterau 1992).

Kinetic studies with these model membranes suggest that MTP transfers lipids by a shuttle mechanism (Aztel 1993). This involves an MTP molecule interacting transiently with a membrane, extracting lipid molecules, dissociating from the membrane, binding transiently with another membrane, delivering lipids to the second membrane, and then becoming available for another cycle of lipid transfer. Kinetic studies suggest that MTP has two lipid binding sites, one fast and one slow. The fast site is implicated in lipid transfer. The slow site appears to be selective for phospholipid (Aztel 1994).

MTP forms a stable complex with a variety of neutral lipids: triglyceride, diacylglycerol and phospholipids as well as cholesterol ester. The binding and transfer of triglyceride by MTP is dependent upon the concentration of triglyceride in the donor membrane. This transfer activity is found to be optimal with neutrally charged and decreased with negatively charged lipids. Transport rates were found by Jamil et al (1995) to be in decreasing order: - triglyceride>cholesterol ester>diacylglycerol>phosphatidylcholine. It is likely that the negatively charged lipids compete with the ionic interactions between apoB and MTP. The removal of fatty acid chains alters the size of the hydrophobic moiety and slows transport dramatically.

The lipid transfer activity of MTP is essential for the assembly and secretion of apoB-containing lipoproteins. In patients with aßlipoproteinaemia, mutations in the M subunit correlate with defective lipid transfer activity and an absence of apoB-containing lipoproteins in plasma. MTP inhibitors of lipid transfer activity decrease
apoB secretion; and co-expression studies of apoB and MTP in non-hepatic and non-intestinal cells demonstrated no secretion in the absence of MTP and degradation of apoB polypeptides.

Rusinol et al (1997) showed in a cell free system that nascent apoB can in fact be assembled into lipoproteins in the absence of MTP. However the longer the apoB peptide the more dependence there is on MTP for lipidation. Another group has shown secretion of apoB-lipoproteins in the absence of MTP (Raabe et al 1999) in a model of hepatocytes from tissue specific knock out mice. However the absence of MTP or MTP inhibition leads to decreased triglycerides in the lumen of the endoplasmic reticulum, indicating a role for MTP in forming lipid droplets for core expansion.

1.6.5 Model of apo B lipoprotein synthesis and secretion

Hussain proposes a sequential model for chylomicron and VLDL assembly in which primordial lipoproteins are first synthesized with minimal lipidation by MTP. MTP associated with lipid vesicles then bind apoB, providing a lipid core for the nascent apoB to incorporate. An alternative but less likely proposal is that free MTP may bind nascent apoB and subsequent to that the apoB-MTP extracts lipid molecules (rather than droplets) onto the apoB lipoprotein.

ApoB is synthesized in excess of its requirements for lipid transport, the excess (about 50%) being degraded within the cell before secretion. When the initial lipidation of apoB by MTP fails because of insufficient lipid levels the inadequate lipid levels
leads to incorrect folding of the protein. This renders it susceptible to conjugation by ubiquitin and the ubiquitin-proteasome reaction occurs.


Synthesis of triglyceride rich droplets is likely to involve MTP as mice deficient in MTP expression do not accumulate lipid droplets in the ER lumen (Raabe 1999). Kulinski et al (2002) used cultured murine hepatocytes to show that MTP inhibition reduced the amount of apoB-free triglyceride in the microsomal lumen.

Gordon et al (1996) using McArdle RH-7777 rat hepatoma cells showed, using pulse labeling and chase techniques that inhibition of MTP after completion of the first step of minimal lipidation of apoB but before core lipidation did not block conversion of high density to very low density lipoproteins. They concluded that MTP is not required for addition of core bulk lipid. Read et al (2000) however have proposed that part of the MTP peptide has fusogenic properties.

Lipid availability also affects secretion of apoB-lipoproteins. Fatty acid supplementation of differentiated Caco-2 cells (enterocyte derived cells that produce
apoB48 and apoB100 lipoproteins) results in changes of particle size secreted (Luchoomun1999).

1.6.6 Determinants of MTP levels and activity

High fat diet

Bennett et al (1995) investigated the hamster model. The Golden Syrian hamster carries an appreciable amount of cholesterol in the LDL fraction, exhibits a hepatic cholesterol synthesis rate comparable to humans, and under appropriate dietary conditions will develop atherosclerotic lesions (insulin resistant model). They found that hepatic MTPmRNA increased in a dose-dependent manner with increasing fat content in a 28 day diet, especially with saturated trinolein and trilinolein rather than unsaturated trinolein and trilinolein. There was a positive correlation between hepatic MTPmRNA and plasma VLDL, LDL, and HDL cholesterol.

Lin et al (1994) gave hamsters one of four diets for 31 days: control low fat, high fat, low fat but high sucrose, or low fat followed by a 48 hour fast. They found that the high fat diet increased MTP large subunit mRNA levels in the liver by 55% and in the intestine by 126%. However this was not accompanied by an increase in apoBmRNA levels. The high sucrose diet caused an increase in hepatic (55%) but not intestinal MTPmRNA levels. MTPmRNA levels were unaffected by fasting but intestinal MTPmRNA was up-regulated within 4 hours after initiation of a high fat diet thus allowing efficient capture of dietary lipids. No response in hepatic MTPmRNA was
observed. A positive correlation was found between intestinal MTPmRNA and triglyceride levels and cholesterol (VLDL+LDL), but not hepatic MTPmRNA.

Lin (1995) showed that in hepG2 cells glucagon or fatty acid supplements (oleate) had no effect on MTPmRNA. However more recently Qiu et al (2005) have defined a region on the MTP promoter in HepG2 cells which may be specific for oleate-mediated increase in MTP gene transcription.

Saturated fatty acids (C14-C16), which are agonists for HNF-4α, regulate MTP expression (Sheena 2005). In contrast, omega-3 fatty acids, which are HNF-4α antagonists, suppress VLDL production (Harris 1990, Brown 1997).

**Dietary triglyceride**

The type of dietary triglyceride may also be important. Taguchi et al (2002) looked at the effects of dietary diacylglycerol and triacylglycerol on hepatic MTPmRNA activity in rats fed a high fat diet. Triacylglycerol increased hepatic MTPmRNA expression and protein activity after 21 days, while dietary diacylglycerol suppressed the high fat induced MTP activity in the liver. Long term ingestion of dietary diacylglycerol has been reported to enhance β oxidation of fatty acids in the liver. This may limit availability of the fatty acids for MTP activity.

Hagan et al (1994) cloned, sequenced and characterized the MTP gene promoters in the human and hamster. They showed that the 5'-promotor region of the MTP gene contains recognition sites for hepatic necrosis factors 1 and 4 (HNF1, 4) and activator protein AP-1. HNF 4 is a transcription factor with ligands including fatty acyl-CoA. The promoter contains a modified sterol response element and a negative insulin response element. Navasa et al (1998) found that endotoxins LPS and cytokines TNF,
IL1 and IL6 cause a reduction in hepatic MTP mRNA levels in HepG2 cells and the Syrian hamster.

**Dietary cholesterol**

The effect of increasing dietary cholesterol over 4 weeks was looked at by Benret et al (1996) in the Golden Syrian hamster. Both VLDL-cholesterol and triglycerides levels in plasma rose, as did hepatic MTP mRNA concentration. Highly significant correlations were found between hepatic MTP mRNA concentrations and plasma VLDL-lipid concentrations and hepatic cholesterol concentrations. Sterol response element binding proteins (1 and 2) have been studied by Sheng et al (1995) in human and hamster liver cell cultures. These proteins are attached to the endoplasmic reticulum membranes and nuclear envelope. Depending on the sterol milieu, fragments are cleaved from these proteins and enter the nucleus where they bind to the sterol response element in the promoter region of the MTP gene.

**HMGCoA reductase inhibitors**

Wilcox et al (1999) examined the effect of HMGCoA reductase inhibitors on MTP levels in HepG2 cells. Atorvastatin administered for 24 hours, was found to have no effect on the synthesis of apoB but intracellular degradation was increased and apoB secretion was decreased. MTP mRNA levels were also decreased by 22%. Whether this is a direct effect of atorvastatin or an effect of reduced cellular cholesterol on the sterol response element was unclear.
Chong et al (2006) showed in the fructose-fed Syrian golden hamster that rosuvastatin treatment, a HMG-CoA reductase inhibitor was associated with a non significant reduction in hepatic MTP.

**Ethanol**

Lin et al (1997) noted that a single dose of ethanol to rats caused a reduction in both intestinal and hepatic MTPmRNA levels. This is unexpected because of the hyper triglyceridaemic effect of ethanol consumption in humans. The likely reason is that although lipoprotein synthesis and secretion are reduced, so also is clearance which would override this effect.

Sugimoto et al (2002) fed male Sprague-Dawley rats an ethanol-containing diet for 37 days. There was a reduction in hepatic MTPmRNA expression and MTP activity associated with increases in hepatic cholesterol and triglyceride content.

**Other**

Lin et al (2002) found that fresh garlic extract reduced MTP mRNA levels in both the human hepatoma HepG2 cells and Caco-2 cells.

Bile acids have been shown to downregulate MTP activity invitro (Hirokoni et al 2004). The bile acid chenodeoxy-cholic acid is a ligand for the farnesoid x receptor and when incubated with HepG2 cells causes increased transcription of small heterodimer partner (SHP) and subsequent down regulation of HNF1 and 4, both activators of MTP transcription.

1.6.7 MTP in models of diabetes
The diabetic (streptozotocin-induced) rat model has been studied by Lin et al (1994). Eight days after diabetes was induced hepatic MTP large subunit mRNA levels were 65% higher than controls. Insulin treatment normalized hepatic mRNA levels. However intestinal MTP mRNA levels were not affected by insulin in the studies by Wetterau et al (1997). Brett et al (1995) found no significant differences in similar experiments after five days—perhaps not enough time to allow a steady state of MTP to be reached. In human subjects acute insulin has been shown to reduce triglyceride and apoB secretion (Lewis 1995) limiting hepatic lipaemia in the postprandial phase when alimentary lipaemia is at its height.

Our group has previously demonstrated in streptozotocin diabetic rats an increased intestinal MTP mRNA associated with an increase in chylomicron triglyceride and cholesterol content but not particle number (Gleeson 1999).

The insulin resistant Zucker obese fa/fa rat has also been studied by our group. Phillips et al (2002) cannulated the lymph ducts and collected lymph for four hours after a high fat meal. In the obese animals plasma cholesterol and phospholipid but not triglycerides were increased. Obese animals secreted more lymph CM apoB48 (mg/hr), triglyceride and phospholipid. Chylomicron composition was similar (except for more phospholipid in the obese animals). Intestinal and hepatic MTP mRNA expression was significantly greater in the obese rats. Therefore in this animal model, insulin resistance was associated with increased MTP, which was associated with increased chylomicron particle number.

Our group has also studied the male New Zealand white rabbits, a model which develops atherosclerosis (Phillips et al 2002). Intestinal MTP activity and mRNA
were higher in the diabetic animals. There was no significant difference in MTP activity or mRNA expression in the liver, unlike in the case of the fa/fa rats.

The diabetic animals were found to have increased triglycerides and decreased HDL cholesterol. They were found to secrete more lymph CM apoB48 and apoB100, and more CM cholesterol/hour. The lymph CM in diabetic animals as compared to controls contained less lipid/apoB.

Au et al (2003) examined the regulation of the MTP gene by insulin in HepG2 cells. They found that insulin decreases MTP mRNA levels mainly through transcriptional regulation in the HepG2 cells. They showed that this occurred through the MAPK<sup>ERK</sup> cascade but not through the PI 3 kinase pathway. Fine tuning of MTP gene transcription is thought to occur by the counterbalancing effect of MAPK<sup>ERK</sup> and MAPK<sup>p38</sup>. The protein kinase cascade is shown below. Pointed arrows denote stimulation; blunted arrows denote inhibition. Raf-1 and MEK are growth-related stimuli. MAPK<p38> is primarily activated by stress-related signals such as heat and osmotic shock, UV irradiation, and proinflammatory cytokines.
Insulin

MAPK$^{erk}$ cascade

Raf-1

MEK-1/-2

ERK-1/-2 (p44/p42)

MAPK$^{p38}$

MTP gene transcription

1.6.8 Promoter region polymorphisms

There has been recent interest in promoter region polymorphisms of the MTP gene, and the effect of these on the plasma lipid profiles in human subjects. There are conflicting results among the 15 publications to date with differences possibly due to the modulating effects of ethnic background and visceral obesity.

Caucasian studies

Karpe et al (1998) showed in non-diabetic subjects homozygous for the T allele of the MTP-493 polymorphism a lower number of lipid-rich VLDL particles and significantly lower total cholesterol and LDL cholesterol. Heterozygotes for the T allele (G/T) had no differences in VLDL or LDL triglyceride or cholesterol from GG subjects. Using minipromoter constructs these authors showed increased promoter activity by the T-variant.

Ledmyer et al (2002) showed that subjects homozygous for -493 T, and -164 C alleles had lower plasma total and LDL cholesterol levels and plasma LDL apoB levels, and also significantly higher body mass index (BMI) and plasma insulin levels compared with carriers of the common alleles. The associations between plasma total cholesterol and MTP -493 genotype was verified in a cohort consisting of control subjects of the West of Scotland Coronary Prevention Study (WOSCOPS).

However the Framingham Offspring Study showed no association between the MTP-493 G/T polymorphism and lipid and lipoprotein levels Couture et al (2000). It has been suggested that the T allele may interact with visceral obesity and hyperinsulinaemia in non-diabetic subjects (St Pierre et al 2002).
**Other polymorphisms**

Other polymorphisms in the promoter region of the MTP gene have been identified, including -164 T/C, and -400 A/T (Karpe et al 1998, Hermann et al 1998, Ledmyer et al 2002).

Hermann et al failed to find any association between either of these polymorphisms and plasma lipid levels.

Ledmyer et al found a significant association between the rare homozygous -164 C/C genotype and plasma total and LDL cholesterol. They suggested that this might be due to the almost complete allelic association between this polymorphism and the -493 G/T polymorphism. The -164 T/C promoter polymorphism shows sequence homology to a sterol regulatory element (SRE) in the same vicinity (bp -171 to -164).

Two studies in familial hypercholesterolaemia have shown that subjects with the MTP-493 T/T variant had lower LDL cholesterol (Bertolini 2004) and fasting triglycerides (Lundahl 2000) than G/G subjects.

**Noncaucasian studies**

A cross-sectional analysis of 585 young healthy black men followed up in the CARDIA study indicated that the T/T genotype was associated with higher levels of total cholesterol, LDL cholesterol, triglycerides, and apoB (Juo et al 2000).

In a Chinese population of diabetic subjects the MTP-493 T/T diabetic group had significantly higher triglyceride, VLDL cholesterol and smaller LDL particle size than
the MTP-493 common GG group. However there were no differences between G/T and G/G groups (Chen et al 2003).

**Postprandial studies and MTP promoter region polymorphisms**

The only study to date that has examined the influence of the -493 G/T polymorphism on postprandial lipoproteins revealed that subjects homozygous for the T/T polymorphism showed an increase in apoB48 in the smallest triglyceride-rich lipoprotein fraction postprandially without an increase in postprandial triglycerides or in fasting total or LDL cholesterol (Lundahl et al 2002).

**Hepatic steatosis and MTP -493 genotype**

In Type 2 diabetes, the common -493 G/G MTP polymorphism has been associated with surrogate markers of non-alcoholic hepatic steatosis (NASH) (Bernard et al 2000).

Namikawa et al (2004) found in a group of non-diabetic Japanese patients with NASH that the frequency of the G/G genotype was higher than in controls, and that the G/G genotype was associated with more severe NASH than G/T.

**Cardiovascular effects of MTP promoter region polymorphisms**

Yamada et al (2006) looked at over 2000 Japanese subjects randomly recruited to a prospective cohort study of aging and age-related diseases. There was no difference in the serum concentrations of total cholesterol, HDL, LDL, or triglycerides among -493 MTP genotypes for men or for women. However in postmenopausal women the T
allele was associated with lower blood pressure \( (p=0.04) \) with the TT genotype protective against hypertension.

The effect of the -493 G/T polymorphism on the risk of cardiovascular disease has been evaluated in one prospective study to date, as part of the West of Scotland Coronary Prevention Study. Ledmyr et al (2004) found that MTP-493T carrier status was associated with significantly increased risk of CHD despite a small reduction in total cholesterol, with the excess being eliminated by pravastatin treatment. This was confirmed using the Uppsala Longitudinal study of adult men (ULSAM), a 20 year follow-up study of coronary heart disease. Eighteen heart muscle biopsies showed a MTP-493T genotype-specific depression of MTP mRNA expression.

1.6.9 Aβlipoproteinaemia

Aβlipoproteinaemia is a rare autosomal recessive disease. Subjects have fat malabsorption with steatorrhea, hepatosteatosis, fat soluble vitamin deficiencies (A, D, E, K), spino-cerebellar degeneration, peripheral neuropathies, and retinitis pigmentosa.

The underlying defect is in functional MTP. This causes a defect in the assembly and secretion of hepatic and intestinal apolipoprotein B containing lipoproteins, VLDL and CM.

Subjects have only traces of apoB in plasma with total plasma cholesterol levels of ~40 mg/dl. The subjects investigated to date and reported in the literature have all been found to have major abnormalities in the MTP M subunit (Wetterau 1992; Narcissi 1995).
1.6.10 MTP inhibitors

At least seven MTP inhibitors have now been developed which have been tested on human cell cultures (HepG2 cells), mice and rat models (review Shoulders et al 2005). They have also been used in phase 1 and 2 trials with significant reductions in blood lipid levels. However they have also been associated with fat accumulation in the liver and also high levels of intestinal fat malabsorption.

Ueshima et al (2004) have shown in male apo E knockout mice, a model of atherosclerosis, that a western-type diet led to up-regulation of MTP expression. In a further publication the authors demonstrated that an inhibitor of MTP reduced atherosclerosis in this mouse model through reduction in both total cholesterol and triglyceride levels (Ueshima 2005).

An inhibitor which would affect intestinal MTP but not hepatic MTP would avoid hepatic lipid accumulation. However MTP inhibitors may be more problematic than previously thought as they may have detrimental effects on the myocardium and the immune system.

Naturally occurring MTP inhibitors have been discovered in flavanoids, a class of plant polyphenols. For example naringenin, found in grapefruit juice, has been found in invitro studies with hepatoma cell lines to lower MTP mRNA levels and lower lipid transfer activity. It was also found to downregulate ACAT2 expression and increase LDL receptor expression via an increase in sterol response element binding protein -1 (SREBP-1) (Borradaile et al 2003).
1.6.11 Aims of the thesis

1. There is no information, to our knowledge, on intestinal MTP mRNA levels in human subjects. The aim of this study was to examine the relationship between intestinal MTP mRNA and postprandial triglyceride-rich lipoprotein composition in Type 2 diabetic and non-diabetic subjects.

2. There is little information to date on the effect, if any, of HMGCoA reductase inhibitors on human intestinal MTP mRNA levels. Reduction in cholesterol synthesis with a statin would be expected to be associated with lower MTP levels through the effect of cholesterol on the sterol response element in the MTP gene. This may result in altered chylomicron particle number and/or composition. Therefore the aim of the thesis was to examine the relationship between intestinal MTP mRNA levels, fasting and postprandial lipoproteins and use of HMGCoA reductase inhibitors in a group of nondiabetic and diabetic subjects.

3. A rare polymorphism on the promoter region of the gene (-493 G/T) for MTP synthesis has been found to affect plasma lipids and lipoproteins. Non-diabetic subjects, homozygous for the T allele of the -493 G/T MTP polymorphism, have decreased numbers of VLDL particles and significantly lower total and LDL cholesterol, while those heterozygous for the T allele show no such changes. Since Type 2 diabetes is associated with major disturbances in the triglyceride-rich lipoproteins, and in animal studies, with an increase in MTP expression, our aim was to assess for possible amplification of the effect of the T allele at the -493 MTP promoter region in diabetes.
4. Little evidence exists on the effect of the -493 G/T polymorphism on the actual risk of cardiovascular disease. One prospective study has shown a detrimental effect of the TT polymorphism despite lower LDL cholesterol. The aim of the study was to characterize this cohort of diabetic patients for evidence of atherosclerotic disease.
Chapter 2

Materials and Methods
2.1 Specialized 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 polyacrilamide gradient gels (4-15%)

Unicam Ltd., York St., Cambridge, UK. 8625 UV/VIS Spectrometer

2.2 Subjects and Ethics

A series of 82 Type 2 diabetic subjects were selected and underwent a high fat meal. MTP genotyping was performed by PCR- RFLP.

A series of 15 adult patients with Type 2 diabetes and 17 adult controls undergoing diagnostic gastroscopy were selected for the meal test and intestinal MTPmRNA estimation. We excluded those found at gastroscopy to have malignant or coeliac disease. Patients with uncontrolled thyroid disease or familial hyperlipidaemias were also excluded.

Ethics committee approval was gained and informed written consent was obtained.

Four extra duodenal (D2) biopsies were taken and stored in RNA later and frozen at -70°C. The other two routine biopsy samples were collected in formalin and examined histologically to exclude inflammatory bowel disease, coeliac disease and malignancy in the usual way.
2.3 **Blood sampling**

Blood for isolation of lipoproteins and plasma lipids were drawn into lithium heparin glass tubes. Blood for glucose determination was drawn into fluoride oxalate tubes and for HbA1c was drawn into ethylenediaminetetra-acetic acid (EDTA) tube. Samples were taken after an overnight fast and 6 hours after the test meal. A Roche Diagnostics autoanalyser (Roche Hitachi 917) was used to determine fasting total cholesterol, triglyceride and HDL-cholesterol. LDL-cholesterol was calculated using the Friedwald formula.

2.4 **Serum analysis**

**Lipids**

Total cholesterol and triglyceride content of lipoprotein fractions were measured using the commercially available enzymatic colorimetric kits from Boehringer Mannheim GmBH (Mannheim, Germany).

Phospholipid determination was made using a colorimetric assay kit from BioMerieux, (Charbonaires les bains, France). Phospholipid standards and samples were prepared in duplicate. Tubes were incubated at 37°C for 15 minutes and absorbencies measured at 505 nm.

**Glucose**

Venous blood glucose levels were determined according to an enzymatic colorimetric method using a commercially available diagnostic kit (Boehinger Mannheim GmBH, Mannheim, Germany).

**HbA1c**
Blood HbA1c was determined using an enzyme immunoassay kit, containing monoclonal antibody that is specific for HbA1c (Novo Nordisk, Cambridge, UK). The normal value is taken to be <5.8% and was DCCT compatible.

2.5 Test meal

The patients were fasted from 10 pm the night before. Those patients who had duodenal biopsies taken had their meal within 1 week of the biopsies.

The high fat meal consisted of 1100 calories and was made up of the following:

A milk shake consisting of:
- 125 mls ice-cream
- 100 mls full cream milk
- 25 mls Flora sunflower cooking oil

This was followed by:
- 2 medium sized fried eggs (cooked in sunflower oil)
- 2 slices of toasted white bread
- 2 sachets of butter/sunflower margarine
- Cup of tea/coffee

The meal comprised 55% of calories as fat, 25% as carbohydrate, and 20% as protein and included 0.6 g cholesterol. The breakdown of the fat content was saturated fat 22.5g, polyunsaturated fat 34.1g, and monounsaturated fat 26.4g. Blood was drawn at time = 0 and 6 hours.

2.6 Lipoprotein isolation
Blood was drawn into heparinised tubes and centrifuged for 10 minutes at 3000 revolutions per minute within 1 hour of collection, to separate plasma and cells. The following preservatives and protease inhibitors were added to the plasma to prevent oxidation and degradation of apoB: (PPACK) D-phenylalanyl-L-prolyl-L-arginine chlormethyl ketone di-hydrochloride (1 mmol/l), phenyl methyl sulphonyl fluoride (PMSF) (0.1 mmol/l), aprotinin (0.005% (v/v)), sodium azide (0.02%, w/v) and EDTA (0.1 mg/ml).

The chylomicron fraction was isolated as previously described by Curtin et al (1996) by overlaying the plasma with an equal volume of saline (density 1.006 g/ml). This was centrifuged for 30 minutes at 20,000 rpm at 4°C in a Beckman L7-55 ultracentrifuge using a fixed angle rotor. The chylomicron fraction was removed from the top of the tube with a stretched Pasteur pipette. The density of the infranate was then adjusted to 1.006 g/ml and the solution was centrifuged at 40,000 rpm for 18 hours at 4°C after which the supranate was pipetted to isolate the very low density lipoprotein fraction. Lipoprotein fractions were stored at 4°C and lipoproteins measured within 1 week.

2.7 Particle Compositional Analysis

Quantification of apoB in the chylomicron and VLDL fraction was done by gradient gel electrophoresis, staining with Coomassie Brilliant Blue 250 and densitometric scanning was used to determine the concentration relative to known standards.

The gel used was sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separation is made on the basis of size, the smaller peptides traveling further
down the gel. Gradient gels were used to optimize separation of proteins in a wide molecular weight range - from 500,000 Da for apoB100 to 8,800 Da for apoC.

2.8 Standard proteins for quantification of sample apoproteins

ApoB100 was isolated from human LDL using the method of Phillips et al (2000). Fasting blood was drawn from a healthy, normolipidaemic volunteer into heparinised tubes. Plasma was isolated and preservatives and antiprotease were added as described above to prevent degradation of apoB100.

Plasma density was raised to 1.025 g/l and triglyceride-rich lipoproteins were removed following ultracentrifugation of the plasma for 24h at 4°C. The density of the infranate was raised to 1.025 g/ml and centrifuged for 24 hours and LDL was removed from the top of the tube. The LDL was dialyzed overnight at 4°C against 0.15M NaCl, 0.05% EDTA and 0.02% NaAC pH7.4. LDL protein was determined by the Lowry method using 4 separate standard curves.

Purification and integrity of the protein was confirmed by SDS-PAGE. Two dilutions of protein were made (0.1 mg/ml and 1.5 mg/ml) in sample buffer (2.0% (v/v) B-mercaptoethanol, 4% (w/v) SDS, 0.01% (v/v) bromophenol blue, 0.1 mmol/l Tris-HCL, 20% (v/v) glycerol, pH6.8) and stored at -20°C for use throughout the study.

2.9 Determination of apoB100 and apoB48 in chylomicron and VLDL fractions
Chylomicron and VLDL apoB100 and apoB48 were separated by SDS-PAGE using 4-15% gradient gels (Biorad, Hercules, Ca. USA) as previously described (Curtin et al 1996).

Non-delipidated lipoprotein samples (28 μl) were reduced in SDS sample buffer (2.0%(v/v) B-mercapto ethanol, 4%(w/v) SDS, 0.01% (v/v) bromophenol blue, 0.1 mmol/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 (20 μl) were loaded onto the gel and electrophoresed at 60mA constant current in 0.019 mol/l Tris and 0.192mmol/lglycine until the tracker dye reached the bottom of the gel. Gels were stained for 1 hour with Coomassie Brilliant Blue staining solution and de stained in water: acetic acid: methanol (5:1:4) with several changes of solvent, until gel was clear. The chromogenicity of apoB48 has been shown to be similar to that of apoB100 (Karpe et al 1994).

Four concentrations of LDL apoB100 were applied to each gel. High range molecular weight marker from Bio-Rad Laboratories was loaded into the last lane of each gel for identification of apoproteins. The bands were quantified by densitometry as described below. Madigan et al (2000) demonstrated apoB100 staining to be linear within the range 0.1-20μg protein, facilitating the measurement of both apoB48 and apoB100 both in the same sample.

2.10 Quantitative apoprotein determination by densitometric scanning

Gels were stained with Coomassie Blue-stained and apo B48 and apo B100 were quantified using the Viber Lourmat gel documentation system by comparison of
sample to the known LDL standards (Vilber Lourmat Biotechnology, Marne La Valee, France). A video image of each gel was generated and imported into Bio 1D version 6.32 software (Viber) for analysis. Density values were assigned to the purified apoB100 standard bands and a standard curve constructed. Values were recalculated by linear regression and those curves with a correlation coefficient of >0.95 were accepted. The concentration of apoB48 and apoB100 were then determined from this standard curve. Results were expressed as micrograms per millilitre of plasma.

The intra-assay coefficient of variations for apoB48 was 2.8% and 3.9% in the chylomicron and VLDL fractions and 4.8% and 6.8% for apoB100 in the two fractions. The inter-assay coefficient of variations was 5.0% and 7.0% for apoB48, and 8.0% and 8.6% for apoB100 in the chylomicron and VLDL fractions respectively.

2.11 Intestinal biopsy RNA extraction

Total RNA from human biopsies was isolated at 4°C using the Ultra spec™ RNA isolation system (Biotecx, Houston, Tex., USA). Intestinal biopsies (10-100 mg) were homogenized on ice in Ultra spec using 1 ml of Ultraspec RNA reagent using a mortar and pestle. Chloroform 0.2 mls per 1 ml of Ultraspec was added, shaken and centrifuged at 12000g for 15 minutes at 4°C. The aqueous supranatant layer was pipetted off, taking care not to disturb the interphase with the organic layer. RNA was precipitated from this supranatant with isopropranol 0.5 volumes and 0.05 volumes of RNA tack resin which was vortexed and spun down to form a pellet. The supranatant was then discarded. The pellet was washed twice with 1 ml of 75% ethanol, vortexed, spun and the supranatant again discarded. Traces of ethanol were removed by a water suctioned pipette tip, and the pellet allowed to dry.
The pellet was then re-suspended in 1 volume (=40 μl) of DEPC water, vortexed and spun, and the supernatant was transferred to a fresh tube, leaving the resin behind. This allowed for the final preparation of un-degraded RNA to be free of DNA and proteins.

Total RNA was quantified by absorbance at 260 nm assuming that 1 OD = 40 μg RNA/μl.

2.12 Quantification of MTP mRNA by Ribonuclease Protection Assay:

overview

A radiolabelled single-stranded probe that is complementary to part of the target RNA to be analysed was synthesised by transcription from a T3 or T7 promoter site. This radiolabelled probe was then mixed with sample intestinal RNA and incubated under conditions that favour hybridization of complementary transcripts.

Single stranded unhybridized RNA was digested with RNAse while the hybridized fragments were protected from digestion. The hybridised fragments were then separated by gel electrophoresis and visualised by phosphorimaging.

2.13 Transcription vector construction

Oligo (dT)-primed first strand cDNA synthesis was performed on total RNA extracted from human intestinal tissue. The reverse transcription product was then amplified by PCR using primers designed from conserved homologous regions across different species. Primers 5'-TCAGCAGAGAGAGAAGAGC-3' AND 3'-TATCTACCTCATGGGTGGTCC-5' which flank a 229 base pair fragment corresponding to nucleotides 365-594 of the human MTPcDNA sequence were used in this PCR. The PCR was carried out (50°C for 30 seconds, 72°C for 45 seconds,
93°C for 30 seconds) for 35 cycles and the 229 base pair product was blunt end cloned into the EcoRV site of pBlue script II KS vector (Stratagene Ltd). The clone was then sequenced to confirm its identity and orientation

2.14 Synthesis of probes

The Bluescript KS vector with template DNA was required to be linearised by the following technique:

2.5 μg of plasmid DNA, 10 μl of buffer, 10μl of Bam HI or Hind III enzyme were mixed and made up to 100 μl with DEPC H₂O, spun and incubated at 37°C for 3 hours. The same volume of phenol:chloroform:isoamyl alcohol at a ratio of 50:49:1 was added, vortexed, and spun for 5 minutes. The upper aqueous phase was transferred to a new DEP treated Eppendorf tube and the following added: 10 μl DEPC treated 3M NaAC (pH5.2), 260 μl ethanol(100%), 1 μg glycogen, vortexed and incubated at -20°C for >30 minutes. This was subsequently spun at high speed (12000-14000) for 10 minutes and the supranatant was removed with a needle and water suction to leave a pellet. The pellet was washed twice with 260 μl 70% ethanol, and spun down. The pellet was then resuspended in 10 μl DEPC H₂O.

To ensure that the plasmid was indeed cut and thereby linearized, a minigel was run with 0.7% agarose. This was done by heating 1g of agarose with 100 mls 1XBE until boiling. Then this was poured into the plate to dry flat. When dry and firm (15 minutes) this was covered by 1X BE to keep the gel moist before the sample was added. 2 μl of the Bam and Hind digest sample was mixed with 2μl of loading dye (bromophenol blue) then entered into the gel along with a lane of uncut plasmid. This was run on 40 MAmp, 80 -90 volts for 1 hour till the dye was seen near the bottom of
the gel. Then the gel was stained with ethinyl bromide and observed in the dark room with UV light to observe the bands. The digests were stored at 4°C for up to 7 days.

2.15 Probe labelling

$[^{32}P]$-labelled cRNA probe and synthetic mRNA standard were synthesized by transcription of linearized template DNA cloned into the Blueprint KS vector, using a T3/T7 transcription kit (Stratagene Ltd), according to the manufacturer's instructions as below.

**Synthetic cold mRNA standard**

DEPC $\text{H}_2\text{O}$ 23 $\mu$l was added to 5X transcription buffer 10 $\mu$l and 10mM 2 $\mu$l of each of the following nucleotides: ATP, GTP, CTP and UTP; 1 $\mu$l RNAse inhibitor, 2 $\mu$g of BAMI digested plasmid, 2 $\mu$l of DTT and lastly 2 $\mu$l (10 U/$\mu$l) of T3 polymerase. This was mixed and spun and incubated at 37°C for 50 minutes. Subsequently RNAse free DNAsse 2.5 $\mu$l was added, mixed, spun and incubated at 37°C for 15 minutes.

DEPC $\text{H}_2\text{O}$ 50 $\mu$l was added to make up to 100 $\mu$l. Then 100$\mu$l of phenol:chloroform:isoamyl alcohol at ratio of 50:49:1 was added and spun on high for 5 minutes. The aqueous phase was then pippetted of and loaded onto the centre of Sephadex G50 columns (which were pre-prepared by spinning at 750 rpm for 1 minute) and spun for 4 minutes at 1100 rpm at 4°C resulting in 100 $\mu$l of mRNA.

**Measuring quantity of mRNA standard**

75$\mu$l of cold probe was made up to 1000$\mu$l and measured using a densitometry scanner at A 260 absorbance. The concentration was then calculated and expressed in
μg / μl. Based on this the cold probe is serially diluted to 1000 atm, 500 atm, 250 atm, 125 atm, 62.5 atm and 31.25 atm.

**Hot radiolabelled cRNA probe**

DEPC H$_2$O 4.5 μl was added to 5X transcription buffer 5 μl and 10mM 1 μl of each of the following nucleotides: ATP, GTP, and UTP; 1 μl RNAse inhibitor, 2 μg of HIND III digested plasmid, 1 μl of DTT, 0.05mM CTP, 7.5μl [P$^{32}$]-CTP and lastly 1 μl (10 U/μl) of T7 polymerase. This was mixed and spun and incubated at 37°C for 45 minutes. Subsequently RNAse free DNase 1.25 μl was added, mixed, spun and incubated at 37°C for 15 minutes.

DEPC H$_2$O 75 μl was added to make up to 100 μl. Then 100μl of phenol:chloroform:isoamyl alcohol at ratio of 50:49:1 was added and spun on high for 5 minutes. The aqueous phase was then pipetted off and loaded onto the centre of Sephadex G50 columns (which were pre-prepared by spinning at 750 rpm for 1 minute) and spun for 4 minutes at 1100 rpm at 4°C resulting in 100 μl of cRNA. This removed unincorporated nucleotides.

**Measuring quantity of radiolabelled probe**

2μl of hot complementary cRNA probe was added to 4ml of scint fluid and measured using a scintillation-counter.

Based on this the hot probe was diluted to 100,000 cpm/2μl.

**Hybridization: standards, control and samples**

MTPmRNA levels were quantified by solution hybridisation nuclease protection assay using an RPA III RNase protection kit (AMS Bitech Technology Ltd). Total intestinal RNA (10μg) and synthetic mRNA (50-1000 amol) were hybridized
overnight at 42°C with 1x10^5 cpm of cRNA in 10 μl hybridization buffer. Samples were then treated with RNase and precipitated in accordance with the manufacturers instructions.

**Standards**

Six standards were set up consisting of 1 μl of mRNA serially diluted (1000, 500, 250, 125, 62.5, 31.25 atomoles); to this was added 2.3 μl ammonium acetate, 21 μl H₂O, 45 μl ethanol, 2μl yeast tRNA and 2μl of radiolabelled probe added last. These were vortexed, spun down and frozen at -20°C for 30 minutes. Subsequently the samples were spun at 13500 rpm for 30 minutes. The supranatant was removed leaving the pellet which was dissolved in 10μl of hybridization buffer which had been pre-heated to 99°C.

The samples were vortexed repeatedly while keeping at 95°C in a heat block. Then they were incubated in a water bath at 42°C overnight. Then RNase (150 μl) was added and incubated at 37°C for 30 minutes, with revortexing after 15 minutes.

Inactivation buffer (150μl) was added with glycobluue dye, spun for 30 minutes and frozen at -20°C for 30 minutes.

**Controls**

Positive and negative controls were made up for each batch of samples. The positive control had no RNase and therefore the full length of the probe was very radioactive, allowing bands to appear on the gels.

The negative controls did have RNase added and this allowed for complete digestion with absence of band on gel.

The following was made up for controls: 2.3 μl ammonium acetate, 21 μl H₂O, 45 μl ethanol, 2μl yeast tRNA and 2μl of radiolabelled probe added last.
These were vortexed, spun down and frozen at -20°C for 30 minutes. Subsequently the samples were spun at 13500 rpm for 30 minutes. The supranatant was removed leaving the pellet which was dissolved in 10μl of hybridization buffer which had been pre-heated to 99°C.

The samples were vortexed repeatedly while keeping at 95°C in a heat block. Then they were incubated in a water bath at 42°C overnight. Then RNase (150 μl) was added to the negative control only and incubated at 37°C for 30 minutes, with revortexing after 15 minutes.

Inactivation buffer (150μl) was added with glycolblue dye, spun for 30 minutes and frozen at -20°C for 30 minutes.

**Intestinal Samples**

Intestinal sample RNA 20μg, made up to 100μl H2O was added to 260μl ethanol, 10μl ammonium acetate and 2μl of hot radiolabelled probe.

These were vortexed, spun down and frozen at -20°C for 30 minutes. Subsequently the samples were spun at 13500 rpm for 30 minutes. The supranatant was removed leaving the pellet which was dissolved in 10μl of hybridization buffer which had been pre-heated to 99°C.

The samples were vortexed repeatedly while keeping at 95°C in a heat block. Then they were incubated in a water bath at 42°C overnight. Then RNase (150 μl) was added and incubated at 37°C for 30 minutes, with revortexing after 15 minutes.

Inactivation buffer (150μl) was added with glycolblue dye, spun for 30 minutes and frozen at -20°C for 30 minutes.
2.17 Gel

The RNAse-resistant products i.e. those protected by hybridization were separated by electrophoresis on a 6% TBE polyacrilamide sequencing gel containing 7 mol/l urea, 2 mmol/l EDTA and 90 mmol/l Tris-borate, pH8.0. The gel was run at 1500 volts for 30 minutes at 50°C, then the samples were loaded on alternate lanes and run for 90 minutes. After disconnecting 3M filter paper was pressed onto the gel and dried for further 90 minutes. When the gels were dried down they were analyzed on a Fugifilm Molecular Imager FLA-2000 phosphorimager system using Aida/2D densitometry version 2.0 (Raytestgerate, GmbH).

Increasing amounts of synthetic mRNA were used to construct standard curves from which the amount of MTP mRNA in terms of amol MTP mRNA/μg total RNA was calculated.

Intra- and inter-assay variation (n=4) for quantification of human MTP mRNA expression using this method were 4.5% and 13.65% respectively.

2.18 Mtp gene analysis

Genomic DNA was isolated using the Puregene®DNA isolation kit (Gentra Systems Inc., MN, USA). The method for detecting the −400 and −493 G/T polymorphisms was adapted from the method of Karpe et al (1998). A 109-bp DNA product, encompassing the −493 site, was generated by PCR with a 5’ mismatched primer MTP493 1 (5’-GGATTTAAATTTAAACTGTTAATTCATAC) and a 3’primer MTP493 2(5’-AGTTTCACACATCAAGGACAATCATCCTA). The PCR was performed in 50 μl containing 0.2 mM of each dNTPs, 2.5 U Taq DNA polymerase, 40 pmol of each primer and 10X Taq DNA polymerase buffer (50 mM KCl (pH8.3), 10 mM Tris-HCl and 1.5 mM MgCl₂). The reactions were carried out on a DNA engine...
thermal cycler (MJ Research, Inc. MA). After an initial denaturation step (3 min 94°C) the reactions were incubated as follows: 94°C for 1 minute, 47°C for 1 minute and 72°C for 1 minute for 35 cycles followed by an elongation step at 72°C for 5 minutes. PCR products were incubated overnight at 37°C with the restriction enzyme HphI (1U). Restriction fragments were then separated on 12% polyacrilamide and sized by comparison to a DNA step ladder Puc 18 (Promega). This restriction digestion gives rise to 1 full length fragment of 109 bp for homozygotes for the T variant, 2 fragments of 89 and 20 bp for homozygotes for the G variant and 3 fragments of 109, 89, and 20 bp for homozygotes for the GT heterozygotes.

Primers MTP400 1 (5'-CCCTCTTAATCTCTTTTCTAGAA) and MTP400 2 (5'-AAGAATCATATTGACCAGCAATC) were used for genotyping of the −400 A/T polymorphism. The MgCl₂ concentration was increased to 2 mM and the PCR was further optimized by changing the procedure to 35 cycles at 94°C for 0.5 min, 55°C for 1 minute and 72°C for 1 minute, followed by a final elongation step of 72°C for 5 minutes. PCR products were then incubated overnight at 37°C with the restriction enzyme Ssp1 (4U). The −400A allele gives rise to a full-length fragment (838 bp), whereas the −400 T allele gives rise to a cutting site, such that 1 full length fragment of 838 bp for homozygotes for the A variant, 2 fragments of 494 and 344 bp for homozygotes for the T variant and 3 fragments of 838, 494 and 344 bp for the AT heterozygotes.

Genotyping for the MTP −164 T/C polymorphism was performed as previously described (Karpe 1998). The MgCl₂ concentration was 2 mM. Primers used were MTP 164 1 (5'-GGTTTGGTTTAGCTCTCTAAAGT) and MTP164 2 (5'-AGTGAGGAGTGACCCCTCTTC). The amplification cycle started with
Denaturation at 94 °C for 3 minutes, followed by 40 cycles at 94°C for 45 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by a final elongation step of 72°C for 5 minutes. PCR products were digested with Bsr I (10 U).

This restriction digestion gives rise to 1 full length fragment of 220 bp for homozygotes for the TT variant, 2 fragments of 176 and 44 bp for homozygotes for the CC variant and 3 fragments of 220, 176 and 44 bp for TC heterozygotes.

2.19 ApoE polymorphism

Genotyping for the ApoE polymorphism was performed using the Amplification Refractory Mutation System (ARMS) as previously described by Wenham et al (1991).

2.20 Ultrasonography and image analysis

Carotid ultrasonography was performed as described in the Epidemiology of Diabetes Interventions and Complications study (EDIC). A single longitudinal lateral view of the distal 10 cm of the right and left common carotid arteries and the three longitudinal views in different planes of each internal carotid artery was defined as including both the carotid bulb, identified by the loss of the parallel wall present in the common carotid artery and the 10mm segment of the internal carotid artery distal to the tip of the flow divider that separates the external and internal carotid arteries. The maximum IMT of the common carotid artery was defined as the mean of the maximum IMT for near and far walls in both left and right sides. The maximum IMT of the internal carotid artery was defined in the same way and the results from the three scans averaged (anterior, lateral and posterior views on both sides).
2.21 Exercise stress test

The exercise stress test was performed on a treadmill with the patient attached to a 12 lead running ECG, according to the standard Bruce protocol. The results were reviewed by a cardiologist, graded and appropriate action taken.

2.22 Statistical analysis

For normally distributed data, groups were compared using the 2 tailed- Student t-test. Non parametric tests were used for unequal distributions. Correlation coefficients were measured by linear regression analysis. The results were expressed as the mean ± standard error of the mean (SEM) in tables and graphs. Interassay and intrassay variation is expressed as the standard deviation/ mean x100. A p value of <0.05 was regarded as statistically significant. Allele frequencies were calculated by gene counting. The normalized linkage disequilibrium coefficients were calculated using Arlequin Software.
Chapter 3

Intestinal microsomal triglyceride transfer protein in Type 2 diabetic and non-diabetic subjects (not on statins): The relationship to triglyceride-rich postprandial lipoprotein composition
Abstract

Microsomal triglyceride transfer protein (MTP) is a lipid transfer protein responsible for the assembly of chylomicrons in the intestine and very low density lipoproteins (VLDL) in the liver. There is one apoB48 per chylomicon and it is the apoB48 which defines the intestinally derived particle and allows these particles to be quantified. This group has previously reported an increase in postprandial apo B48 in human Type 2 diabetes, with some evidence to suggest that synthesis of chylomicrons as well as clearance may account for the abnormality. Also we have previously shown in animal models of diabetes an increase in intestinal levels of MTPmRNA. In this study we examined the relationship between intestinal MTP mRNA and postprandial triglyceride-rich lipoprotein composition in 15 Type 2 diabetic and 17 non-diabetic subjects.

The diabetic patients were shown to have increased levels of intestinal MTP mRNA expression (25±6.25 vs 13.1±1.9 amol/μg RNA (mean± SEM, p<0.05). This is in keeping with similar studies of animal models of insulin resistance and diabetes.
In the diabetic patients there was a significant positive correlation between MTP mRNA and postprandial chylomicron cholesterol/apoB, phospholipids/apoB, and triglyceride/apoB (p<0.01).

To our knowledge this is the first time that intestinal MTPmRNA has been quantified in humans and therefore also the first time that intestinal MTP levels in diabetics have been reported to be higher than in controls.

3.2 Introduction

Microsomal triglyceride transfer protein (MTP) is a lipid transfer protein responsible for the assembly of chylomicrons in the intestine and very low density lipoproteins (VLDL) in the liver.

The promoter region of the MTP gene has been shown to contain a negative insulin response element and a positive cholesterol response element (Hagan 1994).

Our group has previously looked at three different models of diabetes and intestinal MTPmRNA expression.

Firstly, in streptozotocin diabetic rats, a model of Type 1 diabetes, increased intestinal MTP mRNA was found, and associated with an increase in chylomicron triglyceride and cholesterol but not particle number (Gleeson 1999).

Secondly, in Zucker obese rats, a model of insulin resistance, our group demonstrated an increase in intestinal and hepatic MTP mRNA expression with an increase in the number of chylomicron particles compared to lean animals (Phillips 2002).

Thirdly, the male New Zealand white rabbit, a model which develops atherosclerosis was also studied (Phillips et al 2002). Diabetes was induced and the lymph ducts cannulated. Intestinal MTP activity and mRNA were higher in the diabetic animals.
There was no significant difference in MTP activity or mRNA expression in the liver, unlike in the case of the fa/fa rats. The diabetic animals were found to have increased triglycerides and decreased HDL cholesterol. They were found to secrete more lymph CM apoB48 and apoB100, and more CM total and esterified cholesterol/hour. The lymph CM in diabetic animals as compared to controls contained less lipid/apoB.

A fourth animal model has been used by Lewis et al (2005) who have shown in the fructose-fed insulin resistant hamster, an increase in the protein mass of intestinal MTP and an association with over secretion of intestinally-derived apo B48 lipoproteins. The authors went on to show that rosiglitazone, an insulin sensitizer, was associated with a reduction in the MTP mass and activity and a reduction in apo B48 and triglyceride secretion.

In human Type 2 diabetes our group has previously reported an increase in postprandial apo B48, with some evidence to suggest that synthesis as well as clearance may account for the abnormality (Curtin 1994, 1996).

The importance of the postprandial phase in the genesis of atherosclerosis is becoming more evident both because chylomicron cholesterol has been shown to deposit in the atherosclerotic plaque (Pal 2003, Proctor 2004) and also because the triglyceride-rich lipoproteins determine the size and density of the LDL particle (Blackburn 2003, Takayanagi 2004). Lipid peroxidation is also thought to play a major part in the atherosclerotic process and recently Rumley et al (2004) have shown a strong relationship between plasma lipid peroxides and non-fasting serum triglycerides with a much weaker relationship to cholesterol.
There is no information, to our knowledge, on intestinal MTP mRNA levels in human subjects. The aim of this study was to examine the relationship between intestinal MTP mRNA and postprandial triglyceride-rich lipoprotein composition in Type 2 diabetic and non-diabetic subjects.

3.3 Subjects, material and methods

Subjects

A series of subjects undergoing routine biopsy for non-malignant disease were asked to donate 4 extra second part of duodenum (D2) biopsies for MTP mRNA determination. Seventeen control subjects and 15 Type 2 diabetic patients who were found to have normal duodenal mucosa on histological examination and were not taking lipid lowering agents were included in the study. Subjects with coeliac, thyroid, renal or hepatic disease, were excluded. Hospital ethics committee approval was obtained and all subjects gave informed consent. None were on statin treatment.

Intestinal biopsies

Biopsies were obtained from the second part of the duodenum during gastroscopy. Two biopsy samples were collected in formalin and examined histologically to exclude coeliac, inflammatory bowel disease or malignancy. Four separate biopsy samples were collected in RNAlater and stored frozen at −70 for MTP mRNA determination.
**Test Meal**

Within 1 week of the biopsies all patients attended fasting, blood was collected and plasma was separated for lipoprotein measurement. Patients were given an 1100 Kcal high fat test meal. The meal consisted of 2 fried eggs, 2 slices of toast with flora margarine, a milk shake containing 25 ml flora cooking oil, 100 ml full cream milk and 125ml ice cream, and a cup of tea or coffee. Blood samples were repeated 6h postprandially. After separation of plasma the following preservatives were added to prevent oxidation and degradation of apoB: PPACK (1mmol/l), PMSF (0.1mmol/l), sodium azide (0.02% w/v), aprotinin (0.05 TIU), EDTA (0.1%).

**Lipoprotein Isolation**

Chylomicrons and VLDL were isolated by sequential ultracentrifugation. Plasma was overlaid with a 1.006 g/ml density solution and centrifuged at 20,000 rpm at 4°C for 30 min in a Beckman L7-55 ultracentrifuge using a fixed angle rotor. Chylomicrons were carefully removed from the top of the tube with a stretched Pasteur pipette. The density of the infranate was then adjusted to 1.006 g/ml and the solution was centrifuged at 40,000 rpm at 4°C for 18 h to isolate VLDL. Lipoprotein fractions were stored at 4°C and lipoproteins measured within one week.

**Biochemical Analyses**

Venous blood glucose levels were determined according to an enzymatic colorimetric method using a commercially available diagnostic kit (Boehringer Mannheim GmBH, Mannheim, Germany). Blood HbA₁c was determined using an enzyme immunoassay kit, containing monoclonal antibody that is specific for HbA₁c (normal value < 5.8%).
(Novo Nordisk, Cambridge, UK). Commercially available diagnostic kits were used to determine lipoprotein composition. Total cholesterol and triglyceride content of lipoprotein fractions were measured by an enzymatic colorimetric method using kits from Boehringer Mannheim GmbH (Mannheim, Germany) and phospholipids were assayed using a kit from Biomérieux (Marcy l'Etoile, France).

**Chylomicron and VLDL Apolipoprotein B48 and B100 Determination**

Chylomicron and VLDL apolipoprotein B48, and apo B100 were separated by SDS-polyacrylamide gel electrophoresis using 4-15% gradient gels (Biorad, Herculas, Ca. USA) as previously described. Non-delipidated lipoprotein samples (40 µg of protein) were reduced and applied to 4-15% SDS-polyacrylamide gels. Following electrophoresis gels were stained with Coomassie Brilliant Blue. An apoB100 standard was prepared from LDL (density 1.025-1.063 g/ml) of a single individual and was stored at -20°C and used throughout the study. Since the chromogenicity of apoB48 has been shown to be similar to that of apoB100, the concentration of both apoB48 and apoB100 could be determined from this standard. ApoB48 and apoB100 staining was linear within the range 0.1-20 µg of protein. The bands were quantified by densitometry using Vilber Lourmat equipment (Vilber Lourmat Biotechnology, Marne La Valée, and France) and Bio1D v6.32 software (Viber) for analysis. Density values were assigned to the apo B100 band of the human LDL and a standard curve constructed, the values were recalculated by linear regression. Curves with a correlation coefficient >0.95 were accepted. The intra-assay variations were 2.8% and 3.9% for apoB48 in the chylomicron and VLDL fractions and 4.8% and 6.8% for apo B100 in the two fractions. The inter-assay coefficient of variations were 5% and 7%.
for apoB48 and 8% and 8.6% for apoB100 in the chylomicron and VLDL fractions respectively.

**Total RNA extraction**

Total RNA from human biopsies was isolated using the Ultraspec™ RNA isolation system (Biotecx, Houston, Tex., USA). Intestinal biopsies were homogenized on ice in Ultraspec using a mortar and pestle and chloroform extracted. RNA was precipitated with isopropanol and washed with 75% ethanol. Total RNA was quantified by absorbance at 260 nm assuming that 1 OD = 40 μg RNA/μl.

**Transcription vector construction**

Oligo (dT)-primed first strand cDNA synthesis was performed on total RNA extracted from human intestinal tissue. The reverse transcription product was then amplified by PCR using primers designed from conserved homologous regions across the different species. Primers 5'-TCAGCAGAGGGAGAGC-3' and 3' TATCTACCTCATTGGTGGTTCC-5' which flank a 229 base pair fragment corresponding to nucleotides 365 to 594 of the human MTP cDNA sequence were used in this PCR. The PCR was carried out (50°C for 30 sec, 72°C for 45 sec, 93°C for 30 sec) for 35 cycles and the 229 base pair product was blunt-end cloned into the EcoRV site of pBluescript II KS vector (Stratagene Ltd). The clone was then sequenced to confirm its identity and orientation.

**Ribonuclease Protection Assay (RPA)**

A radiolabelled single-stranded probe that is complementary to part of the target RNA to be analyzed was synthesized by transcription from a T3 or T7 promoter site, then mixed with sample RNA and incubated under conditions that favour hybridization of complementary transcripts. Single-stranded, unhybridized RNA was digested with RNase. Protected, hybridized fragments were then separated by gel electrophoresis.
and visualized by phosphorimaging.

**Synthesis of riboprobe and synthetic mRNA standard**

[32P]-labeled cRNA probe and synthetic mRNA standard were synthesized by transcription of linearised template DNA cloned into the Bluescript KS vector, using a T3/T7 transcription kit (Stratagene Ltd.), according to the manufacturer's instructions. Unincorporated nucleotides were removed using Sephadex G50 spun columns. Complementary cRNA probe was counted by liquid scintillation and mRNA standard was quantified by absorbance at 260 nm.

**Quantitation of MTP mRNA by Ribonuclease Protection Assay**

MTP mRNA levels were quantified by solution hybridization nuclease protection assay using an RPA III RNase protection kit (AMS Biotechnology Ltd.). Total intestinal RNA (10μg) and synthetic mRNA (50-1000 amol) were hybridized overnight at 42°C with 1x10⁵ cpm of cRNA in 10 μl hybridization buffer. Samples were then treated with RNase and precipitated in accordance with the manufacturers instructions. The RNase-resistant products were separated by electrophoresis on a 6% acrylamide gel containing 7mmol/l urea, 2mmol/l EDTA and 90 mmol/l Tris-borate, pH8.0. Gels were dried down and analyzed on a Fugifilm Molecular Imager FLA-2000 (phosphorimager) system using Aida/2D densitometry version 2.0 (Raytestgeräte, GmbH). Increasing amounts of synthetic mRNA were used to construct standard curves from which the amount of MTP mRNA in terms of amol MTP mRNA/μg total RNA was calculated. All mRNA values are expressed in amol MTP mRNA/μg total RNA. Intra- and inter-assay variation (n=4) for quantification of human MTP mRNA expression using this method were 4.5% and 13.65% respectively.
Statistical Analysis

Statistical analysis was performed using the Student's t-test. Non-parametric tests were used for triglyceride analysis. Correlation coefficients were measured by linear regression analysis. Results are expressed as the mean±standard error of the mean (SEM) and a p value of <0.05 was regarded as statistically significant.
Patient characteristics

Subject data is presented in Table 1. There were fifteen diabetic and seventeen control patients. The control group consisted of five men and twelve women and the diabetic group consisted of nine men and six women. All were Caucasian. Both groups had an average body mass index of greater than 25 with the diabetic group having a tendency to be heavier and older. Diabetic subjects had moderate glycaemic control (HbA1c 7.4±0.3%). Plasma total cholesterol, LDL and HDL cholesterol and triglyceride were similar between the 2 groups. There was a tendency for the HDL to be higher in the control group as expected though the standard deviations were large at 0.4 for the control group and 0.3 for the diabetic group.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>M/F</td>
<td>5/12</td>
<td>9/6</td>
</tr>
<tr>
<td>Age</td>
<td>50.0±3.3</td>
<td>62.9±3.6</td>
</tr>
<tr>
<td>BMI kg/m$^2$</td>
<td>26.0±1.8</td>
<td>32.9±2.4</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.2±0.4</td>
<td>8.6±1.2</td>
</tr>
<tr>
<td>Plasma triglyceride (mmol/l)</td>
<td>1.5±0.2</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td>Range</td>
<td>0.8-4.7</td>
<td>0.7-3.5</td>
</tr>
<tr>
<td>Median</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/l)</td>
<td>5.6±0.2</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.4±0.2</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.6±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>HbA1c</td>
<td></td>
<td>7.4±0.3</td>
</tr>
</tbody>
</table>

Mean±SEM.
**Intestinal biopsies.**

Five of the non-diabetic subjects had no abnormality on gastroscopy, 7 had mild gastritis, 2 had mild oesophagitis and 3 mild gastritis and oesophagitis. Diabetic patients had a similar distribution of mild histological abnormalities in the oesophagus and stomach.
3.4 Results

Intestinal MTP mRNA was significantly higher in the diabetic subjects compared to non-diabetic subjects (25±6.25 vs 13.1±1.9 amol/µg RNA (p<0.05) (Fig 1). There was no significant difference between male and female intestinal MTPmRNA levels in either diabetic or non-diabetic groups.

Fig. 1. Intestinal MTP mRNA in type 2 diabetic and non-diabetic subjects. Data is expressed mean±SEM. * p <0.05 compared to non-diabetic subjects.
There was no correlation between MTPmRNA and the following: HbA1c, fasting glucose, BMI, age, fasting serum triglycerides, total cholesterol, LDL and HDL. There was also no correlation between MTPmRNA and chylomicron or VLDL apoB48 or apoB100 either fasting or postprandially.
Chylomicron composition is shown in Table 2.

There were no significant differences between the diabetic and nondiabetic group in the chylomicron fraction, either in the number of particles (as measured by apoB 48 and apoB100) or in the composition of the particles.

**Table 2** Chylomicron composition (mg/ml plasma) in diabetic and non-diabetic subjects. Mean±SEM

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>Postprandial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>4±1</td>
<td>14±2</td>
<td></td>
</tr>
<tr>
<td>Apo B100</td>
<td>8±1</td>
<td>20±5</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20±4</td>
<td>61±20</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>40±6</td>
<td>217±37</td>
<td></td>
</tr>
<tr>
<td>Phospholipids</td>
<td>44±13</td>
<td>69±7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fasting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>4±1</td>
<td>10±2</td>
<td></td>
</tr>
<tr>
<td>Apo B100</td>
<td>6±1</td>
<td>17±3</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>17±5</td>
<td>46±10</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>40±10</td>
<td>230±41</td>
<td></td>
</tr>
<tr>
<td>Phospholipids</td>
<td>17±4.2</td>
<td>63±11</td>
<td></td>
</tr>
</tbody>
</table>
VLDL composition is shown in Table 3. There were no significant differences between the diabetic and nondiabetic group in the VLDL fraction, either in the number of particles (as measured by apoB 48 and apoB100) or in the composition of the particles.

Table 3 VLDL composition (mg/ml plasma) in diabetic and non-diabetic subjects

<table>
<thead>
<tr>
<th></th>
<th>Control Fasting</th>
<th>Control Postprandial</th>
<th>Diabetes Fasting</th>
<th>Diabetes Postprandial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B48</td>
<td>3±1</td>
<td>5±2</td>
<td>3±1</td>
<td>5±2</td>
</tr>
<tr>
<td>Apo B100</td>
<td>28±7</td>
<td>43±10</td>
<td>34±9</td>
<td>42±7</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>64±20</td>
<td>93±22</td>
<td>74±11</td>
<td>107±19</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>219±50</td>
<td>371±131</td>
<td>317±60</td>
<td>447±89</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>96±35</td>
<td>133±38</td>
<td>150±27</td>
<td>161±26</td>
</tr>
</tbody>
</table>
Correlations in the postprandial period

In the diabetic group there was a positive correlation between intestinal MTPmRNA and the cholesterol content of the postprandial particles in the chylomicron fraction as measured by cholesterol/total apo B ($r = 0.63, p < 0.01$). The diabetic patients also had a positive correlation between intestinal MTPmRNA and postprandial chylomicron phospholipid/total apo B ($r = 0.66, p < 0.01$), and triglyceride/total apo B ($r = 0.68, p < 0.01$).

Figure 2

![Diabetic patients (n=15)](image)
Postprandial Chylomicron fraction
3.5 Discussion

There were no differences found between controls and diabetic subjects in fasting plasma triglycerides, total cholesterol, LDL cholesterol or HDL cholesterol. This was surprising and suggested that the group of diabetic subjects enrolled in the study were at the least deranged end of the spectrum for dyslipidaemia. None of these patients were on statin treatment at the time of the study as this was an exclusion criteria of the study. As lipid abnormalities are routinely screened for and aggressively treated in our diabetic clinics, the patient population from which we could draw for this study would have been skewed for those patients with almost normal fasting lipids.

Despite the similarities in the fasting lipid profile, the diabetic patients were shown to have increased intestinal MTP mRNA expression. This is in agreement with animal studies previously done by our group in the insulin resistant Zucker rat (Gleeson 1999), the streptozocin treated rat and the alloxon treated diabetic New Zealand rabbit. (Phillips 2002, 2002).

These results may be explained by the fact that the promoter region of the MTP gene contains a negative insulin response element (Hagan 1994). More recently, Au et al (2003) have demonstrated that insulin down-regulation of MTP gene transcription occurs through the mitogen activated protein kinase (MAPK)\textsuperscript{erk} signaling cascade. Intestinal MTPmRNA levels have been found to be positively correlated with insulin levels in the insulin resistant Zucker rat and negatively correlated with the glucose/insulin ratio (Phillips et al 2002). It is uncertain how much of this difference is due to the relative or actual insulin deficiency of the diabetic subjects, leading to loss of suppression through the insulin
response element and how much due to disturbed fatty acid metabolism which occurs in diabetes and which has activity at the MTP promoter region (Phillips et al 2005). However we did not measure insulin levels or assess for degree of insulin resistance in this study which would have been useful. The number of particles as measured by apoB48 and apoB100 in the chylomicron and VLDL fraction were similar between the two groups. This was surprising as in the insulin resistant Zucker rat (Phillips et al 2002) and in the diabetic alloxan treated New Zealand rabbit (Phillips et al 2002) there was an increase in the number of chylomicron particles. Also Curtin et al (1996) has demonstrated in subjects with Type 2 diabetes an increase in plasma apoB48 post-prandially. However in the streptozocin rat there was no change in the number of intestinally derived particles compared to controls (Gleeson et al 1999). A disadvantage of our study was the inability to differentiate synthesis from catabolism. The animal studies described above had negated the effect of catabolism and clearance by the technique of cannulation of the lymphatic duct. Such invasive studies in humans are unfortunately not feasible.

There was a preponderance of males in the diabetic group unlike the control group and they were tending to an older age. The effect of age and gender on intestinal MTP levels in humans has not been reported in the literature. However Ameen et al (2003) reported higher levels of hepatic MTPmRNA in female compared to male rats and that gonadectomy abolished this sex difference.

In the chylomicron and VLDL fractions we found no significant differences in the cholesterol, triglyceride or phospholipid level between diabetic and control subjects.

There was a tendency for the phospholipid content of the chylomicrons in the diabetic group to be lower though this was not significant. This was a little surprising as mtp
would be expected to increase the lipidation of the chylomicron fraction. Postprandially the levels approached more closely those of the control group which may underline the importance of mtp in the postprandial period. Previous studies by our group have shown differing results in phospholipid levels. In the insulin resistant rat there was a significant increase in the lymph chylomicron phospholipids compared to controls (Phillips 2002) while in the diabetic rabbit in 2 separate studies there was no significant difference in the lymph chylomicron phospholipid level compared to controls (Phillips 2002). Plasma chylomicron phospholipids levels were also similar in the diabetic and control rabbits. Dietary preferences may also be a reason for differences in the phospholipids levels although Madigan (2000) demonstrated that a linoleic diet was associated with a higher fasting and postprandial phospholipid level compared to an oleic diet but only in the VLDL fraction and only in a diabetic group.

It was not possible in our laboratory to measure the composition of apo B48 and apo B100-containing particles separately within each fraction. Forty to 50% of the chylomicron fraction is made up of apo B100 particles, the other 50-60% being apo B48-containing particles whereas the VLDL fraction contains mostly apo B100-containing particles.

The correlations between particle composition and MTP mRNA observed in the diabetic patients reflect previous cellular and animal studies. MTP has been found in cultured murine hepatocytes to increase the lipidation of apo B (Kulinski 2002). Our group has previously demonstrated an association between increased intestinal MTP mRNA levels and particle composition in animal models of diabetes. In the streptozocin rat (Gleeson et al 1999) there was an increase in the triglyceride and cholesterol content of the chylomicron particles with a positive correlation between intestinal MTPmRNA and lymph cholesterol and triglycerides. In the alloxan New
Zealand rabbit (Phillips et al 2002) there was a delipidation of the chylomicron particles. However in the insulin resistant Zucker rat (Phillips et al 2002) the chylomicron particle composition was similar to controls.

We were unable to show any correlations between intestinal MTPmRNA and particle composition in the non-diabetic subjects. This may have been due to a difference in particle clearance between diabetic and non-diabetic subjects which has been previously been demonstrated (Phillips et al 2002). The level of apoE is a determinant in the efficiency of clearance of chylomicrons and diabetes is associated with apo E-deficient chylomicron particles.

The promoter region of the MTP gene contains a positive sterol response element as well as an insulin response element and it has been shown that sterol depletion results in a decrease in MTP mRNA (Sato 1999). Diabetes is associated with increased synthesis of intracellular cholesterol and this may partly explain the upregulation of MTP gene expression. Although we found no positive correlation between serum cholesterol and intestinal MTPmRNA, it may be that higher levels of cholesterol would correlate with MTP levels.

The present study was not designed to examine specific diets but a number of studies have demonstrated that fatty acids modulate MTP at the transcriptional level (Lin 1994, Bennett 1995, Hertz 1998). Saturated fatty acids (C14-C16), which are agonists for HNF-4α, regulate MTP expression (Sheena 2005). In contrast, omega-3 fatty acids, which are HNF-4α, antagonists, suppress VLDL production (Harris 1990, Brown 1997). Qui et al (2005) have shown that oleic acid, but not palmitic, linoleic or arachadonic acid, stimulates MTP transcription in HepG2 cells, in a sterol response element independent manner.
It is becoming more evident that the postprandial triglyceride-rich lipoproteins play an important part in atherogenesis particularly in diabetes (Mero 2000, Wilhelm 2003). While both chylomicron size and number have been shown to be important determinants of clearance (Martins 1996), the relationship between chylomicron particle size and atherosclerosis has not been explored. A number of studies however have reported that the triglyceride-rich lipoproteins determine the size and density of the LDL particle (Blackburn 2003, Takayanagi 2004). We found a relationship between lipid enrichment of particles in chylomicrons and MTP mRNA in the diabetic subjects. A reduction in MTP through improved diabetic control and statin therapy might be an effective strategy to reduce atherogenic LDL.

Ueshima et al (2004) have shown in male apo E knockout mice, a model of atherosclerosis, that a western-type diet led to up-regulation of MTP expression. In a further publication the authors demonstrated that an inhibitor of MTP reduced atherosclerosis in this mouse model through reduction in both total cholesterol and triglyceride levels (Ueshima 2005). The development of an intestinal inhibitor of MTP that does not affect hepatic MTP and therefore would not cause hepatic steatosis could be beneficial in improving the postprandial lipid profile and attenuating atherosclerosis in humans.
3.6 Conclusion

It is now generally agreed that the postprandial lipoproteins play an important part in atherogenesis (Mero 2000, Wilhelm 2003). MTP regulates the formation of chylomicron particles in the intestine (Hussein 2000).

Intestinal levels of MTPmRNA levels were significantly higher in patients with Type 2 diabetes compared to the control subjects. This is in keeping with similar studies of animal models of insulin resistance and diabetes.

To our knowledge this is the first time that intestinal MTPmRNA has been quantified in humans and therefore also the first time that intestinal MTP levels in diabetics have been reported to be higher than in controls.

Intestinal MTPmRNA in the diabetic patients correlated with the cholesterol, triglyceride and phospholipid content of chylomicrons.

Further studies are needed to define the relationship between intestinal and hepatic MTP but it is possible that a selective inhibitor of intestinal MTP would be beneficial in improving the postprandial lipid profile and attenuating atherosclerosis. This may be of particular importance in the diabetic patient whom MTP mRNA has been shown to be increased in this study. Such inhibitors will also need to be tested for any detrimental effects at level of the myocardium and immune system, where MTP has been recently found also to be active.
Chapter 4

Relationship between intestinal microsomal triglyceride transfer protein and statin use in diabetic and nondiabetic subjects
4.1 Abstract

Microsomal triglyceride transfer protein (MTP) is responsible for the assembly of the triglyceride rich lipoproteins (TRLs) and is increased in diabetic animal models. The MTP promoter region is known to have a sterol response element.

3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) is converted to mevalonate by the action of HMGCoA reductase which is the rate limiting step in cholesterol synthesis. Although the major effect of the statin family of drugs is suppression of hepatic cholesterol synthesis, in patients with Type 2 diabetes statins also have an effect on fasting triglyceride and postprandial TRLs. There is no information to date on the effect, if any, of HMGCoA reductase inhibitors on intestinal MTP levels in human subjects. Reduction in cholesterol synthesis with a statin would be expected to be associated with lower MTP levels through the sterol response element in the promoter region of the MTP gene. This study examined the relationship between intestinal MTPmRNA levels, fasting and postprandial lipoproteins and use of HMGCoA reductase inhibitors in a group of nondiabetic and diabetic subjects.

MTPmRNA was measured by a ribonuclease protection assay.

MTPmRNA was significantly higher in the diabetic patients compared to the nondiabetic patients in the non-statin group (25.0 ±6.25 vs 13.1±1.4 amol/µg total RNA p<0.05) and in the statin-treated group (17.7±2.1 vs 5.8±1.4 amol/µg total RNA p<0.05) respectively. Among the nondiabetic subjects, those on statin therapy had significantly lower levels of MTPmRNA than those not on statins (p<0.05).
Statin therapy may therefore be of particular benefit in diabetic patients who have a significantly higher level of intestinal MTP, in order to curb the postprandial dyslipidaemia which is so marked in diabetes.

There was a positive correlation between intestinal MTPmRNA and cholesterol content of chylomicrons in the group as a whole (r = 0.36, p<0.01). There was also a weak positive correlation between intestinal MTPmRNA and lipid content of the VLDL particles in the diabetic group (r = 0.5, p<0.05). The correlations found between MTPmRNA and chylomicron and VLDL particle composition would be consistent with the previously shown lipidation effect of MTP on apoB.

This study confirms the suggestion that the sterol response element of the MTP gene is an important regulator of MTP transcription in diabetes.
4.2 Introduction

3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) is converted to mevalonate by the action of HMGCoA reductase which is the rate limiting step in cholesterol synthesis. The reaction is regulated by negative feedback of the end product cholesterol. This occurs both in the liver but also to a lesser extent in the intestine.

The effect of HMGCoA reductase inhibitors (or statins) in lowering fasting lipids has been well described. The 4S study (Pyorala et al 1997) showed statins to be associated with a 30% reduction in LDL cholesterol with lesser reductions in triglycerides and an increase in HDL. A prospective meta-analysis of data from over 90,000 participants in 14 randomised trials of statins (Baigent et al 2005) demonstrated a reduction in 5 year incidence of major coronary events, coronary revascularisation and stroke by about 20% per mmol/L reduction in LDL cholesterol, irrespective of the initial fasting lipids or other presenting characteristics including diabetes. Therefore it appears that statins have no enhanced effect in diabetes per say, but as diabetic patients are at greater absolute risk of atherosclerotic disease, the benefit of LDL cholesterol reduction is greater in this group.

Although the major effect of the statin family of drugs is suppression of hepatic cholesterol synthesis, this group has previously shown that in patients with Type 2 diabetes cerivastatin decreased fasting plasma triglyceride and postprandial triglyceride and apo B48 and apo B100 in the chylomicron fraction (Betula 2000).

In patients with combined hyperlipidaemia atorvastatin significantly reduced both large and small chylomicron remnants, using retinyl palmitate as a marker of chylomicron remnants (Westphal 2003). Using more specific markers, atorvastatin has been shown to reduce fasting chylomicron apo B48 and postprandial AUC for apo
B48 in normolipidaemic CAD subjects (Dane-Stewart 2003). In normolipidaemic subjects atorvastatin significantly reduced the postprandial area under the curve (AUC) for triglyceride in the large triglyceride-rich lipoproteins (Parhofer 2000).

Isley et al (2006) compared the effect of high dose simvastatin (80mg for 12 weeks) in patients with type 2 diabetes versus control subjects. They found that TRL-triglyceride secretion was almost 2-fold higher in the diabetic subjects and was unaffected by simvastatin therapy. However, TRL-triglyceride clearance was significantly increased in the diabetic subjects during simvastatin treatment compared with placebo, with an increase in LPL activity resulting in accelerated delipidation of TRL particles.

In contrast Myerson et al (2005) demonstrated in subjects with Type 2 diabetes on high dose simvastatin no effect on the catabolic rates of VLDL-apoB or VLDL-triglyceride but a decrease in VLDL-triglyceride secretion. They postulated that statins may activate peroxisomal proliferator-activated receptor (PPAR) leading to increased hepatic oxidation of fatty acids and less synthesis of triglyceride for VLDL assembly.

There is no information to date on the effect, if any, of HMGCoA reductase inhibitors on intestinal MTP levels. Reduction in cholesterol synthesis with a statin would be expected to be associated with lower intestinal MTP levels through the effect of cholesterol on the sterol response element in the MTP gene (Hagan 1994). This may result in altered chylomicron particle number and/or composition. Therefore the aim of this part of the thesis was to examine the relationship between intestinal MTPmRNA levels, fasting and postprandial lipoproteins and use of HMGCoA reductase inhibitors in a group of nondiabetic and diabetic subjects.
4.3 Methods

There were a series of 24 nondiabetic participants undergoing routine gastroscopy; 7 of whom were on various statin preparations. There were a series of 27 diabetic subjects undergoing routine gastroscopy, 12 of whom were on statin treatment. We excluded those found at gastroscopy to have malignant or coeliac disease. Ethics committee approved was gained and informed written consent was obtained. Subjects were asked to donate 4 extra second part of duodenum (D2) biopsies for MTP mRNA determination. Subjects with thyroid, renal or hepatic disease, were excluded.

Intestinal biopsies

Biopsies were obtained from the second part of the duodenum during gastroscopy. Two biopsy samples were collected in formalin and examined histologically to exclude coeliac, inflammatory bowel disease or malignancy. Four separate biopsy samples were collected in RNALater and stored frozen at -70 for MTP mRNA determination.

Test Meal

Within 1 week of the biopsies all patients attended fasting and blood was collected and plasma separated for lipoprotein measurement. Patients were given an 1100 Kcal high fat test meal. The meal consisted of 2 fried eggs, 2 slices of toast with flora margarine, a milk shake containing 25 ml flora cooking oil, 100 ml full cream milk
and 125ml ice cream, and a cup of tea or coffee. Blood samples were repeated 6h postprandially. After separation of plasma the following preservatives were added to prevent oxidation and degradation of apoB: PPACK (1mmol/l), PMSF (0.1mmol/l), sodium azide (0.02% w/v), aprotinin (0.05 TIU), EDTA (0.1%).

**Lipoprotein Isolation**

Chylomicrons and VLDL were isolated by sequential ultracentrifugation. Plasma was overlaid with a 1.006 g/ml density solution and centrifuged at 20,000 rpm at 4°C for 30 min in a Beckman L7-55 ultracentrifuge using a fixed angle rotor. Chylomicrons were carefully removed from the top of the tube with a stretched Pasteur pipette. The density of the infranate was then adjusted to 1.006 g/ml and the solution was centrifuged at 40,000 rpm at 4°C for 18 h to isolate VLDL. Lipoprotein fractions were stored at 4°C and lipoproteins measured within one week.

**Biochemical Analyses**

Venous blood glucose levels were determined according to an enzymatic colorimetric method using a commercially available diagnostic kit (Boehringer Mannheim GmBH, Mannheim, Germany). Blood HbA1c was determined using an enzyme immunoassay kit, containing monoclonal antibody that is specific for HbA1c (normal value < 5.8%) (Novo Nordisk, Cambridge, UK). Commercially available diagnostic kits were used to determine lipoprotein composition. Total cholesterol and triglyceride content of lipoprotein fractions were measured by an enzymatic colorimetric method using kits from Boehringer Mannheim GmBH (Mannheim, Germany) and phospholipids were assayed using a kit from Biomerieux (Marcy l'Etoile, France).
Chylomicron and VLDL Apolipoprotein B48 and B100 Determination

Chylomicron and VLDL apolipoprotein B48, and apo B100 were separated by SDS-polyacrylamide gel electrophoresis using 4-15% gradient gels (Biorad, Herculas, Ca. USA). Following electrophoresis gels were stained with Coomassie Brilliant Blue. An apoB100 standard was prepared from LDL (density 1.025-1.063 g/ml) of a single individual and was stored at -20°C and used throughout the study. Since the chromogenicity of apoB48 has been shown to be similar to that of apoB100, the concentration of both apoB48 and apoB100 could be determined from this standard. ApoB48 and apoB100 staining was linear within the range 0.1-20µg of protein. The bands were quantified by densitometry using Vilber Lourmat equipment (Vilber Lourmat Biotechnology, Marne La Valee, France) and Bio1D v6.32 software (Viber) for analysis. Density values were assigned to the apo B100 band of the human LDL and a standard curve constructed, the values were recalculated by linear regression. Curves with a correlation coefficient >0.95 were accepted. The intra-assay variations were 2.8% and 3.9% for apoB48 in the chylomicron and VLDL fractions and 4.8% and 6.8% for apo B100 in the two fractions. The inter-assay coefficient of variations were 5% and 7% for apoB48 and 8% and 8.6% for apoB100 in the chylomicron and VLDL fractions respectively.

Total RNA extraction

Total RNA from human biopsies was isolated using the Ultraspec™ RNA isolation system (Biotecx, Houston, Tex., USA). Intestinal biopsies were homogenized on ice in Ultraspec using a mortar and pestle and chloroform extracted. RNA was precipitated with isopropanol and washed with 75% ethanol. Total RNA was
quantified by absorbance at 260 nm assuming that 1 OD= 40 μg RNA/μl.

Transcription vector construction
Oligo (dT)-primed first strand cDNA synthesis was performed on total RNA extracted from human intestinal tissue. The reverse transcription product was then amplified by PCR using primers designed from conserved homologous regions across the different species. Primers 5'-TCAGCAGAGAGGAGAGAAGAGC-3' and 3' TATCTACCTCATTGGTGTTCC-5' which flank a 230 base pair fragment corresponding to nucleotides 365 to 594 of the human MTP cDNA sequence were used in this PCR. The PCR was carried out (50°C for 30 sec, 72°C for 45 sec, 93°C for 30 sec) for 35 cycles and the 230 base pair product was blunt-end cloned into the EcoRV site of pBluescript II KS vector (Stratagene Ltd). The clone was then sequenced to confirm its identity and orientation.

Ribonuclease Protection Assay (RPA)
A radiolabelled single-stranded probe that is complementary to part of the target RNA to be analyzed was synthesized by transcription from a T3 or T7 promoter site, then mixed with sample RNA and incubated under conditions that favour hybridization of complementary transcripts. Single-stranded, unhybridized RNA was digested with RNase. Protected, hybridized fragments were then separated by gel electrophoresis and visualized by autoradiography or phosphorimaging.

Synthesis of riboprobe and synthetic mRNA standard
[32P]-labelled cRNA probe and synthetic mRNA standard were synthesized by transcription of linearised template DNA cloned into the Bluescript KS vector, using a
T3/T7 transcription kit (Stratagene Ltd.), according to the manufacturer's instructions. Unincorporated nucleotides were removed using Sephadex G50 spun columns. Complementary cRNA probe was counted by liquid scintillation and mRNA standard was quantified by absorbance at 260 nm.

Quantitation of MTP mRNA by Ribonuclease Protection Assay

MTP mRNA levels were quantified by solution hybridization nuclease protection assay using an RPA III RNase protection kit (AMS Biotechnology Ltd.). Total intestinal RNA (10 μg) and synthetic mRNA (50-1000 amol) were hybridized overnight at 42°C with 1x10⁵ cpm of cRNA in 10 μl hybridization buffer. Samples were then treated with RNase and precipitated in accordance with the manufacturers instructions. The RNase-resistant products were separated by electrophoresis on a 6% acrylamide gel containing 7mmol/l urea, 2mmol/l EDTA and 90 mmol/l Tris-borate, pH8.0. Gels were dried down and analyzed on a Fugifilm Molecular Imager FLA-2000 (phosphorimager) system using Aida/2D densitometry version 2.0 (Raytestgeräte, GmbH). Increasing amounts of synthetic mRNA were used to construct standard curves from which the amount of MTP mRNA in terms of amol MTP mRNA/μg total RNA were calculated. All mRNA values are expressed in amol MTP mRNA/μg total RNA. Intra- and inter-assay variation (n=4) for quantification of human MTP mRNA expression using this method were 4.5% and 13.65% respectively.
Statistical Analysis

Statistical analysis was performed using the Student's t-test. Non-parametric tests were used for triglyceride analysis. Correlation coefficients were measured by linear regression analysis. Results are expressed as the mean±standard error of the mean (SEM) and a p value of <0.05 was regarded as statistically significant.
4.4 Results

Five of the non-diabetic, non-statin subjects had no abnormality on gastroscopy, 7 had mild gastritis, 2 had mild oesophagitis and 3 had mild gastritis and oesophagitis. In the group of 7 non-diabetic subjects on statin 2 had no abnormality, 2 had mild oesophagitis and 3 had mild gastritis. Diabetic patients had a similar distribution of mild histological abnormalities in the oesophagus and stomach.

Subject characteristics

The patient characteristics are listed in the table below.

Table 1

<table>
<thead>
<tr>
<th>Subjects characteristics</th>
<th>Non-Diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No statin</td>
<td>statin</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>5/12</td>
<td>5/2</td>
</tr>
<tr>
<td>Age</td>
<td>50.0±2.0</td>
<td>65.6±0.4</td>
</tr>
<tr>
<td>BMI kg/m</td>
<td>26.0±0.8</td>
<td>29.8±1.5</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.2±0.1</td>
<td>6.1±0.4</td>
</tr>
<tr>
<td>Plasma triglyceride (mmol/l)</td>
<td>1.5±0.1</td>
<td>2.5±0.9</td>
</tr>
<tr>
<td>Range</td>
<td>0.8-4.7</td>
<td>0.8-6.9</td>
</tr>
<tr>
<td>Median</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/l)</td>
<td>5.6±0.1</td>
<td>5.0±0.6</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.4±0.1</td>
<td>2.0±0.3**</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.6±0.1</td>
<td>1.1±0.2</td>
</tr>
</tbody>
</table>

** p<0.002
Mean + SEM
There was no significant difference between the four groups in BMI but the mean BMI was in the overweight range for all groups. There were no significant differences between the four groups in fasting triglyceride, HDL and glucose. However fasting LDL cholesterol was significantly lower in the nondiabetic group taking HMG CoA reductase inhibitors. There were non-significant trends for the control subjects not on statins to be younger with a lower BMI and lower triglycerides. Diabetic subjects were in moderate glycaemic control, with a mean HbA1c of 7.4±0.3% in subjects not on statin and a mean HbA1c of 9.1±1.0% in statin-treated diabetic subjects (not significant).
Figure 1: Intestinal MTPmRNA levels in Type 2 diabetes (black) and non-diabetic (grey) subjects on statin therapy and not on statins. Data is expressed as amol/μg total RNA (mean ± SEM)

* p < 0.05 compared to non-diabetic subjects in the corresponding group

§ p < 0.05 statin vs. no statin in the non-diabetic group
In the untreated diabetic group compared to the untreated non-diabetic patients intestinal MTP mRNA was 25.0 ±6.25 vs 13.1±1.4 amol/μg total RNA (p<0.05). In the statin-treated diabetic group compared to statin-treated non-diabetic subjects MTPmRNA was 17.7±2.1 vs 5.8±1.4 amol/μg total RNA (p<0.05). Therefore in either statin category MTPmRNA was significantly higher in the diabetic group compared to the nondiabetic group. Among the nondiabetic subjects, those on statin therapy had significantly lower levels of MTPmRNA than those not on statins (p<0.05). Among the diabetic subjects there was a non-significant trend for MTPmRNA to be lower in those on statin therapy than those not on statins.

There were no significant differences between male and female MTP levels in any group.
Chylomicron composition analysis

Chylomicron composition is shown in Table 2 below.

**Table 2**

Chylomicron composition (mg/ml plasma) in control and diabetic subjects untreated or treated with statin

<table>
<thead>
<tr>
<th>Chylomicron</th>
<th>No Statin</th>
<th>Statin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td>postprandial</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>4+0.5</td>
<td>14+2.4</td>
</tr>
<tr>
<td>Apo B100</td>
<td>8+1.2</td>
<td>20+4.4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20+3.9</td>
<td>61+12.4</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>40+4.9</td>
<td>217+41.5</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>44+7.0</td>
<td>69+7.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48</td>
<td>4+0.8</td>
<td>10+1.6</td>
</tr>
<tr>
<td>Apo B100</td>
<td>6+1.3</td>
<td>17+3.6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>17+5.7</td>
<td>46+10.9</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>40+10.3</td>
<td>230+40.1</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>17+3.9</td>
<td>63+10.6</td>
</tr>
</tbody>
</table>

Mean±SEM

*p<0.001 different from control non-statin

There were no significant differences in chylomicron composition either fasting or postprandially between the two nondiabetic groups, except in the postprandial phospholipid. The two diabetic groups had similar chylomicron composition.
VLDL composition is shown in Table 3 below:

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Statin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fasting</td>
<td>postprandial</td>
</tr>
<tr>
<td>Apo B48</td>
<td>3±0.5</td>
<td>5±0.7</td>
</tr>
<tr>
<td>Apo B100</td>
<td>28±3.4</td>
<td>43±5.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>64±4.6</td>
<td>93±9.2</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>219±17.5</td>
<td>371±48.5</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>96±6.1</td>
<td>133±10.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B48</td>
<td>3±0.8</td>
</tr>
<tr>
<td>Apo B100</td>
<td>34±8.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>74±11.1</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>317±57.9</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>150±26.4</td>
</tr>
</tbody>
</table>

Mean±SEM
Analysis of the VLDL composition showed no significant differences either fasting or postprandially between the two nondiabetic groups or between the two diabetic groups. There was however a trend towards lower levels of apoB100 fasting and postprandially in the statin treated control group as compared to the control group not on statins.
Correlations

There were no significant correlations between intestinal MTPmRNA and total cholesterol, HDL cholesterol, triglycerides, and glucose either in the group as a whole or in the four subgroups separately.

Examination of the relationship between the composition of the chylomicron particle and MTP in all the subjects by linear regression analysis revealed a positive correlation between MTP mRNA and chylomicron cholesterol/B48 postprandially (r=0.36, p<0.01 at 6 hours).

Relationship between ln MTP mRNA and ln chylomicron cholesterol/apo B48 6 h after a high fat breakfast in the whole group of diabetic patients (■) and control subjects(□) (r = 0.36, p < 0.01).
However there was no correlation between MTP in the whole group and chylomicron cholesterol/total apoB. There was a stronger positive correlation between intestinal MTPmRNA and the chylomicron cholesterol/apoB48 postprandially in the whole group of subjects when statin-treated patients withdrawn from the analysis ($r = 0.45$, $p < 0.01$). However again there no correlation when total apoB was substituted for apoB48.

Linear regression analysis was also performed between MTP mRNA and VLDL particle composition in the whole group. There was a weak correlation between MTP mRNA and total lipid/total apo B ($r = 0.5$, $p < 0.05$).

There was no correlation between MTP mRNA and apo B or particle composition in the non-diabetic patients as a group.
4.5 Discussion

The patients in this study were from a series passing through our endoscopy suite. Most participants have routine diagnostic endoscopies done only once, and to repeat an invasive procedure for research purposes would have been difficult to justify ethically. We would have liked to have measured intestinal MTPmRNA levels before and after HMGCoA reductase inhibition in our subjects. In this way each participant would have been their own control with the only variable being statin use. Nevertheless it may be reasonable to assume that those on statin treatment had the usual 20% reduction in cholesterol with a smaller effect on triglycerides.

The promoter region of the MTP gene contains a positive sterol response element and it has been shown that sterol depletion results in a decrease in MTPmRNA (Satto 1999). These observations would support the prediction that statin therapy would be associated with reduced MTP expression. In the current study we found decreased levels of intestinal MTPmRNA in both diabetic and nondiabetic subjects on statin therapy.

In addition to suppression of hepatic cholesterol synthesis, statins also have a strong triglyceride lowering activity (Betula et al 2000, Westphal et al 2003, Dane-Stewart et al 2003, Parhofer et al 2000). It is reasonable therefore to speculate that the reduction in MTPmRNA was at least in part, due to the effect of the statins on triglyceride metabolism. We do not have full data on cholesterol or triglyceride levels in statin treated patients prior to statin therapy but assume a lowering effect on triglycerides. There was no difference in the composition of the chylomicron particle (other than the postprandial phospholipid) when patients on statin were compared with to those not on statin.
This was not expected as our group has previously shown that cerivastatin reduces cholesterol, triglyceride and phospholipids in the chylomicron particle (Batulla et al 2000). A drawback to the present study is that this is a cross-sectional study and it is possible that factors other than statin therapy have come to bear. However this is unlikely to be the case with MTP levels since similar differences in MTP were observed in diabetic and non-diabetic groups on and off statins.

We did not measure the composition of apoB48 and apoB100 containing particles separately. Forty to fifty percent of the chylomicron fraction is made up of apoB100-containing particles, the other 50-60% being made up of apoB48-containing particles. In the present study we observed little difference between diabetic and non-diabetic subjects in the chylomicron and VLDL composition but this may be due to the fact that our patients not on statin therapy had particularly low lipoprotein levels since our policy now is to treat even mild dyslipidaemia of diabetes aggressively with statins.

We did not have the opportunity to measure hepatic MTP mRNA in this study, but have previously shown an increase in intestinal but not hepatic MTPmRNA in diabetic rabbits compared to control animals (Phillips et al 2002), whereas in the insulin-resistant fatty Zucker rat both intestinal and hepatic MTPmRNA were increased compared to control animals (Phillips et al 2002). In this study, analysis of the VLDL fraction, which contains mostly apoB100-containing particles demonstrated a weak relationship between MTP and the lipid composition of the particle in the diabetic subjects suggesting that intestinal MTP may reflect hepatic MTP activity.
MTP increases the lipidation of apoB (Kulinski et al 2002, Hussein 2000) and this would be consistent with the correlations found between MTPmRNA and chylomicron and VLDL particle composition. It is becoming more evident that the postprandial triglyceride rich lipoproteins play an important part in atherogenesis (Taskinen 2001, Mero et al 2000, Wilhelm et al 2003), particularly in diabetes. While both chylomicron size and number have been shown to be important determinants of clearance (Martins et al 1996), the relationship between chylomicron particle size and atherosclerosis has not been explored. A number of studies however have reported that the triglyceride rich lipoproteins determine the size and density of the LDL particle (Blackburn et al 2003, Takayanagi et al 2004, Lahdenpera et al 1996). In the present study we found a relationship between lipid per particle, in both chylomicrons and VLDL, and MTPmRNA in the diabetic subjects. Poorly controlled diabetic patients are at risk of having small dense LDL and since large VLDL particle size is associated with the proatherogenic small dense LDL phenotype (Taskinen 2001, Lahdenpera et al 1996, Koba et al 2003), it is possible to speculate that there may be a relationship between increased MTP and small dense LDL.

Ueshima et al (2004) have shown in male apoE knockout mice, a model of atherosclerosis, a western type diet led to up-regulation of MTP expression. In a further publication the authors demonstrated that an inhibitor of MTP reduced atherosclerosis in this mouse model through reduction in both total cholesterol and triglyceride levels (Ueshima et al 2005). Our results illustrate that diabetic patients have increased intestinal MTPmRNA expression and that statin therapy was associated with a lower MTPmRNA levels in both diabetic and non-diabetic subjects.
4.6 Conclusion

Intestinal MTPmRNA was significantly higher in the diabetic group compared to the nondiabetic group in either statin category. Among the nondiabetic subjects, those on statin therapy had significantly lower levels of MTPmRNA than those not on statins. The correlations found between MTPmRNA and chylomicron and VLDL particle composition would be consistent with the previously shown lipidation effect of MTP on apoB. Further studies are needed to define the relationship between intestinal and hepatic MTP. It is possible that intestine specific inhibitors of MTP with little effect on hepatic MTP (which would avoid hepatic steatosis) would improve the postprandial lipid profile and attenuate atherosclerosis. This may be of particular importance in the diabetic patient whose lipid metabolism is particularly disturbed and whose MTPmRNA has been shown to be increased.

The newly discovered lipid transport functions of MTP in the myocardium and the immune system may have implications for the development of MTP inhibitors. Since it has recently emerged that MTP is an ancient protein which is widely distributed throughout invertebrate and vertebrate species, its functions of intracellular lipid transport are likely to be even more widespread than thus far appreciated. Therefore it may be that inhibition of one of the functions of MTP, namely the MTP-apoB interaction but not of other functions such as the lipid transfer activity will be the direction of future drug development.
Chapter 5

The effect of a common promoter region polymorphism of microsomal triglyceride transfer protein gene on fasting and postprandial lipoproteins in Type 2 diabetes
5.1 Abstract

Non-diabetic subjects homozygous for the T allele at position -493 of the promoter region of the Mtp gene have been shown to have decreased numbers of lipid-rich VLDL particles and significantly lower total cholesterol and LDL cholesterol. We examined the effect of the common -493 G/T gene polymorphism, and also the MTP -400 A/T and -164 T/C polymorphisms on postprandial triglyceride-rich lipoproteins and LDL in 82 subjects with Type 2 diabetes. Forty-five subjects (55%) were homozygous for the MTP -493 G allele, 33 (40%) were heterozygous G/T and four were homozygous for the T allele (5%). LDL cholesterol was significantly lower in subjects with the T allele ($p < 0.05$), and total cholesterol was reduced by 8% in carriers of the T allele ($p < 0.058$).

Diabetes appears to amplify the effect of MTP polymorphisms on the assembly of apoB-containing lipoproteins with an alteration in the postprandial lipoprotein composition resulting in lower LDL.

The -400 A/T substitution gave very similar lipoprotein results but there was significant linkage disequilibrium between the two polymorphisms. No association was found between the -164 T/C polymorphism and either plasma lipids or postprandial lipoproteins. ApoE genotype was also examined but did not influence the above results.
5.2 Introduction

Cardiovascular disease is up to four times more common in diabetes, and up to 50% of patients admitted to the coronary care unit have diabetes or impaired glucose tolerance. Hypercholesterolaemia, and in particular raised levels of LDL cholesterol, are an important cardiovascular risk factor for both diabetic and non-diabetic patients. There is considerable alteration in the triglyceride-rich lipoproteins in the postprandial period in diabetes (Tomkin et al 2001) and these particles have an important influence on LDL composition and size (Koba et al 2003, Packard et al 2000). We and others have demonstrated an increase in the intestinally-derived chylomicron particles in diabetes (Duvillard et al 2000, Curtin et al 1996, Taggart et al 1997, McEneny et al 2000) and we have shown reduction in these levels following improved diabetic control (Phillips et al 2000). Recently, the role of postprandial lipoproteins in atherogenesis has become more apparent, and Mero et al (2000) have demonstrated a relationship between postprandial apoB48 and the severity of coronary heart disease in Type 2 diabetic patients. Microsomal triglyceride transfer protein (MTP) regulates the production of the chylomicrons in the intestine, and VLDL in the liver, by influencing the assembly of the lipoprotein particle (Hussein 2000). ApoB48 defines the intestinally-derived particle and apoB100 the hepatically-derived particle. In animal studies we have demonstrated an increase in intestinal MTP mRNA expression and MTP protein in diabetes (Gleeson et al 1999, Phillips et al 2002) and also in non-diabetic animals with insulin resistance (Phillips et al 2002). The increase in MTP mRNA was associated with an increase in chylomicron particle number, a potentially atherogenic alteration (Mamo et al 1998).
In Type 2 diabetes, the common -493 G/T MTP polymorphism has been associated with surrogate markers of non-alcoholic hepatic steatosis (Bernard et al 2000). The MTP -493 T/T polymorphism has been shown to be associated with a higher concentration of small LDL in Type 2 diabetic patients of Chinese origin, but there was no effect in the heterozygous subjects (Chen et al 2003). It has been suggested that the T allele may interact with visceral obesity and hyperinsulinaemia in non-diabetic subjects (St Pierre et al 2002).

In non-diabetic subjects heterozygous for the T allele, no changes in VLDL or LDL triglyceride or cholesterol have been found, whereas the few subjects homozygous for the T allele had decreased numbers of lipid-rich VLDL particles and significantly lower total cholesterol and LDL cholesterol (Karpe et al 1998).

Examination of fasting lipids in a healthy young Black male population demonstrated that the rare T/T genotype was associated with a higher mean level of apoB related lipids, suggesting possible racial differences (Juo et al 2000).

The only study to date to examine the influence of the -493 G/T polymorphism on postprandial lipoproteins, (Lundahl et al 2002), revealed that subjects homozygous for the T/T polymorphism showed an increase in apoB48 in the smallest triglyceride-rich lipoprotein fraction postprandially, without a difference in postprandial triglycerides or in fasting plasma total cholesterol and LDL cholesterol. However there was a trend towards lower cholesterol and LDL cholesterol.

Other polymorphisms in the promoter region of the MTP gene have been identified, including the MTP -164 T/C, and the MTP -400 A/T (Hermann et al 1998, Havel et al 1955). Information on the -164 T/C and -400 A/T polymorphisms is scant (Karpe et al
The study by Herrmann et al (1998) failed to find any association between either of the polymorphisms and plasma lipid levels. More recently, Ledmyer et al (2002) found a significant association between the rare homozygous -164 T/C genotype and plasma total cholesterol and LDL cholesterol. They suggested that this might be due to the almost complete allelic association between this polymorphism and the -493 G/T polymorphism.

Since Type 2 diabetes is associated with major disturbances in the triglyceride-rich lipoproteins, and since in animal studies diabetes is associated with an increase in MTP expression, our hypothesis was that diabetes may amplify the effect of MTP polymorphisms on the assembly of apoB-containing lipoproteins, and that an alteration in the postprandial lipoprotein composition would result in lower LDL. The aim of the study was to examine the effect of the common -493 G/T gene polymorphism, and also the MTP -400 A/T and -164 T/C polymorphisms on postprandial triglyceride-rich lipoproteins and LDL in Type 2 diabetes.
5.3 Methods

Patients

Patients were recruited from the hospital diabetes unit. Inclusion criteria were that the patients were Caucasian, and in stable diabetic control, treated with diet or oral therapy. Exclusion criteria included abnormal renal or hepatic function, familial hypercholesterolaemia, unstable thyroid function, or being on lipid-lowering therapy. Eighty-two patients were both suitable and agreed to take part in the study. Ethics Committee approval was obtained for the study, and all subjects gave informed consent.

Design

On the day of the study, fasting blood was collected and plasma separated for lipoprotein measurement. Cells were stored frozen for MTP polymorphism determination and apoE genotyping. Patients were given an 1100 kcal high-fat test meal, and blood samples were taken at 6 h postprandially. After separation of plasma, the following preservatives were added to prevent oxidation and degradation of apoB: PPACK (1 mM), PMSF (0.1 mM), sodium azide (0.02% w/v), aprotinin (0.05 TIU) and EDTA (0.1%).

Lipoprotein isolation

Lipoproteins were separated from 10 ml plasma. Plasma was overlaid with a 1.006 g/ml density solution, and centrifuged at 20 000 rpm at 4°C for 30 min in a Beckman L7-55 ultracentrifuge using a fixed-angle rotor. Chylomicrons were carefully removed from the top of the tube with a stretched Pasteur pipette, and the volume was
measured. The density of the infranate was then adjusted to 1.006 g/ml, and the solution was centrifuged at 40,000 rpm at 4°C for 18 h to isolate VLDL. Lipoprotein fractions were stored at 4°C and lipoproteins measured within one week.

**Chylomicron and VLDL apolipoprotein B48 and B100 determination**

Chylomicron and VLDL apolipoprotein B48, and apo B100 were separated by SDS-polyacrylamide gel electrophoresis using 4–15% gradient gels (Biorad). Non-delipidated lipoprotein samples (40 μg of protein) were reduced and applied to 4–15% SDS-polyacrylamide gels. Following electrophoresis, gels were stained with Coomassie Brilliant Blue. An apoB100 standard was prepared from LDL (density 1.025–1.063 g/ml) from a single individual, and was stored at -20°C and used throughout the study. Since the chromogenicity of apoB48 has been shown to be similar to that of apoB100, the concentrations of both apoB48 and apoB100 could be determined from this standard. ApoB48 and apoB100 staining was linear within the range 0.1–20 μg of protein. The bands were quantified by densitometry using Vilber Lourmat equipment (Vilber Lourmat Biotechnology) and BioID v6.32 software (Vilber) for analysis. Density values were assigned to the apo B100 band of the human LDL and a standard curve constructed, with the values being recalculated by linear regression. Curves with a correlation coefficient > 0.95 were accepted. Results were expressed as μg/ml plasma. The intra-assay variations were 2.8% and 3.9% for apoB48 in the chylomicron and VLDL fractions and 4.8% and 6.8% for apo B100 in the two fractions. The inter-assay coefficient of variations were 5% and 7% for apoB48 and 8% and 8.6% for apoB100, in the chylomicron and VLDL fractions, respectively.
Biochemical analyses

Venous blood glucose levels were determined by an enzymic colorimetric method using a commercially available diagnostic kit (Boehringer Mannheim). Blood HbA1c was determined using an enzyme immunoassay kit, containing monoclonal antibody specific for HbA1c (Novo Nordisk). The normal value was taken as < 5.8%.

Commercially available diagnostic kits were used to determine lipoprotein composition. Total cholesterol and triglyceride content of lipoprotein fractions were measured by an enzymic colorimetric method using kits from Boehringer Mannheim, and phospholipids were assayed using a kit from Biomerieux.

DNA analysis

Genomic DNA was isolated using the Puregene DNA isolation kit (Gentra Systems). The method for detecting the -400 and -493 G/T polymorphisms was adapted from the method of Karpe et al (1998). A 109-bp DNA product, encompassing the -493 site, was generated by PCR with a 5' mismatched primer MTP493 1 (5'-GGATTTAAATTTAAACTGTATATCATCAG) and a 3' primer MTP493 2 (5'-AGTTTCACACTCAAGGACAATCTCAG). The PCR was performed in 50 µl containing 0.2 mM of each dNTPs, 2.5 U Taq DNA polymerase, 40 pmol of each primer and 10× Taq DNA polymerase buffer (50 mM KCL (pH 8.3), 10 mM Tris-HCl and 1.5 mM MgCl2). The reactions were carried out on a DNA engine thermal cycler (MJ Research). After an initial denaturation step (3 min at 94°C) the reactions were incubated as follows: 94°C for 1 min, 47°C for 1 min and 72°C for 1 min for 35 cycles, followed by a final elongation step at 72°C for 5 min. PCR products were incubated overnight at 37°C with the restriction enzyme HphI (1U). Restriction fragments were then separated on 12% polyacrylamide and sized by comparison to a DNA step ladder Puc 18 (Promega). This restriction digestion gives rise to one full-
length fragment of 109 bp for homozygotes for the T variant, two fragments of 89 and 20 bp for homozygotes for the G variant, and three fragments of 109, 89 and 20 bp for GT heterozygotes.

Primers MTP400 1 (5'-CCCTCTTAAATCTCTTTCCCTAGAA) and MTP400 2 (5'-aagaatcattgaccgcaatc) were used for genotyping of the -400 A/T polymorphism. The MgCl₂ concentration was increased to 2 mM and the PCR was further optimized by changing the procedure to 35 cycles at 94°C for 0.5 min, 55°C for 1 min and 72°C for 1 min, followed by a final elongation step of 72°C for 5 min. PCR products were then incubated overnight at 37°C with the restriction enzyme Ssp1 (4U). The -400 A allele gives rise to a full-length fragment (838 bp), whereas the -400 T allele gives rise to a cutting site, giving one full-length fragment of 838 bp for homozygotes for the A variant, two fragments of 494 and 344 bp for homozygotes for the T variant, and three fragments of 838, 494 and 344 bp for AT heterozygotes.

Genotyping of the MTP -164 T/C polymorphism was performed as previously described (Bernarde et al 2000). The MgCl₂ concentration was 2 mM. Primers used were MTP164 1 (5'-GGTTTGGTTTAGCTCTCAAAAGTG) and MTP164 2 (5'AGTGAGGGAGTGACCCTCTTTCCCT). The amplification cycle started with denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 45 s, 60°C for 30 s and 72°C for 1 min, followed by a final elongation step of 72°C for 5 min. PCR products were digested with BsrI (10U). This restriction digestion gives rise to one full-length fragment of 220 bp for homozygotes for the TT variant, two fragments of 176 and 44 bp for homozygotes for the CC variant and three fragments of 220, 176 and 44 bp for TC heterozygotes.
Genotyping for the ApoE polymorphism was performed using the Amplification Refractory Mutation System (ARMS) as previously described by Wenham et al (1991).

Statistics

Statistical analysis used the Student's t-test for comparison of fasting and postprandial levels in diabetic subjects. Non-parametric tests were used for unequal distributions. Results are expressed as the mean±SEM. Inter- and intra-assay variation is expressed as SD/mean x 100. A p value of < 0.05 was regarded as statistically significant. Allele frequencies were calculated by gene counting. The normalized linkage disequilibrium coefficients were calculated using Arlequin Software.
5.4 Results

MTP -493 G/T polymorphism

Forty-five subjects (55%) were homozygous for the MTP -493 G allele, 33 (40%) were heterozygous G/T and four were homozygous for the T allele (5%). Patient details are given in Table 1. There was no difference in the sex ratio between the groups.

Serum LDL cholesterol was significantly lower in subjects with the T allele: the GG group had a mean level of 3.5 ± 0.1 mmol/L compared to GT group with a mean level of 3.0 ± 0.2 mmol/L ($p < 0.05$). Total cholesterol was reduced by 8% in carriers of the T allele, not quite reaching statistical significance ($p < 0.058$).

Table 1 MTP -493 G/T polymorphism: patient characteristics and plasma lipids

<table>
<thead>
<tr>
<th>Variant</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>45 (55%)</td>
<td>33 (40%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.0 ± 2</td>
<td>60 ± 3</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30 ± 1</td>
<td>29 ± 1</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>8.8 ± 0.5</td>
<td>8.2 ± 1.5</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.6 ± 0.3</td>
<td>7.3 ± 0.4</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.4 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.9 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Median</td>
<td>1.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Range</td>
<td>0.5–5.6</td>
<td>0.5–4.6</td>
<td>1.1–2.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 ± 0.05</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.5 ± 0.1</td>
<td>3.0 ± 0.2*</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>

*p < 0.05, compared to GG
In the chylomicron fraction (Table 2) there were no significant differences in apo B48 or apo B100 at any time point between the G/T and G/G subjects. There was a significant reduction in triglyceride content of the fasting chylomicron fraction in the GT group.

**Table 2** Effect of MTP -493 G/T polymorphism on chylomicron composition

<table>
<thead>
<tr>
<th>Variant ...</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>33</td>
<td>4</td>
</tr>
</tbody>
</table>

**Fasting chylomicron composition**

<table>
<thead>
<tr>
<th></th>
<th>GG ± SD</th>
<th>GT ± SD</th>
<th>TT ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B48 (µg/ml plasma)</td>
<td>3.4 ± 0.4</td>
<td>3.5 ± 0.5</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Apo B100 (µg/ml plasma)</td>
<td>8.4 ± 0.9</td>
<td>8.4 ± 1.0</td>
<td>8.3 ± 1.3</td>
</tr>
<tr>
<td>Cholesterol (µg/ml plasma)</td>
<td>20.2 ± 3.0</td>
<td>18.4 ± 3.8</td>
<td>21.1 ± 5.4</td>
</tr>
<tr>
<td>Triglyceride (µg/ml plasma)</td>
<td>96 ± 17</td>
<td>52 ± 12*</td>
<td>89 ± 24</td>
</tr>
</tbody>
</table>

**6 h chylomicron composition**

<table>
<thead>
<tr>
<th></th>
<th>GG ± SD</th>
<th>GT ± SD</th>
<th>TT ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B48 (µg/ml plasma)</td>
<td>11.5 ± 2.1</td>
<td>15.0 ± 3.0</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>Apo B100 (µg/ml plasma)</td>
<td>28 ± 4</td>
<td>27 ± 3</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>Cholesterol (µg/ml plasma)</td>
<td>61 ± 6</td>
<td>63 ± 9</td>
<td>72 ± 12</td>
</tr>
<tr>
<td>Triglyceride (µg/ml plasma)</td>
<td>437 ± 54</td>
<td>400 ± 91</td>
<td>487 ± 114</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to GG.
In the VLDL fraction (Table 3) there was a significant increase in apo B48 postprandially \((p < 0.05)\) in the G/T group. There was also a significant reduction in the cholesterol concentration \((p < 0.05)\) and cholesterol/apo B postprandially \((3.9 \pm 0.9 \text{ vs. } 2.7 \pm 0.6, p < 0.05)\).

**Table 3** Effect of MTP -493 G/T polymorphism on VLDL composition

<table>
<thead>
<tr>
<th>Variant</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>45</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td><strong>Fasting VLDL composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48 ((\mu)g/ml plasma)</td>
<td>2.1 ± 0.4</td>
<td>3 ± 0.6</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>Apo B100 ((\mu)g/ml plasma)</td>
<td>42 ± 5</td>
<td>33 ± 5</td>
<td>14.2 ± 3.5</td>
</tr>
<tr>
<td>Cholesterol ((\mu)g/ml plasma)</td>
<td>108 ± 14</td>
<td>74 ± 9.0</td>
<td>33 ± 13</td>
</tr>
<tr>
<td>Triglyceride ((\mu)g/ml plasma)</td>
<td>375 ± 38</td>
<td>326 ± 42</td>
<td>149 ± 57</td>
</tr>
<tr>
<td><strong>6 h VLDL composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B48 ((\mu)g/ml plasma)</td>
<td>3.5 ± 0.5</td>
<td>6.4 ± 1.0*</td>
<td>6.0 ± 1.7</td>
</tr>
<tr>
<td>Apo B100 ((\mu)g/ml plasma)</td>
<td>66 ± 9</td>
<td>56 ± 6</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>Cholesterol ((\mu)g/ml plasma)</td>
<td>143 ± 16</td>
<td>100 ± 9*</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>Triglyceride ((\mu)g/ml plasma)</td>
<td>632 ± 68</td>
<td>592 ± 85</td>
<td>239 ± 35</td>
</tr>
</tbody>
</table>

* \(p < 0.05\) compared to GG
MTP -400 A/T polymorphism

Patient characteristics and plasma lipids are shown in Table 4. Forty-one patients (50%) were homozygous for the A allele, 34 (40%) were heterozygous and eight (10%) were homozygous for the T allele. There was no difference in blood glucose, HbA1c, triglycerides or BMI between the groups. Plasma cholesterol was lower, and LDL cholesterol significantly lower, in the A/T group ($p < 0.05$) compared to the wild type A/A. In the chylomicron fraction, there was no significant difference between the MTP -400 A/T polymorphism groups in apo B48, apo B100, cholesterol or triglyceride at any time point. In the VLDL fraction, A/T polymorphism heterozygotes, when compared to wild type -400 A/A, showed increased apoB48, both fasting ($3.9 \pm 0.8$ vs. $2.0 \pm 0.4$, $p < 0.05$) and at 6 h ($7.4 \pm 1.2$ vs. $3.5 \pm 0.6$, $p < 0.005$) with a tendency to lower apoB100 at all time points in carriers of the T allele. VLDL cholesterol was significantly lower fasting ($57 \pm 9$ vs. $108 \pm 15$, $p < 0.01$) in heterozygous carriers of the T allele, and it was also lower at 6 h ($88 \pm 10$ vs. $149 \pm 17$, $p < 0.005$). Eighty percent of the -400 A/T subjects also carried the -493 G/T polymorphism. All significant differences were obliterated when we re-analyzed the few patients with -400 A/T polymorphism who did not also have the -493 G/T polymorphism.
Table 4 MTP -400 A/T polymorphism: patient characteristics and plasma lipids

<table>
<thead>
<tr>
<th>Variant</th>
<th>AA (n=41, 50%)</th>
<th>AT (n=33, 40%)</th>
<th>TT (n=8, 10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>58 ± 2</td>
<td>59 ± 2</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30 ± 1</td>
<td>30 ± 1</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>8.8 ± 0.6</td>
<td>8.0 ± 0.5</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.6 ± 0.3</td>
<td>7.4 ± 0.4</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.4 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Median (mmol/l)</td>
<td>1.6</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Range</td>
<td>0.5–5.6</td>
<td>0.5–4.5</td>
<td>1.0–3.5</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.5 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>2.8 ± 0.01</td>
</tr>
</tbody>
</table>

*p < 0.05, compared to AA.
MTP -164 T/C polymorphism

No differences in fasting plasma lipids or fasting and postprandial chylomicrons and VLDL were observed in either heterozygous or homozygous carriers of the C allele.

Apo E genotype

ApoE genotype distribution among the -493 G/T and -400 A/T groups is shown in Table 5; 61% of subjects had apoE 3/3, 22% had apoE 3/4, 16% had apoE 3/2, and one patient had apoE 2/4. There was no significant difference in the distribution of the apoE genotype between each of the different MTP genotype groups. Examination of the influence of apoE genotype on the plasma lipids revealed the expected lower levels of LDL cholesterol in apoE 3/2 subjects ($p < 0.02$) and higher LDL cholesterol in the 3/4 subjects ($p < 0.005$). Blood glucose, HbA1c, triglycerides, HDL cholesterol and BMI were similar across groups. ApoE genotype did not significantly alter the postprandial profile. Exclusion of those who did not have the apoE 3/3 genotype did not change the results.
Table 5 Distribution of apoE genotype between MTP polymorphism groups (%)

<table>
<thead>
<tr>
<th>ApoE genotype</th>
<th>3/2</th>
<th>3/3</th>
<th>3/4</th>
<th>2/4</th>
</tr>
</thead>
<tbody>
<tr>
<td>-493 GG</td>
<td>22</td>
<td>57</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>-493 GT</td>
<td>11</td>
<td>61</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>-493 TT</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>-400 AA</td>
<td>22</td>
<td>56</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>-400 AT</td>
<td>0</td>
<td>67</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>-400 TT</td>
<td>40</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Relationship between -493 G/T polymorphism and intestinal MTPmRNA levels

Sixteen diabetic subjects agreed to both genotyping and intestinal MTPmRNA biopsies. Seven patients were G/G, 6 were G/T and 3 were T/T. There was no significant difference between the homozygous G carriers and carriers of the T variant (see Graph 1 below).

Graph 1

![Graph showing intestinal MTPmRNA according to -493 G/T genotype in diabetic subjects](image-url)
5.5 Discussion

In this Type 2 diabetic population, the heterozygous -493 G/T substitution (present in 40%) was associated with a significantly lower LDL cholesterol suggesting that the effect of the T allele is magnified in diabetes.

We did not have enough subjects to analyze the T/T variant separately, but have included the data for comparison.

This is in contrast to studies in non-diabetic subjects. Ledmyr et al (2002) examining 564 healthy white men in Sweden and 1117 healthy men from the WOSCOP study found that serum cholesterol was significantly lower in the rare T/T variant, and LDL cholesterol was also lower, compared to G/G subjects. However they found no difference between the common heterozygous G/T form and the G/G form.

St Pierre et al (2002) examined the role of the -493 G/T polymorphism in patients without diabetes, hypertension or CHD. They combined the homozygous and heterozygous T carriers and demonstrated that the T allele was associated with significantly lower cholesterol in subjects with low visceral adipose tissue or low fasting insulin, when compared to those subjects with high adipose tissue or insulin. They suggested that visceral obesity and hyperinsulinaemia modulate the effect of the MTP -493 G/T polymorphism.

Our patients were obese, with no difference in BMI between the groups, and presumably insulin-resistant, although we did not measure insulin levels. It would be interesting to repeat the study in lean diabetic patients.

Chen et al (2003) examining Chinese patients, found that in Type 2 diabetes the rare homozygous T/T was associated with higher concentrations of LDL3, whereas no
effect was seen in controls. Although we did not sub fractionate LDL or examine the composition, the lack of difference in serum triglyceride or in chylomicron or VLDL triglyceride suggests that LDL size may not have been different.

The postprandial phase is metabolically abnormal in diabetes (Tomkin et al 2001) and we hypothesized that the major affect of the T allele might be on the postprandial lipoproteins, and that this might lead to a reduction in LDL cholesterol. In the Stockholm study, examination of a subset of 60 healthy 50-year-old white men, all heterozygous for the -493 T allele, did not reveal any significant alterations in the postprandial lipoproteins (Lundahl et al 2002). In the postprandial state, there was however a significant increase in 3 h-postprandial apoB48 in the lowest density fraction in the T/T subjects, but no difference in the G/T group.

In this study, examination of the postprandial phase showed a significant increase in apoB48 in the VLDL fraction. This was accompanied by a significant decrease at 6 h in VLDL cholesterol content of these particles. Since VLDL is the precursor of LDL, these results would explain the significantly lower LDL cholesterol in the G/T group.

The distribution of the -493 G/T MTP gene polymorphism in our patient population was similar to that found in France in diabetic patients, and in non-diabetic patients as part of the WOSCOPS study (Bernard et al 2000, Ledmyer et al 2002). The distributions of the -164 T/C and -400 A/T polymorphisms were also similar to those previously reported in a non-diabetic population (Herrmann et al 1998, Ledmyer et al 2002). The -164 T/C, and to a greater extent the -400 A/T polymorphism, were in linkage disequilibrium with the -493 G/T polymorphism. The -400 A/T polymorphism had a normalized linkage disequilibrium coefficient (D') of 0.72 ($p < 0.0001$) and the -164 T/C polymorphism, a D' value of 0.27 ($p < 0.0001$). It is not
surprising therefore, that we found similar lipoprotein alterations in the postprandial lipoproteins in the -400 A/T polymorphism. We had too few patients with the -400 A/T polymorphism who did not also have the -493 G/T substitution to comment on the influence of the -400 A/T substitution in diabetes. Examination of subjects heterozygous for both polymorphisms compared to the -493 G/G group did not alter any results, suggesting that the -400 A/T polymorphism may be silent.

The -164 T/C did not have any significant influence on the lipoprotein patterns in either the fasting or postprandial state in our patients, but the linkage disequilibrium with the -493 polymorphism was less pronounced.

ApoE is known to affect clearance of VLDL through a number of mechanisms. Carriers of the E2 allele have decreased VLDL clearance, leading to higher VLDL and lower LDL cholesterol, whereas carriers of the E4 allele have accelerated clearance of VLDL with lower triglyceride and a higher LDL cholesterol. The apoE phenotype of the patients was therefore examined in this study. The apoE distribution was similar to that reported in the general Irish population (Sheehan et al 2000). Exclusion of those who did not have the apoE 3/3 phenotype did not change the findings for the MTP polymorphisms.

This group has previously shown an increase in MTP mRNA and protein levels in animals with diabetes (Gleeson et al 1999, Phillips et al 2002). In the diabetic rabbit model, the MTP increase was associated with more small particles (Philips et al 2002).

There were more small intestinally-derived particles suggesting that the MTP -493 G/T polymorphism may up-regulate MTP activity. This is in agreement with
expression studies in Hep G2 cells, which showed marked enhancement of MTP transcription with the T variant (Karpe et al 1998). However in the small group of diabetic subjects who agreed to both intestinal MTPmRNA levels and genotyping (n = 16) there were no significant differences in intestinal MTPmRNA levels between the homozygous G variants and T carriers. In this subset of subjects who had intestinal MTPmRNA levels there were no differences in fasting lipid levels, HbA1c or glucose levels between G/G and G/T. Interestingly Ledmyer et al (2004) found in 18 subjects in the WOSCOPs study who were genotyped for the MTP-493 site and had a heart biopsies that the T variants (n=6) had a significantly lower level of MTPmRNA in the atria than the homozygous G variants (p<0.05). Hepatic MTPmRNA in human subjects have not been as yet measured or related to MTP -493 polymorphisms but Namikawa et al (2004) showed that the frequencies of the MTP-493 G allele and of the MTP-493 G/G genotype were higher in Japanese patients with non-alcoholic steatohepatitis compared to controls.

The finding that the T allele in our study was associated with lower LDL cholesterol may be explained by synthesis of the cholesterol-depleted VLDL particles that was found. Small VLDL particles form large buoyant LDL, which is cleared more quickly from the circulation (Gianturco et al 1998).

The postprandial lipoproteins are gaining favour in the aetiology of atherosclerosis, particularly since it has been shown that these particles can enter the sub-endothelial space, and because a specific apoB48 receptor has been identified on the macrophage (Taskinen 2003), which may also help to attract these particles into the atherosclerotic plaque. Analysis of MTP genotype in large prospective studies is needed to discover
whether the common -493 G/T polymorphism is associated with decreased risk of cardiovascular disease.

5.6 Conclusion

The MTP -493 G/T polymorphism is associated with a decrease in the cholesterol content of the postprandial VLDL particles and lower LDL cholesterol. This suggests that the effect of the -493 T allele is magnified in diabetes and that this may confer protection against atherosclerosis in Type 2 diabetes. The -400 A/T substitution gave very similar lipoprotein results but there was significant linkage disequilibrium between the two polymorphisms. No association was found between the -164 T/C polymorphism and either plasma lipids or postprandial lipoproteins. ApoE genotype was also examined but did not influence the above results.
Chapter 6

Atherosclerosis and MTP polymorphisms
6.1 Abstract

The effect of the -493 G/T polymorphism of the promoter region of the MTP gene on the risk of cardiovascular disease has been evaluated in one prospective study to date, showing that MTP-493T carrier status was surprisingly associated with increased risk of CHD despite a small reduction in total cholesterol, with the excess being eliminated by pravastatin treatment.

The cohort of 82 diabetic patients genotyped for the -493 G/T polymorphism was characterized for evidence of atherosclerotic disease. This was done by a chart review, exercise stress tests and carotid artery ultrasonography. Information was available in 63/82 patients.

In this small cohort of diabetic patients the prevalence of atherosclerotic disease was similar in the GG and GT variants of the -493 G/T polymorphism at over 20%.

Of the 4 patients with the TT polymorphism one had a history of coronary artery bypass grafting and a second had a positive coronary angiogram. The other 2 subjects had stress tests which were negative. One of the patients with a negative stress test had a resting ECG with right bundle branch block.

Although a small study, it is in keeping with the finding of Ledmyer et al (2004) and suggests that the TT polymorphism, rather than being protective against atherosclerosis, may paradoxically be a risk factor.
6.2 Introduction

Reports of the effect of promoter region polymorphisms of the MTP gene on the incidence of atherosclerotic disease are scanty. Surrogate markers of atherosclerotic disease have been used more frequently. Non-diabetic subjects homozygous for the T allele at position -493 for the MTP gene have been shown to have lower total cholesterol and LDL cholesterol (Karpe et al 1998). In Chapter 5 of this thesis the effect of the -493 T allele was demonstrated to be magnified in diabetes with the heterozygotes having lower LDL cholesterol and a lower cholesterol content of the postprandial VLDL particles than the G/G subjects.

The effect of the -493 G/T polymorphism on the risk of cardiovascular disease has been evaluated in one prospective study to date, as part of the West of Scotland Coronary Prevention Study. Ledmyr et al (2004) found that MTP-493T carrier status was associated with significantly increased risk of CHD despite a small reduction in total cholesterol, with the excess being eliminated by pravastatin treatment. In eighteen heart muscle biopsies there was a MTP-493T genotype-specific depression of MTP mRNA expression.

Yamada et al (2006) looked at over 2000 Japanese subjects randomly recruited to a prospective cohort study of aging and age-related diseases. There was no difference in the serum concentrations of total cholesterol, HDL, LDL, or triglycerides among -493 promoter region MTP genotypes for men or for women. However in postmenopausal women the T allele was associated with lower blood pressure (p=0.04) with the TT genotype protective against hypertension.
Nielsen et al (1998) have demonstrated that the myocardium in both mice and man express the genes for apoB and MTP and suggested that the secretion of lipoproteins by the heart may represent a pathway by which cardiac myocytes can unload surplus fatty acids. They further demonstrated in human myocardium a negative association between MTP mRNA levels and triglyceride content (2002).

The aim of this part of the study was to evaluate for the prevalence of atherosclerotic disease in the cohort of diabetic subjects genotyped for the -493 G/T polymorphism of the MTP gene.
6.3 **Subjects and methods**

The 82 patients who were screened for the MTP promoter region polymorphisms were evaluated in the following way: a chart review for evidence of atherosclerotic disease was carried out to look for documented evidence of myocardial infarction, coronary artery angiogram showing significant atherosclerotic disease, coronary revascularization, cerebrovascular events confirmed on cerebral imaging, or other revascularization procedures.

Those with a negative history were offered a standard Bruce protocol exercise stress test to assess for evidence of silent coronary artery ischaemia. Those who were unable or unwilling to do an exercise stress test, and those who had an adequate but negative stress test were offered carotid artery ultrasonography to assess for evidence of atherosclerosis at this site.

**Ultrasonography of carotid arteries**

This method was used as a noninvasive marker for atherosclerosis. In the general population strong correlations have been demonstrated between intima-media wall thickness (IMT) and cardiovascular risk factors and coronary artery disease (O Leary et al 1992).

The imaging protocol used for this study is that of the EDIC study (2003). Single longitudinal lateral views of the distal 10mm of the right and left common carotid arteries were taken. For the internal carotid arteries three longitudinal views in different imaging planes were taken.

The internal carotid artery was defined as including both the carotid bulb and the 10mm segment of the internal carotid artery distal to the tip of the flow divider that
separates the internal and external carotid arteries. The bulb was identified by the loss of the parallel wall present in the common carotid artery.

The maximum IMT of the common carotid artery was defined as the mean of the maximum IMT for near and far walls in both left and right sides (4 measurements). The maximum IMT of the internal carotid artery was defined in the same way, and the results of the 3 scans were averaged (anterior, lateral and posterior views on both sides) (12 measurements).

Melidonis et al (2003) found in patients with Type 2 diabetes that composite IMT values $\geq 0.925\text{mm}$ were associated with a relative risk of coronary artery disease of 25. They did not find an association with the Gensini score which denotes the level of artery obstruction at angiography. O'Leary et al (1999) found that non-diabetic subjects had a CVA or MI relative risk of 1 with a maximal CCA IMT of $<0.87\text{mm}$, while this increased as a continuum to a RR of 1.29 at 0.97mm and to 1.76 at 1.06mm. Results for ICA were similar with a CVA or MI relative risk of 1 with a maximal ICA IMT of $<0.90\text{mm}$, while this increased as a continuum to a RR of 1.95 at 1.11 mm and to 2.47 at 1.81mm.

**Statistics**

Chi-square test was used to test for significant differences in cardiovascular disease (positive history or EST) between the groups.

Carotid artery intima-media thickness was expressed as mean $\pm$ standard error of the mean.
6.4 Results

Relevant histories and medical notes were available in 63/82 subjects. Results of chart reviews, exercise stress tests and carotid artery intima-media thickness are shown in Table 1. The prevalence of atherosclerotic disease in this cohort of subjects is high at over 20%, as expected in a group of middle aged Type 2 diabetic subjects. There was no significant difference in the prevalence of atherosclerotic disease as measured by positive history and exercise stress test (chi-square) between the groups as defined by their -493 G/T genotype. When arbitrary cut off values were used to assign presence of carotid artery disease and this was included in the calculations there was still no significant change between GT and GG.

GG variants

Of the 45 GG variants, documented medical history was available in 39 subjects. In 9 subjects there was a positive history of cardiovascular events. Those with a negative history were offered an exercise stress test. Twenty one subjects agreed and none had a positive result. Overall 9/39 subjects with the GG genotype had demonstrable cardiovascular disease (23%). Carotid artery ultrasonography was agreed to by 16 subjects out of a possible 30 who did not otherwise have demonstrable cardiovascular disease. The average maximum IMT CCA (mean±sem) was 0.965±0.75. The average maximum IMT ICA was 0.975±0.90. Nine subjects had a CCA measurement over 0.87. Eight subjects had a ICA measurement over 0.90.
GT variants

Of the 33 GT variants, documented medical history was available in 20 subjects. There was a positive history of cardiovascular events in 4 cases. Ten subjects with a negative history agreed to an exercise stress test and 2 were positive. Overall 6/20 (33%) had demonstrable disease.

Carotid artery ultrasonography was agreed to by 4 subjects out of a possible 14 who did not have otherwise demonstrable disease. The average maximum IMT CCA was 0.994±0.17. Three subjects had a CCA level >0.87 mm. The average maximum IMT ICA was 1.216±0.32. Two subjects had an ICA level >0.9 mm.

TT variants

In the 4 TT subjects all charts were available and 2 had a positive history. One had a history of coronary artery bypass grafting and a second had a positive coronary angiogram. The other 2 subjects had stress tests which were negative. One of the patients with a negative stress test had a resting ECG with right bundle branch block. Carotid artery ultrasonography was agreed to by the other subject who had a negative history and EST. In this subject the maximum IMT CCA was 1.025. The maximum IMT ICA was 0.700.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total numbers</td>
<td>45</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>History of cardiovascular event(s) or positive coronary angiogram in which information was available</td>
<td>9/39</td>
<td>4/ 20</td>
<td>2/4</td>
</tr>
<tr>
<td>Positive exercise stress test</td>
<td>0/21</td>
<td>2/10</td>
<td>0/2</td>
</tr>
<tr>
<td>Carotid ultrasonography done</td>
<td>16</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Average max IMT CC(mean±sem)</td>
<td>0.965± 0.75</td>
<td>0.994± 0.17</td>
<td>1.025</td>
</tr>
<tr>
<td>Average max IMT ICA(mean±sem)</td>
<td>0.975± 0.9</td>
<td>1.216± 0.32</td>
<td>0.700</td>
</tr>
</tbody>
</table>
The cohort of diabetic patients genotyped for the -493 G/T polymorphism of the promoter region of the MTP gene were characterized for evidence of atherosclerotic disease. There was no significant difference in the prevalence of atherosclerotic disease as measured by positive history and exercise stress test between the groups as defined by their -493 G/T genotype. When cut off values were used to assign presence of carotid artery disease and this was included in the calculations there was still no significant difference between GT and GG.

Ledmyr et al (2004) found in the prospective WOSCOPs study that the MTP-493T carrier status was associated with significantly increased risk of CHD despite a small reduction in total cholesterol.

Two of the four patients in our cohort with TT genotype had established coronary artery disease. Although this group is very small in number as expected, the increased level of atherosclerosis is in keeping with Ledmyer's observation. However given the very low numbers it is difficult to assess the significance of these results.

The underlying mechanism of increased risk in the homozygote TT variant may be related to plasma lipoprotein changes (Lundahl et al 2002). Alternatively the MTP polymorphism may be linked with another unknown polymorphism which renders the patients at increased atherosclerotic risk. (Ledmyer et al 2004). It is also possible that the polymorphism has differing effects on MTP activity in the intestine, liver and myocardium. The MTPmRNA levels in human myocardial biopsies have been found
to be depressed in subjects with the -493 T allele. Also human myocardium MTP mRNA levels have been shown to be negatively associated with triglyceride content (Nielson et al 2002).

6.6 Conclusion

In this small cohort of diabetic patients the prevalence of atherosclerotic disease was similar in the GG and GT variants of the -493 G/T polymorphism of the promoter region of the MTP gene. Of the 4 patients with the TT polymorphism 2 had documented evidence of significant cardiovascular disease. This is in keeping with the paradoxical findings by others that the T allele confers susceptibility to atherosclerotic disease despite a reduction in LDL cholesterol. Though this was a small pilot study, it does suggest that a large prospective study in diabetic patients would be useful in assessing the effect of the T allele at the -493 G/T promoter region of the MTP gene.

Limitations of the work

This MD had a number of limitations. As we were recruiting from the endoscopy department of the hospital those patients with no gastro-intestinal complaints were not represented in the cohort which may produce selection bias. Also the fact that the statin study was a cross sectional cohort also introduces possible bias. Unfortunately it would be difficult to obtain ethical approval for a before and after study as most patients in general who undergo diagnostic endoscopy require no follow up
endoscopy. The composition of postprandial chylomicrons change as they move from the lymphatic circulation into the peripheral circulation. Therefore the plasma sampling undertaken in this study introduces the possibility of clearance as well as formation affecting chylomicron composition. This is easily overcome in the animal studies previously employed by our group. Unfortunately lymph vessel cannulation would be too invasive to be ethical in a human cohort.

The study of the MTP polymorphisms and the relationship to prevalence of atherosclerotic disease is severely limited. It was clear from the outset that the number of patients was too small for this section to be anything more than a small pilot study.
Conclusions

Microsomal triglyceride transfer protein is responsible for the assembly of chylomicrons in the intestine and VLDL in the liver. It is now generally agreed that the postprandial lipoproteins play an important part in atherogenesis. MTP gene expression and protein activity is increased in diabetic animal models.

This study showed that intestinal MTPmRNA were significantly higher in subjects with Type 2 diabetes compared to the controls. This is in keeping with similar studies of animal models of insulin resistance and diabetes. This is the first time that intestinal MTPmRNA has been quantified in humans and therefore also the first time that intestinal MTP levels in diabetics have been reported to be higher than in controls. It confirms the suggestion that the insulin response element in the promoter region of MTP is important regulator of intestinal MTP.

Intestinal MTPmRNA in the diabetic patients correlated with the cholesterol, triglyceride and phospholipid content of chylomicrons in keeping with the previously demonstrated lipidation effect of MTP.

Further studies are needed to define the relationship between intestinal and hepatic MTP because it is possible that selective inhibitors of intestinal MTP would be beneficial in improving the postprandial lipid profile and attenuating atherosclerosis without causing hepatosteatosis. This may be of particular importance in the diabetic patient whom MTP mRNA has been shown to be increased in this study. Such inhibitors will also need to be tested for any detrimental effects on the myocardium and immune system, where MTP has been recently found also to be active.
The major effect of the statin family of drugs is suppression of hepatic cholesterol synthesis by inhibiting HMGCoA reductase. However in patients with Type 2 diabetes statins also have an effect on fasting triglyceride and postprandial TRLs. There is no information to date on the effect, if any, of HMGCoA reductase inhibitors on intestinal MTP in human subjects. Reduction in cholesterol synthesis with a statin would be expected to lower MTP through the sterol response element in the promoter region of the MTP gene.

This study found that intestinal MTPmRNA was significantly higher in the diabetic patients compared to the non-diabetic subjects in both the non-statin group and in the statin-treated group. Among the non-diabetic subjects, those on statin therapy had significantly lower MTPmRNA than those not on statins. Statin therapy may therefore curb the postprandial dyslipidaemia which is so marked in diabetes partly by suppression of MTP synthesis. The cross sectional study in this MD has obvious limitations which would be overcome by a before and after study of the effect of statins on intestinal MTPmRNA levels.

There was a positive correlation between intestinal MTPmRNA and cholesterol content of chylomicrons in the group as a whole (diabetic, non-diabetic, on and not on statins). There was also a weak positive correlation between intestinal MTPmRNA and lipid content of the VLDL particles in the diabetic group. The correlations found would be consistent with the previously shown lipidation effect of MTP on apoB. This study confirms the suggestion that the sterol response element of the MTP gene is an important regulator of MTP transcription.
Non-diabetic subjects homozygous for the T allele at position -493 of the promoter region of the MTP gene have been shown to have decreased numbers of lipid-rich VLDL particles and significantly lower total cholesterol and LDL cholesterol. Common polymorphisms (G/T) of the MTP promoter region do not affect lipoproteins in non-diabetic subjects. Their effect in diabetes has not been described in a Caucasian population.

This study examined the effect of the common -493 G/T gene polymorphism, and also the MTP -400 A/T and -164 T/C polymorphisms on postprandial triglyceride-rich lipoproteins and LDL in 82 subjects with Type 2 diabetes. Forty-five subjects (55%) were homozygous for the MTP -493 G allele, 33 (40%) were heterozygous G/T and four were homozygous for the T allele (5%).

LDL cholesterol was significantly lower in subjects with the T allele and postprandially in the VLDL fraction there was an increase in apoB48, and a decrease in the cholesterol content and cholesterol/apoB.

Type 2 diabetes therefore appears to amplify the effect of the -493 G/T MTP polymorphism on the assembly of apoB-containing lipoproteins with an alteration in the postprandial lipoprotein composition resulting in lower LDL.

The -400 A/T substitution gave very similar lipoprotein results but there was significant linkage disequilibrium between the two polymorphisms. No association was found between the -164 T/C polymorphism and either plasma lipids or postprandial lipoproteins. ApoE genotype was also examined but did not influence the above results.
This suggests that in Type 2 diabetes the T allele at position -493 may be protective from risk of atherosclerosis via its effect on LDL. However prospective studies looking at the effect of this polymorphism on atherosclerotic risk in diabetes are lacking and would be useful to pursue.

This cohort of diabetic patients was characterized for the prevalence of atherosclerotic disease. The prevalence of atherosclerotic disease was similar in the GG and GT variants of the -493 G/T polymorphism. Of the 4 patients with the TT polymorphism the prevalence of atherosclerotic disease was high. Although a small study, it is in keeping with the finding of Ledmyer et al (2004) and suggests that the TT polymorphism, rather than being protective against atherosclerosis, may paradoxically be a risk factor.

A large prospective study in diabetic patients would be useful to assess whether the T allele at the -493 promoter site is worth screening for in the future in order to more effectively target cardiovascular treatments.

This study above showed that the MTP -493 G/T polymorphism was associated with more small intestinally-derived particles suggesting an up-regulation of MTP activity. Sixteen diabetic subjects agreed to both MTP genotyping and intestinal MTPmRNA biopsies. Seven patients were G/G, 6 were G/T and 3 were T/T. However there was no significant difference in the level of intestinal MTPmRNA between the homozygous G carriers and carriers of the T variant. This is unlike the findings in expression studies in Hep G2 cells which has shown marked enhancement of MTP transcription with the T variant (Karpe et al 1998). Of particular interest Ledmyer et
al (2004) found in 18 subjects in the WOSCOPs study who were genotyped for the MTP-493 site and had a heart biopsies that the T variants (n=6) had a significantly lower level of MTPmRNA in the atria than the homozygous G variants (p<0.05). Hepatic MTPmRNA in human subjects have not been as yet measured or related to MTP-493 polymorphisms but Namikawa et al (2004) showed that the frequencies of the MTP-493 G allele and of the MTP-493 G/G genotype were higher in Japanese patients with non-alcoholic steatohepatitis compared to controls.

It may well be the case that MTP polymorphisms have different effects on the level of mtp expression at different sites which are all important to overall cardiovascular risk. This is also a good reason for a large study looking at cardiovascular risk in relation to mtp genotype.
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