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Differential Gene Expression in Diabetes: Effects on Postprandial Lipoproteins

Sean Lally B.Sc. (Biomedical Science)

Submitted to the Faculty of Medicine, University of Dublin, Trinity College in fulfilment of the requirement for the degree of Ph.D

Department of Clinical Medicine, University of Dublin, Trinity College

Research was carried out under the direction and supervision of Professor Gerald H. Tomkin MD., F.R.C.P.I., F.R.C.P., F.A.C.P.

Department of Diabetes and Endocrinology, Adelaide and Meath Hospital, Dublin

April 2006
Declaration

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

Sean Lally
Summary

Diabetes is responsible for a large increase in cardiovascular risk which in many studies has not been reduced by improvement in blood sugar. The postprandial period in diabetes is associated with considerable disturbance in fatty acid and triglyceride metabolism and this may be an important factor in atherosclerosis development. In particular the postprandial period in diabetes is associated with increased apolipoprotein B48, the solubilising protein for transport of intestinal cholesterol and triglyceride in the blood stream. These apo B48-containing particles may be particularly atherogenic. Reduced clearance of these particles plays a part in the raised levels but it has been demonstrated that there is an increase cholesterol synthesis in the intestine in animal models of diabetes and human studies have suggested that the early rise in apo B48 is probably due to increased particle synthesis rather than delayed clearance. Microsomal triglyceride transfer protein (MTP) is responsible for the assembly of cholesterol, triglyceride and phospholipids together with one apo B48 molecule to form the chylomicron particle in the intestine and one apo B100 molecule to form VLDL in the liver. The cholesterol in the chylomicron particle may either be absorbed from the diet, absorbed from recycled biliary cholesterol or derived from de novo cholesterol in the intestine. In diabetes cholesterol synthesis is increased as evidenced by the increase in HMGCoA reductase, the rate-limiting enzyme for cholesterol synthesis. The ATP binding cassette proteins (ABC) G5 and G8 regulate cholesterol absorption from the intestine by their ability to work in tandem to excrete cholesterol and plant sterols from the enterocyte back into the intestinal lumen. Impairment in function is associated with increased cholesterol and plant sterol absorption. The Niemann Pick C1-like 1 protein (NPC1-L1) and gene have recently been described. Ezetimibe works by inhibiting Niemann PickC1-like1 protein. Thus this protein appears to play an important role in regulating intestinal cholesterol absorption. The 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (statins) lower cholesterol and triglycerides. They reduce postprandial chylomicrons in the blood and have been shown in diabetic and non-diabetic patients to make an important contribution to reducing the risk of myocardial infarction. Inhibition of cholesterol synthesis, results in up-regulation of the LDL receptor thus improving
clearance not only of the LDL particle but also of the chylomicron and VLDL particles. These inhibitors also play a part in regulating chylomicron synthesis and lower intestinal MTP expression both in diabetic and non-diabetic subjects in patients taking statin therapy for hypercholesterolemia has been shown in preliminary studies. The relationship between MTP, ABCG5, ABCG8 and NPC1-L1 and chylomicron particle composition has not been examined but is of considerable interest in view of drugs which are already on the market or under investigation which affect the above proteins. This is particularly the case in diabetes in view of the compositional alteration in the chylomicron particle and its probable importance in the accelerated atherosclerosis which occurs in diabetes. Some of the aims of the present study were to examine the relationship between chylomicron composition, MTP, ABCG5, ABCG8 and Niemann Pick C1-Like 1 mRNA expression in diabetic and non-diabetic patients. It was also aimed to examine the effect of statin therapy on intestinal expression of the above proteins. The results from this study show that MTP and NPC1L1 mRNA are increased in diabetes, in both the human study and the animal studies. There were strong positive correlations between the expression of MTP mRNA and the levels of postprandial chylomicron cholesterol. A strong positive correlation was also seen between the expression of NPC1L1 mRNA and MTP mRNA in the diabetic condition. ABCG5 and ABCG8 mRNA expression was seen to be consistently reduced in diabetes, and there was a negative correlation between the levels cholesterol content of the chylomicron and the mRNA expression of these two proteins. The present study also aimed to investigate the effect of pioglitazone as compared to insulin on chylomicron and VLDL levels and to correlate these changes to the mRNA expression of several genes involved in cholesterol metabolism. It was seen that not only did the pioglitazone reduce the postprandial triglyceride content of the chylomicron and VLDL particles, but it also aided normalise blood sugar levels and drastically reduced the mRNA expression of MTP, NPC1L1 and HMGCoA reductase toward levels of those found in the control animals, as well as increase the mRNA expression of ABCG5 and G8 which were found to be reduced in diabetes. It is hoped that the results will help to shed some light on the abnormal cholesterol metabolism that is associated with diabetes and may aid in the process of finding some therapeutic interventions for this disease.
Acknowledgements

I would firstly like to thank my supervisor, Professor Gerald Tomkin, for making it possible to carry out this research. He guided me through the whole process, giving help whenever it was asked for and he ensured that my studies were thorough and interesting. He also gave me many opportunities throughout my PhD to present at various national and international conferences, giving me much needed experience and exposure to research at the highest level. His enthusiasm and love for his work was infectious and was a great source of inspiration as I progressed through my studies. I am most grateful to have been given the opportunity to study under his tutelage and thank him sincerely.

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Publications

Papers
Genes that affect cholesterol synthesis, cholesterol absorption and chylomicron assembly. The relationship between the liver and intestine in control and streptozotosin diabetic rats. Atherosclerosis (in press)
**Lally S, Owens D, Tomkin GH.**

**Lally S, Tan CY, Owens D, Tomkin GH.**

Postprandial lipoprotein production in diabetes: The effect of pioglitazone as compared to insulin on intestinal and hepatic MTP, Niemann Pick C1-like1, HMGCoA reductase and ABCG5/G8. Diabetologia (in press)
**Lally S, Owens D, Tomkin GH.**

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Lally S, Tomkin G, Owens D. Comparison of the effect of pioglitazone and insulin on postprandial lipoprotein composition and expression of genes affecting cholesterol absorption, synthesis and chylomicron assembly. Diabetologia 2006

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Lally S, Tan C, Owens D, Tomkin GH. The expression of intestinal ATP binding cassette proteins G5 and G8, the regulators of cholesterol homeostasis, is reduced in diabetes. Diabetologia 2004; 47; A12

Lally S, Tan C, Owens D, Tomkin GH. Genes in the intestine regulating lipoprotein metabolism in diabetes. Atherosclerosis 2004; 175; S4
Abbreviations

ACAT  Acyl-coenzyme A: cholesterol acyltransferase
ABCG5  ATP Binding Cassette G5
ABCG8  ATP Binding Cassette G8
Apo  Apolipoprotein
BMI  Body Mass Index
BSA  Bovine Serum Albumin
CAD  Coronary Artery Disease
CETP  Cholesteryl Ester Transfer Protein
CV  Coefficient of Variation
EDTA  Ethylenediamine tetra acetic acid
HbA1c  Haemoglobin A1c
HDL  High Density Lipoprotein
HL  Hepatic Lipase
HMGCoA  3-hydroxy-3-methylglutaryl coenzyme A
HNF  Hepatic transcription factor
IDL  Intermediate density factor
IU  International Unit
Kg  Kilogramme
LCAT  Lecithin:cholesterol acyltransferase
LDL  Low Density Lipoprotein
Lp(a)  Lipoprotein (a)
LPL  Lipoprotein Lipase
M  Molar
Mg  Milligram
Ml  Millilitre
Mmol  Millimolar
mRNA  Messenger Ribonucleic Acid
MTP  Microsomal Triglyceride Transfer Protein
MW  Molecular Weight
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PPARs</td>
<td>Peroxisome Proliferator Activated Receptors</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element-Binding Proteins</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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<tr>
<td>Vol</td>
<td>Volume</td>
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<tr>
<td>Wt</td>
<td>Weight</td>
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Research

Broadening your mind and brightening your horizons
The hunger for knowledge is what the world relies on
    Individually unique and collectively even stronger
The improving of our lives, so to live for even longer

Steps made so far brought discoveries pure amazing
Continuing towards success, the trail’s been left a blazing
    Fortuitous at times, lady luck was often smiling
But with loyalty and dedication, we’ll continue with this rising

The new challenges that now face us, seemingly too rough
But the human brain and spirit are undoubtedly real tough
So the future does look bright pushing forward from this perch
We’ll continue to persevere with this thing we call Research!

Sean Michael Lally
Copyright ©2005 Sean Lally.
To give anything less than your best is to sacrifice the gift

Steve Prefontaine
To Mam and Dad
Chapter 1

Introduction
1.1 DIABETES MELLITUS

1.1.1. Definition

Diabetes mellitus refers to a disease characterized by hyperglycaemia either in the fasting and postprandial states, resulting from an absolute or relative deficiency in insulin action. Fasting venous blood glucose levels greater than 7mmol/L, or greater than 11.1 mmol/L, 2 hours after an oral load of 75g of glucose, have been designated by the World Health Organisation (1998) to be indicative of diabetes mellitus.

1.1.2. History

It is important to include the history of diabetes as I believe it affords a better understanding of contemporary issues and clearer vision as we look to the future. It was Thomas Willis’ observations of diabetes in 1674 and Mathew Dobson’s experiments in 1776 that conclusively established the diagnosis of diabetes in the presence of sugar in the urine and blood. Diabetes was no longer considered a rare ailment as was previously suggested by the ancient Greeks and Hindus. Willis referred to diabetes as the “pissing evil” and noted that in patients with diabetes, “the urine is wonderfully sweet, as if it were imbued with honey or sugar”. He claimed that diabetes was primarily a disease of the blood and not the kidneys. Willis proposed that the sweetness first appeared in the blood and was later found in the urine. William Cullen, one of Britain’s foremost clinicians, consultant and educators was the first to distinguish between diabetes mellitus and diabetes insipidus. In 1769, Cullen published an elaborate classification of human diseases. In this classification, we see for the first time a distinction between diabetes (mellitus), with the urine of “the smell, colour and flavour of honey” and diabetes (insipidus), with limpid but not sweet urine. It was Cullen who added the descriptive adjective mellitus, from the Latin word for honey. The experimental period in the history of diabetes began in the first half of the 19th century with the experiments of Claude Bernard. Bernard discovered that the liver releases a substance that affects blood sugar levels. In 1857, he isolated a starch-like substance that he called “glycogen”, which was
the precursor of glucose, “the internal secretion” of the liver. This observation established the liver’s role as a vital organ in diabetes.

Paul Langerhan’s most famous histological finding, the pancreatic islets, was presented in his doctoral dissertation at the University of Berlin in 1869. Langerhan acknowledged that he did not know the function of these ductless cells, which were later named “islets of Langerhans,” in his honour by the French histologist Laguesse. At the close of the 19th century, Oscar Minkowski demonstrated conclusively that removal of the pancreas from a dog results in the production of fatal diabetes. This was the turning point in determining the endocrine function of the pancreas.

The discovery and isolation of insulin at the University of Toronto in 1921-22 was a great event in the history of medicine. Insulin therapy would soon commute the death sentence associated with the diagnosis of type 1 diabetes. Banting and Best are the figures that history has most closely associated with the discovery of insulin.

It would be incomplete to finish the history of diabetes without acknowledging the influence on the treatment and care of diabetes that Elliott P. Joslin held on the subject. It was his belief that the key to managing diabetes lay with patient involvement, education and empowerment. This is still the core of the treatment that is shown to patients today, where the Joslin Diabetes Centre is in operation in Boston, and there are many satellites and affiliates across the United States and around the world.

1.1.3. Classification

Diabetes mellitus is a heterogeneous disorder which is currently classified into several major categories. The first classification system was established by the National Diabetes Data Group (1979). In June 1997, an international expert committee released a report with new recommendations for the classification and diagnosis of diabetes mellitus (Report, Diabetes Care, 1997). These new recommendations were the result of more than two years of collaboration among experts from the American Diabetes Association and
the World Health Organization (WHO). The use of classification systems and standardized diagnostic criteria facilitates a common language among patients, physicians, other health care professionals and scientists. The new classification system identifies four types of diabetes mellitus: type 1, type 2, "other specific types" and gestational diabetes as can be seen in table 1.1 below. Arabic numerals are specifically used in the new system to minimize the occasional confusion of type "II" as the number "11." Each of the types of diabetes mellitus identified extends across a clinical continuum of hyperglycaemia and insulin requirements.

Table 1.1

<table>
<thead>
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<th>Classification of Diabetes Mellitus:</th>
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Secondary and other types of diabetes may be induced by a variety of factors: destruction or removal of the pancreas, excessive concentrations of drugs such as diuretics, nicotinic acid and thiazides or hormones with insulin-antagonistic effects such as cortisol, glucagon and epinephrine, by association with complex genetic syndromes such as Myotonic dystrophy and Huntington’s chorea, or it may be due to other endocrine diseases such as Acromegaly or Cushing’s syndrome. Gestational diabetes complicates approximately 4% of all pregnancies in the United States and in a study by Ferrara et al., (2002) the prevalence of gestational diabetes was found to be “5.0 and 7.4% in Asians, 3.9 and 5.6% in Hispanics, 3.0 and 4.0% in African-Americans, and 2.4 and 3.8% in whites”. Between 10 and 15 percent of adults in the United States alone have IGT or impaired fasting glucose. Impaired glucose tolerance is defined as two-hour glucose levels of 7.8 to 11.0 mmol (140 to 199 mg per dl) on the 75-g oral glucose tolerance test, and impaired fasting glucose is defined as glucose levels of 5.6 to 6.9 mmol per L (100 to 125 mg per dL) in fasting patients (Alberti and Zimmet 1998). These glucose levels are
above normal but below the level that is diagnostic for diabetes. Patients with impaired glucose tolerance or impaired fasting glucose have a significant risk of developing diabetes and thus are an important target group for primary prevention.

1.1.4. Type 1 Diabetes Mellitus (T1DM)

T1DM is an auto-immune disease caused by the destruction of the insulin-secreting β-cells of the pancreas. Most patients with T1DM present young and most frequently in children between 12 and 15 years of age, but it can occur at any age. Symptoms of the disease include thirst, tiredness and weight loss and patients with the condition are usually hyperglycaemic and ketoacidotic. By definition patients with T1DM require insulin treatment and there are a variety of different types of insulins and insulin treatments available to try and enable the patient to live as normal a life as possible.

The incidence of diabetes in North America is comparable to that of the UK, Ireland and central Europe. There are endless statistics on the number of people with T1DM. The current prevalence of T1DM is about 0.25%, and this accounts for up to 20% of total diabetic cases. T1DM accounted for 28% of new cases of diabetes in Australia 1999-2002 (The National Diabetes Register, Australia’s Health 2004, AIHW). However, the prevalence of T1DM shows marked geographical variation, with a 35-fold difference between the Nordic countries, in particular Finland, and the Far Eastern countries of Japan and China, the countries with the highest and lowest frequencies. A study conducted on the Italian island of Sardinia (Casu et al 2004), using Bayesian analysis of the geographical distribution of prevalence shows the highest rate of the incidence of T1DM in the Southern part of the island (Cagliari province) and the lowest in the north-western part (Sassari province). A similar picture was produced by data from the Eurodiab Registry in Sardinia (1989 ongoing), yet there appears to be no reasonable explanation for these results. Tuomilehto et al (1992) reported a large difference in the occurrence of T1DM between five populations around the Baltic Sea (Finland, Estonia, Latvia, Lithuania and Poland). Among these five populations, the incidence increased with the latitude. It remains to be established whether the variation in the occurrence of
T1DM within Europe can be attributed to variation in genetic susceptibility or to differential distribution of environmental disease determinants or both.

At least 18 genetic loci have been found that are related to T1DM. They appear to involve abnormal interaction among normal genes, mostly those known as class I and II major histocompatibility genes, which affect the immune response. The odds of inheriting the disease, however, are only 10% if a first-degree relative has diabetes, and even in identical twins, one twin has only a 33% chance of having T1DM if the other has it (Simon, 2002). Also children are more likely to inherit the disease from a father with type 1 diabetes than from a mother with the disorder. Genetic susceptibility to the disease is also associated with genes in the class II human leucocyte associated (HLA) antigens on chromosome 6, in particular HLA DR 3 and HLA DR 4 have been linked with Type 1 diabetes (Michelson and Lernmark, 1987). Although 90-96% of T1DM patients have either of these genes, they are not specific to T1DM since they are found in 60% of the general population (Nerup et al., 1987). It is believed by some that one or more viral infections may trigger the disease in genetically susceptible individuals. The scenario whereby an infection introduces a viral protein that resembles a beta-cell protein and the T cells and antibodies are tricked by this resemblance into attacking the beta protein as well as the virus has been suggested.

It is unknown what first starts the cascade of immune events that leads to the destruction of the beta cells, but there are certain factors that are thought to be important in the process. White blood cells called T lymphocytes produce immune factors called cytokines that attack and gradually destroy the beta cells of the pancreas (Karges et al., 2005). Progression from the first stage, known as insulitis, to full-blown diabetes can take seven years or longer. Unfortunately by the time a person is aware that something is wrong and goes to the doctor with symptoms of T1DM, about 80-90% of the beta cells have been destroyed. It should also be noted that more than half of those with insulitis do not develop diabetes.
1.1.5. Type 2 Diabetes Mellitus (T2DM)

T2DM results from an imbalance between insulin secretion and tissue sensitivity to insulin. Unlike T1DM, where the absolute deficiency of insulin is due to autoimmune-mediated destruction of the β-cells, the state of relative insulin deficiency found in T2DM results from the failure of the β-cells to maintain sufficiently high levels of insulin to compensate for the state of insulin resistance. The condition is usually preceded by a long period of a mild elevation of fasting or postprandial glucose levels, referred to as impaired glucose tolerance (IGT). Patients with T2DM, at least initially, do not require insulin treatment to survive. The incidence of T2DM has doubled every 20 years since 1945 and it accounts for 80-90% of all cases of diabetes in the world (Barnett 1998). The prevalence of T2DM is highly age-dependant, increasing 2-3 fold every 10 years after the age of 40 years of age. In 2004, according to the World Health Organization, more than 150 million people worldwide suffer from diabetes. Its incidence is increasing rapidly, and it is estimated that by the year 2025 this number will double. Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence rate is, however, expected to occur in Asia and Africa, where most of the diabetic patients will be seen by 2025. The increase in incidence of diabetes in the developing countries follows the trend of urbanisation and lifestyle changes. Diabetes is in the top 10, and perhaps the top 5, of the most significant diseases in the developed world, and is gaining in significance. For at least 20 years, diabetes rates in North America have been increasing substantially. In 2002 there were about 18.2 million diabetics in the United States alone. The Centres for Disease Control has termed the change an epidemic. The National Diabetes Information Clearinghouse estimates that diabetes costs $132 billion in the United States alone every year. The economic burden of diabetes on society is enormous; 2% of the UK population with diabetes consume 5% of the health service budget, by comparison the 12% with arthritis who consume just 1.9% (Leese 1995). With the increasing prevalence of the condition, these figures are only going to escalate.

More so than in T1DM, there appears to be a strong genetic factor in the aetiology of T2DM. The genetic impact is illustrated by the finding that the concordance rate in
monozygotic twins approaches 80% over a lifetime (Kobberling et al., 1990). The complex non-Mendelian mode of inheritance of the disease has complicated the search for susceptibility genes. Maturity-onset diabetes of the young (MODY) has autosomal dominant inheritance. Pedigree studies have been limited owing to a scarcity of families, but already abnormalities at several genetic loci on different chromosomes have been identified. The most common form is associated with mutation on chromosome 12 in a hepatic transcription factor (HNF)-1α (Vaxillaire et al., 1995). Five different types of maturity-onset diabetes of the young (MODY) have been genetically identified until now but mutation screening suggests that more MODY genes exist (Baier et al., 2000). Mutations in genes encoding transcription factors essential for normal development and function of pancreatic β cells has recently become important in studying the genetics of T2DM. With this in mind, a study was conducted in Iceland by Kristinsson et al (2001). Patients with MODY and their families were screened for mutations in the transcription factor genes. It was found that a gene known as NeuroD1 gene was responsible for the sixth type of MODY.

There is considerable evidence supporting environmental influences. A special predisposition to the disease has also been observed in migrant workers from developing countries of the world in rich Western countries. In the UK, the prevalence of T2DM is 1-2% amongst the white population, compared to 11% amongst those of Indian origin and 9% amongst African-Carribeans. Likewise in the USA, migrant Hispanic groups (such as Mexicans and Cubans) have higher rates of the disease (12-20%) compared to non-Hispanic white Americans (3-8%). Amongst westernised Micronesians of Nauru and the Aboriginals of Australia an even higher incidence has been recorded. In an attempt to understand the complex nature of T2DM, it was decided to study the disease within populations that have limited genetic and environmental variability. The Pima Indians of Arizona have the highest reported prevalence of diabetes of any populations in the world (Knowler et al., 1979). This population has minimal European admixture (Williams et al., 1992), and their diabetes appears to be exclusively T2DM, with no evidence of the autoimmunity characteristic of T1DM, even in very young subjects with an early onset of the disease (Dabelea et al., 1998). The absence of T2DM and the minimal admixture in
this population may indicate limited genetic and environmental variability in the aetiology of T2DM in the Pima Indians, making this population more amenable to the identification of susceptible loci. Diabetes in Pima Indians is also familial, and the degree of familiality is greater at younger ages of onset compared with older ages of onset (Hanson et al., 1995). The degree of heritability of T2DM and its intermediate phenotypes cannot be assessed by studies in twins in Pima Indians, as there are not enough pairs in this population to make such studies feasible. However, an estimate of heritability can be calculated from the analysis of familial resemblance. Using maximum likelihood methods, such estimates have been obtained for several intermediate phenotypes in non-diabetic Pima Indians (Sakul et al., 1997). For example, 38-49% of the variance in insulin action, independent of the effect of obesity, is familial in Pima Indians. The acute insulin response (AIR) has also been shown to be highly familial.

According to the US Centers for Disease Control and Prevention (CDC), rates of T2DM have tripled in the last 30 years. This is due largely to the global epidemic of obesity, a major risk factor for developing T2DM. The connection between obesity and diabetes is so visible (a vast majority of all cases of T2DM are caused by excessive weight) that some health experts have coined a new term, Diabesity. The word encapsulates the diabetic/weight connection and is a step towards the wider cluster of symptoms of Insulin Resistance Syndrome, a frequent precursor of coronary heart disease (http://www.annecollins.com/obesity/diabetes-diabesity.htm).

Although obesity is a powerful risk factor for T2DM and cardiovascular diseases across populations, substantial heterogeneity exists in the relationship between metabolic and cardiovascular abnormalities and the degree of obesity (Kissebah et al., 1982). A significant minority of subjects who are defined as obese by current guidelines do not develop insulin resistance; conversely, insulin resistance can be present in lean individuals (Reaven, 1988). Genetic and environmental factors may have a major impact on the metabolic and cardiovascular consequences of obesity, although the mechanisms by which genetic factors modify the effects of obesity are largely unknown. The Metabolic Syndrome is a clustering of atherosclerotic cardiovascular disease risk factors characterized by visceral adiposity, insulin resistance, low HDL cholesterol (HDL-C),
and a systemic proinflammatory state (Ford et al., 2002). A major challenge for Metabolic Syndrome (MetSyn) research remains the identification of features of adiposity that best reflect increased risk of developing the MetSyn.

The current clinical approach to the MetSyn uses sex-specific waist circumference criteria to define the body mass component contributing to the MetSyn. The rationale for the use of waist criteria arises partly from data showing that measures of overall obesity, such as body mass index, are relatively insensitive indicators of the risk for metabolic and cardiovascular complications of obesity, as compared with measures of central or abdominal adiposity (Abate et al., 1995). Waist circumference reflects both abdominal subcutaneous adipose tissue (SAT) and abdominal visceral adipose tissue (VAT) and is a general index of central (trunk) fat mass. VAT has been proposed as the major determinant of metabolic and cardiovascular complications of obesity (Nieves et al., 2003). However, this remains controversial, and it is unclear whether more accurate measures of total body fat, trunk fat mass, or specific abdominal SAT or VAT compartments (including CT and MRI) provide superior information regarding obesity complications (Smith et al., 2001). Alternatively, the use of novel biochemical measures of adipose mass and function may be a more practical way to incorporate additional adipose readouts into large epidemiological studies and clinical practice. Adipose tissue is an active secretory organ that elaborates a variety of molecules known as adipocytokines, including tumor necrosis factor, interleukin-6, leptin, adiponectin, and resistin, that may mediate many of the metabolic changes in the MetSyn (Steppan et al., 2003). Some of these fat-derived factors may be directly atherogenic. Plasma leptin, which is largely derived from adipose tissue, increases in obesity and insulin-resistant states. Leptin deficiency in mice protects against atherosclerosis despite causing massive obesity (Hasty et al., 2001) and plasma leptin levels were found to be predictive of cardiovascular events, independent of traditional risk factors, body mass index and C-reactive protein (CRP) levels (Wallace et al., 2001). In contrast, plasma levels of adiponectin are reduced in obesity and DM-2, and early evidence suggests that this molecule may have antiatherosclerotic properties in mice models and in humans (Okamoto et al., 2002; Ouchi et al., 2001). Whether these measures of adipose tissue hormonal activity will be
superior markers of cardiovascular risk over anatomic measures of obesity remains to be
determined.

The 'thrifty gene' hypothesis was proposed in 1962 by a geneticist, James Neel. The
theory is that some populations which relied on farming and fishing for food for
thousands of years, were used to alternating periods of famine or plenty. This lifestyle
collide with our genome, which was most likely selected in the late Palaeolithic era
(50,000 - 10,000 BC) by criteria that favoured survival in an environment that was
characterized by fluctuations between periods of feast and famine. To adapt to these
extreme changes, these people developed a thrifty gene, which allowed them to store fat
in times of plenty so that they can survive during famine. In time, these populations have
gone on to adopt a life style, wherein they always have access to plenty of high-fat food
and have less physical activity. A recent study by Halberg et al (2005) undertakes an
experiment which was the first in humans to show that intermittent fasting increases
insulin mediated glucose uptake rates and the findings are compatible with the thrifty
gene concept. Although Neel’s view as to how this “thrifty” gene may lead to diabetes
was modified over the years (King 1995), a constant part of his argument was “that the
basic difference between those who developed diabetes and those who did not was a
‘quick insulin trigger’ in response to hyperglycaemia”. The survival benefit of this
phenotype was to minimise urinary glucose loss when fasting was replaced by feasting.
This would have benefited the hunter/gatherer, who was very active, consumed a diet low
in refined carbohydrates and fat and may have had to endure sporadic periods of fasting.
Neel proposed that in a Western society of lower levels of physical activity combined
with foods rich in refined carbohydrate and fat, the increased demand for insulin would
exceed the capacity for production, abolishing the protective genotype and promoting
obesity, insulin resistance and the development of diabetes. During the same period of
time, Cahill et al., (1966; 1967) published a series of experiments that led to quite a
different view of how primitive man survived famine. They believed that the more
efficient one is at conserving muscle protein, the better the chance of survival. In
agreement with this hypothesis Reaven (1998) in a thought provoking debate proposed
insulin resistance in muscle rather than ‘a quick insulin trigger’ as a central, genetically determined factor in the aetiology of T2DM. He proposed that muscle insulin resistance conserves glucose utilisation by the central nervous system, decreasing the amount of muscle protein needed to be converted to glucose. As a result, the muscle mass is preserved, thereby increasing the likelihood of a successful search for food. At some point the combined burden of muscle insulin resistance and lifestyle can no longer be overcome by compensatory hyperinsulinaemia and diabetes may then occur.

1.1.6. Insulin

The insulin molecule contains 51 amino acids and is made up of two polypeptide chains linked by disulphide bonds. In man the A chain consists of 21 amino acids while the B chain consists of 30 amino acids. Insulin is synthesised on the ribosomes of the rough endoplasmic reticulum (RER) of the β-cell as a single precursor chain called preproinsulin. This consists of two chains, A and B, linked together by a connecting peptide called the C peptide. The molecule begins with a pre-N-terminal sequence, a signal peptide that facilitates the binding of the ribosome to the membrane of the RER. This prepeptide is rapidly cleaved as the molecule crosses the membrane. Proinsulin, composed of insulin and the C-peptide, then migrates to the Golgi apparatus where it is transformed into insulin, by a highly specific proteolytic system which cleaves the C-peptide. Over 95% of proinsulin is converted to insulin but some insulin is secreted as a single chain proinsulin. Proinsulin has only 5% of the biological activity of insulin.

During high insulin concentrations, such as those found in the β-cell, insulin molecules aggregate. Initially two monomers form a dimer and the in the presence of zinc, three dimers associate to form a hexamer. Insulin is stored in the pancreatic granules in this form but as soon as insulin is released into the blood the aggregated forms dissolve due to the lower concentrations. It is the monomer that binds to the insulin receptors and constitutes the physiological form of insulin. Insulin is secreted by the process of exocytosis. Under the influence of an increase in blood glucose, the contents of the granules, consisting of equimolar amounts of insulin and C-peptide, are liberated in the circulation, where the unbound monomer has a half life of only five minutes. The liver is
the main target organ of insulin and more than 50% is metabolised on its first pass, the remainder along with the C-peptide is degraded by the kidney. Insulin release is pulsatile and is mainly stimulated by glucose. There is an initial response which occurs within one minute of a rise in blood glucose and lasts for 5-10 minutes. Then a secondary release of insulin occurs, which lasts as long as the stimulus continues.

1.1.7. The Insulin Receptor

The insulin receptor is a tetramer of 2 alpha and 2 beta subunits. The alpha and beta subunits are coded by a single gene and are joined by disulfide bonds, a mechanism parallel to that of the ligand, insulin (Rubin, 1984). Mutation in either the structural gene or some of the processing steps may lead to insulin resistance. Ullrich et al. (1985) deduced the entire 1,370-amino acid sequence from a cDNA clone. The precursor starts with a 27-amino acid signal sequence, followed by the receptor alpha subunit, a precursor processing enzyme cleavage site, then the beta subunit containing a single 23-amino acid transmembrane sequence. Seino et al. (1989) found that the INSR gene spans more than 120 kb and has 22 exons. All introns interrupt protein coding regions of the gene. The 11 exons encoding the alpha subunit are dispersed over more than 90 kb, whereas the 11 exons encoding the beta subunit are located together in a region of about 30 kb. Three transcriptional initiation sites were identified, located 276, 282, and 283 bp upstream of the translation initiation site. There is heterogeneity of insulin receptors in different tissues.

1.1.8. Glucose

Blood glucose is derived from the diet, by gluconeogenesis and by glycogenolysis. All biological tissues require glucose as a source of energy, with the brain and erythrocyte requiring substantial amounts. In the post-absorptive state, the concentration of blood glucose in humans is set within the range 4.5-5.5 mmol/L. After the initial ingestion of a carbohydrate meal it may rise to 6.5-7.2 mmol/L and during fasting, levels can drop to 3.3-3.9 mmol/L. The maintenance of stable levels of glucose in the blood is one of the most finely regulated of all homeostatic mechanisms, with levels in the normal healthy
subject rarely straying outside the normal range, but fasting levels of 20-40mmol/L may be found in diabetic patients in poor metabolic control. Both hypoglycaemia and hyperglycaemia have serious metabolic consequences. The former being associated with convulsions, coma, brain damage and death, while the latter eventually leads to ketoacidosis, coma and death. The major organ of glucose homeostasis is the liver, which postprandially absorbs and stores glucose as glycogen and releases glucose when required to compensate for utilisation by the tissues. About 200g of glucose is produced and utilised daily, with more than 90% of this being of hepatic origin (75% from glycogenolysis and 25% from gluconeogenesis). Glucose is continually filtered by the glomeruli and is normally all reabsorbed by the renal tubules. The capacity of the tubular system to reabsorb glucose is limited to about 350 mg/min. When blood glucose levels are elevated, the glomerular filtrate may contain more glucose than can be reabsorbed, with the excess passing into the urine to produce glycosuria.

1.1.9. Glycation and Glycosylation

Extended exposure of long-lived molecules such as collagen, intracellular proteins and nucleic acids to hyperglycaemia results in the non-enzymatic condensation of glucose with the primary amines to form Schiff bases, at a rate proportional to the concentration of the sugar. These glucose-protein adducts can spontaneously undergo ‘Amadori’ rearrangements to form early glycation products (Brownlee et al., 1984). Additional reactions, rearrangements, dehydration and cleavage result in the formation of insoluble cross-linked complexes called advanced glycosylation end (AGE) products. The concentration of AGE-products increases with age and it has been observed that their formation is accelerated in diabetes (Lyons et al., 1991). Most investigations done to assess the role of AGE-products in animal models of diabetic neuropathy have used aminoguanidine as a prototypic inhibitor. Preventive or intervention experiments have shown treatment benefits for motor and sensory nerve conduction velocity, autonomic nitric neurotransmission, nerve morphometry, and nerve blood flow. The latter depends on improvements in nitric oxide-mediated endothelium-dependent vasodilation and is responsible for conduction velocity improvements (Cameron et al., 2005).
Human haemoglobin is also subject to glycosylation and various glycosylated forms have been identified (HbA1a, HbA1b, HbA1c). Elevated levels of these glycosylated forms are found in diabetic subjects, where levels may be 2-3 time higher than those found in non-diabetic subjects. Measurement of the degree of glycosylation of haemoglobin, in particular HbA1c, can be used as a long-term index of glycaemic control in diabetic patients. In non-diabetic patients normally 4-6.5% of the total haemoglobin exists as HbA1c but in poorly-controlled diabetic patients this figure may be as high as 20% or more.

1.1.10. Glycaemic Regulation

Although people with both type 1 and type 2 diabetes develop an increased prevalence of atherosclerosis, which leads to more heart attacks and strokes, direct evidence that this is mediated by hyperglycemia is lacking. In part, this is because additional atherogenic factors such as lipid abnormalities and hypertension accompany the diabetes (UKPDS, 1998; DCCT, 2002). Efforts to demonstrate the presumed toxic effects of hyperglycemia in experimental animals have been similarly hindered (Goldberg, 2004). In a recent study, chow-fed Ldlr−/− diabetic mice had more atherosclerosis at the aortic root, but when these mice were fed cholesterol-containing diet, the atherosclerosis correlated with plasma cholesterol and not glucose (Renard et al., 2004). Thus, as in many previous reports, the more advanced vascular lesions in these mice were attributable to greater hyperlipidemia and not hyperglycemia or defective insulin actions. The lack of animals that develop diabetic macrovascular disease has led to the establishment of an NIH-sponsored program to produce such models, the Animal Models of Diabetic Complications Consortium.

On the other hand, Balkau et al., (1998) published the results of a twenty year follow-up study of non-diabetic men, from three studies originally included in the International Collaborative Group, namely the Whitehall Study, the Paris Prospective Study and the Helsinki Policeman Study. Men in the upper 20% of 2hr glucose level distribution and those in the upper 2.5% for fasting glucose levels had significantly higher risk for coronary heart disease.
Aldose reductase (AR) catalyzes the reduction of glucose, an aldehyde-containing sugar, to sorbitol. AR mediates 1 of several pathways thought to accelerate diabetic complications via production of excess ROS. Although previous clinical trials using AR inhibitors to prevent or treat diabetic complications have been disappointing (Pfeifer et al., 1997; Martyn et al., 1987), more recent studies suggest that AR inhibition is beneficial for diabetic neuropathy (Bril et al., 2004; Gabbay, 2004). AR has been implicated as a cause of increased cardiac ischemic injury in rats (Ramasamy 1997), and AR inhibitor therapy was associated with improvement of human cardiac function (Johnson et al., 2004). Transgenic AR expression amplifies the pathological response to ischemia/reperfusion in mice (Hwang et al., 2004). Studies comparing protein levels have shown that mice normally have much lower levels of AR (Hwang et al., 2004) than humans (Markus et al., 1983; Tanimoto et al., 1998). Vikramadithyan et al., (2005) showed that human aldose reductase expression accelerates diabetic atherosclerosis in transgenic mice, giving a possible alternate reason to higher levels of atherosclerosis seen in diabetes that are not directly linked to a higher glycaemic levels.

It is therefore unclear from the literature whether or not hyperglycaemia per se represents an important risk factor for macrovascular complications in Type 2 diabetes. What is shown is many studies however, including The UKPDS study (1998) is that improvement in glycaemic control resulted in a 25% reduction in microvascular complications, thus showing the undoubted benefits to the patient of controlling blood glucose levels.

1.1.11. Complications of Diabetes

According to the WHO Multinational Study (Stephenson et al., 1995) diabetes ranks as the seventh leading cause of death although some reports have now promoted it to the sixth leading cause of death. Amazingly, diabetes has overtaken poverty-related infections to become the leading cause of death in Mexico, according to a new report that adds weight to a World Health Organization warning that a devastating global diabetes epidemic is looming. The disease has a marked impact on the prognosis and quality of life. It accounts for 12% of all new cases of blindness, 25% of all cases of end-stage renal failure and 40% of all foot or leg amputations among adults. Given these considerations
along with the increasing worldwide incidence of the disease the economic burden of diabetes on society is enormous. The majority of patients with diabetes develop complications, which are present in up to 50% even at the time of diagnosis. The major complications associated with diabetes are classed as either microvascular or macrovascular.

Microvascular complications include retinopathy which is the commonest cause of blindness in the UK. Most diabetic patients will show evidence of retinopathy after 20 years of the disease and this will be sight threatening in 30-40% of cases (Barnett et al., 1996). Diabetic nephropathy affects about 25% of all patients with Type 1 and Typ2 diabetes and is now the most common reason for renal dialysis in the Western world. Nephropathy is associated with a vastly increased cardiovascular risk of between 25 and 100 fold (Hannson et al., 1998). Diabetic nephropathy presents in its earliest stage with low levels of albumin (microalbuminuria) in the urine. The most practical method of screening for microalbuminuria is to assess the albumin-to-creatinine ratio with a spot urine test. Results of two of three tests for microalbuminuria should be more than 30 mg per day or 20 mcg per minute in a three- to six-month period to diagnose a patient with diabetic nephropathy. Slowing the progression of diabetic nephropathy can be achieved by optimizing blood pressure (130/80 mm Hg or less) and glycemic control, and by prescribing an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker. Patients with diabetes and isolated microalbuminuria or hypertension benefit from angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. In the event that these medications cannot be prescribed, a nondihydropyridine calcium channel blocker may be considered (Thorp, 2005). Microalbuminuria is a major predictor of overt nephropathy in Type 1 diabetes. It is a less strong predictor of nephropathy in Type 2 diabetes but is an indicator of increased cardiovascular risk. Once overt nephropathy is present, end stage renal disease usually occurs within 10 years (Bakris et al., 1996).

There are 3 major types of diabetic nephropathy: chronic sensory neuropathy, autonomic neuropathy and acute neuropathy. Chronic sensory neuropathy is the most common form associated with diabetes and may be present with loss of sensation or parasthaesia in the feet. Preventative measures are vital as the condition predisposes to neuropathic
ulceration of the feet which may in turn lead to infection and even necessitate amputation.

Macrovascular disease is up to five times more prevalent in diabetic compared to non-diabetic patients (Turner et al., 1998). In Type 2 diabetes, macrovascular complications are at least twice as common as microvascular complications (Green et al., 1997) and are the single most important cause of premature mortality, accounting for up to 75% of all deaths. The influence of diabetes on the pathogenesis of atherosclerosis is discussed in detail in the atherosclerosis section of the introduction.
1.2 ATEROSCLEROSIS

1.2.1. What is atherosclerosis?

Atherosclerosis is a vascular disease, coming from the Greek words: athera meaning mush, and sclerosis meaning hardness. The disease is slow in its progression and it begins in childhood and does not manifest itself until middle-age or later. It is a complex process which causes stenosis of the large and medium sized arteries due to the formation of atheromatous plaques. Atherosclerosis and its complications, myocardial infarction, stroke and peripheral vascular disease, are responsible for up to 50% of mortality in Westernised society. The early lesions, the ‘fatty streak’, are commonly found in children and it has been reported that among autopsies performed in children between the ages of 10 and 14, half showed the presence of these arterial fatty streaks. In fact it is known that by 10 years of age, these early lesions consist of an aggregation of lipid-rich macrophages (foam cells) and T lymphocytes within the innermost later of the artery wall, the intima (Stary, 1989). The lesions are characteristically yellowish in colour, slightly raised and are most commonly found in the aortic branches of bifurcations. The advanced atheromatous lesion may be evident around the third decade of life. Advanced lesions consist of an extensive inflammatory, fibroproliferative response that intrudes into the lumen of the affected artery and compromises the flow blood and oxygen to the affected part. The final stage in plaque development, the formation of the ‘fibrous plaque’ is a structure covered by a dense cap of connective tissue with embedded smooth muscle cells that usually overlay a core of cholesterol, cholesterol ester, phospholipids and necrotic debris. In advanced fibrous plaques, the endothelium may fissure. Plaque fissures result in haemorrhage into the plaque, followed by thrombosis and acute occlusion of the arterial lumen. Thus plaque rupture is considered to be the key event that triggers abrupt arterial occlusion and ischemia (Rodriguez-Granillo et al., 2005). The resulting tissue ischemia can be the first end point that results in clinical demonstration of a process that has been evolving over decades.
1.2.2. Atherosclerotic Progression

There are many factors involved in the development of the atherosclerotic plaque and this adds to the difficulty in understanding the pathogenesis of this widespread disease. Rayer, in the 1820s, likened atherosclerosis to inflammation, a concept which was developed further by Virchow in 1856. "The Response to Injury" hypothesis was proposed by Ross in 1973, bringing together a wide range of studies and observations in the field of atherosclerosis. The hypothesis was based on the observations that the proliferation of smooth muscle cells and lesions resembling atheromatous plaques could be induced in experimental animals by endothelial denudation (lesion development was accentuated by hypercholesterolemia) and also that a platelet-derived growth factor could induce smooth muscle growth in vitro. The basis of the initial hypothesis was that injury to the endothelium caused by mechanical, chemical, toxic, viral or immunological agents caused the endothelial denudation and this was followed by platelet adhesion and aggregation and consequent release of PDGF, in turn leading to the migration and proliferation of smooth muscle cells in the arterial intima and secretion of connective tissue components. This theory has been tested, modified, retested and re-modified over the past twenty five years. The modifications take into account the observation that the endothelium can respond to a variety of stimuli leading to endothelial dysfunction but not necessarily causing denudation of the endothelium. It was also postulated that growth promoting factors from cell types other than platelets (i.e. macrophages) might regulate smooth muscle accumulation in the arterial intima. Since monocytes and macrophages were the major cellular components of the lesion, it was reasonable to suggest that they had a possible role to play in its initiation and evolution (Ross 1986).

Several different sources of injury to the endothelium (free radicals caused by cigarette smoking, hypertension, diabetes, elevated homocysteine, infectious micro-organisms and agents such as oxidised low density lipoprotein) (Ross 1999) can lead to endothelial cell dysfunction which in turn leads to increased adherence of monocyte/macrophages and T lymphocytes. Migration of these cells into the subendothelial space is increased and formation of adhesive cell-surface glycoproteins by the endothelium is induced. These
cells may migrate further beneath the surface as the process continues and attach to vascular cell adhesion molecules under the influence of growth regulatory molecules released from platelet granule contents such as PDGF and epidermal growth factor (EGF). Following the entry of monocytes and lymphocytes into the arterial intima, the monocytes become activated to macrophages, which accumulate lipid and become foam cells (Shaskin et al., 2005). These foam cells together with lymphocytes become the fatty streak. The fatty streak can then progress to an intermediate, fibrofatty lesion and ultimately to a fibrous plaque.

In summary the processes involved in the development of atherosclerosis include: endothelial injury and dysfunction, plasma lipid infiltration into the artery wall, platelet adherence to the exposed endothelium with the concomitant release of platelet granule constituents, monocyte and smooth muscle cell migration and proliferation, immune and inflammatory responses, thrombosis at vascular injury sites and the formation of lipid-filled foam cells. The major cell types implicated in the development of the atherosclerotic lesion are endothelial cells, smooth muscle cells, monocyte/macrophage cells, platelets and lymphocytes (Ross 1999).

Endothelial cells play a crucial role in atherogenesis, since endothelial injury is the initial event in the process. They play numerous functional roles including: (1) the provision of a non-thrombogenic environment that in non-adherent to leucocytes and platelets (2) a permeability barrier through which there is exchange and active transport of substances into the artery wall, (3) maintenance of vascular tone by release of vasodilatory molecules such as Nitric oxide (NO) and prostacyclin (4) formation and secretion of growth-regulatory molecules and cytokines (5) maintenance of connective tissue matrix, including the basement membrane upon which it lies (6) the ability to modify (oxidise) lipoproteins as they are transported into the artery wall (Ross 1995). Alteration in one or more of these functions may represent the earliest manifestations of endothelial dysfunction and atherogenesis.
Smooth muscle cells located in the media constitute the majority of the normal artery wall. By maintaining their attachments to neighbour cells and surrounding connective tissue matrix, they provide the tonus of the artery that normally dampens the differences between diastole and systole. Smooth muscle cells contribute to the fibroproliferative component of atherogenesis in two ways. First, they can respond to vasoactive agents such as NO and alter vascular tone (Moncada et al., 1990). NO acts as an intra- and intercellular signaling molecule in vascular and gastrointestinal smooth muscle and is an important mediator in numerous physiological and inflammatory processes (Stark et al., 1992). The mechanism of NO action is not fully understood, but many of its actions are mediated by the activation of guanylate cyclase, which results in an increase in the concentration of cyclic guanosine 3',5'-monophosphatate (cGMP) in smooth muscle (Ignarro et al., 1986). cGMP is thought to act directly on ion channels, and to activate protein kinase, which regulates the activity of proteins through phosphorylation (Robertson et al., 1993). NO and NO donors (e.g., sodium nitroprusside SNP) cause relaxation of vascular smooth muscle through the accumulation of cGMP (1) or through the direct activation of K+ channels (Bolotina et al., 1994; Carl et al., 1995). Several studies have suggested that NO might decrease the intracellular Ca2+ level or reduce the Ca2+ sensitivity of the contractile elements, which results in smooth muscle relaxation (Karaki et al., 1997). Furthermore, the relaxing action of NO has been indicated indirectly by inhibiting the release of the neurotransmitters acetylcholine and substance P (Mang et al., 2002). In gastrointestinal smooth muscle, NO or NO donors evoke different responses, including relaxing, contractile effects, relaxations followed by contractions or contractions followed by relaxations, which depend on the compound, tissue and species (Izzo et al., 2002). Secondly they express genes involved in growth regulation such as PDGF, EGF and monocyte-colony stimulating factor (M-CSF) (Libby et al., 1988). It is through the latter mechanism that smooth muscle cells contribute to the development of the atherosclerotic plaque. Smooth muscle cells accumulate in the sub-endothelial space, and although present in fatty streaks, are more abundant in the fibrous space, where synthesis of collagen by smooth muscle cells stabilises the plaque.
Macrophages act normally as antigen-presenting cells to T-lymphocytes and as a source of growth-regulatory molecules and cytokines and are the main inflammatory mediator cells in the atheromatous plaque microenvironment. Monocyte-derived macrophages are the principal source of foam cells in the lesions because they take up lipid and oxidised LDL through both scavenger receptors and a putative LDL receptor (Goldstein et al., 1979). The exposure of macrophages to agents such as oxidised LDL results in the secretion of growth factors and cytokines, in particular PDGF (Ross et al., 1990), interleukin-1 (Libby et al., 1989) and tumour necrosis factor α (TNFα) (Libby et al., 1986). The action of these molecules can further enhance the movement of monocytes into the developing plaque and also stimulate smooth muscle cell proliferation. This together with the finding of Gianturco et al., (1998) that monocyte-derived macrophages possess an apoB-48 receptor, could significantly contribute to their role in atherogenesis.

Platelets are also involved in the progression of atherosclerosis. Initially, they interact with the irregular endothelium overlying subendothelial fibrofatty proliferation and also with components in the exposed subendothelial space causing the formation of intramural thrombi. Platelets also release at least two mitogens, EGF and PDGF, which stimulate smooth muscle and endothelial cell proliferation (Ross, 1986). T-lymphocytes have been observed within lesions (Ross, 1993) suggesting that there may be an immunological component of atherosclerosis.

1.2.3. Growth factors and Cytokines in Atherosclerosis

Growth factors and cytokines are both involved in the induction and regulation of numerous critical cell functions. During the process of atherosclerosis, they may act in cell recruitment and migration, cell proliferation and in the control of lipid and protein synthesis. They are also implicated in vascular events, such as vasodilation, vasoconstriction and coagulation. The terms growth factor and cytokine are often used interchangeably, although the cytokines were originally considered as mediators implicated in immunity and inflammation, while the growth factors were thought to be responsible for the proliferation and chemotaxis of cells in organs and tissues. With
regard to atherosclerosis, these two terms are closely related, with the same mediator sometimes acting as both an inflammatory and growth regulator, depending on the target site. The growth-regulatory molecules can have multiple and sometimes divergent effects. They can stimulate or inhibit cell proliferation and many of the proliferative agents can act as chemoattractants. Some of the growth regulatory molecules are potentially important in cell proliferation; these include: PDGF, insulin-like growth factor (IGF-1), TNFα, transforming growth factor β (TGFβ) and interleukin-1 (IL-1). These are all capable of inducing smooth muscle cell proliferation and while they are not generally expressed in the normal artery, they are upregulated in lesions of atherosclerosis.

Chemotaxis is a critical event in lesion development being necessary to bring leukocytes into the artery wall, and at some sites, smooth muscle cells into the arterial intima. Monocyte chemotactic protein-1 (MCP-1), oxidised LDL and TGFβ can each induce monocyte chemotaxis and endothelial transmigration, whereas PDGF and IGF-1 can induce smooth muscle chemotaxis. Among the cytokines, IL-1, TNFα, interferon-γ, together with CSFs are modulators of the inflammatory response that occurs once the endothelium has been exposed to noxious agents (Ross 1993).

Clearly these growth factors and cytokines play important roles in the development of atherosclerosis, working through a network of cellular interactions, the release of one molecule leading to the expression of a second molecule in a target cell that can either stimulate itself in an autocrine way, or its neighbour in a paracrine way. The full extent of the involvement of growth factors and cytokines in the atherosclerotic process is not yet fully understood, but their complete elucidation would further our understanding of the pathogenesis of this complex disease.

1.2.4. Risk Factors for Atherosclerosis

There are several risk factors that have been implicated in the pathogenesis of atherosclerosis. These factors act independently and are additive when they co-exist. They may be separated into modifiable and non-modifiable risk factors. The non-modifiable risk factors are age, gender and genetic factors including family history.
Amongst the modifiable risk factors include hypertension, diabetes, smoking, lipoprotein abnormalities, hormonal changes, dietary influences, obesity and lack of exercise. Harjai et al (1999) have added to this list of risk factors, with factors including hyperhomocysteinemia, lipoprotein (a) excess, oxidative stress, hyperfibrinogemaemia (among other thromogenic factors), left ventricular hypertrophy, hypertriglyceridaemia, markers of inflammation (such as C-reactive protein and serum amyloid A), infectious agents (such as Helicobacter pylori and Chlamydia pneumoniae) and pro-coagulant substances (such as plasminogen and plasminogen-activator inhibitor-1) all emerging as possible new cardiac risk factors. Burnett et al (2005) suggest the potential role of resistin in atherogenesis, showing increased serum levels in patients with CAD (coronary artery disease), and an increase in the expression of resistin in atherosclerotic plaques.

1.2.4.1 Age as a Risk Factor

The incidence and extent of atherosclerosis generally increases with age. The disease can be thought of as a slow continuous process that evolves as the result of interaction of genetic and environmental factors over time. However, Brown and Goldstein (1984) have reported that individuals homozygous for familial hypercholesterolemia have severe atherosclerosis and myocardial infarcts as early as two years of age. Post-mortem examination of the coronary arteries of over 300 young soldiers killed in Korea showed that more than 75% had some degree of CAD, varying from fibrous thickening to complete occlusion of one or more of the main branches (Enos et al., 1986).

1.2.4.2 Gender as a Risk Factor

It has been well documented that males are more likely to develop cardiovascular than women in general. Wilson et al (1987) reported that pre-menopausal women appear to have added protection but this only lasts until the onset of menopause when CHD increases sharply, although never quite reaching the high levels found in men. The mechanism behind these differences is unknown but probably reflects the higher HDL
cholesterol levels and lower triglycerides found in women. In diabetes however, this
gender associated atheroprotection is abolished.

1.2.4.3. Genetic Factors as Risk Factors

The most common genetic causes of premature CHD are familial hypercholesterolemia,
hyperbetalipoproteinaemia and familial combined hyperlipidaemia. The two former
disorders can occur in homo- and heterozygous forms. Apo E genetic variation also
influences the progression of atherosclerosis. Apo E genotyping has revealed three
common alleles in the population: E2, E3 and E4. In an autopsy study of more than 500
young male trauma victims, the E3/2 genotype (the E3 protein is synthesised from one
parental allele and the E2 from the other) was associated with reduced atherosclerosis
relative to E3/3 (Hixson et al., 1991). Family and twin studies indicate that half the
population variance in LDL cholesterol is genetic, with approximately 7% of the variance
explained by factors such as LDL receptor, apo B and apo E mutations, with
preponderance unexplained (Breslow 1991). Individuals differ in their response to dietary
fat and cholesterol, and genes controlling diet response may explain much of the variation
in LDL cholesterol. In Caucasians, hypertriglyceridaemia is strongly associated with a
polymorphism in the gene encoding apo C-III, an inhibitor of lipoprotein lipase (LPL).
Association of this and other polymorphisms in the apo A1-CIII-AIV gene cluster with
CAD among subjects with a family history has been reported (Price et al., 1989) and
more recently a mutation in the apo E-CI-CIV-CII gene cluster encompassing both the
apo C-II and apo C-IV genes, resulting in a deficiency of apo C-II has been shown to be
associated with familial chylomicronaemia (de Graff et al., 2000).

1.2.4.4. Dietary Influences as a Risk Factor

Foods are composed from six basic nutrients: (a) carbohydrates, (b) fat, (c) proteins, (d)
vitamin, (e) minerals and (f) water. Its balance on daily dietary patterns has more or less
related to the prevalence of many metabolic disorders, like hypertension, dyslipidaemia,
obesity and diabetes, as well as to increased risk of atherosclerotic disease.
The Diet, Nutrition, and the Prevention of Chronic Diseases report of the World Health Organization recommend that 55% to 60% of the daily caloric intake should be obtained from carbohydrates. Forty-five to fifty percent of these calories should come from complex carbohydrates, and natural sugars found in fresh fruits and vegetables and no more than 10% from refined and processed sugars [WHO study group, 2003]. Nevertheless, the consumption of carbohydrates in people with the metabolic syndrome remains controversial and needs further investigation.

Grundy and Denke (1990) are among several authors to point out that substantial data exists that indicates the influence of diet on the development of atherosclerosis. Fibre is an organic compound found in plants and the role of dietary fibre in the prevention of cardiovascular disease has received increasing attention as data have accumulated. Recent cohort studies have found a consistent protective effect of dietary fibre on cardiovascular disease outcomes, prompting many leading organizations to recommend increased fibre in the daily diet.

There is strong evidence that an increase in serum cholesterol levels increases the risk of CVD (Martin et al., 1986). While there are strong genetic determinants of serum cholesterol levels, dietary consumption of cholesterol has been shown to be influential, with the mass of dietary cholesterol absorbed increasing with intake and causing elevated serum levels (Miettinen et al., 1989; McNamara et al., 1987). Dietary cholesterol has also been shown to alter the postprandial lipoprotein response in both Type 2 diabetic (Taggart et al., 1997) and non-diabetic subjects (Dubois et al., 1994) by producing cholesterol-enriched chylomicrons, which may be potentially pro-atherogenic particles. It would still appear that a reduction in total fat and an increase in dietary fibre and unsaturated vegetable oils is still prudent advice (Gotto, 1991). There is clear evidence from animal studies to show that meals rich in polyunsaturated fatty acids (PUFA’s) result in an attenuated postprandial triglyceride response compared with meals rich in saturated fatty acids (SFA’s) (Groot et al., 1988). In these studies chylomicrons from animals fed PUFA’s and re-injected into recipient animals, showed a faster rate of
clearance and uptake into the liver than animals administered chylomicrons from SFA fed animals (Groot et al., 1988; Bravo et al., 1995).

During the last decades there is increasing scientific evidence that there are protective health effects from diets, which are high in fruits, vegetables, legumes and whole grains and which include fish, nuts, and low-fat dairy products. Such diets need not to be restricted in total lipid intake fat as long as there is not an excess of energy intake over expenditure calories and emphasize predominantly vegetable oils that there are low in saturated fats and partially hydrogenated oils (WHO study group, 2003). The “Mediterranean Diet” which is rich in monounsaturated fatty acids (MUFA’s) (Willett et al., 1995) and a diet rich in omega-3 fatty acids (Rapp et al., 1991) have been shown to modify oxidative stress, a potential risk factor for CAD. This dietary pattern is mainly characterized by daily olive oil consumption.

In Type 2 diabetic subjects, it has been shown that a diet rich in oleic acid compared to linoleic acid results in reduced numbers of intestinally-derived apo B-48 particles (Madigan et al., 2000), improves insulin sensitivity and restores endothelium-dependent vasodilation (Ryan et al., 2000) suggesting another mechanism for an anti-atherogenic effect of at least one aspect of a Mediterranean style diet.

1.2.4.5. Smoking as a Risk Factor

Cigarette smoking is the most important preventable cause of premature death in the United States. It accounts for more than 440,000 of the more than 2.4 million annual deaths. Cigarette smokers have a higher risk of developing a number of chronic disorders. Peto et al (1992) reports that cigarette smoking is one of the major modifiable risk factors for CHD, accounting for about 30% of cardiovascular deaths. In the Multiethnic Study of Atherosclerosis (MESA), it was shown that “the smoking association with minimal, moderate, and more severe disease was progressive, estimated as equivalent to LDL cholesterol effects of 40, 85 and 238mg/dl respectively” (Sharrett et al., 2005). The 4 principal mechanisms of cardiovascular damage caused by cigarette smoking are
induction of a hypercoagulable state, reduction of oxygen delivery because of carbon monoxide, coronary vasoconstriction, and nicotine-induced hemodynamic effects (Ludvig et al., 2005). Smoking has been shown to increase fibrinogen and factor VIIc coagulant activity (Meade et al., 1987) and this may lead to increased formation of mural plaque platelet thrombi on areas of atheromatous plaque formation. It has been calculated that cigarette consumption leads to a rise of 0.15 g/l of fibrinogen which increase the risk of CHD by 20% (Meade et al., 1987). Smoking is also known to decrease HDL concentrations (Craig et al., 1989) although this effect is reversible within one year of cessation of smoking. Lowe (1987) reported that smoking results in increased blood viscosity, slowing the flow of blood through the vasculature and leaving platelets and monocytes/macrophages in contact with the endothelial wall for longer periods of time.

1.2.4.6. Hypertension as a Risk Factor

Sixty-five million Americans and over 1 billion individuals worldwide have arterial hypertension. Hypertension is one of the few recognized determinants of atherosclerosis. Atherosclerotic vascular disease in its various expressions is a leading cause of death and disability in industrialized countries. Because hypertension is the most common diagnosis for which physicians see and treat patients, it offers the medical profession the greatest opportunity to intervene in a positive manner to improve the health and well being of patients (Standridge, 2005). Hypertension and atherosclerosis are intimately linked by the endothelial cell (Panza et al., 1990). The endothelium forms a layer, one cell in thickness, lining the interior surface of the vascular system. This lumen-vascular wall interface functions as a semipermeable membrane, maintains a nonthrombogenic blood-tissue interface (by regulating thrombosis, thrombolysis, and platelet adherence), modulates vascular tone and blood flow, and metabolizes hormones. Endothelial cells seem to play a central role in coordinating the microcirculatory system and promoting tissue perfusion and oxygen supply (Vallet B, 2002). Additionally, endothelial cells regulate immune and inflammatory reactions (largely by controlling leukocyte interactions with the vessel wall), modify lipoproteins in the arterial wall, and regulate the growth of other cell types, particularly smooth muscle cells.
1.2.4.7. Obesity as a Risk Factor

It is difficult to assess to what extent obesity is an independent risk factor since it is associated with other risk factors for atheromatous disease, such as hypertension, insulin resistance, hyperinsulinaemia, diabetes and altered lipoprotein metabolism. Kissebah et al (1994) suggest that it is the distribution of body fat and not just its absolute mass that is the important factor. A central distribution of body fat has been associated with a higher risk of morbidity and mortality than a more peripheral distribution of body fat. Loss of body fat has been shown to increase HDL in both sexes (Stevenson et al., 1988). The Framingham study has also revealed data that demonstrates an increased risk of CHD with increased levels of obesity (Hubert et al., 1987).

1.2.4.8. The role of Exercise in the risk of CHD

It has been shown by Harper et al., (1991) that even a moderate increase in exercise can reduce the risk of CHD and Morris et al., (1990) showed in a study that in men who took regular vigorous exercise, the risk of CHD was decreased significantly, by less than 50% on average. Even with these results, it is unclear whether it is the development of physical fitness of the exercise itself that is responsible for the protective effect (Slattery et al., 1989). This is evident in the Seven Countries Study where exercise and physical activity were found not to decrease the incidence of CHD in hypercholesterolemic subjects (Keys, 1980). Lamon-Fava et al, (1989) showed that increasing exercise causes increases in HDL cholesterol and that sustained regular exercise causes triglyceride levels to drop. Rauramaa et al., (2004) showed that in a group that excluded men taking statins, the 6-year progression of intima–media thickness, adjusted for smoking and annual measures of low-density lipoprotein cholesterol level, systolic blood pressure, and waist circumference, was 40% less in the exercise group than in the control group. In a study conducted by Bhalodkar et al, (2005) in Asian Indians (who have a greater prevalence and incidence of coronary artery disease than other ethnic groups, despite similar routine lipid profiles), exercise was associated with significantly greater concentrations of total HDL cholesterol, entirely due to significant increases in the cardio protective large HDL
subclass and larger HDL cholesterol particle sizes. A recent study by Gill et al., (2005) looked at the effects of moderate exercise on postprandial lipoproteins, apolipoproteins and lipoprotein remnants in middle aged men and found a significant reduction in postprandial chylomicrons, VLDL1 and VLDL2 particles. They also found that exercise reduced postprandial remnant-like lipoprotein cholesterol and triglyceride concentrations, suggesting that exercise induces compositional changes to lipoprotein species which are likely to influence their metabolism and atherogenicity.

1.2.4.9. Hormones as a Risk Factor

Epidemiological studies provide the principal evidence that high insulin levels are atherogenic (Jarrett 1988). Two prospective studies have reported than insulin is an independent predictor of CHD (Pyorala 1979; Ducimetiere et al., 1980). More recently however, Zethelius et al (2005) reported that Proinsulin provides a better prediction of CHD than insulin. They also showed that insulin resistance measured by the euglycaemic insulin clamp predicts subsequent CHD in elderly men. Howard et al (1996), demonstrated in the Insulin Resistance Atherosclerosis Study a positive association between insulin resistance and atherosclerosis in a large multiethnic population.

Stress is characterized by an increase in plasma catecholamines and cortisol associated with a rise in heart rate and blood pressure (Klein, 2001). The catecholamines, epinephrine, norepinephrine and dopamine, act as neurotransmitters in the central nervous system and as hormones in the circulation. They act by binding to the α and β adrenergic receptors on the cell membrane to produce a variety of hemodynamic and metabolic effects. In animals it has been shown that administration of catecholamines results in an increase in serum very low density lipoproteins (VLDL) and LDL cholesterol and triglycerides (O’Donnell et al., 1988). It has also been reported that LDL receptor activity is depressed by catecholamines (Krone et al., 1988). Chronic stress and increase catecholamine levels have been shown to cause lipoprotein alterations, suggesting that the link between stress and CHD may be lipoprotein-mediated (O’Donnell et al., 1987).
Women in the age group of 20-50 are shown to have much less susceptibility to Coronary Heart Disease (CHD) and other atherosclerotic diseases as compared to men. Exact cause of which is not precisely known and oestrogen is constantly shown to be associated with this phenomenon. Improvement of serum HDL concentration and improvement of endothelial functions are some of the proposed mechanisms through which oestrogen is believed to mediate this effect (Paranjape et al, 2005). Wallace et al (1979) reported that post-menopausal women on oestrogen replacement therapy were found to have increase HDL levels when compared to women of the same age no on this therapy. More recently, in post-menopausal women, oestrogen replacement has been shown to be associated with reductions in plasma fibrinogen (Giri et al., 1998) and lipoprotein (a) levels (Espeland et al., 1998). Owens et al (2000) found that in Type 2 diabetic women, treatment with either oestrogen alone or in combination with norethisterone acetate resulted in reduced levels of both total and LDL cholesterol. Studies on hormone treatment in pre-menopausal women have produced conflicting results. Pre-menopausal women on the combined oestrogen and progesterone oral contraceptive pill were found to have elevated total cholesterol, VLDL and triglycerides (Molitch et al., 1974). The Framingham study in 1985 suggested an increased risk but a study of 50,000 nurses showed that those on oestrogen demonstrated a 50% reduction of major coronary disease (Stampfer et al., 1991).

1.2.5. Cholesterol and Lipoproteins

Hypercholesterolemia is an important risk factor for the development of atherosclerosis (Steinberg et al., 1989) and the benefits of therapeutic intervention have been demonstrated in clinical studies. Intensive lipid-lowering regimes have been shown not only to slow the progression of coronary atherosclerosis and to reduce the risk of mortality (Tyroler, 1987) but also in some cases to lead to absolute regression (Blankenhorn et al., 1987). Elevated serum cholesterol is a major risk factor in CHD. In the Multiple Risk Factor Intervention Trial (MRFIT), which screened more than 356,000 middle-aged men without a history of MI, the relation between serum cholesterol and risk of premature death from CHD was continuous, powerful, and graded (Stamler et al.,
1986). The results of MRFIT as well as such trials as the Framingham Heart Study indicate that the risk of CHD increases by approximately 2% for each 1% elevation in total cholesterol (Kwiterovich, 1998). Recognizing the relationship between elevated cholesterol levels—specifically low-density lipoprotein cholesterol (LDL-C)—and CHD risk, in 1988 the National Cholesterol Education Program (NCEP) issued guidelines for the detection, evaluation, and treatment of high blood cholesterol, then revised the guidelines in 1993 and again in 2001 (Expert Panel, 1988; 1993; 2001). The most recent guidelines emphasize CHD risk-factor stratification in classifying LDL-C levels as optimal, near optimal, borderline high, high, and very high, and in recommending the types and intensity of therapeutic interventions. The guidelines also recognize statins as the most effective treatment for elevated LDL-C. Despite the efforts of the NCEP, the actual number of CHD deaths declined by only 9% between 1987 and 1997 (American Heart Association). The classic experiments of Brown and Goldstein (1986) firmly established the atherogenicity of LDL. They described the LDL receptor pathway for the regulation of cholesterol homeostasis and established it’s importance in cellular cholesterol uptake. Patients with FH have a variety of well-defined gene defects involving the LDL receptor and suffer from premature atherosclerosis primarily due to their elevated plasma LDL levels, which are in turn due to the deficiency in LDL receptors. The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, inhibit HMG-CoA reductase, the rate-limiting enzyme in the synthesis of cholesterol (Knopp, 1999). Statins reduce LDL entry into the circulation. Statins also upregulate hepatic LDL-C receptors, which increases LDL-C removal through the liver. Whereas other agents, including bile acid sequestrants, fibrates, and nicotinic acid, have a role in many patients with dyslipidaemia, the statins are the most effective medications available for lowering elevated TC and LDL-C levels (Blumenthal, 2000; Safeer, Lacivita, 2000). Of the currently available statins, atorvastatin and simvastatin are generally considered to provide the greatest percentage reductions in TC and LDL-C.

The expansion of statin therapy to a broader range of patients is also supported by results from the Medical Research Council/British Heart Foundation Heart Protection Study,
which, with 20,536 subjects, is the largest trial of statin therapy ever conducted (Heart Protection Study, 2002). Importantly, this study demonstrated significant benefits in patients for whom the benefits of statin therapy had not previously been well-established, including women, elderly patients, diabetic or hypertensive patients without prior CHD, patients with below average cholesterol levels (total cholesterol levels <5 mmol/L [193 mg/dl] or LDL-C levels <3 mmol/l [116 mg/dl]), and patients with prior non-coronary vascular disease. Men and women age 40–80 years were recruited if they had a non-fasting total cholesterol greater than 3.5 mmol/l (135 mg/dl) and a substantial 5 year risk of death from coronary heart disease because of a past history of CHD or CHD risk factors. This study found that simvastatin 40 mg significantly reduced total mortality by 13%, vascular mortality by 17%, stroke by 25%, and non-coronary revascularizations by 15%. The Heart Protection Study also found that there appears to be no threshold lipid or lipoprotein value below which statin therapy was not beneficial in high-risk patients, even if those patients already had below average cholesterol levels. The AVERT study also provides evidence that aggressive reduction of LDL-C may provide additional clinical benefits (Pitt et al., 1999).

Oxidation of LDL is proposed to accelerate atherosclerosis. Monocyte/macrophages take up oxidised LDL, via the scavenger receptor, 3 to 10 times more rapidly than native LDL and can therefore generate foam cells (Steinberg et al., 1989). Oxidised LDL differs from native LDL in several ways that could render it more atherogenic. It is chemotactic for circulating monocytes, while inhibiting the motility of tissue macrophages, it is cytotoxic. It is capable of inducing the release of the chemotactic factor MCP-1 from endothelial cells, and it may also alter the vasomotor properties of coronary arteries (Witztum and Steinberg 1991).

HDL cholesterol on the other hand is considered to be anti-atherogenic. More than 25 years ago, Pearson et al. showed that decreasing levels of HDL-C consistently predicted increasing numbers of diseased coronary arteries in both men and women (Pearson et al, 1979). Moreover, low HDL-C levels were associated with left main coronary artery disease in patients with or without triple-vessel disease. The association of low HDL-C and diseased coronary arteries was independent of LDL-C and TG levels. The
Framingham Heart Study found HDL-C to be the most potent lipid predictor of CHD in both sexes after age 49 (Gordon et al., 1977), and in 15 of 19 epidemiologic studies a significant inverse relationship between HDL-C and CHD was demonstrated; for each 1% increase in HDL-C, a 2–3% decrease in CHD risk has been observed (NIH Consensus Development Panel, 1993). The atheroprotective effects of HDL cholesterol may, in part, be explained by its central role in reverse cholesterol transport (net export of cholesterol from the tissue). HDL has been shown to partially suppress LDL oxidation (Parthasarthy et al., 1990) and this antioxidant property may be due to two enzyme systems (paranoxanase and platelet activating factor acetylhydrolase) associated with normal HDL that have been reported to inhibit LDL oxidation in vitro (Mackness et al., 1991; Stafforini et al., 1993). Data from the Quebec cardiovascular study revealed that not only was HDL cholesterol an independent predictor of ischemic heart disease but also that low HDL cholesterol had more of an impact on the atherogenic index (total cholesterol/HDL cholesterol) than raised LDL cholesterol levels at least in middle-aged men (Despres et al., 2000). In addition to its major role in RCT, HDL-C has other important functions that may contribute to its atheroprotective effect, although their elucidation is as yet unclear. These include anti-inflammatory (Wadham et al., 2004; Xia et al., 1999) and antioxidant properties of HDL (Deakin et al., 2004). Endothelial dysfunction characterised by decreased availability of nitric oxide (NO) and increased affinity for leukocytes, is one of the first pathological events in the early stages of atherosclerosis (Halcox et al., 2002; Schachinger, 2000). Endothelial cells undergoing apoptosis are also a major provider of thrombogenic surfaces and a rich source of cytokines and free radicals, which further aggravate inflammatory processes in the arterial wall. In vivo studies have shown that binding of HDL to SR-B1 suppresses vascular inflammation, by inducing activation of NO synthetase, NO release and resulting vasorelaxation of the endothelium, as well as attenuating expression of adhesion molecules and cytokines, which promote leukocyte extravasation, and preventing apoptosis. Clinically, intravenous infusion of HDL in hypercholesterolemic humans normalised endothelium-dependent vasodilation (Spiker et al., 2002). Additionally, increasing evidence suggests that HDL influences endothelial function by counteracting LDL oxidation, which is commonly considered a major event in the initiation and
development of atherosclerosis (Assmann et al., 2003). HDL has also been shown to possess anti-thrombotic properties, as well as affecting platelet activation. In a recent review, Wierzbicki comments on the role of HDL, saying that “the relation between changes in HDL-C and CHD is difficult to ascertain in the major statin trials due to the magnitude of the reduction in LDL-C observed” (Wierzbicki, 2005), although he does go to point out that “patients with low HDL-C continue to have a greater risk than those with higher HDL-C” (Wierzbicki, 2005).

The Paris Prospective Study found that triglycerides (TG) were an independent risk factor for the development of atherosclerosis (Fontbonne et al, 1989). Hulley et al (1980) suggest that high triglycerides merely reflect a low HDL. A recent study by Ahmad et al (2005) in north Indian type 2 diabetic subjects (known to have a very high prevalence rate of premature CHD) showed a strong positive correlation between postprandial TG levels and carotid intima-media thickness (IMT). Patsch et al., (1992) reported that postprandial but not fasting triglyceride levels exhibit and association with CAD that was statistically independent and stronger than that of HDL cholesterol, and in 2001, the Diabetes Atherosclerosis Intervention Study suggested that reduction of triglyceride by fenofibrate treatment reduces the angiographic progression of CAD in Type 2 diabetes (DAIS 2001).

Zilversmit (1979) formulated the original hypothesis that chylomicrons are atherogenic. Evidence that TRL particles might directly cause or exacerbate atherosclerosis came when it was shown that following hydrolysis to their remnant forms, chylomicron and VLDL do indeed penetrate arterial tissue and become trapped within the subendothelial space (Nordestgaard and Tybjerg-Hansen 1992; Procter and Mamo 1996; 1998). The postprandial concentrations of chylomicron remnants have been shown to correlate with the 5-year progression of CAD in young post-infarction patients (Karpe et al., 1994a) and more recently an association between markers of postprandial TRL remnants and intima media thickness – a marker for early atherosclerosis, has been reported in 96 healthy men (Karpe et al., 2001). This association was also found to be independent of both LDL cholesterol and plasma triglycerides. Elevated fasting levels of both apo B-48-containing
chylicmicron remnants and remnant-like particle cholesterol have recently been reported in patients with familial hypercholesterolemia (Dane-Stewart et al., 2001).

TG-rich lipoproteins are involved in atherosclerosis and thrombosis. TG, remnant-like particle (RLP) cholesterol (RLP-C) and RLP-TG increase after fat load and could contribute to atherothrombosis. Postprandial lипaemia is not a uniform abnormality. Its pathophysiology is not yet entirely clarified; possibly, the response to dietary fat is a polygenic phenomenon (Kolovou et al., 2005). Remnant lipoproteins have been shown to be independently associated with abnormal endothelium-dependent vasomotor function in large and resistant coronary arteries in humans (Kugiyama et al., 1998). These observations emphasise the importance of an efficient clearance mechanism of these particles from the circulation. It was proposed by Byrne (1999) that abnormalities in the TRL along with HDL and LDL particles, comprise a particularly atherogenic lipoprotein profile which may cause atherosclerosis through their procoagulant and proinflammatory effects. Expression of cytokines, adhesion molecules and growth factors is regulated by nuclear factors and PPAR agonists such as fibrates and certain fatty acids, may regulate activity. These studies suggest that TRL particles may mediate their potentially proatherogenic effects by a variety of pathways. Although the association of serum triglycerides (TG) and CHD has been controversial, accumulating evidence suggests that hypertriglyceridemia is an important risk factor for CHD especially in sub groups of the population like those with type 2 diabetes or impaired glucose (Antonio, 1998; Jeppesen et al., 1998; Fontbonne et al., 1989). In type 2 diabetes, elevated triglycerides may be a better predictor of CHD than elevated cholesterol levels (Patsch et al., 1990; Haffner, 1998). Postprandial dyslipidaemia is a distinct component of diabetic dyslipidaemia (Ginsberg et al., 2001; DeMan et al., 1996; Taskinen et al., 2001; Syvanne et al., 1994). Several studies have shown that the response of plasma triglycerides to a standard fat load is much greater in type 2 diabetic than in non-diabetic subjects matched for age, sex and BMI (Taskinen et al., 2001; Syvanne et al., 1994). Importantly, the peak concentrations of plasma triglycerides are achieved at 4–6 h after the load. Recently the diurnal profile of plasma triglycerides was followed using an ambulatory plasma TG measurement device in type 2 diabetic patients selected on the basis of normal fasting TG
concentration (Heine et al., 2002). The TG profile showed that the TG concentrations gradually increased after consecutive meals, showing the importance of postprandial dyslipidaemia in diabetes and the pro-atherogenic role it may play.

1.2.6. Diabetes and Atherosclerosis

Atherosclerosis is up to 5 times more common in diabetes (Mero et al., 2000). Tomkin and Owens (2001) in a review article report that the intestinally-derived lipoproteins are particularly abnormal in diabetes. The potentially atherogenic aspects of lipid abnormalities in diabetes have recently been described (Phillips et al., 2000; Tomkin et al., 2001; Curtin et al., 1996), with the apo-B containing particles thought to be particularly atherogenic. Steiner (1985), reports that the most common cause of death among people with diabetes is atherosclerotic cardiovascular disease. Data from the UKPDS have revealed that the major risk factors for CAD in Type 2 diabetic patients are increased LDL cholesterol, decreased HDL cholesterol, raised blood pressure, hyperglycaemia and smoking (Turner et al., 1998). Although obesity did not emerge as an independent risk factor, about 75% of patients with Type 2 diabetes are overweight and obesity has been shown to be independently associated with the above risk factors.

Chronic hyperglycemia has been hypothesized to contribute to coronary heart disease, but the extent to which hemoglobin A(1c) (HbA(1c)) level, a marker of long-term glycemic control, is independently related to CHD risk is uncertain. In a very recent study, Selvin et al. (2005) report that Elevated HbA(1c) level is an independent risk factor for CHD in persons with diabetes. Interestingly, they also report that in persons without diabetes, but with an elevated HbA(1c) level (over 4.6%), there is an also increased risk for CHD.

Epidemiological analysis suggests that the contribution of all the commonly measured risk factors together can account for no more than about 25% of the excess CHD in diabetes (Pyorala et al., 1987). Potential mechanisms of atherosclerosis in diabetes may include dyslipidaemia, insulin resistance, glycation of lipoproteins in the arterial wall, a
procoagulant state, glycooxidation, oxidation and growth factor and cytokine enhanced smooth muscle cell proliferation and foam cell formation.

Insulin resistance is frequently accompanied by hypertension and hyperinsulinaemia. Hypertension is twice as common in diabetic patients as in the general population and is a major risk factor for both small and large vessel disease (Turner et al., 1998). According to Steinberg et al. (1996), raised insulin levels in NIDDM subjects may be atherogenic. Hyperinsulinaemia has been shown to stimulate the hepatic synthesis of VLDL, leading to subsequently raised levels of LDL and triglyceride with decreased HDL levels. However, Kim et al. (2005) have recently reported that insulin administered in vivo to rats resulted in reduced neointimal growth and they suggest that in metabolic syndrome, insulin resistance, rather than hyperinsulinaemia, is the atherogenic risk factor.

Analysis of the diabetic subset in the Multiple Risk Factor Intervention Trial population demonstrated that increasing levels of LDL cholesterol in diabetes are accompanied by an increase in the incidence of CAD (Stamler et al., 1993). However, Scheffer et al. (2005) report that even in people with type 2 diabetes, who have similar levels of LDL cholesterol to their non-diabetic peers, the type 2 diabetic people are “more prone to atheroma”. This is because, they believe, LDL particles are modified in the presence of diabetes to become more atherogenic. These modifications include glycation in response to high plasma glucose levels; oxidative reactions mediated by increased oxidative stress; and transfer of cholesterol ester, which makes the particles smaller and denser. The latter modification is strongly associated with hypertriglyceridaemia. Oxidatively and non-oxidatively modified LDL is involved in plaque formation, and may thus contribute to the accelerated atherosclerosis (Scheffer et al., 2005). This post-synthetic chemical modification of LDL by oxidation or glycosylation is a popular hypothesis. Modified LDL is foreign to the body and results in antibody formation (Salonen et al., 1992). This may explain part of the inflammatory response seen in the atherosclerotic plaque as demonstrated by the large number of lymphocytes accumulating in the plaque (Ross, 1999). Increased levels of LDL antibodies have been reported in Type 2 diabetic subjects (Griffin et al., 1997), with the highest levels found in diabetic patients with CAD. In both
Type 1 diabetic patients with poorly controlled diabetes (Tsai et al., 1994) and Type 2 diabetic patients it has been shown that LDL has a higher susceptibility to oxidation and this increased susceptibility is related to the glycosylation of the LDL (Bowie et al., 1993). Other studies have also reported the occurrence of glycosylation of LDL in diabetes and have shown that there is increased accumulation of cholesterol ester in macrophages exposed to lipoproteins isolated from diabetic patients (Lyons et al., 1987; Lopes-Virella et al., 1988). The uptake of glycated LDL by human monocyte-derived macrophages is directly proportional to the degree of LDL glycation and is not mediated by the scavenger receptor but rather by a separate receptor of lower affinity and higher capacity (Lopez-Virella et al., 1988). Glycated LDL has been shown to be a potent stimulator of thromboxane B₂ release by platelets in Type 1 diabetes and to enhance thrombin-induced platelet aggregation (Watanabe et al., 1988). Most significantly, however, may be the finding that protein glycation increases the rate of free radical formation, resulting in enhanced oxidation (Mullarkey et al., 1990).

Zilversmit in 1973 first proposed that lipoprotein lipase (LPL) may be important in atherogenesis. LPL activity results in the hydrolysis of triglyceride-rich lipoproteins at the endothelial lining of the arteries and may lead to the formation of atherogenic remnants. However, efficient lipolysis of triglyceride-rich lipoproteins generally drives the lipoprotein profile in a non-atherogenic direction, promoting rapid clearance of postprandial lipoproteins and generating material for HDL formation. Apo E is found on the surface of all major circulating lipoproteins and it acts as the ligand for receptor-mediated uptake and clearance of chylomicron remnants, VLDL and LDL by the liver. Genetic variants of LPL and apo E are common in diabetic patients (Semenkovich and Heinecke, 1997) and may result in hypertriglyceridaemia. Heterozygous LPL deficiency is common, at a surprisingly high prevalence of 3-6% in population-based studies (Fisher et al., 1995) and some of these mutations that decrease LPL enzyme activity may increase the risk of vascular disease (Reymer et al., 1995).

Although some earlier cross-sectional and prospective studies demonstrate a significant independent relationship between levels of triglyceride in diabetes and risk of CAD
(West et al., 1983; Fontbonne et al., 1989), interest in triglyceride as an independent risk factor for CAD in diabetes has recently become fashionable (Stampfer et al., 1996; Alaupovic et al., 1997; Gotto 1998; Patsch et al., 1992) and recent results from the Diabetes Atherosclerosis Intervention Study suggest that reduction of triglyceride by fenofibrate treatment reduces the angiographic progression of CAD in Type 2 diabetes (DAIS, 2001). As previously discussed, triglyceride-rich lipoproteins are potentially atherogenic due to their association with the deposition of cholesteryl ester in the arterial wall. Studies have indicated that the postprandial levels of triglyceride-rich lipoproteins in diabetes are higher than those in control populations (O’Meara et al., 1992; Chen et al., 1993; Curtin et al., 1994). It has also been reported that the severity of angiographically demonstrated CAD in men and women with Type 2 diabetes correlates positively with the numbers of postprandial B-48 and B-100-containing lipoproteins (Mero et al., 2000) and this observation was independent of HDL cholesterol (Tkac et al., 1997).

1.2.7. The role of PPARγ agonists in the treatment of Diabetes

People are in a postprandial state most of the day, and this phase is proatherogenic (van Oostrom et al., 2004). Activating leucocytes and/or endothelial cells is necessary for adherence of leucocytes to the endothelium which starts the whole process of atherosclerosis, so inhibiting either of these processes is an interesting target for intervention (van Wijk et al., 2005). One potential intervention is the use of peroxisome proliferator activating receptors (PPARs). PPARs are nuclear receptors (NRs), which activate transcription by binding to specific sites, termed PPAR response elements (PPRE) in the promoters of target genes (Ricote et al., 2004). Three subtypes exist; PPARα, PPARγ and PPARδ (PPARβ, NUC-1), which differ both in their distribution patterns and in their biological roles. Both PPARα and PPARγ have been well characterised for their roles in lipid and glucose metabolism using specific marketed drugs such as the thiazolidinediones, ligands of PPARγ prescribed for the treatment of type 2 diabetes, and the fibrates, PPARα ligands prescribed for their lipid modulating properties (Ricote et al., 2004; Chinetti et al., 2003). In contrast, the biological role and
function of PPARδ (NUC-1, PPARβ), remain relatively unclear (Bishop-Bailey and Wray, 2003)

Various lines of evidence suggest that PPARδ may have a role relevant to the pathogenesis of atherosclerosis. Initial studies indicated a potential role for PPARδ in the elevation of high-density lipoprotein (HDL) in obese and diabetic db/db mice using a synthetic PPARδ agonist (Leibowitz et al., 2000). These data were confirmed in a subsequent report by Oliver et al., (2001) who showed that specific PPARδ ligands were able to elicit increases in HDL together with decreases in LDL and triglycerides in the obese rhesus monkey as a result of increased ATP-binding cassette A1 (ABCA1) expression and reverse cholesterol transport. A role for PPARδ in lipid metabolism is further supported by studies showing that PPARδ is a key regulator of fatty acid oxidation in both skeletal muscle (Wolf, 2003; Dressel et al., 2003; Wang et al., 2003) and cardiomyocytes (Cheng et al., 2004). Furthermore, Chawla et al (2003) have shown that PPARδ acts as a sensor for very low-density lipoprotein (VLDL), a role that implicates the receptor in the regulation of triglyceride homeostasis in peripheral tissues such as the macrophage and the vessel wall. Together, these data support a beneficial role for PPARδ agonists in the treatment of dyslipidaemia, a risk factor central to the pathogenesis of atherosclerosis.

In contrast to the data supporting a role for PPARδ in the regulation of lipid metabolism are a variety of studies showing potential effects of PPARδ activation on processes involved in the development of the atherosclerotic lesion. Several studies suggest a role for PPARδ in the control of cell differentiation, particularly when lipid accumulation may occur and PPARδ has been suggested to play a role in adipose expansion (Hansen et al., 2001) and in lipid uptake into macrophages (Vosper et al., 2001). In addition, Zhang et al., (2002) have shown that PPARδ is expressed in vascular smooth muscle cells where it is suggested to play a role in modulating cell proliferation, an effect that may promote stabilisation of the plaque. PPARδ has also been shown to play a role in inflammation. Initial studies in keratinocytes showed that PPARδ mediates resistance to tumour necrosis factor-alpha (TNFα)-induced apoptosis Tan et al., (2001) while more recent studies have
shown that deletion of PPARδ in macrophages of LDL receptor knockout (LDLR−/−) mice decreases atherosclerosis compared with control animals (Lee et al., 2003). The mechanism for this reduction is suggested to be via an attenuation of inflammation in the macrophage itself. Interestingly, however, PPARδ agonism in cultured macrophages in same study also resulted in an anti-inflammatory effect, indicating that depletion of PPARδ as well as PPARδ agonism may result in the same phenotype. A recent study by Graham et al., (2005) shows that the PPARδ agonist GW0742X reduces atherosclerosis in LDLR−/− mice. It is therefore unclear as to a definitive role of PPARδ, but from a vast array of literature, it seems that it may have an anti-atherosclerotic effect.

The PPARγ gene gives rise to three distinct mRNAs, i.e. PPARγ1, PPARγ2 and PPARγ3, each differing in their 5’ ends and each under control of their own promoter (Tontonoz et al., 1994; Fajas et al., 1997; Zhu et al., 1995). All three PPARγ subtypes contain the common exons 1–6. Human PPARγ1 contains in addition, the exons A1 and A2 at the 5’ end. PPARγ2 contains the B exon, whereas PPARγ3 contains only the A2 exon. PPARγ1 and 3 give rise to the same protein encoded by exons 1–6, since neither the A1 nor the A2 exon are translated. In PPARγ2, the B exon is translated producing a protein with an additional 28 amino acids at its NH2 terminus. In man, PPARγ1 and 3 expression has been found to be highest in large intestine and adipose tissue, although measurable expression was also found in kidney, liver and small intestine (Fajas et al., 1997; Auboeuf et al., 1997). PPARγ2 expression was highest in adipose tissue, with minimal expression in liver, but no expression at any other site (Fajas et al., 1997; Auboeuf et al., 1997; Zhu et al., 1995). Perhaps surprisingly, expression of PPARγ in muscle, which is classically thought to be involved in glucose disposal, was very low (Fajas et al., 1997; Auboeuf et al., 1997). These mRNA expression studies have also been confirmed by immunocytochemistry, showing that transcription and translation coincide. The high level expression of PPARγ in adipose tissue points to the important function of this transcription factor in adipogenesis and adipocyte gene expression.

Adipogenesis involves multiple transcription factors (Spiegelman and Flier, 1996; Auwerx et al., 1996). Adipogenic stimuli such as insulin, dexamethasone, or cAMP are
known to activate the transcription factors C/EBPβ and δ. Both these transcription factors
directly induce the expression of PPARγ (Wu et al., 1993, 1994), which subsequently
stimulates the expression of adipocyte-specific gene such as aP2, LPL and ACS, both
alone and in cooperation with two other transcription factors, C/EBPα and adipocyte
differentiation and determination factor 1 (ADD-1)/sterol response element binding
protein 1 (SREBP-1) (Tontonoz et al., 1993; Kim et al., 1996). This is highly remarkable
because both the activity of PPARγ-RXR (fatty acids, vitamin A) and the ADD-1/SREBP-1 (cholesterol) transcription factors are regulated by different nutrients and
both are involved in adipocyte differentiation (Schoonjans et al., 1997; Spiegelman and
Flier, 1996; Brown and Goldstein, 1997).

The clinical importance of these findings concerning PPARγ mRNA expression, is that
the antidiabetic thiazolidinediones, potent PPARγ ligands, are very unlikely to have an
important direct transcriptional effect on glucose metabolism in the muscle because there
is very little PPARγ expressed there. This implies that the effect of thiazolidinediones on
insulin sensitivity must be due to an indirect effect most likely mediated due to their
actions on PPARγ in adipose tissue. The crucial role of adipose tissue in glucose
homeostasis was supported by the clinical observation that both lipoatrophy (Moller et
al., 1991; Flier, 1995) and obesity are accompanied by insulin resistance (Spiegelman
and Flier, 1996), which indicates that an approximately 'normal' amount of adipose
tissue is required for normal insulin sensitivity. The currently favored hypothesis suggests
that the beneficial effects of PPARγ on glucose homeostasis are caused by the induction
of signalling molecules in adipose tissue, which indirectly would cause an improvement
in muscle glucose disposal.

Two groups of mediators generated in adipose tissue might influence glucose metabolism
in the muscle. On the one hand, adipocyte-derived protein-based mediators such as tumor
tumor necrosis factor α (Hotamisligil et al., 1996), plasminogen activator inhibitor-1
(Shimomura et al., 1996) and leptin (Cohen et al., 1996 interact with insulin signalling
pathways and affect muscle glucose disposal. On the other hand, lipid-derived mediators,
such as free fatty acids (FFAs), are at least equally important determinants of muscle

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insulin sensitivity (Randle et al., 1961). FFAs are released from adipocytes on activation of hormone sensitive lipase (HSL) and are also generated during the lipolysis of triglyceride-rich lipoproteins by LPL. The FFAs are taken up by the cells via a specific fatty acid transporter protein (FATP). In the cells, FFAs are converted into metabolic active acyl-CoA derivatives by the action of ACS. FFAs, are usually equally distributed between adipose tissue and muscle because both tissues express roughly equivalent amounts of the higher mentioned proteins involved in the generation and uptake of FFAs.

Studies with PPARγ activators suggested that the expression of all of these genes involved in FFA metabolism, i.e. LPL, FATP and ACS are affected in such a way as to improve muscle glucose disposal. In fact, in adipose tissue, in 3T3/L1 and Ob1771 adipocyte-like cell lines, activation of PPARγ induces the higher mentioned genes leading to an enhancement of FFA uptake. Hence, treatment with PPARγ activators shifts FFAs into fat cells, an effect corroborated by the observation that PPARγ agonists induce both adipocyte hypertrophy and hyperplasia and weight gain in rodents (de Vos et al., 1996). This change is usually associated with an increased insulin sensitivity of the adipocyte and will hence, also result in a decrease in HSL activity (Groop et al., 1989). Since extremely low levels of PPARγ are expressed in muscle, treatment with a PPARγ agonists does not affect expression of any of the above mentioned genes in this tissue. Therefore, FFA uptake is decreased in the muscle in favor of an uptake in the adipocyte. This decreased availability of FFAs in muscle produced by this adipocyte ‘FFA steal’ results, via the Randle cycle (Randle et al., 1961), in an improvement in muscle insulin sensitivity. The two available thiazolidinedione (TZD) PPAR agonists, pioglitazone and rosiglitazone, both demonstrate efficacy in the treatment of some aspects of diabetic dyslipidemia: each elevates HDL cholesterol concentrations and increases LDL particle size. However, they have variable effects on LDL cholesterol concentrations, and while pioglitazone reduces plasma TG concentrations, rosiglitazone treatment either has no effect or is associated with increases in plasma TG levels (Freed et al., 2002; LaCivita et al., 2002; Boyle et al., 2002).
1.3. CHOLESTEROL METABOLISM

1.3.1. Cholesterol

Cholesterol, which was first isolated from human gallstones more than two hundred years ago, had its name derived from the Greek language. The literal translation of cholesterol is bile solid and it was first called this by Chevreul in 1816. It is a twenty-seven carbon steroid with a hydroxyl group at position 3 and a double bond between carbons 5 and 6 (Figure 3.1). Cholesterol occurs in the body in two forms either as the sterol or in an esterified form attached to a long-chain fatty acid by an ester linkage. It is an essential component of the cell membrane, the ratio of cholesterol to phospholipids determining the extent of membrane fluidity – the larger the ratio the greater the rigidity of the membrane. Cholesterol is also necessary for steroid hormone biosynthesis and is the precursor of bile acids and Vitamin D.

Figure 3.1

Diagram showing the structure of Cholesterol.
There are two main sources of cholesterol in the body – exogenous from dietary sources or endogenous from *de novo* synthesised cholesterol. Cholesterol entering the body must be metabolised or excreted in order to prevent the potentially hazardous accumulation of the sterol. There are two main pathways for the removal of cholesterol from the body. Firstly, it may be lost by desquamation of the cells lining the gastrointestinal tract or through the movement of cholesterol into pancreatic, gastric, intestinal and canicular secretions. Alternatively cholesterol may be converted to bile acids (polar derivatives of cholesterol which act as detergents and by emulsifying lipids facilitate the absorption of dietary fat) and excreted from the body through the gastrointestinal tract.

1.3.2. **Cholesterol Synthesis**

Virtually every tissue in the human body is capable of synthesising cholesterol, but the vast majority of cholesterol is produced in the liver and in the distal portion of the intestine. The steps in cholesterol biosynthesis have been reviewed by Bloch (1965). The first step is the conversion of acetate to acetyl CoA. Acetoacetyl CoA is formed by the action of acetyl CoA-acyl transferase on two acetyl CoA moieties. The addition of a third acetyl CoA molecule, results in the formation of a six carbon molecule 3-hydroxy 3-methylglutaryl coenzyme A (HMG CoA) and this reaction is catalysed by the enzyme HMGCoA synthase. The next step is the production of mevalonate which is controlled by HMG CoA reductase, the rate-limiting enzyme of cholesterol synthesis. This step is, in part, regulated by the negative feedback of the end-product – cholesterol itself. Mevalonate undergoes several enzymatic and isomeric transformations resulting in phosphorylation and decarboxylation and production of farnesyl pyrophosphate. This product, an isoprenoid, can be polymerised by the enzyme squalene synthetase with six isoprenoid units coming together to form squalene. This is then oxidised by an epoxidase to form squalene oxide which then undergoes cyclization by the action of oxidosqualene-lanosterol cyclase to form cholesterol, which has a four ringed steroid nucleus with a hydroxyl group. The cholesterol synthetic pathway is shown in Figure 3.2.
1.3.3. Plasma Cholesterol Levels

The levels of plasma cholesterol levels are dependent on several parameters, including endogenous synthesis, secretion and catabolism of the various plasma lipoproteins (as is discussed in sections 1.4 – 1.7). Other major contributors to the amount of cholesterol entering the body each day include the amount of cholesterol in the diet (Dietschy et al., 1970), and the rate by which the dietary cholesterol is absorbed (Rudel et al., 1994; Wilson et al., 1994). Gylling et al. (1995) showed that a 90% reduction of cholesterol absorption in moderately hypercholesterolemic subjects has been shown to reduce plasma cholesterol and LDL levels by 35%. It was also estimated in the same study that by 100% inhibition of cholesterol absorption, a 68% reduction in plasma cholesterol levels could be achieved (Gylling et al., 1995). There has been much investment from both the academic and pharmaceutical sectors into designing therapeutic regimen to reduce dietary cholesterol absorption and lower the risk of cholesterol-related diseases. Success has been at best limited and the reason for this may be that the diet responsiveness of plasma cholesterol and LDL levels is extremely variable among different individuals (Beynen et al., 1987; Safonova et al., 1993; Gylling et al., 2002). An example of which is the interesting case of the a healthy octogenarian man who consumed 25 eggs per day but maintained normal blood cholesterol levels by compensating for the extra cholesterol load with an absorption of only 18% and a twice normal conversion rate of cholesterol to bile acids (Kern et al., 1991). These variations in dietary cholesterol responsiveness may be ascribed to the complexity of the cholesterol absorption process, which involves the participation of numerous enzymes and transport proteins through multiple steps.

Although our current understanding of the mechanism by which each of these proteins participates in regulating cholesterol absorption efficiency is still incomplete, significant progress has been made in the past few years in identifying key proteins that are involved in the cholesterol absorption cascade which will now be discussed.
1.3.4. **Pancreatic lipolytic enzymes**

Most of the cholesterol in food exists in the unesterified form with only 8-15% as cholesterol esters. The stomach is the start point of fat digestion and emulsification, with partial fat digestion by pre-duodenal lipases and emulsification by peristalsis. The lumen of the duodenum then accepts the crude emulsions which are mixed with bile and pancreatic juice. Therefore, the dietary cholesterol appearing in the lumen of the intestine is usually associated with triglycerides and phospholipids in lipid emulsions, and coexists with cholesterol secreted from the liver into the bile, which is mixed with phospholipids as vesicles or in bile salt micelles (Hennall *et al.*, 1990; Yao *et al.*, 2002). In vitro cell culture studies showed that cholesterol embedded in lipid emulsions cannot be absorbed directly by intestinal cells (Young *et al.*, 1999). The digestion of the phospholipids and triglycerides in the surface and core of the lipid emulsion particles, respectively, is necessary to liberate the dietary cholesterol to phospholipid vesicles and bile salt micelles for its transport to the brush border of the intestine for absorption by the mucosal cells (Young *et al.*, 1999).

The major lipases secreted by the pancreas into the intestinal lumen in response to a meal include the pancreatic triglyceride lipase (PTL), the Group IB phospholipase A2, and carboxyl ester lipase (CEL). Two additional proteins, pancreatic lipase-related protein-1 and -2 (PTLRP-1 and -2), which are similar in sequence and structure to the PTL, are also secreted by the pancreas and present in small amounts in adult pancreatic juice (Lowe, 2002). In vitro studies showed that PTL, upon activation by colipase, is the primary enzyme in the digestive tract that hydrolyzes triglycerides in emulsified lipid particles (Lowe, 2002; Lowe, 1994). Reduced triglyceride hydrolytic activity in the intestinal lumen such as those observed in *PTL*^-/-^ mice significantly reduces dietary cholesterol absorption (Huggins *et al.*, 2003). The mechanism by which reduced fat digestion results in suppression of cholesterol absorption has not been identified, but is most likely related to the delay in fat digestion efficiency in the intestinal lumen. The dietary cholesterol that remains associated with the undigested lipid emulsions in the proximal intestinal lumen cannot be absorbed through the unstirred water layer of the brush border membranes due to the size of the lipid emulsion particles. As triglyceride hydrolysis proceeds in the distal
intestinal lumen, cholesterol liberated from the digested products to bile salt micelles is less well absorbed due to inefficient cholesterol uptake by enterocytes in the distal intestine (Borgstrom, 1960; Sylven and Nordstrom, 1970).

The observation that dietary triglycerides are absorbed normally in $PTL^{-/}$ mice, albeit at a reduced rate, reveals additional enzymes in the digestive tract can compensate for the lack of PTL in catalysing triglyceride digestion and fat absorption in $PTL^{-/}$ mice (Huggins et al., 2003). The structurally related enzyme PTLRP-1 is unlikely the compensatory enzyme for triglyceride digestion since in vitro characterisation of this protein failed to demonstrate its triglyceride hydrolytic activity (Lowe, 2002). However, the other lipase related protein, PTLRP-2, which is the major triglyceride hydrolytic enzyme in neonates prior to the expression of PTL and remains present at very low level in the pancreatic juice of adults (Lowe et al., 1998), may potentially serve this compensatory function. Another candidate enzyme for compensatory triglyceride digestion in the absence of PTL is CEL, which has a wide substrate reactivity, including its hydrolysis of cholesterol esters, triglycerides, phospholipids, lysophospholipids and ceramides (Hui, 1996).

In addition to a compensatory and/or auxiliary role to PTL for triglyceride digestion in the intestinal lumen, CEL secreted by the pancreas has recently been suggested to participate in the regulation of chylomicron assembly in intestinal cells. The lack of CEL in $CEL^{-/}$ mice results in significant reduction in the number of chylomicron particles produced by the intestine after a lipid meal, with most of the intestinal lipoproteins produced by $CEL^{-/}$ mice being VLDL size particles (Kirby et al., 2002). Although the exact mechanism by which CEL participates in chylomicron assembly is unknown, indirect evidence suggests that CEL may have an important role in intracellular lipid trafficking. This hypothesis is based on observations that: (1) CEL secreted by the pancreas is endocytosed by enterocytes into the cell interior (Huang and Hui, 1990; Bruneau et al., 1998; Bruneau et al., 2001) (2) CEL is capable of hydrolysing ceramides (Hui et al., 1993; Nyberg et al., 1998), which are generated as a consequence of cholesterol desorption from the plasma membrane after the initial step of cholesterol
absorption (Field et al., 1993) and (3) ceramides have been shown to disrupt the Golgi apparatus (Fukunaga et al., 2000; Rosenwald et al., 1993). Accordingly, the role of CEL in enterocytes has been postulated to be related to its ability to hydrolyse the ceramides, thereby allowing proper intracellular trafficking of the absorbed lipids to the site of chylomicron assembly and biosynthesis (Kirby et al., 2002).

1.3.5. Role of bile salts in intestinal cholesterol absorption

The digestion of the dietary triglycerides in the intestinal lumen generates liquid crystalline phase at the surface of the emulsion particles (Hernell, 1990). Cholesterol, which is only minimally soluble in aqueous environment (Swell et al., 1958; Holt et al., 1986), needs to be partitioned into bile salt micelles prior to its transport to the brush border membranes where it can be absorbed into the mucosa. The bile salt micelles are derived from liver secretion into the biliary tract, reaching the duodenal lumen where they can serve as detergents for the lipolytic products. The requirement of bile salt micellar solubilization of cholesterol prior to its absorption is evident from studies of disease states, such as cirrhosis, in which the bile acid pools are reduced in proportion to severity of hepatic function impairment. Cholesterol absorption is positively correlated with the total bile acid pool in these patients (Ponz de Leon et al., 1981). Cholesterol absorption can also be reduced in subjects treated with bile acid sequestrants such as cholestyramine (LaRosa, 1989).

The pivotal role of bile acids in cholesterol absorption implies that local bile acid concentration may dictate the efficiency of cholesterol transport to the brush border membrane. Luminal bile acids are derived from liver secretion and they can be reabsorbed from the intestinal lumen back to the liver in a process mediated by the sodium-dependent ileal bile acid transporter (IBAT), which is also named Na\(^+\)-dependent taurocholate IBAT, which is also named Na\(^+\)-dependent taurocholate cotransporting polypeptide or apical sodium-dependent bile acid transporter (ASBT). Thus ASBT activity may influence cholesterol absorption by modulating bile acid pool size in the intestinal lumen. Interestingly, although pharmacological inhibition of ASBT activity has
been shown to be effective in reducing plasma cholesterol levels in several species (Galman et al., 2003; Root et al., 2002), the reduced plasma cholesterol levels observed with ASBT inhibitors are due to increased bile acid synthesis in response to reduce bile acid reabsorption and the consequential increase in catabolism of plasma lipoproteins and not due to reduction of cholesterol absorption (Galman et al., 2003; Root et al., 2002).

The inability of ABST activity to regulate cholesterol absorption under normal conditions can be explained by the fact that cholesterol absorption occurs predominantly in the proximal intestine (Borgstrom, 1960; Sylven and Nordstrom, 1970) and ASBT-mediated uptake of bile acids occurs primarily in the distal intestine. The bile acid pool size in the proximal intestine are also not different between control and ASBT inhibitor-treated animals (Root et al., 2002; Li et al., 2004), due to the compensatory increase in bile acid synthesis by the liver (Galman et al., 2003; Root et al., 2002). Interestingly, in both groups of animals, a graded concentration of bile acid was observed along the gastrocolic axis, with the highest level in the duodenum-jejunum portions of the intestine and the lowest level in the ileum where ASBT is expressed (Root et al., 2002). Intra-luminal bile acid concentration is also highest in the proximal intestine and decreases in the distal intestinal lumen of human subjects (Hepner and Demers, 1977). This difference in bile acid pool size along the gastrocolic axis may explain the graded cholesterol absorption efficiency along the gastrocolic axis (Borgstrom, 1960; Sylven and Nordstrom, 1970). Previously, the transposition of ileum into the upper jejunum of the intestine has been shown to reduce cholesterol absorption without affecting triglyceride absorption in rats (Tsuchiya et al., 1996). Importantly, the decrease in cholesterol absorption is also associated with premature absorption of the bile acids in the proximal intestine. Thus, another variable that may dictate cholesterol absorption efficiency is the concentration of bile salts micelles in the intestinal lumen, a factor that in controlled by bile acid synthetic enzymes and transport proteins.
1.3.6. Role of ABC proteins

Although current literature has clearly established the importance of cholesterol transfer to bile salt micelles prior to its transport to the brush border membranes for absorption by intestinal cells, the mechanism by which micellar cholesterol are taken up by enterocytes independent of bile salt uptake is still enigmatic and being actively investigated. Currently there are two major hypotheses on the mechanism by which cholesterol in micelles is taken up through the brush border membranes. One long standing hypothesis suggests that cholesterol absorption is an energy-independent passive diffusion process in which micellar cholesterol is in equilibrium with monomolecular cholesterol in solution and the monomeric cholesterol is absorbed to the brush border membrane down a concentration gradient (Westergaard and Dietschy, 1976; Chijiiwa and Linscheer, 1987). The amount of cholesterol absorbed can then be regulated by a class of membrane proteins known as ATP binding cassette (ABC) transporters located on the brush border membranes of the intestine. These proteins have been postulated to limit cholesterol absorption by secreting the internalised cholesterol back to the intestinal lumen. The initial suggestion of their involvement in cholesterol absorption was derived from studies with nuclear hormone receptor RXR and LXR agonists, which were shown to increase ABCA1 expression with a concomitant decrease in cholesterol absorption (Repa et al., 2000). However, ABCA1 knockout mice displayed only marginal increase in cholesterol absorption in one study (McNeish et al., 2000) and decrease cholesterol absorption in another study (Drobnik et al., 2001). A recent study showed that ABCA1 is localised on the basolateral instead of apical membrane of the enterocytes (Mulligan et al., 2003), which is inconsistent with it playing a major role in cholesterol absorption. The latter study revealed that the intestinal ABCA1 serves to promote efficient cholesterol efflux from enterocytes to plasma high density lipoproteins (HDL) (Mulligan et al., 2003). ABCA1 is also present in the liver where it promotes biliary cholesterol excretion (Vaisman et al., 2001). Thus this protein may indirectly impact cholesterol absorption through modulation of lipid composition in bile and in the intestinal lumen.
Two additional ABC transporters in liver and intestine that are up-regulated by LXR and RXR ligands and implicated in regulation of cholesterol absorption. They are ABCG5 and ABCG8 (Repa et al., 2002). The genes for these two proteins are located contiguously on human chromosome 2p21 (Patel et al., 1998). Unlike ABCA1 and other ABC transporters which encode proteins with 12 transmembrane domains, ABCG5 and ABCG8 each encode a protein with six transmembrane domains. Thus the two proteins are ABC half-transporters and their heterodimerisation forms a 12-transmembrane protein complex is required for transport activities. Heterodimerisation of ABCG5 and ABCG8 is also necessary for their translocation from endoplasmic reticulum to the plasma membrane (Graf et al., 2002). Mutations affecting the structure and/or function of either ABCG5 or ABCG8 have been found to be the cause of the rare autosomal recessive disorder sitosterolemia in humans (Berge et al., 2000; Lu et al., 2001). These sitosterolemic patients absorbed 15-20% of the dietary plant sterols instead of the typical <5% plant sterol absorption in normal individuals. Interestingly, sitosterolemic patients also absorb a greater fraction of dietary cholesterol and excrete less cholesterol into the bile compared to normal subjects, resulting in hypercholesterolemia (Berge et al., 2000; Lee et al., 2001). Thus these patients display high levels of plant and animal sterols in their plasma, develop tendon and tuberous xanthomas, accelerated atherosclerosis and premature coronary artery disease (Bjorkhem et al., 1995). The increased sterol absorption and decreased biliary sterol excretion observed in sitosterolemic patients with ABCG5 and/or ABCG8 defects suggest that these proteins form a functional complex that limits sterol absorption by secreting sterols from the intestinal epithelium into the lumen and promoting hepatic sterol secretion into the bile.

The exact mechanism by which ABCG5 and ABCG8 may limit cholesterol absorption has been explored with genetically-modified mice. The over-expression of both ABCG5 and ABCG8 in mice increases biliary cholesterol secretion five-fold and reduces fractional absorption of dietary cholesterol by 50% (Yu et al., 2002). Bile acid pool size, composition and faecal excretion rate are not different between control and ABCG5/ABCG8 transgenic mice. Thus ABCG5 and ABCG8 appear to be specific for promoting biliary cholesterol secretion. The reduction in fractional dietary cholesterol
absorption observed in the ABCG5/ABCG8 transgenic mice may be due to either a direct effect on cholesterol efflux from the enterocytes, or an indirect effect of increased biliary cholesterol delivery to the intestinal lumen, resulting in competition with dietary cholesterol for micellar solubilization and cellular uptake (Sehayek, et al., 1998). Regardless of whether ABCG5 and ABCG8 activity influence intestinal cholesterol via direct or indirect mechanisms, variability in their expression level may be another contributing factor for the difference in cholesterol absorption efficiency among different individuals.

1.3.7. Niemann Pick C1-Like1 as the putative cholesterol transporter

An emerging hypothesis regarding the mechanism of cholesterol absorption suggests that cholesterol absorption is a protein-mediated process. In support of this hypothesis was the observation that cholesterol uptake by brush border membrane in vitro follows a second-order reaction kinetics and the reaction reverts to a low affinity first-order kinetics upon proteolytic digestion of proteins on the surface of the brush border membrane (Thurnhofer and Hauser, 1990). The discovery of inhibitors selectively blocking cholesterol absorption at very low dosage and their binding to intestinal mucosa in a specific and saturable manner is viewed as supportive of the protein-mediated cholesterol absorption hypothesis (Detmers et al., 2000; Nernandez et al., 2000; van Heek et al., 2001; Sudhop et al., 2002). Interestingly, antibody blocking studies (Hauser et al., 1998), absorption inhibitor binding (Detmers et al., 2000), and photo-affinity labelling studies (Kramer et al., 2000) have all identified the class B type I scavenger receptor SR-BI as a putative cholesterol transporter in the intestine. The distribution of SR-BI along the gastrocolic axis and on the apical membrane of the brush border is also consistent with its participation in cholesterol absorption. However, studies with SR-BI knockout mice revealed that this protein is not essential for cholesterol absorption (Mardonez et al., 2001). More importantly the cholesterol absorption inhibitor Ezetimibe, which has been shown to label SR-BI in intestinal cells (Altmann et al., 2002) also inhibits cholesterol absorption in SR-BI knockout mice (Altmann et al., 2002), thus indicating that SR-BI is
not the long sought after cholesterol transporter responsible for cholesterol absorption in intestinal cells.

Ezetimibe is a new class of cholesterol lowering therapeutics that act through a different mechanism than the statins, which lower plasma cholesterol by reducing endogenous cholesterol biosynthesis. Ezetimibe decreases plasma cholesterol level by inhibiting intestinal cholesterol absorption without affecting hepatic or intestinal biosynthesis (van Heek et al., 2003). The mechanism by which ezetimibe inhibits cholesterol absorption has been explored extensively in experimental models, with results showing that ezetimibe does not inhibit pancreatic lipolytic enzyme activities in the intestinal lumen, nor affect bile salt micelle solubilization of cholesterol (van Heek et al., 2001). Rather, ezetimibe appears to directly inhibit cholesterol uptake by enterocytes (van Heek et al., 2001; Sudhop et al., 2002; van Heek et al., 2003). Several clinical trials in humans have demonstrated that ezetimibe at 10mg/day can effectively reduce plasma LDL-cholesterol by 17-20% (Ezzet et al., 2001; Knopp et al., 2003). Importantly, the inclusion of ezetimibe to statin therapy at any dosage resulted in an average additional 25% reduction of plasma LDL (Gagne et al., 2002). These observations suggested the possibility that ezetimibe may be used alone or in conjunction with statin therapy to reduce plasma cholesterol in poor statin-responsive subjects. In fact, there has been a study which has demonstrated that patients who are hypo-responders to statin therapy are hyper-responders to ezetimibe therapy (Ziajka et al., 2004). Since hypo-responders to statin therapy are thought to be hyper-absorbers of dietary cholesterol (Naoumova et al., 1996; O’Neill et al., 2001), these results further support the hypothesis that ezetimibe reduces plasma cholesterol levels by inhibiting cholesterol absorption in the gastrointestinal tract.

A recent study by Altmann et al (2004) revealed similar dietary lipid absorption characteristics between ezetimibe-treated mice and mice with targeted inactivation of the Niemann Pick C-1 like-1 (NPC1L1) gene. These observations suggested the possibility that NPC1L1 may be the ezetimibe-inhibitable cholesterol transporter on intestinal brush border membranes. The identification of NPC1L1 as the putative cholesterol transporter was initially accomplished by a genomics-bioinformatics approach, searching an
expression sequence tags cDNA library prepared with RNA obtained from rat jejunal mucosa and enterocytes for transcripts containing predicted features of a cholesterol transporter (Altmann et al., 2004). A rat homolog of the human NPC1L1 gene was found to be the only gene that encodes a protein that contains extra-cellular signal peptide, transmembrane sequences, N-linked glycosylation sites, and a sterol-sensing domain (Davies et al., 2000). Quantitative reverse transcriptase-polymerase chain reaction analysis of 15 tissues from rat, mouse and human revealed the highest level of NPC1L1 mRNA expression in the intestine (Altmann et al., 2004). NPC1L1 mRNA expression was also detectable in the liver, gallbladder, testis and stomach (Altmann et al., 2004). Importantly, the NPC1L1 mRNA expression in the intestine parallels the efficiency of cholesterol absorption along the gastrocolic axis, with the highest level of NPC1L1 expression and cholesterol absorption in the proximal intestine and little or no NPC1L1 expression and cholesterol absorption in the ileum (Altmann et al., 2004). Furthermore, in situ hybridisation and immunohistochemistry analysis of the jejunum for NPC1L1 mRNA and protein expression revealed discrete NPC1L1 localisation to the epithelial later bordering the luminal space along the crypt-villus axis (Altmann et al., 2004), thus lending additional support for the hypothesis that NPC1L1 is the cholesterol transporter responsible for mediating dietary cholesterol absorption in the intestine. An interesting finding recently by Hegele et al., (2005) shows that about one subject in eight in their study of 101 dyslipidaemic subjects lacked the common NPC1L1 haplotype 1735C-25342A-27677T and these subjects had a significantly greater reduction in plasma LDL cholesterol with ezetimibe than subjects with at least one copy of this haplotype.

The NPC1L1 protein is a member of a gene family of proteins that includes the NPC1 and NPC2 proteins (Davies et al., 2000) NPC1L1 and NPC1 share 42% identity and 51% similarity at the protein level, including the presence of a sterol sensing domain in both proteins. Thus the known functions of NPC1 may provide some clues to the mechanism by which NPC1L1 may facilitate cholesterol transport in intestinal cells. NPC1 is an endosomal/lysosomal protein and its defective expression results in cholesterol and sphingolipids accumulation in the lysosomes. Although this phenotype suggests that NPC1 is responsible for cholesterol trafficking from the endosomal/lysosomal pool,
whether the primary defect in NPC1 deficiency is due directly to sterol transport defects or due to impairment of other lipid trafficking pathways is still under debate. For example, NPC1-deficient cells accumulate a variety of glycosphingolipid in addition to cholesterol. The depletion of glycosphingolipids with inhibitors of glycosphingolipid biosynthesis reduces both sphingolipid and cholesterol accumulation, improves endosomal uptake, and normalises lipid trafficking in NPC1-defective cells (Zervas et al., 2001; Lachmann et al., 2004). These latter studies suggested the possibility that cholesterol accumulation in lysosomes of NPC1-defective cells may be secondary to a defect in sphingolipid metabolism.

The NPC1L1 protein differs from NPC1 in that the latter protein contains a carboxyl-terminal dileucine targeting motif LLNF that is indicative of its function in endosomal-lysosomal trafficking (Higgins et al., 1999; Neufeld et al., 1999). In contrast, NPC1L1 contains a YQRL motif implying that it may function as a plasma membrane to trans-Golgi network protein (Rothman and Wieland, 1996). The decrease in cholesterol absorption observed in NPC1L1-deficient mice is consistent with the role of NPC1L1 as a membrane protein responsible for cholesterol transport from extracellular milieu to intracellular compartment where chylomicron assembly occurs. However, it is also possible that NPC1L1 may mediate cholesterol absorption via modulation of membrane sphingolipid transport in the intestine, analogous to the role of NPC1 in intracellular sphingolipid trafficking. Previous studies have clearly demonstrated sphingomyelin inhibition of cholesterol absorption (Chen et al., 1992; Nyberg et al., 2000). Thus the exact mechanism by which NPC1L1 modulates cholesterol absorption remains to be determined. Regardless, it is clear that NPC1L1 plays an important role in cholesterol uptake and its processing in intestinal cells and limits cholesterol transport from intestinal lumen to the circulation. Hence, NPC1L1 may work in concert with the cholesterol efflux proteins ABCG5 and ABCG8 in determining the amount of cholesterol absorbed.
1.3.8. Diabetes and Cholesterol Metabolism

Numerous reports have demonstrated that poor glycaemic control is associated with elevated plasma cholesterol levels in both diabetic humans and diabetic animal models (Feingold and Siperstein 1986). Despite extensive investigation, the mechanism by which diabetes produces hypercholesterolemia is not fully elucidated and many questions remain. *De novo* cholesterol synthesis has been shown to be 2-3 fold higher in the gut of streptozotocin diabetic rats (Feingold *et al.*, 1982; Glesson *et al.*, 2000). In Feingold’s study, the increase occurred in both the small and large intestine, but quantitatively it was the small intestine that was responsible for most of the observed increase. Intestinal HMGCoA reductase activity is increased in the small intestine of streptozotocin- and alloxan-induced diabetic rats (Goodman *et al.*, 1993; Young *et al.*, 1988), in alloxan-induced diabetic rabbits (Devery *et al.*, 1987; O’Meara *et al.*, 1990) and in diabetic Chinese hamsters (Feingold *et al.*, 1994). Animals in these induced-diabetic states are generally thin, insulinopenic and can be mildly ketotic and thus represent an animal model of Type 1 diabetes. Increased intestinal cholesterol biosynthesis has also been observed in the db/db mouse but not in the ob/ob mouse. While both of the animal models are hyperinsulinaemic, insulin resistant and obese, the diabetes in the ob/ob mouse is less severe than in the db/db mouse (Feingold *et al.*, 1984). These models represent Type 2 diabetes and these findings suggest that diabetes results in an increase in intestinal cholesterol synthesis in many animal models.

Diabetes leads to an increase in the size of the intestine. It has been observed that soon after the onset of diabetes, an increased rate of cholesterol synthesis per unit mass is responsible for the increase in total small intestine synthesis whereas, after a longer period of diabetes, the increase in total small intestinal cholesterol synthesis is primarily due to an increase in mass (Feingold *et al.*, 1984).

Some studies have suggested than insulin plays a role in cholesterol production in diabetes. It was found that insulin causes a shift of faecal sterol excretion from faecal bile acids to faecal neutral steroids. When dietary intake was held constant, net sterol
production did not change with insulin treatment (Saudek and Brach 1978), but when
dietary intake was decreased, net sterol synthesis decreased upon insulinisation (Bennion
and Grundy 1977). Insulin regulation of cholesterol synthesis is tissue specific,
enterocyte cholesterol synthesis being suppressed by insulin (Neill et al., 1987) whereas
insulin stimulates cholesterologenesis in hepatic and peripheral tissue (Devery and
Tomkin 1986; Suresh et al., 1986). HMG CoA reductase is increased in the intestine of
untreated diabetic animals (Goodman et al., 1993; Young et al., 1988; Devery et al.,
1987) and insulin treatment of these animals prevents the increase in intestinal HMG
CoA reductase (Young et al., 1988). In both Type 1 and Type 2 diabetic patients it has
been shown that hyperinsulinaemia is associated with stimulation of cholesterol synthesis
(Stinson et al., 1993) and reversal of hyperinsulinaemia by dietary modification in Type 2
diabetic subjects has been shown to be associated with a normalisation of cholesterol
synthesis (Griffin et al., 1998).
1.4 LIPOPROTEINS – AN OVERVIEW

1.4.1. Lipoproteins

Lipids are insoluble in aqueous solution and therefore need to become components of lipoproteins in order to be transported in plasma. Lipoproteins are complex molecules composed of various proportions of protein, phospholipid, triglyceride and cholesterol. The lipoprotein molecule can be thought of as a sphere with a hydrophobic core of triglycerides and cholesterol ester surrounded by the hydrophilic phospholipids in which the apolipoproteins are embedded. Free cholesterol is found between the hydrophobic and hydrophilic portions of the molecule.

Lipoproteins are classified according to their physical characteristics such as size, electrophoretic mobility and density as determined by ultracentrifugation (Table 1.4.1). There are several different types of lipoprotein, the main ones being: chylomicrons, Very Low Density Lipoproteins (VLDL), Intermediate Density Lipoprotein (IDL), Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL) and Lipoprotein (a) (Lp [a]). The protein components of lipoproteins are called apolipoproteins (or apoproteins) and are involved in the distribution, cell recognition and metabolism of the lipoproteins. Figure 1.4.1 shows the structure of a typical LDL.
Table 1.4.1
The size characteristics of the various lipoproteins

<table>
<thead>
<tr>
<th>Type</th>
<th>Diameter (nm)</th>
<th>Density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>100-1000</td>
<td>&lt;0.95</td>
</tr>
<tr>
<td>VLDL</td>
<td>30-90</td>
<td>&lt;1.006</td>
</tr>
<tr>
<td>IDL</td>
<td>25-30</td>
<td>1.006-1.019</td>
</tr>
<tr>
<td>LDL</td>
<td>19-25</td>
<td>1.019-1.063</td>
</tr>
<tr>
<td>HDL</td>
<td>&lt;18</td>
<td>1.063-1.210</td>
</tr>
<tr>
<td>HDL₂</td>
<td>&lt;18</td>
<td>1.063-1.210</td>
</tr>
<tr>
<td>HDL₃</td>
<td>&lt;18</td>
<td>1.125-1.210</td>
</tr>
</tbody>
</table>

Structure of a plasma lipoprotein

![Diagram of structure of plasma lipoprotein](adapted from Lehninger, 2003)

Figure 1.4.1. Diagram of structure of plasma lipoprotein (adapted from Lehninger, 2003)
1.4.2. **Apolipoproteins**

There are a number of different apolipoproteins which are characteristically associated with the major lipoprotein fractions. In addition to their structural role, they are required for the secretion of the lipoproteins from the intestine and the liver. They may also act as activators and inhibitors of enzymes involved in lipoprotein metabolism and may serve as ligands for cell surface lipoprotein receptors. Their function and localisations are summarised in Table 1.4.2.

**Table 1.4.2.** The function and localisation of the different apolipoproteins.

<table>
<thead>
<tr>
<th>Apo</th>
<th>Function</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I</td>
<td>Structural protein in HDL, lecithin cholesterol acyl transferase (LCAT) activator</td>
<td>HDL, chylomicrons</td>
</tr>
<tr>
<td>A-II</td>
<td>Structural protein in HDL</td>
<td>HDL3, chylomicrons</td>
</tr>
<tr>
<td>A-IV</td>
<td>LCAT activator</td>
<td>HDL, chylomicrons</td>
</tr>
<tr>
<td>B-48</td>
<td>Receptor ligand</td>
<td>Chylomicrons</td>
</tr>
<tr>
<td>B-100</td>
<td>Structural protein in VLDL, IDL, LDL</td>
<td>VLDL, IDL, LDL</td>
</tr>
<tr>
<td>C-I</td>
<td>LCAT activator</td>
<td>HDL, chylomicrons, VLDL</td>
</tr>
<tr>
<td>C-II</td>
<td>Lipoprotein lipase (LPL) activator</td>
<td>HDL, chylomicrons, VLDL</td>
</tr>
<tr>
<td>C-III</td>
<td>LPL modulator</td>
<td>HDL, chylomicrons</td>
</tr>
<tr>
<td>D</td>
<td>LCAT activator</td>
<td>HDL</td>
</tr>
<tr>
<td>E</td>
<td>Receptor ligand</td>
<td>Chylomicrons, remnants, VLDL, IDL, HDL</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Combines with B-100</td>
<td>Lp(a)</td>
</tr>
</tbody>
</table>
The human apoA-I molecule is a single polypeptide chain with 243 amino acid residues consisting of a series of homologous 11- and 22-mer amino acid repeats that are often interposed with proline-containing segments. The 22-mer repeats form amphipathic helices (Marcel and Kiss, 2003; Lund-Katz et al., 2003; Brouillette et al., 2001) and investigations of the secondary structure of apoA-I identified helical segments 44–65 and 220–241 in the N- and C-terminal domains, respectively, as being particularly hydrophobic and having the highest lipid binding affinities (Palgunachari et al., 1996). The tertiary structure of apoA-I consists of two domains: a helix bundle domain comprising the N-terminal and central helices (residues 1–186) and a strongly lipid-binding C-terminal domain (residues 187–243) (15). An apoA-I molecule is thought to associate initially with the surface of a membrane via the latter domain, after which the helical bundle opens enhancing lipid-protein interaction (Saito et al., 2003). When apoA-I molecules interact with a cell expressing ABCA1, low levels of cellular FC and PL are removed and used to create various HDL particles (Liu et al., 2003), in a process we have termed membrane microsolubilization (Gillotte et al., 1999; Gillotte et al., 1998; Gillotte-Taylor et al., 2002). The fact that peptides corresponding to the hydrophobic helical regions of the apoA-I molecule can also mediate membrane microsolubilization (Mendez et al., 1994; Remaley et al., 2003; Yancey et al., 1995) implies that apoA-I/lipid interactions play a critical role. The ability of apoA-I to bind lipids is likely to be important in both the acquisition of PL and FC via ABCA1 and in the retention of these lipids to form stable HDL particles. Importantly, hydrophobic interactions between apoA-I and ABCA1 are also likely to be significant in the overall process of PL and FC efflux (Chroni et al., 2004; Fitzgerald et al., 2004).

Apolipoprotein A-IV is a 46-kDa plasma glycoprotein (Weinberg and Scanu, 1983) that is synthesized by the intestinal enterocytes of mammalian species (Weisgraber et al., 1978) during lipid absorption (Hayashi et al., 1990). Apo-A-IV enters circulation as a component of nascent chylomicrons (Green et al., 1979; Green et al., 1980), but rapidly dissociates from their surface (Green et al., 1980) and thereafter circulates primarily as a lipid-free protein (Bisgaier et al., 1985). A broad spectrum of physiologic functions has been proposed for apoA-IV in human lipid metabolism (Lefevre and Roheim, 1984),
including specific roles in intestinal lipid absorption (Kalogeris et al., 1997),
intravascular lipoprotein metabolism (Weinberg and Spector, 1986; Bisgaier et al., 1987;
Goldberg et al., 1990), cellular cholesterol efflux (Steinmetz et al., 1990; Bielicki et al.,
1992) by interaction with the ABCA1 transporter (Fournier et al., 2000; Remaley et al.,
2001), and regulation of the activity of two key proteins involved in the process of
reverse cholesterol transport: lecithin-cholesterol acyltransferase (Steinmetz and
Utemann, 1985; Chen and Albers, 1985) and cholesterol ester transfer protein (CETP)
(Lagrost et al., 1990; Main et al., 1996). Although like all apolipoproteins, apoA-IV has a
high content of amphipathic-helical structure (Weinberg and Spector, 1985; Weinberg,
1987), the amphipathic helices in apoA-IV are very hydrophilic (Weinberg, 1987), are
predominantly of the Y-class (Segrest et al., 1992) and are incapable of deeply
penetrating lipid monolayers (Weinberg and Jordan, 1990; Weinberg et al., 1992). With
increasing surface pressure, these helices are sequentially excluded from the interface
(Weinberg et al., 1990; Weinberg et al., 2000). Consequently, the interaction of apoA-IV
with lipoproteins is very labile and is sensitive to interfacial pressure (Weinberg, 1992). It
has been proposed that these properties enable apoA-IV to act as a barostat which
maintains lipoprotein surface pressure within a critical range required for optimal activity
of lipolytic enzymes and transfer proteins (Weinberg et al., 1994, Weinberg et al., 1995).
In this regard, apoA-IV possesses dynamic interfacial properties that are optimal for
stabilizing surface tension and lipid packing at expanding lipid/aqueous interfaces
(Weinberg et al., 1994).

ApoB-100 is the binding ligand for the LDL- or B/E- receptor. In humans it is
manufactured in the liver but some reports have found that it is also manufactured in the
intestine and brain (Hoeg et al., 1990; Nielson et al., 1998). It has a molecular weight of
550 kDa and contains 4536 amino acids. It is the structural component of chylomicrons,
VLDL, IDL and LDL. Apo B-48, the major protein associated with the chylomicron, is
produced exclusively in the intestine in humans. It consists of the first 2152 amino acids
of apo B-100 and has a molecular weight of 264 kDa. Post-transcriptional modification of
the apo B-100 mRNA results in the premature insertion of a stop codon and therefore apo
B-48 lacks the amino terminal end of apo B-100 and is unable to bind to the LDL
receptor. The B apolipoproteins are highly insoluble in aqueous solutions and, thus, remain with the lipoprotein particle throughout its metabolism (Kane, 1983). Because of the size and insoluble nature of apoB, it has been difficult to confirm the structural motifs responsible for the lipid-associating properties of this non-exchangeable apolipoprotein (Kane et al., 1975; Lee et al., 1981). Circular dichroic (CD) spectroscopy of LDL suggested that apoB-100 has an -helical content of 25% or greater (Scanu, 1968; Gotto, 1968). Amphipathic -helices, the ubiquitous lipid-associating motifs in the exchangeable apolipoproteins, were detected in the sequence of apoB-100 by helical wheel analysis (Wei et al., 1985; Cladaras et al., 1986). Using computer analysis, De Loof et al., (1987) noted two clusters of potential 22-mer amphipathic helical repeats between residues 2,079;–2,428 and 4,150;–4,484. Further, they showed, using comparison matrix analysis, that the regions between residues 2,035;–2,506 and 4,002;–4,527 contained sequence similarity to the exchangeable apolipoproteins.

Yang et al., (1989) used non-dissociability of peptides from trypsin-treated intact LDL to develop a map of the lipid-associating regions of apoB-100 (Fig 1.4.2.). They determined the regions of apoB on the LDL particle that were trypsin releasable, those that were not, and those there were mixed. Based on these criteria, five broad domains of apoB were identified. Their map defined two major apoB-100 lipid-associating domains between residues 1,701;–3,070 and 4,101;–4,536.

There are at least three apolipoproteins C molecules. Apo C-I is the smallest with a molecular mass of 6.6kDa. It is found in chylomicrons and as a minor component of VLDL and HDL. Apo C-II, with a molecular mass of 8.85 kDa is also found in chylomicrons, VLDL and HDL and is an activator of the LPL. Finally, apo C-III is found as a major component of chylomicrons and VLDL and as a minor component of HDL. It has a molecular mass of 8.8 kDa and it plays a role in the uptake of the particles by masking the apo E and thereby interfering with cell surface receptor recognition.

Apo E has a molecular mass of 34.1 kDa and it acts as the binding ligand for the chylomicron remnant receptor or apo E receptor situated on the liver. It is found in
chylomicrons, VLDL, IDL and HDL. A genetic polymorphism results in three main isoforms E2, E3 and E4 determined by three forms of the gene. The crystal structure of Apo E, the 22-kDa amino-terminal fragment, was solved over a decade ago (Wilson et al., 1991). This domain forms a globular -helix bundle that is similar to the structure of insect apolipophorin III (Breiter et al., 1991). In apoE, there are two independently folded domains: the 22-kDa amino-terminal domain contains the low density lipoprotein receptor binding region and the 10-kDa carboxyl-terminal domain has a high affinity for lipid and is responsible for lipoprotein binding (Weisgraber, 1994). Recently, it was shown that these two domains in apoE4 lead to two different lipid-bound conformations, providing a structural rationale for the variable receptor binding activity displayed by lipoprotein-associated apoE (Saito et al., 2001).

1.4.3. Chylomicrons

Chylomicrons are the largest of the lipoproteins and they contain mainly triglyceride with small amounts of cholesterol, phospholipid, cholesterol esters and protein (Hussain et al., 2005). They are the main carriers of triglycerides absorbed from the diet and are in the greatest concentration in the blood after a fat-laden meal (3-5 hours). After a prolonged fast of 12 hours they are virtually undetectable. Their removal from plasma is one of the most efficient systems of clearance with a half-life of less than 10 minutes in normal mammals (Hussain et al., 2005). Chylomicrons are manufactured in the enterocytes of the duodenum and jejunum. Although these particles contain only 1-2% protein, it is nevertheless extremely important. Between 5-30% of it is apo B-48 (the main structural and functional protein of the chylomicron), the remainder being apo A and apo C. The chylomicron fraction has molecules which contain a single copy of apo B-48 or B-100 (Phillips et al., 1997), which is added at the enterocyte level and together with the other apoproteins (A-I, AΠ and A-IV) are produced in these enterocytes (Kane et al., 1980; Glickman et al., 1986).

Studies as far back as those carried out by Ascellius in 1622 indicated that absorbed fat is assembled into chylomicrons which are then secreted in to the mesenteric lymphatics and
then enter the blood stream through the thoracic lymph duct. When the chylomicrons are released from the thoracic duct into the circulation, they come into contact with HDL and acquire apo C-I, C-II, C-III and E (Havel et al., 1983). The triglyceride component of the chylomicron is broken down by the action of LPL, which is activated by apo C-II on the particle. The free fatty acids released from the glycerol in this hydrolysis are taken up by the cells to provide energy or to be re-synthesised back into triglyceride. LPL is found in greatest amounts on the surface of the vascular endothelial cells of muscle and adipose tissue (Havel et al., 1983).

As triglyceride is removed from the chylomicon and apo A-I is transferred back to the HDL, the lipoprotein becomes smaller and contains increased proportions of cholesterol and its esters, phospholipid, apo B-48 and apo-E and is now referred to as the chylomicon remnant. This remnant particle may be avidly taken up by the chylomicon remnant or apo E receptor on the hepatocyte, the classical LDL receptor or by the apo B-48 receptor which is found on the macrophage (Gianturco et al., 1998).

1.4.4. Very Low Density Lipoprotein (VLDL)

VLDL assembly occurs in the endoplasmic reticulum and Golgi apparatus prior to secretion by the hepatocyte (Gotto et al., 1986). These lipoproteins are somewhat smaller and less dense than chylomicrons, but in structure and composition are very similar. They contain less triglycerides and more protein, phospholipid and cholesterol. The protein consists of a mixture of apo C, apo E and apo B-100. VLDL is almost exclusively produced in the liver and whilst they are similar to chylomicron in that their main purpose is to carry triglyceride, they differ in that VLDL transports endogenously synthesised triglycerides (Jeon and Blacklow, 2005). The synthesis of VLDL is stimulated by an increase in the flow of free fatty acids to the liver and after a high carbohydrate meal when the liver itself is actively synthesising fatty acids. It has been suggested that there are two major subclasses of VLDL particles, the large triglyceride-rich VLDL_{1} and the smaller, more dense, VLDL_{2} which have different metabolic
pathways. VLDL₁ are involved in progression via IDL to LDL and VLDL₂ particles are thought to compete for the same lipolytic pathway as chylomicrons (Yagyu et al., 2002).

Each VLDL molecule contains a single apo B-100 component; nonetheless it is essential for VLDL production, function and structure (Elovsen et al., 1988). VLDL has a greater half-life in the circulation than chylomicrons (up to 5 hours), and it’s function is to redistribute fatty acids to the sties in the body where they can be stored or utilised as an energy source (Yagyu et al., 2002). When secreted into the bloodstream VLDL accepts cholesterol ester and apo C from HDL. LPL in the endothelium of the blood vessels hydrolyses the triglycerides of the VLDL molecule to yield fatty acid fuel for the tissues and leaves VLDL remnants, which are denuded of apo A-I, A-II, A-IV and apo C by transfer to HDL (Jeon and Blacklow, 2005).

1.4.5. Intermediate Density Lipoprotein (IDL)

Removal of triglyceride from VLDL, by the action of lipoprotein lipase (LPL), produces smaller particles known as IDL. This lipoprotein contains the original apo B-100 of the VLDL, some of the apo E and a high concentration of cholesterol. IDL can be converted to LDL following total loss of apo E (Eisenberg, 1980) or it can be removed by the apo B/E receptor of the liver (Friedman et al., 1990). Cholesterol ester transfer protein (CETP) can transfer cholesterol to IDL further enriching its cholesterol content. The fact that IDL accumulation occurs in the condition of familial hypercholesterolemia would suggest that it is normally removed by the LDL receptor. The enzyme hepatic triglyceride lipase (HL) catalyses the conversion of IDL to LDL (Gibson and Brown, 1988). Evidence of this comes from data on individuals with HL deficiency in which the accumulation of IDL occurs in their plasma (Goldberg et al., 1982).

1.4.6. Low Density Lipoprotein (LDL)

Of all the lipoproteins, LDL is considered to be the most atherogenic. This may be due to the fact that it is the major carrier of cholesterol in plasma. It is formed, via IDL, from
VLDL and contains much less triglyceride than VLDL and only one of the apoproteins found the VLDL, namely apo B-100 (Beglova et al., 2005). It would seem that each LDL particle is formed from one VLDL molecule (Eisenberg and Levy, 1975). Other than the formation rate of LDL, the other determinant of its concentration in the blood is its clearance of catabolic rate. LDL accepts cholesterol esters from HDL and is composed of 35-45% cholesterol ester, 20-25% phospholipid and 25-30% protein. LDL is the main supplier of cholesterol to metabolically active cells, and although most cells in the body can synthesise cholesterol de novo, they only used this system in conditions of lipoprotein deficiency i.e. in culture (Dietschy, 1987). The majority of LDL is taken into cells by the LDL receptor pathway, but about 10-15% is imported by the receptor independent transport, therefore cells that are completely devoid of the classical LDL receptor can still acquire small amounts of LDL (Dietschy and Spady, 1986).

Structurally the LDL particle has a diameter of approximately 22nm and an average molecular weight of 2500 kDa (Esterbauer et al., 1990). VLDL and LDL consist of distinct, physicochemically heterogenic subclasses (Berneis and Krauss, 2002). A practical characterization of the LDL profile divides it into two major phenotypes: pattern A, characterized by a preponderance of large, buoyant particles, with peak particle diameter 258 Å, and pattern B, characterized by predominance of small dense LDL particles, with peak particle diameter <258 Å. Small dense LDL phenotype and the concurrent metabolic abnormalities (relative hypertriglyceridaemia and low HDL cholesterol) have been designated the atherogenic lipoprotein phenotype (Austin et al., 1990) consistent with its association with an increased risk of coronary artery disease (Austin et al., 1990; Lamarche et al., 1997) Furthermore, pattern B LDL has been recognized as a feature of the metabolic syndrome (Reaven, 2002) and is characteristic for insulin-resistant states, such as type 2 diabetes mellitus (Snideman et al., 2001). It has been reported that presence of small dense LDL is an inherent component of the dyslipidaemia in FCHL (Allayee et al., 1998; Austin et al., 1990; Allayee et al., 2000; Hokanson et al., 1995) and shares genetic determinants with the expression of FCHL (Allayee et al., 2000; Hokanson et al., 1995).
1.4.7. **High Density Lipoprotein (HDL)**

Plasma HDL are small dense, spherical lipid-protein complexes. They consist of approximately 50% lipid and 50% protein. The major lipids present are phospholipid, free cholesterol, cholesterol esters and triglycerides. The main apolipoproteins of HDL are apo A-I, A-II and A-IV, which account for 90% for the HDL apolipoproteins. Apo A-I acts as an activator of LCAT. There are also a variety of minor but metabolically important apoproteins in HDL, namely apo E and Apo C-I, C-II and C-III. HDL is produced in the liver and small intestine during the catabolism of chylomicrons and VLDL by LPL (Tall and Small, 1978). HDL can be subdivided into two sub-classes: HDL$_2$ or Lp A-I:A-II, the large less dense subfraction containing both apoA-I and apo A-II and HDL$_3$ or Lp A-I, the small more dense subfraction containing apo A-I but not apo A-II. Any individual variability in HDL levels in human populations usually reflects different amounts of HDL$_2$. An important function of HDL is to act as a reservoir of apo E and apo C proteins. These proteins transfer onto the triglyceride-rich lipoproteins providing a signal for the activation of lipolysis (LPL is activated by apo C-II) or targeting information that allows uptake of lipolysed remnant lipoproteins by liver receptors (apo E is attracted to chylomicon and VLDL remnants and acts as a ligand for the uptake of these particles by hepatic receptors). The major function of HDL is to act as a receptacle for excess phospholipid and cholesterol, derived form cells of as by-products of lipolysis. These lipids are normally recycled from HDL to the liver in a process called reverse cholesterol transport.

1.4.8. **Lipoprotein (a)**

This lipoprotein is larger and more dense than LDL but is essentially an LDL particle modified by the binding of apolipoprotein (a), a large glycoprotein, by a disulphide bond to the apo B-100 component of LDL. Lp (a) is produced in the liver and its concentration in the serum (range 0-200 mg/dl) is genetically determined. Lp (a) has been shown to increase delivery of cholesterol to the arterial wall (Rath *et al.*, 1989), enhance foam-cell formation (Naruszewicz *et al.*, 1992) and smooth muscle cell proliferation (Grainger *et
al., 1993) and some studies have suggested an independent association between high levels of Lp (a) and CAD (Dahlen et al., 1986). However, just as many studies such as the Helsinki Heat Study (Jauhiainen et al., 1991) and the Quebec Cardiovascular Study (Cantin et al., 1998) have failed to find any association between Lp (a) and vascular disease.

The functions and sites of production of each of the lipoproteins are outlined in Table 1.4.4. on the next page
### Table 1.4.4. The function and sites of production of the lipoproteins.

<table>
<thead>
<tr>
<th>Function</th>
<th>Apolipoprotein</th>
<th>Site of production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-100, E, C-1, Π, ΠΙ.</td>
<td></td>
</tr>
<tr>
<td>VLDL TG, Cholesterol transport</td>
<td>B-100, C-1, C-Π, E</td>
<td>Liver</td>
</tr>
<tr>
<td>(endogenous) precursor of IDL and LDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDL Precursor of LDL</td>
<td>B-100, C-1, E</td>
<td>In plasma from VLDL</td>
</tr>
<tr>
<td>LDL Cholesterol transport</td>
<td>B-100</td>
<td>In plasma from VLDL, liver.</td>
</tr>
<tr>
<td>HDL Reverse cholesterol transport</td>
<td>A-I, A-Π, C-1, C-Π, C-ΠΙ, D, E</td>
<td>Intestine, liver in plasma from chylos, VLDL.</td>
</tr>
</tbody>
</table>
1.5. MICROsomal triglyceride transfer protein (MTP)

1.5.1. Elucidation of MTP

By studying patients with abetalipoproteinemia (ABL) a major advancement was made in how exactly lipids were transported from their site of synthesis to the location in which they interacted with the newly synthesised apo B polypeptide to form chylomicron or VLDL particles in the intestine or liver, respectively. Abetalipoproteinemia is a rare autosomal recessive disorder characterized by defective assembly and secretion of plasma apolipoprotein B-containing lipoproteins. The disorder results from mutations in the gene encoding the microsomal triacylglycerol transfer protein (Wetterau et al., 1992; Narcisi et al., 1995). Abetalipoproteinemia patients have extremely low concentrations of plasma cholesterol and triacylglycerol and an almost complete lack of lipoproteins in the density ranges of chylomicrons, VLDLs, and LDLs. Plasma lipids are carried almost entirely in the HDL class. Typically, patients with abetalipoproteinemia have malabsorption and may present with failure to thrive in early childhood (Sakamoto et al., 2005). A progressive ataxic neuropathic disease and retinopathy that develop in later childhood are attributed, in part, to a deficiency of fat-soluble vitamins, specifically, vitamins E and A (Kayden, 1972; Muller et al., 1977). Both vitamins possess antioxidant properties, and vitamin E is a proven potent suppressor of lipid peroxidation (Berry, 1992; Sies and Stahl, 1995). Treatment with vitamins E and A, currently offered to abetalipoproteinemia patients from the time of diagnosis, is believed to prevent neurologic and retinal lesions, although it does not reverse the abnormalities already present (Bishara et al., 1982; Granot and Deckelbaum, 1989).

Wetterau et al., (1992), localised the defect in abetalipoproteinemia to be the absence of a non-specific lipid transport protein called MTP. The absence of MTP activity and large MTP subunit in intestinal biopsies from four hundred unrelated abetalipoproteinaemic patients provided strong evidence that defects in MTP are the underlying cause of the disease and that the 97 kDa subunit is necessary for lipid transfer activity and TRL assembly. Confirmation of this later came from comparisons of the genomic sequence of
MTP isolated from homozygous abetalipoproteinaemic patients with cDNA from control subjects (Sharp et al., 1993).

MTP facilitates the transfer of lipid, predominantly triglyceride but also cholesterol esters and phospholipids, from the ER membrane to nascent apo B polypeptides in the lumen of the ER. It has been shown that MTP has a specific preference for binding and transporting non-polar lipids compared with phospholipids and within a class of lipid molecules, a decrease in polarity increases its tendency to be transported (Jamil et al., 1995).

1.5.2. Isolation and Characterisation of MTP

MTP was originally isolated from the microsomal fraction of bovine liver by Wetterau and Zilversmit (1984) and was later shown to be present in the lumen of the endoplasmic reticulum of both liver and intestine (Wetterau and Zilversmit, 1986). Human cardiac myocytes also express a small amount of MTP mRNA (Nielsen et al., 1998), a finding consistent with an old observation by Wetterau and Zilversmit (1986) that MTP activity levels in rat hearts were 3% of those in liver.

MTP has been characterised in several species including human, cow, hamster, mouse, chicken and rat. The human MTP large subunit cDNA and gene have been cloned, and the gene, about 55-60kb in length, contains eighteen exons and is localised to position 4q24 on the short arm of chromosome 4 (Sharp et al., 1994). MTP is a heterodimer consisting of a 97 kDa large subunit noncovalently attached to a smaller 59 kDa subunit protein-disulphide-isomerase (PDI), (Wetterau et al., 1986; Sharp et al., 1993).

Expression studies in insect Sf9 cells have shown that when cells were infected with viruses expressing the large subunit of MTP alone, MTP activity was not detectable and western blot analysis revealed that the expressed protein was an insoluble aggregate (Ricci et al., 1995). In contrast, when cells were infected with viruses expressing both PDI and MTP large subunit, MTP activity was detectable and both the PDI and the MTP large subunit were found in the soluble fraction. These findings suggest that PDI is
essential to maintain the structure and activity of MTP. The PDI subunit may also play a role in ensuring the retention of the MTP complex in the lumen of the ER, since PDI possesses the C-terminal tetrapeptide, Kdel (Vouri et al., 1992), which the MTP subunit lacks. This KDEL sequence (LYS-ASP-GLU-LEU) is common to proteins which are retained in the rough ER.

1.5.3. Regulation of MTP

Gene transcription is usually regulated by interaction of proteins, termed transcription factors, with specific DNA sequences in a region termed the promoter, near the site where transcription begins. Molecular studies on the MTP gene have identified a number of potentially important sites within the promoter to which transcription factors may bind and regulate gene expression. Hagan et al., (1994) examined the transcriptional regulation of human and hamster MTP. They found that the promoter contains sequences for binding of the liver-cell specific transcription factors, HNF-1 and -4 and the activator protein AP-1. They also found that the MTP promoter contains a modified sterol regulatory element and a negative insulin response element. Transient transfection analysis of MTP promoter-driven luciferase gene expression showed that expression of the gene was down-regulated by insulin and up-regulated by cholesterol.

Most data concerning the regulation of MTP in vitro are derived from experiments using either the Hep G2 of CaCo-2 cell lines as models of the human hepatocyte and enterocyte. Lin et al., (1995) showed that insulin caused a dose-and time-dependent decrease in MTP mRNA and that this effect was mediated through the insulin receptor. They also showed that glucose only at a concentration of 50mM caused a change in MTP mRNA levels in Hep G2 cells. Normal human fasting plasma glucose levels are around 5mM and levels of 50 mM are only found in very severe diabetic patients. This suggests that glucose plays little or no role in regulating hepatic MTP in vivo. Despite the change in expression of MTP, no difference was observed in MTP activity and this suggested that within the time frame of the experiment (24hours), MTP protein levels were not controlled by MTP mRNA levels.
However, emerging evidence has indicated that the pathophysiology of dyslipidemia observed under insulin resistance/type 2 diabetes is associated with an increased hepatic MTP mRNA level (Kuriyama et al., 1998; Taghibiglou et al., 2000; Taghibiglou et al., 2002; Bartels et al., 2002). Sato et al., (1999) examined whether MTP mRNA and protein were effected by SREBPs in Hep G2 cells. They reported that in the absence of sterols, the MTP mRNA level was reduced by 53% and the MTP protein mass was also reduced by 49%, indicating that intracellular cholesterol affects MTP protein levels in the long term through the regulation of its mRNA levels. Although the SREBP-binding site overlaps the insulin-responsive element, they showed that insulin negatively regulates MTP expression even under sterol-loading conditions, indicating that SREBPs only slightly, if at all, mediate the insulin effects. Cytokines have also been reported to regulate MTP. Navasa et al., (1998) showed that interleukin 1 (II-1) and II-6 decreased MTP large subunit activity and mass in HepG2 cells and in hamsters, they found that IL-1 and TNF-α caused a decrease in hepatic MTP mRNA levels.

Recent evidence from the fructose-fed hamster model of insulin resistance has shown that an increased activity of MTP contributes to the increased secretion/overproduction of hepatic apoB100 lipoproteins (Taghibiglou et al., 2000; Lewis et al., 2005). Furthermore, amelioration of hepatic insulin resistance in this model resulted in normalization of MTP expression and reduction of the overproduction of apoB100-containing lipoproteins (Carpentier et al., 2002). Under normal culture conditions, insulin negatively regulates the expression of MTP in hepatocytes (Lin et al., 1995). Therefore, other compounds that activate intracellular insulin-signalling pathways may also decrease MTP expression and potentially be used in patients to treat dyslipidaemia associated with insulin resistance. Previous studies in cell culture systems and animal models have shown that the grapefruit flavonoid naringenin dramatically decreases the secretion of hepatic lipoproteins (Borradaile et al., 1999, 2002, 2003; Wilcox et al., 2001; Lee et al., 1999, 2001, 2003). It has been reported that naringenin dose-dependently inhibited apoB secretion in HepG2 cells (Wilcox et al., 2001) resulting in increased intracellular apoB degradation via a proteasomal rapid kinetic pathway (Borradaile et al., 2002). Initial mechanistic studies revealed that naringenin inhibited the expression and activity of acyl CoA:cholesterol
acyltransferase-2 and MTP, whereas apoB mRNA was unaffected (Wilcox et al., 2001). Subsequent experiments revealed that decreased acyl CoA:cholesterol acyltransferase activity and cholesteryl ester availability within the ER lumen were not requirements for the naringenin-induced inhibition of apoB secretion (Borradaile et al., 2002). In other studies, treatment of streptozotocin-induced diabetic rats with naringenin 7-O-β-D-glucoside (isolated from a Korean folk remedy) reduced blood glucose, triglycerides, and total cholesterol, indicating that naringenin may have insulin-like properties (Choi et al., 1991). Recently, it was reported that naringenin increases the activity of phosphatidylinositol (PI) 3-kinase independent of insulin receptor substrate (IRS)-1/2 (Borradaile et al., 2003). The increase in PI 3-kinase activity translated to an increase in cytosolic and nuclear sterol regulatory element–binding protein (SREBP)-1 protein levels and LDL receptor (LDL-r) mRNA, an effect that was completely blocked by inhibiting PI 3-kinase with the specific inhibitor wortmannin. These responses contributed to the decrease in apoB100 secretion from hepatocytes, thus supporting the concept that naringenin activates at least one insulin-signalling pathway (Borradaile et al., 2003). However, despite complete abrogation of the increase in SREBP-1 and LDL-r expression, wortmannin blocked the naringenin-induced decrease in apoB100 secretion by only 30%. It was possible that naringenin might also activate other intracellular insulin-signalling cascades involved in regulation of apoB100 secretion. Naringenin potently decreases the activity and expression of MTP, contributing to its ability to inhibit secretion of apoB100 from hepatocytes (Borradaile et al., 2003; Wilcox et al., 2001).

Insulin has been shown to decrease MTP mRNA in hepatocytes (Lin et al., 1995) via activation of the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) (MAPKerk) pathway (Au et al., 2003). The MAPKerk pathway is involved in the intracellular regulation of gene transcription (Whit marsh and Davis, 2000) and it is activated either via IRS-1/2 or the adapter protein Shc, two direct substrates of the insulin receptor (Skolnik et al., 1993). Major kinases in this pathway that are sequentially activated are Ras, Raf-1, MAPK kinase (MEK)-1/2, and ERK1/2 (Widmann et al., 1999). In addition, MAPKp38 modulates the MAPKerk pathway, and activation of this kinase normally functions as a negative regulator of MEK1/2-ERK1/2 signaling, thus providing
fine-tuning of this pathway (Keeton et al., 2002). It was demonstrated by Allister et al.,
(2005) that naringenin, like insulin, inhibits the expression of MTP via MEK1/2
activation and ERK1/2 phosphorylation. Importantly, naringenin and insulin cause an
acute decrease in the secretion of apoB100 from HepG2 cells mediated by MAPK_{erk}.
Furthermore, MAPK^{p38} is directly linked to apoB100 secretion such that blocking its
activity enhances the naringenin- and insulin-induced activation of MAPK_{erk} and
decreases apoB100 secretion.

HNF-4 is a highly conserved member of the nuclear receptor superfamily. It is a liver-
enriched transcription factor that, together with other factors, plays a key role in the
tissue-specific expression of a large number of genes involved in lipid and glucose
metabolism. The active form of HNF-4 is a homodimer, and it does not appear to
heterodimerize with other members of the nuclear receptor family. Recent investigations
have shown that coenzyme A derivatives of certain fatty acids activate the receptor, and
these derivatives thus have been characterized as endogenous ligands for HNF-4 (Hertz et
al., 1998; Petrescu et al., 2002). A crucial role for HNF-4 in metabolic homeostasis was
demonstrated by the finding that mutations in the HNF-4 gene cause the disorder known
as maturity onset diabetes of the young (Yamagata et al., 1996). Conditional HNF-4 gene
knock-out mice, which were produced using the Cre-loxP method with an albumin-Cre
transgene, exhibit a great reduction in serum cholesterol and triglycerides because of the
decreased levels of MTP and several apolipoproteins (Hayhurst et al., 2001). Although
this result indicates that MTP gene expression is under the control of HNF-4, little is
known about the specific step.

It has been shown that the transcriptional activity of HNF-4 is regulated by interaction
with small heterodimer partner (SHP), an atypical negative nuclear receptor lacking a
DNA-binding domain (Seol et al., 1998; Lee et al., 2000). SHP, induced by FXR together
with bile acids, controls the transcriptional activity of several other nuclear receptors
including the constitutive androstane receptor, thyroid receptor, retinoid X receptor
(RXR), retinoic acid receptor, estrogen receptors, peroxisome proliferator-activated
receptors, the liver X receptor, and the liver receptor homolog-1 (Lee et al., 2000; Seol et
al., 1998; Johansson et al., 1999; Masuda et al., 1997; Goodwin et al., 2000; Brendel et al., 2002). Moreover, recent findings have provided evidence that bile acids activate a MAPK pathway (Nakahara et al., 2002; Wang et al., 2002) and reduce the transactivation potential of HNF-4 (22). It was shown by Hirokane et al., (2004) that MTP gene expression is regulated by HNF-4 and HNF-1, which bind to the individual responsive element in the promoter of the human MTP gene. They also demonstrated that bile acids can down-regulate MTP transcription by impairing the transactivation potential of HNF-4 through the interaction with SHP and suppressing HNF-4 gene expression. In response to attenuated HNF-4 activity the transcription of other HNF-4-responsive genes including HNF-1 and apo B was also shown to be reduced, leading to decreased VLDL secretion. Taken together, this evidence suggests that bile acids are able to control lipoprotein synthesis and secretion via the FXR- and HNF-4-mediated pathways.

On the subject of the nuclear receptor superfamily, an interesting co-activation study by Meirhaeghe, Crowley et al (2003) of the nuclear receptor coactivator, Peroxisome-proliferator-activated receptor-γ co-activator 1β (PGC1β), showed that PGC1β may be an important co-activator integrating some of the actions of thyroid hormone in energy expenditure and mitochondrial biogenesis. The tissue distribution of PGC1β mRNA is conserved among species and does not seem to be regulated transcriptionally in vivo raising questions about the role of PGC1β in adaptive thermogenesis or gluconeogenesis (Meirhaeghe, Crowley et al., 2003), and therefore it may play a role in the abnormal metabolism found in the diabetic subject.

As mentioned already, the MTP promoter contains a sterol regulatory element. To examine whether dietary cholesterol affects MTP expression, Bennett et al., (1996) fed hamsters a variety of cholesterol concentrations and at the end of the feeding period, measured hepatic MTP mRNA levels. They found that increased dietary cholesterol caused increased hepatic MTP expression in hamsters and this increase was correlated with VLDL cholesterol and triacylglycerol levels, and also with hepatic cholesterol concentrations. They suggested that the increased hepatic expression of MTP was part of
a coordinated response to hepatic cholesterol accumulation leading to increased VLDL lipid secretion.

The MTP promoter also contains a negative insulin response element which appears to be active in cultured cells. Hepatic overproduction of VLDL in the insulin resistant state may result from direct hepatic effects of insulin as well as indirect metabolic effects such as increased availability of free fatty acids for triglyceride secretion. Several studies have examined the effect of diabetes on MTP expression. In streptozotocin-diabetic rats, Brett et al., (1995) observed no change in hepatic MTP activity or mass despite a markedly reduced VLDL output. Gleeson et al., (1999) investigated the effect of insulin-deficiency on intestinal MTP mRNA levels in the same animal model and found that intestinal MTP expression was almost 4-fold higher in the diabetic rats. In contrast to both of these studies, Wetterau et al., (1997) reported a 65% increase in hepatic MTP mRNA levels in streptozotocin-diabetic rats, but found no change in intestinal levels. Brett et al., (1995) measured MTP only 5 days after treatment, while Wetterau et al., waited for 8 days. Since the half life of MTP has been described as 4.4 days, at least in Hep G2 cells, perhaps it may be that only in Wetterau’s study sufficient time had elapsed to see a change in hepatic MTP levels.

Interest in MTP has been stimulated by the finding that an MTP inhibitor could normalise the levels of atherogenic apo B-containing lipoproteins in Watanabe-heritable hyperlipidaemic rabbits (Wetterau et al., 1998), a model for human homozygous familial hypercholesterolemia. This suggests that MTP inhibitors have potential applications for the therapeutic lowering of atherogenic lipoproteins in humans, which would be of particular interest in conditions such as diabetes in which lipoprotein metabolism is profoundly disturbed. Some of the problems encountered with MTP inhibitors carried out in animal and human studies to date are that treatment results in the accumulation of hepatic triglycerides (Raabe et al., 1999; Chandler et al., 2003; Liao et al., 2003; Shiomi and Ito, 2001). Very recently however, there has been a study which was able to demonstrate that JTT-130, (an MTP inhibitor) has the potential to decrease the prime risk factors of cardiovascular disease, namely plasma TG and LDL-C concentrations in
guinea pigs. The novelty of the drug tested is that there was no significant accumulation of lipid in the liver (Aggarwal et al., 2005). It is interesting that the main target of JTT-130 was the intestine. Because of this, it is possible that due to MTP inhibition, less TG was transferred to the chylomicron particle being packaged in the intestine. As a result a lower concentration of TG was taken up by the hepatocytes through the chylomicron remnant. Thus the VLDL particles secreted from the liver had lower concentrations of TG molecules due to the major inhibitory effect of the MTP inhibitor in the intestine. It seems likely that inhibition of MTP in humans may result in the near future, fulfilling the potential role of an MTP inhibitor in the treatment of high levels of lipoproteins.
1.6 REVERSE CHOLESTEROL TRANSPORT

1.6.1. Reverse Cholesterol Transport (RCT)

The movement of cholesterol from the peripheral tissues through the plasma compartment back to the liver is known as reverse cholesterol transport. This involves a direct pathway where cholesterol is transferred to HDL and then taken up by the liver, the indirect pathway involves transfer of cholesteryl ester from HDL to VLDL, then hepatic uptake of VLDL, or conversion of VLDL to LDL and uptake of LDL by the liver.

1.6.2 Cholesterol Ester Transfer Protein (CETP)

In humans CETP mRNA encodes a polypeptide with a molecular weight of 53kDa, which is n-glycosylated at four sites giving rise to the mature form of CETP with a molecular weight of 74kDa (Drayna et al., 1987). The most abundant sources of CETP mRNA are liver, spleen and adipose tissue, with lower levels of expression in the small intestine and heart.

CETP gene expression is regulated by a variety of factors including lifestyle, cholesterol, drugs and apo E genotype. In animals and humans, CETP is upregulated in response to increased dietary cholesterol (Quinet and Tall 1991; Martin et al., 1993). However, the regulation of CETP by dietary fats is not clearly understood. Interestingly, a recent study by Cheema et al., (2005) has found that addition of cholesterol to a low-fat monounsaturated fatty acid (MUFA) diet increased CETP activity and mRNA expression, whereas addition of cholesterol to a high-fat MUFA diet led to a decrease in CETP activity and mRNA expression. Hypolipidaemic drugs such as fibrates and statins may reduce CETP activity by reducing plasma concentrations of its substrates - the cholesteryl ester acceptors (VLDL and LDL).

The role of CETP in the development of atherosclerosis is still largely unknown. Theoretically, cholesterol ester transfer can be proatherogenic, as high plasma CETP
activity decreases HDL cholesterol levels. On the other hand cholesterol ester transfer can be anti-atherogenic, as CETP plays a role in RCT whereby peripheral cholesterol is removed from the circulation by the liver. High CETP activity has often been considered to be proatherogenic (Bhatnagar et al., 1993). In cholesterol- and fat-fed monkeys, Quinet et al., (1991) found a strong inverse relationship between CETP and HDL cholesterol and a positive correlation with LDL cholesterol. The extent of atherosclerosis was positively correlated with both LDL cholesterol and CETP concentrations. Increased CETP concentration and/or activity has been reported in many human dyslipidaemias associated with increased CAD, such as diabetes (Bagdade et al., 1993; Jones et al., 1996) and familial hypercholesterolemia (Inazu et al., 1992). Ordovas et al., (2000) found that the B2 allele was associated with decreased CETP activity with increased particle size for HDL and LDL. They calculated and odds ratio for CHD of 0.696 and after adjustment for BMI, diabetes, blood pressure, smoking, alcohol, age, total and HDL cholesterol, the ratio increased to 0.735, suggesting that the protective effect of the B2 allele was due in part to is association with HDL cholesterol levels. Several studies have examined the relationship between CETP and LDL particle size (Ambrosch et al., 1998; Talmud et al., 2000) and reported that LDL size was inversely related to CETP activity, thus proving that increasing CETP activity was associated with production of smaller more dense atherogenic LDL particles. A recent study by Vakkilainen et al., (2002) a strong inverse correlation between serum triglycerides and LDL size was found. Although CETP and hepatic lipase (HL) activities did not correlate with LDL size in univariate analyses, they were significantly associated with LDL size in the multivariate regression analysis, i.e., when the influence of triglycerides was taken into account. This may be explained by results of Mann et al (1991)., who discovered that CETP mass and activity are the rate limiting factor in net cholesteryl ester transfer from LDL to VLDL only in hypertriglyceridemic subjects.

The finding that postprandial chylomicron are the most patent ultimate acceptors of cholesterol released from cell membranes and that a low HDL level is not a factor that limits the ability of postprandial plasma to promote cholesterol efflux from cell membranes (Chong et al., 1998) suggests that postprandial chylomicrons may play a
major role in promoting RCT. It is probable that high CETP activity may be an anti-atherogenic factor when these cholesteryl ester-enriched chylomicrons are removed rapidly from the circulation by the liver, but when chylomicron clearance is delayed it may be proatherogenic. Thus, the effect of CETP on atherogenesis may depend on the metabolic context.

1.6.3. Reverse Cholesterol Transport and Diabetes

Cardiovascular risk is known to be increased in patients with diabetes (Pyorala et al., 1987). The precise mechanism for this increased propensity to CAD remains uncertain, but it is very likely that in addition to the effects of diabetes per se, other risk factors such as hyperlipidaemia also operate. As mentioned earlier, the finding of an association between the CETP gene Taq 1B B1 allele with higher plasma CETP concentrations, lower HDL cholesterol concentrations and a significant dose-dependent association with the progression of coronary atherosclerosis (Kuivenhoven et al., 1998), provides strong evidence that CETP can contribute to atherogenesis. This association has also been reported in Type 2 diabetic subjects (Bernard et al., 1998).

However, conflicting reports have been obtained in several studies measuring CETP and LCAT activity in diabetes. This may be due to variations in the methods used in their measurement. Several studies have reported an accelerated rate of cholesteryl ester esterification and transfer in diabetes (Jones et al., 1996; Bhatnagar et al., 1996; Bagdade et al., 1993; Riemans et al., 1998). Jones et al., (1996) found that while CETP mass was not different between control and Type 2 diabetic patients, the activity of both CETP and LCAT were higher in the diabetic patients and that CETP activity was negatively correlated with HDL cholesterol only in the diabetic patients. Whereas Bhatnagar et al., (1996) reported similar LCAT activity between control subjects and diabetic patients, they also found that cholesteryl ester transfer to VLDL and LDL, which was not effected by treatment with diet or sulphonylurea, was higher in the patients and was correlated with glycaemic control as assessed by HbA1c. It has been suggested that this increased
transfer of cholesteryl ester observed in Type 2 diabetic patients may be due to
dysfunction of VLDL and not LDL (Bagdade et al., 1993). In keeping with these
findings, Guerin et al., (2001) have also reported increased cholesteryl ester transfer from
HDL to VLDL and small dense LDL in Type 2 diabetic patients with the degree of
hypertriglyceridaemia being an important determination of CETP activity.

On the other hand, Lottenberg et al., (1996), demonstrated that both fasting and
postprandial CETP concentration and the rates of cholesteryl ester synthesis and transfer
from HDL to the apo B-containing lipoproteins were similar in treated Type 2 diabetic
subjects and controls. In a study using retinyl palmitate as a marker of chylomicron
particles, chylomicron clearance was shown not to be different between control and Type
2 diabetic subjects, but clearance of the chylomicron remnants was slower in the diabetic
subjects (Durlach et al., 1996). They also found that LCAT activity increased
postprandially in both groups, and while fasting CETP activity was 35% higher in the
diabetic subjects, it decreased postprandially, yet HDL$_2$ cholesteryl ester remained
unchanged. Together with the finding that postprandial control HDL$_3$ was phospholipid-
enriched was respect to fasting control and diabetic HDL$_3$, suggests that in control
subjects the combination of phospholipid-enriched HDL$_3$, increased CETP and LCAT
activities, stimulate efflux of cellular cholesterol and transfer of cholesteryl ester to the
apo B-containing lipoproteins which are then efficiently cleared from the circulation. In
diabetes these conditions (phospholipid-enriched HDL$_3$ and increased CETP) fail to
occur and deprive the patient of a potentially efficient mechanism of free cholesterol
clearance.

The effect of glycation of lipoproteins on the activities of LCAT and CETP has been
examined (Passerelli et al., 1997). They found a 50% decrease of the esterification rate of
cholesterol occurred in assays carried out with glycated HDL$_2$ and HDL$_3$ compared with
control HDL$_2$ and HDL$_3$, whereas glycation of the donor (HDL) and acceptor (VLDL and
LDL) lipoproteins increased the transfer rate of cholesteryl esters. These results are in
keeping with those of Cavallero et al., (1995). They found that apo A-I containing HDL
particles, isolated from the plasma of controlled Type 2 diabetic patients, exhibited a
decreased capacity to induce cholesterol efflux from adipose tissue cells both fasting and in the postprandial state. This abnormality was further amplified by the finding that LCAT activity increased by 54% and decreased by 18% postprandially for control and diabetic subjects respectively.

Taken together with the finding that the glycation of HDL enhances its turnover in plasma (Witzum et al., 1982) suggest that uncontrolled diabetes potentially favours the accumulation of cholesterol in the arterial wall, giving strength to the importance given to controlling blood sugar levels in trying to aid in the lessening of the process of atherosclerosis.
Chapter 2

Materials and Methods
2.1. MATERIALS

Materials were obtained from the following suppliers:

**Alpha Technologies (Biorad)**
Precast Ready Gels (4-15%)
Address: Blessington Industrial Estate, Co. Wicklow, Ireland

**Ambion**
RNA-later
Address: Ambion Europe Ltd, Cambridgeshire, UK

**Applied Biosystems**
PCR master-mix, Reverse Transcription Kit, ABI Prism caps and plates, Assay on Demand Primers and Probes for mRNA levels of genes, Reverse Transcription kit, Multiscribe reverse transcriptase,
Address: Warrington, UK

**Beckman Instruments Inc.**
6ml ultracentrifuge tubes.
Address: Naas, County Kildare, Ireland

**BDH Chemicals Ltd**
Comassie Brilliant Blue R-250, copper sulphate, ethylenediaminetetraacetic acid disodium salt, Folin and ciocaulteau’s phenol reagent, potassium hydroxide, potassium tartrate, potassium bromide, sodium azide, sodium hydroxide, tris (hydroxymethyl) methyamine, diethyl ether, hexane, hydrochloric acid, methanol.
Address: Poole, England.
BioMerieux
Diagnostic kit for phospholipid estimation, diagnostic kit for triglyceride estimation
Address: F-69280 Marcy, l’Etoile, France.

Boehringer Mannheim GmbH
Diagnostic kits for the estimation of total and free cholesterol, 10X PBS.
Address: D-68305, Mannheim, Germany

Calbiochem
D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) 2 trifluoroacetate
salt, phenylmethysulfonyl fluoride (PMSF)
Address: Distributed by Merck Biosciences, Nottingham N69 2JR, UK.

Qiagen
5mm Stainless steel balls for mixer mill 300, Rnease Mini Kit for extraction of RNA,
DNA extraction kit,
Address: Crawley, West Sussex, RH10 9NQ, UK

Lennox
Ethanol, Methanol
Address: Naas Road, Dublin, D12, Ireland

Roche
Cholesterol Reagent
Address: Lewes, East Sussex, BN7 1LG, UK

Sarstedt
Storage boxes, Cuvette boxes
Address: Drinagh, Wexford, Ireland.
Sigma
Agar, Agarose, Ammonium persulphate, Aprotinin, Bovine serum albumn (BSA), beta-mercaptoethanol, Bromophenol blue, copper sulphate, chloroform, ethium bromide, formaldehyde, formamide, glycerol, glycine, isopropanol, magnesium chloride, PMSF, sodium acetate, Sodium dodecyl sulphate, sodium chlorate, sodium citrate solution, streptozotocin,
Address: Dublin, Ireland.

Sparks
0.2ml Red-capped tubes, glass pipettes, plastic pipettes, 10ul 200ul, 1000ul sterile and non-sterile eppendorf tips
Address: Dublin, Ireland.

Unitech
Safe-lock micro test tubes, heparin coated tubes
Address: Airton Road, Tallaght, Dublin 24, Ireland.
2.2. HUMAN STUDIES METHODS

2.2.1. Subjects

Type 2 diabetic subjects were recruited from the Diabetic Day Centre in the Adelaide and Meath Hospital. Subjects were of both sexes and ranged in age from 47 to 76 years old. Control subjects were recruited from the same Day Centre and all patients gave informed consent. All studies were approved by the Federated Dublin Hospitals and Saint James Hospital Ethics Committee. Patient groups for the various studies are described in the relevant chapters.

2.2.2. Blood Sampling

Blood from the subjects was drawn into heparinised vacutainers by venepuncture at 8am following a 12 hour fast and at 4 and 6 hours postprandially. Blood was centrifuged within 1 hour to separate plasma and cells.

2.2.3. Serum Analysis

Total serum cholesterol was estimated using a commercially available diagnostic kit, supplied by Boehringer Mannheim. Total serum cholesterol content was assayed using an enzymatic colorimetric method as described by Roschlae et al. (1974). Cholesterol standards (0-80μg) and samples were measured in duplicate. 10μl of sample/standard was mixed with 1ml of the cholesterol assay solution. Standards and samples were then incubated at 37°C for 20 mins. The total cholesterol content of each was sample was then estimated by reference to the constructed cholesterol standard curve.

Serum triglyceride levels were estimated using a commercially available kit supplied by Biomerieux. They were quantified according to the method of Wahlefeld (1974) which involves the enzymatic hydrolysis of the triglyceride and subsequent determination of liberated glycerol by colorimetry.
2.2.4. Plasma Glucose Estimation

Venous blood glucose levels were estimated according to an enzymatic colorimetric method using a commercially available diagnostic kit supplied by Boehringer Mannheim GmBH. Blood samples (50μl) were deproteinised with 500μl of uranyl acetate deproteinising solution. The samples were vortexed and centrifuged for 7min at 800 x g and the supernatant removed. 20μl of supernatant from each sample was assayed in duplicate and a blank, a standard (0.505 mmol/l) and the Dade Moni-Trol I-E control were included in each assay. Glucose reagent solution (1ml) was added to each standard and sample which were then incubated at 37°C for 15min. The absorbances of all samples were read at 610nm.

2.2.5. Measurement of Glycosylated Haemoglobin

Blood HbA1c was determined using an enzyme immunoassay kit, containing monoclonal antibody that is specific for HbA1c, supplied by Novo Nordisk. The normal value is taken to be <4.9%.

2.2.6. Design of the Fat-Test Meal

Following an overnight fast, subjects were given a high fat test meal which they consumed over 30 minutes. The meal consisted of a milkshake containing 250mls of ice-cream, 250mls of milk, and 25mls of flora oil. The rest of meal consisted of 2 slices of toast with 4 sachets of flora butter, 2 fried eggs and a cup of tea or coffee (200mls). The meal contained 1,100 kcal of which 55% of the calories were fat and 25% carbohydrate. This meal was chosen to maximise lipoprotein changes in the diabetic patients. Blood samples were collected from each subject prior to the meal and at 4hrs and at 6 hrs postprandially. Water, but no food, was allowed during the study period.
2.2.7. Lipoprotein Isolation

Blood was collected into heparinised tubes by venepuncture and centrifuged at 3000g at 4°C for 15 min within 1 hr to separate plasma and cells. After separation of plasma, the following preservatives were added to prevent oxidation and degradation of Apo B: PPACK II (1mM), sodium azide (0.02%w/v), aprotinin (0.05 TIU), EDTA (0.1%), PMSF (0.1%). Lipoproteins were separated by the method of Havel et al., (1955). All density adjustments were made with a solution of KBr of density 1.346g/ml according to the equation: 

\[ aX + b(1.346) = (X + b)c \]

where \( a = \) initial density of solution, \( X = \) volume of initial solution, \( b = \) volume of 1.346 g/ml density solution to add, \( c = \) final density.

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 using a fixed angle rotor. Chylomicrons were carefully removed from the top of the tube with an elongated Pasteur pipette (pipette was heated and then stretched for a smaller diameter to make it more selective in taking up the floating chylomicron particles). Infranatant density was then adjusted to 1.006 g/ml and the solution was centrifuged at 40,000rpm at 4°C for 18 hr to isolate VLDL. VLDL was then removed, again with a stretched Pasteur pipette and all lipoprotein fractions were stored at -20°C and used within one week. The volume of each fraction was measured and recorded.

2.2.8. Chylomicron and VLDL Cholesterol and Triglyceride Determination

Chylomicron and VLDL cholesterol and triglyceride levels were estimated using commercially available diagnostic kits from Boehringer Mannheim and Biomerieux respectively as was previously described in section 2.2.3.
2.2.9. Chylomicron and VLDL Apolipoprotein B-48 and B-100 Determination

Chylomicron and VLDL apo B-48 and apo B-100 were separated by SDS-polyacrylamide gel electrophoresis using 4-15% gradient gels (Biorad, Herculas, Ca. USA) as was described by Curtin et al., (1995). For the determination of apo B-100 in the VLDL fraction and both the apo B-48 and B-100 in the chylomicron fraction, 30μl of each sample was used. For the determination of apo B-100 in the VLDL fraction, 10μg of total protein was used. This ensured that all apo B-48 and B-100 bands fell within the range of the standard curve. Non-delipidated lipoprotein samples were reduced in SDS sample buffer (2.0% mercaptoethanol, 4.0% SDS, 0.01% v/v bromophenol blue, 0.1mmol/L Tris-HCL, 20% glycerol, pH6.8) using a 1:1 ratio of sample to buffer for 4 min at 96°C. Samples were applied to the gel and run at 80mA constant current in 0.019mol/L Tris/0.192mol/l glycine. Gels were stained for 1hr with Comassie Brilliant Blue (0.1% in methanol:acetic acid:water 4:1:5) and destained with several changes of the same solvent. Since the chromogenicity of apo B-48 has been shown to be similar to that of apo B-100 (Karpe et al., 1994), a protein 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 for quantification of apo B-48 and apo B-100.

2.2.10. Linearity of the LDL standard

Linearity of apo B-48 and apo B-100 staining was determined by applying a range of LDL standards to a 4-15% gradient gel (figure 2.1). Staining was found to be linear with in the range 0.1-2.0μg of protein, in keeping with the findings of Karpe and Hamsten (1994), Kotite et al., (1995) and Curtin et al., (1996). Three concentrations of LDL apo B-100 within this range, depending on the expected apolipoprotein concentration, were applied to all gels. Any samples that fell above or below this standard were either increased or decreased in concentration respectively, by adjusting the volume put into the well of the gradient gel. Apolipoproteins were identified by comparing the distance they migrated into the gels with that of known molecular weight standards (SDS-PAGE Standards, High Range Biorad). Individual proteins included in the kit were myosin Mr
200,000; β-galactosidase Mr 116,250; phosphorylase b Mr 97,400; bovine serum albumin Mr 66,200 and ovalbumin Mr 45,000. The apo B-100 band co-migrated with the only stainable band in the LDL and the B-48 band was identified as the band lying immediately below the B-100 band and above myosin, the top molecular weight marker. The bands were quantified by densitometry using Video images of the gels were generated and imported into Vilbar Lourmet software for analysis. Density values were assigned to the apo B-100 bands of the human LDL and a standard curve constructed, the values were recalculated by linear regression and curves with a correlation coefficient >0.95 accepted. The concentrations of apo-B48 and B-100 were then determined from the standard curve.

**Figure 2.1.**

Linearity of the LDL standard

<table>
<thead>
<tr>
<th>Lane</th>
<th>Protein (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Apo B-100
2.2.11. Variation of the estimation of Apo B-48 and B-100

In order to calculate the intra-assay coefficient of variation of the quantification of apo B-48 and B-100 in the chylomicron and VLDL fractions by SDS-PAGE and densitometry, a single postprandial plasma sample was taken from a diabetic subject. Chylomicron and VLDL fractions were isolated by sequential ultracentrifugation as described in section 2.2.7. Non-delipidated chylomicron and VLDL samples were treated (as described in section 2.2.9.) and applied to six wells on a 4-15% gradient gel, using a separate gel for each of the two fractions. For determination of the inter-assay variation, non-delipidated chylomicron and VLDL samples were also applied to a single well on six separate 4-15% gradient gels (see figure 2.2 for example). Three concentration of LDL apo B-100 standard in the range of 0.5-2.0μg were applied to all gels. Chylomicron and VLDL apolipoprotein B-48 and apo B-100 were separated by SDS-polyacrylamide gel electrophoresis and quantified by densitometry as described in section 2.2.9. The coefficients of variation are shown in table 2.1. Measurements of apo B by this method provided an intra-assay CV of 3.4% and 3.6% for apoB-48 in the chylomicron and VLDL fractions and 3.0% and 4.5% for apo B-100 in the two fractions. The inter-assay CVs were similar with CV of 4.0% and 4.9% for apo B-48 and 6.4% and 5.8% for apo B-100 in the chylomicron and VLDL fractions respectively. These results are in line with those in the literature (Karpe and Hamsten 1994; Curtin et al., 1996).
Intra-assay variation on the estimation of Apo B48 and B100

Lane: 1 2 3 4 5 6

Apo B-100
Apo B-48

Lanes 1-6: Same sample applied to each lane, showing the inter-assay variation on the estimation of Apo B-100 and Apo B-48.

2.2.12. Reproducibility of Apo B-48 and B-100 quantification

To ascertain the reproducibility of the entire method of apo B quantification, to include preparation of the plasma sample, the ultracentrifugation process, separation by SDS-PAGE and quantification by densitometry, the following experiment was carried out.

A single postprandial blood sample from a diabetic individual was taken. Blood was collected into heparinised tubes by venepuncture and centrifuged at 3000 x g at 4°C for 15 min to separate plasma and cells. After separation of plasma the following preservatives were added to prevent oxidation and degradation of apo B: PPACK (1mM), PMSF (0.1mM), sodium azide (0.02% w/v), aprotinin (0.05 TIU), and EDTA (0.1%). Plasma was then divided into six identical 3ml portions of plasma which were treated as separate samples through all the steps. Samples were ultracentrifuged separately and chylomicrons and VLDL were isolated as described in section 2.2.7. Apo B-48 and B-
100 in both fractions was determined in each of the six aliquots by applying 24μl of each to six separate 4-15% gradient gels. Three concentration of LDL apoB-100 standard in the range of 0.1-2.0μg were applied to all gels. Each of the gels were stained and destained individually and quantified by densitometry on separate occasions. The results of this estimation of reproducibility are displayed in Table 2.2. By treating the six plasma portions as separate samples, the combined error of the preparation of plasma, the ultracentrifugation process and the analytical SDS-PAGE was tested. This yielded CVs of 15.9% and 21.0% for apoB-48 and 15.2% and 14.5% for apoB-100 in the chylomicron and VLDL fractions respectively. The error inherent in the SDS-PAGE and quantification by densitometry was shown to range from 4% to 6.4% in section 2.2.11., therefore it is likely that the major proportion of the analytical error may be accounted for by the procedure for isolating the TRL fractions.

Table 2.1
Intra- and inter assay coefficients of variation for the separation and quantification of chylomicron and VLDL apo B-48 and B-100.

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay CV%</th>
<th>Inter-assay CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Chylomicron apo B-48</td>
<td>3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Chylomicron apo B-100</td>
<td>3.0</td>
<td>6.4</td>
</tr>
<tr>
<td>VLDL apo B-48</td>
<td>3.6</td>
<td>4.9</td>
</tr>
<tr>
<td>VLDL apo B-100</td>
<td>4.5</td>
<td>5.8</td>
</tr>
</tbody>
</table>
Table 2.2  
Reproducibility of methods used in isolation of chylomicron and VLDL fractions from plasma, separation of apo B-48 and B-100 by SDS-PAGE and quantification by densitometry.

<table>
<thead>
<tr>
<th></th>
<th>Reproducibility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
</tr>
<tr>
<td>Chylomicron apo B-48</td>
<td>15.9</td>
</tr>
<tr>
<td>Chylomicron apo B-100</td>
<td>15.2</td>
</tr>
<tr>
<td>VLDL apo B-48</td>
<td>21.0</td>
</tr>
<tr>
<td>VLDL apo B-100</td>
<td>14.5</td>
</tr>
</tbody>
</table>
2.3. MOLECULAR BIOLOGY METHODS

2.3.1. RNA Isolation

All glassware and solutions used in the isolation of RNA were pre-treated with diethlypyrocarbonate (DEPC), a potent inhibitor of ribonucleases. Solutions to which DEPC (0.1% w/v) has been added were incubated at 37°C overnight and autoclaved to inactivate any residual DEPC. Glassware, gloves and equipment were also treated with RNAzap, another potent inhibitor of ribonucleases, in creating an RNase free environment.

RNA was isolated from the tissues of rat liver and intestinal mucosa, and also from the second part of the human intestine, the duodenal mucosa. Rat intestine was washed with phosphate buffered saline. The mucosa was scraped using a sterile blade and approximately 20mg of biopsy tissue was placed immediately in 1ml RNAlater, and stored at -20°C. A sample of approximately 20mg of rat liver was cut and stored in 1ml RNAlater immediately following the sacrifice of the animal. Patients undergoing routine gastroscopy had two biopsy samples, each approximately 10mg, placed separately in 1ml RNAlater and stored at -20°C. 10mg of tissue was homogenized in 600μl RLT buffer (Qiagen) using a Retsch Mixer-Mill 300 and 5mm stainless steel beads were used in RNase free eppendorfs (B-Mercaptoethanol was added to the Buffer RLT before use). The tissue lysate was centrifuged for 3 min at maximum speed in a microcentrifuge. The supernatant was then transferred to a new microcentrifuge tube by pipetting. 1 volume, 600μl, of 70% ethanol was added to the cleared lysate and mixed immediately by pipetting. 700ul of the sample was applied to an RNeasy mini column and places in a 2ml collection tube. At this stage, an RNase-Free DNase Set (Qiagen) was used, providing efficient on-column digestion of DNA. The DNase is efficiently removed in subsequent wash steps. This step is used to ensure that no DNA has inadvertently bound to the silica membrane of the RNeasy column used in the RNA extractions. It is necessary to do so as contaminating DNA will give an inaccurate result when quantifying the RNA in a later step (2.4.3). 350μl buffer RW1 was pipetted into the RNeasy mini column and
centrifuged for 15 sec at 10,000 x g to wash the column. The flow through was discarded. 10 µl DNase 1 stock was then added to 70 µl buffer RDD and mixed gently by inverting the tube. Following this, the DNase 1 incubation mix of 80 µl was pipetted directly onto the RNeasy silica-gel membrane and left on the bench-top at room temperature for 15 min. Another 350 µl buffer RW1 was then pipetted into the RNeasy column, and the column was centrifuged for 15 sec at 10,000 x g. Once again the flow through was discarded. 700 µl buffer RW1 was then added to the RNeasy column and the tube was centrifuged for 15 sec at 10,000 x g in order to wash the column. The flow through was again discarded. After placing the RNeasy column in to a new 2 ml collection tube, 500 µl buffer RPE was pipetted onto the RNeasy column. Once again the tube was centrifuged for 15 sec at 10,000 x g to wash the column and the flow through discarded. Another 500 µl buffer RPE was added to the RNeasy column and centrifuged for 2 min at 10,000 g to dry the RNeasy silica gel membrane. Following this step the RNeasy column was transferred to a new 1.5 ml collection tube. 50 µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and the tube was centrifuged for 1 min at 10,000 g to elute the RNA in the RNase free water. RNA samples were stored in aliquots at -70°C until required.

2.3.2. Assessment of RNA Purity and Integrity

The purity of the final preparation of RNA was determined by spectrophotometric methods and agarose gel electrophoresis. RNA was separated under denaturing conditions on agarose formaldehyde gels. Both the gel and running buffer contained 1x MOPS buffer; (20 mM 3-[N-morpholino] propanesulphonic acid pH 7.0, 50 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde). The RNA sample was prepared as follows: 4.5 µl of RNA, 2 µl of 10x MOPS, 10 µl of formamide and 3.5 µl of formaldehyde were mixed and heated for 15 min at 65°C before addition of 2 µl loading buffer (1 mM EDTA (pH 8), 0.25% (w/v) bromophenol blue, 0.23% (v/v) xylene cyanol, and 50% (v/v) glycerol) and 1 µl ethidium bromide (10 mg/ml). Samples were electrophoresed on a 1.5% (w/v) agarose gel at 20 V for 2 hr. RNA was visualised under UV light displaying a 28 S and 18 S ribosomal band (as is shown in Figure 2.3.1). The high quality of the RNA
preparations is reflected by the integrity of the ribosomal (rRNA) species, with 28s/18s ratios of approximately 2. The UV (ultra violet) absorbance spectrum of pure RNA is such that an absorbance of 1 unit at 260nm and 1cm pathlength indicates the presence of 40μg of RNA/ml. An aliquot of each sample (5μl) was diluted with 995μl deionised water and its UV absorbance at 260nm and 280nm was measured. The ratio of absorbance 260nm/280nm also gives an indication of purity. Undegraded pure RNA has an absorbance 260nm/280nm ratio of approximately 1.8-2.0. Samples with an absorbance ratio of 1.7 or over were used and considered to have a high purity.

**Figure 2.3.1.**

RNA visualised under UV light displaying a 28S and 18S ribosomal band

Lane 1: DNA marker
Lane 2: Blank
Lane 3: RNA sample
2.3.3. Assessment of RNA Quantity

Although the spectrophotometric method of quantifying RNA has been widely used previous to this study, it was noted in the literature of a more sensitive for measuring the amount of RNA extracted from a sample, using the Quant-iT™ RiboGreen® reagent and assay kit. The major disadvantages of the previously used absorbance-based method are the large relative contribution of proteins and free nucleotides to the signal, the interference caused by contaminants commonly found in nucleic preparations and the relative insensitivity of the assay. The use of sensitive, fluorescent nucleic acid stains alleviates many of these problems. The sensitivity achieved using this RiboGreen probe method exceeds that achieved by ethidium bromide-based assays by 200 fold and exceeds that achieved with ultraviolet absorbance determination by 1000-fold (Anal Biochem, 1998).

The T.E. buffer is prepared fresh each time the assay is carried out and an RNA standard curve is produced following the instructions given with the kit. A high range assay (measuring from 20ng/ml to 1μg/ml RNA) or a low range assay (measuring 1ng/ml to 50ng/ml RNA) can be used. The Quant-iT™ RiboGreen® reagent is then prepared and the assay volume used is 200μl in total per well on a 96-well dark microplate. The samples fluorescence is measured using a fluorescence microplate reader and standard fluorescein wave-lengths (excitation of 480nm and emission of 520nm). The fluorescence value of the reagent blank from that of each RNA standard was subtracted and the corrected data was used to generate a standard curve of fluorescence versus RNA concentration. The RNA concentrations of the samples were determined from the generated standard curve using standard linear regression. It is worth noting that the Quant-iT™ RiboGreen® reagent also binds to DNA, therefore making the DNase treating step in the RNA extraction of high importance.
2.3.4. Reverse Transcription of RNA

TaqMan® Reverse Transcription Reagents were used in cDNA synthesis. This is the first step in the two-step RT-PCR (reverse transcription-polymerase chain reaction) protocol, allowing for the employment of the rapid 5' nuclease assay under TaqMan® probe assay development guidelines, which minimizes optimization time. It contains AmpliTaq Gold® DNA polymerase to provide a better yield and a more robust 5' nuclease assay than AmpliTaq® DNA polymerase. Thermal cycling parameters for Two-Step PCR are shown in Table 2.3. The ingredients of the two-step reverse transcription are listed in Table 2.4.

Table 2.3.

Thermal cycling parameters for Two-Step PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Incubation</th>
<th>RT</th>
<th>Reverse Transcriptase Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOLD</td>
<td>HOLD</td>
<td>HOLD</td>
<td>HOLD</td>
</tr>
<tr>
<td>Temperature</td>
<td>25 °C</td>
<td>48 °C</td>
<td>95 °C</td>
</tr>
<tr>
<td>Time</td>
<td>10 min</td>
<td>30 min</td>
<td>5 min</td>
</tr>
</tbody>
</table>
Table 2.4.
Ingredients of the two-step reverse transcription.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Tube (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10µl reaction</td>
<td>100µl reaction</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>see below</td>
<td>see below</td>
</tr>
<tr>
<td>10X TaqMan RT buffer</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>25mM Magnesium Chloride</td>
<td>2.2</td>
<td>22.0</td>
</tr>
<tr>
<td>deoxyNTPs Mixture</td>
<td>2.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Random Hexamers</td>
<td>0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Multiscribe Rev. Tr. (50 U/µL)</td>
<td>0.25</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>6.15</strong></td>
<td><strong>61.5</strong></td>
</tr>
</tbody>
</table>
2.3.5. Real-Time RT-PCR

Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In contrast, an endpoint assay (also called a “plate read assay”) measures the amount of accumulated PCR product at the end of the PCR cycle.

Initially, intercalator dyes were used to measure real-time PCR products. The primary disadvantage to these type of probes is that they detect accumulation of both specific and non-specific PCR products. Real-time systems for PCR were improved by the introduction of fluorogenic-labelled probes that use the 5’ nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products. The development of fluorogenic labelled probes also made it possible to eliminate post-PCR processing for the analysis of probe degradation.

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5’end and a quencher dye on the 3’end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space. If the target is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5’ nuclease activity of Taq DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye, increasing the reporter dye signal. It also removes the probe from the target strand, allowing primer...
extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced. TaqMan MGB (minor groove binding) probes were used in this study. They contain a nonfluorescent quencher at the 3’ end – the SDS instrument can measure the reporter dye contributions more precisely because the quencher does not fluoresce. They also contain a minor groove binder at the 3’ end – the minor groove binder increase the melting temperature (Tm) of probes, allowing the use of shorter probes. Consequently, the TaqMan MGB probes exhibit greater differences in Tm values between matched and mismatched probes, which provides more accurate allelic discrimination.

The Assays-on-Demand Gene Expression products were used for the gene expression quantitation, as the second step in the two-step RT-PCR. An ABI Prism 7000 machine was used in this process, which is optimised to work with TaqMan Universal PCR Master Mix and complimentary DNA (cDNA). The universal thermal cycling parameters which were used are outlined in table 2.5. The commonly used $2^{-\Delta\Delta CT}$ method of relative quantification was used in determining the relative levels of mRNA gene expression in relation to the housekeeping gene, Glyeraldehyde-3-phosphate dehydrogenase (GAPDH).
Table 2.5.

Thermal Cycling Conditions for use in the ABI Prism 7000 machine:

<table>
<thead>
<tr>
<th>Thermal Cycler</th>
<th>Initial Setup</th>
<th>Each of 40 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Denature</td>
</tr>
<tr>
<td>ABI Prism 7000 Sequence Detec. Syst.</td>
<td>HOLD</td>
<td>CYCLE</td>
</tr>
<tr>
<td></td>
<td>10 min 95°C</td>
<td>15 sec 95°C</td>
</tr>
</tbody>
</table>

The Assay on Demand Gene Expression products consist of a 20X mix of unlabelled PCR primers and TaqMan MGB (Minor Groove Binding) probe (FAM dye-labelled). These assays are designed for the detection and quantitation of specific human/rat genetic sequences in RNA samples that have been converted to cDNA. To prepare the reaction components for a single 25μl reaction on a 96 well plate, the different volumes used are shown in Table 2.6.

Table 2.6.

Reaction components and volumes in a 25μl reaction for real time PCR.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume/well</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Universal PCR Master Mix (2X)</td>
<td>12.5</td>
<td>1X</td>
</tr>
<tr>
<td>20X Assays on Demand Gene Expression Assay Mix</td>
<td>1.25</td>
<td>1X</td>
</tr>
<tr>
<td>cDNA diluted in RNase-free water</td>
<td>11.25</td>
<td>-</td>
</tr>
<tr>
<td>Total (μl)</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>
2.4 ANIMAL STUDIES METHODS

2.4.1. Animal Maintenance

Two groups of animal were used in the study. Zucker fatty rats and Sprague Dawley rats (streptozotocin injected) were used as models of diabetes and non-fatty Zucker rats and buffer only injected Sprague Dawley rats were used for comparative controls. The rats were housed in groups of 2 or 3 and allowed to acclimatize for two weeks, with free access to standard chow and water. Food and water intake of each rat was monitored daily and each rat was weighed daily to monitor its well-being. All animals were housed under license from the Department of Health and experiments carried out according to Irish law as administered by the Department of Health.

2.4.2. Induction and treatment of diabetes

Following the two weeks acclimatization, diabetes was induced in the Sprague Dawley rats by subcutaneous injection of streptozotocin (60mg/kg, in sodium citrate buffer, pH=4) in the neck region. The rats were allowed to stabilize for 24 hours and the blood glucose was monitored frequently (every 2 hours) for the first 12 hours in case of hypoglycaemia, caused by insulin release from necrotic beta cells in the pancreas. Diabetes was confirmed within 30 hours, with blood glucose > 20 mmol/L. There were concerns that the acidic nature of the injected solution (pH=4) may cause the skin to crack following the injection, but these fears were not realised. Extra bedding was also supplied for the diabetic rats due to the increased production of urine (polyuria), one of the classic symptoms of diabetes. Control rats were injected with sodium citrate buffer only, (pH=4). Diabetic control was monitored daily by taking a tail vein blood sample, and the sample was measured on a blood glucose monitor (the tail was heated on a thermal block to increase blood flow). Food and water intake of each rat was monitored before and after the induction of diabetes, and each animal was weighed on a daily basis. Animals were diabetic for 10 days prior to experiment.
2.4.3. Blood Sampling and Biopsy taking

Animals were sacrificed by cardiac puncture and cervical dislocation. Blood was drawn from the heart while the animal was under halothane gas anaesthetic for the duration. The blood was then spun at 3000 x g for 10min in lithium heparin 10ml tubes to isolate the serum. The serum was then treated with 5% volume of an anti-protease mixture containing Aprotinin (0.05 TIU), NaN3 (0.02% w/v), EDTA (0.1% w/v), PPACK ß (1mmol/L) and PMSF (0.1mmol/L) to prevent oxidation and degradation of the lipoproteins. The small intestine and liver were removed and weighed. Both the liver and the small intestine were washed with ice-cold PBS containing EDTA. Two small biopsy samples of the liver were taken (approximately 10mg) using a sterile blade and placed in RNAlater. The intestine was laid out on a plastic plate, cleaned down with RNAzap. It was then divided into 3 equal portions, representing the duodenum, the ileum and the jejunum. The intestinal mucosa was scraped, following the washing with PBS, with a sterile blade and two biopsy samples from each portion were placed separately in RNAlater. All biopsy samples were stored at -70°C until required.

2.4.4. Isolation of Chylomicrons and VLDL

Chylomicrons were isolated from the animal serum as was described in section 2.2.7. From one practice sample it could be seen that there were high volumes of chylomicrons being collected, so only 2mls of serum was underlaid beneath 4 mls of infranatant instead of the usual 3mls of serum beneath 3mls of infranatant (density of 1.006g/ml) in order to make it easier to collect all the sample. VLDL particles were collected using the same method as was described in section 2.2.7.

2.4.5. Lipoprotein Cholesterol and Triglyceride Determination

Chylomicron and VLDL cholesterol and triglyceride levels were estimated using commercially available diagnostic kits from Boehringer Mannheim and Biomerieux respectively as was previously described in section 2.2.3.
2.4.6. Chylomicron and VLDL Apolipoprotein B-48 and B-100 Determination from animal serum samples

As in section 2.2.9., chylomicron and VLDL apolipoprotein B-48 and B-100 were separated by SDS-polyacrylamide gel electrophoresis using 4-15% gradient gels. A standard curve containing 0.5, 1.0, 2.0 and 3.0 μg of protein were used on each gel, in order to make sure that all the reading fell between the upper and lower standards.

2.4.7. Time Course Study

A time course study, study number 2, was included as part of the overall animal studies, whereby two groups of ten Sprague Dawley rats were fasted for a period of 6 hours and 12 hours respectively before sacrifice. Water was supplied during this time and all rats were monitored for any adverse effects.

2.4.8. Drug Administration

The third animal study involved treating two groups of 10 Zucker fatty rats: one group was treated with insulin and one group was treated with pioglitazone. One quarter of a unit of insulin was administered on a daily basis for 14 consecutive days and there were no hypoglycaemic events. The other group were administered with 10mg pioglitazone, mixed in 1ml water and gavaged to the animals on a daily basis for 14 consecutive days. There were no hypoglycaemic events in any of the animals treated with pioglitazone during this period. Blood glucose was monitored at least once daily in each animal and all animals were monitored for adverse side effects for the duration of the study.
Chapter 3.

Postprandial lipoprotein production in diabetes: The effect of pioglitazone as compared to insulin on intestinal and hepatic MTP, Niemann Pick C1-like1, HMGCoA reductase and ABCG5/G8.
3.1. **Introduction**

Diabetes is associated with an increase in atherosclerosis. The major risk factors include dyslipidaemia and in particular alterations in triglyceride and HDL. It is for these reasons that the postprandial phase in diabetes is thought to be particularly atherogenic (Tanaka *et al.*, 2004; Wilhelm *et al.*, 2003). Chylomicron and VLDL clearance are altered in diabetes due to a delay in triglyceride hydrolysis. Increase in apolipoprotein (apo) C3 has also been shown to be a factor in the delayed clearance of triglycerides (Mann *et al.*, 1997). A reduction in apo E has been demonstrated on the chylomicrons of both diabetic animals and patients which would contribute to the delayed clearance (Levy *et al.*, 1985; Madigan *et al.*, 2005). It has been suggested that the early increase in apo B48 in diabetic patients may be due to increase in synthesis of chylomicron particles (Hiukka *et al.*, 2005) and this has been confirmed to some extent by demonstrating an increase in microsomal triglyceride transfer protein (MTP) mRNA in diabetic patients (Curtin *et al.*, 1994). Synthesis of the chylomicron particle depends on the availability of both cholesterol and triglyceride in the intestine. Intestinal cholesterol synthesis has been shown to be increased in diabetes whereas cholesterol absorption is usually normal or may be reduced (Gylling *et al.*, 1997).

The recent discovery of Niemann Pick C1-like 1 (NPC1-L1) protein which regulates sterol absorption (Altmann *et al.*, 2004) and the ATP binding cassette proteins (ATP) G5 and G8 which work in tandem to excrete enterocyte cholesterol back into the lumen of the intestine (Graf *et al.*, 2003) has given us new tools to explore chylomicron and VLDL synthesis in diabetes. The peroxisome proliferator activated receptor (PPAR) agonists, the thiazolidinediones, improve insulin sensitivity and dyslipidaemia by altering fat storage and lipid metabolism. The thiazolidinediones however have many other effects and alter more than 100 genes (Yki-Jarvinen, 2004). It has been suggested in large prospective studies that they improve cardiovascular risk factors. They have been shown to increase lipolysis of VLDL by increasing lipoprotein lipase mass and decreasing plasma levels of apo CIll (Schoonjans *et al.*, 1996).
The purpose of the present study was to investigate the effect of pioglitazone as compared to insulin on postprandial chylomicron and VLDL levels in an animal model for T2DM. It was also aimed to correlate any changes in the production of these lipoproteins to the intestinal and hepatic expression of HMGCoA reductase, MTP, ABCG5 and G8 and NPC1-L1, all proteins involved in whole body cholesterol homeostasis.
3.2 Animals

Thirty male zucker diabetic fatty rats and 10 lean littermates (non-diabetic) were investigated. Animals were acclimatised for one week, with free access to standard chow and water. Diabetes was confirmed when the rats were 9 weeks old by a blood sugar of \( >7.5 \text{ mmol/l} \). Ten of the diabetic rats were then treated with a dose of 10 mg/kg body weight of pioglitazone for 2 weeks and ten were treated with insulin (0.5 units per day was found to be more effective in bringing blood sugar levels into the normal range than 0.25 units per day, and there were no hypoglycaemic events). At the end of this period all of the rats were exsanguinated in the postprandial phase and chylomicrons and VLDL were isolated from the plasma. The liver was removed and samples stored in RNAlater and the intestine was washed, mucosa scraped and samples stored in RNAlater for MTP, HMGCoA Reductase, ABCG5 and G8 and NPC1-L1 mRNA determination as is described in detail in section 2.4.3.

3.3 Methods

Chylomicrons and VLDL were isolated from the plasma by sequential ultracentrifugation as described in section 2.4.4. Venous plasma glucose levels were determined according to an enzymatic colorimetric method using a commercially available diagnostic kit (Boehringer Mannheim GmbH, Mannheim, Germany). Total cholesterol and triglyceride content of lipoprotein fractions were measured by an enzymatic colorimetric method using kits from Boehringer Mannheim GmbH (Mannheim, Germany). Chylomicron and VLDL apolipoprotein B48 and B100 were separated by SDS-PAGE and quantified by densitometry as described in section 2.4.6.

RNA was quantified using the Quant-iT Ribogreen RNA Assay Kit (Molecular Probes). An RNA standard curve was constructed using dilutions of a supplied, known amount of RNA, and read on a dual band plate reader with an excitation wavelength of 500nm and an emission wavelength of 525nm. Samples were calculated using linear regression from this standard curve.
RNA was then reverse transcribed as part of the 2-step real-time RT-PCR to cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). A GeneAmp 2400 PCR System (Applied Biosystems) was used and the conditions were: 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. Eight hundred ng of each sample in 100μl was reverse transcribed to cDNA, with 80ng of the cDNA to be used in the next step.

10μl of the cDNA containing 80ng was used in the real-time PCR analysis. The rat-specific primers and probes for the genes of interest were purchased from Applied Biosystems using the Assay On Demand system. Thermal cycling conditions and volumes of ingredients are already optimised using this system. The thermal cycling conditions used were: stage 1: 50°C for 2 min, stage 2: 95°C for 10 min, stage 3: 40 repetitions of 95°C for 15 sec alternating with 60°C for 1 min. A sample volume of 25μl was used in each well. Primers and probes were used from a 20X stock solution and the final concentration was 1X. A 2X master mix solution was added to each well at a final concentration of 1X. 2μl cDNA containing 80ng was added separately into each well and a standard deviation on ct value of <0.3 was accepted.

GAPDH was used as the housekeeping gene and all reactions were done as single-plex reactions on a 96-well plate. A TaqMan Universal PCR Master Mix was used (Applied Biosystems) as the mix for the rest of the PCR ingredients. Analysis was run on an ABI-prism 7000 and the $2^{\Delta\Delta CT}$ method of relative gene expression was employed. Results were expressed as arbitrary units in comparison to GAPDH.

3.4 Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test, and the software used was SSPS. Analysis of Variance (ANOVA) was employed for statistical analysis between the different treatment groups in the study. Non-parametric tests were used for triglyceride analysis. Correlation coefficients were measured by linear regression.
analysis. Data are expressed as the mean ± standard deviation and mean ± of the mean (SEM) and a p value of <0.05 was regarded as statistically significant.

3.5 Results

The characteristics of the 4 groups are shown in Table 3.1. The lean littermates were significantly lighter than the diabetic animals whether untreated or treated with insulin or pioglitazone. There was no significant difference in weight between the 3 diabetic groups. The untreated diabetic animals had the highest plasma glucose (9.7±1.7 mmol/l) while the other 3 groups had similar blood sugars.

Table 3.1
Characteristics of the four groups

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
<th>Blood sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>zucker diabetic rats</td>
<td>423.7 ± 45.7</td>
<td>9.7 ± 1.7</td>
</tr>
<tr>
<td>lean litter mates</td>
<td>295.6 ± 17.3</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>pioglitazone treated</td>
<td>438.6 ± 56.7</td>
<td>7.9 ± 0.4</td>
</tr>
<tr>
<td>insulin treated</td>
<td>427.8 ± 33.8</td>
<td>7.0 ± 1.4</td>
</tr>
</tbody>
</table>

Chylomicron triglyceride was highest in the diabetic untreated rats (474±145mg/ml plasma) as can be seen in Table 3.2. The pioglitazone-treated animals had similar chylomicron triglyceride to the lean littermates (74±31 v 81±44 mg/ml plasma). The insulin-treated animals had a chylomicron triglyceride of 264±72 mg/ml plasma which was significantly higher (p<0.01) than that for the pioglitazone-treated animals and significantly lower (p<0.02) than the diabetic untreated animals. Chylomicron cholesterol was lowest in the lean littermates 28±16 mg/ml plasma and significantly lower (p<0.01) than the other three groups. Chylomicron cholesterol in the pioglitazone group was significantly lower than that in the insulin treated group (78±20 v 152±33 mg/ml plasma, p<0.0001). Apo B48 was significantly lower in the lean litter mates
compared than in the other 3 groups (p<0.0005). The pioglitazone treated animals had significantly lower chylomicron apo B48 compared to the other two diabetic groups (p<0.001). Chylomicron apo B100 was also lowest in the lean animal (p<0.0001) compared to the other 3 groups. There was no significant difference between the chylomicron apo B100 in the pioglitazone rats as compared to the insulin-treated rats but both were significantly lower than the untreated animals.

Table 3.2
Chylomicron analysis

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>ApoB48</th>
<th>ApoB100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker diabetic rats</td>
<td>282.57 ± 67.9</td>
<td>474.40 ± 145.3</td>
<td>167.72 ± 33.94</td>
<td>101.11 ± 36.54</td>
</tr>
<tr>
<td>Lean litter mates</td>
<td>27.75 ± 16.48</td>
<td>81.19 ± 44.22</td>
<td>13.89 ± 4.49</td>
<td>6.95 ± 4.38</td>
</tr>
<tr>
<td>Pioglitazone treated</td>
<td>77.79 ± 20.43</td>
<td>74.51 ± 31.49</td>
<td>56.82 ± 17.82</td>
<td>41.08 ± 12.12</td>
</tr>
<tr>
<td>Insulin treated</td>
<td>152.63 ± 33.26</td>
<td>263.68 ± 72.10</td>
<td>108.50 ± 26.59</td>
<td>43.91 ± 13.85</td>
</tr>
</tbody>
</table>

VLDL triglyceride was highest in the untreated diabetic animals as can be seen in Table 3.3. The VLDL triglyceride was significantly higher in the insulin-treated compared to the pioglitazone-treated animals (p<0.0001) but both were significantly less than the untreated animals (p<0.0001). VLDL cholesterol was lowest in the lean littermates and highest in the untreated diabetic animals (p<0.0001) as it was in the chylomicron fraction. VLDL cholesterol was significantly lower in the pioglitazone-treated compared to insulin treated animals (p<0.0001) and in both groups the levels were significantly less than in the untreated diabetic animals (p<0.0001). Apo B48 was lowest in the lean littermates and highest in the diabetic untreated animals (p<0.0001) with no significant difference between the pioglitazone and the insulin-treated animals. Apo B100 on the other hand was significantly lower in the pioglitazone-treated as compared to the insulin-treated and
untreated animals \((p<0.0001)\). The insulin-treated rats were not significantly different from the untreated diabetic animals.

Table 3.3
VLDL Analysis

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>ApoB48</th>
<th>ApoB100</th>
</tr>
</thead>
<tbody>
<tr>
<td>zucker diabetic rats</td>
<td>427.19 ± 96.97</td>
<td>448.80 ± 73.42</td>
<td>85.32 ± 28.56</td>
<td>253.92 ± 69.55</td>
</tr>
<tr>
<td>lean litter mates</td>
<td>57.20 ± 23.82</td>
<td>93.03 ± 52.74</td>
<td>10.19 ± 3.42</td>
<td>47.97 ± 14.21</td>
</tr>
<tr>
<td>Pioglitazone treated</td>
<td>111.14 ± 33.97</td>
<td>115.62 ± 35.19</td>
<td>26.49 ± 20.17</td>
<td>102.87 ± 28</td>
</tr>
<tr>
<td>insulin treated</td>
<td>253.20 ± 55.41</td>
<td>281.73 ± 49.28</td>
<td>38.11 ± 13.02</td>
<td>205.50 ± 50.68</td>
</tr>
</tbody>
</table>

Table 3.4 shows the intestinal mRNA expression of the 5 different proteins in the 4 different animal groups. Intestinal HMGCoA reductase expression in the intestine was significantly lower in the lean littermates compared to the diabetic groups \((p<0.0002)\) with no significant difference between any of the diabetic groups, as can be seen in figure 3.1. MTP mRNA was lowest in the lean littermates and highest in the untreated diabetic group \((p<0.0001)\), see figure 3.2. Both the insulin and pioglitazone treated rats had significantly higher MTP mRNA than the lean litter-mates \((p<0.02)\) and lower than the untreated animals \((p<0.001)\) with no difference between the 2 treated groups. ABCG5 and G8 mRNA were significantly lower in the untreated diabetic animals than in the lean littermates \((p<0.02)\) with no significant difference between the 3 diabetic groups, as can be seen in figure 3.5. Intestinal NPC1-L1 mRNA was lowest in the lean animals \((p<0.0001)\) with no significant difference in the 3 diabetic groups (figure 3.3)
### Table 3.4
Intestinal mRNA gene expression

<table>
<thead>
<tr>
<th></th>
<th>ABCG5</th>
<th>ABCG8</th>
<th>NPC1L1</th>
<th>MTP</th>
<th>HMGC0A</th>
</tr>
</thead>
<tbody>
<tr>
<td>zucker diabetic rats</td>
<td>16.69 ±11.57</td>
<td>13.44 ±8.74</td>
<td>72.86 ±40.00</td>
<td>189.53 ±53.80</td>
<td>52.09 ±25.46</td>
</tr>
<tr>
<td>lean litter mates</td>
<td>44.76 ±30.40</td>
<td>26.71 ±11.90</td>
<td>7.69 ±6.25</td>
<td>36.75 ±18.57</td>
<td>11.62 ±9.36</td>
</tr>
<tr>
<td>pioglitazone treated</td>
<td>19.97 ±10.72</td>
<td>17.32 ±11.15</td>
<td>68.09 ±22.58</td>
<td>55.30 ±13.35</td>
<td>38.01 ±17.87</td>
</tr>
<tr>
<td>insulin treated</td>
<td>21.62 ±18.99</td>
<td>22.09 ±23.71</td>
<td>86.28 ±19.06</td>
<td>62.07 ±6.63</td>
<td>46.33 ±19.96</td>
</tr>
</tbody>
</table>

### Figure 3.1
Intestinal expression of HMGC0A mRNA in the 4 groups

Diabetic
Lean
Pioglitazone Treated
Insulin Treated
Figure 3.2
Intestinal expression of MTP mRNA in the 4 groups

Figure 3.3
Intestinal expression of NPC1L1 mRNA in the 4 groups

Figure 3.4
Intestinal expression of ABCG8 mRNA in the 4 groups
Figure 3.5
Intestinal expression of ABCG8 mRNA in the 4 groups

![Intestinal ABCG5 mRNA expression](image)

Diabetic
Lean
Pioglitazone Treated
Insulin Treated

HMGCoA reductase mRNA was significantly higher in the liver of the 3 diabetic groups compared to the lean animals (p<0.0001), see Table 3.5. The highest value was found in the diabetic rats on pioglitazone and this was significantly higher than the insulin-treated diabetic rats (p<0.001). Hepatic MTP mRNA was lowest in the lean rats and highest in the untreated diabetic animals (p<0.0001). MTP mRNA was significantly lower in the pioglitazone-treated rats compared to insulin-treated and untreated animals (p<0.0001). ABCG5 and G8 mRNA were significantly lower in the untreated diabetic as compared to the lean rats (p<0.0001). Diabetic rats on pioglitazone had significantly higher ABCG5 and G8 expression than either of the other diabetic groups (p<0.0001). NPC1-L1 mRNA was significantly higher in all 3 diabetic groups as compared to the lean animals (p<0.0001). Pioglitazone-treated animals had significantly lower NPC1-L1 mRNA levels compared to the untreated and insulin-treated rats (p<0.0001).
Table 3.5
Hepatic mRNA gene expression

<table>
<thead>
<tr>
<th></th>
<th>ABCG5</th>
<th>ABCG8</th>
<th>NPC1L1</th>
<th>MTP</th>
<th>HMGCoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>zucker diabetic rats</td>
<td>12.97 ±</td>
<td>12.63 ±</td>
<td>13.51 ±</td>
<td>269.15 ±</td>
<td>142.95 ±</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>3.04</td>
<td>4.09</td>
<td>55.04</td>
<td>48.25</td>
</tr>
<tr>
<td>lean litter mates</td>
<td>75.74 ±</td>
<td>63.06 ±</td>
<td>1.96 ±</td>
<td>30.79 ±</td>
<td>37.38 ±</td>
</tr>
<tr>
<td></td>
<td>19.87</td>
<td>16.99</td>
<td>0.33</td>
<td>5.99</td>
<td>18.9</td>
</tr>
<tr>
<td>pioglitazone treated</td>
<td>72.84 ±</td>
<td>63.27 ±</td>
<td>8.11 ±</td>
<td>107.60 ±</td>
<td>157.78 ±</td>
</tr>
<tr>
<td></td>
<td>28.68</td>
<td>25.81</td>
<td>2.94</td>
<td>22.64</td>
<td>26.49</td>
</tr>
<tr>
<td>insulin treated</td>
<td>21.33 ±</td>
<td>20.15 ±</td>
<td>14.86 ±</td>
<td>195.51 ±</td>
<td>115.53 ±</td>
</tr>
<tr>
<td></td>
<td>5.71</td>
<td>4.07</td>
<td>3.31</td>
<td>33.53</td>
<td>21.51</td>
</tr>
</tbody>
</table>

In the lean animals there was no correlation between intestinal and hepatic NPC1-L1 mRNA levels but there was a significant correlation between chylomicron cholesterol and NPC1-L1 mRNA in the intestine \((r=0.81, p<0.01)\) and between VLDL cholesterol and NPC1-L1 mRNA in the liver \((r=0.83, p<0.001)\).

Figure 3.6
Relationship between Chylomicron Cholesterol and NPC1L1 mRNA Expression in lean animals

![Chylo Cholesterol v Intestinal NPC1L1 mRNA Expression](image-url)
3.6 Discussion

Reduction in the increased risk of atherosclerosis found in diabetes poses a major challenge. The reduction in atherosclerotic risk induced by statin therapy and from hypotensive therapy has been important but it is disappointing that there is so little evidence to confirm the benefit of improved glycaemic control in the prevention of atherosclerosis. It is for this reason that more and more attention is being paid to understanding the lipoprotein disturbance that occurs in the postprandial phase. In this context it may be relevant that subcutaneous insulin has important differences to the more physiological delivery of insulin into the peritoneum. Pro-insulin, which is converted to insulin in the liver, has been shown to alter triglyceride metabolism in a significantly different way to subcutaneous insulin (Winocour et al., 1991). The importance of insulin resistance rather than hyperglycaemia in mediating the dyslipidaemia of diabetes has recently been addressed by Annuzzi et al (2004) who showed that insulin resistance was a better predictor of postprandial lipoprotein abnormalities than hyperglycaemia. The
thiazolidinediones are now widely used in the treatment of type 2 diabetes and these PPARγ agonists improve insulin sensitivity. Pioglitazone, which also has PPAR alpha activity, has been shown to also decrease triglyceride levels and reduce liver fat (Galloway et al., 1992).

The present study was designed to explore the regulation of postprandial triglyceride-rich lipoproteins in diabetes and in particular to compare the effects of pioglitazone with insulin. The study shows that chylomicron triglyceride was very significantly improved with pioglitazone treatment compared to insulin and this improvement was unrelated to glycaemic control as there was no significant difference in blood glucose between insulin-treated and pioglitazone-treated animals. Chylomicron triglyceride levels were reduced by pioglitazone to a level indistinguishable from that of the lean littermates. Lewis et al (2005) examined the fructose-fed Syrian golden hamster and showed that rosiglitazone normalised chylomicron triglyceride production rates in this non-diabetic model of insulin resistance, in the fasting state but no reduction in the postprandial state.

This study, chylomicron cholesterol was also significantly reduced by insulin but a further two-fold reduction occurred with pioglitazone. However this was still almost 3-fold more than the chylomicron cholesterol for lean rats. Unlike humans, rats do not completely edit apo B100 in the intestine thus the chylomicron particle in the rat contains both apo B48 and B100 particles. The chylomicron fraction includes the largest particles produced both in the intestine and liver. Interestingly apo B48 was significantly lower in the rats on pioglitazone compared to those on insulin whereas treatment with both insulin and pioglitazone significantly reduced chylomicron apo B100 to a similar extent. Lewis et al (2005) showed a 50% reduction in the overproduction of apo B48 in villus enterocytes isolated from fructose-fed hamsters treated with rosiglitazone and a reduction in MTP mass and activity. The results for our VLDL fraction were very similar to those for the chylomicron fraction except that apo B100 was significantly lower in the diabetic rats on pioglitazone compared to untreated rats whereas insulin did not significantly affect apo B100. Apo B48 was significantly reduced to a similar extent by both treatments.
Changes in genes regulating cholesterol synthesis, absorption and assembly of chylomicrons in the intestine and VLDL in the liver were looked for in order to explain the findings, although recognising that the particles would also be influenced by catabolism once in the circulation. In the intestine HMCoA reductase expression was increased in the 3 diabetic groups suggesting an increase in cholesterol synthesis. It has been previously shown that there is an increase in HMCoA reductase activity and mRNA in the intestine of diabetic animals (O'Meara et al., 1991; O'Meara et al., 1990; Feingold et al., 1994). In the present study neither pioglitazone nor insulin significantly affected HMCoA reductase in the liver or intestine. Inhibition of NPC1-L1 has been shown to up-regulate HMCoA reductase (Altmann et al., 2004). It was found that there was a significant reduction in HMCoA reductase in the insulin-treated rats compared to pioglitazone-treated animals. This may reflects a reduction in cholesterol absorption in the pioglitazone group which had a markedly reduced NPC1-L1 in the liver.

MTP assembles the chylomicron particle in the intestine and VLDL in the liver (Wetterau et al., 1992). It has been previously shown that MTP expression is raised in the intestine of both Zucker obese insulin-resistant non-diabetic rats and streptozotocin diabetic rats (Gleeson et al., 1999; Phillips et al., 2002). It has also been shown that MTP expression is increased in human intestinal biopsies from Type 2 diabetic patients (Phillips et al., 2004). In the present study the increase in chylomicron cholesterol in the diabetic rats was associated not only with increased MTP expression but also with increased HMCoA reductase. The promoter region of the MTP gene carries a positive sterol response element and a negative insulin response element (Hagan et al., 1994; Lin et al., 1995) explaining the increase in MTP in the diabetic animals. In the intestine there was no significant difference in the reduction of MTP between the insulin and pioglitazone groups but in the liver insulin was much less effective than pioglitazone in reducing MTP mRNA.

The study also examined factors affecting cholesterol absorption. ABCG5 and G8 work in tandem to excrete cholesterol from the intestinal cells back into the lumen and from the liver into the bile (Berge et al., 2000). Intestinal ABCG5 was significantly lower in the
diabetic animals compared to lean animals. Treatment did not make a significant
difference although there was a suggestion of increase in the 2 treatment groups. In the
liver ABCG5/G8 expression were lowest in the untreated diabetic rats suggesting that the
dyslipidaemia of diabetes is at least in part due to a defect in the regulation of cholesterol
excretion from the liver into the bile.

Cholesterol absorption is also regulated by the NPC1-L1 protein. The diabetic animals
had significantly higher levels of NPC1-L1 mRNA and treatment did not appear to make
any difference. The significantly increased levels of NPC1-L1 in the liver is of interest
particularly since, although there was no correlation between hepatic and intestinal
NPC1-L1 levels, there was a strong positive correlation between chylomicron cholesterol
and NPC1-L1 mRNA in the intestine, and in the liver between NPC1-L1 mRNA and
VLDL cholesterol. Compounds with PPARδ activity significantly decrease cholesterol
absorption and NPC1-L1 levels in the intestine (van der Veen et al., 2005). It is not
thought that pioglitazone has PPARδ activity; however, this study suggests that it might
have some PPARδ activity since rats on pioglitazone showed a marked reduction in
NPC1-L1 compared to those on insulin. This study suggests that NPC1-L1 plays an
important part in regulating VLDL cholesterol from the liver. In view of the availability
of effective inhibitors of NPC1-L1 further studies need to be done to confirm the results
of the importance of hepatic NPC1-L1 in regulating VLDL composition.

Yu et al (2005) showed that mice transfected with human ABCG5 and G8 had a
significant increase in HMGCoA reductase expression in the liver in both male and
female animals and in the intestine in the female animals with increase in neutral sterol
excretion in the faeces. It appears therefore that diabetes disturbs the relationship between
diabetic rats, a model of type 1 diabetes, found reduced ABCG5 and G8 in the liver and
intestine and the doubling of the apparent calculated cholesterol absorption. Our increase
in chylomicron cholesterol in the diabetic animals would appear to be due to increased
intestinal synthesis and increased absorption and a reduction in cholesterol re-excretion.
Our study does not explain the intestinal function as being the cause of the difference
between chylomicron cholesterol on pioglitazone and insulin. In the liver however the
pioglitazone had a major effect on ABCG5 and G8 as compared to insulin. This perhaps
was the reason for the difference in the chylomicron cholesterol. One might explain the
significantly increased HMGCoA reductase mRNA in the liver compared to insulin-
treated and lean rats as being a response to the increased cholesterol excretion through the
ABCG5/8.

The VLDL fraction also demonstrated a significant reduction in cholesterol and
triglyceride on pioglitazone as compared to insulin with a significantly less apo B100.
These changes are likely to reflect the significant reduction in MTP mRNA in the liver in
the rats on pioglitazone as compared to those on insulin. The role of NPC1-L1 in the liver
may reflect reduced trafficking of cholesterol in the hepatocyte in rats treated with
pioglitazone as compared to those on insulin. Repa et al (2005) found that inhibition of
NPC1-L1 with ezetimibe was associated with increased hepatic cholesterol synthesis. In
this study the reduction in hepatic NPC1-L1 by pioglitazone as compared to insulin was
associated with the highest hepatic HMGCoA reductase mRNA expression, a level that
was significantly higher than that for the animals treated with insulin, whereas in the
intestine NPC1-L1 and HMGCoA reductase were similar in the diabetic animals treated
with pioglitazone or insulin. These results favour the liver as being an important target
for NPC1-L1 inhibition.

In conclusion the study shows marked changes in chylomicron and VLDL composition in
diabetic rats as compared to lean littermates. For a similar reduction in blood sugar
pioglitazone appears much more effective than insulin in normalising chylomicron and
VLDL composition. The study suggests that these changes are in part due to alteration in
the assembly of the lipoprotein particles by MTP and through increase in cholesterol
excretion by the ABCG5 and G8 proteins. NPC1-L1 which regulates cholesterol
absorption was increased in diabetes however there was no significant difference between
the insulin-treated and pioglitazone treated animals in the intestine. The significant
decrease in NPC1-L1 in the liver may play an important role in the changes in lipoprotein
particles, recognising that the chylomicron fraction contains large VLDL particles and the
VLDL fraction small chylomicron particles. The study suggests that it might be useful to explore the role of pioglitazone with insulin in the treatment of dyslipidaemia in Type 2 diabetes.
Chapter 4.

Genes that affect cholesterol synthesis, cholesterol absorption and chylomicron assembly. The relationship between the liver and intestine in control and streptozotosin diabetic rats.
4.1 Introduction

Alteration in cholesterol metabolism plays a major part in the development of atherosclerosis (Tailleux et al., 2005). Cholesterol absorption through the intestinal villi is controlled to some extent by the Niemann Pick C1-Like protein (Altmann et al., 2004). Cholesterol absorbed by the intestine may come from dietary cholesterol or biliary cholesterol as part of the enterohepatic circulation. Newly synthesised cholesterol in the intestine through HMGCoA reductase may also require the NPC1-L1 protein. NPC1-L1 is also found in the liver but its role has not been clearly defined (Altmann et al., 2004). Cholesterol absorption in type 2 diabetes has not been shown to be increased in human studies using surrogate markers whereas cholesterol synthesis is increased (Gylling et al., 1997). In streptozotocin diabetic rats, a model of type 1 diabetes, cholesterol absorption has been shown to be increased (Young et al., 1988) and in type 1 diabetic patients it has recently been shown that cholesterol absorption is increased with a reduction in markers of cholesterol synthesis (Miettinen et al., 2004). ATP binding cassette proteins, ABCG5 and G8, work in tandem to re-excrete some of the absorbed cholesterol and virtually all plant sterols back into the intestine from the intestinal villi and in the liver to excrete cholesterol into the bile (Graf et al., 2003). Streptozotocin induced diabetes in rats has been shown to affect intestinal cholesterol transporters with reduced hepatic and intestinal ABCG5 and G8 mRNA expression (Blocks et al., 2004). Insulin supplementation normalised these changes of mRNA expression of ABCG5 and G8 (Van Warde et al., 2002). Microsomal triglyceride transfer protein (MTP) is responsible for the assembly of the chylomicron particle in the intestine and the VLDL in the liver. Diabetes is a condition in which dyslipidaemia is a common finding resulting at least in part, in the increased burden of atherosclerosis. It has recently been demonstrated that an increase in MTP mRNA in both liver and intestine in diabetic rabbits. Lewis et al (2005) found an increase in MTP mRNA in isolated enterocytes from fructose fed hamsters a model of insulin resistance, which was associated with over-secretion of apo B48like proteins. In human studies it has been shown that there is an increase in MTP mRNA in the intestine of fasting human subjects (Phillips et al., 2005). The purpose of the present study was to explore the relationship between the expression of genes regulating intestinal cholesterol
absorption and excretion and to examine mRNA expression levels of several different proteins in the postprandial state in the streptozotocin diabetic rats. The effect of fasting on the mRNA expression of these proteins will also be investigated to ascertain if there is a difference between the fed and the fasting states. Postprandial lipoprotein profiles of all subjects will be conducted and results correlated with mRNA expression from both the liver and the intestine.

4.2 Animals

Sixty Sprague Dawley rats were examined in this study. These were split into 2 subsections: a chow diet group of 20 rats, named “Group A” and a group of rats fed a high fat diet, named “Group B”. Following two weeks of acclimatisation 10 rats from group A and 10 rats from group B were made diabetic with streptozotocin as described below. All animals from group A were fed a standard low fat, chow diet. All animals from group B were fed a high fat (40% fat), high cholesterol chow diet (manufactured by Harlan Teklad). Twenty percent of the fat was palmitic acid, 25% stearic acid, 29% oleic acid and 26% linoleic acid with 0.5% cholesterol. The duration of both diets was 2 weeks and at the end of this period all 60 of the rats were exsanguinated and chylomicrons and VLDL were isolated from the plasma. The liver was removed and samples stored in RNAlater and the intestine was washed, mucosa scraped and samples stored in RNAlater for MTP, HMGCoA Reductase, ABCG5 and G8 and NPC1-L1 mRNA determination as is described in detail in section 2.4.3.

4.3 Study Design

Group A: 10 diabetic Sprague Dawley rats (STZ induced) on a standard chow diet
  10 non-diabetic Sprague Dawley rats on a standard chow diet
Group B: 10 diabetic Sprague Dawley rats (STZ induced) on a high fat diet
  10 non-diabetic Sprague Dawley rats on a high fat diet
  10 non-diabetic Sprague Dawley rats on a high fat diet (fast for 6hrs)
  10 non-diabetic Sprague Dawley rats on a high fat diet (fast for 12hrs)
The 20 rats in group A were used as 2 extra control groups (a diabetic and a non-diabetic group) for group B, so that it could be seen whether it was the diabetes or it was the high fat diet (or both) that was driving changes in gene expression and the postprandial lipoprotein profile of the animals. To investigate the effect of feeding in group B on the intestinal and hepatic expression of Niemann Pick C1-like 1 and ABCG5/G8, HMG CoA reductase and MTP mRNA, the three groups of 10 non-diabetic Sprague Dawley Rats each were examined. Ten of the rats were sacrificed after a 12h fast (overnight), 10 after 6h fast (2-8pm) and 10 were sacrificed after ad lib feeding at 11am. The 20 rats from group A were sacrificed after ad lib feeding at 11am. The rats were exsanguinated and chylomicrons and VLDL isolated from the plasma. The liver was removed and samples stored in RNAlater and the intestine was washed, mucosa scraped and samples stored in RNAlater for MTP, HMGCoA Reductase, ABCG5 and G8 and NPC1-L1 mRNA determination as is described in detail in section 2.4.3. In order to examine the role of diabetes, on chylomicron and VLDL production diabetes was induced in ten of the rats by subcutaneous injection of streptozotocin (60mg/kg, in sodium citrate buffer, pH 4). The rats were allowed to stabilize for 24 hours. Diabetes was confirmed within 30 hours, with blood glucose > 20 mmol/L (2 rats did not survive). Diabetic control was monitored in tail vein blood samples. Food and water intake of each rat was monitored before and after the induction of diabetes, and each animal was weighed on a daily basis. Animals had confirmed diabetes for 10 days prior to experiment. Rats were sacrificed at the end of the feeding phase at 10am. The rats were exsanguinated and chylomicrons and VLDL isolated from the plasma. The liver was removed and samples stored in RNAlater and the intestine was washed, mucosa scraped and samples stored in RNAlater for NPC1-L1, ABCG5 and G8, NPC1-L1 and HMGCoA reductase mRNA determination as previously described.

4.4 Methods

Chylomicrons and VLDL were isolated from the plasma by sequential ultracentrifugation as described in section 2.4.4. Venous plasma glucose levels were determined according to
an enzymatic colorimetric method using a commercially available diagnostic kit (Boehringer Mannheim GmBH, Mannheim, Germany. Total cholesterol and triglyceride content of lipoprotein fractions were measured by an enzymatic colorimetric method using kits from Boehringer Mannheim GmBH (Mannheim, Germany). Chylomicron and VLDL apolipoprotein B48 and B100 were separated by SDS-PAGE and quantified by densitometry as described in section 2.4.6.

RNA was quantified using the Quant-iT Ribogreen RNA Assay Kit (Molecular Probes). An RNA standard curve was constructed using dilutions of a supplied, known amount of RNA, and read on a dual band plate reader with an excitation wavelength of 500nm and an emission wavelength of 525nm. Samples were calculated using linear regression from this standard curve.

RNA was then reverse transcribed as part of the 2-step real-time RT-PCR to cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). A GeneAmp 2400 PCR System (Applied Biosystems) was used and the conditions were: 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. Eight hundred ng of each sample in 100μl was reverse transcribed to cDNA, with 80ng of the cDNA to be used in the next step.

10μl of the cDNA containing 80ng was used in the real-time PCR analysis. The rat-specific primers and probes for the genes of interest were purchased from Applied Biosystems using the Assay On Demand system. Thermal cycling conditions and volumes of ingredients are already optimised using this system. The thermal cycling conditions used were: stage 1: 50°C for 2 min, stage 2: 95°C for 10 min, stage 3: 40 repetitions of 95°C for 15 sec alternating with 60°C for 1 min. A sample volume of 25μl was used in each well. Primers and probes were used from a 20X stock solution and the final concentration was 1X. A 2X master mix solution was added to each well at a final concentration of 1X. 2μ cDNA containing 80ng was added separately into each well and a standard deviation on ct value of <0.3 was accepted.
GAPDH was used as the housekeeping gene and all reactions were done as single-plex reactions on a 96-well plate. A TaqMan Universal PCR Master Mix was used (Applied Biosystems) as the mix for the rest of the PCR ingredients. Analysis was run on an ABI-prism 7000 and the $2^{-\Delta\Delta CT}$ method of relative gene expression was employed. Results were expressed as arbitrary units in comparison to GAPDH.

4.5 Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test, and the software used was SSPS. Analysis of Variance (ANOVA) was employed for statistical analysis between the different groups in the study. Non-parametric tests were used for triglyceride analysis. Correlation coefficients were measured by linear regression analysis. Data are expressed as the mean ± standard deviation and mean ± of the mean (SEM) and a p value of <0.05 was regarded as statistically significant.

4.6 Results

The difference in mRNA expression and lipoprotein profile between the fed and fasting state:

There was no difference in the weight of the 3 groups of control rats. The time course for chylomicron and VLDL for the rats is shown in Table 4.1. There was a significant reduction in chylomicron and VLDL triglyceride, cholesterol, apo B48, and apo B100 (p <0.02) after the 12h fast.
Table 4.1.
Time course for lipoproteins in fat-fed animals

<table>
<thead>
<tr>
<th></th>
<th>Chylomicron (mg/ml plasma)</th>
<th>VLDL (mg/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No fast 6 hr fast 12 hr fast</td>
<td>No fast 6 hr fast 12 hr fast</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>10 9 10</td>
<td>10 9 10</td>
</tr>
<tr>
<td><strong>Triglyceride</strong></td>
<td>91±30 78±35 53±24 **</td>
<td>99±43 90±35 62±12 *</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>45±12 42±8 29±8 **</td>
<td>58±27 52±27 30±9 **</td>
</tr>
<tr>
<td><strong>Apo B48</strong></td>
<td>19±9 17±6 7±2 ***</td>
<td>9±5 9±4 4±2 **</td>
</tr>
<tr>
<td><strong>Apo B100</strong></td>
<td>17±8 19±8 6±3 ***</td>
<td>27±11 24±6 14±4 **</td>
</tr>
</tbody>
</table>

Mean ± S.D. *p<0.02, **p<0.01, ***p<0.001

Table 4.2 shows the time course for the intestinal and liver mRNA.

Table 4.2.
Effect of fasting on hepatic and intestinal mRNA expression

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No fast 6 hr fast 12 hr fast</td>
<td>No fast 6 hr fast 12 hr fast</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>10 9 10</td>
<td>10 9 10</td>
</tr>
<tr>
<td>HMGCoA Reductase</td>
<td>45±27 51±24 46±23 95±26 76±12 80±13</td>
<td></td>
</tr>
<tr>
<td>MTP</td>
<td>47±40 53±27 48±27 78±12 61±10§** 41±9**</td>
<td></td>
</tr>
<tr>
<td>ABCG5</td>
<td>84±35 82±41 106±58 72±28 77±22 72±22</td>
<td></td>
</tr>
<tr>
<td>ABCG8</td>
<td>4±4 3.5±2 3.4±1.6 66±34 70±14 64±21</td>
<td></td>
</tr>
<tr>
<td>NPC1L1</td>
<td>51±68 104±64 36±43§§ 15±5 15±5§* 11±3*</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.001 different from fasting  §p<0.05  §§p<0.02 different from 6hr
There was no significant change from the fed state for intestinal HMGCoA reductase MTP, NCP1-L1 or ABCG5/G8 mRNA after 12h fasting. NPC1-L1 and ABCG8 both rose at 6h however, this rise was significant for NPC1-L1 (p<0.02). There was no change in liver HMGCoA reductase over the 12h period. Hepatic MTP fell significantly in step-wise fashion (p<0.05, and P<0.001). NPC1-L1 and ABCG5/G8 were similar fed and at 12h but NPC1-L1 increased significantly at 6h (p<0.05).

Effects of diabetes and diet on mRNA expression and lipoprotein profiles on the animals in the fed state

As can be seen from Table 4.3, the diabetic rats on the high fat diet had a mean blood glucose of 32±4mmol/l compared to 5.3±1.2mmol/l for control rats and they were significantly lighter than control animals (260±41 vs 428±30g). Triglyceride, cholesterol apo B48 and apo B100 in both the chylomicron and VLDL fractions were very significantly higher in the diabetic rats. Intestinal and hepatic HMGCoA reductase were increased in the diabetic rats compared to controls but the increase was significant only in the liver (p<0.01). MTP was significantly increased in both the intestine and the liver P<0.005. NPC1-L1 was also increased significantly in both intestine and liver while ABCG/G8 were reduced in both intestine and liver (p<0.05) – see Table 4.4.

Table 4.3.
Effect of diabetes and diet on lipoprotein composition

<table>
<thead>
<tr>
<th></th>
<th>Chylomicron (mg/ml plasma)</th>
<th>VLDL (mg/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>91±30</td>
<td>496±206***</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>45±12</td>
<td>357±141***</td>
</tr>
<tr>
<td>Apo B-48</td>
<td>19±9</td>
<td>208±74***</td>
</tr>
<tr>
<td>Apo B-100</td>
<td>17±8</td>
<td>158±43***</td>
</tr>
</tbody>
</table>

*** p<0.0001 different from control
Table 4.4.
Effect of diabetes on intestinal and hepatic mRNA expression

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>HMGC0A Reductase</td>
<td>45±27</td>
<td>67±23</td>
</tr>
<tr>
<td>MTP</td>
<td>47±40</td>
<td>266±156***</td>
</tr>
<tr>
<td>ABCG5</td>
<td>84±35</td>
<td>20±19***</td>
</tr>
<tr>
<td>ABCG8</td>
<td>4±4</td>
<td>0.9±1.4*</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>51±68</td>
<td>231±181*</td>
</tr>
</tbody>
</table>

Mean ± S.D. * p<0.05 ** p<0.01, *** p <0.0005 different from fed control rats

Figure 4.1
Intestinal mRNA expression levels of NPC1L1, MTP and HMGC0A reductase in rats on a high fat diet
Relationship between liver and intestinal mRNA expression of ABCG5/G8, HMGCoA reductase, MTP and NPC1-L1

As expected, there was a strong positive correlation between ABCG5 and G8 in both the intestine (r=0.75, p<0.001) and liver (r=0.92, p<0.0001), as can be seen in figures 4.5 and 4.6. There was a significant positive correlation between intestinal and Hepatic ABCG5 (r=0.54, p<0.05) and ABCG8 (r=0.68, p<0.001). There was no statistically significant correlation between intestinal and hepatic HMGCoA reductase, MTP or NPC1-L1.

Figure 4.5
Hepatic ABCG5 v ABCG8 mRNA expression in rats on a high fat diet
Correlation between apolipoprotein lipid content and intestinal and hepatic mRNA expression of the genes measured

There was a strong correlation between intestinal HMGCoA reductase and chylomicron cholesterol when both groups were analysed together but the significance was lost when the groups were analysed separately. There was no correlation between hepatic HMGCoA reductase and VLDL cholesterol. Intestinal MTP mRNA strongly correlated with chylomicron triglyceride, chylomicron cholesterol, chylomicron apo B48 and apo B100 (p<0.001). The significance remained when the diabetic and control groups were analysed separately (p<0.05). There was a significant correlation between hepatic MTP mRNA expression and VLDL triglyceride, cholesterol apo B48 and apo B100 (p<0.001). The significance remained when each group was analysed separately. There was a strong positive correlation between intestinal NCP1-L1 mRNA expression and chylomicron cholesterol (p<0.001). This correlation
remained when the fed and fasting groups were analysed separately – see figures 4.7 and 4.8.

**Figure 4.7**
Intestinal NPC1L1 v MTP mRNA expression in rats on a high fat diet
Figure 4.8
Chylomicron Cholesterol v Intestinal NPC1L1 mRNA expression in rats on high fat diet

Figure 4.9
VLDL Cholesterol v Hepatic NPC1L1 mRNA expression in rats on high fat diet
4.7 Discussion

The present study was designed to investigate the effect of feeding on the intestinal and hepatic expression of Niemann Pick C1-like 1 and ABCG5/G8, HMG CoA reductase. The study shows that a high fat or western diet very significantly increases the cholesterol and triglyceride content of both the chylomicron and VLDL particles in the diabetic and non-diabetic animals. These confirm the finding of the studies where dietary cholesterol has been shown to alter the postprandial lipoprotein response in both Type 2 diabetic (Taggart et al, 1997) and non-diabetic subjects (Dubois et al., 1994) by producing cholesterol-enriched chylomicrons, which may be potentially pro-atherogenic particles. There is clear evidence from animal studies to show that meals rich in polyunsaturated fatty acids (PUFA's) result in an attenuated postprandial triglyceride response compared with meals rich in saturated fatty acids (SFA's) (Groot et al., 1988). The high fat diet was rich in SFA's and this was reflected in the higher triglyceride levels in the both the diabetic and non-diabetic subjects compared with the animals on the low fat diet.

There were highly elevated blood sugar levels in the diabetic subjects, and those diabetic subjects on the low fat diet had an increase in the chylomicron fraction of Apo B-48 and Apo B-100 and the VLDL fraction of Apo B-48 and Apo B-100. This reflects a higher production of these lipoprotein particles in the diabetic animals. This postprandial response was exaggerated in the animals on the high fat diet, showing just how important a balanced and low-fat diet is in diabetes. Whether it was the presence of the excess sugar or the absence/reduction of insulin in these animals that resulted in this hyper-production of postprandial lipoproteins is unclear.

The genetic response of these animals was again very interesting. There was an increase in the expression of NPC1L1 mRNA in both sets of diabetic animals in the liver and the intestine, although the greatest expression was in the intestine. There was also an increase in the mRNA expression of MTP and HMGCoA and a decrease in the expression of ABCG5 and ABCG8 in the diabetic animals when compared with the controls, in both the chow diet and the high fat diet.
The mRNA expression of these proteins was exaggerated in the animals on the high fat diet. There was a strong positive correlation between the expression of MTP mRNA and the chylomicron cholesterol levels. There was also a strong positive correlation between the expression of NPC1L1 mRNA and MTP mRNA. This would indicate that in the diabetic condition where it was found that the expression of these proteins is increased, the excess cholesterol being absorbed by the NPC1L1 protein may provide the impetus for the increased expression of MTP, leading to an increase in the number of chylomicron particles produced. The source of this cholesterol may be a reabsorption of biliary cholesterol and possibly of de novo synthesised cholesterol in the intestine itself as previous studies have shown that the absorption of dietary cholesterol is not increased in diabetes (Phillips et al., 2002; Glesson et al., 2000; Gylling et al., 1997).

Of course this animal model is more representative of type 1 diabetes, and there is comparatively little information available on cholesterol absorption and synthesis in this type of diabetes. There is generally less advanced dyslipidaemia associated with type 1 diabetes (Winocour et al., 1986), but obliterating arterial disease often develops, and myocardial infarction is also an important cause of death in these patients (Roper et al., 2002). The relatively normal lipid pattern might be the reason why the metabolism of cholesterol is less studied in patients with type 1 than type 2 diabetes. In streptozotocin-induced diabetes in experimental animals, cholesterol absorption is elevated and synthesis down-regulated, but these alterations can result not only from lack of insulin but also from gut hypertrophy that is present in these animals (Young et al., 1988). It was recently reported that cholesterol absorption is higher in type 1 than in type 2 diabetes (Miettinen et al., 2004). Perhaps this accounts for the much elevated expression of NPC1L1 found in this study and the strong positive correlation it has with the largely increased chylomicron cholesterol content. It is also known that increased amounts of dietary cholesterol increase LDL cholesterol more effectively in type 1 diabetic than in matched control subjects (Romano et al., 1998). However, reduction of dietary cholesterol with modification of fat intake has been observed (Kaufman et al., 1975) to effectively reduce...
serum cholesterol in type 1 diabetic subjects. In this study, it seems that chylomicon and VLDL cholesterol content is reduced on a low fat diet, in line with the study of Kaufman.

This study found that the expression of ABCG5 and G8 was reduced significantly in diabetes, both in the liver and the intestine, with dramatically lower levels in the intestine. These two proteins work in tandem to excrete almost 100% of the plant sterols that are ingested in the diet (Berge et al., 2000). They also excrete some cholesterol back out into the lumen of the intestine (Berge et al., 2000). In transgenic mice or in over-expression of the two genes in mice, serum plant sterol levels and sterol absorption are reduced and cholesterol synthesis and biliary and faecal secretion of cholesterol are increased (Yu et al., 2002). But on the other hand, knockout of ABC G5 and G8 genes in mice changes the serum sterol pattern (Yu et al., 2002) to resemble that seen in hereditary sitosterolemia (Bhattacharyya et al., 1974), a metabolic disease caused by mutations of the ABC G5 and G8 genes (Berge et al., 2000; Lee et al., 2001). Kojima et al., (1992) measured serum plant sterols, campesterol and sitosterol, in these streptozotocin-induced diabetes animals, and they were increased, and this group also found elevated serum plant sterols in a group of poorly controlled patients with type 1 diabetes, and the levels were reduced during intensified insulin treatment (Kojima et al., 1999). In a recent study by Gylling et al (2004) in cholesterol metabolism in type 1 diabetes, they found increased ratios of sterol markers for cholesterol absorption and decreased ratios of sterol markers for cholesterol synthesis in type 1 diabetics compared with control subjects. According to the author, this can be interpreted to indicate high absorption and low synthesis of cholesterol in patients with type 1 diabetes compared with non-diabetic control subjects (Gylling et al., 2004). They also state that their findings showed that the difference between the two groups in free, esterified, and total non-cholesterol sterols was also present in different lipoproteins and that the sterol ratios in serum were only modestly different from those in different lipoproteins (Gylling et al., 2004). As the mRNA expression of these proteins has been shown to be consistently reduced in diabetes in these studies, it is likely that these proteins have an important role to play in the dyslipidaemia of diabetes. Perhaps they play a larger role in type 1 diabetes, where the conventional lipid profile is often normal, but the detection of the absorption of plant sterols is not carried out. If the plant sterols
are so destructive in the condition of sitosterolemia, where cholesterol levels are often only slightly raised (Bhattacharyya et al., 1974) and the onset of atherosclerosis is premature and very severe, perhaps these plant sterols have a malevolent role to play in diabetes.

The other aspect of this study was to investigate the affect of fasting on the mRNA levels of expression of the genes involved in cholesterol metabolism. It was seen that they did not vary significantly in general from the non-fasting state to the 6-hour and 12-hour fasts. Naturally, the lipoprotein levels did fall following these periods of fasting, but it was interesting to note that the mRNA expression levels of NPC1L1, MTP, HMGCoA and ABCG5 and G8 remained unaltered. This may be due to the fact that the body is constantly ready for the intake of food. The NPC1L1 mRNA levels did increase after 6 hours of fasting but they were reduced again to a non-significant difference between the ad lib feeding after a 12-hour fast.

As a result, it is possible to deduce that undergoing an overnight fast before the duodenal biopsy tissue is taken, is unlikely to effect the mRNA expression of the proteins involved in cholesterol homeostasis.

The results of this study will hopefully shed some more light on the dysregulation in lipid metabolism and the significant role that the intestine has to play in the synthesis of the atherogenic chylomicron particle and altered cholesterol metabolism in diabetes.
Chapter 5.

Messenger RNA levels of genes involved in dysregulation of postprandial lipoproteins in type 2 diabetes: the role of Niemann-Pick C1-like 1, ATP-binding cassette, transporters G5 and G8, and of microsomal triglyceride transfer protein.
Chapter 5: Human Studies

Introduction:

Diabetes is responsible for a large increase in cardiovascular risk which in many studies has not been reduced by improvement in blood sugar. The postprandial period in diabetes is associated with considerable disturbance in fatty acid and triglyceride metabolism (Geluk et al., 2004) and this may be an important factor in atherosclerosis development (van Wijk et al., 2005). In particular the postprandial period in diabetes is associated with increased apolipoprotein B48 the solubilising protein for transport of intestinal cholesterol and triglyceride in the blood stream (Curtin et al., 1994). These apo B48-containing particles may be particularly atherogenic (Karpe et al., 1994; Mero et al., 2000). Reduced clearance of these apo B48-containing particles plays a part in the raised levels of cholesterol (Phillips et al., 2002; Dane-Stewart et al., 2003) but we have also demonstrated increased cholesterol synthesis in the intestine in animal models of diabetes (O Meara et al., 1991) and in human studies have suggested that the early rise in apo B48 is probably due to increased particle synthesis rather than delayed clearance (Curtin et al., 1994; Phillips et al., 2002).

Microsomal triglyceride transfer protein (MTP) is responsible for the assembly of cholesterol, triglyceride and phospholipids together with one apo B48 molecule to form to the chylomicron particle in the intestine and one apo B100 molecule to form VLDL in the liver (Hussain, 2000; Hussain et al., 2005). The cholesterol in the chylomicron particle may either be absorbed from the diet, absorbed from recycled biliary cholesterol or derived from de novo cholesterol in the intestine. It has been suggested that 25% of newly synthesised cholesterol is derived from the intestine. In diabetes cholesterol synthesis is increased as evidenced by the increase in HMGCoA reductase, the rate-limiting enzyme for cholesterol synthesis (O Meara et al., 1991; Feingold et al 1994). The ATP binding cassette proteins (ABC) G5 and G8 regulate cholesterol absorption from the intestine by their ability to work in tandem to excrete cholesterol and plant sterols from the enterocyte back into the intestinal lumen (Lee et al., 2001). Impairment
in function is associated with increased cholesterol and plant sterol absorption. Genetic mutations in the ABC genes have been described which result in early severe atherosclerosis (Berge et al., 2000). The Niemann Pick C1-like 1 protein (NPC1-L1) and gene have recently been described following the discovery of the drug ezetimibe, a drug which has a cholesterol lowering effect due to reduction of cholesterol absorption. It works by inhibiting Niemann PickC1-like1 protein (Altman et al., 2004). Thus this protein appears to play an important role in regulating intestinal cholesterol absorption (Davis et al., 2004). The 3-hydroxy-3methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (statins) lower cholesterol and triglycerides. They reduce postprandial chylomicrons in the blood and have been shown in diabetic and non-diabetic patients to make an important contribution to reducing the risk of myocardial infarction (Schaefer et al., 2004). Inhibition of cholesterol synthesis, results in up-regulation of the LDL receptor thus improving clearance not only of the LDL particle but also of the chylomicron and VLDL particles (Van Wijk et al., 2005; Dane-Stewart et al., 2003). These inhibitors also play a part in regulating chylomicron synthesis and, in preliminary studies, it has been shown that there is lower intestinal MTP expression both in diabetic and non-diabetic subjects in patients taking statin therapy for hypercholesterolemia (Mullan et al., 2003).

The relationship between MTP, ABCG5, ABCG8 and NPC1-L1 and chylomicron particle composition has not been examined but is of considerable interest in view of drugs which are already on the market or under investigation which affect the above proteins. This is particularly the case in diabetes in view of the compositional alteration in the chylomicron particle and its probable importance in the accelerated atherosclerosis which occurs in diabetes.

The aims of the present study were to examine the relationship between chylomicron composition, MTP, ABCG5, ABCG8 and Niemann Pick C1-Like 1 in diabetic and non-diabetic patients. A secondary aim was to examine the effect of statin therapy on intestinal expression of the above proteins.
5.2. Subjects

Subjects undergoing routine gastroscopy and biopsy for non-malignant disease were asked to donate 4 extra biopsies (each approximately 10mg) of the second part of duodenum (D2) for MTP, ABCG5 and G8 and Niemann Pick C1-like 1 mRNA determination. Fifteen type 2 diabetic subjects and 17 non-diabetic subjects 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. Eleven type 2 diabetic subjects and 4 non-diabetic subjects with normal D2 biopsies who were treated with statins for hypercholesterolemia were also examined. Subjects had no evidence of coeliac, thyroid, renal or hepatic disease. The study was conducted according to the principles outlined in the Declaration of Helsinki. Hospital ethics committee approval was obtained and all subjects gave informed consent.

5.3. Intestinal Biopsy Sample Collection

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. Three of the non-diabetic subjects had no abnormality on gastroscopy, 8 had mild gastritis, 6 had mild oesophagitis and 3 mild gastritis and oesophagitis. In the group of 4 non-diabetic subjects on statin 2 had no abnormality, 1 had mild oesophagitis and 1 had mild gastritis. Ten of the diabetic patients not on statin had mild oesophagitis and mild gastritis. Two had no abnormality and 2 had oesophagitis and one had gastritis only. Of the 11 diabetic patients on statin therapy 4 had no abnormality, 3 had gastritis and oesophagitis, one had gastritis only and 3 had oesophagitis. Four separate biopsy samples were collected in RNAlater and stored frozen at -70°C for MTP mRNA determination.
5.4. Study Design

Within 1 week of the biopsy collection patients were invited to attend fasting for a test meal. Seventeen diabetic patients and 12 controls agreed to attend for the test meal. Blood (40mls) was collected fasting 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 125 ml ice cream, and a cup of tea or coffee. Blood samples were repeated 4 and 6h after the meal. 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%).

5.5. Methods

Lipoproteins were isolated as described in section 2.2.7 and chylomicron and VLDL apoB48 and apo B100 were determined as detailed in section 2.2.9. Biochemical analysis of lipoproteins was carried out using commercially available kits as described in section 2.2.8. Serum was analysed as per section 2.2.4. Sections 2.3.1 – 2.3.5 explain the process of how the RNA was extracted from the biopsy samples and quantified. The RNA was then reverse transcribed and real-time PCR was carried out on the newly synthesised cDNA as described in section 2.3.5.

5.6. Statistical Analysis

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

Subject’s characteristics are shown in table 5.1. There were 15 diabetic and 17 non-diabetic control subjects who were not taking statin therapy. Eleven diabetic and 4 control subjects were taking statin for hypercholesterolemia. The ages of the 4 groups were similar but the BMI was significantly higher in the diabetic patients (both statin-treated and non-statin-treated). The diabetic patients were well controlled with HbA1 of 6.6±1.1% (normal value <5.8%) v 5.3±0.3% for control subjects. There were more females in the control groups than in the diabetic groups.

Table 5.1
Subjects Characteristics, including statin status, HbA1C, Age and BMI.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Subjects</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>61.67 ± 10.85</td>
<td>64.86 ± 13.25</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.03 ± 7.98</td>
<td>165.99 ± 6.51</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>85.19 ± 13.67</td>
<td>67.11 ± 9.13</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>6.6 ± 1.1</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>BMI</td>
<td>30.07 ± 3.26 *</td>
<td>24.27 ± 2.64</td>
</tr>
<tr>
<td>Number on a Statin</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Number not on a Statin</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

*p<0.01 compared to control subjects

MTP, ABCG5, ABCG8, and NPC1-L1 in statin-treated and un-treated diabetic and control subjects are shown in Table 5.2. Looking at the non-statin treated subjects, diabetic patients had a significant increase in intestinal MTP mRNA compared to control subjects (8.76±5.65 v 4.87±3.24 p<0.02) (fig 5.1). ABCG5 and G8 were both significantly lower in diabetic patients not on statin compared to controls (0.12±0.07 v 0.17±0.08 p<0.04 and 0.05±0.02 v 0.1±0.07 p<0.05) (fig 5.2). There was a close
correlation between ABCG5 and G8 in both the diabetic and non-diabetic patients (r=0.84 and r=0.83 p<0.001 respectively). There was a strong correlation between the expression of ABCG5 and ABCG8 intestine mRNA in the diabetic and control subjects combined, not on statin treatment (r=0.73, p<0.02) (fig 5.14) Niemann PickC1-Like 1 was significantly higher in diabetic patients not on statin compared to control subjects (2.47±1.42 vs 1.39±1.78 p<0.02) (fig 5.3).

Table 5.2

<table>
<thead>
<tr>
<th></th>
<th>Intestinal mRNA Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTP</td>
</tr>
<tr>
<td>Diabetics on statin</td>
<td></td>
</tr>
<tr>
<td>4.80 ± 2.47</td>
<td>0.21 ± 0.12 ^</td>
</tr>
<tr>
<td>Diabetics no statin</td>
<td>8.76 ± 2.59 *</td>
</tr>
<tr>
<td>Controls on statin</td>
<td></td>
</tr>
<tr>
<td>4.87 ± 4.90</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>Controls no statin</td>
<td></td>
</tr>
<tr>
<td>5.04 ± 1.65</td>
<td>0.24 ± 0.08</td>
</tr>
</tbody>
</table>

* p<0.02, ** p<0.05 for difference between diabetic no statin and control no statin
^ p<0.05, ^^ p<0.02 for difference between diabetic on statin and control on statin
**Figure 5.1**
Intestinal MTP mRNA expression between diabetic and control subjects not on statin intervention.

![Intestinal MTP mRNA - no statin treatment](image)

**Figure 5.2**
Intestinal ABCG5 and G8 mRNA expression between diabetic and control subjects not on statin intervention.

![Intestinal ABCG5 and G8 mRNA - no statin treatment](image)
There were 11 statin-treated diabetic patients. These patients had significantly less MTP mRNA than those not on statin (4.8±2.47 v 8.76±5.65 p<0.05) (fig 5.4). ABCG5 was almost double in the diabetic patients on statin (0.21±0.12 v 0.12±0.07 p<0.02) and there was also a significant increase in ABCG8 (0.07±0.03 v 0.05±0.02 p<0.05). There was a 40% reduction in NCP1-L1 in diabetic patients on statin but this did not reach statistical significance (1.49±0.71 v 2.47±1.49 p<0.06). There were only 4 statin-treated control patients and there were no statistically significant differences in the mRNA of the intestinal regulatory proteins studied compared to those not on statin.
Combining diabetic and non-diabetic subjects who were not on statin treatment there was a weak negative correlation between MTP and ABCG5 (r=-0.40, p<0.05). Analysing the two groups separately in the diabetic patients there was a negative correlation between MTP and ABCG5 (r=-0.53, p<0.05) whereas in the non-diabetic subjects the correlation did not reach statistical significance. Between MTP and ABCG8, there was a weak negative correlation in the combined diabetic and control patients (r = -0.37, p<0.05) but it didn’t reach statistical significance in the diabetic patients or control subjects. There was no correlation between MTP and ABCG5 or G8 in the diabetic patients on statin.

There was a strong positive correlation between MTP and NPC1-L1 in the combined group of diabetic patients and control patients not on statin (r=0.78, p<0.001). In the diabetic patients alone the correlation was r=0.79, p<0.001 (fig 5.6) and in the control subjects r=0.85, p<0.001 (fig 5.7). In neither the diabetic nor control patients on statin
was there a correlation between MTP and NPC1-L1. There was a negative correlation between ABCG5 and NPC1-L1 \((r=-0.60, p<0.001)\) in the combined diabetic and control patients not on statin and a similar correlation between ABCG8 and NPC1-L1 in the combined group \((r=-0.52, p<0.001)\). In the diabetic patients alone, the correlation between ABCG5 and NPC1-L1 was also significant \((r=-0.52, p<0.05)\) but the correlation with ABCG8 did not reach statistical significance. There was no correlation between ABCG5 or G8 and NPC1-L1 in the control group alone. In the diabetic patients on statin there was no correlation between either ABCG5 or G8 and Niemann Pick C1-L1.

Figure 5.6

Intestinal mRNA expression in diabetic subjects not a statin, MTP v NPC1L1
Chylomicron and VLDL composition for the diabetic and control patients who agreed to a meal is given in table 5.3. The diabetic patients not on statin had significantly more apo B48 in the postprandial chylomicron fraction compared to control subjects not on statin (p<0.05). There was a 60% increase in chylomicron cholesterol at 4h (ns), but at 6h levels were similar. There was a significant increase in apo B100 in the chylomicron fraction in the diabetic patients at both 4 and 6h (p<0.05). Chylomicron triglyceride was significantly greater at 4h (p<0.05) and was 20% greater at 6h though this did not reach statistical significance.

Diabetic patients not on statins had significantly more apo B100 than the statin-free control subjects postprandially but apo B48 was not significantly different. In the diabetic patients postprandial apo B100 was significantly less in those patients on statin.
<table>
<thead>
<tr>
<th></th>
<th>NO STATIN</th>
<th>STATIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting 4h 6h</td>
<td>Fasting 4h 6h</td>
</tr>
<tr>
<td>Diabetic</td>
<td>N = 15</td>
<td>N = 11</td>
</tr>
<tr>
<td>ApoB48 (ug/ml plasma)</td>
<td>3.9 ±2.8 21 ±15 *^</td>
<td>16±10</td>
</tr>
<tr>
<td></td>
<td>3.5±2.4</td>
<td>17±9</td>
</tr>
<tr>
<td></td>
<td>8±6</td>
<td></td>
</tr>
<tr>
<td>ApoB100 (ug/ml plasma)</td>
<td>1.5±1.3 7.3±6.6 *^</td>
<td>5.1±4.6 *^</td>
</tr>
<tr>
<td></td>
<td>1.0±1.1</td>
<td>5.4±4.4</td>
</tr>
<tr>
<td></td>
<td>4.2±4.1</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/ml plasma)</td>
<td>22±17 99±53 ^</td>
<td>54±21</td>
</tr>
<tr>
<td></td>
<td>25±5</td>
<td>69±21</td>
</tr>
<tr>
<td></td>
<td>52±10</td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/ml plasma)</td>
<td>59±26 264±110 *^</td>
<td>169±107</td>
</tr>
<tr>
<td></td>
<td>54±17</td>
<td>163±60</td>
</tr>
<tr>
<td></td>
<td>135±66</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N = 4</td>
<td>N = 17</td>
</tr>
<tr>
<td>ApoB48 (ug/ml plasma)</td>
<td>1.7±1.3 6.3±5.3</td>
<td>9.2±5.3</td>
</tr>
<tr>
<td></td>
<td>1.3±0.4</td>
<td>10±7</td>
</tr>
<tr>
<td></td>
<td>7.5±6.5</td>
<td></td>
</tr>
<tr>
<td>ApoB100 (ug/ml plasma)</td>
<td>0.6±0.6 2.7±1.7</td>
<td>2.0±1.1</td>
</tr>
<tr>
<td></td>
<td>0.3±0.2</td>
<td>3.0±1.7</td>
</tr>
<tr>
<td></td>
<td>1.1±0.6</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/ml plasma)</td>
<td>21±15 64±35</td>
<td>54±27</td>
</tr>
<tr>
<td></td>
<td>29±8</td>
<td>69±16</td>
</tr>
<tr>
<td></td>
<td>49±19</td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/ml plasma)</td>
<td>34±14 140±124</td>
<td>119±91</td>
</tr>
<tr>
<td></td>
<td>60±19</td>
<td>196±90</td>
</tr>
<tr>
<td></td>
<td>138±66</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD, *p<0.05 diabetic v control, ^p<0.05 diabetic statin v no statin.
There was a significant positive correlation in the combined diabetic and non-diabetic
patients not on statin treatment between MTP mRNA and postprandial chylomicron
cholesterol at 4h (r=0.83, p<0.001) (fig 5.8) and 6h (r=0.78, p<0.01) (fig 5.9) and apo
B48 at 4h (0.83, r=0.001) (fig 5.10) and 6h (r=0.63 p<0.01) (fig 5.11). There was also a
positive correlation between MTP and chylomicron apo B100 at 4h (r=0.79, p<0.001)
and 6h (r=0.6, p<0.01) and between MTP and chylomicron triglyceride (r=0.75, p<0.001)
for both 4 and 6h.

**Figure 5.8**
Diabetic and Control subjects combined showing relationship between intestinal MTP
mRNA expression and Chylomicron Cholesterol, (t=4), with no statin intervention.
Figure 5.9
Diabetic and Control subjects combined showing relationship between intestinal MTP mRNA expression and Chylomicron Cholesterol, (t=6), with no statin intervention.

Correlations between chylomicron composition and intestinal mRNA for the combined diabetic and control patients not on statin showed that there was a significant negative correlation between chylomicron cholesterol and ABCG5 at 4h ($r=-0.45$, $p<0.05$) (fig 5.12) and at 6h ($r=0.60$, $p<0.01$) (fig 5.13) and ABCG8 at 4h ($r=-0.46$, $p<0.05$), but the correlation at 6h did not reach significance. There was a positive correlation between chylomicron cholesterol and NPC1-L1 at 4h ($r=0.69$, 0.01) and at 6h ($r=0.60$, $p<0.01$).
Figure 5.12
Diabetic and Control subjects combined showing relationship between intestinal ABC G5 mRNA expression and Chylomicron Cholesterol, (t=4), with no statin intervention.

![Intestinal ABCG5 v Chylo Chol (t=4), diabetics and controls, no statin](image)

$R = -0.45$
$p<0.05$

Figure 5.13
Diabetic and Control subjects combined showing relationship between intestinal ABC G5 mRNA expression and Chylomicron Cholesterol, (t=6), with no statin intervention.

![Intestinal ABCG5 v Chylo Chol (t=6), diabetics and controls, no statin](image)

$R = -0.60$
$p<0.01$
There was a significant positive correlation between postprandial chylomicron cholesterol and triglyceride at 4h \((r=0.72, p<0.001)\) and 6h \((r=0.74, p<0.001)\).

The only significant correlations in the statin treated combined group were MTP and triglyceride at both 4 and 6h \((r=0.71\) and \(r=0.64, p<0.01)\), and cholesterol at 6h \((r=0.68, p<0.01)\) but not at 4h. There was also a positive correlation between chylomicron cholesterol and NPC1-L1 at 6h.

Combining the diabetic and control subjects not on statins there was a significant correlation between MTP and both apo B48 and apo B100 at 4 and 6h in the VLDL fraction \((p<0.001)\). There was also a significant positive relationship between MTP and VLDL cholesterol at 4 and 6h postprandially \((p<0.01)\). As in the chylomicron, there was a significant correlation between ABCG5 and postprandial VLDL cholesterol \((p<0.01)\). The correlations between NPC1-L1 and VLDL was similar to that found in the chylomicron fraction with significant positive correlations to cholesterol at 4 and 6h \((p<0.01\) and \(p<0.001)\).

There was a positive correlation between VLDL triglyceride and cholesterol postprandially \((VLDL\ at\ 4h\ 0.78,\ p<0.001\ and\ 6h\ r=0.64,\ p<0.01)\).

In the diabetic patients on statin therapy, there was a weak correlation between MTP and postprandial VLDL apo B48 at 4h \((p<0.05)\) and apo B100 at 4h \((p<0.01)\). There was also a weak correlation between NPC1-L1 and postprandial VLDL apo B48 at 6h \((p<0.05)\).
The following figures are an illustrative aid for reference within the discussion which is the next section.

**Figure 5.5**
Intestinal mRNA expression in diabetic subjects not on a statin, ABCG5 v NPC1L1

![Intestinal mRNA Expression, Diabetes, no Statin](image)

**Figure 5.10**
Diabetic and Control subjects combined showing relationship between intestinal MTP mRNA expression and Chylomicron apo B48, (t=4), with no statin intervention.

![Intestinal MTP mRNA v Chylo apoB48 (t=4) in Diabetic and controls, no statin](image)
Figure 5.11
Diabetic and Control subjects combined showing relationship between intestinal MTP mRNA expression and Chylomicron apo B48, (t=6), with no statin intervention.

![Intestinal MTP mRNA v Chylo apoB48 (t=4) in Diabetic and Control, no statin](image)

R = 0.63
p<0.01

Figure 5.14
Intestinal ABCG5 v ABCG8 mRNA expression in diabetic and control subjects not on statin treatment.

![Intestinal ABCG5 v ABCG8 mRNA expression in diabetic and control subjects not on statin treatment](image)

R = 0.73
p<0.02
This cross-sectional study demonstrates increased intestinal MTP mRNA in diabetic subjects and a positive correlation between postprandial chylomicron and VLDL composition and MTP mRNA. These results are similar to a previous study on a separate group of subjects using the RNase protection assay to measure MTP mRNA (Mullan et al., 2003). There are no previous reports of intestinal ABCG5 and G8 and Niemann Pick C1-L1 mRNA expression in human subjects. In the present study there was a close correlation between ABCG5 and G8 in both diabetic and control subjects (Fig 5.14) which was expected since these proteins are known to work in tandem to expel cholesterol from the enterocyte (Davis et al., 2004). Diabetic patients had significantly lower ABCG5 and G8 mRNA expression suggesting an impairment in the re-excretion of cholesterol from the enterocyte back into the lumen in diabetic patients. The negative correlation between MTP mRNA and both ABCG5 and G8 mRNA suggests that the increase in available cholesterol may stimulate MTP expression leading to a significant increase in the number of chylomicron particle. This is supported by the negative correlation between both postprandial chylomicron cholesterol and apo B48 and ABCG5 and G8. NPC1-L1 is responsible for transporting cholesterol across the membrane (Davis et al., 2004). It is uncertain whether the protein also regulates the absorption of cholesterol synthesised in the intestine. NPC1-L1 was significantly higher in the intestine in diabetic patients compared to control subjects (fig 5.3) suggesting that diabetic patients have an increase in absorption of cholesterol. Since in both animal studies (Phillips et al., 2002; Glesson et al., 2000) and in human studies (Gylling et al., 1997) have not demonstrated an increase in dietary cholesterol absorption in Type 2 diabetes but have shown rather an increase in intestinal cholesterol synthesis, we suggest that the increase in NPC1-L1 results in increased absorption of biliary cholesterol and possibly of newly synthesised cholesterol in the intestine.

This study demonstrates a significant positive relationship between MTP mRNA and NPC1-L1 expression in the intestine (fig 5.5). This again suggests that MTP, which has a positive sterol response element (Hagan et al., 1994), is up-regulated in response to the
increased cholesterol absorption. The negative correlation between NPC1-L1 and ABCG5 and ABCG8 suggests a co-ordinated mechanism to regulate cholesterol homeostasis. Thus in times of cholesterol need, absorption is increased through up-regulation of NPC1-L1 and down-regulation of ABCG5 and G8. In Type 2 diabetes it would appear that the increase in NCP1-L1 and decrease in ABCG5 and G8 leads to the increase in chylomicron cholesterol. The relationship of the above mRNA levels to chylomicron composition which we observed in this study and which we have previously reported for MTP mRNA (Phillips(a) et al., 2002; Phillips(b) et al., 2002) suggests that the mRNA does affect the activity although in this study we were unable to measure protein or activity directly. The promoter region of the MTP gene has a negative insulin response element (Linn et al., 1995), it is speculated that this may influence the raised levels of MTP which we find in diabetes. It is interesting to see that polymorphisms in the Q604E allele of the ABCG5 gene in men were associated with insulin resistance (Gylling et al., 2004). In streptozotocin-induced diabetes in rats it has been shown that intestinal mRNA expression of ABCG5 and G8 and ABCG5 protein content were reduced in these animals as was the ABCG5 and G8 expression in the liver (Bloks et al., 2004).

This study demonstrates that lowering cholesterol by inhibition of HMGCoA reductase, which reduces cholesterol synthesis, down-regulates MTP mRNA again demonstrating the importance of cholesterol in regulating MTP. It was expected that inhibition of cholesterol synthesis would increase NPC1-L1 since NPC1-L1 deficiency results in up-regulation of HMGCoA reductase Davis et al (2004) have recently shown that NPC1-L1 null mice had increased HMGCoA synthase expression. In acute experiments it has been shown that inhibition of NPC1-L1 by ezetimibe does not affect acute intestinal or hepatic cholesterol synthesis in rats (van Heek et al., 2003) but there doesn’t appear to be any other studies on the relationship between HMGCoA reductase inhibition and NPC1-L1 mRNA. This study showed a 40% reduction in NCP1-L1 mRNA in diabetic patients treated with statin, but this decrease did not reach statistical significance, suggesting that statin treatment in type 2 diabetes may not decrease cholesterol absorption. There are no published studies in diabetic patients that have measured cholesterol absorption before
and after statin therapy. In non-diabetic patients Matthan et al. (2003) have shown an increase in cholesterol absorption in CAD patients with low HDL taking Simvastatin with niacin. However Ntanios et al. (1999) examined sterol absorption in hypercholesterolemic subjects using camostero! as a marker and found that Simvastatin reduced plasma camostero! and Miettinen and Gylling (2002) in the same year examining intestinal absorption and synthesis of cholesterol in coronary patients found that statin therapy decreased cholesterol synthesis but also reduced cholesterol absorption. In a further paper in 2003, the authors demonstrated increased fractional and mass absorption of cholesterol in patients treated with high dose atorvastatin for 6 months (Miettinen and Gylling, 2003). Watts et al. (2003) demonstrated an increase in cholesterol absorption in patients with the metabolic syndrome in patients on 40mg Atorvastatin, using camostero!/cholesterol as a surrogate marker, making the picture rather confusing. The results from this study would suggest that if there is an increase in cholesterol absorption in patients taking statin therapy, this increase is not through the NPC1-L1 pathway. The results also demonstrate an increase rather than decrease in ABCG5 and G8 in statin treated patients and suggest that perhaps there is an increase in sterol absorption which up-regulates the ABC pathway in an attempt to limit the increased sterol absorption load. It may be, as has been suggested, that the net result of statin therapy, with regard to the balance of decreased synthesis and altered cholesterol absorption may depend on the dose of statin and whether the patients are high or low cholesterol absorbers. It is possible that the method of measuring cholesterol absorption by measuring the ratio of plant sterol to cholesterol is no longer reliable when cholesterol synthesis is inhibited by drugs such as statins since, particularly in patients who are low absorbers of sterols, there would be very major changes in the ratio of plant sterol to cholesterol even if there was no change in cholesterol absorption (Matthan et al., 2004).

There was good evidence from these results to support the role of cholesterol in stimulating MTP mRNA. The reduction in MTP mRNA in patients on statin therapy suggests a major role of synthesised rather than absorbed cholesterol in regulating MTP. However it was also found that there was a strong correlation between MTP and chylomicron and VLDL triglyceride. This confirms the inter-dependence of cholesterol
and triglyceride in the postprandial phase. The next step was therefore to look to see whether there was a correlation between cholesterol and triglyceride in the postprandial chylomicron and VLDL fractions after the high fat meal and found significant positive correlations in both chylomicron and VLDL fractions. MTP and ABCG5/G8 play an important role in cholesterol homeostasis in the liver. There is no information on the relationship between intestinal and hepatic mRNA levels in diabetic or non-diabetic subjects. The positive correlation between MTP and apo B100 in the chylomicron fraction and the negative correlation between ABCG5 and chylomicron apo B100 suggests that intestinal levels may reflect hepatic mRNA levels. The strong correlation between NPC1-L1 and apo B100 may just reflect the inter-dependence between apo B48 and apo B100 in uptake by the B/E receptor which accepts apo B48 chylomicron particles in preference to apo B100 particles.

Postprandial lipoproteins are now believed to play an important role in atherosclerosis (Tanaka et al., 2004). In diabetes, a condition with accelerated atherosclerosis, LDL cholesterol is frequently normal although compositional differences have been described (Kraus et al., 2004). The postprandial lipoproteins do influence LDL composition (Ladenperra et al., 1996) but are also atherogenic in their own right (Karpe et al., 1994) and it is probably of considerable importance that there is a specific apo B48 receptor on the macrophage (Gianturco et al., 1998). The present study confirms previously described alterations in chylomicron and VLDL composition in type 2 diabetes and explains for the first time the mechanisms that result in the production of an abnormal chylomicron particle. There is substantial evidence to suggest that the chylomicron is atherogenic and can deliver significant amounts of cholesterol to the atherosclerotic plaque. The alteration of intestinal proteins regulating cholesterol absorption and chylomicron formation in type 2 diabetes may result in a less atherogenic postprandial chylomicron particle leading to a reduction in atherosclerosis.
Chapter 6.

Final Discussion
It is well established that the risk of cardiovascular disease is up to four times greater in patients with Type 2 diabetes compared with normoglycaemic individuals (Pyorala et al., 1987; Laakso and Lehto, 1997). The same factors that put the general population at risk for developing atherosclerotic heart disease are applicable to the diabetic population but are even more lethal (Stamler et al., 1993; Gu et al., 1999; Kannel et al., 1985). These well-identified risk factors include hypertension, tobacco use, high levels of low-density lipoprotein (LDL), low levels of high-density lipoprotein (HDL), age and family history of cardiovascular disease. According to Gu et al. (1998), more than 50% of deaths in people with diabetes are due to cardiovascular disease. The risk of coronary artery disease is heightened in Type 2 diabetes, and it has been demonstrated that patients with Type 2 diabetes but no prior history of myocardial infarction have a similar risk of having a cardiac event as patients without diabetes but who are known to have underlying coronary disease (Haffner et al., 1998).

Abnormalities in lipid metabolism may, in part, account the increased risk associated with type 2 diabetes. The postprandial state in Type 2 diabetes is metabolically the most abnormal period over the 24 hours and the chylomicron remnant, a postprandial particle, has been shown to be of particular importance in atherosclerosis in both diabetic and non-diabetic subjects (Mero et al., 2000; Karpe et al., 1994). Highly sensitive C-reactive protein (CRP) is an important newly identified potential risk factor, which provides a strong prediction of future risk for cardiovascular events (Ridker et al., 1997).

Plasminogen activator inhibitor, type 1 (PAI-1) is an inflammatory marker that works against tissue plasminogen activator (tPA). Hence, higher PAI-1 levels leads to diminished fibrinolysis and more thromboembolic events. PAI-1 levels are elevated in the vessel walls in patients with insulin resistance and diabetes (Gray et al., 1993). Matrix metalloproteinases (MMPs) degrade collagen and destroy the integrity of the fibrous cap. This causes plaque destabilization and rupture, which leads to thrombosis. Higher levels of MMP's cause a greater risk of plaque rupture and subsequent cardiovascular events (Kai et al., 1998). Elevated MMP levels are also linked to the metabolic syndrome.
MMP-9 can be measured in the serum; although this is primarily used in research settings, it may be a useful tool in the future. Other emerging cardiovascular risk factors associated with insulin resistance include several of the adipokines secreted from adipose tissue such as adiponectin, leptin and resistin, which have been shown to directly affect endothelial function as well as insulin action. A better understanding of these novel signalling networks will help in appreciating their potential role in insulin sensitivity, vasculature function and clinical cardiac endpoints.

Multiple clinical trials show that reduction of cholesterol with statins is important for risk reduction of coronary disease in both primary and secondary prevention (4S, CARE, AFCAPS and PROSPER) (4s, 1994; Sacks et al., 1996; Downs et al., 1998; Shepard et al., 2002). In the PROVE-IT trial, patients recently hospitalized with an acute coronary syndrome receiving a high-dose statin (atorvostatin 80 mg) had an average LDL of 3.41 mmol/L compared with a moderate-dose statin (pravastatin 40 mg) leading to an average LDL of 5.23 mmol/L. Those with the high-dose statin and average LDL of 3.41mmol/L had a 16% composite reduction in cardiovascular end points compared to the moderate statin therapy group. This study suggests that additional benefit may be obtained by reducing LDL-C to well below 5.5mmol/L (Cannon et al., 2004). This data is further supported by the finding that intensive lowering of LDL-C to well under 5.5mmol/L will reduce progression of coronary atherosclerotic lesions as measured by intravascular ultrasound (Nissen et al., 2004). In the Heart Protection Study, patients who had both diabetes and known coronary vascular disease were at the highest risk of future cardiac events. This group of patients obtained the greatest benefit from statin therapy. The results of this trial support the starting of statin therapy, especially in patients with both known diabetes and coronary disease, regardless of baseline LDL-C levels (Heart Protection Study Group, 2002). In a study by van Wijk, Cabezas et al., (2005), high-dose Simvastatin (80mg/day) has beneficial effects in normotriglyceridemic patients with premature CAD, due to improved chylomicron remnant clearance, besides effective lowering of LDL-cholesterol. In addition, the lipoprotein subfractions became more cholesterol-poor, as reflected by the increased TG/cholesterol ratio, which potentially makes them less atherogenic.
Because of the wealth of clinical experience with the statins, they are the mainstay of therapy in diabetic dyslipidaemia. However, clinical trials with fibrates have also shown a therapeutic role of this class of medications in the prevention of cardiac events (Rubins et al., 1999). The choice of an agent depends on baseline lipid profile. In general fibrates are preferred when the predominant lipid abnormality is high triglycerides, and statins are preferred for LDL lowering (Rosenberg et al., 2005).

The importance of tight glycaemic control for the prevention of microvascular complications is widely recognized. However, its value in preventing macrovascular disease has been disputed. Although the UKPDS did show that a decrease in HbA1c from a mean of 7.9 to 7.0% was associated with a 16% reduction in myocardial infarction, this change was not statistically significant (Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes, 2000). One important epidemiological study revealed a continuous correlation between HbA1c levels and mortality from all causes. Interestingly, this relationship continued even for values in the non-diabetic range (Khaw et al., 2001). As was previously mentioned, a recent study showed that chow-fed Ldlr−/− diabetic mice had more atherosclerosis at the aortic root, but when these mice were fed cholesterol-containing diet, the atherosclerosis correlated with plasma cholesterol and not glucose (Renard et al., 2004). Thus, as in many previous reports, the more advanced vascular lesions in these mice were attributable to greater hyperlipidaemia and not hyperglycaemia or defective insulin actions.

In chapter 3, ten zucker obese rats were treated with insulin, and ten were treated with pioglitazone. Both groups had lower blood glucose levels than the untreated group, and this resulted in a lower postprandial lipoprotein profile, with chylomicron and VLDL cholesterol and triglyceride levels both significantly decreased when compared with the untreated group. However, it is not clear whether it is: 1. the presence of an exogenous source of insulin, a hormone which has multi-systemic effects; 2. the actions of pioglitazone, a drug known to improve insulin sensitivity (Peterson et al., 2000) and improve the secretory function of β-cells (Finegood et al., 2001; Lister et al., 1999) or 3.
the actual lowering of blood glucose that is the main contributor to the lipid lowering effects seen. It is likely that these lipid lowering effects with the treatment of either insulin or pioglitazone are as a result of the down regulation of MTP mRNA, the protein involved in packaging the chylomicron particle. The results from chapter 3 show that MTP mRNA was significantly increased in the diabetic animals, and the treated animals showed a marked reduction in this expression in both the liver and the intestine. It is known that the promoter region of the MTP gene contains a positive sterol response element and a negative insulin response element (Hagan et al., 1994; Lin et al., 1995) explaining the increase in MTP in the diabetic animals and it has been previously shown that MTP expression is raised in the intestine of both Zucker obese insulin-resistant non-diabetic rats and streptozotocin diabetic rats (Gleeson et al., 1999; Phillips et al., 2002). It has also been shown that increased MTP expression in human intestinal biopsies from Type 2 diabetic patients (Phillips et al., 2004). The debate on whether or not hyperglycaemia per se represents an important risk factor for macrovascular complications in Type 2 diabetes remains.

As discussed, the thiazolidinediones (TZDs) are selective and potent agonists of peroxisome proliferator-activated receptor (PPAR) γ, a nuclear transcription factor expressed in target tissues for insulin action, especially adipose tissue (Vidal-Puig et al., 1997). When activated, PPARγ modulates the expression of genes involved in lipid metabolism, adipose-tissue differentiation and insulin action. The major metabolic effect is to enhance the sensitivity to insulin in peripheral tissues, primarily skeletal muscle, and at higher doses, the TZDs also reduce hepatic glucose production (Peterson et al., 2000). PPAR-γ is also present in many cells that play important roles in atherosclerosis including endothelial cells, vascular smooth muscle cells, monocytes and macrophages, and some of the clinical effects of the TZDs on vascular function are likely to be due to effects at these sites.

So why should TZDs be used in the treatment of diabetes? It is known that TZDs have significant positive effects on many of the risk factors for the development of coronary heart disease, including decreased CRP, MMP-9 and PAI-1 levels (Sidhu et al., 2003).
TZDs increase adiponectin levels in the circulation, which may be a major mediator of the effects of insulin sensitivity as well as vascular endothelial function (Phillips et al., 2003). In some studies, TZDs have had beneficial effects on the dyslipidemic profile in type 2 diabetes, increasing HDL by 10-30%, changing LDL from small, dense particles to more buoyant ones and decreasing triglycerides (Freed et al., 2002; Olefsky et al., 2000; Ovalle et al., 2002). In another study, TZDs also lowered blood pressure, another major risk factor for atherosclerotic disease (Raji et al., 2003). TZDs have been shown to affect fat distribution, by increasing subcutaneous peripheral fat deposits and reducing intrahepatic and visceral fat, which appears to contribute to the improvement in insulin sensitivity and the decrease in circulating FFA and adipocytokines (Akazawa et al., 2000; Kelly et al., 2002; Miyazaki et al., 2002). It has been demonstrated that TZDs decrease microalbuminuria, which in addition to being a marker of microvascular complication on the kidney, is also independently correlated with the development of cardiac disease (Bakris et al., 1999). Another potential benefit of TZDs is related to the effect of these medications on pancreatic -cell function. TZDs may improve the secretory function of β-cells and reduce β-cell apoptosis by reducing both lipid accumulation in the islets and chronically elevated FFAs that are characteristic of the diabetic state (Yang et al., 2003). In diabetes-prone, obese rodents, TZDs can restore β-cell insulin content and prevent loss of β-cell mass (Finegood et al., 2001; Lister et al., 1999).

In general, improvement in glycaemic control with decreased glycosuria and caloric retention and storage may result in expanded adipose tissue and body weight. Several studies (Akazawa et al., 2000; Kelly et al., 2002; Miyazaki et al., 2002) have shown that the weight gain with TZDs may be associated with an increase in subcutaneous fat. However, at the same time, there is a reduction in visceral fat and an overall decrease in the ratio of visceral to subcutaneous fat (Akazawa et al., 2000; Kelly et al., 2002; Miyazaki et al., 2002). This change in fat distribution seems to underlie the improvement in glycaemic control despite an overall increase in body weight. The average weight of the pioglitazone treated group of animals in chapter 3 was slightly higher than the insulin treated group and the untreated group, but this was not statistically different. Perhaps a
longer duration of treatment would be needed to see larger increase as the rats were only treated for 14 days.

In chapter 3 the change in gene expression for some of the proteins involved in cholesterol metabolism in the intestine and the liver was investigated, and it was seen that there was an increase in HMGCoA reductase mRNA expression, MTP mRNA expression and also in the expression of NPC1L1 mRNA in all 3 diabetic groups when compared with the control group. Treatment with pioglitazone, a TZD, reduced the expression of HMGCoA mRNA in the liver, the protein involved in the de novo synthesis of cholesterol, but it was not statistically significant, and there was no difference in the mRNA expression in the liver when compared with the untreated group. There was a significant reduction in HMGCoA reductase mRNA in both the intestine and the liver in the insulin-treated rats compared to pioglitazone-treated animals. The expression of NPC1L1 mRNA was markedly reduced in the liver of the pioglitazone treated group, thus this reduction in HMGCoA mRNA probably reflects a reduction in cholesterol absorption in the pioglitazone group.

Intestinal ABCG5 mRNA expression levels were significantly lower in the diabetic animals compared to lean animals. You will remember that ABCG5 and G8 work in tandem to excrete cholesterol from the intestinal cells back into the lumen and from the liver into the bile (Berge et al., 2000). Although not statistically significant, there was a small increase in the expression of these mRNA transcripts in both of the treated groups. The lowest expression was in the untreated diabetic group and this would suggest that the reduction in the expression of ABCG5 and G8 in diabetes plays a role in the abnormal cholesterol metabolism associated with diabetes, both in the intestine and in the liver where these proteins play a role in the excretion of cholesterol into the bile. By promoting the expression of ABCG5 and G8, this intervention may aid in the balancing of altered cholesterol metabolism commonly seen in diabetes.

Interestingly, the animals on pioglitazone showed a marked reduction in the expression of NPC1-L1 mRNA compared to those on insulin, which may support the idea that NPC1-
L1 plays an important part in regulating VLDL cholesterol from the liver. This is suggested by the finding that there was a strong positive correlation between chylomicron cholesterol and NPC1-L1 mRNA in the intestine, and in the liver between NPC1-L1 mRNA and VLDL cholesterol. Chawla et al (2003) have shown that PPARδ acts as a sensor for very low-density lipoprotein (VLDL), a role that implicates the receptor in the regulation of triglyceride homeostasis in peripheral tissues such as the macrophage and the vessel wall, and this supports a beneficial role for PPARδ agonists in the treatment of dyslipidaemia, and a reduction in the expression of NPC1L1 may also help reduce the burden of atherosclerosis in diabetes.

In trying to normalise the postprandial dyslipidaemia seen in the diabetic rats, pioglitazone was shown to have reduced the chylomicron and VLDL levels more than the insulin treatment. However, it would be interesting to see the effects of treating the animals with both insulin and pioglitazone. As both treatments are seen to try and bring mRNA expression levels towards those of the non-diabetic rats, perhaps a combination of insulin and pioglitazone would result in a better postprandial and fasting lipid profile in the diabetic rats. Indeed there has very recently been a review article which has looked at the treatment of thiazolidinediones with insulin as opposed to metformin with insulin, as some patients can suffer from significant gastrointestinal problems (Strowig and Raskin, 2005) and it is suggested that metformin should not be used in patients with elevated serum creatinine or those being treated for congestive heart failure. The thiazolidinediones in combination with insulin have also been effective in lowering blood glucose levels and total daily insulin dose (Strowig and Raskin, 2005). This may be particularly useful in the treatment of type 1 diabetic subjects who need large doses of daily insulin to control blood sugar levels.

Chapter 4 looked at the effects of gene expression in a different model of diabetes, where the animals were injected with streptozotocin in order to induce diabetes. This study provided a more significant/pronounced difference in the levels of blood glucose between the diabetic and non-diabetic groups than were observed in the chapter 3 or in chapter 5. Forty of the animals in this study were also fed a high fat, western-diet, in order to
investigate the changes in gene expression in the liver and the intestine and in the lipid profile when a higher load of cholesterol was ingested. Twenty of the animals were fed a standard chow diet, so as to see if the changes could be attributed to diet or diabetes. The other question being examined was the time-dependent response of fasting on the expression of the genes in focus in this study.

There was a dramatic difference in the chylomicron and VLDL cholesterol and triglyceride levels between the diabetic and control groups on the high fat diet, with highly elevated levels seen in the diabetic groups. The high fat diet also resulted in the control groups on the high fat diet having an increase in the chylomicron and VLDL cholesterol and triglycerides. From the results, it seems that the high fat diet, when combined with highly elevated blood sugar levels, greatly exacerbated the postprandial dyslipidaemia seen in these diabetic animals. It was interesting to see that the expression of NPC1L1 in the intestine of the diabetic animals on the high fat diet (group B) was dramatically increased when compared with the diabetic animals on the chow diet (group A) and there is a greater increase again when compared with the non-diabetic subjects, confirming it’s role in cholesterol absorption, predominantly it seems in the intestine as the hepatic mRNA levels of NPC1L1 were relatively low.

The results of the time-dependent response of fasting on the expression of the various genes investigated were eagerly awaited. The reason for this was that in the human studies, all the patients who took part in the study had undergone an overnight fast before the gastroscopy procedure the following morning, in order to obtain the duodenal biopsy samples. It was seen that, with the exception of NPC1L1 which was different after 6 hours fasting but not 12 hours fasting, there was no statistically significant changes in the expression profile when the rats were fasting compared to when they were feeding. This would suggest that as the body spends most of the day in the postprandial period (van Oostrom, Cabezas et al., 2004), the up/down-regulation of the genes involved in cholesterol metabolism is not a rapid response process. As a result, calculated postprandial lipoproteins can be correlated with mRNA expression levels in the patients from the human study without fear of interference of the fasting from the night before.
It is clear from the results obtained in chapter 4 that a high fat or western diet in diabetic subjects is potentially deleterious. There were high levels of postprandial lipoproteins, for both the chylomicron and VLDL subfractions, in the diabetic animals on the chow diet but there was a further dramatic increase in these lipids when the diabetic animals were put on the high fat diet. It also becomes apparent that a low-fat diet alone in diabetic subjects is not enough of a therapeutic intervention in trying to treat postprandial dyslipidaemia. Cholesterol and triglycerides levels were both increased, and these correlated positively and strongly with the expression of NPC1L1 and MTP respectively. Pharmacological intervention is almost a necessity in these subjects in order to normalise the dyslipidaemia that is typically associated with diabetes.

Chapter 5 deals with the expression of the genes involved in regulating cholesterol homeostasis in human subjects, both type 2 diabetics and non-diabetics. The results obtained for the mRNA of MTP are similar to results on a separate group of subjects obtained previously using the RNase Protection Assay (Mullan et al., 2003). In this study however, the real time, 2-step, RT-PCR method was employed for the relative determination of the mRNA levels for each of the genes involved.

The results show that there was an increase in NPC1L1 mRNA expression in both the liver and the intestine. It is already known that NPC1-L1 is responsible for transporting cholesterol across the membrane (Davis et al., 2004). What is uncertain though is whether the protein also regulates the absorption of cholesterol synthesised in the intestine. The fact that NPC1-L1 mRNA was found to be significantly higher in the intestine in diabetic patients compared to control subjects would suggest that diabetic patients have an increase in absorption of cholesterol. In both animal studies (Phillips et al., 2002; Glesson et al., 2000) and in human studies (Gylling et al., 1997) there has been no demonstration of an increase in dietary cholesterol absorption in Type 2 diabetes. However, since these studies have shown rather a potential increase in intestinal cholesterol synthesis perhaps it is that the increase in NPC1-L1 which results in increased
absorption of biliary cholesterol and possibly of newly synthesised cholesterol in the intestine.

There is a significant strong positive correlation between the expression of MTP and NPC1L1 mRNA in this study. This would suggest that a possible increase in biliary cholesterol resulting from the increased NPC1L1 intestinal expression provides the stimulus for an increase in the production of MTP to package more chylomicron particles for the transport of this excess cholesterol and triglyceride in the lymphatic system.

It was also found that there is a decrease in the mRNA expression of the heterodimeric proteins ABCG5 and G8 in the diabetic subjects. As these function in transferring plant sterols and cholesterol back out into the lumen, a decrease in their expression may result in more cholesterol being available for MTP to package. The fact that there is a negative correlation between the expression of MTP mRNA and both ABCG5 and ABCG8 mRNA strengthens this argument. There was also found to be a negative correlation between chylomicron cholesterol levels and the expression of G5 and G8, meaning that the lower the expression of these genes, the higher the levels of cholesterol which would fit in with this hypothesis.

Statins were used to lower cholesterol by inhibition of HMGCoA reductase, which reduces cholesterol synthesis. This treatment resulted in the down-regulation of MTP mRNA, again demonstrating the importance of cholesterol in regulating MTP. It was expected that inhibition of cholesterol synthesis would increase NPC1-L1 since NPC1-L1 deficiency results in up-regulation of HMGCoA reductase. Davis et al (2004) have recently shown that NPC1-L1 null mice had increased HMGCoA synthase expression. In acute experiments it has been shown that inhibition of NPC1-L1 by ezetimibe does not affect acute intestinal or hepatic cholesterol synthesis in rats (van Heek et al., 2003) but there doesn’t appear to be any other studies on the relationship between HMGCoA reductase inhibition and NPC1-L1 mRNA. This study showed a reduction in NCP1-L1 mRNA in diabetic patients treated with statin, but this decrease did not reach statistical
significance, suggesting that statin treatment in Type 2 diabetes may not increase cholesterol absorption.

It is clear that the intestine plays an integral part in regulating cholesterol metabolism. These results may help in our understanding of the postprandial period in diabetes, a condition in which there is considerable postprandial dyslipidaemia. These studies are the only known studies which have investigated the expression of NPC1L1 mRNA and ABCG5 and G8 in diabetes in human subjects. They confirm the increase in expression of MTP and HMGCoA reductase and they suggest that combination therapy of statins to inhibit HMGCoA reductase, ezetimibe to inhibit NPC1L1 and the development of an intestinally specific MTP inhibitor may help to alleviate the burden of atherosclerosis in diabetes.
Chapter 7.

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